UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

INSTITUTO DE FISIOLOGÍA CELULAR
PROGRAMA DE DOCTORADO EN CIENCIAS BIOMÉDICAS

Mecanismos de neurodegeneración y neuroprotección en modelos experimentales *in vivo* de esclerosis lateral amiotrófica

T E S I S

que para obtener el grado de

D O C T O R E N C I E N C I A S

P R E S E N T A:

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Ciudad Universitaria, México D.F. Diciembre 2009

El presente trabajo se realizó bajo la dirección del Dr. Ricardo Tapia Ibargüengoytia en la División de Neurociencias del Instituto de Fisiología Celular de la Universidad Nacional Autónoma de México con los apoyos del Consejo Nacional de Ciencia y Tecnología (42668 y 60322) y de la Dirección General de Asuntos del Personal Académico de la UNAM (IN213703, IN201204 e IN209807).

Durante mis estudios en el Programa de Doctorado en Ciencias Biomédicas recibí la beca nacional del CONACyT (194983) y realicé una estancia doctoral de julio a diciembre de 2008 en el Institut de Biologie du Développement de Marseille Luminy del Institut National de la Santé et de la Recherche Médicale (INSERM) en Marsella, Francia, con el apoyo de la beca mixta del CONACyT (187311), de la Dirección General de Estudios de Postgrado de la UNAM y del INSERM (Francia).

Agradezco al Dr. Ricardo Tapia Ibargüengoytia la tutoría que me ha brindado durante el tiempo que he trabajado en su grupo, la dirección de esta tesis y de los proyectos emprendidos de cuyos resultados aquí se presenta una parte. Especialmente agradezco su interés en mi trabajo, mi desempeño profesional, y en las oportunidades académicas que se me han presentado en estos años. Las enseñanzas que he recibido del Dr. Tapia durante mi estadía en su laboratorio constituyen la cualidad más grande de mi formación doctoral.

Agradezco también las contribuciones de los Dres. Iván Velasco Velázquez y Miguel Ángel Morales Mendoza, miembros de mi Comité Tutoral, para el mejoramiento de mi proyecto doctoral.

Asimismo, expreso mi agradecimiento por la revisión y comentarios hechos a esta tesis del Jurado de Grado integrado por los Dres. Ricardo Tapia Ibargüengoytia, Lourdes Massieu Trigo, Clorinda Arias Álvarez, José Fernando Peña Ortega y Abel Santamaría del Ángel.

Durante el desarrollo de este proyecto se contó con la colaboración del Bioterio y de las unidades de Cómputo, Histología y Microscopía del Instituto de Fisiología Celular de la UNAM. Agradezco especialmente el apoyo técnico y la asesoría de Francisco Pérez Eugenio de la Unidad de Cómputo.

Finalmente agradezco a la Universidad Nacional Autónoma de México la inigualable oportunidad que me ha brindado para la realización de mis estudios tanto profesionales como de postgrado y el espacio otorgado para mi desarrollo académico.

Índice

Resumen

La esclerosis lateral amiotrófica (ALS) es un padecimiento neurodegenerativo generado por la muerte selectiva y progresiva de las motoneuronas de la médula espinal, el tallo cerebral y la corteza motora. Se caracteriza principalmente por una parálisis progresiva de los músculos esqueléticos que ocasiona la muerte del paciente en un promedio de 2 a 5 años después de haber sido diagnosticada. Se ha clasificado a la ALS en dos tipos, familiar y esporádica, un porcentaje de los pacientes con ALS familiar presentan mutaciones en la enzima antioxidante superóxido dismutasa 1 (SOD1), y la expresión de la SOD1 humana mutada en ratones transgénicos ha permitido el desarrollo de un modelo de ALS familiar *in vivo*. Existen diferentes hipótesis sobre los mecanismos fisiopatológicos que dan origen a la ALS, entre ellos se ha considerado que una transmisión sináptica excitadora excesiva causa la muerte de las motoneuronas a través de un fenómeno conocido como excitotoxicidad. En esta tesis se presentan evidencias que indican que los ratones que expresan la SOD1 humana mutada no tienen una susceptibilidad incrementada a la muerte neuronal inducida por excitotoxicidad en regiones del cerebro que pudieran ser vulnerables como el hipocampo o la corteza motora. También, que un incremento crónico y significativo de la concentración de glutamato extracelular en la médula espinal de la rata silvestre, inducido mediante el bloqueo del transporte de glutamato no es suficiente para inducir la muerte de las motoneuronas. Por el contrario, la administración del agonista glutamatérgico AMPA a través de su infusión crónica en la médula espinal de la rata adulta genera la muerte de las motoneuronas y en consecuencia la presentación de alteraciones motoras que terminan como una parálisis total del tren posterior, constituyendo un modelo de neurodegeneración espinal no genético. Se demuestra también que el factor de crecimiento vascular endotelial (VEGF), cuyo efecto terapéutico contra la muerte de la motoneuronas ha sido comprobado en modelos familiares de ALS, es altamente efectivo para el bloqueo de la muerte excitotóxica de las motoneuronas inducida por AMPA, y que este efecto es logrado a través de la activación directa de uno de sus receptores específicos expresado en las motoneuronas el cual induce la activación de la vía de sobrevivencia celular mediada por las cinasas PI3-K/Akt. Nuestros resultados demuestran que la disminución de la actividad de los transportadores de glutamato no es suficiente para inducir la muerte excitotóxica de las motoneuronas, y por lo tanto su participación no es un evento clave para la generación de la ALS, y que no obstante la causa de la muerte de las motoneuronas, el VEGF es capaz de prevenir la muerte neuronal convirtiéndose en una alternativa terapéutica cuyo potencial es interesante analizar.

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative condition generated by the selective and progressive death of motoneurons in the spinal cord, brainstem and motor cortex. ALS is mainly characterized by a progressive paralysis of skeletal muscles which causes the demise of the patient in an average of 2-5 years after been diagnosed. ALS is classified in two groups, familial and sporadic, a fraction of patients with the familial form harbor mutations in the antioxidant enzyme superoxide dismutase 1 (SOD1), and the expression of human mutant SOD1 in transgenic mice has allowed the generation of an *in vivo* model of familial ALS. There are different hypotheses on the pathophysiological mechanisms that cause ALS, among them it is considered that an excessive excitatory synaptic transmission causes the death of motoneurons through a mechanism known as excitotoxicity. In this thesis, I present experimental evidences that indicate that transgenic mice harboring human mutant SOD1 have no increased susceptibility to excitotoxic neuronal death in brain regions that might be prone to such a stimulus, like hippocampus and motor cortex. I also show that a chronic and significant augment of extracellular glutamate concentration due to glutamate transport blockade in the spinal cord of wild type rats is not sufficient to induce the death of motoneurons. On the other hand, the administration of the glutamatergic agonist AMPA through its chronic infusion in the spinal cord of the adult rat causes the death of motoneurons and in consequence rats show motor alterations that end up in a complete paralysis of the rear limbs, constituting a non-genetic model of spinal neurodegeneration. I also show that vascular endothelial growth factor (VEGF), whose therapeutic effect has been proven against motoneuron death in familial models of ALS, is highly effective to prevent AMPA-induced motoneuron excitotoxic death, and that this protective effect is achieved by means of the direct activation of a specific VEGF receptor expressed in motoneurons, which induces the activation of the intracellular survival pathway PI3-K/Akt. Taken together these results show that the decrease of glutamate transporters activity is not enough to induce the excitotoxic death of motoneurons and therefore its involvement in ALS may not be a key event, and that despite the cause of motoneurons death, VEGF is capable to prevent neuronal death making this factor a therapeutic alternative for ALS of interesting potential.

Abreviaturas

ALS esclerosis lateral amiotrófica
AMPA α-amino-3-hidroxi-5-metil-4-isoxazol propionato
ATP adenosín trifosfato
EAAC acarreador de aminoácidos excitadores
EAAT transportador de aminoácidos excitadores
ERK cinasa regulada por señal extracelular
FALS esclerosis lateral amiotrófica familiar
Flk-1 cinasa de hígado fetal 1
Flt-1 cinasa de tirosina similar a fms 1
GABA ácido γ-aminobutírico
GDNF factor neurotrófico derivado de glia
GLAST transportador de glutamato/aspartato
GLT transportador de glutamato
HIF factor inducido por hipoxia
HPLC cromatografía líquida de alta resolución
HRE elemento de respuesta a hipoxia
IGF-1 factor de crecimiento similar a insulina 1
KDR receptor con dominio de cinasa
MAPK proteína cinasa activada por mitógeno
NMDA N-metil-D-aspartato
PCR reacción en cadena de la polimerasa
PDC pirrolidín-dicarboxilato
PGE resistencia del asimiento de la pata
PI3-K fosfatidilinositol trifosfato cinasa
PlGF factor de crecimiento placentario
ROS especies reactivas de oxígeno
RTK receptor con actividad de cinasa de tirosina
SALS esclerosis lateral amiotrófica esporádica
SNC sistema nervioso central
SOD1 superóxido dismutasa 1
TBOA DL-treo-β-benziloxiaspartato
VEGF factor de crecimiento vascular endotelial
VPF factor de permeabilidad vascular

Organización de la tesis

Este trabajo está dividido en ocho secciones: Introducción, Antecedentes, Planteamiento del problema, Objetivos, Diseño y procedimientos experimentales, Resultados, Discusión y Conclusiones.

En la Introducción, se presenta un marco teórico breve sobre la esclerosis lateral amiotrófica (ALS), la médula espinal que es el órgano principalmente afectado en esta enfermedad y las motoneuronas cuya muerte da origen a la ALS. Asimismo, se describe brevemente la transmisión sináptica glutamatérgica, la cual se ha pensado que se encuentra alterada en la ALS, y los mecanismos de neurodegeneración. La investigación realizada en el presente trabajo se basa principalmente en el posible papel de la excitotoxicidad como mecanismo causante de la ALS, por ello se describe este fenómeno de manera particular y se presentan las evidencias que lo involucran en la ALS en el artículo *"Excitotoxicity as a mechanism of motoneuron death in amyotrophic lateral sclerosis"*. Finalmente se describen en esta sección los mecanismos de sobrevivencia como la activación de vías de señalización intracelular, particularmente aquellos mediados por el factor de crecimiento vascular endotelial. En la sección de Antecedentes se presenta el artículo *"Cerebral neurons of transgenic ALS mice are vulnerable to glutamate release stimulation but not to increased extracellular glutamate due to transport blockade"*, trabajo con el cual comenzamos el estudio del probable origen de estímulos excitotóxicos en la ALS en un modelo transgénico de la enfermedad.

Enseguida se presenta el planteamiento del problema y se enlistan los objetivos de este trabajo. Para el diseño y los procedimientos experimentales se presenta en el artículo *"Experimental models for the study of neurodegeneration in amyotrophic lateral sclerosis"*, una revisión integral de los modelos actualmente usados para el estudio de la ALS y de sus mecanismos así como de sus posibles terapias, donde se presentan los modelos empleados en este trabajo. A continuación se describe el procedimiento experimental utilizado para el desarrollo de un modelo crónico de neurodegeneración espinal *in vivo*, las pruebas de conducta motora utilizadas para la caracterización de tal modelo y la técnica de microdiálisis utilizada en diversos experimentos de este proyecto.

En la primera parte de Resultados se presenta el artículo *"Chronic elevation of extracellular glutamate due to transport blockade is innocuous for spinal motoneurons*

in vivo", en el que describimos el efecto del bloqueo crónico de los transportadores de glutamato en la médula espinal de la rata silvestre. A continuación, en la segunda parte de los Resultados, se presentan dos artículos más de investigación original sobre la protección contra la excitotoxicidad mediada por el VEGF; *"Vascular endothelial growth factor prevents paralysis and motoneuron death in a rat model of excitotoxic spinal cord neurodegeneration"* y *"VEGF protects spinal motoneurons against chronic excitotoxic degeneration in vivo by activation of PI3-K pathway"*.

Finalmente, se ofrece una discusión integral de los hallazgos del presente trabajo sobre las alteraciones del transporte de glutamato en la ALS y del VEGF como una terapia alternativa para la misma y se presenta el artículo *"Glutamate excitotoxicity and therapeutic targets for amyotrophic lateral sclerosis"* como un marco de referencia sobre las actuales estrategias terapéuticas para la ALS. Este trabajo termina con la sección de Conclusiones donde presento una visión personal del panorama actual de los avances hechos sobre la ALS y sus posibles implicaciones clínicas.

Introducción

Esclerosis lateral amiotrófica

Hace 140 años el fisiólogo francés Jean-Martin Charcot describió por primera vez un padecimiento neurodegenerativo caracterizado principalmente por una atrofia muscular progresiva, al cual se llamó por algunos años la esclerosis de Charcot (Charcot y Joffory 1869). Hoy en día esta condición es conocida como esclerosis lateral amiotrófica (ALS, por sus siglas en inglés), ya que como describió Charcot, los pacientes presentan además de la atrofia muscular, un endurecimiento o esclerosis de las columnas laterales de la médula espinal (Cleveland y Rothstein 2001). A esta enfermedad también se le conoce en algunos lugares con el popular nombre de enfermedad de Lou Gerigh, en honor al jugador de pelota afectado por la ALS en la cumbre de su carrera deportiva en los años 30 del siglo pasado.

A pesar de que la enfermedad se conoce desde hace más de 100 años, hasta ahora no hay un tratamiento efectivo para detener o retrasar significativamente su progreso. La característica fisiopatológica más importante en la ALS es la muerte selectiva de las neuronas encargadas del movimiento, las motoneuronas, ubicadas a lo largo de la columna del asta ventral de la médula espinal y el tallo cerebral (motoneuronas inferiores) y en la corteza motora (motoneuronas superiores) (McHanwell y Watson 2009). Dos formas de ALS han sido descritas según la región del SNC donde las motoneuronas son afectadas inicialmente, si la degeneración ocurre en las motoneuronas inferiores de las porciones bajas de la médula espinal la ALS se clasifica como lumbar, en cambio si ocurre en las motoneuronas del tallo la ALS se clasifica como bulbar (Cleveland y Rothstein 2001). De hecho, esta característica determina los síntomas iniciales que el paciente presentará. En general, la muerte neuronal genera una debilidad muscular progresiva, espasticidad, fasciculaciones y en consecuencia parálisis; otros síntomas pueden incluir disfagia y disartria (Kuhnlein et al. 2008). Debido a la selectividad de la muerte neuronal en la ALS, los pacientes no sufren discapacidades intelectuales, excepto en casos atípicos donde la enfermedad se asocia con demencia frontotemporal y parkinsonismo (Wilhelmsen 1997; Hosler et al. 2000). Sin embargo, se reconoce que algunos pacientes presentan deterioros cognitivos como cambios de personalidad, irritabilidad, conductas obsesivas, y un desempeño pobre en pruebas de funciones ejecutivas (Phukan et al. 2007). Generalmente los pacientes mueren como resultado de un fallo respiratorio debido a la contracción inadecuada de los músculos respiratorios después de un promedio de 3 a 5 años de haber sido diagnosticados (Cleveland y Rothstein 2001).

La incidencia mundial de la ALS típica es alrededor de 2 a 5 por cada 100,000 individuos y ocurre en la adultez media entre los 40 y 60 años aunque puede comenzar a edades más tempranas (Cleveland y Rothstein 2001). Dependiendo de su origen se reconocen dos formas principales de ALS, la esporádica (SALS) y la familiar (FALS). La primera es la más común y cuenta con ~90% de los casos, mientras que la segunda comprende al ~10% restante y es heredada de manera autosómica dominante (Cleveland y Rothstein 2001).

En 1993 Rosen y colaboradores (Rosen et al. 1993) encontraron que una fracción de los pacientes con FALS, aproximadamente el 20%, presenta mutaciones de sentido equivocado ó "missense" en la enzima superóxido dismutasa 1 (SOD1). Las formas mutantes de la SOD1 son tóxicas debido a la ganancia de una nueva función catalítica que genera especies reactivas de oxígeno (ROS) pero no debido a que pierden su actividad catalítica normal de dismutación del anión superóxido (Revisado en Boillee et al. 2006). Casi inmediatamente después de este descubrimiento se generaron ratones transgénicos que expresan una forma mutante de la SOD1 humana que demostraron sufrir neurodegeneración espinal y parálisis (Gurney et al. 1994). Como se discute en la parte de diseño y procedimientos experimentales estos animales son el modelo más utilizado para el estudio de la ALS *in vivo*, a pesar de que reproducen únicamente el mecanismo de neurodegeneración que ocurre en la menor proporción de los pacientes, aquellos con la forma familiar de la enfermedad causada por mutaciones en la SOD1.

Debido a la variabilidad de los síntomas que presentan los pacientes, el diagnóstico de la ALS no es una tarea fácil y requiere de una serie de evaluaciones clínicas, electrofisiológicas y de neuroimagenología que cumplan con los criterios internacionalmente acordados en El Escorial, España, en 1990 (Brooks 1994). Los criterios para el diagnóstico de la ALS incluyen la presencia y el progreso de signos de degeneración tanto de las motoneuronas inferiores como de las superiores y la ausencia de evidencia de otras enfermedades que puedan explicar los síntomas observados (Brooks 1994). Cuando la degeneración de las motoneuronas inferiores o superiores ocurre de manera independiente, el síndrome es considerado como alguna enfermedad de las motoneuronas pero no necesariamente ALS (Brooks 1994).

La médula espinal

La médula espinal es un órgano que constituye parte del sistema nervioso central. Tiene una forma cilíndrica ligeramente achatada en el eje dorso-ventral y ocupa los dos tercios superiores del canal de la columna vertebral (Watson y Kayalioglu 2009).

Sus funciones principales son el control de los músculos esqueléticos de las extremidades y del tronco, y la recepción de la información sensitiva de estas regiones. También controla a la mayoría de las vísceras y a los vasos sanguíneos del tórax, abdomen y pelvis (Watson y Kayalioglu 2009).

La médula espinal está cubierta por 3 membranas denominadas meníngeas. De adentro hacia afuera éstas son: pía madre, aracnoides y dura madre y están separadas una de otra por los espacios subaracnoideo y subdural. El líquido cefalorraquídeo fluye en el espacio subaracnoideo, entre la aracnoides y la pía madre. Entre la dura y el periostio de la columna vertebral existe un espacio llamado epidural, que contiene tejido adiposo y linfático, pequeñas arterias y un plexo venoso (Watson y Kayalioglu 2009).

Longitudinalmente, la médula espinal en los humanos tiene 31 segmentos: 8 cervicales, 12 torácicos, 5 lumbares, 5 sacros y 1 coccígeo. En la rata hay 34 segmentos: 8 cervicales, 13 torácicos, 6 lumbares, 4 sacros y 3 coccígeos (Watson y Kayalioglu 2009).

Transversalmente, en la superficie ventral hay una fisura en la línea media llamada fisura media ventral o anterior, formada por la comisura ventral blanca, que se extiende cerca de un tercio a lo largo del eje rostro-caudal y contiene una prolongación de la pía madre y ramas de la arteria espinal anterior. En el lado dorsal, hay una hendidura somera llamada surco medio dorsal o posterior. El septo dorsal se extiende desde la base de este surco hasta la materia gris comisural. En las caras laterales, existen surcos ventro- y dorso-laterales que corresponden a las líneas de origen de las raíces ventrales y dorsales (Watson y Kayalioglu 2009).

Los segmentos más anchos de la médula espinal se encuentran en la región cervical, seguidos de las regiones lumbar, torácica y sacra (Watson y Kayalioglu 2009). La materia blanca ocupa casi toda el área transversal al nivel del alargamiento cervical, mientras que al nivel de los plexos branquial y lumbosacral, que inervan a las extremidades, el área ocupada por la materia gris crece. Los alargamientos de las regiones que inervan a los miembros son llamados ensanchamiento cervical y ensanchamiento lumbar, y son directamente proporcionales al tamaño y desarrollo de las extremidades (Ranson y Clark 1953).

En la médula espinal puede distinguirse transversalmente la materia blanca en la parte más externa y la materia gris en la parte más interna, en un arreglo en forma de mariposa o de letra H dependiendo del segmento. La barra horizontal de la H, llamada sustancia gris comisural rodea al canal central, las proyecciones dorsales de la materia gris se conocen como astas dorsales y las proyecciones ventrales se llaman astas ventrales. En la región torácica y en las porciones superiores de la región lumbar, hay una proyección lateral de la materia gris comisural que se llama asta intermediolateral y contiene células del sistema nervioso autónomo. El asta dorsal se extiende hasta la superficie de la médula espinal donde entran las raíces de los nervios dorsales. El asta ventral no tiene contacto con la superficie de la médula espinal (Watson y Kayalioglu 2009).

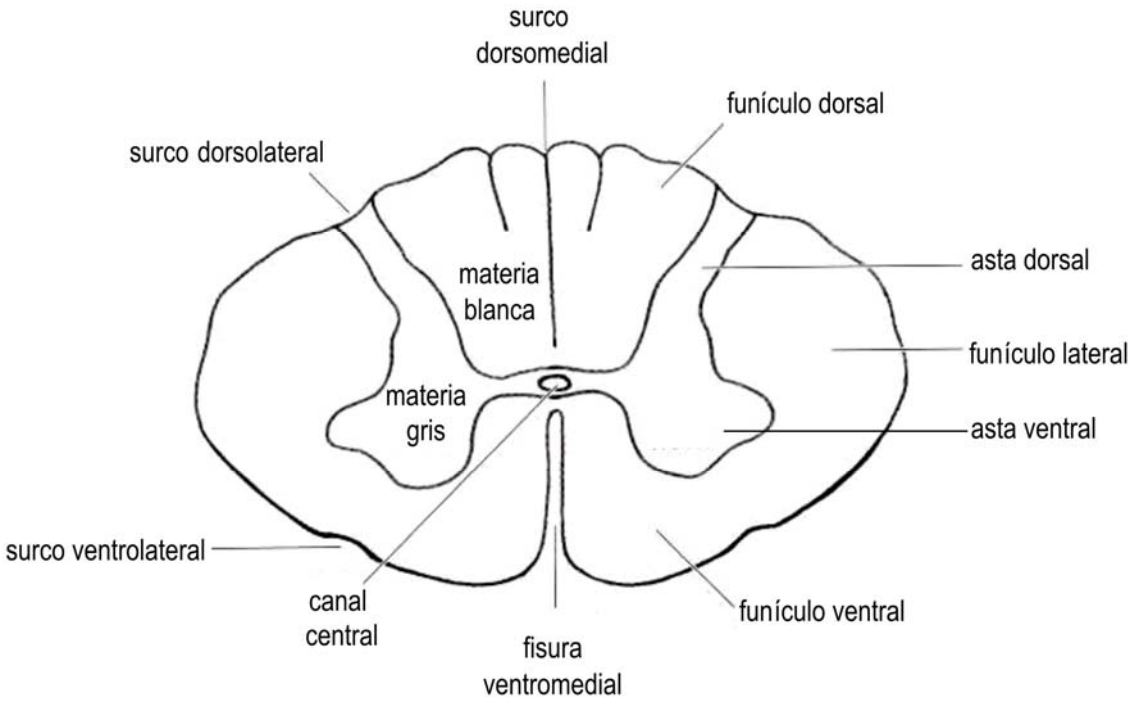

Figura 1. Esquema de un corte transversal de la médula espinal en la porción cervical.

El flujo sanguíneo a la médula espinal es suministrado por una arteria espinal ventral y dos arterias espinales dorsales. La primera se genera a partir de la arteria vertebral y desciende dentro de la fisura media ventral. Las arterias dorsales se originan de la arteria vertebral o de su rama cerebelar inferior posterior y descienden por el surco dorsolateral de la médula espinal. Ramas segmentales de las arterias vertebral, cervical profunda, intercostal y lumbar anastomosan con las arterias espinales dorsales y ventral. Las venas de la médula espinal forman un plexo de superficie que

desemboca rostralmente en las venas cerebelares y en los senos craneales y a través de las venas intervertebrales y los plexos venosos externos al sistema ácigos (Watson y Kayalioglu 2009).

Las motoneuronas

Las motoneuronas en la médula espinal pertenecen a dos grupos funcionales: somático y visceral. Las motoneuronas somáticas, que son las que mueren en la ALS, inervan los músculos esqueléticos mientras que las viscerales a los músculos lisos y a las glándulas (McHanwell y Watson 2009).

Las motoneuronas somáticas se ubican en el asta ventral de la médula espinal e inervan a los músculos estriados del esqueleto axial y de las extremidades superiores e inferiores. La mayoría de éstas son motoneuronas alfa que inervan a las fibras musculares extrafusales del músculo esquelético. También existe una población más pequeña de motoneuronas gamma que inervan fibras musculares intrafusales dentro de los husos musculares y motoneuronas beta que son mucho menos numerosas que las alfa y gamma y envían sus proyecciones dentro del músculo a fibras intrafusales y extrafusales. Las motoneuronas beta inervan principalmente a los músculos distales de las extremidades (McHanwell y Watson 2009).

Existe un grupo diferente de motoneuronas somáticas que no son afectadas en la ALS, que se ubican en el núcleo de Onuf, en la parte ventrolateral del asta ventral de la región caudal lumbar y rostral sacral. El núcleo de Onuf inerva a los músculos perineales, a los esfínteres anal y uretral y al cremaster (McHanwell y Watson 2009; Watson y Kayalioglu 2009).

Transmisión sináptica glutamatérgica

El glutamato es el neurotransmisor excitador más abundante del SNC de los mamíferos; a pesar de que su concentración es relativamente alta y su distribución es uniforme a lo largo del cerebro y la médula espinal, una fracción del glutamato funciona como neurotransmisor y cumple con las 4 características de éstos: localización presináptica, liberación por un estímulo fisiológico, acción idéntica a la de un transmisor endógeno y un mecanismo de rápida terminación del efecto biológico. Las otras pozas metabólicas de glutamato están destinadas para diversas funciones, como las que participan en la síntesis de GABA y glutamina, el ciclo de los ácidos tricarboxílicos, y la incorporación a las proteínas (Broman et al. 2000). El glutamato

participa en diversos procesos del funcionamiento del SNC como la cognición, el aprendizaje y la memoria, la sinaptogénesis, la organización de los circuitos neuronales, la plasticidad sináptica y la migración y diferenciación celulares (Mattson 2008).

La formación y degradación del glutamato son parte del metabolismo energético, ya que éste se sintetiza principalmente a partir de la glucosa sérica. El metabolismo del glutamato en el SNC ocurre en ciclos coordinados entre las neuronas y las células gliales. El glutamato que participa en la transmisión puede ser sintetizado a través de distintas vías que no necesariamente son las mismas entre diferentes poblaciones de neuronas. Una de las vías es la conversión del α-cetoglutarato a glutamato por transaminación. Otra alternativa es a través de la conversión de glutamina a glutamato mediante la acción de la glutaminasa. La glutamina se sintetiza en la glia teniendo como precursor al glutamato recapturado después de su liberación sináptica, este glutamato es convertido a glutamina por amidación a través de la glutamina sintetasa; la glutamina es posteriormente liberada al espacio extracelular y recapturada por las neuronas quienes la convierten en glutamato en lo que constituye el ciclo glutamato-glutamina (Broman et al. 2000).

El glutamato transmisor es almacenado en vesículas sinápticas a través de transportadores vesiculares con la ayuda de un gradiente electroquímico que es generado por una bomba de protones dependiente de ATP y magnesio (Broman et al. 2000). Las vesículas sinápticas contienen concentraciones elevadas del neurotransmisor y en respuesta a un potencial de acción, se fusionan en la zona activa de la membrana presináptica y liberan su contenido por exocitosis al espacio sináptico. El glutamato activa receptores específicos ubicados en la pre- y la postsinapsis, desencadenando diferentes respuestas fisiológicas. Se conocen distintos tipos de receptores de glutamato y se han dividido en dos grandes grupos en base a su clonación molecular, propiedades bioquímicas y electrofisiológicas y su antagonismo farmacológico. El primer grupo está integrado por proteínas que forman canales iónicos operados por ligando permeables a cationes como Na^+, K^+ y Ca^{2+}, que se encuentran generalmente en las membranas postsinápticas y se clasifican dependiendo del agonista exógeno análogo al glutamato por el cual también pueden ser activados. Estos receptores se llaman ionotrópicos y son de los tipos N-metil-D-aspartato (NMDA), y no-NMDA. Dentro de éstos últimos se encuentran los tipo α-amino-3-hidroxi-5-metil-4-isoxazol propionato (AMPA) y los tipo kainato (KA). Los receptores de tipo NMDA son sensibles al voltaje transmembranal porque en condiciones de reposo el

canal se encuentra bloqueado por un ión Mg^{2+} que es removido por la despolarización de la membrana. Esta despolarización puede ser causada por la activación de receptores no-NMDA que median una transmisión sináptica más rápida (McDonald y Johnston 1990). El segundo grupo está constituido por los receptores metabotrópicos que se encuentran acoplados a proteínas G heterotriméricas y activan cascadas de señalización intracelular por medio de segundos mensajeros como inositol trifosfato, diacilglicerol y monofosfato de adenosina cíclico; estos receptores se ubican con mayor frecuencia en la presinapsis (Meldrum 2000).

En el asta ventral de la médula espinal hay una expresión relativamente alta de las subunidades que componen a los receptores tipo AMPA, en comparación con las subunidades de los receptores NMDA (Petralia et al. 2000). Los receptores AMPA están conformados por 4 subunidades denominadas GluR1-GluR4. La subunidad GluR2 tiene un papel fundamental con respecto a la permeabilidad del receptor al calcio ya que en su secuencia peptídica hay una glutamina en el segundo dominio intramembranal, que mediante la edición postranscripcional del mRNA es sustituida por una arginina confiriéndole al canal la impermeabilidad al calcio. Si la subunidad GluR2 no está presente o no está editada, el canal es permeable al catión (revisado en Corona et al. 2007). Como se discutirá más adelante en este trabajo, esta característica es de especial importancia dado el papel que juega el calcio en la generación de la muerte neuronal excitotóxica.

El mecanismo de terminación de la transmisión sináptica glutamatérgica es la recaptura del glutamato a través de transportadores de alta afinidad dependientes de sodio (Kanner 1989). Se han clonado cinco diferentes transportadores de glutamato: EAAC1, localizado en neuronas glutamatérgicas; EAAT2 ó GLT1 en roedores y EAAT3 ó GLAST, ambos localizados en la glia, específicamente en astrocitos; EAAT4 en las dendritas de las células de Purkinje; y EAAT5 en las células de Müller de la retina (revisado en Gadea y López-Colomé 2001). El EAAT2 es el transportador de glutamato más abundante en el SNC, y se localiza exclusivamente en los astrocitos, aunque se ha identificado una forma de splicing alternativo en las neuronas (Schmitt et al. 2002). Este transportador es responsable de la recaptura del ~90% del glutamato liberado y su importancia *in vivo* ha sido demostrada a través de la generación de ratones knockout de GLT1 que sufren crisis epileptiformes y lesiones corticales y no sobreviven más que algunas semanas (Tanaka et al. 1997).

Inervación glutamatérgica de la médula espinal

Las señales motoras se comunican a las motoneuronas espinales desde la corteza motora a través del tracto corticoespinal. Aunque todos los mamíferos poseen esta estructura existen variaciones considerables en la posición del tracto en la médula espinal (Watson y Harvey 2009). En los roedores el tracto corticoespinal de ubica en la columna dorsal (Brown 1971; Elger et al. 1977) y en los primates en la columna lateral (Petras 1969). En primates la mayor porción de las fibras del tracto corticoespinal provienen directamente de la corteza motora (Nudo y Masterton 1990a, b), mientras que en mamíferos más primitivos las fibras provienen de la corteza sensorimotora (Lende 1963), ya que en éstos la corteza motora como tal no es una región bien definida (Watson y Harvey 2009). Las fibras que originan el tracto corticoespinal en los mamíferos adultos provienen principalmente de las áreas corticales motora, premotora y somatosensorial y se derivan de neuronas piramidales corticales medianas a grandes de la capa 5 (Watson y Harvey 2009).

La inervación glutamatérgica del tracto corticoespinal a las motoneuronas espinales se ha demostrado a través de diversos medios. Por ejemplo, en el inicio de la locomoción se ha probado *in vitro* la participación de aminoácidos excitadores (Jordan et al. 2008), y se ha demostrado que la administración intratecal de antagonistas glutamatérgicos bloquean la locomoción inducida por la estimulación eléctrica de la región locomotrora mescencefálica del cerebro medio del gato (Douglas et al. 1993), de la misma manera la administración de NMDA produce el inicio de la locomoción en gatos (Chau et al. 2002). En la rata también se ha demostrado que la estimulación eléctrica del funículo dorsal, que contiene axones corticoespinales descendientes, desencadena potenciales postsinápticos excitadores, que son bloqueados con CNQX, un antagonista de receptores glutamatérgicos del tipo no-NMDA (Hori et al. 2002).

Mecanismos de neurodegeneración

Los mecanismos exactos que dan origen a las enfermedades neurodegenerativas no se conocen con precisión, sin embargo, algunos procesos celulares y moleculares que llevan a la muerte de las neuronas se tienen bien estudiados. En general, estos eventos ocurren muy lentamente, sin embargo, en la ALS este proceso es relativamente rápido en comparación con otras enfermedades como las de Parkinson o Alzheimer (Kanazawa 2001). A continuación, se mencionan algunos de los procesos comunes que participan en la muerte neuronal como el estrés oxidante, la pérdida de la homeostasis de calcio y la formación de complejos proteicos macromoleculares generados por la agregación de proteínas mal plegadas. En la siguiente sección se

discute con detalle un mecanismo importante de muerte neuronal producido por una transmisión sináptica excitadota excesiva conocido como excitotoxicidad.

Estrés oxidante

El estrés oxidante es generado por una producción incrementada de especies reactivas de oxígeno (ROS) o radicales libres, o una disminución en las defensas antioxidantes celulares (Davies 2000). En la ALS, este tipo de alteraciones tiene especial importancia debido a que la única causa probada de esta enfermedad son las mutaciones de la SOD1, que le confieren la ganancia de una función catalítica tóxica para las motoneuronas (Brown 1995; Wiedau-Pazos et al. 1996). El estrés oxidante genera alteraciones químicas en todas las macromoléculas celulares: ácidos nucléicos, lípidos y proteínas (Valko et al. 2007) y participa en la inducción de la muerte neuronal a través de diferentes procesos como apoptosis, autofagia y necrosis (Maycotte y Morán 2007).

Alteraciones en la homeostasis de calcio

La homeostasis de calcio es mantenida en las neuronas a través de la regulación de la entrada y salida del catión a la célula, de su amortiguamiento y de su almacenaje intracelular. La regulación de la concentración citosólica de calcio es estricta ya que este catión gobierna muchas funciones celulares a través de mecanismos en los que participa como segundo mensajero o modulador alostérico, y bajo condiciones fisiológicas puede dirigir múltiples procesos de manera independiente dentro de la misma célula. Sin embargo, un exceso en la concentración de calcio citosólico lleva a una activación inapropiada de los procesos dependientes de calcio generando alteraciones metabólicas y eventualmente la muerte celular (Arundine y Tymianski 2003). Entre las consecuencias de tal incremento están la activación de nucleasas, proteasas, lipasas y fosfatasas dependientes de calcio (Bano y Nicotera 2007), el hinchamiento de las mitocondrias con la consecuente pérdida de la polaridad de su membrana interna, la deficiencia de ATP, la generación de ROS y la activación del poro de transición mitocondrial (revisado en Hernández-Fonseca et al. 2007), y la fosforilación de cinasas activadas por estrés que participan en procesos de muerte celular como p38MAPK (Semenova et al. 2007).

Agregación de proteínas

La agregación de proteínas mal plegadas y la formación de complejos supramoleculares son fenómenos comunes en las enfermedades neurodegenerativas

que ocurren ya sea debido a mutaciones específicas en las proteínas agregadas, como en el caso del la SOD1 en la ALS (Durham et al. 1997; Johnston et al. 2000; Wang et al. 2002a), o a modificaciones postraduccionales como las que ocurren en el caso de la proteína β-amiloide que se agrega en la enfermedad de Alzheimer (Selkoe 2001). El mal plegamiento de las proteínas y su polimerización forman inclusiones insolubles intracelulares en la mayoría de los casos, aunque en algunas ocasiones estos agregados se acumulan fuera de las células. Aún cuando no se sabe con precisión si la acumulación de agregados protéicos induce la muerte neuronal, o por el contrario, participa en la protección de las neuronas, este fenómeno causa una serie de alteraciones celulares, principalmente el bloqueo del sistema de degradación proteasoma, que pueden tener consecuencias severas en otros procesos celulares como la regulación transcripcional (Berke y Paulson 2003). En la ALS se han encontrado además, la presencia de inclusiones de neurofilamentos en las motoneuronas en casos relacionados o no a mutaciones en la SOD1 (Hirano et al. 1984; Rouleau et al. 1996). La acumulación de estos neurofilamentos ocurre principalmente en los axones y su toxicidad se puede deber a la estrangulación del transporte axonal (Julien 2001).

Excitotoxicidad

Como se mencionó anteriormente uno de los mecanismos de muerte neuronal es la hiperactivación de los receptores de glutamato. A continuación, se presenta el capítulo "*Excitotoxicity as a mechanism of motoneuron death in amyotrophic lateral sclerosis*" publicado en el libro "*The neurochemistry of neuronal death*" Research Signpost, Kerala, India, donde se discuten los mecanismos intracelulares que conducen a la muerte neuronal y se detallan las evidencias que indican la participación de este fenómeno en la ALS, teniendo como causa posible el mal funcionamiento de los transportadores de glutamato.

Research Signpost
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

The Neurochemistry of Neuronal Death, 2007: 223-234 ISBN: 81-308-0086-1
Editors: Lourdes Massieu, Clorinda Arias and Julio Morán

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Excitotoxicity as a mechanism of motoneuron death in amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neuromuscular disorder characterized by the selective and progressive death of motoneurons in the spinal cord and the cerebral motor cortex. Whereas familial ALS with genetic alterations represents about 10% of all cases, the remainder 90% of cases are sporadic, and the etiology and mechanisms are unknown. Therefore, no effective treatments are available. Glutamate-mediated excitotoxicity may play a fundamental role in the pathogenesis of this disease, since several pieces of evidence indicate that disruption of glutamate metabolism and transport are pathophysiological features present in some ALS patients. A series of limited

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in vivo and in vitro models have been established to study the contribution of excitotoxicity and other mechanisms that have been considered to participate in motoneuron death in ALS. The most exploited is the use of transgenic rodents that bear a mutant form of the human superoxide dismutase 1 (SOD1), the only proven cause of ALS, although this model applies only to familial ALS. While some investigators sustain that glutamate transport failure is the key mechanism that produces excitotoxicity resulting in motoneuron death, there is evidence that an elevation in endogenous extracellular glutamate by transport blockade does not cause neuron damage. On the other hand, it is increasingly clear that spinal motoneurons are particularly susceptible to AMPA receptor-mediated excitotoxicity in a process that involves the entrance of Ca^{2+} and the consequent elevation of its intracellular concentration. In this chapter we will review the experimental evidence that proposes glutamate-mediated excitotoxicity as the foremost pathway to motoneuron degeneration in ALS, in the light of the contributions and limitations of the models employed. We will also discuss the participation of other factors that seem to be relevant for the understanding of the intricate mechanisms of motoneuron death.

Introduction

As for the majority of the neurodegenerative diseases affecting the central nervous system (CNS), the mechanisms leading to neuronal death in ALS remain poorly understood. ALS has been known for more than 130 years and still no effective treatment is available to either halt or significantly retard its progression. The prominent pathophysiological feature of ALS is the selective death of neurons that are in charge of movement (motoneurons) and are located in the ventral horns of the spinal cord (lower motoneurons), the brainstem and the cerebral motor cortex (upper motoneurons). Several forms of ALS have been described, depending on which type of motoneurons (upper or lower) are primarily affected. In fact, this characteristic determines the initial symptoms that patients will present. In general, the neuronal loss causes progressive muscle weakness, spasticity, fasciculation and consequently paralysis; other symptoms can include dysphagia and dysarthria. Due to the selectiveness of neuronal death in ALS, patients do not suffer intellectual disabilities, except in atypical cases where the disease is associated with frontotemporal dementia and parkinsonism [1,2]. Generally, patients die as a result of respiratory failure due to improper respiratory muscle contraction, 3-5 years after diagnosis.

Worldwide incidence of typical ALS is about 2-5 per 100,000 individuals and occurs during mid adulthood, although it can start at younger ages. Two main forms of ALS are recognized, the sporadic (SALS) and the familial (FALS). The former is the commonest, accounting for ~90% of cases, whereas

the latter comprises the remainder ~10% and is inherited in an autosomal dominant manner. In 1993 Rosen and colleagues [3] found that a fraction of patients with FALS, nearly 20%, had missense mutations in the enzyme superoxide dismutase 1 (SOD1). Mutant SOD1 is toxic not because of the loss of activity but by the gain of a new catalytic activity that produces reactive oxygen species (ROS) (for review see [4]) and as a consequence induces motoneuron damage. Soon after the discovery of SOD1 mutations, transgenic mice and rats expressing human SOD1 mutants were generated and shown to undergo spinal neurodegeneration and paralysis [5,6]. These animals are the most utilized model for studies of ALS in vivo, although they reproduce only the neurodegenerative mechanism of the smallest portion of ALS patients, those with the familial disease caused by SOD1 mutations.

Because of the variability of signs that patients present, diagnosis of ALS is not always an easy task and requires a series of clinical, electrophysiological and neuroimaging evaluations with results that meet the criteria internationally agreed in El Escorial, Spain, in 1990 [7]. The criteria to diagnose ALS include the presence and progression of lower and upper motor neuron degeneration signs and the absence of evidence of other diseases that may explain the observed symptoms [7]. Whenever upper or lower motor neuron degeneration occurs separately form each other the syndrome is considered as a motor neuron disease, not necessarily ALS [8].

Excitotoxicity

What kills motoneurons in ALS? Several hypothesis on the mechanisms responsible for neuronal damage in ALS have been proposed, all of them probably acting synergistically. Among the mostly considered are protein misfolding and aggregation, mitochondrial dysfunction, axonal transport impairment, oxidative stress, inflammation and apoptosis, as amply discussed elsewhere [4,8-11]. Nevertheless, glutamate-mediated excitotoxicity has long been postulated as the foremost mechanism leading to motoneuron degeneration in both SALS and FALS.

Excitotoxicity is a process leading to neuronal death as a consequence of the intense depolarization of neurons subjected to an over-stimulation of their postsynaptic membrane receptors to excitatory neurotransmitters, of which glutamate seems to be the most important. Excess activation of these receptors allows a massive influx of Ca^{2+} into the neuron, triggering the uncontrolled activation of calcium-dependent endonucleases, proteases, lipases, phosphatases and the generation of ROS, that eventually produce membrane destruction and cell death [12].

It is well known that glutamate-dependent excitotoxicity may be responsible for the neuronal death in acute CNS injuries including stroke, trauma and ischemia, in which a rapid elevation in the concentration of extracellular

glutamate, resulting from the initial cellular destruction due to oxygen deprivation, takes place. The increased extracellular glutamate diffuses in the tissue surrounding the initial site of the lesion (the penumbra), causing progressive excitotoxic death in the course of a few hours. In contrast, in the case of chronic neurodegenerative diseases, excitotoxicity must develop during long time periods, and in addition must occur in a selective way in order to generate the death of certain neuronal populations, motoneurons in the case of ALS. We will briefly review the physiology of glutamate synapses and then discuss the experimental data that have led to the suggestion that glutamate-mediated excitotoxicity might be an important mechanism for motoneuron death in both familial and sporadic ALS.

At glutamatergic synapses, glutamate is released from a presynaptic terminal upon stimulation and elicits the depolarization of the postsynaptic neuron through the binding to its high affinity postsynaptic membrane receptors. Two types of glutamate receptors have been identified, the ligand-gated ion channels ionotropic receptors and the G protein-coupled metabotropic receptors. The former type comprises the N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-isoxazolepropionate (AMPA) and kainate receptors, and the latter is constituted by three main subtypes with several subgroups, that direct second messenger pathways.

Glutamatergic synaptic transmission is terminated by the uptake of the neurotransmitter form the synaptic cleft into either glial or neural cells. This uptake is carried out by transporter proteins in a process driven by symport or antiport co-transport of ions [13]. Five high affinity glutamate transporters have been identified and cloned: excitatory amino acid transporter 1 (EAAT1), also known in non-human mammalian tissues as glutamate/aspartate transporter (GLAST), EAAT2 or glutamate transporter 1 (GLT1) in rodents, EAAT3 or excitatory amino acid carrier 1 (EAAC1), EAAT4 and EAAT5. Each transporter has different pharmacology and distribution throughout the CNS (for review see [14]). EAAT2 is the most abundant glutamate transporter in the CNS, and is located exclusively in astrocytes, although an alternative spliced variant has been found in neurons [15]. This protein carries probably ~90% of the released glutamate and its importance in vivo has been shown by the finding that GLT1 knockout mice suffer seizures, show cortical lesions and die at few weeks of age [16]. The remainder of the EAATs are important during development or in CNS regions that are spared in ALS.

As mentioned earlier, intracellular Ca^{2+} is a prime mediator of several cellular processes leading to neuronal death. In this respect, motoneurons are particularly vulnerable to intracellular calcium overload because they have a low buffering capacity. Intracellular free Ca^{2+} is normally regulated by several means including chelation by calcium trapping proteins, mitochondrial and endoplasmic reticulum storage, and extrusion by Ca^{2+}-ATPases. Motoneurons

in rats [17] and healthy or diseased humans [18,19] are devoid of the two principal Ca^{2+} binding proteins calbindin D-28k and parvalbumin. Consistently, in the FALS mouse with mutant SOD1 there is a lesser expression of these proteins in comparison to non-transgenic controls, even at early presymptomatic stages [20]. Thus, it has been postulated that the selective death of motoneurons could be in part related to a poor capacity for calcium handling. Interestingly, subsets of motoneurons that are not affected in ALS, as those of the Onuf's and oculomotor nuclei, do express the calcium-binding proteins [17-20]. This argues in favor of the possibility that calcium buffering is a key player in the selectivity of neuronal death in ALS. In accordance, double transgenic mice expressing the mutant SOD1 and overexpressing parvalbumin have improved intracellular calcium buffering capacity and this retards the progression of the disease, although it does not prevent the neuronal death [21].

Calcium entry into spinal motoneurons is likely to be mediated by AMPA receptors. This kind of glutamatergic receptors are distributed throughout the CNS and are normally Ca^{2+}-impermeable. They are constituted by different heterotetrameric combinations of four subunits denominated GluR1-GluR4. GluR2 plays a fundamental role with regard to calcium permeability of this receptor-channel. When at least one GluR2 subunit composes the AMPA receptor, a positive-charged arginine in its structure impedes Ca^{2+} flux through the pore, but if this subunit is absent the channel is permeable to the cation. GluR2 sequence has an uncharged glutamine residue at the second intramembranal domain (site Q/R), and postranscriptional modifications edit this site by substituting the glutamine for arginine, thus providing the Ca^{2+} impermeability to the edited GluR2 contained in AMPA receptors.

In spinal motoneurons, however, there is a low expression of GluR2 in AMPA receptors. In normal human motoneurons GluR2 mRNA was practically undetected, whereas the rest of the subunits were clearly present [22], suggesting that the majority of AMPA receptors in this cell type are calcium-permeable. Furthermore, it has been described that in some ALS patients motoneurons fail to edit the Q/R site of GluR2, so that the genetically-encoded gultamine is not exchanged with arginine, whereas other neuronal types like Purkinje cells, as well as motoneurons from healthy individuals, can normally edit the GluR2 Q/R site [23]. In cultured motoneurons, calcium entry through Ca^{2+}-permeable AMPA receptors in response to their activation by kainate has been demonstrated, and this was correlated with neuronal death [24]. In addition, it was shown that this calcium influx was indeed mediated by Ca^{2+}-permeable AMPA receptors and not by voltage-gated Ca^{2+} channels [25]. Nevertheless, it is important to note that in these in vitro experiments the concentration of Ca^{2+} in the culture medium had to be raised from the physiological 2 mM to 10 mM in order to observe the kainate-induced neuronal death. This is extremely relevant when extrapolating these results to the situation in vivo.

In vivo, AMPA receptor overstimulation leads to unselective neuron death in the spinal cord when the AMPA receptor agonists kainate and quisqualate are administered intrathecally [26,27], or microinjected in the cervical spinal cord [28]. Furthermore, when AMPA, but not NMDA, is perfused by means of microdialysis in the rat lumbar spinal cord, motoneurons are remarkably damaged, and in consequence the hindlimbs get paralyzed [29]. It is worth noticing that these effects were prevented by the AMPA receptor antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX). With these experimental paradigm we demonstrated that the gradual administration of AMPA by means of reverse microdialysis directly in the rat spinal cord caused the degeneration of motoneurons within few hours. In other recent experiments we extended to several days the period over which this neurodegeneration occurs, by infusing AMPA in the spinal cord with a slow delivery system using osmotic minipumps (A. Zepeda, L.B. Tovar-y-Romo and R. Tapia, submitted), thus generating a chronic model of spinal motoneuron death that resembles the characteristics of neurodegeneration and paralysis that are present in both ALS patients and FALS rodents.

In the same line, in cultured motoneurons modified to express mutant SOD1 the AMPA/KA receptor antagonists 6-cyano-7-nitroquinoxaline-dione disodium (CNQX) and the Joro spider toxin, a GluR2-lacking AMPA receptor antagonist, prevented mutant SOD1-induced neuronal death [30]. On the other hand, in vivo administration of the AMPA receptor antagonist NBQX to FALS mice only modestly retarded the progression of the paralysis and prolonged survival [31]. As well, we have recently found in the rat in vivo that both the blockade of Ca^{2+}-permeable AMPA receptors with a synthetic analog of the Joro spider toxin, and the intracellular chelator BAPTA-AM [1,2-bis(2-aminophenoxy)ethane-tetra-acetic acid tetrakis-acetoxymethyl ester] protect against motoneuron loss and paralysis induced by the microdialysis perfusion of AMPA in the spinal cord [32]. These results clearly show that increased Ca^{2+} cytoplasmic concentration due to the entrance of the cation through AMPA receptors is an important factor in spinal motoneuron death in vivo. The relevance of these findings is noteworthy because, differently from the experiments in vitro mentioned above, the neuronal death due to increased cytoplasmic Ca^{2+} occurs under the physiological extracellular concentration of the cation. Altogether, these data suggest that AMPA receptors may have an important role in the development of ALS, albeit, as discussed below, convincing evidence that this occurs in the human disease has not yet been obtained.

The excitotoxic hypotheses in ALS is supported by the abnormalities in glutamate metabolism that have been observed in various patients. Measurement of glutamate content in SALS patients indicate that the amino acid is increased in plasma [33] and in CSF [34], although other studies have

not confirmed these findings, neither in plasma [35,36] nor in CSF [35]. Later studies in larger cohorts demonstrated that only a subset of about 40% SALS patients have about 2-fold augmented glutamate in CSF [36,37]. Interestingly, one of these studies [37] correlated the high glutamate levels to lower neuron symptoms and poor limb function. Nevertheless, given the fact that not all patients have this alteration, elevated glutamate does not seem to be the triggering factor for motoneuron death in SALS.

CSF glutamate levels have been also measured in the mutant SOD1 mouse model of FALS, and they were found unaltered [38]. In these mice it has been reported that glutamate is elevated in the cerebral cortical extracellular fluid, as detected by microdialysis [39], a finding that we failed to confirm in a recent work [40]. SOD1 mutations, therefore, are not necessarily related to high glutamate concentration in extracellular fluids.

Glutamate transport and motoneuron death

Glutamate can be elevated in the extracellular tissue fluid as a result of an increased release from synaptic terminals or due to a deficiency in its transport. Even when these two mechanisms ultimately augment extracellular glutamate, the final result can be strikingly different depending on the source of glutamate. When the neurotransmitter is released in excess directly from synaptic endings, neuronal hyperexcitation and excitotoxicity, leading to neuronal death, occur almost invariably, as we have shown in cerebral brain regions with the use of the K^+ channel blocker 4-aminopyridine. This excitotoxicity is due to the stimulation of glutamate release, with the consequent overactivation of glutamate receptors, because the excitotoxic effects of 4-aminopyridine are prevented by different glutamate receptor antagonists, which are effective depending on the cerebral region studied [41-44]. In contrast to the excitotoxic effects of glutamate release stimulation, we have demonstrated that the blockade of glutamate transport in several brain regions like the hippocampus and the striatum is innocuous for neurons, in spite of great elevations of extracellular glutamate [45,46]. This indicates that glutamate transport deficits do in fact generate increased levels of extracellular glutamate but that such increase does not result in overactivation of its postsynaptic receptors. More closely related to ALS, the acute inhibition of glutamate transport in the rat spinal cord in vivo, using the non-specific transport blocker L-2,4-trans-pyrrolidine-dicarboxylate (PDC), is innocuous for the motoneurons, under experimental conditions identical to those producing neuronal death with AMPA [29]. Moreover, if this inhibition is prolonged over several days extracellular glutamate is increased ~3-fold but neuronal death does not occur and the animals motor behavior is not affected (A. Zepeda., L.B. Tovar-y-Romo and R. Tapia, submitted).

We have also studied the neuronal vulnerability of cerebral cortex and hippocampal neurons to glutamate transport failure in the FALS transgenic mice bearing a mutant human SOD1. Our results show that when the extracellular glutamate is augmented ~10-fold by PDC perfused by microdialysis in this brain areas, there is no sign of excitotoxicity, either in the mice carrying the mutant SOD nor in the control animals [40]. It seems, therefore, that extracellular glutamate elevations due to transport deficiency does not overactivate glutamate receptors and does not produce excitotoxicity, and that this innocuousness is preserved in the mouse FALS model with mutant SOD1.

It is noteworthy that the lack of effect described above occur only in vivo, because when glutamate transport is blocked in cultured cerebral or spinal neurons death is quite evident [24,47,48]. This discrepancy between in vivo and in vitro might reflect the importance of the topographical location of glutamate as related to the synaptic cleft: whereas, as discussed above, in vivo the dysfunction of glutamate transport leads to increased glutamate far from the postsynaptic receptors, in vitro such limitation disappears because the normal cytoarchitecture and the glia-synapse structural relationships have been lost [44].

In ALS, decreased glutamate transport was found in synaptosomes prepared from the spinal cord of patients, where the efficiency of glutamate uptake was reduced without alterations in the affinity [49]. Later, decreased EAAT2 expression was found in the motor cortex and spinal cord of ALS patients, assessed by immunohistochemistry [50] and by autoradiography using tritium-labeled D-aspartate [51]. In the FALS mouse model there are controversial data. Some reports describe a certain loss of GLT1 [38,52], while other groups have failed to find such reduction in transporter expression [39,53,54]. In this regard, it is worth noting that the sole overexpression of the human SOD1 in mice, even the wild type enzyme, down-regulates GLT1 without altering the mRNA levels [55].

In vitro, interestingly the expression of glial glutamate transporters is increased when astrocytes are cocultured with neurons, suggesting the involvement of soluble factors released by neurons in the expression of EAAT2 [56]. Consistently, immunoreactivity of EAAT2 in the spinal cord of ALS patients correlated with the degree of neuronal loss in the spinal cord: the patients with mild neuronal depletion showed EAAT2 immunoreactivity similar to that of controls, whereas in patients with severe neuronal loss, EAAT2 expression was clearly reduced [57]. Therefore, the loss of glutamate transporters in tissue from ALS patients might be a consequence, not a cause, of neuronal loss.

Even if the amount of glutamate transporters is not affected, their function may be compromised by the alteration of cellular environment possibly

occurring in ALS, like oxidative damage. Glutamate transporters are regulated by the redox state of conserved cysteines in their structure. The conformational structure of the protein is altered when disulphide bonds are formed by oxidation, and the Vmax of the transport is reduced [58,59]. Indeed, glutamate transporters are vulnerable to oxidative stress and can be inhibited within seconds in the presence of peroxinitrite [60]. Moreover, when cortical astrocyte cultures are exposed to free radical generators like xanthine/xanthine oxidase or H_2O_2, the uptake capacity of the transporters decreases rapidly and the function of the oxidized transporters can be restored by the addition of antioxidants like SOD1, catalase, glutathione or dithiothreitol [61]. Remarkably, when both mutant human SOD1 and EAAT2 are coexpressed in Xenopus oocytes, the production of ROS by mutant SOD1 catalysis triggered by H_2O_2 inhibits glutamate transport by oxidation [62]. Apparently EAAT2 is prone to oxidative damage in its intracellular face, where there may be also oxidation of its aromatic residues. In this respect, motoneurons seem to have a rate of ROS generation greater than other spinal cells, as has been observed in in vitro studies in the presence of glutamate and kainate, where ROS can be released and oxidize neighboring glial transporters [63]. Similarly, there are oxidative alterations in the areas surrounding motoneurons in the spinal cord of FALS mice [63]. Altogether, these data suggest that in FALS there may be a decreased efficiency of glutamate transport, which in turn may contribute to motoneuron damage. However, as discussed above, cerebral neurons of FALS mice are not vulnerable to increased extracellular glutamate due to the blockade of its transport.

Blockade of excitotoxicity as a possible treatment of ALS

The evidence discussed in the present article suggests that the use of anti-glutamatergic agents to treat ALS is worth trying. In fact, there have been a series of clinical trials testing the effects of anti-excitotoxic compounds. Nowadays the only approved treatment for ALS is riluzole, a drug that inhibits glutamate release form nerve endings possibly by stabilizing the inactive state of voltage-dependent sodium channels and by a G protein-coupled intracellular pathway that can be inhibited with pertussis toxin (for review see [64]). In some clinical trials riluzole has been shown to moderately improve survival of patients [65,66], although most recent reports address mainly the safety and the good tolerance of riluzole by ALS patients, but no further beneficial effects of the treatment [67,68]. In this regard, it is of interest that, although riluzole did reduce the release of glutamate induced by 4-aminopyridine in rat hippocampus, it also reduced the release of other transmitters like GABA. In these experiments riluzole not only failed to block, but even enhanced, the intense convulsant action 4-aminopyridine, thus indicating that the clinical use of riluzole should be considered with caution [43].

Experimental data in the FALS mouse have shown that riluzole, at the therapeutic dose used to treat ALS, diminish the increased neuronal excitability of motoneurons bearing the mutant SOD1 to the baseline of the control motoneurons [69]. Riluzole administration to FALS mice prolongs their survival but does not delay the onset of the disease [70], which might be related to distinct mechanisms participating in the onset and development of ALS and indicate that excitotoxicity in not necessarily involved in the first stages. It is notable that nearly all anti-excitotoxic drugs that have been tested in clinical trials, like gabapentin, lamotrigine, dextromethorphan, memantine and NBQX, (reviewed in Refs. 71 and 72), have resulted ineffective as well. This might reflect the lack of a major participation of excitotoxicity in ALS.

Other anti-excitotoxic drugs currently being tested are the antiepileptic compounds topiramate [71] and talampanel [72]. Topiramate protects motoneurons in organotypic spinal cord slices against the toxicity of glutamate transport blockade, but it has no effect on the FALS mouse model [47], probably because in vivo glutamate transport blockade is innocuous, as we discussed earlier [40].

In conclusion, there is little doubt that overactivation of AMPA receptors and increased intracellular Ca^{2+} result in motoneuron death. Nevertheless, in spite of this certainty and of the great collection of experimental data that has emerged over the past years, there is no clear evidence that glutamate-mediated excitotoxicity is the triggering factor of ALS, nor whether its participation is a cause or a consequence in the pathology of this neurodegenerative process. As previously mentioned, there are several other factors beyond glutamate metabolism in CNS that appear to participate in the etiology of ALS. Interestingly, it was recently described that the deletion of the hypoxia response element present in the promoter of the vascular endothelial growth factor (VEGF) in mice causes a motor syndrome reminiscent of ALS that involves the loss of motoneurons as well [73]. In addition, the intramuscular injection of viral vectors encoding VEGF produces one of the best reported improvements in delaying the progress of the paralysis and in the survival of FALS mice [74]. These interesting data open a new route in the search for the mechanisms underlying motoneuron death in ALS and in the design of preventive or therapeutic procedures.

Acknowledgments

This work was supported by CONACyT (project 42668) and DGAPA, UNAM (project IN213703).

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Mecanismos de sobrevivencia. Activación de vías de señalización intracelular

Las motoneuronas son células postmitóticas que se encuentran en un arresto del ciclo celular en la fase G_0 y establecen conexiones sinápticas con sus blancos musculares y neuronas eferentes que se mantienen relativamente estables a lo largo de toda la vida de los individuos, por lo que a diferencia de otros tipos celulares, su recambio es prácticamente inexistente. Por lo mismo, su sobrevivencia depende principalmente de un buen estado energético, un balance redox adecuado, la capacidad de superar los daños inducidos por estímulos nocivos (por ejemplo, la eficiencia de sus sistemas de recambio de proteínas y de reparación del DNA) y de la capacidad de responder a factores tróficos. Estos últimos funcionan en la mayoría de las células a través de la activación de cascadas de señalización intracelular que estimulan la sobrevivencia, y en otros casos la proliferación celular, mediante la transcripción de diversos genes, muchos de ellos aún desconocidos, y mediante la supresión de eventos que conducen a la muerte neuronal como la apoptosis.

Existen diversas vías de sobrevivencia celular activadas en respuesta a factores tróficos, dos de las más importantes son la vía de fosfatidilinositol 3 cinasa (PI3-K)/Akt y la vía de cinasas reguladas por señal extracelular (MEK)/ERK (Kennedy et al. 1997).

PI3-K/Akt

Las PI3-K son una familia de enzimas que fosforilan el grupo hidroxilo en la posición D3 de los fosfoinositidos para generar fosfatidilinositol-3-fosfato, fosfatidilinositol-3,4-difosfato, y fosfatidilinositol-3,4,5-trifosfato. En los mamíferos existen 3 clases diferentes de PI3-K, de las cuales, sólo la clase I produce fosfatidilinositol-3,4,5-trifosfato que es responsable de la activación del efector Akt, también conocido como proteína cinasa B (PKB), que a su vez es el principal efector biológico de los factores de sobrevivencia (Franke et al. 1997). Las PI3-K de clase I están constituidas por subunidades catalíticas (p110) y subunidades reguladoras (p85 y p55). La subunidad reguladora p85 estabiliza a la subunidad catalítica p110 inhibiendo su actividad de cinasa, hasta que, como consecuencia de un estímulo, las PI3-K son reclutadas por un receptor con actividad de cinasa de tirosina (RTK) a través del dominio SH2 de p85, liberando a p110 de la inhibición (Geering et al. 2007). Los efectos de PI3-K son terminados por medio de la actividad del receptor al que se unen y a través de la fosfatasa PTEN (homólogo de tensina suprimido del cromosoma 10) (Gericke et al. 2006). Akt activada fosforila una serie de proteínas blanco, entre ellas Bad y GSK3β (cinasa de glucógeno sintasa), que están implicadas en la modulación de la apoptosis

(Datta et al. 1997; Crowder y Freeman 2000; Hetman et al. 2000). Adicionalmente, Akt también fosforila e inhibe factores de transcripción de la familia "forkhead" que promueven la expresión de proteínas proapoptóticas como FasL (Brunet et al. 1999).

MEK/ERK

La vía MEK/ERK está constituida por miembros de la familia de proteínas cinasas activadas por mitógeno (MAPK) y participa primordialmente en la regulación del crecimiento celular, desarrollo y diferenciación (Chambard et al. 2007). Existen dos MEK; MEK1 y MEK2, codificadas por genes diferentes con una homología de 80% (Zheng y Guan 1993), que son activadas río abajo de la fosforilación de Ras y Raf, y a su vez fosforilan a las cinasas reguladas por señal extracelular ERK1 y ERK2 (Chambard et al. 2007). ERK1/2 son las proteínas efectoras de la vía, tienen pesos moleculares de 44 y 42 kDa respectivamente por lo que también se les conoce como p44 y p42, y tienen una homología de 85% (Boulton et al. 1990; Boulton et al. 1991). Los blancos de ERK1/2 son muy diversos, incluyendo proteínas de citoesqueleto, factores de transcripción, proteínas antiapoptóticas y promotores de sobrevivencia. Entre ellos están factores de transcripción que forman el complejo AP-1 como c-*fos* y c-*jun* (Chen et al. 1992), y proteínas que tienen como blanco molecular a CREB (proteína de unión al elemento de respuesta a AMPc) que participa en diversos procesos de plasticidad sináptica, diferenciación celular y sobrevivencia (Xing et al. 1996; Frodin y Gammeltoft 1999; Riccio et al. 1999).

La unión de factores tróficos a RTK's activa en muchos casos tanto a la vía PI3-K/Akt como a MEK/ERK (Kaplan y Miller 2000; Xue et al. 2000; Li et al. 2003b). En la mayoría de los casos, las funciones relacionadas con la sobrevivencia celular son mediadas a través de la vía PI3-K/Akt de manera independiente de la activación de MEK/ERK (Creedon et al. 1996; Virdee y Tolkovsky 1996; Klesse et al. 1999), aunque sí se ha descrito la participación de MEK/ERK en la promoción de la sobrevivencia neuronal, por ejemplo en modelos *in vitro* con células granulares de cerebelo (revisado en Xifró et al. 2007).

El factor de crecimiento vascular endotelial

El factor de crecimiento vascular endotelial (VEGF) es una proteína que participa en la promoción y regulación de la angiogénesis y la vasculogénesis. Este factor, también conocido como VEGF-A, pertenece a una familia de factores de crecimiento de la que también forman parte el factor de crecimiento placentario (PlGF), VEGF-B, VEGF-C y VEGF-D (Ferrara et al. 2003). Las funciones del VEGF sobre la regulación de la

proliferación y sobrevivencia de las células endoteliales y de las células derivadas de la médula ósea, y sobre la regulación de la permeabilidad vascular (por la cuál también se le conoce como factor de permeabilidad vascular ó VPF) se conocen con gran detalle (revisado en Ferrara et al. 2003). Recientemente, se ha identificado al VEGF como un importante promotor de la sobrevivencia neuronal en diversos modelos de enfermedades neurodegenerativas, teniendo un papel de especial importancia en la sobrevivencia de las motoneuronas como se discutirá más adelante en esta tesis.

En el humano el VEGF está codificado en la región p21.3 del cromosoma 6 (Mattei et al. 1996; Vincenti et al. 1996) y está organizado en 8 exones (Houck et al. 1991; Tischer et al. 1991). Se han identificado diferentes formas de VEGF-A generadas por splicing alternativo, con extensiones de 121, 145, 165, 183, 189 y 206 aminoácidos (Neufeld et al. 1999). La forma más frecuente y la que posee la mayor actividad biológica es $VEGF_{165}$, la cual carece del exón 6 (Ferrara et al. 2003). En su forma nativa, el VEGF es una glicoproteína homodimérica de 45 kDa con sitios de unión a heparina y heparán sulfato, es termoestable y resistente al medio ácido (Ferrara y Henzel 1989), y tiene afinidad por la matriz extracelular; en consecuencia, es fácilmente secuestrado por ésta, especialmente en sus formas más largas ($VEGF_{189}$ y $VEGF_{206}$). La forma $VEGF_{121}$ es completamente soluble y no se une a la matriz extracelular, mientras que la forma $VEGF_{165}$ tiene una afinidad media y puede encontrarse de manera soluble o unida a la matriz (Houck et al. 1992; Park et al. 1993).

La región promotora del gen *Vegf* posee un elemento de respuesta a hipoxia que es activado por el factor inducido por hipoxia HIF-1. Éste responde a cambios en la tensión de oxígeno mediante un mecanismo por el cual el oxígeno promueve la hidroxilación de un residuo de prolina (Safran y Kaelin 2003). Adicionalmente, la expresión del VEGF se incrementa en repuesta a señales de otros factores de crecimiento, como el factor de crecimiento epidérmico, los factores de crecimiento transformantes α y β, el factor de crecimiento de queratinocitos, el factor de crecimiento similar a insulina-1, el factor de crecimiento de fibroblastos y el factor de crecimiento derivado de plaquetas (Bequet Romero y López Ocejo 1999; Ferrara et al. 2003) y en respuesta a una serie de citocinas, oncogenes, mutantes de genes supresores y proteínas de cascadas de señalización y segundos mensajeros (Bequet Romero y López Ocejo 1999; Ferrara et al. 2003).

EL VEGF ejerce su actividad biológica a través de su unión a dos receptores de alta afinidad de tipo RTK clase III que son: VEGFR1 ó Flt-1 (fms-like tyrosine kinase 1) y VEGFR2 ó KDR/Flk-1 (kinase domain receptor/fetal liver kinase 1; homólogo murino). Estos receptotes tienen un paso transmembranal, 7 dominios parecidos a inmunoglobulina en la cara extracelular y en la parte intracelular un dominio cinasa de tirosina truncado por una región denominada inserto de cinasa, rica en residuos de tirosina fosforilables (Terman et al. 1992). Existen además otros receptores similares que pertenecen a la misma familia, sin embargo no tienen como ligando al VEGF-A (Karkkainen et al. 2002). Otros ligandos de la familia de VEGF, como PlGF y VEGF-B también se unen al VEGFR1 pero no al VEGFR2 (Park et al. 1994; Olofsson et al. 1998). En las células endoteliales el VEGFR2 desencadena la respuesta mitogénica, mientras que el VEGFR1 regula las interacciones de éstas células entre sí y con la matriz extracelular (Bequet Romero y López Ocejo 1999). EL VEGFR1 también es considerado como un modulador negativo de la actividad del VEGFR2, ya que una forma soluble generada por splicing alternativo que carece de la región carboxilo terminal tiene la capacidad de secuestrar al ligando (Park et al. 1994; Terman y Dougher-Vermazen 1996; Thomas 1996). Además de los RTK's, el VEGF interactúa con una familia de correceptores conocidos como neuropilinas, cuya función incrementa la afinidad del ligando al VEGFR2, especialmente de la forma $VEGF_{165}$ (Soker et al. 1998).

En respuesta a la unión del ligando, el VEGFR2 se dimeriza y transfosforila desencadenando la activación de diversos mediadores de vías de señalización intracelular, como PI3-K/Akt, fosfolipasa Cγ (PLCγ), y MEK/ERK (Bequet Romero y López Ocejo 1999; Ferrara et al. 2003). Recientemente se ha descrito la expresión del VEGFR2 en motoneuronas de la médula espinal de roedores en etapas embrionarias (Oosthuyse et al. 2001; Tolosa et al. 2008), y en motoneuronas espinales de humanos (Brockington et al. 2006), y como se presenta en la parte de Resultados, nosotros hemos encontrado la expresión del VEGFR2 en las motoneuronas espinales de la rata adulta.

Antecedentes. Probable origen de estímulos excitotóxicos en la ALS (modelo transgénico)

Cerebral neurons of transgenic ALS mice are vulnerable to glutamate release stimulation but not to increased extracellular glutamate due to transport blockade

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Experimental Neurology 2006, **199**:281-290

Resumen

Los mecanismos de la pérdida de las motoneuronas en la ALS se desconocen, pero se ha postulado que la excitotoxicidad debida a una neurotransmisión glutamatérgica excesiva causada por la deficiencia del transporte de glutamato puede estar involucrada tanto en la FALS como en la SALS. Usando microdiálisis *in vivo*, probamos los efectos del inhibidor del transporte de glutamato L-*trans*-pirrolidin-2,4-dicarboxilato (PDC) y de la 4-aminopiridina (4-AP), que estimula la liberación del glutamato desde las terminales nerviosas, en el hipocampo y en la corteza motora de ratones silvestres (WT) y ratones transgénicos con la mutante G93A de la SOD1 humana, un modelo establecido de FALS. La perfusión de 4-AP indujo convulsiones, la expresión de la proteína de choque térmico HSP70 que es un marcador inducible de estrés, y la pérdida neuronal hipocampal. Estos efectos fueron similares tanto en los ratones WT como en los $SOD1^{G93A}$, y en ambos grupos fueron prevenidos por la administración sistémica previa del antagonista de receptores NMDA MK-801. En contraste, la perfusión de PDC resultó en un incremento grande y duradero (2 h) del glutamato extracelular, pero no en convulsiones, daño neuronal o expresión de HSP70, en los ratones WT ni en los $SOD1^{G93A}$. Nuestros resultados demuestran que la $SOD1^{G93A}$ no potencia la vulnerabilidad a la excitotoxicidad mediada por glutamato endógeno en el cerebro debida al bloqueo su transporte ni por la estimulación de su liberación. Por lo tanto, estos datos no apoyan la posibilidad de que la deficiencia del transporte de glutamato pueda ser un factor importante para la degeneración neuronal del cerebro en la FALS.

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Experimental Neurology 199 (2006) 281–290
Experimental Neurology
www.elsevier.com/locate/yexnr

Cerebral neurons of transgenic ALS mice are vulnerable to glutamate release stimulation but not to increased extracellular glutamate due to transport blockade

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Received 15 August 2005; revised 21 October 2005; accepted 1 November 2005
Available online 20 December 2005

Abstract

Mechanisms of motor neuron loss in amyotrophic lateral sclerosis (ALS) are unknown, but it has been postulated that excitotoxicity due to excessive glutamatergic neurotransmission by decreased efficiency of glutamate transport may be involved in both familial (FALS) and sporadic ALS. Using microdialysis in vivo, we tested the effects of the glutamate transport inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) and of 4-aminopyridine (4-AP), which stimulates glutamate release from nerve endings, in the hippocampus and motor cortex of wild type (WT) and transgenic SOD1/G93A mice, an established model of FALS. Perfusion of 4-AP induced convulsions, expression of the inducible stress-marker heat-shock protein 70 (HSP70) and hippocampal neuronal loss. These effects were similar in both WT and G93A mice, and, in both groups, they were prevented by the previous systemic administration of the NMDA receptor antagonist MK-801. In contrast, perfusion of PDC resulted in a large and long-lasting (2 h) increase of extracellular glutamate, but no convulsions, neuronal damage or HSP70 expression were observed in either the WT or the G93A mice. Our results demonstrate that SOD1 G93A mutation does not enhance the vulnerability to endogenous glutamate-mediated excitotoxicity in brain, neither by blocking glutamate transport nor by stimulating its release. Therefore, these data do not support the possibility that glutamate transport deficiency may be an important factor of brain neuronal degeneration in familial ALS.

Keywords: Amyotrophic lateral sclerosis; Excitotoxicity; G93A mice; Glutamate; Hippocampus; HSP70; Microdialysis; Motor cortex

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating disease of the central nervous system characterized by the selective motor neuron death in spinal cord, brain stem and motor cortex that leads to a relentless paralysis and the death of the individual 2–5 years after onset. About 90% of ALS cases are sporadic, and, in the remaining 10%, this syndrome is inherited in a dominant autosomal fashion. This last form is known as familial ALS, and it has been found that 20% of these familial cases are associated with missense mutations in the gene that encodes the antioxidant enzyme superoxide dismutase 1 (SOD1) (Rosen et al., 1993). Rather than inactivating the enzyme, this sort of mutations convert it into a potent oxidative source because of the gain of an aberrant catalysis that produces reactive oxygen species (ROS) (Brown, 1995; Cleveland, 1999; Liochev and Fridovich, 2003). Transgenic mice that express mutant forms of the human SOD1, such as G93A, become paralyzed because of motor neuron loss (Gurney et al., 1994). Therefore, these animals are considered a trustworthy model for studies of ALS in vivo.

Even though the molecular and cellular mechanisms that underlie motor neuron loss in ALS are not quite clear, glutamate-mediated excitotoxicity has been postulated as a cause of ALS. This hypothesis is sustained on the finding that 40% of ALS patients have an increased glutamate level in the CSF (Shaw et al., 1995; Spreux-Varoquaux et al., 2002). In addition, in the motor cortex and the spinal cord of ALS patients analyzed post-mortem, a reduction in the content of the glutamate transporter protein EAAT2 (GLT1) has been found.

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doi:10.1016/j.expneurol.2005.11.005

Other transporters like GLAST and EAAC1 were not affected (Rothstein et al., 1992, 1995). It was recently reported that there might be some compensatory changes of the glutamate transporters in ALS by upregulation of a splice variant of EAAT2, although this does not fully avoid the reduction in transport activity in the motor cortex of ALS patients (Maragakis et al., 2004).

In the G93A mouse model, there are controversial data. It has been observed that the basal content of glutamate and aspartate in the extracellular fluid of the prefrontal cortex is significantly elevated with respect to non-transgenic mice (Alexander et al., 2000) and that the concentration of glutamate and glutamine in the tissue is elevated (Andreassen et al., 2001). However, these increments were not observed in the CSF of the transgenic mouse (Bendotti et al., 2001). Although in this work a progressive diminishment of GLT1 in the ventral horn of the spinal cord was observed, this change started after the symptomatic stage, which argues against this as a causal factor of the disease. Other groups have shown that there are no changes in GLT1, GLAST or EAAC1 at any of the G93A mouse life stages, neither in the spinal cord (Sasaki et al., 2001; Deitch et al., 2002) nor in the motor cortex (Alexander et al., 2000; Deitch et al., 2002). Besides the excitotoxic injury, it has been shown that glutamate and kainate induce the generation of ROS in motor neurons in vitro, that these species can be released and oxidize neighboring glial glutamate transporters and that a higher index of oxidative alterations occurs in the vicinity of spinal cord motor neurons of G93A mice (Rao et al., 2003).

These observations led to the suggestion that, in the G93A mouse, there is a glutamate transport failure because of oxidative damage and that, as a consequence, the extracellular glutamate accumulates and motor neurons die by excitotoxicity. If this is true, at the final stage of the pathological process, the transgenic mice should have a greater susceptibility to glutamate-induced neurodegeneration in the brain, in comparison to wild type (WT) mice. The main objective of the present work was to test this possibility, and, for this purpose, we used two strategies designed to increase the concentration of extracellular endogenous glutamate: first, we perfused by means of microdialysis in the hippocampus and motor cortex of G93A and WT mice the non-selective inhibitor of glutamate transporters L-*trans*-2,4-pyrrolidin-dicarboxylic acid (PDC), and, second, we stimulated the release of glutamate from synaptic terminals using the potassium channel blocker 4-aminopyridine (4-AP). This drug has been shown to induce excitotoxicity and neurodegeneration through this mechanism in rat striatum (Morales-Villagran and Tapia, 1996) and hippocampus (Peña and Tapia, 1999, 2000). Hippocampus was chosen because of its high vulnerability to excitotoxic damage (Tapia et al., 1999; Peña and Tapia, 2000; Ayala and Tapia, 2003) and motor cortex because it is involved in the pathology of ALS. We evaluated the effects of these drugs on the motor behavior of the animals and on the neuronal morphology and expression of the inducible cellular stress-marker heat-shock protein 70 (HSP70) in their brain.

Materials and methods

Transgenic mice B6SJL-TgN(SOD1-G93A)1Gur that express G93A human SOD1, and WT mice of the same strain (B6SJL), which were used as controls, were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA). Breeding was carried out by pairing transgenic G93A mice with the WT mice. F1 generations were genotyped by PCR using two sets of primers, one of them amplifying a fragment of the fourth exon of the human SOD1 and the second one a fragment of the murine interleukin-2 as positive control. In some preliminary experiments, we assessed the effect of the drugs used in this work in transgenic mice that express the normal human SOD1 (B6SJL-TgN(SOD1)2Gur, obtained also from Jackson Laboratories). No differences between these animals and WT mice were observed. All animals were kept in a temperature- and light-controlled room, with water and food ad libitum, and under veterinarian surveillance. Throughout the experiments, animals were used and handled in accordance with the Rules for Research in Health Matters (Mexico), with approval of the Local Animal Care Committee. G93A mice were used at the time when they underwent paralysis at both hindlimbs but were still able to wander around their cages using their forelimbs and were also able to feed themselves regularly (at 18–20 weeks of age).

G93A mice or control age-matched littermates were anesthetized with 0.4–1% halothane in a 95% O_2/5% CO_2 mixture and were placed in a stereotaxic mouse frame (D. Kopf, Tujunga, CA, USA). Microdialysis probes with a 1 mm membrane length, 0.5 mm outer diameter and molecular cut-off of 20 kDa (CMA/12, Solna, Sweden) were implanted unilaterally in either the hippocampus or motor cortex with the following coordinates, from bregma: hippocampus, AP −1.9, L +1.3 and V −2.3; motor cortex, AP +1.0, L +1.3 and V −1.2 (Franklin and Paxinos, 1997). Animals were maintained under low anesthesia (~0.4% halothane) throughout the experiment. A Ringer–Krebs medium (118 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1.18 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose and 2.5 mM $CaCl_2$, pH 7.4) was perfused at a flux rate of 2 μl/min, and eight fractions of 25 μl (12.5 min) were continuously collected after 1 h of equilibrium. After the first three fractions, which were used for the determination of the basal levels of the amino acids, 25 mM PDC or 17.5 mM 4-AP was perfused during the time periods indicated in Results. A group of animals was injected i.p. with (+)-5-methyl-10,11-dihydro-5*H*-dibenzo(a,d)cyclohepten-5,10-imine maleate (MK-801, 1 mg/kg) 30 min before 4-AP perfusion. At the end of the experiment, the skin was sutured, anesthesia was discontinued and the animals were observed during the next few hours for behavioral changes; at 24 h, they were fixed for histological analysis as described below.

Amino acid content in the dialysates was determined by HPLC as described earlier (Salazar et al., 1994; Massieu et al., 1995). Briefly, each collected fraction was derivatized with *o*-phthaldialdehyde, and, after 3 min, 20 μl was injected into a Beckman liquid chromatograph equipped with an ODS column (25 cm × 0.4 cm i.d.). A mixture of 0.1 M potassium acetate pH 5.5/methanol was used as mobile phase and was run at a flux rate of 1.5 ml/min in a 25% to 75% methanol linear

gradient. Amino acid quantification was made by comparison with a standard mixture of aspartate, glutamate, glutamine, glycine, taurine, alanine and GABA, processed in the same way. The values obtained were not corrected for the efficiency of the dialysis membrane, which is 10–12% (Morales-Villagran and Tapia, 1996).

Twenty four hours after surgery, animals were anesthetized with sodium pentobarbital and transcardially perfused with 50 ml of ice-cold 0.9% NaCl solution followed by 150 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.4. Brains were removed and postfixed for 24 h and then dehydrated in a sucrose gradient from 10% to 20% to 30% (24 h each). Brain coronal sections (50 μm thick) were obtained in a cryostat, and alternate sections from the same brain were used for cresyl violet stain or HSP70 immunohistochemistry. For the latter, the sections were placed in 0.1 M PBS and refrigerated until processed. Sections were treated in 0.1 M PBS/0.3% Triton-X-100 for 1 h then were blocked in 5% bovine albumin diluted in 0.1 M PBS for 2 h and incubated with a mouse monoclonal α-HSP70 antibody (1:66, Santa Cruz Biotechnology, CA, USA) for 48 h. Sections were washed twice with PBS/Triton for 10 min and incubated with the secondary goat anti-mouse antibody conjugated with fluorescein isothiocyanate (FITC; 1:150, Zymed, San Francisco, CA, USA) for 2 h. Finally, they were washed twice for 10 min with PBS/Triton, placed on a slide and covered with Vecta-Shield (Vector, Burlingame, CA, USA) and observed in a Nikon microscope equipped with an epifluorescence attachment.

Statistical analysis of amino acid changes in the dialysates was carried out using paired Student's t test and ANOVA. A value of $P < 0.05$ was considered statistically significant.

Results

Glutamate accumulation by blocking glutamate transport does not induce neuronal death nor HSP70 expression in G93A and control mice

Hippocampus

In the hippocampus, the basal concentration of extracellular glutamate was similar in the G93A and the control animals (13.9 ± 2.8 and 11.5 ± 3.5 pmol/10 μl, respectively; the difference was not significant). In both groups, perfusion of 25 mM PDC elevated 8- to 11-fold the extracellular concentration of the amino acid for more than 1 h (Fig. 1). Aspartate basal levels were practically undetectable and in both groups

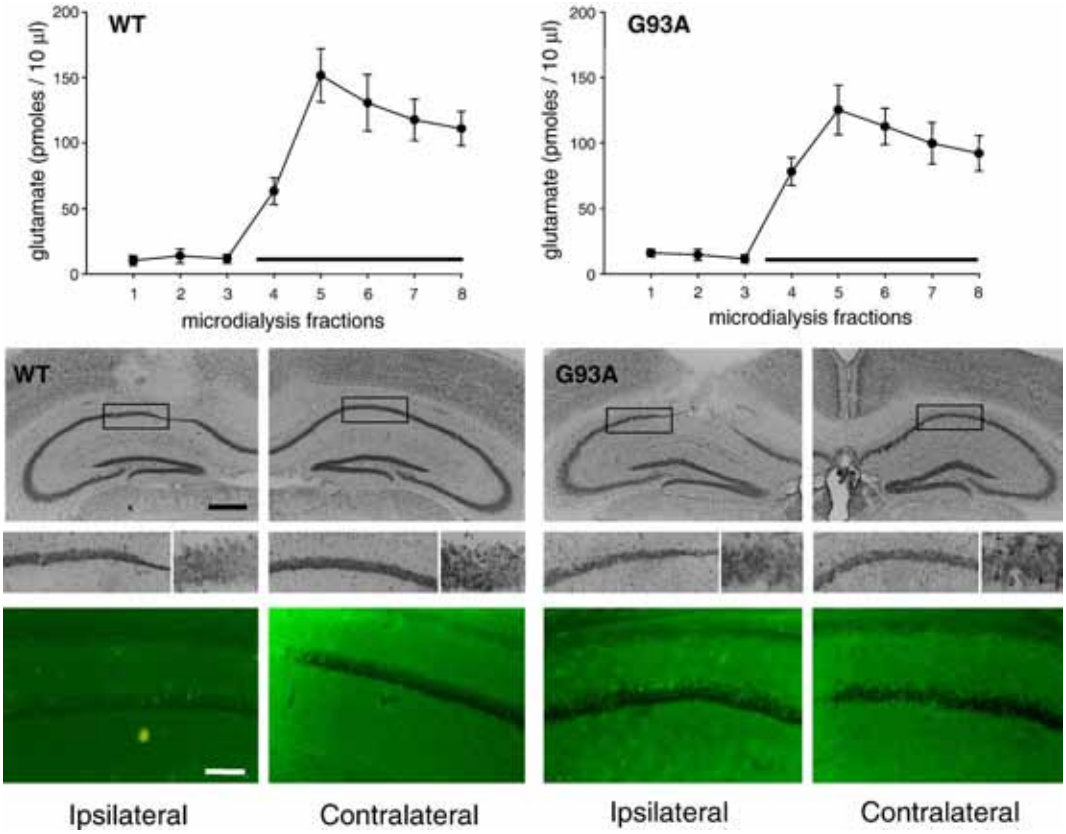

Fig. 1. Effect of PDC perfusion in the hippocampus of WT and G93A mice. Top row, time course of the changes in glutamate concentration during PDC perfusion for 5 microdialysis fractions (horizontal bars; each fraction corresponds to 12.5 min). Means ± SEM for 5 animals in each group. Middle row, representative micrographs (cresyl violet staining) of the ipsilateral PDC-perfused hippocampus (left) and contralateral (right) hippocampus, 24 h after the experiment. Two higher magnifications of the areas inside the rectangle are shown below each low magnification micrograph. No damage was observed either in the WT or in the G93A mice, besides the mechanical injure due to probe insertion in the ipsilateral hippocampus. Bottom row, HSP70 immunohistochemistry in sections from the same brains, showing that no expression of this protein can be detected in either of the groups (compare with the positive HSP70 expression in Figs. 4 and 5). Scale bar in the cresyl violet sections = 500 μm, 200 μm and 50 μm in the lowest, middle and highest magnification, respectively. Scale bar in the immunostaining = 100 μm.

increased to peak values up to 32.4 ± 2.8 pmol/10 μl in G93A and 41.9 ± 4.9 pmol/10 μl in control mice, whereas glutamine and taurine were slightly augmented and glycine and alanine were unchanged (data not shown). All these changes were similar in both groups, and the differences were not significant. No behavioral alterations were noted at any time after recovery from anesthesia.

As shown in Fig. 1, despite the large increase in extracellular glutamate due to its transport blockade, no neuronal damage besides the mechanical lesion caused by the probe was observed 24 h after the experiment, neither in the G93A nor in the control mice. Furthermore, no expression of HSP70 could be observed, indicating the lack of neuronal stress (Fig. 1).

Motor cortex

In both the G93A and control mice, the basal values of extracellular glutamate in the motor cortex were similar to those in the hippocampus, with no significant differences between the two groups, and the perfusion of PDC induced also a remarkable increase in the amino acid concentration (12- to 16-fold) (Fig. 2). The increment was higher in the control group than in the G93A mice ($P < 0.05$), although this difference may not be meaningful because of the huge increments. The basal level of aspartate was very low, and PDC increased it to values similar to those observed in the hippocampus, whereas the extracellular concentration of the other amino acids measured did not change significantly (data not shown). None of the animals showed behavioral abnormalities at any time after recovery from anesthesia.

Also, similarly to the results in the hippocampus, in spite of the great increment of extracellular glutamate induced by PDC, histological examination of the motor cortex tissue did not reveal apparent alterations as compared to the control tissue treated only with medium (Fig. 2). In both groups of animals, neuronal somas of the cortex adjacent to the microdialysis probe were negative for HSP70 immunostaining, indicating that, as in the hippocampus, PDC treatment did not induce the expression of this chaperone protein (Fig. 2, bottom).

4-AP perfusion induces epilepsy, neurodegeneration and HSP70 expression in G93A and control mice

Hippocampus

In some pilot experiments, we found that mice were more susceptible to the epileptogenic action of 35 mM 4-AP, as compared to our previous study in rats (Peña and Tapia, 1999).

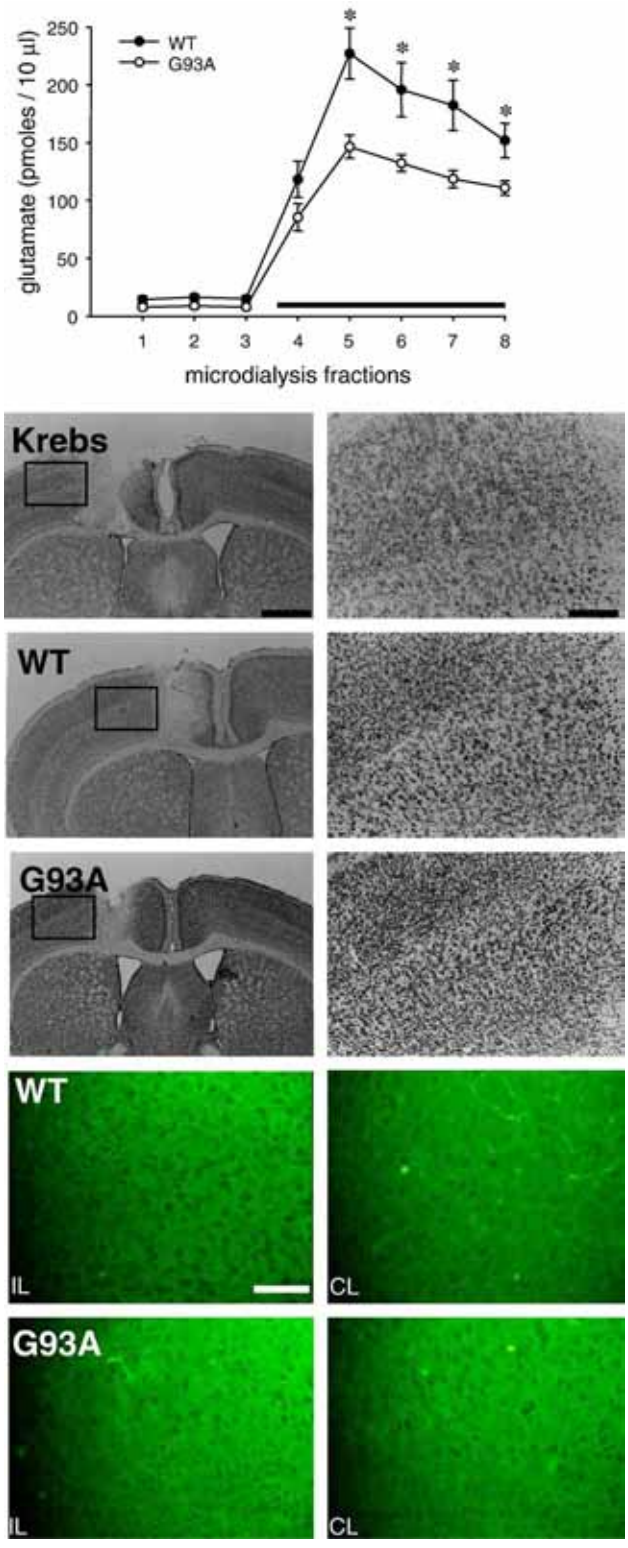

Fig. 2. Effect of PDC perfusion in the motor cortex of WT and G93A mice. Top graph, time course of the changes in glutamate concentration during PDC perfusion for 5 microdialysis fractions (horizontal bar, each fraction corresponds to 12.5 min). Means ± SEM for 5 animals in each group (*$P < 0.05$ with respect to the corresponding G93A fraction). Middle part, representative micrographs (cresyl violet staining) of the perfused area from a WT mouse treated only with Krebs medium and of WT and G93A mice 24 h after PDC perfusion. A higher magnification of the area inside the rectangle is shown at the right of each low magnification micrograph. No damage was observed either in the WT or in the G93A mice, besides the mechanical injure due to probe insertion, as can be observed in the control tissue perfused only with Krebs medium. Bottom part, HSP70 immunohistochemistry in the ipsilateral cortex (IL, in the vicinity of the probe insertion site) and in the corresponding contralateral (CL) cortex of WT and G93A mice from the same brains, showing that no expression of this protein can be detected in neither of the groups (compare with the positive HSP70 expression in Figs. 4 and 5). Scale bar in the cresyl violet sections = 1 mm and 200 μm in the lowest and highest magnification, respectively. Scale bar in the immunostaining = 100 μm.

We therefore chose a lower concentration of 4-AP for hippocampal and motor cortex perfusion (17.5 mM). In both species, perfusion with this dose of 4-AP did not induce a significant increase in extracellular glutamate in the microdialysis fractions, but there was a reduction of extracellular glutamine to about half the baseline level. This decrease was observed in the hippocampus and the motor cortex of both G93A and control mice (Figs. 3 and 6) and was similar to that previously observed in the rat hippocampus (Peña and Tapia, 1999). No significant changes in the concentration of aspartate, glycine and alanine were detected, and taurine showed a small reduction (data not shown). At the end of the experiment, after recovery from anesthesia, the WT mice showed epileptic behavior characterized by frequent generalized tonic–clonic convulsions, which lasted for about 2.5 h; after this time, the seizures gradually disappeared, and, at 24 h, all animals looked normal. In contrast, the G93A mice showed more intense tonic–clonic seizures, which lasted much longer (over 5 h); in addition, 3 of the 9 animals died within 12 h, and those that survived (6 of 9) were lying down, not able to walk and with frequent clonic convulsions, until the time of fixation for histology (24 h).

Similarly to our previous experiments in rat hippocampus, with the dose of 4-AP used, no significant increase in extracellular glutamate could be detected by our microdialysis procedure, albeit with higher doses, a notable increment is readily observed (Peña and Tapia, 1999). However, we have demonstrated that the epileptogenic effect of this drug is indeed due to overactivation of NMDA receptors since antagonists of this receptor type protected against 4-AP-induced seizures (Fragoso-Veloz and Tapia, 1992; Peña and Tapia, 2000; Ayala and Tapia, 2003). To test if this occurred also in our mice, we injected i.p. MK-801, an antagonist of NMDA receptors, 30 min before 4-AP perfusion. This treatment totally prevented the convulsions produced by 4-AP, in both WT and G93A animals (n = 5 in each group); this protective effect was striking in the G93A mice because of the severity of the seizures occurring in those not treated with MK-801.

Twenty four hours after treatment, 4-AP markedly damaged the neurons of the ipsilateral hippocampus of WT mice. The damage was notable in the CA1 region, in which a thinning of the pyramidal layer and the presence of numerous neurons with pyknotic nucleus were observed, whereas CA2 region was preserved. This neuronal loss was very similar in the five WT

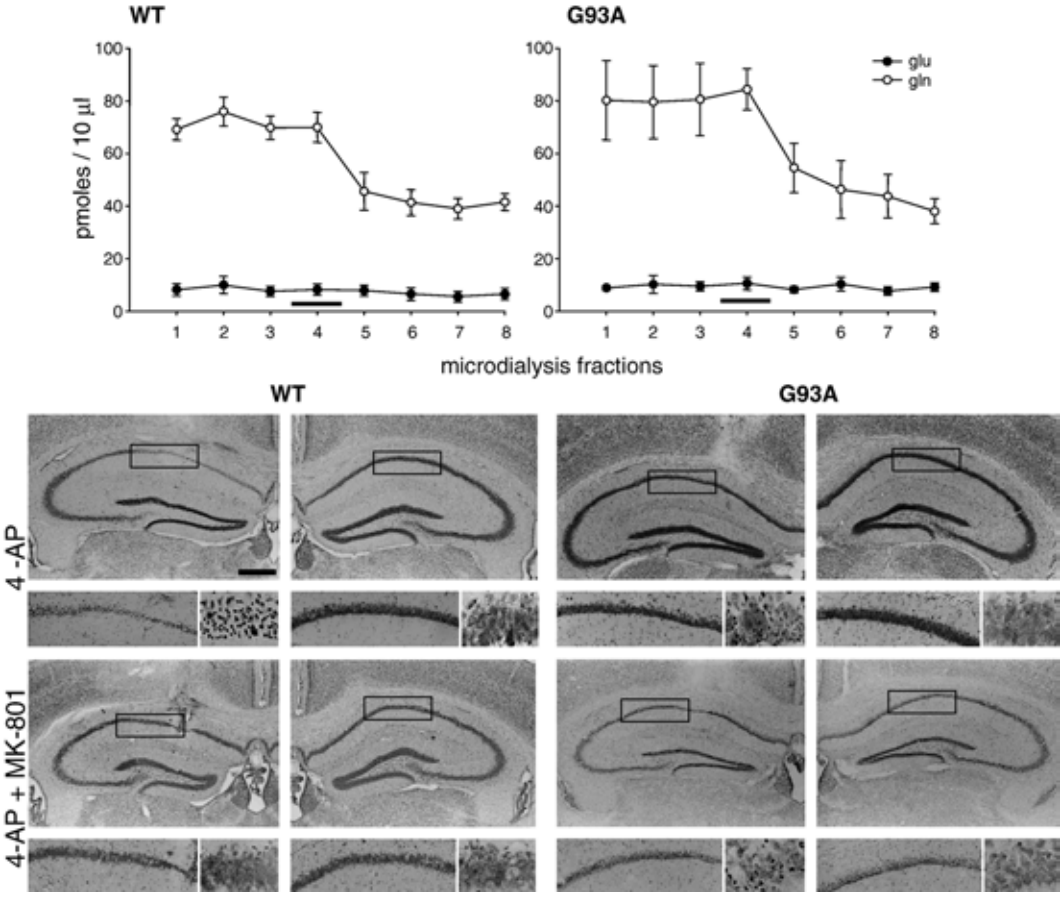

Fig. 3. Effect of 4-AP perfusion in the hippocampus of WT and G93A mice and prevention of the neuronal damage by prior systemic injection of MK-801. Top row, time course of the changes in glutamate and glutamine concentration during 4-AP perfusion for one microdialysis fraction (horizontal bar, each fraction corresponds to 12.5 min). Means ± SEM for 5 animals in each group. The micrographs (cresyl violet staining) show the damage produced by 4-AP in the ipsilateral CA1 region, 24 after the experiment, in WT and G93A mice (top row micrographs) and the protection by the prior systemic injection of MK-801 (bottom row micrographs). Micrographs are representative of 5 WT mice and of the 3 G93A mice that survived for 24 h and showed damage (see text), treated only with 4-AP, and of 5 animals in each group treated with MK-801 and 4-AP. Scale bars and magnification details, as in Fig. 1.

mice treated with 4-AP and was totally prevented by the systemic administration of MK-801 in the five mice pretreated with this antagonist (Fig. 3). Surprisingly, in spite of the much more intense and long lasting convulsant action of 4-AP in the G93A animals, the hippocampal neurodegeneration caused by this drug was less pronounced than in control mice and was observed only in 3 of the 6 mice that survived 24 h. Due to this variability, in these animals, the protection exerted by MK-801 against this damage was less clear than in the WT mice since some pyknotic nuclei were still observed (Fig. 3).

Differently from PDC, 4-AP induced a notable HSP70 immunostaining in both animal groups, especially in the G93A mice (Fig. 4). Such expression was observed in several brain regions: it was not very intense in the CA1 field of the perfused hippocampus, whereas it was remarkable in the CA3 region and hilus of both hippocampi, as well as in the somatosensory, auditory and ectorhinal cortices, and in the amygdala. HSP70 immunostaining was also very intense in the hippocampus and cortex of the WT mice, mainly in the contralateral side (Fig. 5). Similarly to the protective action of MK-801 against epileptic seizures and neurodegeneration, this antagonist blocked the induction of HSP70 expression in all brain areas in the two groups of mice perfused with 4-AP (Figs. 4 and 5).

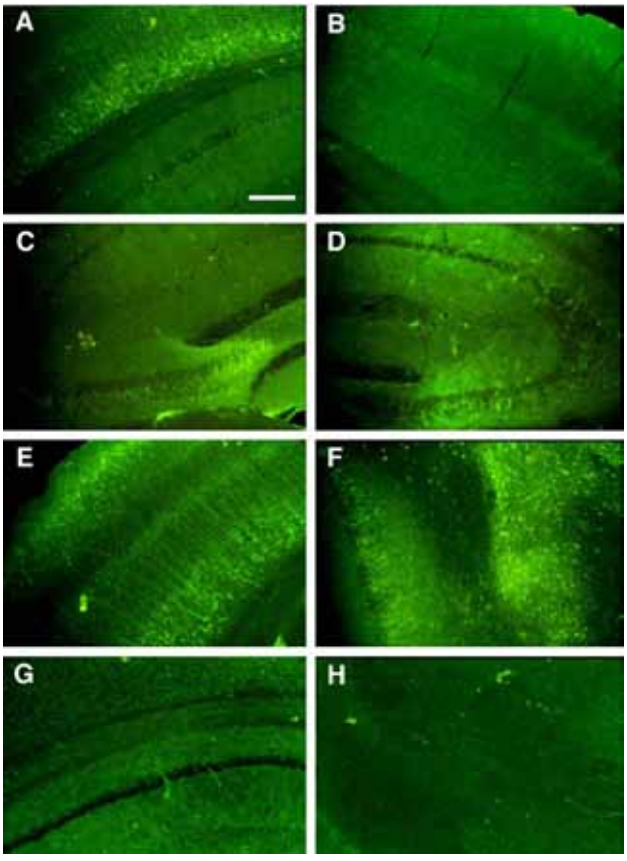

Fig. 4. 4-AP perfusion in the hippocampus induces the expression of HSP70 in several brain regions of G93A mice, and this expression is prevented by prior systemic injection of MK-801. Twenty four hours after the experiment, numerous neurons are labeled in the contralateral somatosensory cortex (A), and this labeling was blocked by MK-801 (G); however, no HSP70 staining was seen in the ipsilateral somatosensory cortex (B). Neither the contralateral (C) nor the ipsilateral (D) hippocampal CA1 and CA2 regions were labeled, whereas CA3 and hilar neurons are stained mainly in the contralateral hippocampus (C and D). The contralateral secondary somatosensory, auditory and ectorhinal cortices (E) and the contralateral amygdala (F) are heavily stained, and this was also prevented by MK-801 (shown only for the cortex in H). Micrographs are representative of 5 mice treated only with 4-AP and 5 animals treated with MK-801 and 4-AP. Scale bar = 200 μm.

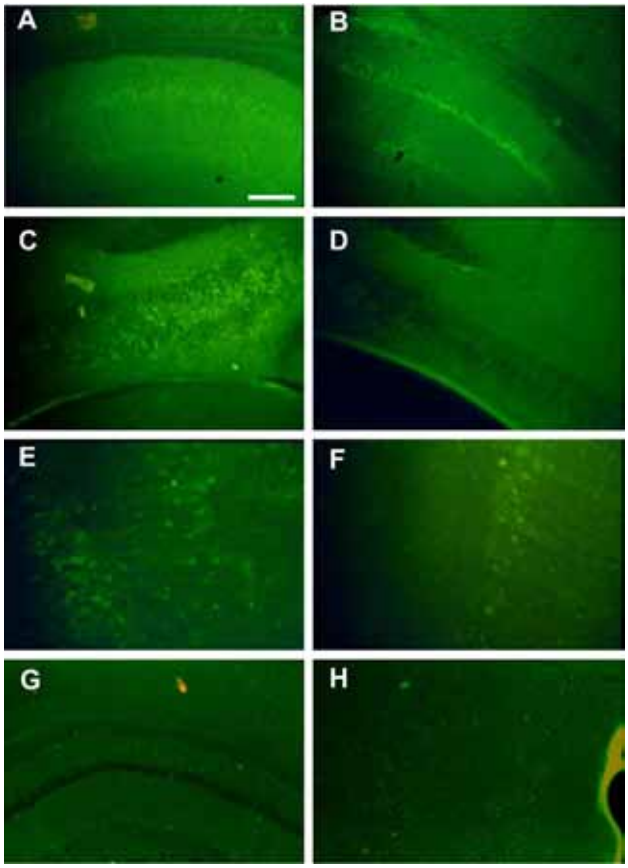

Fig. 5. 4-AP perfusion in the hippocampus induces the expression of HSP70 in several brain regions of WT mice, and this expression is prevented by prior systemic injection of MK-801. In general, HSP70 immunostaining in these animals was less intense than in G93A mice (compare with Fig. 4). Several labeled pyramidal neurons can be seen in the contralateral (A) and ipsilateral (B) hippocampal CA1 region, and MK-801 blocked this expression (G). On the other hand, the contralateral hilus (C) and perirhinal cortex (E) show heavier labeling than the corresponding ipsilateral regions (D and F). As in the hippocampus, HSP70 expression in the contralateral cortex was blocked by MK-801 (H). Micrographs are representative of 5 mice treated only with 4-AP and 5 animals treated with MK-801 and 4-AP. Scale bar = 200 μm in A, B and G, and 100 μm in C, D, E, F and H.

Motor cortex

As already mentioned, similarly to the results in the hippocampus, perfusion of 17.5 mM 4-AP in the motor cortex did not significantly modify the content of extracellular glutamate but produced a 50% reduction of extracellular glutamine (Fig. 6). No significant differences in extracellular glutamate or glutamine were found between the two animal groups ($P > 0.05$). Aspartate, glycine and alanine were unchanged, and taurine levels were slightly reduced with respect to its basal value (data not shown).

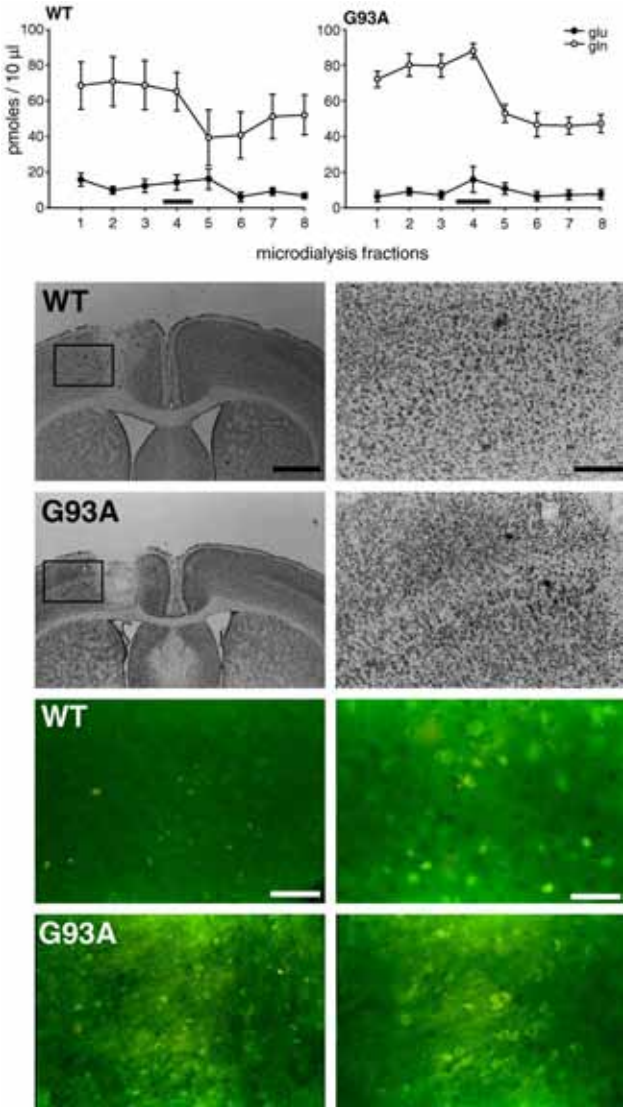

Fig. 6. Effect of 4-AP perfusion in the motor cortex of WT and G93A mice. Top row, time course of the changes in glutamate and glutamine concentration during 4-AP perfusion for one microdialysis fraction (horizontal bar, each fraction corresponds to 12.5 min). Means ± SEM for 5 animals in each group. Middle part, representative micrographs (cresyl violet staining) of WT and G93A mice, 24 h after the experiment. A higher magnification of the area inside the rectangle is shown at the right of each low magnification micrograph. No damage was observed either in the WT or in the G93A mice, besides the mechanical injure due to probe insertion (compare with Fig. 2, Krebs perfusion). Bottom part, HSP70 immunohistochemistry in the contralateral secondary somatosensory cortex, showing some labeled neurons, in both the WT and the G93A mice (the right side micrographs are magnifications of those at the left). Scale bar in the cresyl violet sections = 1 mm and 200 µm in the lowest and highest magnification, respectively. Scale bars in the immunostaining = 100 and 50 µm.

When animals from both groups recovered from anesthesia, they showed convulsions similar to those after hippocampal 4-AP perfusion, characterized by generalized tonic–clonic seizures that lasted approximately 2.5 h. However, in contrast with the hippocampal perfusion, there were no differences in the intensity and duration of the seizures between the control and the G93A mice, and all animals recovered completely at about 3 h after the experiment and survived until the time of fixation (24 h).

Histological examination of the perfused tissue showed that, differently from the hippocampus, 4-AP did not cause neurodegeneration of the motor cortex in the G93A nor in the control mice (Fig. 6); the only damage observed was the mechanical lesion caused by the cannula, as in the tissue perfused with medium only. Twenty four hours after 4-AP perfusion, HSP70 immunoreactivity was observed in small areas of the contralateral cortex (Fig. 6), but it was not as intense as that seen after perfusion in the hippocampus; it was slightly stronger in the G93A group than in the WT mice.

Discussion

The main aim of this work was to test whether SOD1 mutations might enhance excitotoxic neuronal damage in transgenic ALS mice, consequent to increased extracellular endogenous glutamate. For this purpose, we used a microdialysis procedure that allows both the perfusion of drugs and the determination of changes in the extracellular concentration of glutamate. Because of the small diameter of the mouse spinal cord, microdialysis is not possible in this structure–the mostly affected in ALS–and therefore we carried out the experiments in the hippocampus and the motor cortex. The former was chosen because of its high vulnerability to excitotoxic damage (Tapia et al., 1999; Peña and Tapia, 2000) and the latter because it is involved in the pathology of ALS. Our results show that the accumulation of glutamate consequent to its transport blockade is innocuous for both the WT and the G93A mice, whereas the stimulation of glutamate release produces neurodegeneration in susceptible cerebral regions.

The paralysis that G93A mice develop as a result of the expression of human mutant SOD1 is progressive; it begins at the rearlimbs and follows all the way up as motor neurons in the spinal cord are dying. We performed these experiments at a paralysis stage when both hindlimbs had a very noticeable motor deficit, but animals were still capable to ramble around their cages using their forelimbs and were also able to eat and drink normally. Although this means that the neurodegeneration at the time of the experiment had not progressed farther than the lower spinal cord, the hippocampus and the motor cortex had been exposed to the harmful effects of mutant SOD1 throughout the life of the G93A mice so that, as discussed next, at this stage their neurons could be at a higher risk for undergoing excitotoxicity-induced degeneration, in comparison with the neurons of WT mice.

One of the prevailing hypothesis of the mechanisms of neurodegeneration in ALS is the malfunction of glutamate transporters, which might be caused by an oxidative environment produced by a mutant SOD1. As has been shown in cortical astrocyte cultures exposed to the ROS generator system xanthine/xanthine oxidase or to H_2O_2, the function of glutamate transporters is vulnerable to oxidation and recovers by the addition of SOD1, catalase or reducing agents such as glutathione or dithiotreitol (Volterra et al., 1994). In addition, peroxynitrite is capable to inhibit GLT1, GLAST and EAAC1 function within seconds (Trotti et al., 1996), and, in spinal cord synaptosomes from SOD1/G93A transgenic rats, there is a

progressive reduction in the V_{max} of the sodium-dependent glutamate uptake (Dunlop et al., 2003). Furthermore, in *Xenopus laevis* oocytes coexpressing the human WT SOD1 or the mutants A4V or I113T, and the human GLT1, the activity of this transporter is inhibited by H_2O_2 only in the oocytes that express the mutant forms of the SOD1; the activity of EAAC1 was also evaluated, but it was not altered (Trotti et al., 1999).

In addition, in ALS, as well as in other neurodegenerative diseases, there is an energetic dearth, which is enhanced by the oxidative damage of mitochondria (Beal, 2000). In this respect, G93A mice show a characteristic vacuolization in mitochondria in spinal cord as an early sign of the disease (Wong et al., 1995). Furthermore, SOD1, which is normally cytosolic, accumulates and forms aggregates inside mitochondria, both in the spinal cord (Higgins et al., 2002) and in the brain (Vijayvergiya et al., 2005) of G93A mice. Thus, it should be expected that, when a major energetic deficit exists, the membrane potential, maintained by Na^+/K^+ ATPases, could decay easier, causing that even small increases in the glutamate concentration keep the postsynaptic membrane depolarized, activating NMDA receptors faster and thus generating greater excitotoxic damage. Thus, the neurons under these energetic conditions should be more susceptible to an overstimulation caused by increased extracellular endogenous glutamate in comparison with the neurons of WT mice.

With this in mind, we hypothesized that, under the cellular conditions just discussed, which occur in the G93A mice, the blockade of glutamate transporters could result in neuronal hyperexcitation and loss of the neurons exposed to the accumulation of extracellular glutamate. This expectation, however, was not fulfilled since perfusion of PDC did not cause motor hyperactivity or neuronal death in the hippocampus and the motor cortex, neither in the WT nor in the G93A mice, in spite of a remarkable and long lasting elevation of extracellular glutamate. The lack of damage in the motor cortex is noteworthy because, in compliance with the selectivity of the neuronal death in ALS, this region should be more prone to such a challenge. These results are in agreement with the lack of lower motor neuron damage in rat spinal cord after the accumulation of extracellular glutamate produced by PDC perfusion (Corona and Tapia, 2004), as well as with the lack of neuronal degeneration or hyperexcitation in the rat striatum and hippocampus (Massieu et al., 1995; Massieu and Tapia, 1997; Obrenovitch et al., 1996). A possible explanation for this phenomenon is that the increased extracellular glutamate arising from the blockade of its transport is not capable of reaching the postsynaptic receptors (Tapia et al., 1999; Corona and Tapia, 2004). It has been previously reported that a low concentration of PDC (1 mM), perfused in the prefrontal cortex of G93A mice during 40 min, induces an elevation of glutamate slightly higher than in WT mice, but no behavioral alterations are mentioned and no histological analysis was reported (Andreassen et al., 2001).

Confirming the lack of deleterious effect of PDC, this drug did not induce HSP70 expression. The induction of this protein occurs in cells submitted to intense and potentially lethal stress, including that due to excitotoxicity even when cells survive (Gonzalez et al., 1989; Sloviter and Lowenstein, 1992; Tomioka et al., 1993; Ohtsuka and Suzuki, 2000; Ayala and Tapia, 2003). Thus, the lack of immunohistochemical detection of HSP70 indicates that neurons did not become stressed during or after the PDC-induced elevation of extracellular glutamate, not even in the G93A mice. Since the lack of PDC effects was observed at 24 h, it could be argued that a deleterious effect arising from transport blockade may require a longer time. Although with the present data we cannot completely discard this possibility, the effects of 4-AP under similar experimental conditions clearly indicate that glutamate-induced excitotoxicity occurs at 24 h, as we shall discuss next.

To examine the effect of extracellular glutamate excess due to stimulation of its release, we used 4-AP, a K^+ channel blocker that induces NMDA-receptor-dependent seizures, neurodegeneration and HSP70 expression when perfused in the rat hippocampus (Peña and Tapia, 2000; Ayala and Tapia, 2003). As already mentioned, similarly to previous studies in rat hippocampus (Peña and Tapia, 1999), with the 4-AP concentration used (17.5 mM), the increase in extracellular glutamate level in the hippocampus and motor cortex could not be detected by the microdialysis technique used. However, the notable decrease in glutamine concentration and, most important, the convulsant effect of 4-AP in the two groups of mice suggest that there was in fact a stimulation of glutamate release from the synaptic terminals, capable of overactivating postsynaptic receptors. This interpretation is supported by the total protection exerted by the NMDA receptor blocker MK-801 against the convulsions induced by the hippocampal perfusion of 4-AP, which was similar to the effect previously reported in rats (Tapia et al., 1999; Peña and Tapia, 2000; Ayala and Tapia, 2003). It is remarkable that, despite the fact that the G93A mice suffered more intense and long lasting convulsions than the WT animals, MK-801 was equally effective in the two groups. This suggests that the transgenic mice are more sensitive to seizures produced by the overstimulation of NMDA receptors.

In contrast to the lack of effect of PDC on HSP70 expression, in both groups of mice, the immunostaining of this protein was intense after 4-AP treatment and was observed not only in neurons of the contralateral hippocampus but also in regions far from the perfused tissue, similarly to previous observations in the rat (Ayala and Tapia, 2003). This expression was blocked by MK-801 in both WT and G93A mice, which is a further indication that the neuronal stress and hyperexcitation induced by 4-AP are mediated by overactivation of NMDA receptors. The immunoreactivity of HSP70 in distal zones from the site of perfusion may be consequent to transynaptic stimulation to homologous regions of the contralateral hippocampus through the commissural fibers arising from CA3 (Ayala and Tapia, 2003).

Perfusion of 4-AP in the motor cortex did not produce morphologically detectable neurodegeneration in the perfused area, neither in the WT nor in the G93A mice. However, the convulsions shown by these animals and the expression of HSP70, although reduced in comparison to that seen after perfusion in the hippocampus, are evidence that the excitatory and stressful effects of 4-AP occur also in the cortex. The lack of

damage in this structure suggests, firstly, that it is less vulnerable to the excitotoxic damage with respect to the hippocampus and, secondly, that the mutant SOD1 and the consequent oxidative damage do not make this region more susceptible to excitotoxic death as compared to the WT mice. In compliance with our data, SOD1 mutations do not increase motor neuron susceptibility to AMPA receptor activation (Raoul et al., 2002), unless this receptor lacks the Ca^{2+} permeable subunit GluR2 (Van Damme et al., 2005).

We conclude that SOD1 G93A mutation does not increase the sensitivity of cerebral neurons to endogenous glutamate-mediated convulsions, cellular stress and neurodegeneration, induced by stimulation of glutamate release by 4-AP. In addition, in view that under the same experimental conditions the accumulation of extracellular glutamate due to blockade of its transport was innocuous, in both WT and G93A mice, our results do not support the possibility that glutamate transport deficiency may be a key factor involved in brain neuronal degeneration in familial ALS.

Acknowledgments

This work was supported by DGAPA, UNAM (projects IN213703 and IN201204) and CONACYT (project 42668). The authors wish to thank Federico Jandete for cresyl violet staining procedure.

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Planteamiento del problema

Entre las hipótesis que se tienen sobre la muerte neuronal en la ALS incluyendo: estrés oxidante, mal plegamiento y agregación de proteínas, alteraciones del transporte axonal, alteraciones mitocondriales, inflamación y apoptosis (Cleveland y Rothstein 2001; Julien 2001; Bruijn et al. 2004; Shaw 2005; Boillee et al. 2006; Pasinelli y Brown 2006), se ha planteado que la pérdida de las motoneuronas en este padecimiento puede ser debida a la excitotoxicidad.

Como ya se mencionó en la revisión *"Excitotoxicity as a mechanism of motoneuron death in amyotrophic lateral sclerosis"* la hipótesis de la excitotoxicidad como causa de la ALS se fundamenta principalmente en que algunos pacientes con SALS, aproximadamente el 40% de acuerdo con los estudios más extensos (Shaw et al. 1995; Spreux-Varoquaux et al. 2002), tienen un nivel de glutamato elevado en el líquido cefalorraquídeo. Además se ha encontrado, en análisis post mortem de tejidos de pacientes, que hay una disminución en el contenido de EAAT2 en las regiones del SNC afectadas en la ALS (Rothstein et al. 1995). Entonces, se ha planteado un modelo de neurodegeneración que involucra la pérdida de los transportadores de glutamato, tal vez debido a una elevación en la producción de ROS, lo cual resultaría en un incremento de la concentración de glutamato extracelular y éste a su vez produciría la degeneración de las motoneuronas por excitotoxicidad.

Se han llevado a cabo diferentes estudios en sistemas tanto *in vitro* como *in vivo* con la intención de dilucidar hasta dónde la pérdida de los transportadores puede generar la muerte de las motoneuronas. Mientras que en los modelos *in vitro* es muy claro que la disfunción del transporte de glutamato causa muerte neuronal, aún es tema de controversia si este efecto *in vitro* puede reproducir los fenómenos *in vivo* (Rattray y Bendotti 2006). Se ha demostrado, por ejemplo, que *in vitro* la reducción del transporte de glutamato es letal para las motoneuronas (Rothstein et al. 1993; Carriedo et al. 1996; Okazaki et al. 1996). Sin embargo, *in vivo* el bloqueo del transporte de glutamato en la médula espinal (Corona y Tapia 2004), así como en otras estructuras cerebrales (Massieu et al. 1995; Massieu y Tapia 1997; Obrenovitch y Urenjak 1997) no genera muerte neuronal. Por otra parte, en los modelos transgénicos de FALS algunos estudios señalan que existe una disminución en la expresión de los transportadores de glutamato (Bendotti et al. 2001; Dunlop et al. 2003), y se ha propuesto también que estos ratones tienen una concentración de glutamato incrementada en el líquido extracelular (Alexander et al. 2000; Andreassen

et al. 2001). Sin embargo, en este modelo, la pérdida de los transportadores de glutamato se presenta después de la etapa sintomática lo que descarta que su desaparición sea la causa de la neurodegeneración (Bendotti et al. 2001).

Las motoneuronas son particularmente susceptibles a la estimulación excesiva mediada a través de receptores glutamatérgicos de tipo AMPA. Esta susceptibilidad fue inicialmente descrita en modelos *in vitro* (Carriedo et al. 1996) y se ha reproducido mediante la aplicación de diferentes agonistas de este tipo de receptores glutamatérgicos en la médula espinal de la rata (Hugon et al. 1989; Urca y Urca 1990; Ikonomidou et al. 1996). Cuando se administra AMPA a ratas adultas en la médula espinal por microdiálisis, éstas desarrollan parálisis ipsilateral en la extremidad posterior, como consecuencia de la muerte de las motoneuronas en el asta ventral (Corona y Tapia 2004). Este modelo no involucra la participación de alteraciones genéticas y por lo tanto es representativo de un mayor número de casos de ALS, pero, su principal desventaja es que la neurodegeneración ocurre entre 3 y 6 horas después de la administración de AMPA, lo que constituye un modelo agudo de una enfermedad que se desarrolla de forma crónica.

Por otra parte, la utilización de factores tróficos es uno de los enfoques terapéuticos más recientes en la ALS (Kotzbauer y Holtzman 2006). La parálisis que desarrollan los ratones transgénicos de FALS se puede retrasar y la vida del ratón se puede prolongar, mediante la administración de factores tróficos como el factor neurotrófico derivado de glia (GDNF) (Wang et al. 2002b), el factor de crecimiento similar a insulina (IGF-1) (Kaspar et al. 2003) y el VEGF (Azzouz et al. 2004; Zheng et al. 2004; Storkebaum et al. 2005; Wang et al. 2007). De los factores probados mediante este procedimiento en el modelo transgénico, hasta ahora el VEGF ha sido el que confiere una protección mayor (Greenberg y Jin 2004).

En relación al VEGF, un estudio demostró que en ratones la eliminación del elemento de respuesta a hipoxia (HRE) presente en el promotor de este factor causa una parálisis extraordinariamente similar a la que sufren los ratones con la SOD1 mutada (Oosthuyse et al. 2001). Estos animales ($VEGF^{\delta/\delta}$) desarrollan el mismo patrón de muerte de las neuronas motoras en la médula espinal, lo que sugiere que el VEGF puede jugar un papel primordial en la viabilidad de estas neuronas.

Dada la importancia del VEGF en la fisiología de las motoneuronas, nosotros consideramos relevante analizar la posible protección del VEGF en un modelo de

neurodegeneración espinal excitotóxica que no involucra alteraciones genéticas y que, por lo mismo, es representativo de una mayor proporción de casos de neurodegeneración de la ALS humana. Al mismo tiempo, diseñamos y desarrollamos un modelo *in vivo* que permitiera correlacionar la muerte neuronal espinal con sus efectos sobre la parálisis muscular.

Objetivos

Objetivo general

Estudiar la participación de la excitotoxicidad en el desarrollo de la neurodegeneración en modelos experimentales de ALS *in vivo* y evaluar el efecto neuroprotector del VEGF.

Objetivos particulares

- Analizar el efecto del bloqueo crónico de los transportadores de glutamato sobre la viabilidad de las motoneuronas de la médula espinal de ratas silvestres, como una posible causa de ALS.

- Generar un modelo crónico de neurodegeneración espinal *in vivo*, no relacionado con alteraciones genéticas, que permita la correlación de la muerte de las motoneuronas con sus respectivos efectos sobre la motricidad.

- Conocer si el VEGF puede actuar como protector contra la neurodegeneración espinal en un modelo experimental crónico de excitotoxicidad que semeje a la SALS.

- Dilucidar el mecanismo intracelular de la protección mediada por el VEGF contra la muerte excitotóxica de las motoneuronas espinales.

Diseño y procedimientos experimentales

En esta sección se describen los modelos experimentales de ALS empleados en este trabajo de tesis, así como los procedimientos experimentales que se emplearon para el desarrollo de uno de ellos. También, se menciona el fundamento de la técnica de microdiálisis utilizada en algunos experimentos de este proyecto y las pruebas usadas para la evaluación de la conducta motora que permitieron caracterizar el inicio y progresión de la parálisis inducida experimentalmente en ratas.

El resto de los materiales y métodos empleados (procedimientos quirúrgicos, PCR, HPLC, western blotting, inmunohistoquímica y análisis histológicos) se encuentran debidamente descritos en las secciones correspondientes en los artículos que se presentan en la siguiente sección de Resultados.

Modelos para el estudio de la ALS

Los modelos experimentales de las enfermedades proveen el conocimiento de los mecanismos patofisiológicos y permiten el diseño y ensayo de estrategias terapéuticas siempre y cuando mimeticen de manera tan fiel como sea posible los síntomas y progresión temporal de la enfermedad humana. Para el estudio de los mecanismos de la ALS, existen diversos modelos *in vitro* e *in vivo* que reproducen la muerte de las motoneuronas. Sin embargo, sólo algunos de los modelos *in vivo* permiten la correlación de la muerte neuronal con las alteraciones musculares progresivas características de la ALS. Dentro de estos modelos el más común es el ratón transgénico que expresa una mutante humana de SOD1. La principal desventaja de este modelo es que reproduce la causa de muerte neuronal que ocurre en la menor proporción de los pacientes con la forma familiar de la enfermedad dependiente de las mutaciones en la SOD1.

En el curso de este proyecto empleamos 2 modelos experimentales de ALS *in vivo*: los ratones transgénicos con la SOD1 humana mutada y un modelo crónico de neurodegeneración espinal crónica que desarrollamos para este fin. En el siguiente artículo de revisión "*Experimental models for the study of neurodegeneration in amyotrophic lateral sclerosis*" publicado en Molecular Neurodegeneration, revisamos estos y otros modelos experimentales de ALS tanto *in vitro* como *in vivo* y ofrecemos un análisis de su utilidad.

Molecular Neurodegeneration

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Review

Experimental models for the study of neurodegeneration in amyotrophic lateral sclerosis

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Published: 20 July 2009

Molecular Neurodegeneration 2009, **4**:31 doi:10.1186/1750-1326-4-31

Received: 12 May 2009
Accepted: 20 July 2009

This article is available from: http://www.molecularneurodegeneration.com/content/4/1/31

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of unknown cause, characterized by the selective and progressive death of both upper and lower motoneurons, leading to a progressive paralysis. Experimental animal models of the disease may provide knowledge of the pathophysiological mechanisms and allow the design and testing of therapeutic strategies, provided that they mimic as close as possible the symptoms and temporal progression of the human disease. The principal hypotheses proposed to explain the mechanisms of motoneuron degeneration have been studied mostly in models in vitro, such as primary cultures of fetal motoneurons, organotypic cultures of spinal cord sections from postnatal rodents and the motoneuron-like hybridoma cell line NSC-34. However, these models are flawed in the sense that they do not allow a direct correlation between motoneuron death and its physical consequences like paralysis. In vivo, the most widely used model is the transgenic mouse that bears a human mutant superoxide dismutase 1, the only known cause of ALS. The major disadvantage of this model is that it represents about 2%–3% of human ALS. In addition, there is a growing concern on the accuracy of these transgenic models and the extrapolations of the findings made in these animals to the clinics. Models of spontaneous motoneuron disease, like the wobbler and pmn mice, have been used aiming to understand the basic cellular mechanisms of motoneuron diseases, but these abnormalities are probably different from those occurring in ALS. Therefore, the design and testing of in vivo models of sporadic ALS, which accounts for >90% of the disease, is necessary. The main models of this type are based on the excitotoxic death of spinal motoneurons and might be useful even when there is no definitive demonstration that excitotoxicity is a cause of human ALS. Despite their difficulties, these models offer the best possibility to establish valid correlations between cellular alterations and motor behavior, although improvements are still necessary in order to produce a reliable and integrative model that accurately reproduces the cellular mechanisms of motoneuron degeneration in ALS.

Introduction

Effective treatments for practically all diseases can only result from the knowledge of their cellular and molecular pathophysiological mechanisms. This is particularly evident in the case of diseases whose cause is still unknown in spite of the remarkable progress of biomedicine in the recent decades, such as devastating neurodegenerative diseases, including Alzheimer's disease and amyotrophic lat-

eral sclerosis (ALS). For the purpose of gaining insights into such mechanisms, the design and use of experimental models is essential. In general, such studies are carried out in vitro, in cell cultures, slices or organotypic cultures, and in vivo. Whereas the former can give very useful information regarding cellular and molecular mechanisms, the experiments in whole living animal models obviously reflect more closely the human disease, provided that the symptoms and their development during time mimics as close as possible those of the human disease. In this framework, the purpose of the present article is to review the available experimental animal models of ALS.

Amyotrophic lateral sclerosis, described in 1869 by the French neurologist Jean-Martin Charcot, is a fatal adult-onset neurodegenerative disease characterized by the selective and progressive death of both upper and lower motoneurons, leading to a progressive paralysis, respiratory depression and death usually within 2–5 years after onset. Based on which type of motoneurons are primarily affected, whether lower motoneurons, located in the ventral horns of the spinal cord, or upper motoneurons, located in the brainstem and the cerebral motor cortex, ALS can be classified in two forms: spinal onset (~75% of cases), characterized by muscle weakness and atrophy, cramps, fasciculations, spasticity and paralysis, and bulbar-onset (~25% of cases), characterized by progressive dysphagia and dysarthria, spasticity and hyperreflexia [1]. Because the neuronal loss in ALS is selective, the disease generally does not cause major cognitive impairments such as those occurring in other neurodegenerative diseases like Alzheimer's and Huntington's. However, some ALS patients may present changes in personality, irritability, obsessions, poor insight and deficits in frontal executive tests [2]. In the majority of ALS patients, death is due to respiratory failure caused by the denervation of the respiratory muscles and diaphragm. The prevalence of ALS is about 2–6 cases/100,000 and the median age of onset is 55 years, although it can start at younger ages [3].

The disease occurs in sporadic and familial forms with very similar clinical courses and common pathological features, such as the presence of abnormal accumulations of neurofilaments in degenerating motoneurons [4]. The familial form of ALS (FALS) accounts for 5–10% of cases and has an autosomal dominant pattern of inheritance, whereas the sporadic form (SALS) accounts for the majority of ALS cases (~90%). Among the FALS cases, about 20% are caused by missense mutations in the *SOD1* gene that codes for the enzyme $Cu^{2+}Zn^{2+}$ superoxide dismutase 1 (SOD1) [5]. However, the cause of most ALS cases is still unknown and several hypotheses have been proposed to account for the selective death of upper and lower motoneurons. These include oxidative damage, axonal strangulation and transport impairment, disorganization of neurofilaments, protein misfolding and toxicity from intracellular aggregates, mitochondrial dysfunction, inflammation, apoptosis, and excitotoxic death arising from the mishandling of glutamate [4,6-10]. Because the clinical course of the disease is highly variable, the mechanism of motoneuron death may arise from the unfortunate convergence of multiple factors rather than from a single alternative.

The pathogenesis of ALS has been studied in autopsy samples, but this has not yielded reliable information in terms of the pathophysiological mechanisms of motoneuron degeneration during the progressive clinical stage, from disease onset to the death of the patients. The pathological hallmarks that have been found in the spinal cord of autopsied ALS patients include the atrophy of dying motoneurons with notable swelling of the perikarya and proximal axons, intracytoplasmic neurofilament abnormalities, and the presence of Bunina bodies, spheroids and strands of ubiquitinated material in degenerating axons and in cell somas; this motoneuron pathology is often accompanied by reactive gliosis [11].

Under these circumstances, experimental in vitro and in vivo models have been developed to improve our understanding of the disease and have allowed the testing of possible therapeutic strategies. However, these models have many limitations and have not succeeded in designing effective treatments to stop the course of the disease. Therefore, an integrative model that reproduces the chronic progressive motoneuron death and the main characteristics of the disease is still needed.

Based on the multiple events considered to contribute to the selective loss of motoneurons as targets for therapy, many different drugs have been tested on their capacity to alleviate or retard the symptoms of ALS patients and to prolong their survival, but none has proved to be effective. The only currently used compound that slightly slows disease progression and prolongs the survival of ALS patients, with no improvement in muscle function, is riluzole. This drug limits glutamate release from nerve endings possibly by stabilizing the inactive state of voltage-dependent sodium channels and by a G protein-coupled intracellular pathway [12-16].

Mutations in superoxide dismutase 1 as cause of one form of familial ALS

Cu^{2+}/Zn^{2+} superoxide dismutase 1 (SOD1) is an ubiquitously expressed cytoplasmic enzyme that catalyzes the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide and molecular oxygen and is an important free radical scavenging enzyme that protects cells against oxidative stress. The copper atom is alternately reduced and oxidized by superoxide, providing a reactive center for its dismutation, while the zinc atom gives structural stability to the protein; both cations are buried at the bot-

tom of an active site channel [17-19]. The disease-causing SOD1 mutations are scattered throughout the primary structure of the protein. More than 100 mutations have been found [20-22], and all but one, $SOD1^{D90A}$ [23,24], cause the dominantly inherited disease. Superoxide radical is a very reactive intermediate formed by the reduction of O_2 in the respiratory chain and is a powerful oxidant; it is normally converted to hydrogen peroxide before it can undergo other free-radical reactions. Hydrogen peroxide is converted to water by catalase or glutathione peroxidase, but it can also be decomposed to hydroxyl radical in the presence of iron; this radical is highly reactive and can damage lipids, proteins or nucleic acids.

It has been demonstrated that SOD1-mediated toxicity in ALS is not due to the loss of its catalytic activity but instead to a gain of function which confers one or more toxic properties that are independent of the levels of dismutase activity [25]. The main arguments against the importance of loss of dismutase function are that SOD1 knockout mice do not develop motoneuron disease [26] and that levels of SOD1 activity do not correlate with disease in mice or humans. In fact, some mutant enzymes retain full dismutase activity [27,28], and chronic increase in the levels of wild-type SOD1 (and dismutase activity) has no effect on the disease [29] or even accelerates it [30]. The acquired toxic property likely disrupts several basic cellular functions in neurons, including protein breakdown by the ubiquitin-proteasome system, slow anterograde transport, fast retrograde axonal transport, calcium homeostasis, mitochondrial function, and maintenance of the cytoskeletal architecture [7]. The toxicity can arise either through aberrant chemistry, mediated by the misfolded aggregated mutants, which can disregulate the redox equilibrium [31] or produce loss or sequestration of essential cellular components, for example by saturating the protein-folding chaperones and/or the protein-degradation machinery [7,8,31]. This includes endoplasmic reticulum stress and accumulation of the mutant SOD1 in microsomes [32,33]. The discovery of prominent cytoplasmic inclusions in motoneurons and, in some cases, within the astrocytes surrounding them in the SOD1 ALS mouse model [29,30,34] and in autopsy samples from patients with SALS and FALS [35-37] led to the hypothesis of toxic protein aggregation. These inclusions are commonly detergent-insoluble elements dispersed in the cytosol, which have been characterized by ubiquitin and SOD1 staining [38,39]. The toxicity to motoneurons generated by SOD1 mutants seems to be non-cell autonomous, since mutant damage occurs not just within motoneurons but also in non-neuronal cells, suggesting that neuronal death depends, at least in part, on a contribution from surrounding astrocytes and possibly other cell types [40-42].

Other mutations as cause of familial ALS

A series of other mutant genes have been reported to cause ALS in both familial and sporadic cases (see [43,44] for comprehensive reviews), but the number of patients harboring these mutations is substantially low, and therefore these mutations are not commonly used for modeling ALS, although there are few exceptions such as alsin.

Alsin, the product of the *ALS2* gene coded in chromosome 2q33 [45], is a protein with three putative guanine nucleotide exchange factor domains that has been found altered in some FALS cases [46,47], with a higher prevalence in Tunisian and Pakistani populations [45,48]. Most *ALS2* mutations are deletions caused by abnormal stop codons that produce a truncated dysfunctional protein [49]. Homozygous expression of mutant alsin is responsible for early onset FALS, also known as juvenile ALS or ALS2; this form progresses slower than the adult onset forms [50]. Since juvenile ALS is inherited in a recessive manner it is assumed that the proper function of alsin is an elemental component of motoneuron physiology. Unlike SOD1 mutations that cause a rather homogeneous phenotype independently from the amino acid substitution, although subtle differences exist, *ALS2* truncated products cause a diversity of clinical outcomes depending on the form generated by the specific mutation. Furthermore, mutations in alsin are not only responsible for provoking ALS but for at least two other types of motoneuron disease: primary lateral sclerosis and hereditary spastic paraplegia (reviewed in [51]).

Recently, it was reported that mutations in the RNA/DNA binding protein TDP-43 cause classical ALS with an autosomal recessive inheritance pattern (see [52] for review), and shortly after this discovery another nucleic acid binding protein was found to be mutated in a British family with FALS [53,54]. The discovery of these mutations may allow the development of new experimental models for ALS, although as we shall discuss later, such models might be limited to reproduce the causes of motoneuron death related to those specific mutations.

Glutamate-mediated excitotoxicity as a causal factor of ALS

Glutamate-mediated excitotoxicity generated by an excessive glutamatergic synaptic transmission is considered a probable mechanism leading to motoneuron degeneration in both SALS and FALS. Excitotoxicity involves a massive influx of Ca^{2+} through glutamate receptors, triggering the uncontrolled activation of deleterious processes that eventually produce neuronal death [55,56]. Motoneurons are highly vulnerable to intracellular calcium overload due to their low calcium buffering capacity [57-60].

In spinal motoneurons, calcium entry is likely to occur through calcium-permeable AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate)-type receptors. Functional AMPA receptors are composed of four subunits, GluR1-GluR4, in various combinations [61]. Subunit GluR2 determines the calcium permeability because the presence of at least one GluR2 in the receptor structure makes it impermeable to the cation [62,63]. In addition, the posttranscriptional modifications that edit the Q/R site of the GluR2 impede the calcium flux through the pore [64]. Therefore, activation of AMPA receptors lacking the GluR2 subunit or without its posttranscriptional edition may have significant pathophysiologic consequences that could be involved in ALS. In fact, spinal motoneurons are particularly vulnerable to agonists of the AMPA-type receptors probably because of a large calcium influx, both in vitro [56,65,67-72] and in vivo [73,74].

Glutamatergic synaptic transmission is rapidly terminated by the neurotransmitter uptake from the synaptic cleft into neurons and glia, a process carried out by high affinity glutamate transporters. Five of these transporters have been identified and cloned, and their location in the central nervous system has been determined. Excitatory amino acid transporter 2 (EAAT2), also called glutamate transporter 1 (GLT1) in rodents, is the most abundant and is present almost exclusively in astrocytes [75]. In ALS patients, post-mortem analysis of the motor cortex and the spinal cord revealed a reduction in the content of EAAT2 [69,76,77]. These findings led to the hypotheses that malfunction of EAAT2 might be an important cause of motoneuron death in ALS, but it is still unknown if the loss of glutamate transporters in the tissue of ALS patients is a cause or a consequence of neuronal loss [78]. In addition, even when increased levels of glutamate in the cerebrospinal fluid and plasma of SALS patients have been described [79,80], this increase occurs in only ~40% of patients [81,82], suggesting that elevated glutamate does not seem to be the triggering factor for motoneuron death in SALS. Furthermore, recent findings from our laboratory do not support this hypothesis, because the acute [73] and chronic [83] pharmacological blockade of glutamate transport in the rat spinal cord in vivo, which results in increased concentrations of extracellular glutamate, failed to cause motoneuron death or motor deficits. Also, no neuronal damage was observed in the hippocampus and motor cortex of transgenic FALS mice in which extracellular glutamate was elevated by transport blockade [84].

Oxidative stress in ALS

There are many data supporting the involvement of oxidative stress in ALS pathogenesis. Analysis of post mortem tissue from ALS patients has revealed an increased oxidative damage of cell components as compared to controls, like oxidized DNA [85,86] and formation of carbonyl [85,87] and nitrotyrosine [88-90] derivatives in proteins. Furthermore, lipid peroxidation and protein glycoxidation were found increased in spinal cord motoneurons and glial cells [91]. Markers of oxidative damage have also been analyzed in cerebrospinal fluid and plasma from living ALS patients during the course of the disease, showing enhanced DNA oxidative damage [92,93], lipid peroxidation [94-97] and elevation of nitrotyrosine [98], although the latter result is controversial [99]. Increased oxidative damage in macromolecules has also been demonstrated in the transgenic mutant SOD1 mouse, [100-104] suggesting that oxidative stress could be involved in FALS pathogenesis. In models in vitro, spinal motoneurons exposed to an excitotoxic insult by stimulation of AMPA receptors produced a mitochondrial calcium overload that triggered mitochondrial depolarization and generation of ROS [105].

All these data support the hypothesis of oxidative stress as a mechanism that contributes to motoneuron injury in ALS, but it is still unclear whether oxidative stress is a cause or a consequence of the disease, since it may result from other cellular processes like excitotoxicity, mitochondrial dysfunction or protein aggregation.

Protein aggregation in ALS

The aggregation of misfolded proteins leads to cellular degenerative processes that ultimately cause neuronal death. This kind of disturbances is well characterized for neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's diseases, whereas in ALS, besides the previously discussed aggregation of mutant SOD1 in FALS, some toxic intracellular inclusions have been described in both SALS and FALS. The best described are changes in neurofilament composition that generate alterations in perykaria and proximal axons of motoneurons, a pathogenic characteristic of ALS described several years ago [106]. In some cases mutations were found in the heavy subunit of neurofilaments [107-109], and transgenic mice harboring mutant or overexpressed neurofilament subunits H and L show a motoneuron pathology reminiscent of that occurring in ALS [110,111].

Experimental models for the study of ALS

The rationale of the foregoing review on the advances in the knowledge of the mechanisms of motoneuron death and the hypotheses on the pathophysiology of ALS is to provide a framework for analyzing the experimental approaches that have been developed when attempting to create valid experimental models of the disease. These include experiments in vitro using diverse spinal cord preparations, and whole animal experiments, which include animals with spontaneous motoneuron degeneration, transgenic rodents, and animals in which spinal motoneuron death was produced with pharmacological tools.

In vitro models

Spinal cord cultures

Primary spinal cord cultures have been established and used to study the morphological, biochemical and electrophysiological characteristics of motoneurons for many years [112]. Generally, the tissue for cellular culture is taken from 12–14 days-old rodent embryos or 6–7 days-old chicken embryos, the spinal cord is dissociated by mechanical and enzymatic procedures and then plated on matrix-coated dishes. Motoneurons are relatively easy to identify in culture due to their large size (25 to 30 μm of diameter), but because they are present in very small quantities in the spinal cord (in a transversal section at the lumbar segment of the rat spinal cord there are less than 25 motoneurons in each side), motoneuron enriched cultures are often used instead of mixed primary cultures. Motoneuron enrichment is achieved by several methods. For example, the cellular suspension obtained after spinal cord homogenization is centrifuged in a metrizamide cushion, that separates cell bodies by cellular densities; motoneurons have a relatively low cellular density and the enriched fraction is identified by biochemical analyses of acetylcholine production [113].

Further purification of the motoneuron population can be achieved by the immuno-recognition of the nerve growth factor receptor p75 that motoneurons express since early embryonic stages. A specific antibody for the p75 receptor is immobilized in a Petri dish on which a suspension of cells is poured, and motoneurons specifically adhere to the antibody coated surface increasing their concentration [114]. Because motoneurons require a large variety of trophic factors to survive, the enriched cultures are generally seeded on top of a spinal glial feeder layer, usually obtained from the same spinal tissues that motoneurons came from. Enriched motoneuron cultures can also be obtained by flow cytometry. Motoneurons are labeled with fluorescent tracers that are injected in the developing muscle and retrogradely transported by the axons to the neuronal somas in the spinal cord, and then the labeled cells can be sorted [115]. Another way to obtain these cultures is to express an enhanced green fluorescent protein under a specific motoneuron promoter in embryonic stem cells; marked cells can be sorted and then differentiated into motoneurons in culture [116].

Modeling a complex disease in such a reduced and limited system has a series of inconveniences, but still, important information on some intracellular mechanisms can be obtained by studying the physiology of motoneurons. For example, using these systems it was first demonstrated that motoneurons are particularly vulnerable to glutamatergic excitotoxicity trough AMPA receptors [66], that the toxicity underlying this process is mediated by Ca^{2+} [71,105] and that glutamate preferentially stimulates the production of reactive oxygen species in motoneurons in comparison with other neuronal types of the spinal cord [117].

A major shortcoming of these systems is that the conditions in which motoneurons exist must be substantially modified. For instance, the mentioned particular vulnerability of motoneurons to AMPA in culture is only seen when extracellular calcium concentration is raised from physiological 2 mM to 10 mM [66,71,105]. Also, the fact that the ratio motoneurons:glia is altered is not trivial, since non-neuronal cell types play a fundamental role in the pathogenesis of this disease and accumulating experimental evidence indicates that ALS is a non cell-autonomous disease, meaning that its origin may be localized not only in motoneurons, but in the surrounding spinal cells as well [40,41,118-120]. Thus, although cultures of glial cells have helped to elucidate the participation of other cellular types in the development of ALS, like micro and astroglia toxicity due to the expression of mutant SOD1, the information obtained from these studies is limited to the particular experimental conditions employed.

NSC-34 cells

Establishing a cell line of immortalized neurons in culture is a difficult task, mainly because of the intrinsic properties of the neuronal lineage, including their null capacity to proliferate when they are fully differentiated. In an attempt to circumvent this problem, a hybrid cell line (NSC-34) of neuroblastoma (a highly proliferative neuronal cell type) and spinal cord motoneurons from enriched primary cultures, was produced by fusing the two cell types [121]. In this hybrid line some of the motoneuron characteristics are present, like acetylcholine synthesis, storage and release, action potential generation, expression of neurofilament proteins and association with neuromuscular synapse-specific basal lamina glycoproteins.

The NSC-34 line expressing mutant SOD1 is considered a cellular model of ALS. In these cells some features of motoneuron alterations have been described, for example, Golgi apparatus fragmentation [122], and mitochondrial dysregulation [123]. They have also been used to study in vitro some of the mechanisms of mutant SOD1 toxicity [124-126]. Nonetheless, NSC-34 cells also retain characteristics of the neuroblastoma linage, like an enhanced N-myc action [121]. N-myc is an oncogene that directs diverse cellular responses, especially those involved in proliferation [127]. For the study of the mechanisms of neuronal death and its prevention by possible therapeutic agents the effects of N-myc could obstruct the underling mechanisms, making this model faulty.

Organotypic cultures

All neurons exist in a specific tissue context where the surrounding cellular types shape the biochemical, electro-

physiological and morphological characteristics of each neuron. Motoneuron excitatory or inhibitory inputs and outputs from their afferences and interneurons, as well as trophic support from surrounding glia and its reactivity in response to neuronal death, modulate important characteristics of the cellular and molecular processes that occur in ALS. When spinal cord is disgregated and plated on dishes, all these important cellular interactions are lost. One way to preserve the tissue structure for in vitro analyzes is to cultivate an entire slice of the spinal cord in an organotypic culture. With this method, spinal cord slices obtained from neonate pups are chopped into 400 μm thick sections that can be cultured for as long as 3 months. Motoneurons in this type of preparations retain their metabolic characteristics like choline acetyltransferase and acetylcholinesterase activities [128].

Slow motoneuron degeneration in organotypic cultures has been induced by the chronic exposure to the glutamate transporter blocker threo-β-hydroxyaspartate (THA) [129]. Using this system it has been reported that neurotrophic factors are able to protect motoneurons from excitotoxic death [130,131]. As with the cell cultures, a major shortcoming of organotypic systems is that they do not always accurately reproduce what would be happening in an in vivo system and even less in an ALS patient. For example, while the glutamate transport blockade is highly toxic for motoneurons in organotypic cultures [129-131], as already mentioned the blockade of glutamate uptake in vivo is innocuous for motoneurons [73,83,84].

In vivo models

Spontaneous genetic defects that cause motoneuron degeneration

The oldest known model of spontaneous motoneuron alterations is the wobbler mouse. This mouse harbors a mutation in the *wr* locus that has been mapped in chromosome 11 [132]. It was recently found that this gene that codes for the vacuolar-vesicular protein sorting 54 (Vps54) has the missense mutation L967Q in the wobbler mouse [133]. Homozygous expression of the mutant *wr* gene causes the death of motoneurons in the cervical portion of the spinal cord, affecting mainly the somas, and in consequence, causing a proximal axonopathy [134]. The pathology presented by these animals may have a glial origin, since they develop astrocytic defects and an increased astrocyte reactivity that seems independent from motoneuron death [135].

The mechanisms of neurodegeneration in the wobbler mice are so far unidentified. Excitotoxic processes seem not be involved, since glutamate transporters are expressed normally, extracellular glutamate levels are unchanged [136], and AMPA receptor antagonists have no effect on motor behavior and neuronal loss [137]. Also, results of TUNEL staining of fragmented DNA [138], and activation of caspases suggest that apoptosis is not involved in neuronal death in these mice [139].

Another model of spontaneous motoneuron death is a mouse that suffers a progressive motoneuronopathy (pmn), caused by the homozygous expression of the mutant tubulin-specific chaperone E [140,141]. These animals develop a progressive caudo-cranial degeneration of the motor axons and die at few weeks after birth [142]. The main pathological characteristic of the pmn mouse is a distal axonopathy with minor alterations of neuronal somas, an alteration different from the ALS pathology [143]. Although the pathophysiology of motoneuron death in these animals is not the same as in ALS, the neuroprotective potential of some agents, like certain trophic factors [144,145] or anti-excitotoxic compounds [146] have been shown to be partially effective.

The wasted mutant mouse with genotype *wst*/*wst* is another murine model of spontaneous spinal neurodegeneration. These animals develop a hindlimb paralysis, not necessarily due to motoneuron loss but to cell vacuolization [147]. There are reported cases of spontaneous motoneuron disease in larger animals. A spontaneous disease of the horse, known as equine motoneuron disease, is characterized by a generalized weakness, progressive muscle atrophy and loss of motoneurons in the spinal cord and brainstem [148]. In dogs, a condition named hereditary canine muscular atrophy is caused by a mild loss of motoneurons in spinal cord and brainstem and neurofibrillary swellings in proximal axons [149]. These animals may mimic in some way the pathology underlying sporadic ALS, but their use in experimental studies is clearly complicated.

Genetically modified models

- Alsin knock out mice

Human mutations in the alsin protein encoded by the *ALS2* gene in some FALS cases were mentioned in a previous section. *ALS2* knock out mice have been developed and shown to be deficient in motor coordination and motor learning [150], to have abnormalities in endosome trafficking [151,152], as well as axonal degeneration [153-155] and increased susceptibility to oxidative stress [150]. Although no overt motoneuron degeneration is present, these animals appear to be a good in vivo model for the study of the alterations of motoneuron physiology that take place before cellular death such as axonal impairment, but, as in the case of mutant SOD1 transgenic mice described next, the processes studied may mimic only those occurring in patients with this type of genetic alterations.

- Transgenic mutant SOD1 rodents

A major breakthrough in the ALS research was the discovery that ~20% FALS cases were due to mutant SOD1 [5].

Almost immediately after this finding, transgenic mice that express mutant human SOD1 were developed. The initial mutations produced the substitution of glycine for alanine at position 93 (G93A), and of alanine for valine at position 4 (A4V) [156]. Other SOD1 mutations have been expressed in transgenic mice. The commonest, in addition to G93A and A4V, are glutamate for arginine substitution at positions 37 (G37R) [157] and 85 (G85R) [158]. The phenotype expressed by these mice, regarding the age at symptoms onset and their severity is directly proportional to the amount of mutant protein expressed in the tissue [159], lending further support to the hypothesis of the acquired toxic function of mutant SOD1 mentioned before.

The disease in transgenic mice begins with hindlimb weakness, impaired leg extension and shortened stride length, and continues to complete paralysis of the limbs, principally the rear ones, within few days [156]. Cellular alterations are mainly characterized by vacuolar degeneration of motoneurons and their processes at the early stages, followed by neuronal loss and atrophy of the ventral horns in the spinal cord at the late stages; the most damaged tissue is the spinal cord, but the medulla, pons and midbrain are affected as well [34]. In addition, mice expressing the G85R mutation in the murine SOD1 develop paralysis due to motoneuron loss similar to that presented by the transgenic expression of human mutant SOD1 [160]. Transgenic rats expressing mutant human SOD1 have also been developed and display a similar pathology [161,162].

Since no symptomatic or pathological evident differences exist between FALS and SALS, it has been assumed that the mechanisms underlying both types of the disease are shared. Therefore, transgenic SOD1 animals have been extensively used in numerous studies related to ALS, principally because the phenotype they display is elicited by the only proven cause of the disease. In this FALS model all the primary hypothesis on ALS origin have been put to challenge, including oxidative stress [31,163,164], excitotoxicity [84,165], apoptosis [39,166,167], protein aggregation [39], axonal dysfunction [168], mitochondrial failure [40,166,167], endoplasmic reticulum stress [32,33,169], and practically every other suspected mechanism. This model has also been the golden standard for drugs and therapies testing at experimental stages. Nonetheless, the major weakness of this FALS model is that it reproduces the mechanism of neuronal death that occurs in only ~2% of ALS cases. Therefore, it is not completely accurate to extrapolate the findings made in this model to the processes occurring in patients that do not bear mutations in SOD1 [165]. In addition, a meta-analysis of a vast number of data on the therapeutic action of many drugs in SOD1 mutant mice revealed that most of these experiments show serious methodological problems, such as a bias for reporting beneficial effects, small number of animals, lack of randomization and blinding and the initiation of the treatment prior to symptoms onset, something that cannot be done in human ALS [170]. These problems may be the reason for the lack of reproducibility of the therapeutic effect of several drugs when tested in large cohorts and in blind manner, as was described in detail in another recent study [171]. This clearly urges the necessity to test these potential treatments in non familial related models of motoneuron degeneration.

Models of non-genetic spinal motoneuron degeneration

There are much less animal models for SALS than for FALS, in spite of the fact that it accounts for >90% of the cases. Taking advantage of the knowledge on motoneuron death by excitotoxicity discussed above, our group has developed some acute and chronic experimental models that should be useful for studying the mechanisms of spinal motoneuron death and for testing potential therapeutic agents. The objective of the first experiments in this effort was to test whether the excitotoxicity produced by N-methyl-D-aspartate and AMPA/kainate receptor agonists or by endogenous glutamate might induce spinal motoneuron death and paralysis in the living rat. The experimental procedure used was microdialysis in the lumbar spinal cord, which permits the perfusion of drugs and at the same time the collection of extracellular fluid for the measurement of glutamate and other amino acids. Using this method we found that increasing the concentration of endogenous extracellular glutamate through the inhibition of glutamate transport failed to cause motor alterations or motoneurons loss, results that do not support the hypothesis of glutamate-mediated excitotoxicity resulting from the loss of glutamate transporters. On the other hand, perfusion of AMPA produced permanent paralysis of the ipsilateral hindlimb and a remarkable loss of spinal motoneurons, which started ~3–6 h after the beginning of the perfusion and progressed until reaching a maximum at 12 h, when practically all motoneurons in the spinal segment studied were lost. All these effects were prevented by the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) and by an inhibitor of proteases [73,172].

The fact that the motoneuron death in our model begins only 3–6 h after the infusion of AMPA, and becomes complete at 12–24 h led to the hypothesis that the entry of calcium, probably through the calcium-permeable AMPA receptor channel, induces a delayed deleterious process leading to motoneuron death. To test this hypothesis, AMPA was co-applied with 1-naphthyl acetyl spermine (NAS) a selective blocker of the AMPA receptor that lacks the GluR2 subunit [67,173-175]. This compound significantly prevented the selective spinal motoneuron loss and

the subsequent paralysis [74], indicating that rat spinal cord motoneurons possess functional calcium-permeable AMPA receptors lacking GluR2 and suggesting that the cellular process leading to motoneuron death in this model, in vivo, is triggered by an increase of intracellular calcium via these receptors. The hypothesis that such an increase is responsible for the damage was confirmed by the co-perfusion of the intracellular calcium chelator 1,2-bis-(o-aminophenoxy)-ethane-N,N,-N',N'-tetraacetic acid tetraacetoxy-methyl ester (BAPTA-AM) with AMPA, which was as effective as NAS in the prevention of the motoneuron damage and the paralysis [74]. The relevance of these findings is noteworthy because, differently from the experiments in vitro mentioned in a previous section, the neuronal death due to increased cytoplasmic Ca^{2+} occurs under the physiological extracellular concentration of the cation (2 mM). Altogether, these data suggest that AMPA receptors may have an important role in the development of ALS.

The main limitation of the model discussed above is that the paralysis develops within a few hours, whereas motoneuron death in ALS is a chronic process that takes lengthened time periods. For this reason, we designed a different experimental approach, allowing the continuous slow infusion of AMPA in the spinal cord during several days using osmotic minipumps. This procedure generated a chronic model of spinal motoneuron degeneration induced by excitotoxicity, because it produced progressive motor impairment and motoneuron death along several days, depending on the AMPA concentration, resembling the characteristics of neurodegeneration and paralysis that are present in both ALS patients and FALS rodents [176]. Interestingly, we demonstrated that the coinfusion of vascular endothelial growth factor (VEGF) with AMPA remarkably protected against the deleterious chronic effect of the latter, indicating that this chronic model may indeed be useful for testing therapeutic strategies for ALS [176].

We have thus developed in vivo models of acute and chronic spinal motoneuron degeneration, in the absence of altered genetic components, which are useful for studying the mechanisms of this degeneration and for assaying potential neuroprotective compounds. As discussed throughout this article, this is relevant in view of the need of experimental animal models that reproduce motoneuron degeneration in processes such as sporadic ALS, which are not related to genetic alterations and occur in the great majority of ALS cases. The main limitation of our model is that, although glutamate-mediated overactivation of AMPA receptors may be a relevant mechanism of spinal motoneuron degeneration in ALS, convincing evidence that this occurs in the human disease has not yet been obtained.

Conclusion

In spite of the high complexity of ALS, it is clear that great progress has been made regarding the mechanisms of motoneuron death. However, one of the strongest obstacles is the insufficient information on the cause of the selectivity of motoneuron degeneration, because many of the postulated mechanisms are common for the death of other types of neurons located in several brain regions. This is the case, for example, of Parkinson's and Alzheimer's diseases. The intracellular Ca^{2+}-dependent neuronal death via overactivation of Ca^{2+}-permeable AMPA receptors may be an important factor for this selectivity, because of the abundance of this type of glutamate receptors in spinal cord motoneurons.

It is also evident that valuable data have been obtained from experimental approaches in vitro, and that the combination of these results with those generated in models in vivo may lead to a better understanding of the pathophysiology of the disease and therefore to the design of effective therapeutic measures. Nevertheless, it appears that the limitations of the models in vitro are far greater than those of in vivo models, despite the difficulties of the experiments in vivo and their interpretation, correlations between cellular alterations and motor behavior can only be obtained in the whole animal.

Most of the experimental models of ALS are in fact FALS models, because they are transgenic rodents expressing human mutant SOD1. It is therefore very relevant to develop in vivo models of SALS, responsible for >90% of all ALS cases. Such models can be generated by applying the limited knowledge on the mechanisms already available, such as those related to excitotoxicity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LBTR, LDSC and RT reviewed the literature, wrote, read and approved the final manuscript.

Acknowledgements

This work was supported by CONACYT, Mexico (project 60322) and DGAPA, UNAM (project IN209807).

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Desarrollo del modelo crónico de neurodegeneración espinal excitotóxica *in vivo*

El fundamento de este modelo consiste en administrar AMPA de manera crónica directamente en la médula espinal de la rata, e inducir la muerte de las motoneuronas espinales por excitotoxicidad. Para este fin implantamos en el parénquima dorsal de la región lumbar de la médula espinal una cánula conectada a una minibomba osmótica que permite la infusión continua de una sustancia. El procedimiento fue variado en algunos experimentos para permitir la infusión de sustancias diferentes contenidas en dos minibombas a través de la misma cánula y para permitir también la inserción de una cánula de microdiálisis para evaluar la concentración extracelular de diferentes aminoácidos después de un periodo determinado de tratamiento. La metodología experimental esencial para el implante de la cánula en la médula espinal se describe a continuación.

Ratas Wistar adultas se anestesiaron con 5% halotano en una mezcla de 95% O_2/5% CO_2 y se colocaron en un aparato estereotáxico. Se realizó una incisión longitudinal de la piel en la región lumbar del dorso del animal y los músculos adyacentes fueron retraídos para exponer la porción lumbar de la columna vertebral. Se hizo una perforación de 1 mm de diámetro en la lámina izquierda de la segunda vértebra lumbar y se colocó un tornillo de acero inoxidable (1 mm de diámetro; 3.7 mm de largo) que sirvió posteriormente como ancla para el implante. Se realizó una laminectomía de ~2 mm en el lado derecho de la misma vértebra y se estimuló ligeramente la médula espinal en esa porción, causando la contracción de la pata ipsilateral, para cerciorarse de que el implante estuvo en la región correcta. Posteriormente se hizo un pequeño corte de las meninges y se introdujo la punta de una cánula (2 mm de largo y 0.24 mm de diámetro) en la región dorsal del parénquima de la médula espinal. La laminectomía se selló con gelfoam y se colocó acrílico dental sobre el tornillo y el soporte plástico de la cánula. La cánula se conectó utilizando un sistema de catéteres a la minibomba osmótica (Alzet, modelo 2004, con una capacidad aproximada de 200 µL y un flujo de 0.25 µL/h; Durec, Cupertino, CA; Figura 2) la cual se implantó de manera subcutánea. Finalmente la incisión de la piel se cerró con grapas quirúrgicas y las ratas recibieron una dosis de penicilina. Como controles experimentales se implantaron minibombas cargadas con medio Ringer-Krebs. Ninguna de las ratas control presentó alteraciones motoras ni daño neuronal, indicando que el procedimiento quirúrgico del implante no tiene un efecto deletéreo en el animal.

La caracterización conductual del efecto de la infusión crónica de AMPA, así como la neurodegeneración causada por la misma se describen detalladamente en el artículo V en la sección de Resultados.

Figura 2. Minibomba osmótica.

Microdiálisis

La microdiálisis es un método empleado en diversos estudios del SNC para determinar la liberación de diferentes neurotransmisores bajo distintas condiciones experimentales. La microdiálisis funciona a través del implante en el tejido de una doble cánula concéntrica (Figura 3) que tiene en su extremo una membrana de copolímero de poliéter-policarbonato semipermeable, que permite la difusión pasiva de agua, solutos y moléculas de relativamente bajo peso molecular, los cuales fluyen a través de su gradiente de concentración. La cánula se conecta a un sistema de bombeo que permite perfundir líquidos a un flujo constante y recuperar un volumen en un tiempo determinado. El porcentaje de recuperación es inversamente proporcional a la velocidad de perfusión.

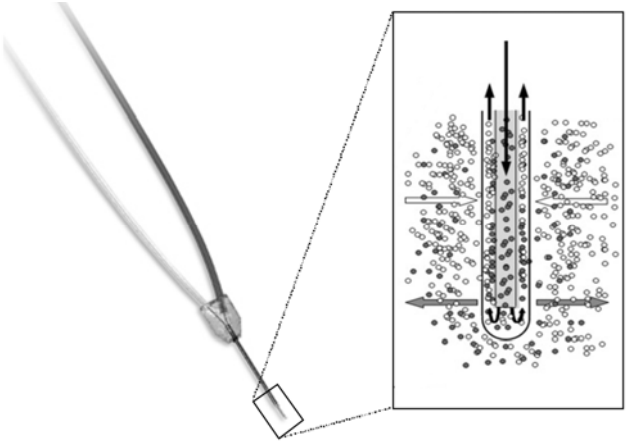

Figura 3. Cánula de microdiálisis. En el recuadro de la derecha se esquematiza el flujo a través de la membrana de microdiálisis. La flecha negra larga indica el flujo de perfusión y las cortas el de recuperación. Las flechas abiertas indican los intercambios de solutos entre el medio extracelular y el medio de perfusión a través de la membrana de diálisis.

Evaluación de la conducta motora

Para la caracterización conductual del modelo crónico de neurodegeneración espinal excitotóxica y para la evaluación de los efectos de los tratamientos administrados mediante las minibombas osmóticas, se emplearon 3 pruebas conductuales que se describen a continuación. Para estas pruebas todas las ratas fueron entrenadas dos días antes del implante de la cánula el cual se consideró como el día cero.

Rotarrod

El rotarrod es una prueba que permite la evaluación de la coordinación de los 4 miembros del animal. Consiste en un aparato con un eje cilíndrico elevado que gira con una velocidad constante y se determina el tiempo que los animales permanecen caminando sobre él antes de caer. Para la prueba de rotarrod, las ratas caminaron en un carril individual sobre el cilindro giratorio con una velocidad inicial de 10 rpm y una aceleración constante de 0.2 rpm/s. En cada punto los animales se evaluaron tres veces y se registró el mayor tiempo antes de que cayeran con un límite superior de 120 segundos.

Paw grip endurance

En la prueba de asimiento de la pata (PGE, por sus siglas en inglés) los animales se colocan en una superficie que gira verticalmente a la cual pueden asirse y trepar para alcanzar una plataforma estable cuando no existen problemas motores asociados a un estado de parálisis (Weydt et al. 2003). Para esta prueba, las ratas se colocaron individualmente sobre una rejilla de 30 $\times$ 19 cm orientada horizontalmente y conectada a un rotor mecánico. La rejilla se giró delicadamente (3 rpm) hasta que alcanzó una posición vertical. El tiempo que las ratas tomaron para trepar a la cima de la rejilla y alcanzar una superficie estable a partir de que la rejilla alcanzó la posición vertical o el tiempo que tardaron en caer de la rejilla cuando no pudieron trepar fue registrado con un límite máximo de 30 segundos. Los datos presentan el mejor de 3 intentos en cada punto y están graficados en los resultados con escalas de "tiempo para trepar" y "latencia para caer" como un continuo, porque los animales que son capaces de trepar pueden alcanzar la cima de la rejilla dentro de 30 segundos, de otra forma se sujetan de la rejilla y finalmente caen.

Patrón de la zancada

Para una evaluación cualitativa del inicio y el progreso de la parálisis en estos experimentos analizamos el patrón de la zancada del tren posterior de las ratas. Para este fin se tiñeron las plantas de las patas traseras de las ratas con tinta china y se hizo a los animales caminar sobre una pasarela de papel. Sobre el patrón de la zancada no se hicieron determinaciones cuantitativas.

Resultados I. Probable origen de estímulos excitotóxicos en la ALS (rata silvestre)

Chronic elevation of extracellular glutamate due to transport blockade is innocuous for spinal motoneurons in vivo

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Neurochemistry International 2009, **54**:186-191

Resumen

Se ha considerado que la excitotoxicidad mediada por glutamato juega un papel importante en el mecanismo de muerte neuronal en la ALS, y que la excitotoxicidad puede ser debida al decremento del transporte de glutamato su consecuente elevación extracelular. Nosotros hemos mostrado previamente que incrementos poco duraderos del glutamato extracelular, debidos a la administración del bloqueador no selectivo del transportador de glutamato PDC por microdiálisis en la médula espinal de la rata, no inducen daño a las motoneuronas. En este trabajo examinamos el potencial involucramiento del bloqueo crónico del transporte de glutamato como un factor causante de la muerte de las motoneuronas espinales y la parálisis *in vivo*. Usando minibombas osmóticas, infundimos directamente en la médula espinal hasta por 10 días PDC y otro bloqueador del transporte de glutamato, DL-treo-β-benziloxiaspartato (TBOA), y medimos a través de microdiálisis y HPLC la concentración extracelular de glutamato y de otros aminoácidos. Encontramos que después de la infusión tanto de PDC como de TBOA la concentración del glutamato extracelular endógeno era de 3 a 4 veces más alta que la de los controles. Aún así, a pesar de ésta elevación no se observaron ni degeneración de las motoneuronas ni gliosis, como se determinó mediante la evaluación histológica y la inmunohistoquímica para colin-acetiltransferasa y la proteína ácida fibrilar glial (GFAP). De acuerdo con esta falta de efectos tóxicos, no se observaron déficits motores como se determinó a través de 3 pruebas de actividad motora. Dado que nosotros probamos que bajo condiciones experimentales idénticas la infusión de AMPA induce la muerte progresiva de las motoneuronas y parálisis (como se verá en el siguiente artículo en esta tesis), concluimos que una elevación prolongada del glutamato extracelular debida al bloqueo de su transporte *in vivo* es inocua para las motoneuronas espinales y por lo tanto estos resultados no apoyan la hipótesis de que la deficiencia del transporte de glutamato juega un rol crucial como factor causante de la degeneración de las motoneuronas espinales en la ALS.

Neurochemistry International 54 (2009) 186–191

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Chronic elevation of extracellular glutamate due to transport blockade is innocuous for spinal motoneurons in vivo

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ARTICLE INFO

Article history:
Received 14 July 2008
Received in revised form 24 September 2008
Accepted 30 September 2008
Available online 3 December 2008

Keywords:
Spinal cord
Glutamate transport
Amyotrophic lateral sclerosis
Motoneurons

ABSTRACT

Glutamate-mediated excitotoxicity has been considered to play an important role in the mechanism of spinal motoneuron death in amyotrophic lateral sclerosis (ALS), and some reports suggest that this excitotoxicity may be due to a decreased glutamate transport and the consequent elevation of its extracellular level. We have previously shown that short lasting increments in extracellular glutamate due to administration of the non-selective glutamate transport blocker L-2,4-trans-pyrrolidine-dicarboxylate (PDC) by microdialysis in the rat spinal cord do not induce motoneuron damage. In the present work we examined the potential involvement of chronic glutamate transport blockade as a causative factor of spinal motoneuron death and paralysis in vivo. Using osmotic minipumps, we infused directly in the spinal cord for up to 10 days PDC and another glutamate transport blocker, DL-threo-β-benzyloxyaspartate (TBOA), and we measured by means of microdialysis and HPLC the extracellular concentration of glutamate and other amino acids. We found that after the infusion of both PDC and TBOA the concentration of endogenous extracellular glutamate was 3–4-fold higher than that of the controls. Nevertheless, in spite of this elevation no motoneuron degeneration or gliosis were observed, assessed by histological examination and choline acetyltransferase and glial fibrillary acidic protein immunocytochemistry. In accord with this lack of toxic effect, no motor deficits, assessed by three motor activity tests, were observed.

Because we had previously shown that under identical experimental conditions the infusion of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) induced progressive motoneuron death and paralysis, we conclude that prolonged elevation of extracellular glutamate due to its transport blockade in vivo is innocuous for spinal motoneurons and therefore that these results do not support the hypothesis that glutamate transport deficiency plays a crucial role as a causal factor of spinal motoneuron degeneration in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective and progressive loss of motoneurons in the spinal cord, brainstem and motor cortex. ALS occurs in two main forms, the familial (FALS) that accounts for about 10% of cases and the sporadic (SALS) that comprises the remainder 90%. To date, the precise mechanisms that cause the selective death of motoneurons are not understood, but there is evidence suggesting that motoneuron degeneration in ALS may be related to disturbances of glutamatergic neurotransmission leading to excitotoxicity. Abnormalities in glutamate metabolism (Plaitakis, 1990; Rothstein et al., 1990), and elevated glutamate levels in the cerebrospinal fluid of SALS patients (Shaw et al., 1995; Spreux-Varoquaux et al., 2002) have been found by some groups, but not by others (Perry et al., 1990). Furthermore, there is evidence for a reduction of the glutamate transporter EAAT2 in motor cortex and spinal cord of SALS patients (Rothstein et al., 1992, 1995). Thus, it has been suggested that deficiencies in the expression and function of the glutamate transporter system may result in elevated levels of extracellular glutamate, which would lead to neuronal death through excitotoxicity. However, we have previously demonstrated that the infusion by means of reverse microdialysis of the glutamate transport blocker L-2,4-trans-pyrrolidine-dicarboxylate (PDC) in the rat lumbar spinal cord in vivo results in a remarkable increase in the endogenous extracellular glutamate concentration (about 5-fold) and that this increase elicited no damage to motoneurons and no motor alterations (Corona and Tapia, 2004). As the accumulation of

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doi:10.1016/j.neuint.2008.09.015

glutamate in these acute experiments lasted for only 1 h, which was the period of PDC infusion, it is possible that a neurotoxicity did not occur because of the short duration of the exposure to increased glutamate, in spite of the fact that an even shorter exposure to α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) induced paralysis and motoneuron death by overactivation of AMPA-type glutamate receptors permeable to Ca^{2+} (Corona and Tapia, 2004, 2007). Therefore, we have now studied the effects of prolonged blockade of glutamate transport on the levels of extracellular glutamate and neurodegeneration in the rat spinal cord, and on the rat motor behavior.

For this purpose, we used two inhibitors of glutamate uptake, the transportable blocker PDC, which may exchange with intracellular glutamate, and the non-transportable blocker DL-threo-β-benzyloxyaspartate (TBOA). These compounds were continuously infused in the rat lumbar spinal cord with the help of osmotic minipumps, and the motor behavior of the rats was studied and correlated with spinal motoneuron histology. Using this system of continuous delivery through osmotic minipumps, we have previously shown that the chronic infusion of AMPA induces a progressive and permanent bilateral motor paralysis due to the death of spinal motoneurons (Tovar-y-Romo et al., 2007), thus validating this procedure.

2. Materials and methods

2.1. Animals and surgical procedures

Adult male Wistar rats (290–310 g) were used in all the experiments. They were housed in a laboratory environment with a 12 h artificial light/dark cycle and with food and water ad libitum. Procedures were performed in accordance with the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care Committee, and all efforts were made to minimize animal suffering and reduce the number of animals used.

Implantation of osmotic minipumps was performed as described (Tovar-y-Romo et al., 2007). Briefly, rats were anesthetized with halothane in a 95% O_2/5% CO_2 mixture and placed in a stereotaxic spinal unit. Lumbar vertebrae were exposed and the spinous process of the second lumbar vertebra was removed. Two ~1 mm holes were drilled on the left (contralateral) side of the lamina, and stainless-steel screws (1 mm diameter; 3.7 mm long) were inserted without reaching the surface of the spinal cord in order to anchor the implant. A laminectomy of the right side of the same vertebra was made and after cutting the meninges the tip of the inner cannula (2 mm long and 0.24 mm diameter) of a microdialysis probe (described below), from which the dialysis membrane and the outer cannula were removed, was inserted. A cannula guide was placed rostrally to the infusion cannula and was lowered 0.3 mm down the surface of the spinal tissue. Dental cement was poured over the screws, the plastic support of the infusion cannula, and the base of the cannula guide. The osmotic minipump (Alzet, Model 2004, approximate capacity 200 μL, flow rate 0.25 μL/h; Durec, Cupertino, CA, USA) was placed subcutaneously in the back of the rat, caudally to the skin incision, and its tubing was attached to the cannula. Finally, the skin incision was closed with surgical clips and rats received an i.m. dose of penicillin and were returned to their cages.

The minipumps were filled with Ringer–Krebs medium of the following composition (in mM): 118 NaCl, 4.5 KCl, 2.5 $MgSO_4$, 4.0 NaH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 10 glucose, pH 7.4. In control animals the pump contained only this medium; in the experimental groups 50 mM PDC (Tocris, Ellisville, MO, USA) or 50 mM TBOA (Tocris) was added to the medium and NaCl concentration was reduced to 93 mM to maintain iso-osmolarity. Pumps were filled 24 h before the surgery and immersed in a sterile saline solution at 37 °C for stabilization.

2.2. Assessment of motor function

Motor assessment was performed as previously described (Tovar-y-Romo et al., 2007). Rats were trained for 2 days prior to the surgery on two motor tests: a variation of the paw grip endurance (PGE) task (Weydt et al., 2003) and a rotarod test (Columbus Instruments, Columbus, OH, USA). After implantation animals were evaluated in each test routinely until the time of fixation. For the PGE test, rats were placed on a horizontally oriented grid (30 cm × 19 cm) attached to a mechanical rotator that turned the grid to a vertical position. The time taken by the rats for climbing to the top of the grid, or the latency to fall from the grid if they were unable to climb was scored with a cutoff time of 40 s. Data are presented in the figure as time to climb because none of the animals developed motor deficits (see Section 3). For the rotarod test, rats walked on an accelerating (0.2 rpm/s) rod, starting from 10 rpm for three trials, the time before falling was scored with a cutoff of 120 s. In addition to the motor tests described, a qualitative evaluation was performed on the overall stride pattern analyzing the rear footprints obtained after staining with black ink the hindpaws and making the rats walk along a paper runway.

2.3. Microdialysis and amino acid analysis

To assess the concentration of extracellular glutamate, rats were subjected to spinal cord microdialysis 7–10 days after the beginning of infusion of PDC, TBOA or Krebs medium (control). This time period was chosen because infusion with AMPA by the same osmotic minipump procedure resulted in a dose-dependent severe progressive motor deficit starting 2–5 days after the beginning of the infusion, and by days 6–12 all rats showed partial or complete hindlimb paralysis (Tovar-y-Romo et al., 2007). Animals were anesthetized with 5% halothane in a 95% O_2/5% CO_2 mixture and placed in the stereotaxic spinal unit. They were maintained under low anesthesia (1–2% halothane) throughout the experiment. The surgical clips were removed and the skin incision was pulled apart to expose the implant; then a microdialysis probe (dialysis membrane 2 mm long and 0.24 mm diameter, CMA/7, Stockholm, Sweden) was inserted through the previously implanted cannula guide. The probes were perfused continuously with Ringer–Krebs, at a flux rate of 2 μL/min, using a microsyringe mounted on a microinjection pump (model CMA/100, Carnegie, Sweden). Ten consecutive fractions of 25 μL (12.5 min) of perfusate were collected. At the end of the experiment, rats were transcardially fixed for histology and immunohistochemistry, as described below.

Glutamate was measured in the dialysates by HPLC, as previously described (Massieu et al., 1995; Salazar et al., 1994). Briefly, the 25 μL collected fractions were derivatized with the same volume of *o*-phthaldialdehyde and injected into a Beckman liquid chromatograph system. An ODS column (25 cm × 4 mm internal diameter) was used, and the column effluent was monitored with a fluorescence detector. The mobile phase was methanol/potassium acetate (0.1 M, pH 5.5) and was run at a rate of 1.5 mL/min in a 25–75% methanol linear gradient (15 min duration). Besides glutamate this procedure allows the measurement of aspartate, glutamine, glycine, taurine and alanine.

2.4. Histology and immunohistochemistry

For histological and immunohistochemical analyses, immediately after the microdialysis rats were perfused transcardially with 250 mL of ice-cold 0.9% saline, followed by 250 mL of ice-cold 4% paraformaldehyde in phosphate buffer pH 7.4. Spinal cords were removed, postfixed at 4 °C, and successively transferred to sucrose solutions (up to 30%). Transverse 40 μm sections of the lumbar region, where the infusion was made, were obtained in a cryostat. Alternate sections were mounted on gelatin-covered slides and processed for histology (cresyl violet staining), or left free-floating on pH 7.4 phosphate-buffered saline 0.9% (PBS) for double immunohistochemistry for choline acetyltransferase (ChAT) and glial fibrillary acidic protein (GFAP). The latter sections were incubated in 5% bovine serum albumin and normal rabbit serum (1:25) in PBS–Triton X-100 (0.3%) for 2 h, and then incubated with a goat polyclonal anti-ChAT antibody (1:100, Chemicon, Temecula, CA, USA) and a mouse anti-GFAP antibody (1:1000 Sigma) for 48 h with shaking at 4 °C. Sections were then washed 3 times for 10 min each in PBS–Triton X-100 (0.3%) and incubated with a biotynil-conjugated anti-goat antibody (1:200, Vector, Burlingame, CA) for 1 h. After 3 washes in PBS–Triton X-100, sections were incubated for 2 h with avidine–Texas Red conjugate (1:200, pH 8.2, Vector) and a fluorescein-conjugated anti-rabbit antibody (1:250 Zymed, San Francisco, CA) for 2 h. Finally, sections were washed 3 times for 10 min in PBS and mounted on silane (γ-methacryloxypropyltrimethoxysilane; Sigma)-covered slides and coverslipped with fluorescent mounting medium (DAKO, Carpinteria, CA). Cross-reactivity was excluded by appropriate controls incubated in the absence of primary antibodies; these control sections showed no immunostaining.

Sections were visualized under confocal microscopy (Olympus IX81) and merged images are the overlay of 2 laser sections in the Z plane merged for the FITC and Texas Red channels with the Olympus Fluoview 1.6 Viewer. Morphologically undamaged motoneurons (i.e. >25 μm diameter with a distinguishable nucleus) stained with cresyl violet were counted in a 10× microscopic field. The number of cells was determined in sections where the trace of the infusion cannula was evident; five sections per rat were analyzed and the values were averaged.

2.5. Statistical analysis

Comparisons regarding changes in amino acid levels in dialysates and number of motoneurons were made with ANOVA followed by a Fisher's post hoc test. A value of $p < 0.05$ was considered statistically different.

3. Results

3.1. Chronic blockade of glutamate transport does not induce motor alterations

As previously described (Tovar-y-Romo et al., 2007), none of the control rats infused with Ringer–Krebs medium ($n = 4$) showed

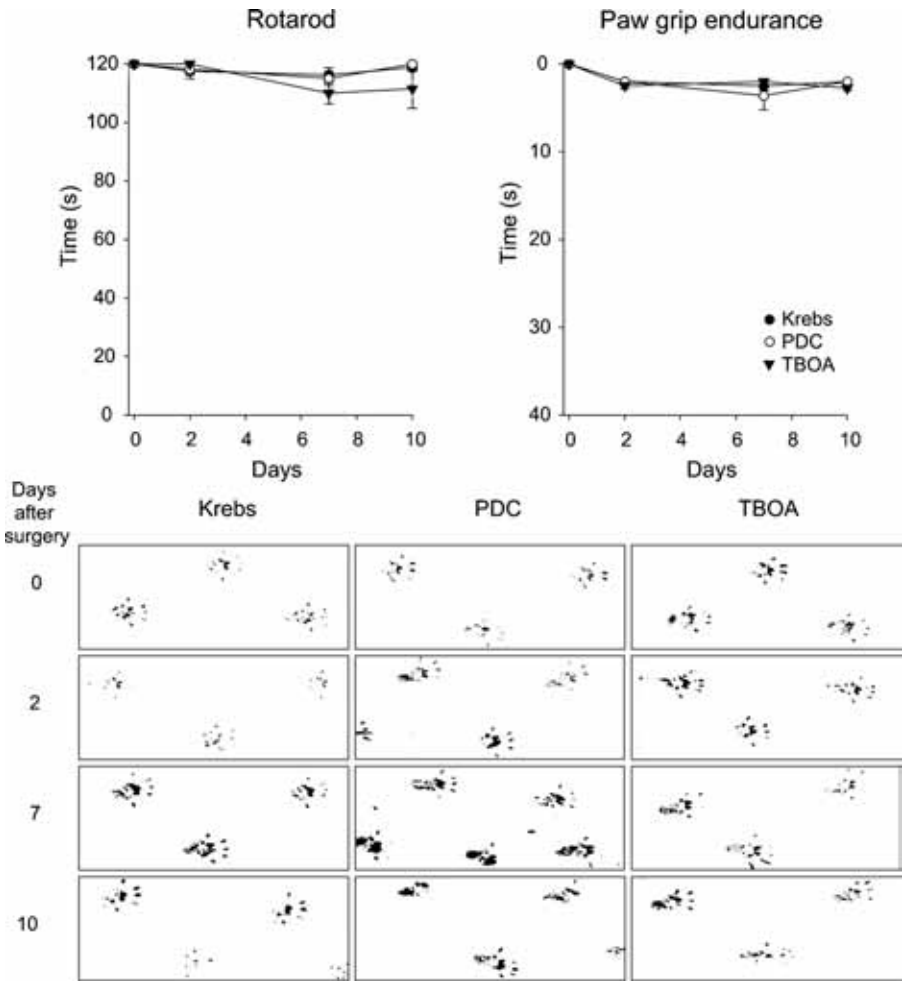

Fig. 1. Chronic blockade of glutamate transport does not induce motor deficits. Upper panel, time course of the Rotarod and paw grip endurance performances of rats infused with Krebs medium alone (control, $n = 4$), PDC ($n = 7$) and TBOA ($n = 4$). There were no statistical differences between the scores of the groups in both tests. Lower panel, representative hindpaw footprints of rats chronically infused with Krebs medium (control), PDC and TBOA, at different days after minipump implantation. Note that the footprint patterns of the experimental groups are not different from baseline or from that with Krebs medium alone.

motor dysfunctions at any time up to 10 days, the maximum period studied. Rats in this group were able to walk normally after the recovery from anesthesia until the time they were subjected to spinal microdialysis (Fig. 1). None of the rats infused with PDC showed any alteration in the walking pattern, or any motor deficit in the motor behavioral tests (Fig. 1). In the group treated with TBOA, 2 out of 6 rats presented paralysis of the rearlimbs, similar to that observed in rats treated with AMPA (not shown) (Tovar-y-Romo et al., 2007). The other four animals in this group behaved as those treated with Krebs medium or PDC, since they did not show any sign of motor alterations up to the time of microdialysis, 7–10 days after implantation (Fig. 1). However, as will be described in the next section, in these rats the concentration of extracellular glutamate was elevated whereas in the two affected rats this concentration was normal.

3.2. Continuous PDC or TBOA infusion leads to increased levels of extracellular glutamate

In order to assess whether extracellular glutamate had indeed been elevated in the spinal cord of the rats infused with PDC or TBOA, we measured the extracellular levels of this amino acid by microdialysis, 7–10 days after the beginning of the blockers infusion, as described in Section 2. In the control and the PDC- or TBOA-treated rats the values in the first 12.5 min were relatively high because the collection of fractions for amino acid analysis started immediately after insertion of the microdialysis cannula. This was done because it was important to know the glutamate basal levels as soon as the probe was in place, even before an equilibration period. Thereafter, in the control rats the concentration stabilized at the normal extracellular values of 10–20 pmoles/10 μL of glutamate (Corona and Tapia, 2004), indicating that the surgical procedure had not damaged the tissue. Remarkably, once that stabilization occurred (40–120 min, Fig. 2) in both the PDC-treated and in the four TBOA-treated rats that did not show motor alterations the level of extracellular glutamate was steadily 3 and 4-fold higher as compared to the controls, respectively (Fig. 2). This increase seems to be relatively specific, since only a slight non-significant increase in aspartate was observed and glutamine, taurine and alanine levels did not change (Fig. 3). Glycine concentration incremented significantly, although much less than glutamate (about 90%), in the TBOA-treated rats but not in those infused with PDC (Fig. 3). This effect of TBOA on extracellular glycine has been previously reported in the striatum in vivo (Montiel et al., 2005). Surprisingly, in the two rats treated with TBOA that were paralyzed the concentration of extracellular glutamate was similar to the control rats (data not shown) and, as mentioned below, motoneurons were damaged.

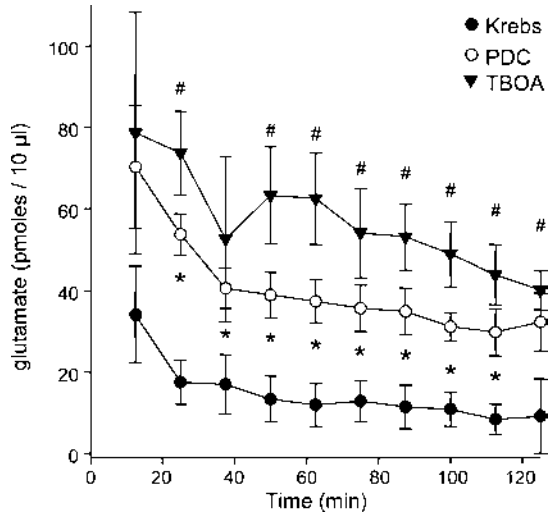

Fig. 2. Chronic blockade of glutamate transport augments the extracellular concentration of glutamate. Extracellular glutamate concentration was measured by microdialysis and HPLC as described in Section 2, 7–10 days after osmotic pump implantation and continuous Ringer–Krebs medium (control, $n = 4$), PDC ($n = 7$) and TBOA ($n = 4$; in the other two rats of this group the amino acid changes were not significant) infusion. Each point is the value determined in the corresponding microdialysis fraction obtained every 12.5 min (mean ± S.E.M.). Note that the steady state concentration of glutamate was about 3 and 4-fold higher in the PDC and TBOA-treated rats, respectively, than in the control animals. $^{*}p < 0.05$ for PDC and $^{\#}p < 0.05$ for TBOA, as compared to the corresponding control value (ANOVA).

3.3. Elevated extracellular glutamate due to transport blockade does not induce spinal motoneuron damage

The lack of behavioral effects of PDC infusion was in agreement with the histological and immunohistological observations, which showed that the number of motoneurons in the area of infusion was normal, that they appeared as healthy as those of the control rats, and that no glial reaction was developed (Fig. 4; quantitative data is shown in Fig. 5). In addition, no neuronal damage was observed in the dorsal horn (not shown). These observations indicate that no cell damage occurred as a consequence of glutamate transport blockade by PDC infusion.

Similarly to the PDC-treated animals, in the four rats treated with TBOA that did not present motor alterations and had a 4-fold increment in extracellular glutamate, no glial reaction and no histological or immunocytological alterations were observed (Fig. 4; quantitative data is shown in Fig. 5). In the two rats treated with TBOA that showed motor deficits a notable loss of motoneurons was observed (not shown), similar to that produced by chronic AMPA infusion (Tovar-y-Romo et al., 2007).

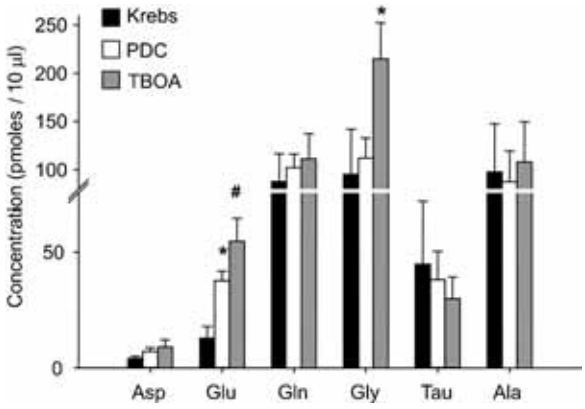

Fig. 3. Extracellular levels of different amino acids after chronic treatment with glutamate transport blockers. The extracellular concentration of aspartate, glutamate, glutamine, glycine, taurine and alanine were measured as described in Fig. 2. Each bar represents the mean ± S.E.M. of the averaged values in the microdialysis fractions 2–10 (minutes 25–120, Fig. 2) of extracellular levels of amino acids. $^{*}p < 0.05$ and $^{\#}p < 0.01$ as compared with controls (ANOVA). No significant changes were observed in the other amino acids.

4. Discussion

In this study we demonstrate that the chronic infusion of the glutamate transport inhibitors PDC and TBOA directly in the lumbar spinal cord elevates the extracellular concentration of glutamate but does not result in neuronal damage. This lack of deleterious effect cannot be ascribed to an insufficient diffusion of the inhibitors into the ventral horn, because we have previously demonstrated that under identical experimental conditions the infusion of AMPA induces in a dose-dependent manner a progressive degeneration of the spinal motoneurons, leading to paralysis (Tovar-y-Romo et al., 2007).

Although in spinal cord cultures the blockade of glutamate transport by PDC produces neuronal damage after long exposure periods (Carriedo et al., 1996; Matyja et al., 2005; Rothstein et al., 1993; Velasco et al., 1996), we have previously shown that in vivo the acute infusion of PDC by reverse microdialysis causes a several fold increase of the neurotransmitter in the extracellular fluid without generating neuronal death, neither in the spinal cord (Corona and Tapia, 2004) nor in the hippocampus or striatum (Massieu et al., 1995; Massieu and Tapia, 1997). Furthermore, PDC induced also a large elevation of glutamate and was innocuous when infused in the motor cortex and hippocampus of a transgenic ALS mouse in vivo (Tovar-y-Romo and Tapia, 2006). These findings, however, did not rule out the possibility that an extended inhibition of glutamate transport may have deleterious effects on neurons (Rattray and Bendotti, 2006).

In our present experiments with PDC and TBOA we detected a 3 and 4-fold increase in extracellular glutamate respectively, up to 10 days after its continuous infusion in the spinal cord. Because PDC administered by microdialysis induced a notable elevation of glutamate within 20 min, that persisted for as long as PDC was perfused (Corona and Tapia, 2004), it is reasonable to conclude that in the present experiments the concentration of glutamate increased promptly from the beginning of the infusion and remained constant thereafter, up to the time of the microdialysis procedure. The increase in glutamate concentration after infusion of PDC may be due not only to the blockade of glutamate transport, but also to heteroexchange (Volterra et al., 1996; Waagepetersen et al., 2001). Nonetheless, it is remarkable that in spite of this significant increase in endogenous glutamate, none of the animals receiving PDC showed any behavioral alteration in motor performance at any time, and the histological and immunocytochemical analyses revealed no cellular abnormalities.

TBOA is a potent non-transportable competitive glutamate uptake blocker that impedes the function of the transporters as well as heteroexchange (Shimamoto et al., 1998, 2000). In the present experiments, in four of six rats the infusion of TBOA induced an even larger increase in extracellular glutamate than that produced by PDC, and similarly to PDC this increase did not cause any motor behavioral alterations or motoneuron loss. We cannot offer an explanation for the fact that two rats treated with TBOA showed paralysis associated to motoneuron loss, whereas four rats behaved exactly as the PDC-treated animals. However, because in the latter four animals glutamate levels were elevated, whereas in the former two rats glutamate concentration did not increase, it seems possible that TBOA might produce a direct excitotoxic effect on motoneurons. In support of this possibility, it was recently found that the intrahippocampal injection of TBOA

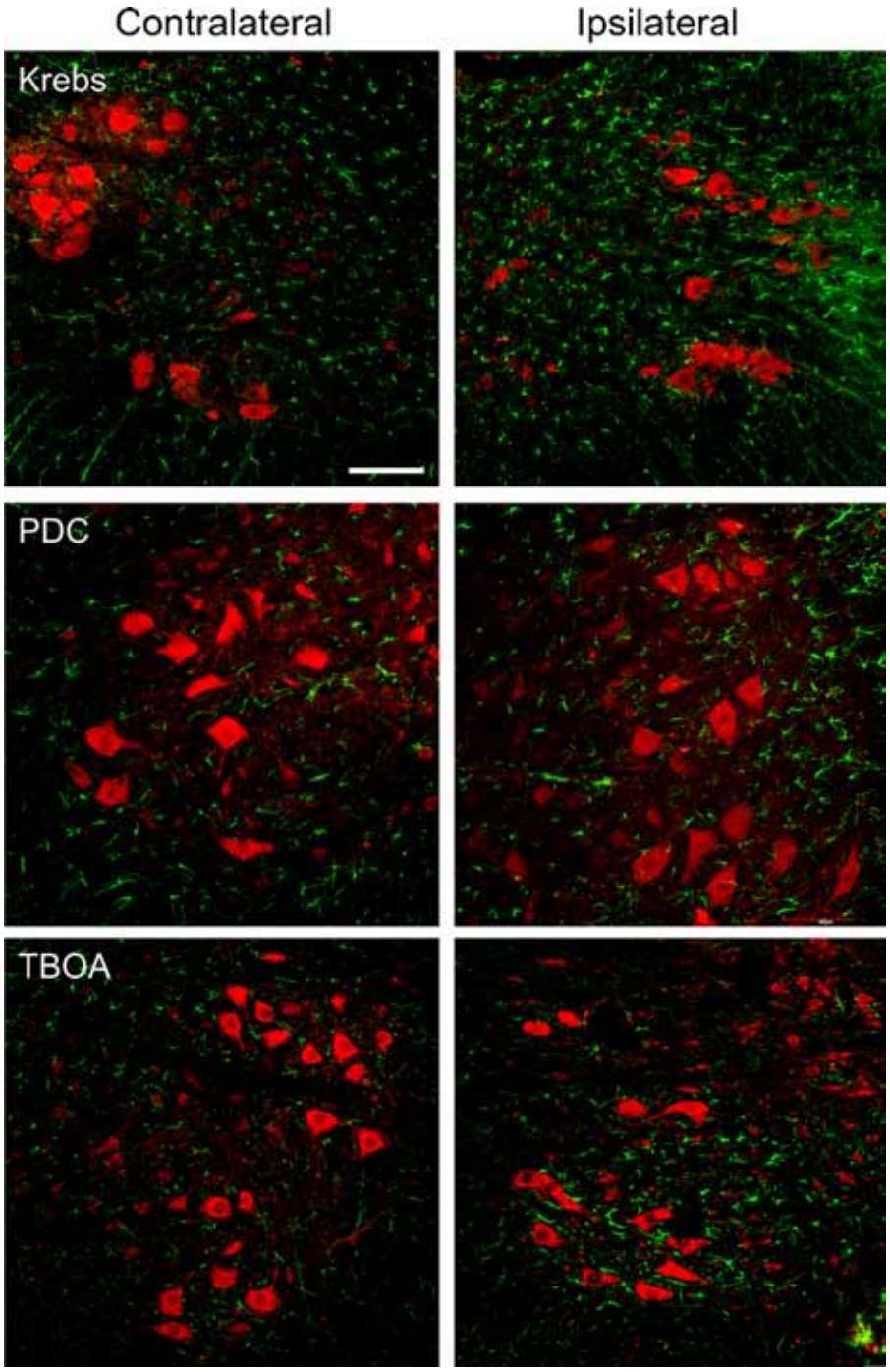

Fig. 4. Chronic blockade of glutamate transport does not induce motoneuron death. Representative micrographs of ChAT (red) and GFAP (green) immunohistochemistry in sections of the lumbar spinal cord, 7–10 days after pump implantation and continuous infusion with Krebs medium (control), PDC and TBOA. Motoneurons look healthy and gliosis is absent in all groups. Scale bar = 100 μm.

induced epilepsy and neurodegeneration in rat hippocampus, while PDC was innocuous, and that this effect was blocked by MK-801 and NBQX (Montiel et al., 2005), suggesting that TBOA might activate glutamate receptors. In rat brain membranes TBOA binds with high affinity to NMDA receptors and with less affinity to non-NMDA receptors (Shimamoto et al., 1998, 2000), but no data are available in the spinal cord. So, the lack of neurotoxicity observed in the TBOA-infused rats showing elevated extracellular glutamate concentrations, together with the results in the PDC experiments, leads us to conclude that even a prolonged accumulation of extracellular endogenous glutamate arising from its transport blockade is not capable of overactivating glutamate receptors and therefore do not cause spinal motoneuron death in vivo. This conclusion is also supported by previous work showing that the infusion of PDC in the striatum and the hippocampus induces a substantial elevation of extracellular glutamate but does not induce neuronal damage, in rats and mice, including ALS transgenic mice (Corona and Tapia, 2004; Massieu et al., 1995; Massieu and Tapia, 1997; Tovar-y-Romo and Tapia, 2006; Tapia et al., 1999). It might be argued that a 7–10 days period is not longer enough to discard the possibility that a more extended inhibition of glutamate transport could be detrimental for motoneurons. However, our experiments with the chronic infusion of AMPA using osmotic minipumps show that the motor

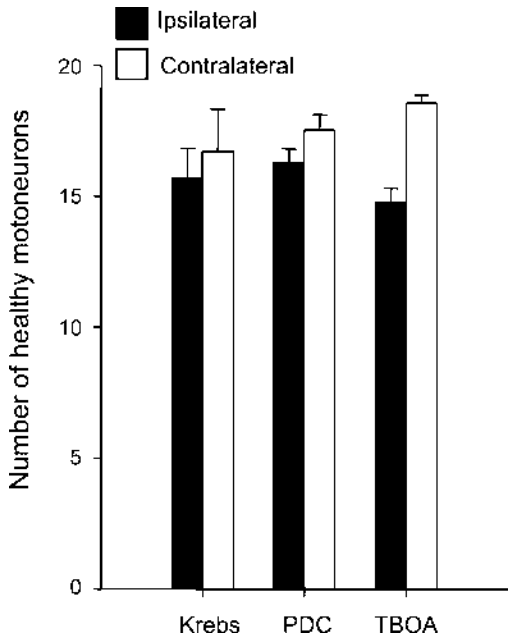

Fig. 5. Motoneuron number does not change after PDC or TBOA chronic administration. Number of healthy motoneurons in the ipsilateral and contralateral ventral horns of rats infused with Ringer–Krebs medium (control, $n = 4$), PDC ($n = 7$) and TBOA ($n = 4$). Five 40 μm sections from each rat were analyzed. Values are means ± S.E.M.

performance deficiencies occur within the first 2–6 days of infusion (Tovar-y-Romo et al., 2007).

In this respect, it is noteworthy that, although reductions in glutamate transporter EAAT2 have been reported in ALS transgenic rodents and in the spinal cord of ALS patients, the majority of these data are more consistent with the possibility that these changes are a consequence rather than a cause of ALS (see Rattray and Bendotti, 2006, for a review on this point). For example, in SOD1/G93A ALS mice, when GLT1 is decreased in heterozygous GLT1± animals the onset of paralysis is not modified in comparison with SOD1/G93A mice that have the normal amount of the transporter protein, implying that loss of GLT1 is not an initiator of the disease (Pardo et al., 2006).

It is also worth mentioning that the 3–4-fold elevation of extracellular concentration of glutamate that we found in our experiments is higher than that observed in the CSF of a subset of SALS patients, which in most cases was less than twice the upper limit parameter (Spreux-Varoquaux et al., 2002).

In conclusion, our results show that the persistent increased levels of extracellular glutamate in the spinal cord during several days due to transport deficit are not capable of eliciting excitotoxicity and therefore these data do not support the hypothesis that a deficiency in glutamate transport is causally involved in motoneuron degeneration in ALS.

Acknowledgements

This work was supported by DGAPA-UNAM (project IN209807) and CONACYT (project 60322). LBTR and LDSC are recipients of a fellowship from CONACYT.

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Resultados II. Protección mediada por el VEGF contra la excitotoxicidad

Vascular endothelial growth factor prevents paralysis and motoneuron death in a rat model of excitotoxic spinal cord neurodegeneration

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Journal of Neuropathology and Experimental Neurology 2007, **66**:913-922

Resumen

El VEGF retrasa el inicio y progreso de la enfermedad en los modelos de roedores de FALS. Dado que la mayoría de los casos de ALS son esporádicos, es importante determinar si el VEGF puede proteger a las motoneuronas en un paradigma experimental de ALS no transgénico. Nosotros probamos esta posibilidad en un nuevo modelo de neurodegeneración espinal excitotóxica crónica en la rata. Usando minibombas osmóticas, infundimos continuamente AMPA directamente en la médula espinal en la región lumbar. El efecto de este tratamiento sobre la conducta motora se determinó a través de 3 pruebas de desenvolvimiento motor, y la neurodegeneración fue evaluada a través de análisis histológicos e inmunohistoquímicos. La infusión de AMPA produjo déficits motores progresivos de las extremidades posteriores de manera dosis-dependiente, llegando a un estado de parálisis bilateral completa en ~10 días, lo cual fue correlacionado con la pérdida de las motoneuronas espinales. El VEGF administrado junto con AMPA previno completamente los déficits motores, y la muerte de las motoneuronas fue reducida en más del 75%. Por lo tanto, hemos desarrollado un modelo progresivo *in vivo* de muerte de motoneuronas espinales debida a la hiperactivación de receptores AMPA. El hallazgo de que el VEGF protegió a las motoneuronas de la excitotoxicidad mediada por receptores AMPA sugiere que este factor trófico puede constituir un agente terapéutico para la SALS.

J Neuropathol Exp Neurol
Vol. 66, No. 10
October 2007
pp. 913–922

ORIGINAL ARTICLE

Vascular Endothelial Growth Factor Prevents Paralysis and Motoneuron Death in a Rat Model of Excitotoxic Spinal Cord Neurodegeneration

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Abstract

Vascular endothelial growth factor (VEGF) delays disease onset and progression in transgenic rodent models of familial amyotrophic lateral sclerosis (ALS). Because most cases of ALS are sporadic, it is important to determine whether VEGF can protect motoneurons in a nontransgenic ALS paradigm. We tested this possibility in a new model of chronic excitotoxic spinal neurodegeneration in the rat. Using osmotic minipumps, we continuously infused the glutamate receptor agonist α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) directly in the lumbar spinal cord. The effect of this treatment on motor behavior was assessed with 3 motor performance tests, and neurodegeneration was evaluated by histologic and immunohistochemical analyses. AMPA infusion produced dose-dependent progressive hindlimb motor deficits, reaching complete bilateral paralysis in ~10 days, which was correlated with the loss of spinal motoneurons. VEGF administered together with AMPA completely prevented the motor deficits, and the motoneuron death was reduced by more than 75%. Thus, we have developed an in vivo model of progressive spinal motoneuron death due to overactivation of AMPA receptors. The finding that VEGF protected motoneurons from this AMPA receptor-mediated excitotoxic death suggests that it may be a therapeutic agent in sporadic ALS.

Key Words: Amyotrophic lateral sclerosis, Excitotoxicity, Motoneuron degeneration, Spinal cord, Vascular endothelial growth factor.

INTRODUCTION

Motoneuron death in spinal cord, brainstem, and motor cortex is the cause of the devastating disease amyotrophic lateral sclerosis (ALS). Although ALS was described more than 130 years ago, the molecular and cellular processes that cause motoneuron loss have not been completely defined. Currently, the only proven cause for ALS is the presence of mutant superoxide dismutase 1 (SOD1) in only ~2% of the cases (1), although several studies have demonstrated an involvement of Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors in spinal motoneuron degeneration (2–9). In a previous study we showed that microdialysis administration of AMPA, but not of *N*-methyl-D-aspartate, in the lumbar spinal cord of rats induces motoneuron death and hindlimb paralysis that develops within a few hours (10). Because motoneuron death in processes such as those occurring in ALS develops over lengthened time periods, we have now evaluated whether chronic administration of AMPA leads to progressive motor impairment associated with spinal neurodegeneration, and we have thus generated a model of excitotoxic motoneuron death.

Potential treatments of neurodegenerative syndromes include growth factors (11). Previous studies in the familial ALS mouse model, generated by the transgenic expression of human mutant SOD1 (12), have shown that intramuscular injections of viral vectors encoding glial cell line-derived neurotrophic factor (13), insulin-like growth factor (14), and vascular endothelial growth factor (VEGF) (15), resulted in delay of the paralysis and an increase in survival. Interestingly, removal of the hypoxia response element present in the promoter sequence of VEGF in the mouse causes a motor syndrome associated with selective spinal motoneuron death strikingly similar to ALS (16). Thus, VEGF seems to play a fundamental role in the normal physiology of spinal motoneurons. This discovery has led some groups to explore the potential benefit of VEGF administration in familial models of ALS (17–19), but the assessment of the capability of VEGF to modify neuronal death induced by excitotoxicity, a mechanism that has been considered to be involved in ALS, is lacking. Therefore, we tested whether the administration of exogenous VEGF was able to protect motoneurons from excitotoxic AMPA receptor overactivation in the chronic model of spinal excitotoxic motoneuron death mentioned above.

MATERIALS AND METHODS

Adult male Wistar rats (290–310 g) were used in all the experiments. Procedures were performed in accordance

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This work was supported by La Dirección General de Asuntos del Personal Académico, Universidad Nacional Antónoma de México (project IN209807) and Consejo Nacional de Ciencia y Tecnologia (CONACYT), Mexico (project 42668). L.B.T.R. is recipient of a scholarship from CONACYT.

with the Rules for Research in Health Matters (Mexico), with approval of the local animal care committee. Animals were housed in a laboratory environment with a 12-hour light/dark cycle and with food and water ad libitum.

For minipump implant, rats were anesthetized with 5% halothane in a 95% O_2/5% CO_2 mixture and placed in a stereotaxic spinal unit (David Kopf Instruments, Tujunga, CA). Anesthesia was then lowered and maintained to 1% to 2% halothane during surgery. A longitudinal incision of the skin was made in the lumbar region, and muscles surrounding lumbar vertebrae were retracted. On the second lumbar vertebra the spinous process was removed, a ~1-mm hole was drilled on the left side of the lamina, and a stainless-steel screw (1-mm diameter; 3.7-mm long) was inserted in the hole without reaching the surface of the spinal cord. A ~2-mm hole was drilled on the right side of the lamina of the same vertebra, and the tissue underneath was gently pressed with the tip of a small forceps to elicit the contraction of the hindlimb and thus ensure that drug infusion was in the correct spinal segment. A small cut of the meninges was made to insert the tip of the inner cannula (2-mm long and 0.24-mm diameter) of a CMA/7 microdialysis probe (CMA Microdialysis, Acton, MA) into the dorsal parenchyma, from which the dialysis membrane and the outer cannula had been removed. The infusion cannula was not inserted farther in the spinal cord to avoid mechanical injury of the ventral horn. The laminectomy was sealed with Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI), and dental cement was poured over the screw and the plastic support of the cannula in such a way that the tubing protruding from the plastic support of the cannula remained free to be connected to the minipump tube. The osmotic minipump (Alzet model 2004, approximate capacity 200 μL, flow rate 0.25 μL/hour; Durect Corp., Cupertino, CA) was implanted subcutaneously in the back of the rat, caudal to the skin incision, and its tubing was attached to that of the cannula. Finally, the skin incision was closed with surgical stainless-steel clips, and rats received an intramuscular dose of penicillin.

The minipumps were filled with control Ringer-Krebs medium (118 mM NaCl, 4.5 mM KCl, 2.5 mM $MgSO_4$, 4.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 10 mM glucose, pH 7.4), or with AMPA (Tocris, Bristol, UK; the concentrations used were 4, 7.5, 9.5, or 11.2 mM) dissolved in Ringer-Krebs medium. In the experiments with VEGF, we used a recombinant rat $VEGF_{164}$ obtained from Sigma Chemical (St. Louis, MO), which was reconstituted in phosphate-buffered saline (PBS)-0.1% bovine serum albumin (BSA) and then mixed with the AMPA solution at a concentration calculated to deliver 24 ng/day through the minipump. A control group was infused with VEGF only. Each pump was weighed before and after filling to ensure that they were completely full. Pumps were stabilized by a 40-hour incubation in sterile saline at 37°C before implantation. The free flux of the pump cannula was corroborated immediately at the end of each experiment.

Assessment of Motor Function

All rats were trained for 2 days before the surgery on 2 motor tests: a variation of the paw grip endurance (PGE) task (20) and the Rotarod (Columbus Instruments, Columbus, OH). Animals were then evaluated in each test routinely, as described in the Results section, until the time of fixation for histologic analysis.

For the PGE test, rats were placed individually on a horizontally placed grid (30 × 19 cm) attached to a mechanical rotator. The grid was gently turned (3 rpm) until it reached a vertical position. The time taken by the rats to climb to the top of the grid and reach a stable position or the latency to fall from the grid when they were unable to climb was scored with a cutoff time of 30 seconds. Each rat was tested 3 times, and the shortest time for climbing or the longest before falling was recorded. Data are presented in the figures as "time to climb" and "latency to fall" as a continuum, because animals that were able to climb could reach the top of the grid within 30 seconds; otherwise they would hold on to the grid and finally fall. For the Rotarod test, rats walked individually on an accelerating (0.2 rpm/second) rod, starting from 10 rpm, for 3 trials and the longest latency to fall, with a cutoff of 120 seconds, was recorded. In addition to the motor tests described, a qualitative evaluation was performed on the overall stride pattern analyzing the rear footprints obtained after ink staining of the hindpaws and making the rats walk along a paper runway.

Histology and Immunohistochemistry

For histologic and immunohistochemical analyses, rats infused with AMPA were transcardially fixed when they reached the lowest scores on the behavioral tests. Control rats that did not show any motor abnormality were fixed 10 days after the surgery; at this time all AMPA infused animals had already shown signs of paralysis (see Results section). Rats receiving only VEGF were fixed 25 days after pump implantation, because they did not score low in motor tests by this time and Alzet osmotic minipumps used have a mean duration of 28 days. For fixation, animals were anesthetized with barbiturate and perfused transcardially with 250 mL of ice-cold 0.9% saline, followed by 250 mL of ice-cold 4% paraformaldehyde in phosphate buffer pH 7.4. Spinal cords were removed, postfixed at 4°C, and successively transferred to sucrose solutions (up to 30%).

Transverse 40-μm sections of the lumbar region, where the infusion cannula had been implanted, were obtained in a cryostat. Alternate sections were stained with cresyl violet or double immunostained for choline acetyltransferase (ChAT) and glial fibrillary acidic protein (GFAP). Free-floating sections were blocked with 5% bovine serum albumin and normal rabbit serum (1:25) in PBS-Triton X-100 (0.3%) for 3 hours and then incubated with goat polyclonal anti-ChAT (1:100; Chemicon, Temecula, CA) and rabbit anti-GFAP antibodies (1:1000; DAKO, Carpinteria, CA) for 48 hours at 4°C. Sections were then washed 3 times for 10 minutes each in PBS-Triton X-100 and incubated with a biotinyl-conjugated mouse anti-goat IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 hour. After 3 washes in PBS-Triton, sections were incubated for 1 hour with preincubated avidin-Texas Red conjugate (1:200, pH 8.2; Vector Laboratories). Sections were washed 3 times in PBS-Triton and then incubated with an FITC-conjugated anti-rabbit antibody

(1:250; Zymed Laboratories, South San Francisco, CA) for 2 hours. Finally, sections were washed 3 times for 10 minutes in PBS and placed on silane (γ-methacryloxypropyltrimethoxysilane; Sigma Chemical)-covered slides and coverslipped with fluorescent mounting medium (DAKO).

To study the possible effect of VEGF on tissue vascularization in some experiments with VEGF alone and with VEGF plus AMPA, immunostaining was performed for rat endothelial cell antigen 1 (RECA-1). Sections were processed as described above, using a mouse monoclonal

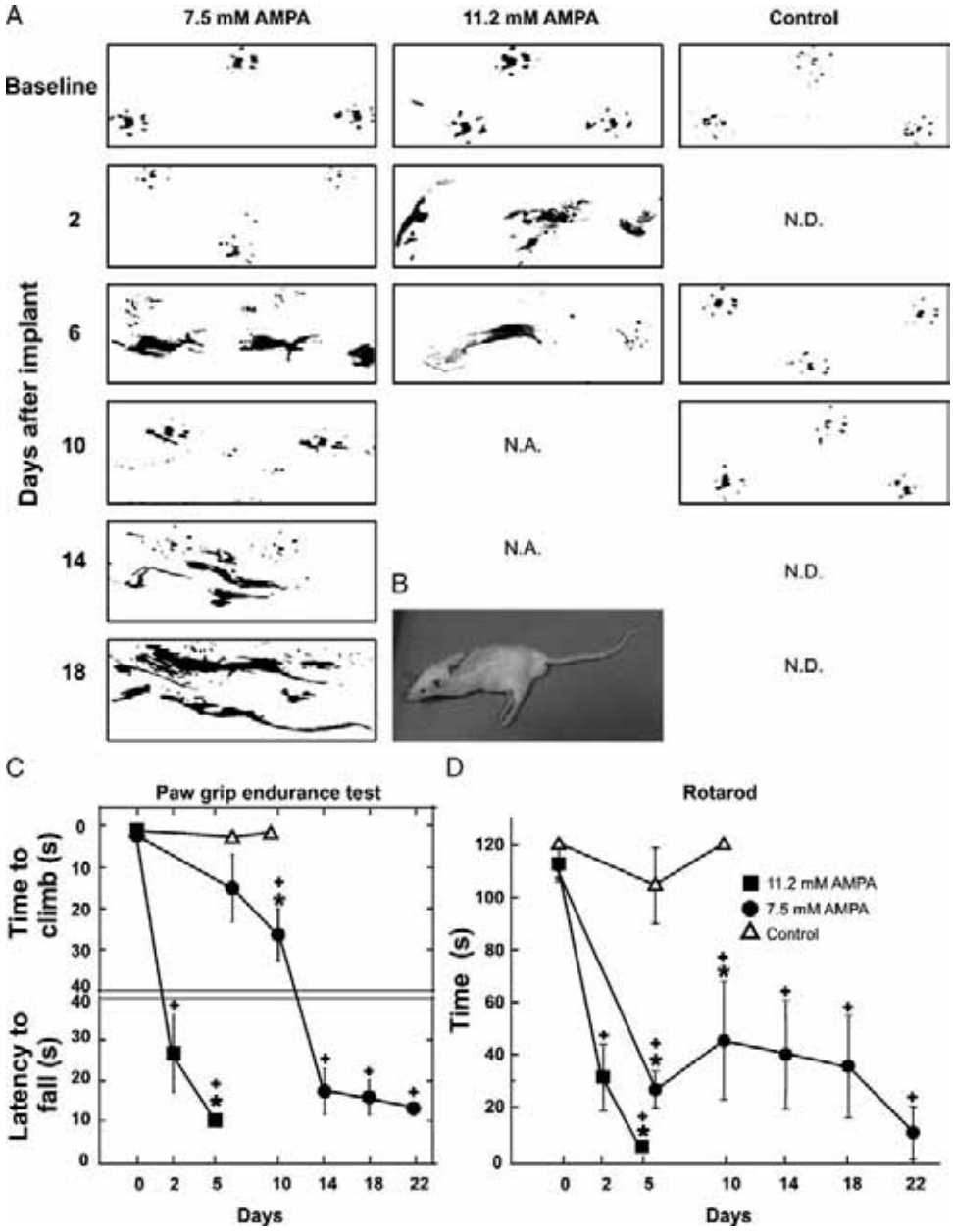

FIGURE 1. Chronic infusion of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) induces progressive development of motor paralysis. **(A)** Representative hindpaw footprints of rats chronically infused with 7.5 mM AMPA (left column), 11.2 mM AMPA (middle column), or Ringer-Krebs medium (control, right column). N.A., not applicable; N.D., not determined. **(B)** Representative photograph of a rat after 18 days of 7.5 mM AMPA infusion; notice the abnormal posture and the rigidity of the hindlimbs. **(C)** Time course of the PGE test performance of the control rats (△) and of the rats infused with 7.5 mM (●) and 11.2 mM (■) AMPA. Time to climb and latency to fall were recorded and are presented as a continuum because rats that were able to climb could reach the top of the grid within the cutoff time of 30 seconds; otherwise they would hold on to the grid and finally fall. None of the control rats fell at any time point. **(D)** Time course of the performance in the Rotarod test. Each point is the mean ± SEM for 7 rats treated with 11.2 mM AMPA, 6 rats treated with 7.5 mM AMPA, and 5 control rats treated with Krebs medium. +, $p < 0.02$ compared with the score before implant (day 0) (*t*-test); *, $p < 0.02$ compared with the corresponding day of the control group (ANOVA followed by Fisher test).

anti-RECA-1 as the primary antibody (1:500; Serotec, Oxford, UK) and revealing with anti-mouse antibody conjugated with fluorescein isothiocyanate. Cross-reactivity was excluded by appropriate controls and incubated in the absence of primary antibodies; these control sections showed no immunostaining.

Sections were visualized using a Nikon microscope equipped with an epifluorescence attachment or a confocal LaserScan microscope (MRC1024; Bio-Rad, Hercules, CA). Confocal images were imported into the Confocal Assistant Program version 4.02 (T. C. Brelje, University of Minnesota). Each image was projected in the *z* plane (4 optical sections), and maximal values of pixels were integrated to produce single images containing the information of the 4 optical sections. Morphologically undamaged motoneurons (i.e. >25 μm diameter, heavily immunolabeled for ChAT, with a distinguishable nucleus) were counted in a 10x microscopic field. Numbers of cells were determined in sections in which the trace of the infusion cannula was evident; 5 sections per rat were analyzed, and the values were averaged. Sections from segments more than 1 mm from the infusion site showed no morphologic or ChAT immunostaining alterations.

Statistical Analysis

Comparisons of number of motoneurons and behavioral scores were made using Student's *t*-test and analysis of variance followed by a Fisher post hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Chronic Infusion of AMPA in Lumbar Spinal Cord Leads to Progressive Hindlimb Paralysis and Motoneuron Death

None of the control rats infused with Ringer-Krebs medium (n = 5) showed motor dysfunctions at any time, up to the maximum period studied (10 days). Immediately after recovering from anesthesia these animals were able to move hind- and forelimbs normally (Fig. 1A). In contrast, 24 to 48 hours after surgery rats infused with 11.2 mM AMPA (n = 7) showed a bilateral hindlimb rigid flexion, which progressed continuously until day 5, when they had developed complete bilateral hindlimb paralysis and were unable to move. At this time rats were fixed for histology (3 rats left a longer time died on the 6th day by an undetermined cause; however, all rats that presented with complete hindlimb paralysis were able to reach food and water by crawling using their forelimbs). The progression of the motor disabilities of these animals can be appreciated in the stride pattern and motor test scores shown in Figure 1. From the 2nd day they showed a significant deficiency in the paw grip endurance (PGE) test compared with the score before the implant ($p < 0.001$), and a similar drop was observed in the ability to

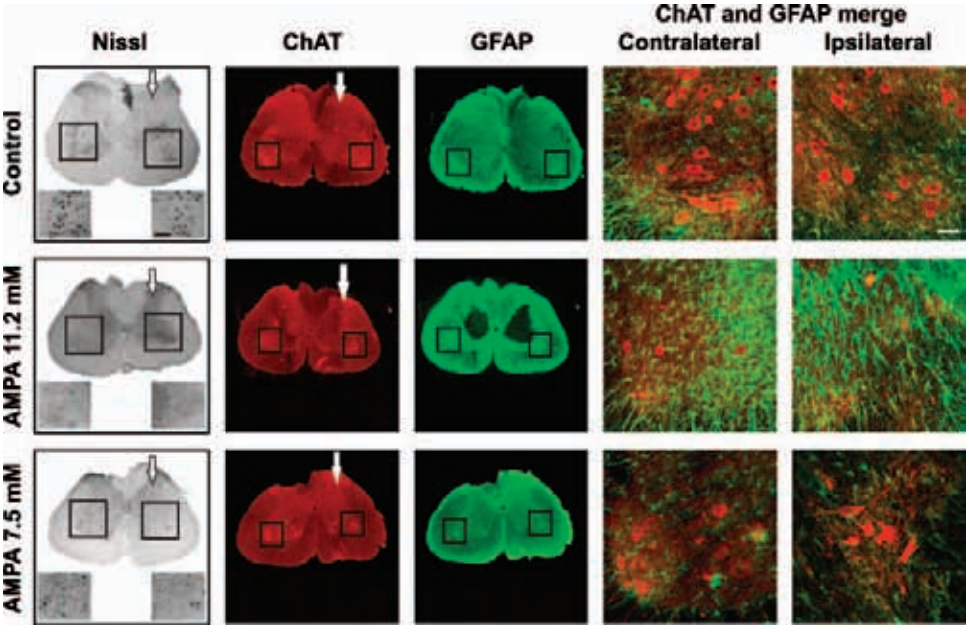

FIGURE 2. Chronic infusion of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) induces motoneuron death. The micrographs are representative transverse sections of the lumbar spinal cord of control rats infused with Ringer-Krebs medium (control) 10 days after pump implant (top row), 11.2 mM AMPA 5 days after implant (middle row), or 7.5 mM AMPA, 18 days after implant (bottom row). Arrows indicate the site of infusion. Nissl staining shows the loss of motoneurons induced by 11.2 and 7.5 mM AMPA, which is more clearly seen in the magnifications (insets, scale bar = 200 μm) and in the choline acetyltransferase (ChAT) immunohistochemistry. See Figure 6 for quantitative analysis. The highest concentration of AMPA induced a dense astroglial reaction, revealed by glial acidic fibrillary protein (GFAP) immunohistochemistry, surrounding a zone depleted of GFAP. Gliosis was less intense with 7.5 mM AMPA. Magnifications of the zone marked by the squares are shown in the 2 last columns, depicted as merged ChAT and GFAP labeling. Micrographs in each row are from the same rat. Scale bar = 50 μm.

walk on the Rotarod. Values in both tasks were statistically different from those obtained in the control group infused with Krebs' medium (Fig. 1C, D).

In view of the fact that the motor deficits that developed with 11.2 mM AMPA occurred within 1 to 3 days and rats did not survive longer than 6 days, we tested 4, 7.5, and 9.5 mM concentrations of the drug. With a concentration of 4 mM AMPA the animals walked normally at all times. Although at day 4 they started to show a slight rigid flexion of the ipsilateral hindpaw, this condition did not progress further during the 25 days studied and did not cause any change in walking or in the Rotarod and PGE scores. Histologic and immunohistochemical analyses of the tissue did not reveal significant alterations in motoneurons or gliosis (data not shown). With 7.5 and 9 mM concentrations (n = 6) the effects were similar and are summarized in Figure 1. Two days after the implant rats still walked normally, and from day 3 they started to show a rigid flexion of the ipsilateral hindpaw, which clearly modified their stride and the Rotarod performance, with less effect in the PGE test. After 6 days they were still able to lift both hindlimbs for walking and from days 7 to 11 the ipsilateral hindlimb slipped and subsequently became paralyzed, thus making the rats drag the limb; by day 18 both hindlimbs were paralyzed (see the strides and the photograph in Fig. 1). All animals were able to climb the grid even after 10 days, although the scores obtained at this time were statistically different from those obtained before the implant or from control animals ($p < 0.001$). By day 14

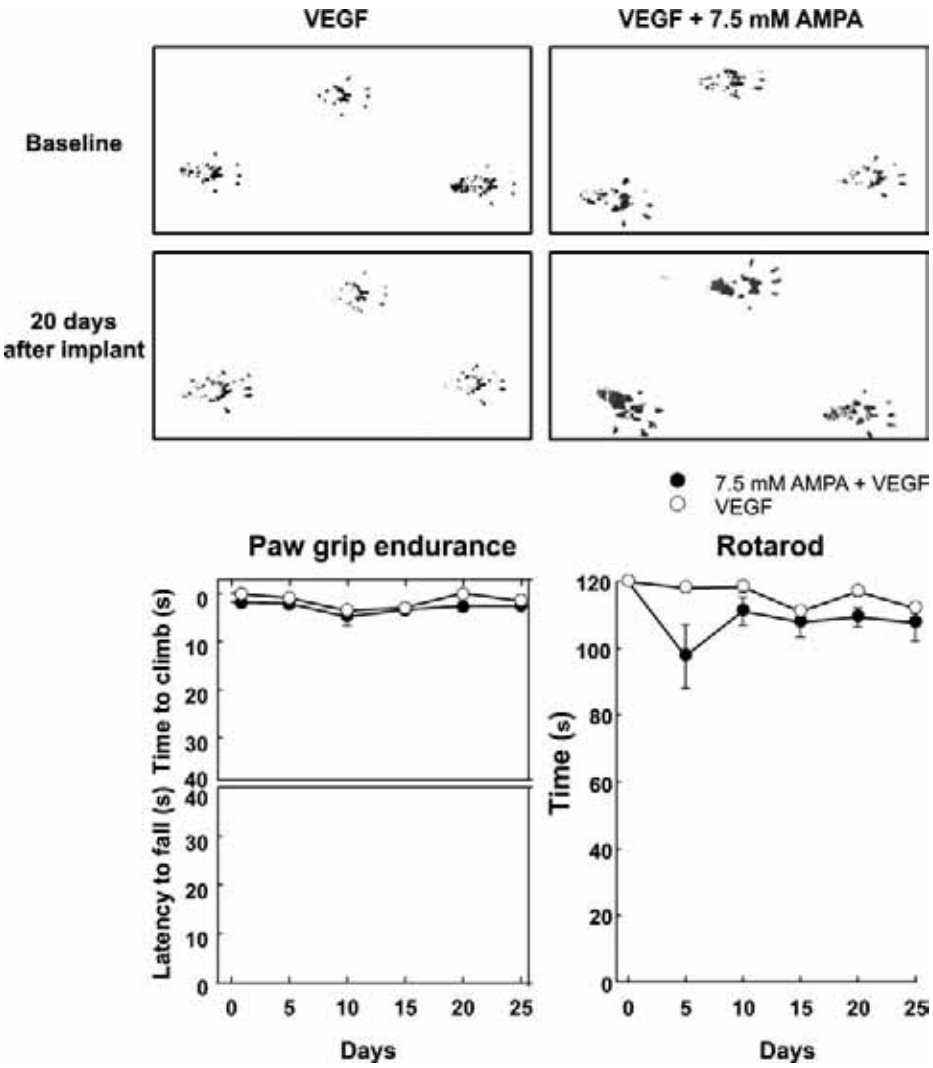

FIGURE 3. Coinfusion of vascular endothelial growth factor (VEGF) prevents the progressive development of motor deficits induced by 7.5 mM α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA). Upper panel, representative hindpaw footprints of control rats chronically infused with VEGF only (left column) and of rats infused with 7.5 mM AMPA + VEGF (right column), 20 days after pump implant. Note that the footprint pattern of the latter is not different from baseline nor from that with VEGF alone or with control Krebs' medium (Fig. 1). Lower panel, time course of the PGE and Rotarod test performances of rats treated with VEGF alone (○) (n = 3) and of rats infused with 7.5 mM AMPA + VEGF (•) (n = 16). There were no statistical differences between the scores of the groups in the paw grip endurance or Rotarod tests.

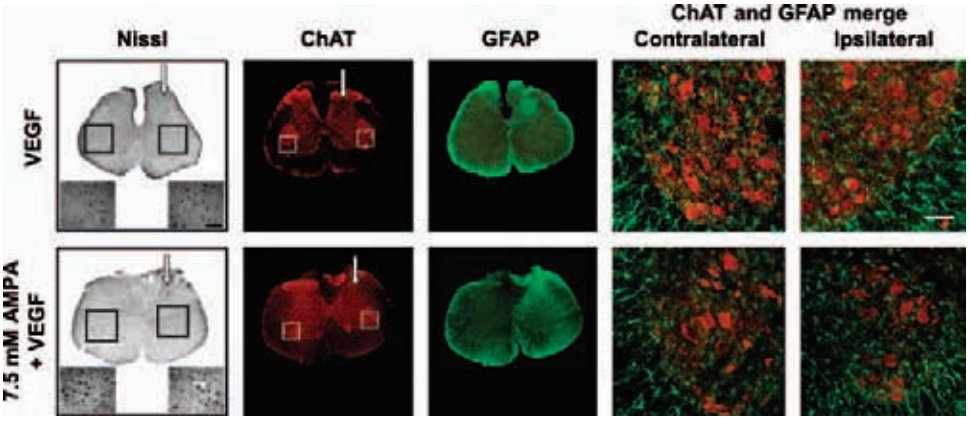

FIGURE 4. Coinfusion of vascular endothelial growth factor (VEGF) prevents the motoneuron death induced by 7.5 mM α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA). The micrographs are representative transverse sections of the lumbar spinal cord of rats infused with VEGF alone (top row) and rats infused with 7.5 mM AMPA + VEGF (bottom row), 25 days after pump implant. Arrows indicate the site of infusion. Nissl staining shows normal appearance of motoneurons in both groups, which is corroborated by choline acetyltransferase (ChAT) immunochemistry. See Figure 6 for quantitative analysis. Gliosis was absent in both groups. Magnifications of the zone marked by the squares are shown in the 2 last columns, depicted as merged ChAT and choline acetyltransferase (glial fibrillary acidic protein [GFAP]) labeling. Micrographs in each row are from the same rat. Scale bar = 50 μm.

the animals were unable to climb, and they rapidly fell although they still gripped to the grid with the forepaws (Fig. 1C). The performance in the Rotarod test dropped drastically after 6 days compared with that for the control group ($p < 0.0001$).

Besides the motor alterations, both 11.2 and 7.5 mM AMPA induced swelling of the hindpaws and an autotomy behavior that animals developed once the paralysis started (1 day with 11.2 mM and about 8 days after 7.5 mM AMPA). This behavior began with biting of the nails and soft tissue, followed in some cases by mutilation of the toes. However, the motor performance deficits described above were clearly due to the paralysis process and not to the autotomy injuries, as shown by the results of the experiments with VEGF described next. Although autotomy behavior may not reflect pain but anesthesia due to denervation (21), rats showing early and intense autotomy were killed and excluded from the analysis.

To establish a cellular correlation between the paralysis produced by AMPA and motoneuron damage we performed Nissl staining and double immunohistochemistry for ChAT and GFAP. As shown in Figure 2, the motoneurons of control animals infused with Krebs' medium appeared healthy, with the cytoplasm heavily labeled for ChAT. The tissue shows general GFAP labeling without signs of reactive astrogliosis. In contrast, 5 days after the implant both the ipsilateral and the contralateral ventral horns of the region infused with 11.2 mM AMPA were depleted of Nissl-stained and ChAT-labeled motoneurons (Figs. 2, 6). In this region a well-delimited zone devoid of GFAP-positive cells surrounded by reactive astroglia, which covered both ventral and dorsal horns, was observed bilaterally (Fig. 2).

Fifteen to 20 days after the implant, the damage observed in the tissue of the rats infused with 7.5 mM AMPA was less intense than that seen 5 days after 11.2 mM AMPA. Whereas an almost total loss of motoneurons was observed in the ipsilateral horn, the contralateral horn showed only about 65% loss (Figs. 2, 6). Except for 1 rat, no GFAP-free zone was observed in these animals, and the GFAP-positive glia surrounding the motoneurons was less dense than that observed in the 11.2 mM AMPA group.

Nissl and GFAP staining revealed that, in addition to motoneuron damage, 11.2 mM AMPA produced a nearly complete depletion of neurons in the dorsal horn, which was filled with reactive astroglia. This damage occurred also with 7.5 mM AMPA but was less intense.

Administration of VEGF in Lumbar Spinal Cord Prevents Hindlimb Paralysis and Motoneuron Death Induced by AMPA

Once we settled the experimental conditions to induce progressive motor paralysis associated with spinal motoneuron death, we tested the possible preventive effect of VEGF coinfused with AMPA. We chose a dose of 24 ng of VEGF per day (1 ng/hour) administered through the minipump directly in the spinal tissue, based on the intracerebroventricular dose (60 ng/day) previously shown to delay onset of symptoms and the death in transgenic mutant SOD1 rats (17) and after proving that this treatment with VEGF alone caused no significant behavioral alterations nor significant deficits in the motor tests scores (Fig. 3). Twelve of the 16 rats treated with 7.5 mM AMPA + VEGF did not show any apparent motor dysfunction at any time, up to the maximum period studied (25 days). Motor tests remained practically unchanged over time (Fig. 3) and stride pattern was identical to that of control rats infused only with Krebs' medium (Fig. 3). The remaining 4 rats of this group presented slight limping in the ipsilateral hindlimb, but this condition was not enough to significantly reduce scores in the motor tests and did not progress to any other motor alterations. Autotomy behavior, however, was not completely prevented by VEGF because 1 week after implant 10 rats

showed biting of hindpaw soft tissue, followed by biting of the hindlimb skin proximal to the body. Nevertheless, mutilation of the toes was absent and the severity of the damage was considerably less than that after 7.5 mM AMPA alone. Consequently, all rats were left until the end of the experiment 25 days after surgery and were considered for the study.

VEGF notably prevented the massive loss of motoneurons in spinal tissue observed after 7.5 mM AMPA alone. In contrast with the 80% loss observed in the latter group, more than 80% of the motoneurons were healthy 25 days after the infusion of VEGF + AMPA, as revealed by Nissl staining and ChAT immunostaining (Fig. 4). In addition, glial reactivity was also greatly reduced, because it was detected only in the dorsal horn where the cannula was inserted, whereas in the remaining spinal tissue the pattern of GFAP staining was similar to that of the control rats treated with Krebs' medium only (Fig. 4).

The protection by VEGF against the higher dose of AMPA used (11.2 mM) was also assessed in 6 rats. Five of these animals did not show any significant deficit in the 3 motor tests used, up to 15 days, when they were fixed for the histologic studies (motor tests scores were similar to those shown in Figure 3 for the protected animals). One rat presented partial hindlimb paralysis that started on day 3 but did not progress further; this rat fell from the Rotarod in less than 15 seconds but could still climb in the PGE test in 7 seconds. VEGF prevented the autotomy induced by 11.2 mM AMPA in a similar way to the protection after 7.5 mM AMPA, except in the rat that was not completely protected from paralysis.

Histologic and immunochemical observations revealed that the number of healthy motoneurons in the 5 fully protected rats was 11.8 ± 0.6 (75% compared with controls), whereas the partially protected animal had only 2.3 ± 0.5 healthy neurons (means of 5 sections).

Inasmuch as the main known function of VEGF is to promote angiogenesis, it was relevant to study whether the exogenous administration of this factor in the spinal tissue induced abnormal growing of vascular structures. The results

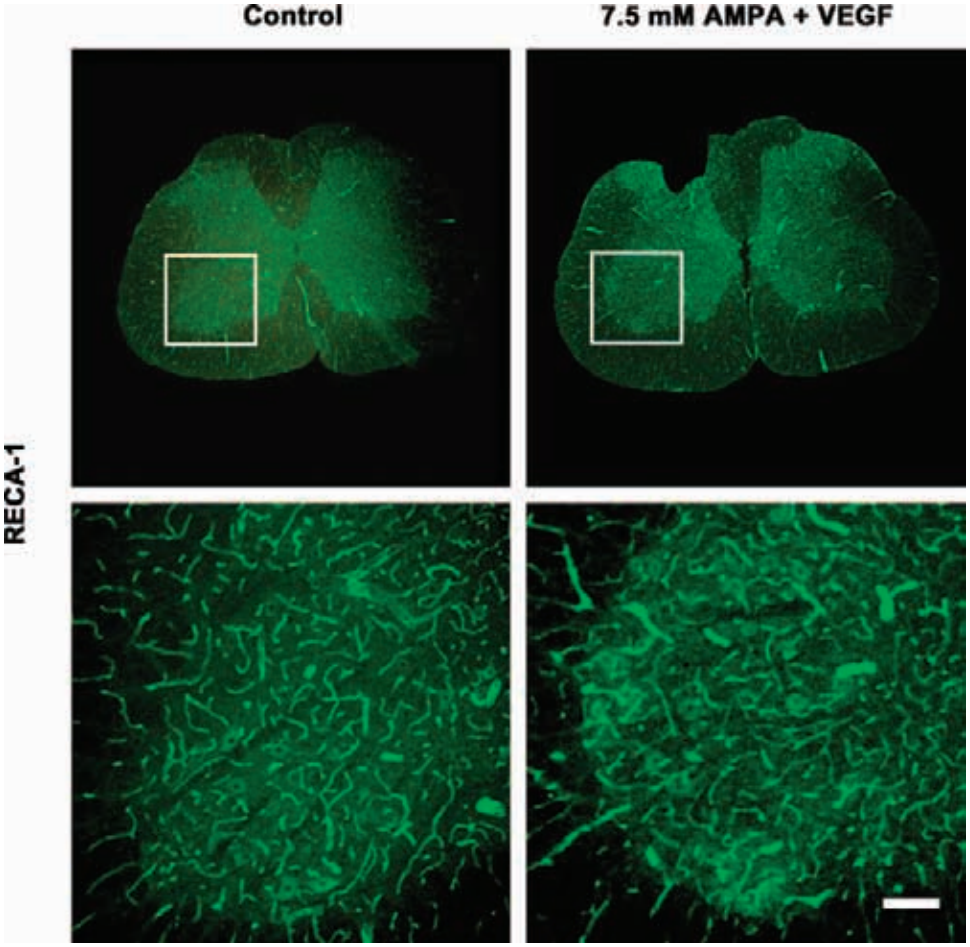

FIGURE 5. Chronic infusion of exogenous vascular endothelial growth factor (VEGF) does not alter the vascular architecture of spinal cord. Representative micrographs of rat endothelial cell antigen 1 (RECA-1) immunostaining in lumbar spinal cord of intact rats (control) and in the spinal cord infused with 7.5 mM α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) + VEGF for 25 days. The RECA-1 pattern is alike in both groups. Lower micrographs are magnifications of the areas in squares in the upper micrographs. Scale bar = 50 μm.

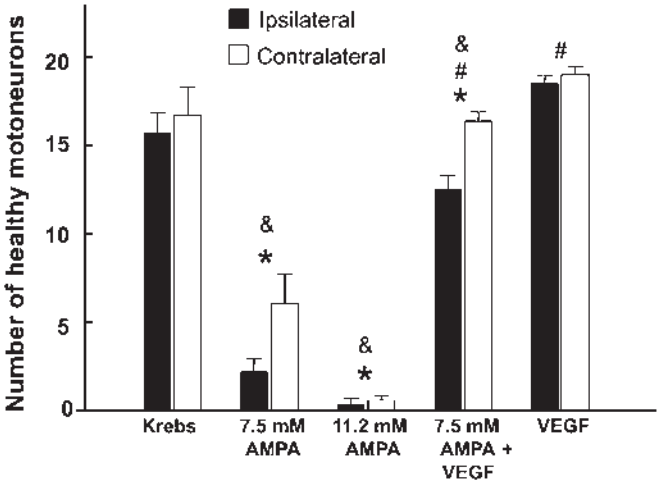

FIGURE 6. Number of healthy choline acetyltransferase (ChAT)-labeled motoneurons in the ipsilateral and contralateral ventral horns of control rats infused with Ringer-Krebs medium (n = 5), 7.5 mM α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) (n = 6), 11.5 mM AMPA (n = 7), 7.5 mM AMPA+ vascular endothelial growth factor (VEGF) (n = 8), and VEGF alone (n = 3). Five 40-μm sections from each rat were analyzed. Values are means ± SEM. *, $p < 0.05$ compared with Krebs medium; #, $p < 0.05$ compared with 7.5 mM AMPA; &, $p < 0.05$ compared with VEGF (ANOVA followed by a Fisher test).

of the immunohistochemistry for the endothelial cell antigen RECA-1 show that the infusion of VEGF alone or with AMPA did not significantly modify the expression pattern of RECA-1, with respect to intact rats (Fig. 5).

DISCUSSION

In this study we show that chronic administration of AMPA directly in the rat lumbar spinal cord causes progressive paralysis associated with motoneuron death, thus generating a chronic model of spinal motoneuron degeneration. This model, in which neuronal death is produced by excitotoxicity due to AMPA-receptor overactivation, was used to assess the action of VEGF, a growth factor previously shown to be protective in genetic models of ALS.

All animals treated with 7.5 or 11.2 mM AMPA developed hindlimb paralysis strikingly resembling that of the mutant SOD1 transgenic mouse and rat models of familial ALS (12, 22, 23). The paralysis correlated with the loss of motoneurons in both ipsilateral and contralateral ventral horns, indicating that AMPA diffused throughout the tissue. Because AMPA was administered locally through an implanted cannula in the lumbar spinal cord, effects outside the infused area are rather unlikely. In fact, the behavioral effects induced by AMPA administration were limited to the rear limbs, according to the innervation areas, and no signs of upper spinal or brain motor alterations were observed at any time. In addition, the histologic appearance of the lumbar spinal cord 1 mm away from the infusion site was normal.

Coadministration of VEGF together with the low dose of AMPA (7.5 mM) completely prevented the decline in the scores of the motor tests at all times after AMPA infusion, even in the few animals that presented ipsilateral hindlimb limping. The protective effect of VEGF was striking because it also notably prevented the rapid onset and progression of the motor symptoms produced by the high AMPA concentration (11.2 mM). This remarkable protection was clearly due to the prevention of cell death, inasmuch as the almost 100% loss of motoneurons after 11.2 mM AMPA was reduced to 25% and the ~86% loss after 7.5 mM AMPA was diminished to 20% (Fig. 6). This finding indicates that a loss of ~25% of motoneurons is not enough to produce motor deficits.

Because the infusion cannula was inserted in the dorsal horn region of the spinal cord, AMPA certainly also acted on neurons in this region. The overactivation of AMPA receptors located in dorsal neurons could thus account for the autotomy behavior observed a few days after pump implantation. In fact, it has been shown that AMPA receptors in the dorsal horn are determinants of activity-induced sensitization in pain pathways (24, 25) and that nociceptive stimuli through non-NMDA receptors (26) or excessive axonal firing reaching dorsal horn neurons (21) can cause autotomy. The fact that rats treated with AMPA + VEGF also presented autotomy behavior, albeit less intense, may indicate that, in contrast to motoneurons, dorsal neurons responded poorly to VEGF. This interpretation is supported by the finding that the deletion of the hypoxia response element in the VEGF promoter causes selective motoneuron death, leaving other neuronal populations unaffected and thus further supports the notion that motoneurons rely on the adequate trophic support of VEGF for survival (16). The differential effect of VEGF on distinct neuron groups could be related to the presence of receptors for VEGF. In fact, VEGF receptor 2 is expressed in somas of spinal motoneurons in both mice (16) and humans (27). Interestingly, this receptor is diminished in spinal motoneurons in ALS (27).

As mentioned, VEGF has been shown to protect against motoneuron death in familial transgenic rodent models (15, 17–19). Two hypotheses have been postulated to explain this protection. One states that VEGF promotes a good vascular niche that motoneurons require to survive (16). The other suggests that VEGF can directly activate its tyrosine kinase receptors expressed on the motoneuron membrane. The latter hypothesis is supported by accumulating evidence linking the activation of VEGF receptors to the phosphatidylinositol 3-kinase/Akt pathway that is required for motoneuron survival (28–30). In this respect, it has been shown that VEGF-dependent activation of phosphatidylinositol 3-kinase/Akt is capable of preventing neuronal death in a mutant SOD1-transfected motoneuron-like cell line (28). In addition, this intracellular pathway is required for the protection by VEGF against excitotoxic death in cultured hippocampal neurons (31). To what extent the neuronal death induced by mutant SOD1 shares molecular mechanisms with the excitotoxic death is still unsolved, but the fact that VEGF can protect motoneurons against both death mechanisms suggests an intersection in the degenerative processes.

We have recently demonstrated, using an in vivo model of spinal acute neurodegeneration, that both the blockade of Ca^{2+}-permeable AMPA receptors and the chelation of intracellular Ca^{2+} prevent motoneuron death produced by AMPA (9), indicating that excessive calcium entry is responsible for this motoneuron death. So, it is reasonable to think that in our present experimental model massive influx of Ca^{2+} entering through AMPA-permeable receptors accumulates inside mitochondria, possibly triggering energetic deficits and the induction of apoptotic processes (32, 33). Thus, the activation of prosurvival intracellular cascades such as phosphatidylinositol 3-kinase/Akt, which causes the inhibition of proapoptotic factors such as bad (30), caspase 9 (34), and caspase 3 (35, 36) might be the key factor responsible for the protection induced by VEGF in this model.

On the other hand, GFAP immunohistochemistry revealed that AMPA produced, besides the death of motoneurons, a notable astrogliosis surrounding the sites of the motoneuron-missing spaces. Remarkably, VEGF also prevented this glial reaction, suggesting that AMPA-induced astrogliosis was probably secondary to the massive motoneuron death.

It is worth emphasizing that at the dose used in this study VEGF per se did not produce any detectable effect on the behavior of the animals. Consistently, no significant changes were observed in the histologic features or in ChAT or GFAP immunochemistry of the spinal cord. In addition, the vascular architecture was not affected, as demonstrated by the results of RECA-1 immunostaining. This lack of angiogenic action is relevant, because abnormal tissue growth might be an undesired effect of the exogenous administration of VEGF. In fact, VEGF inhibitors are promising treatments for neoplastic diseases (37) on the basis of the rationale that growing tumors require continuous angiogenesis.

In conclusion, we have developed an in vivo model of chronic spinal neurodegeneration induced by excitotoxicity that is useful for assaying therapeutic agents that may protect against motoneuron death produced in the absence of altered genetic components. In addition, we found that administration of exogenous recombinant VEGF prevents the excitotoxic neuronal death induced by overactivation of AMPA receptors and the consequent motor alterations and paralysis. This is the first demonstration that VEGF protects against spinal motoneuron degeneration in an excitotoxicity model in vivo. Our results are relevant in view of the need for effective treatments directed to dampen motoneuron degeneration in processes such as sporadic ALS, which are not related to genetic alterations and occur in the great majority of cases of ALS.

ACKNOWLEDGMENTS

Angélica Zepeda held a postdoctoral position and made a fundamental contribution in the implementation of the chronic model of neurodegeneration by intraspinal AMPA infusion.

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VEGF protects spinal motoneurons against chronic excitotoxic degeneration in vivo by activation of PI3-K pathway

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Enviado a publicación

Resumen

Se ha demostrado que el VEGF protege a las motoneuronas espinales en algunos modelos de roedores transgénicos de FALS. En ratas sometidas a la degeneración excitotóxica crónica de las motoneuronas, a través de la infusión de AMPA mediante minibombas osmóticas en la médula espinal, hemos demostrado que el VEGF previene la muerte de las motoneuronas y la consecuente parálisis de las extremidades posteriores. En este trabajo demostramos que el VEGFR2 se encuentra en las motoneuronas de la rata adulta, y que su bloqueo mediante de la infusión de un inhibidor previene la protección mediada por el VEGF contra la muerte excitotóxica de las motoneuronas y sus déficits motores asociados. Además, la inhibición crónica de PI3-K, una cinasa activada por el VEGFR2, también previene completamente esta protección. También, encontramos que la infusión de AMPA resulta en una fosforilación incrementada de la proteína cinasa activada por mitógeno p38 (p38MAPK) y que el VEGF bloquea también este efecto. Estos resultados alumbran los mecanismos del efecto protector del VEGF contra la muerte excitotóxica de las motoneuronas espinales y sugieren que la función del VEGFR2 y la activación de la vía PI3-K/Akt, o la inhibición de p38MAPK pueden ser blancos terapéuticos importantes para la ALS.

VEGF protects spinal motoneurons against chronic excitotoxic degeneration *in vivo* by activation of PI3-K pathway

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Abstract

Vascular endothelial growth factor (VEGF) protects spinal motoneurons in models of familial amyotrophic lateral sclerosis (ALS). We previously demonstrated that VEGF also prevents motoneuron death and hindlimb paralysis in rats subjected to AMPA-induced chronic excitotoxic motoneuron degeneration. Here we show that tyrosine-kinase receptor-2 for VEGF (VEGFR2) is expressed in spinal motoneurons of the adult rat, and that its blockade impedes the VEGF-mediated protection against motoneuron death and paralysis. In addition, inhibition of phosphatidyl-inositol-3-kinase (PI3-K), which is activated by VEGFR2, completely prevented this protection, whereas blockade of mitogen-activated protein kinase kinases resulted only in a partial prevention. We also show that AMPA induces an increased p38MAPK phosphorylation and that VEGF blocks this effect. These results shed light into the mechanisms of the protective effect of VEGF against excitotoxic motoneuron death *in vivo* and suggest that VEGFR2 and activation of PI3-K or inhibition of p38MAPK might be important therapeutic targets for ALS.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective and progressive death of upper and lower motoneurons, with consequent progressive paralysis. The mechanisms of the specific motoneuron death are not fully understood. However, it is known that motoneurons are particularly susceptible to α-amino-3-hydroxy-5-isoxazolepropionate (AMPA) receptor overstimulation, allowing a massive entrance of calcium through AMPA receptors that lack the GluR2 subunit or are not edited in the calcium restrictive Q/R site (Williams *et al.* 1997; Greig *et al*. 2000; Van Den Bosch *et al.* 2000; Kawahara *et al*. 2003; Kawahara *et al*. 2004; Corona and Tapia 2007). We have recently shown that chronic infusion of AMPA in the rat lumbar spinal cord leads to motoneuron death and concomitantly paralysis of the rear limbs, and that these effects are completely prevented by the coadministration of recombinant vascular endothelial growth factor (VEGF) (Tovar-y-Romo *et al.* 2007). This growth factor was previously shown to be one of the most effective protectors of motoneurons in transgenic rodents that bear human mutant superoxide dismutase 1 (SOD1) (Azzouz *et al*. 2004; Zheng *et al.* 2004; Storkebaum *et al*. 2005; Wang *et al.* 2007). However, transgenic models reproduce the mechanism of neuronal death that occurs in only ~2% of ALS patients. The majority of ALS cases are sporadic, not involving mutant SOD1.

VEGF is a growth factor originally described to promote angiogenesis and vasculogenesis, and is a ligand for specific tyrosine kinase membrane receptors. One of them, VEGF receptor 2 (VEGFR2), also known as kinase domain receptor (KDR) or fetal liver kinase-1 (Flk-1), is expressed in motoneurons of human (Brockington *et al*. 2006) and mouse (Oosthuyse *et al*. 2001) spinal cord. Some ALS patients present a reduced expression of this protein (Brockington *et al.* 2006). In endothelial cells, VEGFR2 triggers the phosphorylation of intracellular pathways driven by the phosphatidyl-inositol-3-kinase (PI3-K), phospholipase C-γ, and mitogen-activated protein kinase kinase (MEK) (Ferrara *et al.* 2003). The activation of these intracellular signaling pathways has also been extensively studied in the central nervous system (CNS) (Zachary 2005), and they might be involved in the protection induced by VEGF against motoneuron death.

On the other hand, the participation of stress activated protein kinases like p38MAPK has been previously reported in familial models of the disease (Tortarolo *et al.* 2003; Holasek *et al*. 2005; Veglianese *et al*. 2006; Dewil *et al*. 2007). Furthermore, a role of p38MAPK has been described in a motoneuron specific cell death pathway (Raoul *et al.* 2006), and its inhibition prevents neuronal death *in vitro* (Dewil *et al.* 2007; Tolosa *et al*. 2009), and *in vivo*, although in the latter condition the effects are less clear (Dewil *et al*. 2007).

The aim of the present study was to evaluate the participation of VEGFR2 and of the activation of the survival signaling transduction pathways downstream this receptor, in the protection induced by exogenous VEGF against excitotoxic spinal motoneuron death, as well as its possible interference with the activation of MAPKs that could contribute to neuronal death.

Materials and methods

Animals

Adult male Wistar rats (270-290 g) were used in all the experiments. Procedures were performed in accordance with the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care Committee. Animals were housed in a laboratory environment with a 12 h light/dark cycle and with food and water *ad libitum*.

Surgical implantation of the osmotic minipumps

All drugs were infused into the dorsal horn of the lumbar spinal cord using osmotic minipumps as described previously (Tovar-y-Romo *et al*. 2007), with the following modifications. We implanted a double concentric cannula (CMA/7 Stockholm, Sweden) from which the tip, including the dialysis membrane, was removed. Rats were anesthetized with 5% halothane in a 95% O_2/5% CO_2 mixture and placed in a stereotaxic spinal unit. Anesthesia was lowered and maintained to 1%-2% halothane during surgery. A longitudinal incision of the skin was made in the lumbar region and muscles surrounding lumbar vertebrae were cut and retracted. On the second lumbar vertebra spinous process was removed and a ~1 mm diameter hole was drilled on the left side of the lamina, and a stainless-steel screw (1 mm diameter; 3.7 mm long) was inserted in the hole to anchor the implant. A ~2 mm^2 laminectomy was made in the right side of the lamina of the same vertebra and a small cut of the meninges was made to insert into the dorsal parenchyma the tip of the double concentric cannula. Dental cement was poured over the screw and the plastic support of the cannula. One end of the cannula was connected to an osmotic minipump (Alzet, model 2004, approximate capacity 200 µl, flow rate 0.25 µl /h; Durec, Cupertino, CA) containing AMPA and VEGF in an aqueous solution, and the other end to another minipump containing the inhibitors of VEGFR2, PI3-K and MEK dissolved in dimethylsulfoxide (DMSO). The two pumps were placed subcutaneously in the back of the rat, caudally to the skin incision. Finally, the skin incision was closed with surgical stainless-steel clips and rats received an i.m. dose of penicillin.

Preparation of osmotic minipumps for drug infusion

Of the two minipumps that were implanted in each experiment, one was filled with one of the following solutions: 7.5 mM AMPA (Tocris, Ellisville, MO) dissolved in 0.1 M phosphate buffer (PB) with or without recombinant rat $VEGF_{164}$ (Sigma, Saint Louis, MO) dissolved in 0.1 M PB/2% DMSO at a concentration calculated to deliver 24 ng/d (Tovar-y-Romo *et al*. 2007). The other minipump contained one of the following: vehicle (DMSO), 20 mM tyrphostin SU1498, 20 mM LY294002, 2 mM wortmannin or 20 mM PD98059 (all 4 inhibitors were obtained from LC Laboratories, Woburn, MA). The concentration of the inhibitors was chosen based on preliminary experiments, as indicated in Results. In the experiments regarding p38MAPK only one minipump, containing AMPA with or without VEGF, was used. Pumps were filled 40 h before the implantation and incubated in sterile saline at 37°C for stabilization.

Assessment of motor function

Motor performance was evaluated as described previously (Tovar-y-Romo *et al*. 2007). Briefly, rats were trained for 2 days prior to the surgery on 2 motor tests: a variation of the paw grip endurance (PGE) task and the rotarod (Columbus Instruments, Columbus, OH). Animals were evaluated in each test routinely until fixation.

For the PGE test, rats were placed individually on a horizontally placed grid (30 x 19 cm) attached to a mechanical rotator. The grid was gently turned (3 rpm) until reaching a vertical position. The time taken by the rats for climbing to the top of the grid and reaching a stable position, or the latency to fall from the grid when they were unable to climb was scored, with a cutoff of 40 s. Data are presented in Fig. 2 as "time to climb" and "latency to fall" as a continuum. For the rotarod test, rats walked individually on an accelerating (0.2 rpm/s) rod, starting from 10 rpm with a cutoff of 120 s, and the time on the rod was scored. In addition to the motor tests, a qualitative evaluation of the overall stride pattern was made by analyzing the hind footprints.

Histology and immunohistochemistry

For histological and immunohistochemical analyses, rats were fixed when they reached the lowest scores on the behavioral tests or at 20 days after implantation when they did not score low. For fixation, animals were anesthetized with barbiturate and perfused transcardially with 250 ml of ice-cold 0.9% saline, followed by 250 ml of ice-cold 4% paraformaldehyde in phosphate buffer pH 7.4. Spinal cords were removed, postfixed at 4°C, and successively transferred to sucrose solutions (up to 30%). Transverse 40 µm sections of the lumbar region, where the infusion cannula was implanted, were obtained in a cryostat. Alternate sections were stained with cresyl violet or immunostained for choline acetyltransferase (ChAT) and glial fibrillary acidic protein (GFAP). For the latter, free-floating sections were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)-Triton X-100 (0.3%) for 2 h, and then incubated with goat polyclonal anti-ChAT (1:100, Chemicon, Temecula, CA, USA) and rabbit anti-GFAP antibodies (1:1000; Sigma, Saint Louis, MO), for 48 h at 4°C. Sections were washed 3 times for 10 min in PBS–Triton X-100 and incubated with biotynil-conjugated mouse anti-goat IgG (1:200, Vector, Burlingame, CA) for 1 h. After 3 washes, sections were incubated for 2 h with avidin-Texas Red conjugate (1:200, pH 8.2, Vector) and fluorescein-conjugated anti-rabbit antibody (1:250 Zymed, Carlsbad, CA) for 2 h. Finally, sections were washed and mounted on silane (γ-methacryloxypropyltrimethoxysilane; Sigma)-covered slides and coverslipped with fluorescent mounting medium (DAKO, Carpinteria, CA). Sections were visualized under confocal microscopy (Olympus IX81); merged images are the overlay of 2 laser sections in the Z plane, using the Olympus Fluoview 1.6 Viewer. Morphologically undamaged motoneurons in the Nissl preparations (with a soma diameter >25 µm and a distinguishable nucleus) were counted in a 10X microscopic field. The number of cells was determined in sections where the trace of the infusion cannula was evident; 5 sections per rat were analyzed and the values were averaged.

For VEGFR2 immunohistochemistry, free floating sections from the lumbar spinal cord of an intact rat were obtained and treated as described above but using the following conditions: incubation with primary antibodies for 7 days at 4°C with a rat anti-VEGFR2 monoclonal antibody (1:50, Chemicon, Australia) and SMI-32 antibody (1:2000; Covance, Berkeley, CA), and incubation with secondary antibodies for 48 h at 4°C with fluorescein-conjugated anti-rat antibody (1:100, Jackson ImmunoResearch, West Grove, PA) and Cy5-conjugated anti-mouse antibody (1:200, Zymed, Carlsbad, CA).

For p38MAPK-P immunohistochemistry, rats treated with AMPA or AMPA + VEGF were sacrificed 2 days after surgery in order to assess the immunofluorescence of the phosphorylated p38MAPK at a time when the motoneurons of the rats treated with AMPA alone were still alive. Sections were incubated with an anti-p38MAPK antibody that recognizes phosphorylation at

Thr^{180} and Tyr^{182} (1:500, Calbiochem, Germany). Fluorescence was developed with a tyramide signal amplification system with Cy5 as fluorochrome, following the directions from manufacturer (TSA Cyanine 5 System, PerkinElmer, Waltham, MA). Co-labeling of motoneurons was made with the motoneuron marker SMI-32 and the secondary anti-mouse antibody conjugated with FITC.

In all immunofluorescence analyses cross-reactivity was excluded by appropriate controls incubated in the absence of primary antibodies; these control sections showed no immunostaining.

Western blot analysis

For immunoblot assays of PI3-K and p44/42 MAPK (as target of MEK1/2), treated rats were sacrificed by decapitation 3-4 days after surgery and the spinal cords were removed. The infused area was dissected out and homogenized in ice-cold lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% EDTA, 1% Triton X-100, 1% CHAPS, 0.5% Nonidet P-40, 0.1% BSA and 0.1% SDS) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) and a mixture of phosphatase inhibitors (0.5 mM Na_3VO_4, 1 mM NaF and 1 µM okadaic acid). Homogenates were prepared by sonication and then centrifuged for 5 min at 4500 rpm in a tabletop centrifuge from which the aqueous phase was recovered. Based on a protein concentration curve, a total amount of 10 µg of protein was used for the immunodetection of p44/42 MAPK and 50 µg of protein for PI3-K. Samples were boiled for 3 min in loading Laemmli buffer (Bio Rad, Hercules, CA) containing β-mercaptoethanol, separated by polyacrylamide-gel electrophoresis and transferred to a nitrocellulose membrane (Bio Rad) overnight at 4ºC. Membranes were incubated with 2.5% dry milk in Trizma-base buffer saline/Tween 20 (TBS/T; Trizma Base 0.1 M pH 7.6, NaCl 0.9%, Tween 20 0.1%) during 1 h. Then, membranes were incubated overnight at 4°C with one of the following antibodies in TBS/T-5% BSA: anti-p44/42 MAPK (1:1000), anti- phospho-p44/42 MAPK that selectively recognizes phosphorylation at residues Thr^{202} and Tyr^{204} (1:2000), anti-PI3-K p85 (1:1000) or anti-phospho-PI3-K p85/p55 that selectively recognizes phosphorylation at residues Tyr^{458} in p85 and Tyr^{199} in p55 (1:1000). All antibodies were purchased from Cell Signaling Technology (Anvers, MA). Membranes were washed 3 × 10 min in TBS/T and then incubated with an anti-rabbit IgG HRP-linked antibody (1:2000, Cell Signaling Technology) for 1 h. Finally, membranes were washed 3 × 10 min in TBS/T and incubated with West Pico chemiluminescent substrate (Pierce, Rockford, IL) and exposed to an X-ray film. Levels of immunoreactivity were quantified by densitometry using the image analyzer software ImageJ.

Statistical analysis

Comparisons regarding number of motoneurons were made using ANOVA followed by a Fisher's post-hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

VEGFR2 is expressed in spinal motoneurons of the adult rat

To investigate the participation of VEGFR2 in the protection of motoneurons against excitotoxicity we first determined its expression in spinal motoneurons in intact adult rats. VEGF activates a series of tyrosine kinase membrane receptors like VEGFR1 and VEGFR2, but VEGFR2 seems to be the principal mediator of VEGF neuroprotective effects (Brockington *et al.*, 2006; Ferrara *et al.*, 2003; Zachary, 2005). Double immunohistochemistry for VEGFR2 and SMI-32, a motoneuron marker (Carriedo *et al.* 1996), revealed a co-localization of both markers, thus confirming the presence of VEGFR2 in motoneurons of the adult rat (Fig. 1).

VEGFR2 and PI3-K signaling pathway are key mediators of the neuroprotective effects of VEGF against AMPA-induced paralysis

As shown in Fig. 2, the rats treated with 7.5 mM AMPA + 24 ng/d VEGF in one minipump and control vehicle DMSO in the other one are capable to perform properly in rotarod and PGE up to 20 days after the surgery, and never showed any sign of limping or dragging the rear limbs, whereas animals infused with AMPA + DMSO behaved as those receiving only AMPA (Figs. 2 and 3; Tovar-y-Romo *et al.*, 2007), confirming our previous finding of protection by VEGF and in addition showing that DMSO was innocuous. In contrast, when 20 mM SU1498, a selective VEGFR2 inhibitor (Strawn *et al.* 1996), was infused in addition to AMPA + VEGF all treated rats (n=6) displayed motor alterations that began with flexion of the ipsilateral hindlimb and progressed to a rigid extension of both hindlimbs as soon as 2 days after surgery; by day 7 the posterior train of all rats was fully paralyzed, causing notable rotarod, PGE and stride deficits (Figs. 2 and 3). At this time the animals were fixed for histology. Infusion of 20 mM SU1498 alone did not induce any motor alteration up to 20 days after surgery (n=3).

We then tested the role of the PI3-K/Akt pathway in the protective effect of VEGF against paralysis. For this objective we tried two known inhibitors of PI3-K: LY294002 and wortmannin. Infusion of 2 mM, 7.5 mM and 10 mM LY294002 did not affect the protection by VEGF, since the rats treated with these doses in addition to 7.5 mM AMPA + 24 ng/d VEGF did not show any type of motor alteration. When the concentration of LY294002 was increased to 20 mM, equivalent to an infusion of 1.2×10^{-7} moles in the tissue per day, the treated rats (n=6) displayed ipsilateral limping, observable from day 10 after surgery in the footprints as a wasted mark of the ipsilateral hindpaw (Fig. 3). This alteration, however, was not enough to generate significant deficits in the rotarod or the PGE tests (Fig 2). Control rats treated with 20 mM LY294002 alone for 20 days did not show any motor alteration (n=3).

Wortmannin is a more potent and irreversible inhibitor of PI3-K and is used at concentrations up to 3 orders of magnitude less than LY294002, given the differences in the mechanism of action ((Walker *et al.* 2000). Infusion of 2 mM wortmannin in addition to AMPA + VEGF resulted in the development of a progressive paralysis in the treated rats (n=6) that started few days after

surgery; by day 5 most of the rats were still able to climb in the PGE test but with difficulty, and in the following days they developed a complete paralysis that made them fall (Fig. 2). On the rotarod, the rats performed poorly since 5 days of treatment. Paralysis in this group was evident in the footprints and is remarkably similar to that presented by rats treated with only AMPA (Fig. 3). Rats receiving 2 mM wortmannin alone for up to 10 days did not present motor alterations (n=3).

Finally, we analyzed the participation of the MEK/ERK pathway by inhibiting MEK with PD98059. Infusion of 20 mM PD98059, the most concentrated solution that we were able to infuse because of its poor solubility, along with 7.5 mM AMPA + 24 ng/d VEGF had an effect similar to that of LY294002, since animals did not show a notorious motor deficit in both the PGE and the rotarod tests (n=6) (Fig. 2), but presented limping of the ipsilateral hindlimb by 10 days of treatment that can be observed in the footprints (Fig. 3). Infusion of PD98059 alone did not induce any motor deficiency (n=3).

Since the infusion cannula was placed in one side of the spinal cord, diffusion of the drugs to the contralateral side probably took some time and was not complete. This may explain the differences between right and left hindlimbs in the stride traces (Fig. 3).

The inhibition of the intracellular kinases and their level of phosphorylation were corroborated by western blot analysis. As shown in Fig. 4, VEGF induced an increase in the phosphorylation of PI3-K as compared to control and AMPA conditions, and this was partially blocked by LY294002 and totally by wortmannin. This correlates well with the effects of the two inhibitors on motor behavior described above. Regarding MEK, we observed a high basal level of phosphorylation of p44/42MAPK in control animals, which was not significantly modified either by VEGF or by the MEK inhibitor PD98059 at the concentration used (Fig. 4). This also correlates with the motor behavior of the animals.

PI3-K inhibition prevents the protection against spinal motoneuron death exerted by VEGF

The paralysis presented by the rats treated with AMPA + VEGF + SU1498 or wortmannin is due to the loss of motoneurons in the ventral spinal horn of the perfused lumbar segment, as revealed by the histological and the immunohistochemical analyses of ChAT and GFAP. Infusion of AMPA and vehicle DMSO resulted in a remarkable loss of > 80% of motoneurons in both the ipsilateral and contralateral horns (1.1 ± 0.6 and 3.0 ± 1.2 motoneurons, respectively, vs 15.7 ± 1.1 and 16.7 ± 1.6 in control rats), accompanied by intense glial reaction. Confirming our previous findings, VEGF almost fully prevented motoneuron loss and glial reaction (Fig. 5). In these animals, the number of healthy motoneurons in the ipsilateral horn (12.6 ± 0.8) decreased ~20% as compared to the contralateral side (16.3 ± 0.5) where no damage occurs (Fig. 6). It is important to note that DMSO did not interfere either with the toxic effect of AMPA nor with the protection induced by VEGF against excitotoxicity.

Blockade of VEGFR2 by SU1498 totally prevented the protection by VEGF, since the number of healthy motoneurons was almost identical to that after AMPA alone, and the glial reaction was notable (Figs. 5 and 6). Wortmannin also completely prevented the protective action of VEGF in the ipsilateral horn, although in the contralateral one the prevention was partial (1.9 ± 0.6 and 9 ± 1.1 motoneurons, respectively), and gliosis was intense (Figs. 5 and 6).

Consistently with their effects on motor behavior, the inhibitor of PI3-K LY294002 and the inhibitor of MEK PD98059 only partially reduced the protection by VEGF in the ipsilateral horn (68% and 60% motoneuron loss, respectively) and had no significant effect in the contralateral one. GFAP staining in these animals indicates that glial reaction also occurred in the ipsilateral horn but was not as intense as in the rats totally unprotected (Figs. 5 and 6). As mentioned above, the differences between ipsilateral and contralateral sides probably depend on the rate of drug diffusion in the tissue.

AMPA augments the activation of p38MAPK and VEGF inhibits this increase

To test whether p38MAPK participates in the intracellular processes involved in our excitotoxic model of motoneuron death, we used immunofluorescence with an antibody that recognizes phosphorylated p38MAPK. As shown in Fig. 7, control rats showed some phospho-p38MAPK staining, restricted to the perinuclear area, whereas in the animals infused with AMPA an intense labeling of the motoneuron cytoplasm, spanning throughout the entire soma, was observed. Importantly, when VEGF was co-infused with AMPA the staining of phospho-p38MAPK was considerably reduced and limited to a more localized site around the nucleus, similarly to the control animals (Fig.7).

Discussion

In this work we demonstrate that the pharmacological blockade of VEGFR2 and of PI3-K prevents the protection by VEGF against AMPA-

induced excitotoxic spinal motoneuron death *in vivo*, indicating that activation of VEGFR2 and the consequent triggering of the signaling pathways driven by PI3-K is involved in the protective action of VEGF. In addition, we show that the activation of p38MAPK by AMPA is prevented by VEGF.

A role of VEGF in protecting motoneurons *in vivo* has been determined previously in experimental models of the familial form of ALS (Azzouz *et al*. 2004; Zheng *et al*. 2004; Storkebaum *et al*. 2005; Wang *et al*. 2007), and in our present non-genetic model of spinal neurodegeneration *in vivo* elicited by the overactivation of AMPA type receptors that allows a massive entrance of calcium into the cell (Tovar-y-Romo *et al*., 2007). The degenerative mechanism may involve the uncontrolled activation of lytic enzymes that eventually leads to neuronal death (Corona *et al*. 2007; Tovar-y-Romo and Tapia 2007).

Two hypotheses have been put forward to explain the mechanism of VEGF-mediated neuronal protection; one states that VEGF supports an adequate tissue vasculature that motoneurons require to survive, the other one postulates that VEGF exerts its protective action directly on motoneurons. Our model of excitotoxic neuronal death induced by the chronic infusion of a glutamatergic agonist has no effects on the vasculature per se, and since this is not a genetic modified model it is unlikely that such alterations may participate in the neuronal death process, opposite to the familial models in which vascular changes are present prior to motoneuron death (Zhong *et al*. 2008). Furthermore, we have previously shown that the dose of VEGF used in these experiments has no effects on the vasculature of spinal cord tissue (Tovar-y-Romo *et al*., 2007).

The two main receptors with tyrosine kinase catalytic activity that are activated by VEGF are VEGFR1 and VEGFR2. VEGFR1 is also a receptor for other members of the VEGF family like placental growth factor and VEGF-B, and in endothelial cells is considered to be a negative regulator of VEGF by impeding its union to VEGFR2 (Ferrara *et al*. 2003). On the other hand, VEGFR2 is a promoter of survival for both endothelium and CNS cells (Ferrara *et al*. 2003; Zachary 2005) and, differently from VEGFR1, it is expressed in spinal motoneurons in humans, and its expression is diminished in ALS patients (Brockington *et al*. 2006). Consistently, mouse motoneurons (Oosthuyse *et al*. 2001), and motoneurons of spinal cord organotypic cultures from 8 days old rats (Tolosa *et al*. 2008) also express VEGFR2. As revealed by double fluorescence immunohistochemistry, our present results show the co-localization of the VEGFR2 and SMI-32 markers, thus confirming the presence of the receptor in the motoneurons of adult rats. This finding is of interest because the expression of VEGF and VEGFR2 is high in embryonic stages but declines during development and becomes restricted to discrete regions of the CNS (Millauer *et al*. 1993).

ALS is a non-cell autonomous pathology, meaning that other cellular types besides motoneurons play a fundamental role in the neurodegenerative process. Hence, VEGF might protect motoneurons indirectly by activating its receptors located on other cells. However, it has been demonstrated that VEGFR2 is not expressed in the glia of spinal cord grey matter, in both rodents (Sköld *et al*. 2000; Tolosa *et al*. 2008) and humans (Brockington *et al*. 2006) although, as VEGFR1, is expressed in reactive astrocytes following trauma (Choi *et al*. 2007; Krum *et al*. 2008). However, in our experiments reactive astrocytosis seems a consequence of motoneuron death, since the coadministration of VEGF with AMPA prevents both the motoneuron death and the concomitant glial response. Besides, the blockade of VEGFR2 completely prevents the protection by VEGF, thus suggesting that the participation of VEGFR1 is minimal, if any. Therefore, it is reasonable to conclude that the protective effect of VEGF is mediated by its binding to VEGFR2 located on the motoneuron membrane.

Among the intracellular pathways involved in cellular survival that are activated downstream VEGFR2 are PI3-K/Akt and MEK/ERK. The role of these pathways in the VEGF-mediated neuroprotection has been previously reported *in vitro* (Matsuzaki *et al*. 2001; Li *et al*. 2003a; Koh *et al*. 2005; Tolosa *et al*. 2008). *In vivo*, hypoxia causes motoneuron death through a reduction of VEGFR2 and a decrease in the phosphorylation of PI3-K and MEK (Shiote *et al*. 2005). Furthermore, PI3-K/Akt is required for motoneuron survival and axonal regeneration in spinal cord injury (Namikawa *et al*. 2000). Here we show that inhibition of PI3-K with wortmannin blocks the protection induced by VEGF against the AMPA-induced excitotoxic motoneuron death, thus resulting in motoneuron loss and consequently complete paralysis. However, the blockade of PI3-K with LY294002 resulted in a milder loss of motoneurons, only in the ipsilateral horn of the spinal cord, which was reflected as limping of the corresponding limb without reaching complete paralysis. The different outcomes of the administration of these two PI3-K inhibitors are likely due to their potency. Wortmannin is a more powerful blocker with nanomolar IC_{50} that irreversibly inhibits PI3-K by fitting the active site and inducing covalent modifications and conformational changes of the protein (Wymann *et*

al. 1996; Walker *et al*. 2000). The paralysis and motoneuron loss after blockade of PI3-K were not as intense as after blockade of VEGFR2, suggesting that other molecules besides PI3-K and its downstream effectors participate in the VEGF-mediated neuroprotection. One of these could be the MEK/ERK pathway.

Using the selective inhibitor of MEK PD98059, we found results similar to those obtained after the infusion of LY294002: only a slight motor disturbance was generated after 20 days of treatment, reflected as limping of the ipsilateral hindlimb and a significant, although not total, loss of motoneurons in the ipsilateral horn of the spinal cord. Because the basal level of MEK phosphorylation was high and because the low solubility of the inhibitor impeded the administration of higher concentrations, a clear correlation of these effects with a blockade of the activation of the MEK/ERK pathway cannot be made. Nonetheless, we may conclude that the participation of these kinases is important, but not indispensable, for the neuroprotective action of VEGF, as long as the PI3-K/Akt pathway works properly. In agreement with this conclusion, it has been shown that even when PI3-K/Akt and MEK/ERK pathways are simultaneously activated in response to the same stimulus, their role in survival may be independent from each other (Xue *et al*. 2000; Li *et al*. 2003b).

Our present results also demonstrate that in our experimental model another component of the neuronal death process is the activation of p38MAPK. As mentioned in the Introduction, it has been shown that p38MAPK is involved in other models of motoneuron degeneration, including the transgenic model of familial ALS (Tortarolo *et al*. 2003; Holasek *et al*. 2005; Veglianese *et al*. 2006; Dewil *et al*. 2007). In addition, p38MAPK is a component of a neurodegenerative processes activated by Fas that seems to be unique for motoneurons (Raoul *et al*. 2006). In our model, this mechanism probably involves a calcium-dependent process that activates p38MAPK following excitotoxicity. In this respect, it was found in primary cultures of cerebellar granule neurons that intracellular calcium increase due to glutamate stimulation activates p38MAPK via Rho GTPases (Semenova *et al*. 2007). Consistent with this interpretation, we previously showed that spinal motoneuron death *in vivo* triggered by AMPA receptor overactivation is a calcium-dependent process, because the specific blockade of calcium-permeable AMPA receptors with naphthyl acetyl spermine and the intracellular chelation of calcium impede AMPA-induced motoneuron death (Corona and Tapia 2007). Therefore, another consequence of the massive entrance of calcium to the cell, in addition to the activation of lytic enzymes, would be the activation of death-triggering kinases like p38MAPK. Thus, our finding that VEGF blocks the AMPA-induced phosphorylation of p38MAPK suggests that the suppression of cellular death processes mediated by phospho-p38MAPK might be another mechanism for protection induced by VEGF. In agreement with this interpretation, it was recently demonstrated in spinal cord organotypic slices that the motoneuron death induced by growth factors withdrawal can be reverted by VEGF through a mechanism involving the blockade of p38MAPK activation (Tolosa *et al*. 2009).

In conclusion, our findings strongly indicate an important role of the survival pathway PI3-K/Akt in the mechanism of the protection exerted by VEGF against excitotoxic spinal motoneuron death *in vivo*, and that this mechanism is triggered by the activation of the VEGFR2 present on motoneuron somas. We emphasize that these results were obtained in a non-transgenic model of spinal motoneuron death *in vivo* that permits a correlation of neuronal damage with motor alterations, and that this is the first demonstration of the participation of an intracellular signaling pathway in the mechanism of spinal motoneuron protection in this type of models. Our results shed light into the mechanisms behind the protective effects of neurotrophic factors in spinal motoneurons *in vivo* and suggest that the PI3-K pathway may be an appealing therapeutic target for ALS.

Acknowledgements

This work was supported by DGAPA, UNAM (project IN209807) and CONACYT, Mexico (project 60322). L.B.T.R. is recipient of a scholarship from CONACYT. The authors declare they have no conflict of interest.

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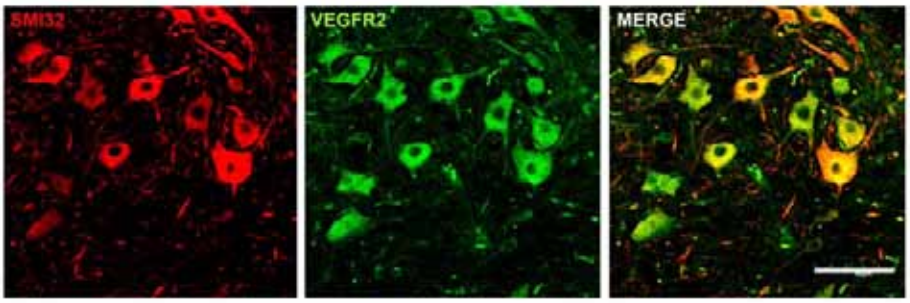

Fig. 1 Spinal motoneurons of the adult rat express VEGFR2. A section from the lumbar spinal cord of an intact rat was immunolabeled with the motoneuron marker SMI-32 (red) and with an anti-VEGFR2 antibody (green). Colocalization of the markers indicates that motoneurons in the spinal cord of the rat express VEGFR2. Scale bar = 100 µm.

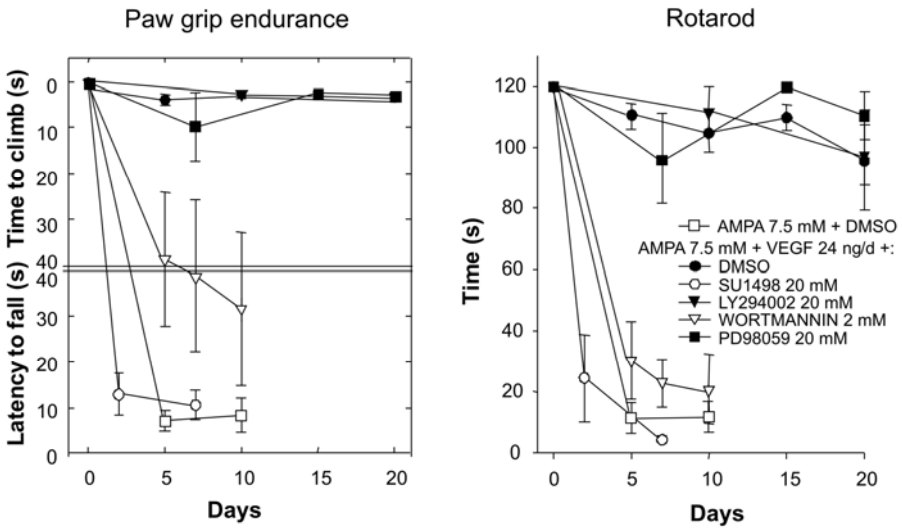

Fig. 2 Chronic blockade of VEGFR2 and PI3-K prevents the protection mediated by VEGF against AMPA-induced paralysis. Time course of PGE and rotarod performances of rats infused with the indicated drugs. Blockade of VEGFR2 with SU1498 and of PI3-K with wortmannin resulted in paralysis in the treated rats, manifested as low scores in the motor tests. In PGE, time to climb and latency to fall were scored and presented as a continuum because rats that were able to climb could reach the top of the grid within the cutoff time of 40 s; otherwise they would hold on to the grid and finally fall. Each group is the mean ± SEM for 6 rats, except for AMPA + DMSO (n=3; this result is very similar to that previously reported after AMPA alone).

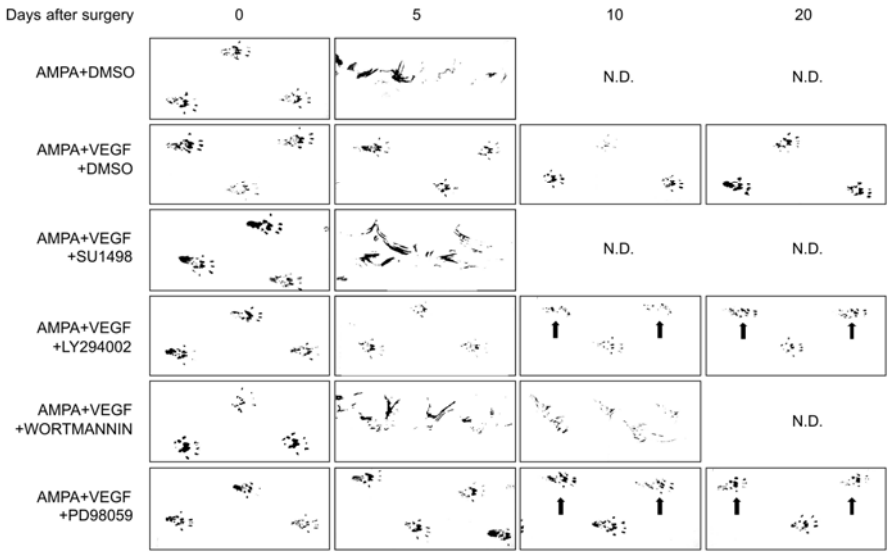

Fig. 3 Representative hindpaw footprints of rats chronically infused with the drugs indicated on the left. Blockade of VEGFR2 with SU1498 and PI3-K inhibition with wortmannin resulted in a complete paralysis of the hindlimbs depicted as dragging marks in the footprinting, similar to that presented by rats treated with only AMPA. Inhibition of PI3-K with LY294002 and of MEK with PD98059 induced a delayed limping of the ipsilateral hindlimb depicted as a shrunken mark of the footprint (arrows) observed since day 10 after surgery.

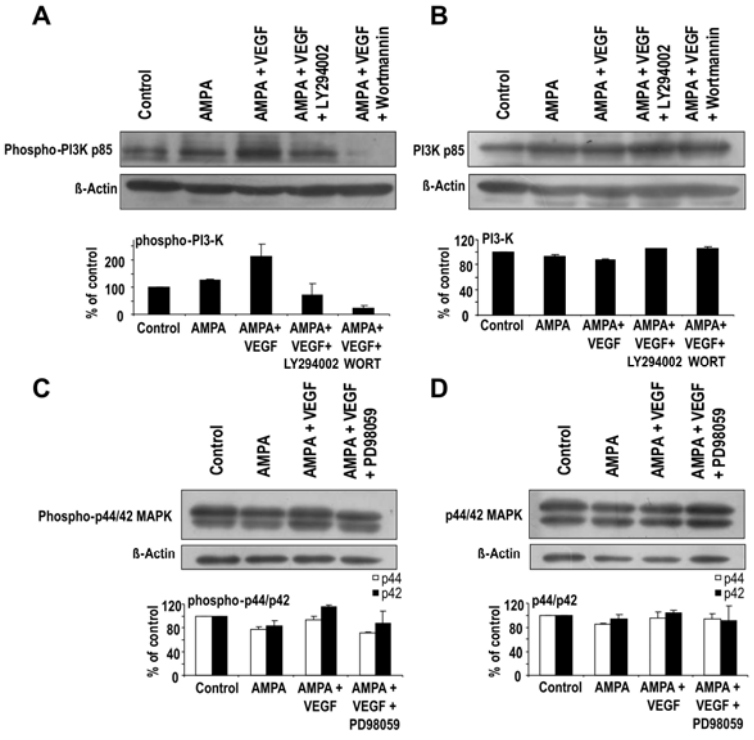

Fig. 4 Immunoblot analysis of phosho-PI3-K and phospho-p44/42 MAPK in the spinal cord of rats treated with AMPA, AMPA + VEGF and AMPA + VEGF in combination with the PI3-K inhibitors LY294002 and wortmannin and the MEK inhibitor PD98059. (A) Phosphorylated PI3-K p85 subunit. (B) Total PI3-K p85

subunit. (C) Phosphorylated p44/42 MAPK. (D) Total p44/42 MAPK. Equal amounts of tissue protein (50 µg for PI3-K (A and B) and 10 µg for p44/42 MAPK (C and D)) were loaded into each lane and subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes for detection with specific antibodies. Membranes were probed with an anti β-actin antibody as control of the total protein loaded per lane. Graphs show the mean ± SEM of densitometric values as percent of controls (two independent experiments in each condition).

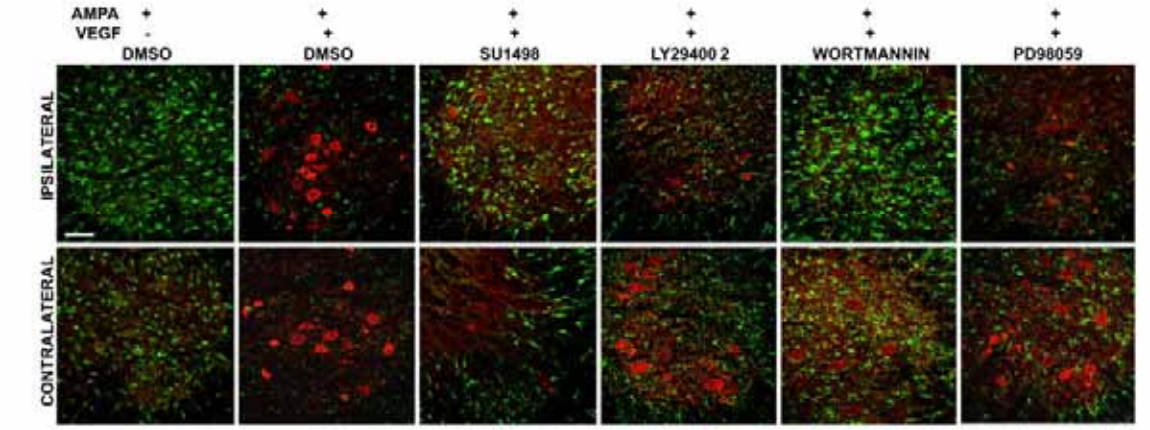

Fig. 5 Chronic blockade of VEGFR2 and PI3-K prevents the protection mediated by VEGF against AMPA-induced motoneuron death. Representative micrographs of the ventral horns immunostained for ChAT (red) and GFAP (green) in the lumbar section of spinal cord of rats treated with the drugs indicated on top. Note the remarkable loss of motoneurons and the glial reaction induced by AMPA in both sides of the spinal cord, and the protection by VEGF. VEGFR2 blockade with SU1498 and PI3-K inhibition with wortmannin completely prevented this VEGF-mediated protection. Note that the changes in gliosis (GFAP) closely inversely parallel those of motoneuron number (ChAT). See Fig. 6 for quantitative data. Scale bar = 100 µm.

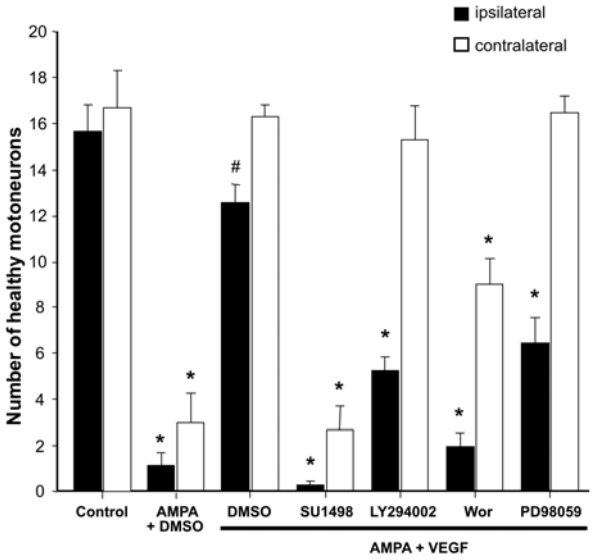

Fig. 6 Number of healthy motoneurons in the spinal cord of rats treated with the drugs indicated. Nissl stained motoneurons in the ipsilateral and contralateral ventral horns of rats treated with the indicated drugs were counted in five 40 µm sections from each rat (n=6 per group). Values are mean ± SEM. *p < 0.0001 as

compared to corresponding side in the control group and in the AMPA + VEGF + DMSO group; #p < 0.01 as compared to the corresponding side in the control group (ANOVA followed by Fisher's post-hoc).

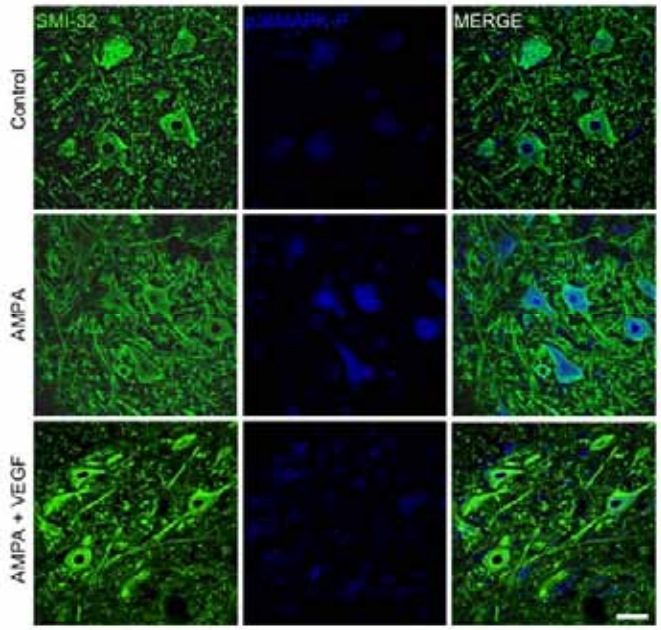

Fig. 7 AMPA infusion stimulates the phosphorylation of p38MAPK and VEGF blocks this stimulation. Representative micrographs of the motoneurons of the ventral horns immunostained for phospho-p38MAPK (blue) and the motoneuron marker SMI-32 (green). Phospho-p38MAPK staining is diffuse throughout the neuronal somas in the rats treated only with AMPA whereas the co-administration of VEGF notably reduced and confined the labeling mainly to the perinuclear zone. Scale bar = 50 μm.

Discusión

La muerte neuronal origina diversas patologías dependiendo del tipo de neuronas que mueran y de la función que desempeñen dentro de las estructuras específicas del SNC donde se encuentren, y las diferentes neuropatologías están caracterizadas por la vulnerabilidad selectiva de un grupo de neuronas. En la ALS, se ha sugerido que la vulnerabilidad de las motoneuronas puede estar relacionada a una alta sensibilidad a la estimulación de receptores tipo AMPA permeables a calcio (Carriedo et al. 1996; Williams et al. 1997; Greig et al. 2000; Van Den Bosch et al. 2000; Vandenberghe et al. 2000; Van Damme et al. 2002; Corona y Tapia 2004; Tateno et al. 2004; Corona y Tapia 2007) y a una pobre capacidad de amortiguamiento del catión que entra a las células a través de estos canales (Ince et al. 1993; Alexianu et al. 1994; Siklos et al. 1998; Palecek et al. 1999). De manera interesante las motoneuronas que no se afectan en la ALS, como las de los núcleos de Onuf y el oculomotor, poseen niveles elevados de las proteínas amortiguadoras de calcio calbindina-D28k y parvalbúmina, en contraste con las motoneuronas alfa de la médula espinal (Alexianu et al. 1994). Otros factores de susceptibilidad descritos incluyen un gran contenido de neurofilamentos (Williamson et al. 1998) y una alta vulnerabilidad al mal funcionamiento mitocondrial (Bergmann y Keller 2004).

Por otra parte, se ha propuesto que el estrés oxidante generado a través de diferentes mecanismos, entre ellos la ganancia de la función tóxica de la SOD1 mutada en algunos casos familiares, afecta de manera selectiva a las motoneuronas o a la glia circundante porque se altera el funcionamiento de diversos sistemas como el de transporte de glutamato (Volterra et al. 1994; Trotti et al. 1996; Trotti et al. 1999; Rao et al. 2003). Con estas bases, hemos estudiado dos fenómenos que podrían estar involucrados en la muerte de las motoneuronas: la excitotoxicidad producida por la pérdida del transporte de glutamato y la estimulación excesiva de los receptores glutamatérgicos, especialmente los de tipo AMPA, mediante la administración del agonista exógeno del receptor. Además, estudiamos el efecto protector ejercido por el VEGF contra la muerte neuronal excitotóxica en la médula espinal de la rata y caracterizamos los mecanismos intracelulares que participan en dicha protección.

Experimentalmente existen muchos medios para abordar el estudio de la muerte neuronal (Tovar-y-Romo et al. 2009a), en este trabajo hemos empleado modelos *in vivo* que permiten la correlación de la muerte neuronal con sus efectos concomitantes como la parálisis. Utilizamos el ratón transgénico con la SOD1 humana mutada con el

fin de caracterizar la vulnerabilidad de las neuronas cerebrales a la excitotoxicidad, y desarrollamos un nuevo modelo de neurodegeneración espinal crónica que permite estudiar los efectos de la excitotoxicidad y la protección mediada por el VEGF en un paradigma experimental libre de alteraciones genéticas, las cuales no están presentes en la mayor porción de los pacientes que sufren ALS.

En esta última parte de la tesis presento una discusión integral sobre las alteraciones del transporte de glutamato en la ALS y sobre la actividad del VEGF en la prevención de la muerte de las motoneuronas, y finalmente presento el artículo "*Glutamate excitotoxicity and therapeutic targets for amyotrophic lateral sclerosis*" que pone en perspectiva al VEGF como una opción terapéutica para el tratamiento de la ALS.

Alteraciones del transporte de glutamato como causa de la ALS

La neurotoxicidad del glutamato como causa de la ALS se ha fundamentado en anormalidades del metabolismo de este neurotransmisor. Los primeros trabajos sobre esta línea comenzaron hace casi 20 años cuando se mostró que existían aumentos en el contenido de glutamato en el líquido cefalorraquídeo de un grupo pequeño de pacientes (Rothstein et al. 1990). Posteriormente, estudios similares se realizaron en cohortes numerosos mostrando que aproximadamente 40% de los pacientes tienen un incremento significativo en la concentración de glutamato en el líquido cefalorraquídeo (Shaw et al. 1995; Spreux-Varoquaux et al. 2002). Desde entonces, una gran cantidad de reportes han presentado evidencias de alteraciones en el transporte de glutamato (como se menciona en la introducción; Tovar-y-Romo y Tapia 2007) generando una de las principales hipótesis sobre el origen de la muerte de las motoneuronas en la ALS. Esta hipótesis sostiene que la neurodegeneración se debe la muerte neuronal excitotóxica causada por la pérdida de los transportadores de glutamato, o por un mal funcionamiento de los mismos, que puede ser causado por el daño oxidante (Rothstein et al. 1995). En este sentido se ha demostrado en cultivos de astrocitos corticales expuestos al sistema generador de ROS xantina/xantina oxidasa o a H_2O_2, que la función de los transportadores es vulnerable a la oxidación y que la adición de SOD1, catalasa o agentes reductores como ditiotreitol rescata dicha función (Volterra et al. 1994). Se ha demostrado también que el peroxinitrito es capaz de inhibir la función de GLT1, GLAST y EAAC1 en segundos (Trotti et al. 1996), y que en sinaptosomas de médula espinal de ratas transgénicas $SOD1^{G93A}$ hay una reducción progresiva en la V_{max} de la recaptura de glutamato dependiente de sodio (Dunlop et al. 2003).

También, en ovocitos de *Xenopus* que coexpresan la SOD1 humana silvestre o las mutantes A4V o I113T y el EAAT2 humano, la actividad del transportador se inhibe con la administración de H_2O_2 en los huevos que expresan las formas mutantes de SOD1, mas no en los que expresan la SOD1 silvestre (Trotti et al. 1996).

La pérdida o disfunción de los transportadores de glutamato se ha tratado de reproducir experimentalmente a través de varios métodos. Aunque en cultivos neuronales el bloqueo del transporte de glutamato por PDC causa daño después de periodos largos (Rothstein et al. 1993; Carriedo et al. 1996; Okazaki et al. 1996; Velasco et al. 1996; Amin y Pearce 1997; Wang et al. 1998; Himi et al. 2003; Matyja et al. 2005), la perfusión *in vivo* de este bloqueador no genera hiperactividad neuronal o muerte en el hipocampo o en la corteza motora de los ratones $SOD1^{G93A}$, a pesar del gran incremento en la concentración del glutamato extracelular. Este resultado va de acuerdo con datos previos que muestran que la perfusión de PDC por microdiálisis no mata a las neuronas espinales (Corona y Tapia 2004) ni a las cerebrales del estriado o del hipocampo (Massieu et al. 1995; Obrenovitch et al. 1996; Massieu y Tapia 1997) y es una fuerte indicación de que la presencia de la SOD1 mutada no hace a las neuronas más susceptibles al daño excitotóxico. Estos resultados concuerdan con análisis *in vitro* que demuestran que la sobre-expresión de la SOD1 mutada no incrementa la sensibilidad de las neuronas a la excitotoxicidad (Raoul et al. 2002; Van Den Bosch et al. 2004)

Sin embargo estos hallazgos no excluyen la posibilidad de que una inhibición más prolongada del transporte de glutamato tenga efectos tóxicos sobre las neuronas (Rattray y Bendotti 2006). Por lo mismo, probamos el efecto de la inhibición continua del transporte de glutamato en la médula espinal de ratas silvestres mediante la administración crónica de los bloqueadores del transporte PDC y TBOA. Mediante este procedimiento logramos inducir una elevación de 3 a 4 veces el nivel basal de glutamato extracelular por un periodo de hasta 10 días, y encontramos que este tratamiento tampoco causa la muerte de las motoneuronas, aún más, ninguno de los animales tratados presentó alteraciones conductuales en las pruebas motoras. Es importante hacer notar que la elevación del glutamato extracelular que se obtuvo experimentalmente es mayor que la medida en el líquido cefalorraquídeo de pacientes con ALS, que en la mayoría de los casos es menor del doble del valor basal de glutamato (Spreux-Varoquaux et al. 2002). Estos resultados llevan a la conclusión de que incluso un incremento sostenido de glutamato extracelular que surge del bloqueo del transporte no es capaz de hiperactivar a los receptores y que por lo mismo no

causa la muerte de las motoneuronas espinales *in vivo*. Si la pérdida de la función de los transportadores fuera el evento clave en el desarrollo de la muerte de las motoneuronas en la ALS, el reemplazo de esta función debería evitar la muerte neuronal, en este sentido, un ratón doble transgénico que sobre-expresa GLT1 junto con la $SOD1^{G93A}$ tiene cierto retraso en la muerte de las motoneuronas espinales, sin embargo los rasgos más importantes de la enfermedad como el inicio y progreso de la sintomatología y la duración de vida no se modifican en comparación a los ratones $SOD1^{G93A}$ (Guo et al. 2003), sugiriendo una vez más que la disfunción de los transportadores no es un evento fundamental para el desarrollo de la ALS.

Una posible explicación sobre la falta de daño neuronal a pesar de la gran cantidad de glutamato acumulado en le espacio extracelular debido al bloqueo de su transporte es que el glutamato no alcanza a sus receptores postsinápticos. Sabemos que la falta de daño no es debida a una difusión limitada de los bloqueadores porque bajo condiciones experimentales idénticas la administración de 4-AP o AMPA si generan la muerte de las neuronas en el hipocampo de los ratones o en la médula espinal de las ratas respectivamente.

Aunque se han encontrado reducciones en la cantidad del transportador EAAT2 en los ratones transgénicos $SOD1^{G93A}$ y en la médula espinal de pacientes con ALS, la mayoría de estos datos son consistentes con la posibilidad de que los cambios sean una consecuencia y no una causa de la enfermedad (Rattray y Bendotti 2006). Por ejemplo, en los ratones $SOD1^{G93A}$ cuando el GLT1 está disminuido en animales heterocigotos $GLT1^{+/-}$, el inicio de la parálisis no se modifica en comparación con los ratones $SOD1^{G93A}$ que tienen la cantidad normal de la proteína transportadora, lo que implica que la pérdida de GLT1 no es un factor inicial de la enfermedad (Pardo et al. 2006). También se sabe que el GLT1 se regula a la baja en cultivos de astrocitos donde no hay la influencia de neuronas sugiriendo que su expresión en la membrana plasmática depende de la interacción con moléculas solubles liberadas por las neuronas (Schlag et al. 1998).

La ausencia de un efecto deletéreo después de la administración del PDC en las condiciones experimentales del ratón transgénico tiene otro punto interesante que analizar. En la ALS así como en otras enfermedades neurodegenerativas existe un déficit energético que es potenciado por el daño a las mitocondrias (Beal 2000). Por ejemplo, los ratones $SOD1^{G93A}$ muestran vacuolizaciones en las mitocondrias espinales como uno de los primeros síntomas de la enfermedad (Wong et al. 1995), y la SOD1

que es normalmente citosólica se acumula y forma agregados mitocondriales en la médula espinal (Higgins et al. 2002) y en el cerebro (Vijayvergiya et al. 2005) de estos animales. Entonces, la hipótesis indicaría que cuando existe un daño energético el potencial de la membrana plasmática, mantenido por las ATPasas Na^+/K^+, se pierde, lo que causaría que incrementos aun pequeños de glutamato mantuvieran a la membrana despolarizada activando a los receptores glutamatérgicos más rápido, generando un daño excitotóxico más severo. Sin embargo, experimentalmente no encontramos tal efecto, por lo que postulamos que la expresión de la SOD1 mutada en las neuronas no es un factor que incremente la susceptibilidad a la muerte neuronal excitotóxica *in vivo*.

La excitotoxicidad mediada por el receptor AMPA permeable a calcio y el desarrollo de un modelo de neurodegeneración espinal progresiva

Como ya se mencionó, a pesar de que no existe una prueba concluyente de que la excitotoxicidad es el mecanismo por el cual se desarrolla la muerte de las motoneuronas y se genera la ALS, se sabe que las motoneuronas expresan receptores AMPA permeables al calcio lo que constituye un factor de susceptibilidad a la muerte neuronal debido, entre otros mecanismos, a desbalances mitocondriales y desequilibrios en la homeostasis del catión (von Lewinski y Keller 2005).

La permeabilidad del calcio a través de los receptores AMPA que no poseen la subunidad GluR2 o que tienen la subunidad no editada en el sito Q/R es un factor determinante en la muerte excitotóxica de estas neuronas. Las propiedades electrofisiológicas de los receptores AMPA que dependen de la abundancia relativa de GluR2, como la sensibilidad a las poliaminas, el índice de rectificación y la permeabilidad relativa al calcio, muestran que las motoneuronas poseen niveles bajos de GluR2 en comparación con otros tipos neuronales (Van Damme et al. 2002). Molecularmente también se ha demostrado que los niveles de ARNm de GluR2 son más bajos en las motoneuronas en comparación con otras neuronas (Van Den Bosch et al. 2006). El sólo hecho de que la subunidad GluR2 esté ausente en la composición del receptor AMPA no es causa suficiente para el desarrollo de ALS, sin embargo, ratones knockout de GluR2 padecen una sintomatología motora (Jia et al. 1996), y la deficiencia de su expresión acelera la patología y reduce la esperanza de vida de los ratones $SOD1^{G93A}$ (Van Damme et al. 2005). Asimismo, la cruza del ratón $SOD1^{G93A}$ con otro que posee una permeabilidad reducida al calcio, dado que sobre-expresa la

subunidad GluR2 bajo el control transcripcional del promotor de la ChAT, retrasa el inicio de la enfermedad, la mortalidad y el desarrollo de los cuadros patológicos (Tateno et al. 2004). La importancia de la edición de la subunidad GluR2 en la sobrevivencia neuronal también ha sido demostrada en varios paradigmas experimentales. Animales transgénicos que no pueden editar el sitio Q/R del mensajero de la subunidad GluR2 desarrollan un fenotipo letal con convulsiones y neurodegeneración aguda (Brusa et al. 1995; Higuchi et al. 2000), mientras que animales transgénicos que expresan una mezcla de GluR2 con un residuo de asparagina en el sitio Q/R, donde la edición del mensajero es imposible, y la GluR2 silvestre poseen receptores AMPA permeables a calcio (Burnashev et al. 1992) y desarrollan una degeneración tardía de las motoneuronas (Feldmeyer et al. 1999; Kuner et al. 2005).

La capacidad de las motoneuronas para amortiguar los incrementos de calcio es limitada debido a la baja expresión de proteínas amortiguadoras. Se ha pensado que esta condición fisiológica puede proveerle a las motoneuronas la capacidad de una señalización rápida a un relativo bajo costo energético, permitiéndoles tiempos más rápidos de relajación de las corrientes transitorias del calcio durante una actividad rítmica de alta frecuencia, pero esta condición sin duda las hace más vulnerables a la excitotoxicidad (von Lewinski y Keller 2005).

La administración del agonista exógeno de los receptores AMPA en la médula espinal de la rata genera, por lo tanto, la muerte de las motoneuronas espinales en un proceso dependiente de calcio. Sabemos que la neurodegeneración de las motoneuronas comienza de 3 a 6 horas después de la administración de AMPA por microdiálisis, y es completa en un periodo de 12 a 24 horas (Corona y Tapia 2004), lo que lleva a la hipótesis de que la entrada de calcio induce un proceso dañino retrasado que termina en la muerte de las motoneuronas. El bloqueo selectivo de los receptores AMPA que no tienen la subunidad GluR2 con naftil-acetil-espermina en la médula espinal de la rata, previene la pérdida de las motoneuronas inducida por AMPA (Corona y Tapia 2007), indicando que las ratas poseen receptores AMPA funcionales permeables a calcio que no poseen la subunidad GluR2 y que el proceso celular que lleva a la muerte neuronal es dependiente de calcio. La administración del quelante de calcio intracelular BABTA-AM junto con AMPA también previne la neurodegeneración espinal *in vivo* confirmando esta hipótesis (Corona y Tapia 2007).

En el presente trabajo, hemos mostrado que la administración crónica de AMPA en la porción lumbar de la médula espinal de la rata causa una parálisis progresiva asociada a la muerte de las motoneuronas. Los animales que reciben una administración continua de AMPA desarrollan una sintomatología motora progresiva del tren posterior similar a la que presentan los ratones transgénicos $SOD1^{G93A}$, y la parálisis se correlaciona con la muerte de las motoneuronas en ambos lados de la médula espinal indicando que el AMPA difunde a través del tejido. Entonces la administración de AMPA en la médula espinal permite el desarrollo de modelos *in vivo* que mimetizan la degeneración de las motoneuronas en un ambiente libre de alteraciones genéticas. La principal limitación de nuestro modelo es que, aunque la sobre-activación de los receptores AMPA mediada por glutamato puede ser un mecanismo importante para la neurodegeneración en la ALS, aún no existe una evidencia convincente de que este proceso ocurre en la enfermedad humana.

Nuestros resultados también demuestran la participación de p38MAPK en el proceso de muerte neuronal inducido por AMPA. Se sabe que esta cinasa está involucrada en la neurodegeneración de los ratones $SOD1^{G93A}$ (Tortarolo et al. 2003; Holasek et al. 2005; Veglianese et al. 2006; Dewil et al. 2007), y es un componente de una vía de señalización de muerte neuronal que parece ser específica de motoneuronas (Raoul et al. 2002). En nuestro modelo este mecanismo probablemente involucra un proceso de activación de p38MAPK dependiente de calcio ya que se sabe que esta cinasa puede ser activada en respuesta a este catión por medio de Rho GTPasas (Semenova et al. 2007). De manera interesante demostramos que el VEGF es capaz de evitar la activación por fosforilación de esta proteína reguladora aunque no elucidamos el mecanismo subyacente.

El VEGF como terapia alternativa para la ALS

Los factores tróficos representan una alternativa terapéutica de utilidad para el tratamiento de las enfermedades neurodegenerativas (Kotzbauer y Holtzman 2006). Entre ellos, el VEGF es de especial importancia en la ALS ya que como se demostró en el ratón $VEGF^{\delta/\delta}$, los animales sobrevivientes a la deleción del HRE del promotor del VEGF, desarrollan una parálisis progresiva de las extremidades posteriores asociada a la muerte de las motoneuronas espinales extremadamente parecida a la parálisis de los ratones $SOD1^{G93A}$ (Oosthuyse et al. 2001), lo que sugiere que el VEGF es un factor importante en la fisiología de las motoneuronas.

Se ha considerado que el VEGF es un modulador del proceso patológico en la ALS y se le ha tratado de relacionar como un biomarcador de la misma. En este sentido, se ha reportado una disminución del VEGF circulante en el plasma de pacientes con ALS en comparación con controles sanos (Lambrechts et al. 2003). Sin embargo, otros estudios no han encontrado tales diferencias en los niveles circulantes de VEGF en plasma (Devos et al. 2004; Moreau et al. 2006; Just et al. 2007). La función del VEGF en el desarrollo y mantenimiento del sistema circulatorio es tan importante que la deleción o mutaciones del gen *Vegf* son letales; esta puede ser una razón por la cual no se han encontrado mutaciones en los pacientes con ALS en la región codificante ni en la región promotora del *Vegf* (Gros-Louis et al. 2003; Lambrechts et al. 2003; Brockington et al. 2005). Sin embargo, un estudio reportó inicialmente haplotipos de riesgo en el promotor o en la secuencia líder del gen *Vegf* en un meta-análisis con alrededor de 1900 pacientes de Bélgica, Suecia e Inglaterra (Lambrechts et al. 2003). Sin embargo, la asociación de estos haplotipos y SALS no fue confirmada en estudios con pacientes británicos (Brockington et al. 2005), holandeses (Van Vught et al. 2005), norteamericanos (Chen et al. 2006), italianos (Del Bo et al. 2008), alemanes (Fernandez-Santiago et al. 2006) ni chinos (Zhang et al. 2006). Más aún, en un meta-análisis con más de 7000 casos analizados de 11 poblaciones de ALS europeas y americanas, no se encontró ninguna asociación entre los haplotipos de riesgo anteriormente reportados y la ALS (Lambrechts et al. 2008). Por otra parte, se ha reportado que en el líquido cefalorraquídeo de pacientes con ALS hay una disminución significativa en el contenido de VEGF en comparación con controles sanos o con otras enfermedades neurodegenerativas (Devos et al. 2004). Estos resultados son también controversiales dado que no han sido replicados por otros grupos (Ilzecka 2004; Nagata et al. 2007).

Recientemente se ha estudiado de manera intensiva la participación del VEGF en la modulación del proceso patológico en el modelo del ratón transgénico $SOD1^{G93A}$. En experimentos de entrecruzamiento entre ratones $SOD1^{G93A}$ y $VEGF^{\delta/\delta}$ los dobles transgénicos mueren más rápido que los $SOD1^{G93A}$ (Lambrechts et al. 2003). Además, en los ratones $SOD1^{G93A}$ hay una regulación a la baja del ARNm del VEGF antes del inicio de la enfermedad (Lu et al. 2007), y en las ratas transgénicas $SOD1^{G93A}$ hay una disminución del VEGF antes del inicio de los síntomas que progresa durante el curso de la enfermedad (Xie et al. 2004). Por otra parte, se ha demostrado de manera consistente en diversos paradigmas experimentales que la administración de VEGF a ratones $SOD1^{G93A}$ alivia de manera importante la sintomatología (Azzouz et al. 2004; Zheng et al. 2004; Storkebaum et al. 2005; Wang et al. 2007). Además, el VEGF ha

sido probado como protector contra la muerte neuronal en modelos de hipoxia/hipoglucemia y de privación de factores tróficos (Van Den Bosch et al. 2004) y en modelos *in vitro* se ha probado que puede proteger a las motoneuronas de una muerte excitotóxica lenta (Tolosa et al. 2008), aunque no de la excitotoxicidad aguda (Van Den Bosch et al. 2004). En este trabajo demostramos que la co-administración del VEGF junto con AMPA previene completamente el deterioro de las funciones motoras y que este efecto protector se debe a la preservación de las motoneuronas ya que el VEGF es capaz de proteger al 75 % de las mismas. Nuestros resultados constituyen el primer dato reportado sobre el efecto protector del VEGF contra la muerte excitotóxica de las motoneuronas espinales *in vivo*, en un modelo de neurodegeneración no inducida por mutaciones de la SOD1.

Existen dos hipótesis sobre como el VEGF ejerce su efecto protector en los modelos de ALS, la primera señala que el VEGF mantiene una estructura vascular adecuada en el tejido espinal que las motoneuronas requieren para sobrevivir. La segunda postula que el VEGF ejerce su acción protectora directamente sobre las motoneuronas. En nuestro modelo la neurodegeneración es causada por la sobre-excitación de los receptores AMPA por lo que la contribución de alteraciones vasculares del tejido como el origen de la muerte neuronal es muy poco probable, contrariamente a lo que sucede en el ratón transgénico $SOD1^{G93A}$ en el que se han reportado alteraciones vasculares que ocurren antes de la muerte de las motoneuronas (Zhong et al. 2008). Hemos demostrado con los presentes experimentos que la neuroprotección en este modelo es mediada directamente a través de la activación del VEGFR2 presente en las motoneuronas de la médula espinal de la rata adulta y que la activación de este receptor desencadena la señalización intracelular de la vía PI3-K cuya inhibición evita la protección inducida por del VEGF contra la neurodegeneración excitotóxica. *In vitro* se ha demostrado la participación de esta vía en la protección contra la neurodegeneración en diferentes modelos experimentales (Matsuzaki et al. 2001; Li et al. 2003a; Koh et al. 2005; Tolosa et al. 2008) e *in vivo*, se ha reportado que la hipoxia causa la neurodegeneración de las motoneuronas a través de la reducción de la fosforilación de PI3-K y MEK (Shiote et al. 2005), y que estas vías se requieren para la sobrevivencia de las motoneuronas y la regeneración axonal en modelos de daño de médula espinal (Namikawa et al. 2000).

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Glutamate excitotoxicity and therapeutic targets for amyotrophic lateral sclerosis

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Two forms of amyotrophic lateral sclerosis (ALS) are known, the familial (FALS), due in part to mutations in superoxide dismutase 1 (SOD1), and the sporadic (SALS), which accounts for > 90% of all cases. The cause of SALS is not known, but excitotoxicity due to overactivation of glutamate receptors may mediate the motor neuron degeneration in the spinal cord, which is the hallmark of this disease. Overactivation of calcium-permeable α-amino-3-hydroxy-5-isoxazole propionate receptors lacking the subunit glutamate receptor 2, leading to an increase in calcium cytoplasmic concentration, seems to play an important role in the mechanism of neuronal death. The knowledge of this mechanism, in addition to other factors, provides several possible targets for therapeutic strategies that are reviewed in this article. Some of these strategies have proven to be partially effective in both human mutant superoxide dismutase 1 transgenic rodents (FALS model) and the few existing *in vivo* models of spinal motor neurodegeneration induced by excitotoxicity (SALS models), although observable benefits are still to be shown in clinical trials.

Keywords: α-amino-3-hydroxy-5-isoxazole propionate receptor, calcium, excitotoxicity, neurodegeneration, spinal cord

Expert Opin. Ther. Targets (2007) 11(11):1415-1428

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive loss of motor neurons, leading to severe motor deficits. Two forms of the disease are known: the sporadic (SALS) and the familial (FALS), although the symptoms and pathology of the two forms are practically identical. FALS accounts for only ~ 10% of the cases, of which 15 – 20% is characterized by mutations in the gene that codes for copper/zinc superoxide dismutase 1 (SOD1) [1]. The cause and mechanisms of the remaining 90% of sporadic cases are unknown, and still no effective treatment is available to either halt or significantly retard its progression. The prominent pathophysiologic feature of ALS is the selective death of the motor neurons located in the ventral horns of the spinal cord (lower motor neurons), the brain stem and the cerebral motor cortex (upper motor neurons). The initial symptoms depend on which type of motor neuron is primarily affected. In general, the neuronal loss causes progressive muscle weakness, spasticity and fasciculation, and consequent paralysis; other symptoms may include dysphagia and dysarthria. Generally, patients die as a result of respiratory failure 3 – 5 years after diagnosis. Worldwide prevalence of typical ALS is ~ 2 – 8 per 100,000 individuals and occurs during mid-adulthood, although it can start at younger ages [2].

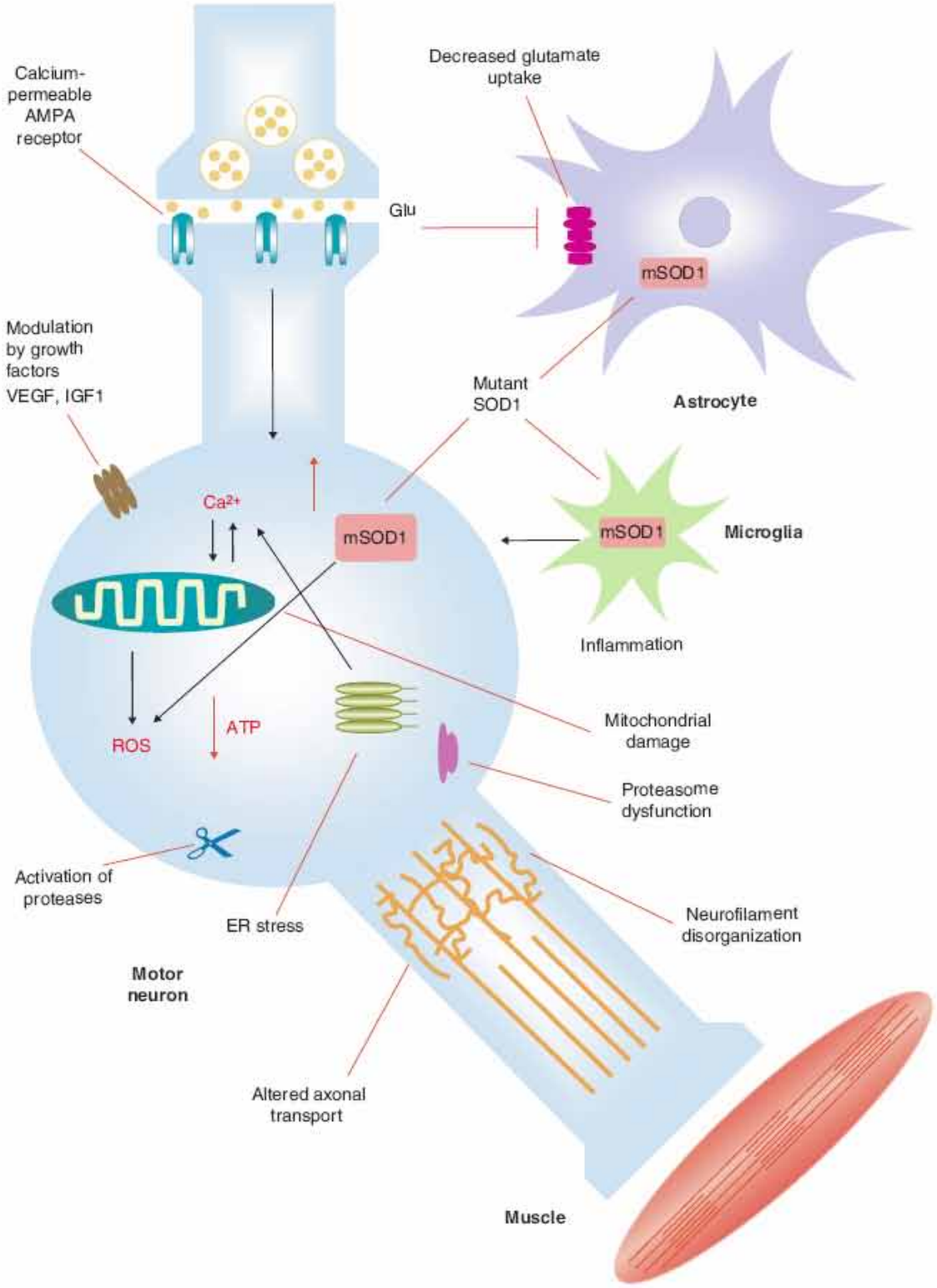

Figure 1. Mechanisms that may contribute to motor neuron damage in ALS and, hence, are targets for therapeutic strategies (continued).

2. Glutamate-mediated neurotransmission and excitotoxicity

Glutamate is the main excitatory neurotransmitter in the mammalian CNS and is involved in many aspects of normal brain function, including cognition, memory and learning, as well as in synaptogenesis, neuronal circuitry organization, synaptic plasticity, cell migration and cell differentiation. However, an excessive synaptic glutamatergic transmission, leading to overactivation of the different types of glutamate receptors, may result in neurodegeneration. As will be discussed below, overactivation of these receptors results in hyperexcitation of neurons by depolarization and, thus, may allow a massive influx of calcium into the neurons, triggering the uncontrolled activation of deleterious processes that eventually produce membrane destruction and cell death [3]. This phenomenon seems to occur after a wide range of neurological insults, including stroke (anoxia-ischemia), trauma, epilepsy and various neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease and ALS [4-6]. In fact, glutamate-mediated excitotoxicity is considered as the foremost mechanism leading to motor neuron degeneration in both SALS and FALS, although several other hypothesis have been postulated, such as protein misfolding and aggregation, mitochondrial dysfunction, axonal transport impairment, oxidative stress, inflammation and apoptosis [7-11].

When glutamate is released from the presynaptic glutamatergic terminal, it binds to its receptors located on the postsynaptic membrane. Two broad categories of glutamate receptors have been recognized on the basis of their molecular cloning, electrophysiologic and biochemic properties, and pharmacologic antagonism: the ionotropic and the metabotropic. The ionotropic receptors are ligand-gated ion channels, whereas the metabotropic receptors do not form ion channels, but are associated to G proteins and coupled to the production of intracellular second messengers [12-14]. The former type comprises the *N*-methyl-D-aspartate, α-amino-3-hydroxy-5-isoxazole propionate (AMPA) and kainate receptors; the latter is constituted by three main subtypes with several subgroups, linked to different second messenger pathways.

The action of glutamate is rapidly terminated by high-affinity glutamate uptake from the synaptic cleft into neurons and glial cells, and this process seems to be essential for maintaining extracellular glutamate below neurotoxic levels. This uptake is carried out by transporter proteins in a process driven by symport or antiport co-transport of ions [15]. Of the five high-affinity glutamate transporters that have been identified, cloned and located throughout the CNS [16,17], excitatory amino acid transporter 2, called glutamate transporter 1 (GLT1) in rodents, is the most abundant glutamate transporter and is present almost exclusively in astrocytes (**Figure 1**) [17]. Under physiologic conditions, GLT1 carries probably ~ 90% of the released glutamate, as suggested by the finding that mice lacking this protein suffer seizures and die prematurely [18]. With regard to ALS, one of the prevailing hypotheses of the mechanisms of neurodegeneration is the malfunction of GLT1. This hypothesis is based mainly on a reduction in the content of the glutamate transporter protein excitatory amino acid transporter (GLT1) that was detected in the motor cortex and the spinal cord of ALS patients analyzed post-mortem [19,20]. In addition, 40% of ALS patients have increased glutamate concentration in the cerebrospinal fluid [21,22]. However, some recent findings do not support this hypothesis, because an increased concentration of extracellular glutamate, due to pharmacological blockade of the transporter in the rat spinal cord *in vivo*, failed to cause motor neuron death or motor deficits [23] [Tovar-y-Romo *et al.*, in preparation] and also failed to cause neuronal death in the hippocampus and motor cortex in transgenic FALS mice [24].

As mentioned earlier, intracellular calcium is a prime mediator of several cellular processes leading to neuronal death. Of the different kinds of glutamate receptors, the NMDA type is normally permeable to this cation, and when overactivated leads to neuronal death in some areas of the brain where it is abundant, such as the hippocampus [25-27]. However, in spinal motor neurons, calcium entry is likely to occur, not through NMDA receptors, but by AMPA receptors, which mediate fast excitatory synaptic neurotransmission. Functional AMPA receptors are homo- or hetero-oligomeric assemblies composed of four subunits, GluR1–GluR4, in various combinations, which are encoded by separate genes and are expressed abundantly and ubiquitously throughout the CNS [12]. The subunits are ~ 900 amino acids in length, show approximately 65 – 75% sequence homology and form tetrameric assemblies

Figure 1. Mechanisms that may contribute to motor neuron damage in ALS and, hence, are targets for therapeutic strategies (continued). An increase of extracellular glutamate concentration in the synaptic space may produce an excessive stimulation of glutamate receptors, principally the Ca^{2+}-permeable AMPA receptors, which results in increased intracellular calcium concentration. Calcium-induced damaging processes include: mitochondrial damage, with the consequent disruption of energy metabolism and ROS generation, besides activation of lytic enzymes such as proteases. Endoplasmic reticulum stress, neurofilament abnormalities and altered axonal transport also occur. In FALS, mutant mSOD1 generates ROS, which can damage the GLT and, consequently, augment the extracellular glutamate levels. The mutant SOD1 can also induce microglial activation that could promote the secretion of toxic cytokines and cause inflammation, thus contributing to motor neuron death.

ER: Endoplasmic reticulum; IGF: Insulin-like growth factor; mSOD1: Mutant superoxide dismutase 1; ROS: Reactive oxygen species; VEGF: Vascular endothelial growth factor.

AMPA receptor	Ca^{2+} permeability	External NAS blockade
Lacking GluR2	Yes	+
+ GluR2 (R)	No	-
+ GluR2 (Q)	Yes	+

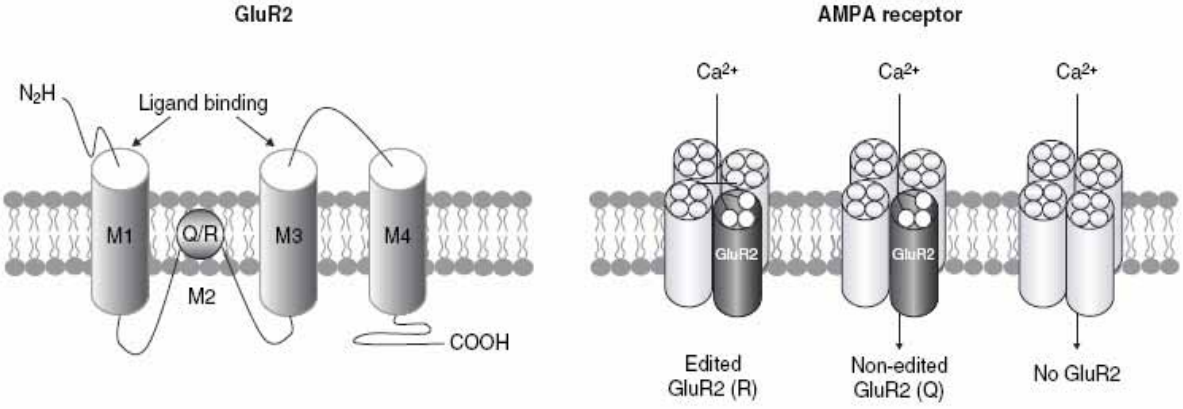

Figure 2. Structure of AMPA receptor GluR subunits showing its functional domains. The figure on the right and the table illustrate how the presence of GluR2 and its Q/R site editing determine the calcium permeability of the receptor. 1-naphthyl acetyl spermine is a specific blocker of the calcium-permeable AMPA receptor that protects against AMPA-induced motor neuron death *in vivo*.
AMPA: α-amino-3-hydroxy-5-isoxazole propionate; GluR: Glutamate receptor; M1-M4: Membrane domain; NAS: 1-napthyl acetyl spermine; Q/R: Glutamate/arginine.

arranged around a central pore. As illustrated in **Figure 2**, each subunit has a large extracellular N-terminal domain, three transmembrane-spanning domains (M1, M3 and M4), one re-entrant loop within the membrane (M2), which contributes to the cation pore channel and is the site for glutamine/arginine (Q/R) RNA editing in the glutamate receptor 2 (GluR2) subunit (see below), a binding domain for the agonist, and an intracellular C-terminal domain that contains phosphorylation sites.

The presence of the GluR2 subunit is known to govern the permeability of the AMPA receptors to calcium. AMPA receptors were considered to be calcium impermeable and it was later shown that this is due to the presence of at least one GluR2 subunit in its structure because AMPA receptors lacking GluR2 subunit are calcium permeable [28,29] (**Figure 2**). This role of the GluR2 subunit in determining calcium permeability not only depends on its presence, but also on the post-transcriptional RNA editing of the Q/R site present in its second membrane domain (M2) [30]. This editing substitutes the uncharged glutamine of this site for a positive-charged arginine, thus providing the calcium impermeability to the AMPA receptors (**Figure 2**). Therefore, changes in the expression or a deficit in the post-transcriptional edition of GluR2 would be expected to have significant pathophysiologic consequences because they would result in calcium entry into the neurons by activation by endogenous glutamate.

3. Calcium: role in neuronal damage

Calcium is an important intracellular messenger that, under physiologic conditions, has several functions, such as the regulation of membrane excitability, exocytosis, signal transduction, cell growth, synaptic activity and differentiation. In the resting state, cytosolic calcium in neurons is maintained at low concentration (~ 100 nM) by means of several homeostatic mechanisms, such as the regulation of influx and efflux, chelation by calcium trapping proteins, storage in mitochondrial and endoplasmic reticulum, and extrusion by calcium-ATPases. Excessive influx of extracellular calcium may elevate the cytoplasmic concentration to levels that exceed the capacity of calcium-buffering mechanisms and this increase can activate enzymes that normally operate at low levels, such as proteases, lipases, phosphatases and endonucleases, thus, causing membrane protein and lipid alterations, generation of toxic reactive oxygen species (ROS), mitochondrial damage, disruption of energy metabolism, and membrane depolarization (**Figure 1**). These events potentiate each other in a cascade manner to produce membrane damage and, consequently, cell death [3,5,31,32]. In this respect, it is worth noting that motor neurons are particularly vulnerable to intracellular calcium overload because they have a low buffering capacity.

Spinal cord motor neurons in rats and mice [33,34], and in both healthy individuals and ALS patients [35,36], contain scant amounts of the two principal calcium-binding proteins

calbindin D-28k and parvalbumin, whereas other motor neurons that are not affected in ALS, as those of the Onuf's and oculomotor nuclei, do express these calcium-binding proteins [33,35-38]. In addition, it has been recently demonstrated in cultures that motor neurons have an insufficient mitochondrial capacity to buffer large calcium concentrations following repetitive AMPA receptor activation by exposure to kainate [39]. Furthermore, in motor neuron cultures capable of spontaneous synaptic activity, AMPA receptor activation produced transient cytoplasmic calcium increases, resulting mainly from calcium-induced calcium release from the endoplasmic reticulum via activation of ryanodine receptors [40]. These data suggest that the vulnerability of motor neurons to AMPA receptor-mediated excitotoxicity may be due to a deficient mechanism of intracellular calcium homeostasis that cannot respond sufficiently to the consequences of overactivation of the calcium-permeable AMPA receptors, which are abundant in spinal motor neurons.

4. Neurodegeneration of spinal motor neurons and AMPA receptors: experimental models

The foregoing data can be correlated with the finding that spinal motor neurons are particularly vulnerable to AMPA or kainate, agonists of the AMPA-type glutamate receptor, both *in vivo* and *in vitro*, probably because of calcium influx through calcium-permeable AMPA receptors lacking the GluR2 subunit or with a non-edited Q/R site in this subunit [41-45]. In cultured rat motor neurons, activation of AMPA receptors by kainate was correlated with calcium entry and neuronal death [41]; this calcium influx is mediated by calcium-permeable AMPA receptors and not by voltage-gated calcium channels [43]. Nevertheless, in order to observe these effects, *in vitro*, the concentration of calcium in the culture medium had to be raised from the physiologic 2 mM concentration to 10 mM, a methodologic point that is often disregarded when extrapolating these results to the situation *in vivo*.

Although the results of the studies on the expression of GluR2 in rat and human spinal motor neurons are controversial [42,46-48], in normal human motor neurons GluR2 mRNA was practically undetected, whereas the rest of the subunits were clearly present, suggesting that most AMPA receptors in these cells are calcium-permeable [49]. It was recently shown that the expression of GluR2 mRNA in single spinal cord motor neurons was lower in comparison with other neuronal populations, but no difference was found between ALS patients and other neurologic patients [49]. The edition of the Q/R site in GluR2 was decreased in the spinal ventral horns of ALS patients [50] and it was later found that the GluR2 editing efficiency was not complete in single spinal motor neurons of some ALS patients, whereas it was 100% complete in motor neurons of non-ALS control patients and > 99% in the cerebellar Purkinje cells of both ALS and non-ALS groups [51,52]. Of interest, the RNA editing efficiency of the GluR2 Q/R site in transgenic rats carrying a mutant human SOD1 was found to be normal [53], suggesting that the mechanisms of motor neuron death in SALS and FALS are different.

As mentioned, most of the studies on the mechanisms of spinal motor neuron death have been carried out *in vitro*, in primary or organotypic cultures. *In vivo*, most of the experiments have focused on FALS, by using transgenic mice and rats carrying human mutant SOD1 that develop selective spinal motor neuron loss with consequent paralysis and death [54-56]. However, there are much less animal models of SALS, which accounts for > 90% of the cases. The authors have therefore developed an experimental model that should be useful for studying the mechanisms of motor neuron death [23]. These experiments were designed to test whether, in the living rat, the excitotoxicity produced by AMPA or by endogenons glutamate might induce spinal motor neuron death and paralysis. The authors found that the perfusion of AMPA, by microdialysis in the rat lumbar spinal cord, produced intense muscular spasms of the ipsilateral rear limb, followed by ipsilateral permanent paralysis and a remarkable loss of spinal motor neurons. These effects were produced by neither kainate nor NMDA, and were prevented by the AMPA receptor antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide, thus demonstrating that the overactivation of AMPA receptors in spinal motor neurons was responsible for their death *in vivo* [23].

Because this motor neuron death occurs only ~ 3 – 6 h after the infusion of AMPA, and becomes complete at 24 h, the authors reasoned that the entry of calcium, probably through the calcium-permeable AMPA receptor channel, induces a delayed deleterious process leading to motor neuron death. To test this hypothesis, the authors studied whether 1-naphthyl acetyl spermine (NAS) a selective blocker of the AMPA receptor that lacks the GluR2 subunit [44,57-59] could prevent the AMPA receptor-dependent motor neuron loss. When co-applied with AMPA by microdialysis, NAS significantly prevented both the selective spinal motor neuron loss and the paralysis [60]. Because NAS was very efficient in preventing neuronal damage and the subsequent paralysis, these results clearly indicate that rat spinal cord motor neurons possess functional calcium-permeable AMPA receptors lacking GluR2 and suggest that the cellular process leading to motor neuron death in this model, *in vivo*, is triggered by an increase of intracellular calcium via these receptors. To test whether such an increase was responsible for the damage, we co-perfused the intracellular calcium chelator 1,2-*bis*-(o-aminophenoxy)-ethane-N,N,-N',N'-tetraacetic acid tetraacetoxy-methyl ester with AMPA and found that this chelator was as effective as NAS for blocking the motor neuron damage and, thus, the paralysis [60]. The relevance of these findings is noteworthy because, differently from the experiments *in vitro* mentioned above, the neuronal death due to increased cytoplasmic calcium occurs under

the physiologic extracellular concentration of the cation. Altogether, these data suggest that AMPA receptors may have an important role in the development of ALS, albeit, as discussed below, convincing evidence that this occurs in the human disease has not yet been obtained.

In other recent experiments the authors extended to several days the period over which spinal motor neuron degeneration occurs, by infusing AMPA in the spinal cord with a slow delivery system using osmotic minipumps [61], thus, generating a chronic model of spinal motor neuron death that resembles the characteristics of neurodegeneration and paralysis that are present in both ALS patients and FALS rodents. This animal model of SALS may be useful to test therapeutic strategies aimed at preventing or rescuing motor neurons damage in ALS.

5. Therapeutic targets related to glutamate-mediated excitotoxicity

5.1 Modulation of excitatory transmission

Despite the enormous amount of experimental data pointing towards excitotoxicity as the main neuronal death mechanism leading to ALS, there are still no certainties that this process triggers motor neuron death. Nonetheless, impeding excitotoxicity is an appealing therapy target for this disease (Figure 1). This approach is so promising that the only approved treatment for ALS, at present, is riluzole, which is thought to inhibit glutamate release from nerve endings by stabilizing the inactive state of voltage-dependent sodium channels and by a G-protein-coupled intracellular pathway that can be inhibited with pertussis toxin [62]. In clinical trials conducted over a decade ago, riluzole only moderately improved survival of patients [63,64]. Later, the same group reported that riluzole is safe and well tolerated by patients, but no significant clinical improvement was mentioned [65,66].

In rodent experiments, riluzole prolonged the survival of FALS mice, although the disease onset was not modified [67]. One interesting effect of riluzole is that it lowered the increased excitability to the basal level that motor neurons show in this transgenic model [68]. In contrast, in a model of excitotoxicity produced by the stimulation of endogenous glutamate release by 4-aminopyridine in rat hippocampus, riluzole did not protect, but rather potentiated the epilepsy produced by this drug [27].

Other antiglutamatergic drugs presently used against epilepsy have also been tested in the ALS experimental models. Topiramate is a drug capable of blocking non-NMDA receptors [69] and it showed protective action when tested in an *in vitro* model of motor neuron death induced by glutamate transport blockade; however, chronic administration of this compound to transgenic ALS mice did not extend survival [70]. Furthermore, topiramate therapy in human patients resulted in a faster decrease in muscle strength and elicited a series of adverse effects [71].

Talampanel (LY-300164), another antiepileptic drug that acts as an antagonist of AMPA receptors, protects against motor neuron death induced by kainate *in vitro* [43]. Epileptic patients tolerated this medication well [72], but whether this drug will function in ALS patients is still to be determined.

If excitotoxicity is considered to arise from glutamate transport dysfunction, pharmaceutical treatments directed to increase glutamate uptake, by stimulating the expression of transporter proteins, seem to be a good choice. Some β-lactam antibiotics, such as ceftriaxone, seem to be stimulators of GLT-1 expression and its administration to FALS mice extended their survival [73], but to the authors' knowledge there are no clinical ceftriaxone trials at present. However, enhancing uptake capacity may not be sufficient to prevent motor neuron death significantly, in as much as glutamate transport deficiency may not be the pivotal mechanism triggering neuronal death in ALS, even in the presence of mutant SOD1 [24,74].

5.2 Energetic metabolism

Massive calcium entrance to the cell, secondary to excitotoxicity, most probably has deleterious effects on energetic metabolism. In fact, there is abundant evidence that mitochondrial damage, possibly related to alterations in calcium homeostasis, is involved in ALS [75-78]. On this rationale, some energy metabolic substrates have been tested in experimental models of both FALS and SALS. Energetic substrates, such as creatine [79,80] and pyruvate [81] improve motor function in SOD1 mutant transgenic mice. In addition, the authors have shown in wild-type rats that AMPA receptor-mediated excitotoxic motor neuron death is reduced when spinal cord is overloaded with pyruvate [60]. Nevertheless, despite these findings, a clinical trial failed to demonstrate that creatine administration to patients has any therapeutic effect [82]. Feeding transgenic mice with coenzyme Q10, an electron carrier in the mitochondrial respiratory chain, moderately extends survival [83]. Although there are some clinical trials demonstrating the tolerability to high doses of coenzyme Q10 [84,85], a large Phase III study proving the benefits of this treatment is still missing. It is worth mentioning that the protective effects of pyruvate and coenzyme Q10 may be mediated by the antioxidant properties of these molecules, rather than or in combination with their metabolic roles.

5.3 Oxidative stress

Oxidative stress can arise as a consequence of several pathologic processes, such as excitotoxicity or mitochondrial malfunction, but whether oxidative stress is a cause or a consequence of ALS is still controversial. The recovery of the normal oxidative balance, which is probably lost in ALS, is an attractive therapeutic target.

In FALS, the evidence involving oxidative stress opens with the production of ROS by the mutant SOD1,

which should elevate oxidative stress in the cell. In *in vitro* models of excitotoxicity, which are independent of genetic alterations, exposure of spinal motor neurons to AMPA or to glutamate caused generation of ROS, possibly due to mitochondrial calcium overload [86,87]. In humans, post-mortem analysis of CNS tissue of ALS patients has shown oxidative damage to macromolecules, demonstrated by increased protein tyrosine nitration [88,89], formation of carbonyl groups in proteins [90,91] and oxidized nucleic acids [90,92].

Classic antioxidants include tocopherols, specifically α-tocopherol (vitamin E), which was one of the first strategies tested in the transgenic ALS mice. This treatment delayed onset and slowed down progression, but did not prolong survival [67]. In ALS clinical trials, vitamin E at high doses [93,94], as well as the antioxidant acetylcysteine [95] that was effective in mice [96], and the inducer of anti-oxidant enzymes selegiline [97], failed to be beneficial. Despite the negative results of these antioxidant trials, a notable delay in paralysis progress and death of transgenic ALS mice was achieved by the continuous administration of the manganese porphyrin AEOL-10150, starting at symptom onset [98]. This porphyrin was intended to mimic SOD1 activity, but the actual mechanism behind neuroprotection may be more related to its ROS scavenging activity. Results of clinical trials with AEOL-10150 are expected in the near future [99].

5.4 Growth factors

Trophic support is a promising strategy for neurodegenerative diseases [100]. There have been some clinical trials administering trophic factors to ALS patients, but the results have been negative in most cases. Subcutaneous injections of ciliary neurotrophic factor, which was effective in the mutant mice models of motor neuron disease *pmn/pmn* [101] and wobbler [102], had no effect on the progression of disease and caused minor adverse side effects [103]. Similarly, ALS patients treated subcutaneous with brain-derived neurotrophic factor [104] or with insulin-like growth factor 1 (IGF-1) [105,106] showed no significant slowing of the disease progress.

On the other hand, intramuscular administration to mutant SOD1 ALS mice of viral vectors encoding growth factors are among the most effective treatments to delay progression of the degenerative process and prolong survival [107-109]. In particular, vascular endothelial growth factor (VEGF) has gained the attention of researchers because it remarkably retarded the progression of the disease and the death of the animals [107,110-112]. Moreover, VEGF seems to be necessary for motor neuron survival under physiologic conditions, as demonstrated by the selective motor neuron death and paralysis, strikingly similar to that of transgenic mutant SOD1 mice, caused by the deletion of the hypoxia response element within the promoter sequence of the VEGF gene [113].

VEGF and its receptors are essential for normal angiogenesis and vasculogenesis, and mutations in their corresponding genes may be lethal. Single nucleotide polymorphisms, downstream of the VEGF gene, may confer an increased susceptibility to ALS. In a large-scale analysis of different European populations of ALS patients, two at-risk haplotypes were identified in the promoter or leader sequence of VEGF; these haplotypes have been linked to reduced expression and availability of VEGF [114].

The mechanism by which VEGF exerts its effects on motor neurons remains to be elucidated. Two hypotheses have been postulated to explain these effects. One states that VEGF promotes a good vascular niche that motor neurons require to survive and the other suggests that VEGF can directly activate its tyrosine-kinase receptors, expressed on the motor neuron membrane. In favor of the latter, there is experimental evidence indicating that activation of VEGF receptors is coupled to the survival pathway PI3K/Akt and this pathway is required for motor neuron survival [115-117]. Excitotoxicity can be counteracted by VEGF through the activation of this pathway in cultured hippocampal neurons [118]. Recent results from the authors' laboratory demonstrate that VEGF was very effective at protecting against chronic AMPA-induced excitotoxic death of spinal motor neurons in the rat *in vivo* [61]. Modulation of intracellular survival pathways, such as PI3-K/Akt constitutes an attractive therapeutic target to explore.

6. Therapeutic targets against non-excitotoxic motor neuron death

Glutamate-mediated excitotoxicity is one of many hypotheses that have long been considered to cause ALS. However, there are some other important factors known to participate in ALS pathology that offer interesting therapeutic targets. The authors shall discuss the possibility of inhibiting mutant SOD1, which theoretically represents a possible cure to the reduced percentage of patients with the FALS form that harbors mutations in this enzyme. In section 7, the authors will review other new targets for therapy, opened by the possible involvement of nonneuronal cell types in the development of ALS pathology.

6.1 Inhibiting mutant SOD1

It is largely accepted that toxicity of mutant SOD1 arises from the gain of a new catalytic activity, rather than from the loss of its normal activity [119]. In support of this, familial cases linked to mutant SOD1 are inherited in an autosomal-dominant fashion, so one mutant allele is sufficient to cause ALS, whereas the other allele remains unaltered. In addition, genetic experiments have demonstrated that despite the important role that SOD1 plays in cellular redox homeostasis, its loss has no serious consequences to motor neuron development and function [120]. The most accepted hypothesis on how mutant SOD1 becomes toxic is that its acquired catalytic activity generates ROS, but independently of whether this is the actual toxic mechanism it is clear that the only proven

cause of ALS is the occurrence of mutations in SOD1. Therefore, molecular strategies intended to silence mutant SOD1 might overcome the problem at least in the very small percentage of the ALS cases (~ 2%) that carry SOD1 mutations.

Approaches to develop this strategy have been made. The administration of lentiviral vectors coding for RNA of interference (RNAi) designed to silence human SOD1 to transgenic ALS mice resulted in a striking extension of nearly 80% of lifespan [121,122]. However, despite this promising result, it is pertinent to consider that among the 20% of FALS patients that bear SOD1 mutations, > 100 missense mutations along the SOD1 sequence have been described and, therefore, a specific RNAi molecule must be designed for each one. Designing a molecule of RNAi that specifically distinguishes between two SOD1 sequences, different in only one base, has been successful [123,124], but it may not be possible to create one RNAi for each of the found mutations. A possible solution could be to non-specifically silence both wild type and mutant SOD1 and co-administer wild type SOD1 RNAi-resistant genes to compensate for the lost function of normal SOD1 [125].

It was recently reported, using different cell lines, including motor neuron-like cells, that mutant SOD1 can be secreted [126,127] and that the secreted enzyme may be neurotoxic [126]. Therefore, an alternative to the above described RNAi technology is the immunization against extracellular mutant SOD1. This strategy has recently been used, *in vivo*, in ALS mice and the results showed that antibodies against extracellular SOD1 delayed the onset and the progress of symptoms [128].

6.2 Inflammation and non-autonomous cell death

There is evidence demonstrating microglial activation in the CNS tissue of ALS patients [129-131] and in the transgenic ALS mouse, microglia activation occurs before the onset of motor symptoms [132]. Once activated, microglia can release neurotrophic factors capable of protecting nearby neurons from noxious stimuli, or a series of pro-inflammatory molecules that ultimately lead to neuronal death [133]. Whether reactive microglial action in ALS is protective or damaging is not completely clarified, but it seems to be involved in the neurodegenerative process because blocking TNF-α-induced microglial activation significantly prolonged survival of transgenic ALS mice [134].

Minocycline is a tetracycline derivative capable of inhibiting microglial activation [135]. *In vitro*, this action of minocycline protects against motor neuron death induced by cerebrospinal fluid obtained from ALS patients [136]. In ALS mice, this drug delayed the onset of disease and extended survival [137-139], effects that could be enhanced by creatine diet supplementation [80]. Still, it is not clear whether this protective effect is totally due to the minocycline anti-inflammatory capacity or to its blocking effect on apoptotic mechanisms [136,139,140]. Cannabinoid receptor agonists can also suppress microglia activation and it is possibly through this mechanism that chronic administration of a selective agonist of the cannabinoid receptor 2 exerted beneficial effects in ALS mice [141].

The importance of the participation of glial cells in the process of motor neuron death is gaining recognition. Transgenic mice that only express mutant SOD1 in neurons or in astrocytes present neither motor neuron death, nor the associated symptoms [142-144], suggesting that some kind of intercellular action is required for neuronal damage. In fact, glia can enhance the toxic effect of kainate in cultures [145] and it was recently shown, *in vitro*, that glia expressing mutant SOD1 induce motor neuron death via the release of unidentified toxic factors through bax activation [146,147]. Moreover, experiments with chimeric mice expressing SOD1 in different neuronal and glial populations showed the participation of non-neuronal cells in aggravating or slowing the motor deficits [148].

7. Expert opinion and conclusions

Most of the studies on the mechanisms of motor neuron death in ALS have been focused on transgenic rodents expressing human mutant SOD1, which constitute a model of FALS. Therefore, the therapeutic strategies designed to target mutant SOD1 expression or the production of ROS due to its toxic acquired catalytic activity probably apply mainly, if not only, to FALS. Therefore, it is relevant to consider targets that may apply also to SALS, responsible for > 90% of cases, and for this purpose, it seems essential to develop *in vivo* models of spinal motor neuron death. In this respect, in spite of the lack of definitive evidence proving that excitotoxicity by excessive synaptic glutamatergic neurotransmission is a cause of the disease, this hypothesis provides a valuable experimental approach to develop such *in vivo* models and has generated relevant knowledge for the understanding of the cellular and molecular mechanisms of motor neuron death. This knowledge permits researchers to identify several targets for the design of therapeutic strategies, as reviewed in this article.

One of the most intriguing questions is the selectivity of the motor neuron degeneration, despite the fact that excitotoxicity seems to be a common mechanism of neuronal death in other neurodegenerative diseases affecting different neuronal populations, such as Alzheimer's and Parkinson's diseases, as well as the zone of penumbra after a brain ischemic episode. The intracellular calcium-dependent neuronal death via overactivation of calcium-permeable AMPA receptors may be an important factor for this selectivity and, therefore, represents an attractive therapeutic target because of the presence of this type of glutamate receptor in spinal cord motor neurons. Treatments aimed at blocking AMPA receptors or restoring the intracellular calcium homeostasis appears promising.

Another relevant question is to what extent the mechanisms of FALS and SALS overlap. No genetic alterations have been demonstrated so far in SALS patients and SOD1 mutations account for only ~ 20% of FALS. However, some of the therapeutic strategies assayed seem to be at least partially effective for both FALS rodent models and *in vivo* excitotoxicity models. Of these strategies, the use of trophic factors, particularly VEGF, appears to be promising, although the cellular and molecular mechanisms of the protective effect of these growth factors are still unclear.

The revised emerging possible role of glial cells in motor neuron damage also constitutes an interesting target for the design of new therapeutic strategies, even when it is not possible to ascribe to the different types of glia a determinant role in these mechanisms at present. Because of the devastating consequences of ALS and other neurodegenerative diseases, for which there are no effective treatments, the knowledge of the mechanisms of neuronal death is indispensable to develop strategies for therapeutic measures.

Declaration of interest

The work of the authors cited in this article was supported by DGAPA, UNAM (IN209807) and CONACYT (project 42668), Mexico. Juan C Corona and Luis B Torva-y-Romo are recipients of a fellowship from CONACYT, Mexico.

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•• In this work in FALS mice the authors demonstrated that motoneuron death is not only caused by alterations in the motoneurons themselves, but that there is an important contribution from other cell types.

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Conclusiones

Ha transcurrido más de un siglo desde la descripción original de Charcot sobre la muerte de las motoneuronas en la ALS y poco se sabe aún de los mecanismos celulares y moleculares que subyacen a la misma. Esta enfermedad afecta a una cantidad significativa de pacientes alrededor del mundo los cuales no cuentan con ningún remedio que les permita detener o retrasar de manera importante el progreso de la patología.

A pesar del gran esfuerzo hecho en décadas de investigación por numerosos laboratorios, el avance en el conocimiento de los mecanismos que causan la ALS es claramente limitado. No obstante, dos descubrimientos son de especial importancia y han permitido desarrollar una cantidad substancial de hallazgos que poco a poco alumbran los mecanismos neurodegenerativos en esta condición. El primero, son las mutaciones de la SOD1, que es hasta ahora la única causa probada de ALS y que permitió el desarrollo del modelo *in vivo* más recurrido para la investigación celular, molecular y fisiológica de la enfermedad y que además ha servido para probar una buena cantidad de estrategias terapéuticas, aunque prácticamente todas sin éxito. El segundo, más reciente, es que el VEGF ejerce un efecto trófico importante sobre las motoneuronas no descrito anteriormente para algún otro factor. Sin duda en los años por venir, el conocimiento de la función del VEGF sobre las motoneuronas permitirá un desarrollo importante del entendimiento de esta devastadora enfermedad y facilitará la identificación de blancos terapéuticos. Recientemente el descubrimiento de 2 proteínas mutadas (TDP43 y Fus, ver Tovar-y-Romo et al. 2009a) en casos de ALS tanto familiares como esporádicos ha captado la atención de los investigadores donde de manera prometedora se podrán identificar mecanismos compartidos entre la SALS y la FALS y, en principio, se podrá hacer un avance importante sobre el entendimiento de su etiología.

Nuestro trabajo presenta la primera demostración de la eficiencia del VEGF como una molécula protectora en un modelo vivo de neurodegeneración espinal libre de alteraciones genéticas. Esto adquiere una gran relevancia dado que la mayoría de los pacientes con ALS sufren la forma esporádica y su patología no tiene ningún vínculo con alteraciones de la SOD1. La acción reguladora del VEGF sobre las motoneuronas puede ser entonces un blanco terapéutico importante en la ALS. Existe una gran necesidad de explorar estas alternativas ya que como se describió, la mayoría de las

estrategias terapéuticas ensayadas en pacientes no han tenido una efectividad significativa hasta ahora.

En cuanto a la excitotoxicidad, que en años anteriores se consideró el mecanismo causal más importante de la ALS, cada día es más claro que su participación dentro del proceso patológico no parece tener la importancia antes pensada, principalmente porque la mayoría de las estrategias terapéuticas antiexcitotóxicas han fallado en su aplicación clínica. En este sentido, nuestro trabajo presenta resultados importantes ya que describe que la función limitada de los transportadores glutamatérgicos no es un evento iniciador de la neurodegeneración en la ALS, tanto la de origen genético causado por mutaciones de la SOD1, como la de probable origen esporádico donde no hay modificaciones genéticas.

A pesar de los cuantiosos reportes científicos publicados constantemente sobre el entendimiento de algunos procesos celulares o moleculares que ocurren en algún modelo de estudio, las preguntas fundamentales sobre el origen de la enfermedad humana quedan aún por contestarse. La investigación básica permitirá eventualmente entender cuál o cuáles son las alteraciones fisiológicas de fondo que conducen a la muerte neuronal específica de las motoneuronas en la ALS. Este conocimiento permitirá, además de entender la fisiología del SNC, generar curas efectivas para esta y posiblemente otras enfermedades degenerativas, indispensables para brindar a los pacientes una buena calidad de vida.

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