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MECANISMOS BIOQUÍMICOS DE LA DEGENERACIÓN DE MOTONEURONAS ESPINALES *IN VIVO*

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RESUMEN

La excitotoxicidad mediada por glutamato, la disfunción mitocondrial y el estrés oxidativo son algunos de los mecanismos que están involucrados en el proceso de degeneración de las motoneuronas en la esclerosis lateral amiotrófica (ALS). Previamente, nuestro grupo demostró que la perfusión por microdiálisis de ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiónico (AMPA) en la región lumbar de la médula espinal de las ratas produce una parálisis permanente de la extremidad posterior ipsilateral y muerte de las motoneuronas mediante un mecanismo dependiente de Ca^{2+}. El objetivo de este trabajo fue el estudiar a fondo el grado de participación de la disfunción mitocondrial, de las deficiencias del metabolismo energético, y del estrés oxidativo en este modelo *in vivo* de degeneración excitotóxica de motoneuronas espinales. Con este propósito, se evaluaron los efectos neuroprotectores de varios sustratos del metabolismo energético y antioxidantes. Se co-perfundieron con el AMPA los sustratos energéticos lactato y creatina, el α-cetobutirato y el β-hidroxibutirato, que pueden suministrar energía y también tienen propiedades antioxidantes, y el ascorbato, el glutatión y el etil éster de glutatión, los cuales son únicamente antioxidantes. Además, se examinaron marcadores de estrés oxidativo durante las etapas iniciales del proceso de degeneración de motoneuronas, y también se estudiaron las propiedades funcionales y morfológicas de las mitocondrias aisladas de las astas ventrales de las médulas espinales lumbares de cuatro grupos de ratas: perfundidas con AMPA, AMPA + piruvato, piruvato + solución Krebs-Ringer, y solución Krebs-Ringer como controles. El piruvato, el lactato, el β-hidroxibutirato, el α-cetobutirato y la creatina redujeron la muerte de las motoneuronas protegiendo entre el 50-65% de las motoneuronas, preservaron la función motora y evitaron por completo la parálisis inducida por AMPA. El ascorbato, el glutatión y el etil éster de glutatión protegieron parcialmente contra los déficits motores y preservaron sólo entre el 30-45% de las motoneuronas sanas. El AMPA no provocó cambios en la oxidación de proteínas (evaluado por medio de la inmunoreactividad a la 3-nitrotirosina) ni en la formación de especies reactivas de oxígeno, durante el inicio del proceso degenerativo de las motoneuronas (estudiados a las 1.5–2 h después de la perfusión de AMPA). Las mitocondrias aisladas (también a estos tiempos) de las médulas espinales de las ratas tratadas con AMPA mostraron disminuciones en el consumo de oxígeno, en el control respiratorio dependiente de ADP, y en el potencial transmembranal. Adicionalmente, las actividades de los complejos I y IV de la cadena respiratoria disminuyeron significativamente en las mitocondrias de las ratas perfundidas con AMPA. Además, las mitocondrias de este grupo de ratas presentaron daño y anormalidades morfológicas, como son hinchamiento, crestas desorganizadas, y membranas rotas. Notablemente, la co-perfusión de piruvato evitó todos estos defectos funcionales y anormalidades morfológicas. De manera interesante, observamos una clara correlación entre las alteraciones de la función motora y el número de motoneuronas dañadas, que sugiere que existe un umbral de aproximadamente 50% en el número de motoneuronas sanas necesario para preservar la función motora. Concluimos que la disfunción y deficiencias energéticas mitocondriales desempeñan un papel crucial en esta degeneración de motoneuronas espinales inducida por la sobreactivación de los receptores tipo AMPA *in vivo*, mientras que el estrés oxidativo parece ser un mecanismo menos relevante, que sin embargo también participa. Estos mecanismos podrían estar involucrados en la degeneración de las motoneuronas en la ALS.

ABSTRACT

Glutamate-mediated excitotoxicity, mitochondrial dysfunction and oxidative stress are involved in motor neuron degeneration process in amyotrophic lateral sclerosis (ALS). Previously, our group showed that the microdialysis perfusion of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) in the lumbar region of the rat spinal cord produces a permanent paralysis of the ipsilateral hindlimb and death of motor neurons by a Ca^{2+}-dependent mechanism. The aim of this work was to study thoroughly the extent of involvement of mitochondrial dysfunction, of energy metabolic deficiencies, and of oxidative stress in this *in vivo* model of spinal motor neurons excitotoxic degeneration. For this purpose, we assessed the neuroprotective effects of various energy metabolic substrates and antioxidants. We co-perfused with AMPA the energy substrates lactate and creatine, α-ketobutyrate and β-hydroxybutyrate, which can supply energy and also have antioxidant properties, and ascorbate, glutathione and glutathione ethyl ester, which are solely antioxidants. Furthermore, we examined oxidative stress markers during the initial stages of the motor neuron degenerating process, and we also studied the functional and ultrastructural properties of mitochondria isolated from the ventral horns of lumbar spinal cords of four groups of rats: perfused with AMPA, AMPA + pyruvate, pyruvate + Krebs-Ringer medium, and Krebs-Ringer medium as controls. Pyruvate, lactate, β-hydroxybutyrate, α-ketobutyrate and creatine reduced motor neuron death protecting 50–65% of healthy motor neurons, preserved motor function and completely prevented the paralysis induced by AMPA. Ascorbate, glutathione and glutathione ethyl ester partially protected against motor deficits and preserved only 30–45% of healthy motor neurons. AMPA did not produce changes on protein oxidation (assessed by 3-nitrotyrosine immunoreactivity) and reactive oxygen species formation, during the initial motor neuron degenerating process (studied at 1.5–2 h after AMPA perfusion). Mitochondria isolated (also at those times) from the AMPA-treated group showed decreased oxygen consumption, ADP-dependent respiratory control and transmembrane potential. Additionally, activities of the respiratory chain complexes I and IV significantly decreased in mitochondria from rats perfused with AMPA. Furthermore, mitochondria of this group of rats were damaged and presented ultrastructural and morphological abnormalities such as swelling, disorganized cristae, and disrupted membranes. Remarkably, pyruvate co-perfusion prevented all these functional deficits and ultrastructural abnormalities. Interestingly, we observed a clear correlation between the alterations of motor function and the number of damaged motor neurons, suggesting that there is a threshold of about 50% in the number of healthy motor neurons necessary to preserve motor function. We conclude that mitochondrial dysfunction and energy deficiencies play a crucial role in this spinal motor neuron degeneration induced by overactivation of AMPA receptors *in vivo*, whereas oxidative stress seems a less relevant mechanism, which however participates as well. These mechanisms could be involved in ALS motor neuron degeneration.

ORGANIZACIÓN DE LA TESIS

Esta tesis está dividida en 9 secciones: Introducción, Antecedentes, Planteamiento del problema, Hipótesis, Objetivos, Resultados, Discusión, Conclusiones y Referencias. No se incluye una sección de Metodología puesto que los materiales y métodos se describen detalladamente en los artículos que forman parte de las secciones de Antecedentes y Resultados.

La Introducción comprende un marco teórico general acerca de la neurodegeneración, de la neurotransmisión mediada por glutamato, y de la excitotoxicidad y el calcio en relación a su participación en los procesos de neurodegeneración en general. En los Antecedentes se describen las principales características de la enfermedad neurodegenerativa esclerosis lateral amiotrófica (ALS), y los factores que muy probablemente contribuyen a la muerte de las motoneuronas. De entre éstos se revisa más detalladamente la excitotoxicidad glutamatérgica, sección en la cual se incluyen dos artículos. El primero es de investigación original titulado "*Chronic elevation of extracellular glutamate due to transport blockade is innocuous for spinal motoneurons in vivo*" en el que se demuestra que el bloqueo crónico del transporte de glutamato en la médula espinal de las ratas eleva la concentración extracelular de glutamato pero no causa daño neuronal. El segundo artículo de esta sección es una revisión titulada "*Experimental models for the study of neurodegeneration in amyotrophic lateral sclerosis*" en la que se describen los modelos experimentales de ALS tanto *in vitro* como *in vivo*, incluyendo los modelos desarrollados en nuestro laboratorio y el que se utiliza en este trabajo, y algunos de los mecanismos que se ha visto que están implicados en la muerte de las motoneuronas. También en la sección de Antecedentes se describen con mayor profundidad la disfunción mitocondrial y el estrés oxidativo como parte de estos mecanismos que participan en la degeneración de las motoneuronas, aquí se incluye el capítulo titulado "*Role of mitochondrial dysfunction in motor neuron degeneration in ALS*" donde se detallan éstos y también abarca otros mecanismos que se han propuesto como hipótesis para explicar la muerte selectiva de las motoneuronas.

En las secciones subsecuentes, se exponen el Planteamiento del problema, las Hipótesis y los Objetivos. Los Resultados engloban los principales hallazgos obtenidos de los experimentos diseñados para cumplir estos objetivos, y se presentan en dos artículos, el primero titulado "*Role of energy metabolic deficits and oxidative stress in excitotoxic spinal motor neuron degeneration in vivo*" que ya ha sido publicado, y el segundo que lleva por título "*Mitochondrial dysfunction during*

the early stages of excitotoxic spinal motor neuron degeneration in vivo" que se enviará próximamente para su publicación.

En la Discusión se integran y complementan las discusiones de los artículos presentados de tal manera que se da una visión más amplia y unificada de la interpretación de los resultados obtenidos. Se discuten así desde esta perspectiva las alteraciones mitocondriales, las deficiencias energéticas, el estrés oxidativo, y la interrelación entre estos mecanismos y con otros que también se ha propuesto participan en el proceso de degeneración de motoneuronas, como la excitotoxicidad y la desregulación en la homeostasis de Ca^{2+}. También se discute acerca de la correlación encontrada entre el número de motoneuronas preservadas y el desempeño motor. Finalmente se presentan las conclusiones de este trabajo y las referencias.

LISTA DE ABREVIATURAS

ALS	Esclerosis Lateral Amiotrófica (por Amyotrophic Lateral Sclerosis)
AMPA	Ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiónico
ATP	Adenosín trifosfato
ChAT	Colina acetiltransferasa
Ciclo TCA	Ciclo de los ácidos tricarboxílicos o ciclo del ácido cítrico o ciclo de Krebs (por tricarboxylic acid cycle)
DCD	Desregulación retardada de Ca^{2+} (por Delayed Calcium Deregulation)
$\Delta\psi_m$	Potencial transmembranal mitocondrial
Δp	Fuerza protón-motriz
EAAT	Transportador de aminoácidos excitadores (por Excitatory Amino Acid Transporter)
FALS	Esclerosis Lateral Amiotrófica Familiar (por Familial Amyotrophic Lateral Sclerosis)
GEE	Etil éster de glutatión (por glutathione ethyl ester)
GLT1	Transportador de glutamato 1 (por Glutamate Transporter 1)
GSH	Glutatión
GTP	Guanosín trifosfato
HPLC	Cromatografía líquida de alta resolución (por High Performance Liquid Chromatography)
KA	Kainato
MPTP	Poro de la transición de la permeabilidad mitocondrial (por Mitochondrial Permeability Transition Pore)
NMDA	N-metil-D-aspartato
$NO^{\bullet}$	Radical óxido nítrico
NOS	Óxido nítrico sintasa (por Nitric Oxide Synthase)
3-NT	3-Nitrotirosina
$O_2^{\bullet-}$	Radical superóxido
$^{\bullet}OH$	Radical hidroxilo
$ONOO^-$	Anión peroxinitrito
PDC	Ácido L-trans-2,4-pirrolidín-dicarboxílico
RCR	Control respiratorio (por Respiratory Control Ratio)
ROS	Especies reactivas de oxígeno (por Reactive Oxygen Species)
SALS	Esclerosis Lateral Amiotrófica Esporádica (por Sporadic Amyotrophic Lateral Sclerosis)
SNC	Sistema nervioso central
SOD	Superóxido dismutasa
TBOA	DL-treo-β-benziloxiaspartato

I. INTRODUCCIÓN

Neurodegeneración

La neurodegeneración es un proceso que se da cuando hay una degradación o deterioro de las funciones neuronales que conduce a la muerte de la célula durante los padecimientos agudos y crónicos del sistema nervioso central (SNC), como son la isquemia (accidentes vasculares cerebrales), hipoxia, hipoglucemia, traumatismos craneoencefálicos y de la médula espinal, epilepsia, y en numerosas enfermedades neurodegenerativas tales como la esclerosis lateral amiotrófica (ALS), las enfermedades de Parkinson, de Alzheimer y de Huntington. La neurodegeneración también puede ocurrir durante el envejecimiento debido a la acumulación de daños a nivel molecular y celular con el paso del tiempo.

Entre las características patológicas que comparten las enfermedades neurodegenerativas se encuentra la pérdida neuronal gradual y selectiva; los grupos neuronales que se afectan primordialmente varían dependiendo del padecimiento. Se han realizado numerosas investigaciones para tratar de entender las causas y los mecanismos celulares y moleculares que participan en el daño y muerte neuronal. Los esfuerzos de los investigadores han sido útiles en cierta medida pues, aunque no se ha tenido éxito en conocer las causas, sí se tiene un gran avance en el conocimiento de los procesos celulares que muy probablemente participan de manera importante en los mecanismos de la neurodegeneración. Algunos de éstos son: la agregación de proteínas mal plegadas, la excitotoxicidad por sobreactivación de los receptores glutamatérgicos, el incremento en la concentración intracelular de Ca^{2+} y alteraciones en sus mecanismos de homeostasis, la disfunción mitocondrial y el estrés oxidativo (Coyle y Puttfarcken 1993; Tapia 1998; Tapia *et al.* 1999). Estos mecanismos están estrechamente relacionados, no son mutuamente excluyentes, y pueden interactuar y cooperar en el establecimiento de un círculo vicioso que resulta en muerte neuronal. Este círculo vicioso dañino puede iniciar alternativamente en sitios diferentes (comenzado por ejemplo por excitotoxicidad o producido por falla energética o estrés oxidativo), pero una vez desencadenado, un proceso puede conducir a o potenciar los otros, dando lugar a mecanismos multifactoriales e interdependientes de muerte celular.

Muchos estudios han demostrado defectos mitocondriales o del metabolismo energético en enfermedades neurodegenerativas como la ALS y las enfermedades de Huntington, Alzheimer y Parkinson (Beal 1992; Beal *et al.* 1993).

No se han encontrado tratamientos efectivos para combatir las enfermedades neurodegenerativas, por lo que generalmente los tratamientos que se dan a los pacientes son paliativos, que pueden ayudar a aliviar en cierta medida los síntomas o a atenuar los efectos negativos de la enfermedad y mejorar así un poco la calidad de vida del paciente, pero no curan, frenan, ni modifican (o lo hacen muy limitadamente) el desarrollo de la enfermedad, por lo que esto sigue siendo uno de los grandes retos para la investigación científica. Uno de los problemas es que cuando aparecen los síntomas y se da el diagnóstico de la enfermedad, la neurodegeneración ya está avanzada, y esta es una de las razones por las cuáles muchos de los compuestos que han funcionado en modelos animales no han ayudado a los pacientes. Por eso también se están realizando esfuerzos para encontrar marcadores para diagnosticar estas enfermedades desde etapas tempranas o incluso asintomáticas y poder así tratarlas desde las fases iniciales. Con estas herramientas, muy probablemente se facilitaría la prevención del desarrollo o ayudaría a retrasar el avance de la enfermedad. Por consiguiente, es de gran importancia seguir investigando para conocer las causas, entender mejor los mecanismos de neurodegeneración, y optimizar el diagnóstico para poder proponer medidas preventivas y encontrar tratamientos efectivos para estos lamentables padecimientos.

Neurotransmisión glutamatérgica, excitotoxicidad y calcio

El glutamato es el principal neurotransmisor que media la transmisión sináptica excitadora en el SNC, y está involucrado en muchos aspectos de la función normal del cerebro. Existen 2 principales categorías de receptores al glutamato: los ionotrópicos y los metabotrópicos. Cuando el glutamato se une a los receptores ionotrópicos, que son canales de cationes, éstos se abren permitiendo el flujo de los iones a través de ellos; mientras que cuando el glutamato se une a los receptores metabotrópicos, éstos activan enzimas intracelulares asociadas con las vías de transducción de señales por medio de proteínas G. Se conocen 3 clases de receptores ionotrópicos: los receptores tipo NMDA (N-metil-D-aspartato), y los no-NMDA que son los tipo AMPA (ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiónico) y KA (kainato), nombrados así por sus agonistas selectivos. Los receptores ionotrópicos son complejos tetraméricos formados por las diversas subunidades individuales de cada tipo de receptor en diferentes combinaciones. Hay muchas variantes de las subunidades relacionadas con el empalme alternativo de los transcritos de RNA o con la edición del RNA, y los patrones de expresión de las subunidades a lo largo del SNC son diversos, lo que da lugar a múltiples subtipos de receptores tipo AMPA, KA y NMDA

funcionales, permitiendo una heterogeneidad de los receptores a glutamato en todo el SNC (Seeburg 1993; Hollmann y Heinemann 1994). Los receptores tipo NMDA permiten el flujo de Ca^{2+} y de Na^{+} hacia el interior, y de K^{+} hacia el exterior de la célula, y la apertura del canal depende tanto del voltaje de membrana como de la unión simultánea de los 2 co-agonistas del receptor NMDA para su activación, el glutamato y la glicina (Dingledine *et al.* 1999). Los receptores tipo AMPA están ampliamente distribuidos en el SNC, sirven como receptores para la neurotransmisión sináptica excitadora rápida mediada por glutamato y son independientes de voltaje, por lo que la despolarización que causan puede activar a los receptores NMDA que sí dependen de voltaje para que sea removido el ion Mg^{2+}, que bloquea el canal en condiciones de reposo. Los receptores tipo AMPA y KA (que también están ampliamente distribuidos) son permeables a Na^{+}, K^{+}, y algunos de ellos son permeables a Ca^{2+} también dependiendo de la composición y edición post-transcripcional de sus subunidades; de esto se hablará con más detalle más adelante.

La transmisión sináptica glutamatérgica normalmente termina rápidamente gracias a la recaptura del neurotransmisor de la hendidura sináptica hacia el interior de las neuronas y las células gliales, manteniendo la concentración de glutamato extracelular por debajo de niveles neurotóxicos. Este proceso es llevado a cabo por los transportadores de glutamato de alta y baja afinidad. La remoción activa del neurotransmisor se da en 2 pasos: unión del glutamato a los transportadores y translocación al interior de las células. La recaptura de glutamato se da en contra de su gradiente de concentración, por lo que necesita de una fuerza motriz, la cual es dada principalmente por el gradiente electroquímico de Na^{+} a través de la membrana plasmática. El glutamato entra a la célula con 3 iones Na^{+} y un H^{+}, mientras que un ion K^{+} es contra-transportado, resultando en la reorientación del transportador de tal manera que el sitio de unión al glutamato es accesible de nuevo al espacio extracelular (Zerangue y Kavanaugh 1996; Levy *et al.* 1998; Danbolt 2001). Se han identificado y clonado cinco de estos transportadores de alta afinidad (EAAT 1-5), y se ha determinado su localización en el SNC. El más abundante de estos es el transportador de aminoácidos excitadores 2 (EAAT2), también llamado transportador de glutamato 1 (GLT1) en roedores, y se encuentra presente casi exclusivamente en astrocitos (Danbolt 2001). Este probablemente transporta ~90% del glutamato liberado, como sugiere el hallazgo de que ratones que carecen de esta proteína sufren convulsiones, presentan lesiones corticales y mueren prematuramente (Tanaka *et al.* 1997).

La transmisión sináptica glutamatérgica excesiva puede producir daño neuronal, y así el glutamato y sus agonistas (como el NMDA, Kainato y AMPA) pueden actuar como potentes neurotoxinas. El término excitotoxicidad describe la neurodegeneración mediada por aminoácidos excitadores, cuyas propiedades tóxicas están relacionadas a las propiedades excitadoras que tienen sobre sus receptores (Olney 1969; Olney 1978), siendo que los receptores ionotrópicos juegan un papel central (Choi 1988a). Así, la excitotoxicidad es un proceso en el cual la sobreactivación de los receptores a glutamato postsinápticos da lugar a una despolarización intensa y prolongada de las neuronas, permitiendo una entrada masiva de Ca^{2+} principalmente a través de los receptores a glutamato permeables a este catión, lo cual desencadena la activación no controlada de procesos perjudiciales que en conjunto provocan muerte neuronal (Arundine y Tymianski 2003).

Los mecanismos celulares de homeostasis mantienen bajas concentraciones citosólicas de Ca^{2+} (~100 nM) en las neuronas en reposo. Durante la neurotransmisión, las elevaciones fisiológicas de la concentración de Ca^{2+} intracelular son significativas tras la activación normal de los receptores postsinápticos. Sin embargo, después de la sobreactivación de los receptores, hay una entrada excesiva de Ca^{2+} extracelular que, junto con la salida de este catión de los compartimentos intracelulares, puede elevar su concentración citoplásmica a niveles que excedan la capacidad de los mecanismos de regulación que mantienen la homeostasis para este catión. Esto desencadena la activación inapropiada de procesos celulares dependientes de Ca^{2+} que normalmente están inactivos u operan a niveles bajos, induciendo alteraciones metabólicas y degeneración neuronal (Tymianski y Tator 1996). La concentración incrementada de Ca^{2+} puede provocar daño a las mitocondrias, despolarización en el potencial transmembranal mitocondrial ($\Delta\psi_m$), trastornos y deficiencias en el metabolismo energético, sobreproducción de especies reactivas de oxígeno (ROS) debido a la activación de enzimas que las producen y por vía mitocondrial, despolarización de membranas, activación de enzimas líticas como proteasas, lipasas, fosfatasas y endonucleasas, lo que ocasiona alteraciones en proteínas, lípidos y ácidos nucleicos, y daño membranal y nuclear; todo esto provoca eventualmente muerte celular (Choi 1988b; Coyle y Puttfarcken 1993; Siesjö 1994; Tapia *et al.* 1999; Arundine y Tymianski 2003). A la pérdida descontrolada e irreversible de la homeostasis de Ca^{2+} citoplásmico se le ha llamado desregulación retardada de Ca^{2+} (DCD). Al parecer, la DCD es resultado de una falla en los mecanismos para extraer el Ca^{2+} de la célula más que un flujo aumentado, y es el punto final irreversible de una secuencia que involucra sobrecarga de Ca^{2+} mitocondrial, generación incrementada de ROS, y daño oxidativo a la célula, dando lugar a esta falla en la salida de Ca^{2+}, que también podría resultar del agotamiento del ATP (Adenosín

trifosfato) citoplásmico (Milani *et al.* 1991; Randall y Thayer 1992; Tymianski *et al.* 1993; Ankarcrona *et al.* 1995; Khodorov *et al.* 1996; Wang y Thayer 1996; Castilho *et al.* 1998).

Se distinguen 2 tipos de excitotoxicidad: la excitotoxicidad clásica que hace referencia a la degeneración neuronal que ocurre después de un incremento de la concentración extracelular de glutamato, y la excitotoxicidad indirecta o lenta que se refiere a la muerte de una neurona postsináptica debilitada en la presencia de niveles normales de glutamato sináptico (Doble 1999; Van Den Bosch *et al.* 2006). La elevación de glutamato extracelular puede darse como consecuencia del aumento en la liberación de las terminales presinápticas o de una recaptura insuficiente de la hendidura sináptica; también puede resultar de la liberación del contenido intracelular de glutamato de neuronas dañadas dando lugar a la muerte excitotóxica de neuronas vecinas, y propagando así la neurodegeneración. En el caso de la excitotoxicidad indirecta, una estimulación normal de los receptores al glutamato puede ser suficiente para causar daño y muerte celular bajo condiciones en las cuáles la neurona postsináptica se encuentra debilitada. Así, trastornos de la función mitocondrial pueden resultar en este tipo de excitotoxicidad, debido a que el estado energético deficiente y la consecuente disminución en los niveles de ATP intracelulares, ocasionan que la neurona no pueda satisfacer las demandas de los muchos procesos dependientes de ATP, y como consecuencia se dañe y finalmente muera. De ahí surge la hipótesis excitotóxica ligada a energía (Novelli *et al.* 1988; Henneberry *et al.* 1989; Beal *et al.* 1993; Greene y Greenamyre 1996; Henneberry 1997), que propone que la correlación entre el daño excitotóxico y la restricción de energía se debe a la despolarización de la membrana plasmática resultante de la limitación energética. La disminución en los niveles de ATP causa una reducción en la función de la Na^+/K^+ ATPasa y de la Ca^{2+} ATPasa disminuyendo la extracción de Na^+ y de Ca^{2+}de la célula. La consecuente despolarización de la membrana plasmática resulta en un flujo de Ca^{2+} hacia el citosol a través de los canales de Ca^{2+} dependientes de voltaje, y por consiguiente aumenta la liberación de glutamato por exocitosis al espacio extracelular, sobreactivando sus receptores. Además, en condiciones de falla energética, los transportadores del glutamato operan en dirección inversa porque el gradiente electroquímico Na^+/K^+ se colapsa debido al decremento de ATP, resultando en la disminución de la captura y en la liberación de glutamato no vesicular al espacio extracelular (Longuemare y Swanson 1995; Jabaudon *et al.* 2000).

Se piensa que en condiciones como epilepsia, accidentes cerebrovasculares (isquemia) y neurotraumatismos, el daño neuronal es inducido por elevaciones agudas de glutamato, mientras

que en enfermedades neurodegenerativas, la excitotoxicidad podría darse por elevaciones crónicas y no tan drásticas de glutamato. Así se ha propuesto una asociación entre la disfunción mitocondrial y sus consecuencias perjudiciales incluyendo amortiguamiento deficiente de Ca^{2+}, generación de radicales libres, activación de la transición de la permeabilidad mitocondrial, y excitotoxicidad secundaria para estas enfermedades (Beal 1998).

II. ANTECEDENTES

Esclerosis Lateral Amiotrófica (ALS)

La enfermedad neurodegenerativa esclerosis lateral amiotrófica se caracteriza por la muerte selectiva y progresiva de las motoneuronas superiores e inferiores localizadas en la corteza motora cerebral, el tallo cerebral y la médula espinal. La pérdida progresiva de las motoneuronas va ocasionando sucesivamente debilidad muscular, fasciculaciones, espasticidad, parálisis gradual, depresión respiratoria y finalmente la muerte. También se da una pérdida anormal de la masa muscular y del peso corporal. Los síntomas de los pacientes con ALS de inicio bulbar (~25% de los casos) comienzan con disfagia y disartria progresivas, espasticidad e hiperreflexia puesto que las motoneuronas principalmente afectadas son las que se localizan en el tallo cerebral, mientras que los de los pacientes con ALS de inicio espinal (~75% de los casos) presentan primero debilidad muscular, calambres, fasciculaciones, espasticidad, atrofia muscular y la consecuente parálisis puesto que las motoneuronas que se localizan en las astas ventrales de la médula espinal son las primeras en degenerar (Cleveland y Rothstein 2001). Por lo general, los pacientes mueren por paro respiratorio provocado por la denervación de los músculos respiratorios y del diafragma, 2-5 años después de que comienzan los síntomas y son diagnosticados. La prevalencia de la ALS es de aproximadamente 2 a 10 casos por cada 100,000 individuos y la edad media de inicio de los síntomas es de ~55 años, aunque puede comenzar a edades menores (Chancellor y Warlow 1992; Huisman *et al.* 2011).

Se conocen dos formas de ALS, la forma familiar (FALS), que tiene un patrón de herencia autosómica dominante y representa sólo un ~5-10% de los casos, de entre los cuáles el ~10-20% están asociados con mutaciones en el gen *SOD1* que codifica para le enzima Cu^{2+}-Zn^{2+} superóxido dismutasa 1 (SOD1) (Rosen *et al.* 1993; Rowland y Shneider 2001). El otro ~90-95% de los casos son de ALS esporádica (SALS), cuya etiología es desconocida. Ambas formas presentan síntomas y desarrollan características clínicas e histopatológicas similares.

Con el objetivo de tratar de entender mejor la etiología y patogénesis de la enfermedad y los factores que contribuyen a la muerte de las motoneuronas, se han analizado muestras de autopsias y biopsias de pacientes con ALS. El problema es que la mayoría de estas muestras se obtienen de pacientes cuando ya está avanzada la enfermedad o en su etapa terminal, lo cual limita el entendimiento de los mecanismos patológicos de la degeneración de las motoneuronas durante las etapas clínicas progresivas de la enfermedad, desde el inicio de los síntomas o aun antes de éstos, hasta la muerte de los pacientes. Las características histopatológicas distintivas que se han encontrado en la médula espinal de pacientes con ALS incluyen la atrofia de motoneuronas dañadas con hinchamiento notable del cuerpo neuronal y axones proximales, alteraciones mitocondriales, anormalidades intracitoplásmicas de los neurofilamentos, y la presencia de cuerpos de Bunina (pequeñas inclusiones intracitoplasmáticas eosinofílicas, de material electrodenso amorfo, de origen lisosomal, inmunorreactivas para cistatina C y transferrina; generalmente se consideran una característica patológica específica de la ALS), inclusiones ubiquitinadas con perfil filamentoso o cuerpos esféricos compactos (parecidas a los cuerpos de Lewy) en los axones afectados y en los cuerpos celulares; esta patología de las motoneuronas es frecuentemente acompañada de gliosis reactiva (Strong *et al.* 2005). También se ha observado que las motoneuronas degeneran con señales de fragmentación de organelos, daño por radicales libres, sobrecarga de Ca^{2+} mitocondrial, transporte axonal defectuoso y acumulación de proteínas en cuerpos de inclusión intracelulares (Grosskreutz *et al.* 2010).

Dado que se desconoce la causa de la muerte selectiva de las motoneuronas en la mayoría de los casos de esta enfermedad, se han propuesto algunos mecanismos que pueden estar contribuyendo a la degeneración de las neuronas motoras basados en la investigación que se ha realizado hasta la fecha tanto en los pacientes como en los modelos experimentales. Algunos de los mecanismos propuestos son: excitotoxicidad glutamatérgica, disfunción mitocondrial y falla energética, estrés oxidativo, desorganización y acumulación anormal de neurofilamentos, estrangulación axonal y deterioro en el transporte axonal, plegamiento anormal de proteínas y toxicidad de agregados intracelulares, deficiencias de factores tróficos, e inflamación (Cleveland y Rothstein 2001; Julien 2001; Rowland y Shneider 2001; Bruijn *et al.* 2004; Shaw 2005; Boillee *et al.* 2006; Robberecht y Philips 2013). Estos mecanismos no son excluyentes uno del otro, por el contrario pueden coincidir, estar interrelacionados y cooperar mutuamente provocando un círculo vicioso que termine en muerte neuronal.

Mecanismos involucrados en la muerte de las motoneuronas

Excitotoxicidad glutamatérgica

De entre los diferentes mecanismos involucrados en la muerte selectiva de las motoneuronas en la ALS, se ha considerado que la excitotoxicidad mediada por glutamato muy probablemente contribuye de manera importante a esta neurodegeneración. Esta hipótesis ha sido ampliamente propuesta debido a la vulnerabilidad selectiva de las motoneuronas a la sobreactivación de los receptores de glutamato, probablemente debido a la entrada masiva de Ca^{2+} a través de los receptores tipo AMPA permeables a este catión, y a la baja capacidad que tienen estas neuronas para amortiguar este ion. Las evidencias que apoyan esto se discuten ampliamente más adelante.

Otra de las razones por las cuáles se postuló que la excitotoxicidad puede estar involucrada en esta enfermedad es porque los análisis post-mortem de corteza motora y médula espinal de pacientes con ALS revelaron una reducción en el contenido de EAAT2 (Rothstein *et al.* 1992; Shaw *et al.* 1994; Rothstein *et al.* 1995). Estos hallazgos dieron lugar a la hipótesis de que la disminución o el mal funcionamiento de EAAT2 pudieran ser un mecanismo causal importante de la degeneración de las motoneuronas en la ALS. Sin embargo, aún no se sabe si la pérdida de los transportadores de glutamato en los tejidos de los pacientes con ALS es una consecuencia o una causa de la pérdida neuronal (Sasaki *et al.* 2000). Además, hallazgos de nuestro laboratorio no apoyan esta hipótesis, dado que el bloqueo farmacológico del transporte de glutamato *in vivo* en la médula espinal de la rata tanto aguda (Corona y Tapia 2004) como crónicamente (Tovar-y-Romo *et al.* 2009b) resulta en concentraciones incrementadas de glutamato extracelular, pero no causa muerte de motoneuronas, ni déficits motores. Tampoco se observó daño neuronal en el hipocampo ni en la corteza motora de los ratones transgénicos SOD1/G93A, un modelo establecido de la FALS, tras la elevación de glutamato extracelular provocada por el bloqueo del transporte de glutamato (Tovar-y-Romo y Tapia 2006).

Enseguida se presenta el artículo titulado "*Chronic elevation of extracellular glutamate due to transport blockade is innocuous for spinal motoneurons in vivo*" publicado en Neurochemistry International, en el cual se muestra que la perfusión continua de dos diferentes inhibidores del transporte encargado de la recaptura de glutamato por medio de minibombas osmóticas, provoca una elevación significativa del glutamato extracelular endógeno; sin embargo, las ratas no presentan ningún déficit motor, ni tampoco hay degeneración de motoneuronas ni gliosis.

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

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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.

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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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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

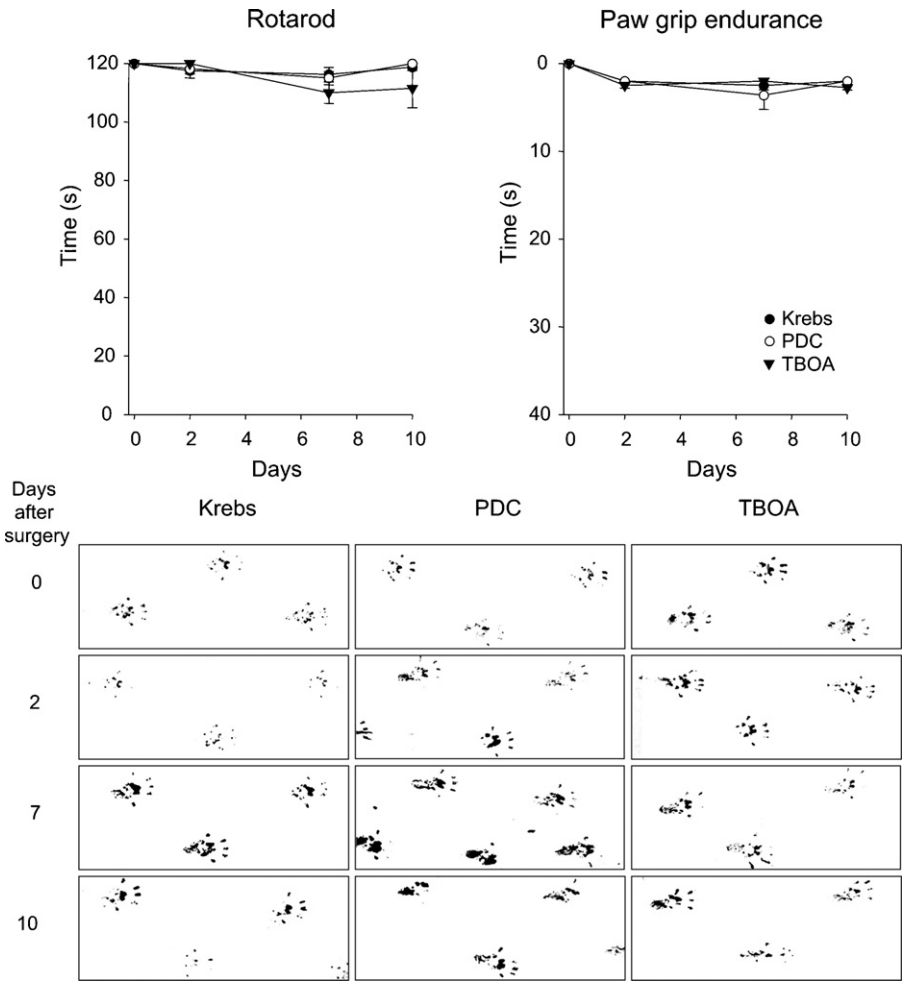

Fig. 1. Chronic blockade of glutamate transport does not induce motor deficits. Upper panel, time course of the Rotarod and paw grip endurance performances of rats infused with Krebs medium alone (control, $n = 4$), PDC ($n = 7$) and TBOA ($n = 4$). There were no statistical differences between the scores 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.

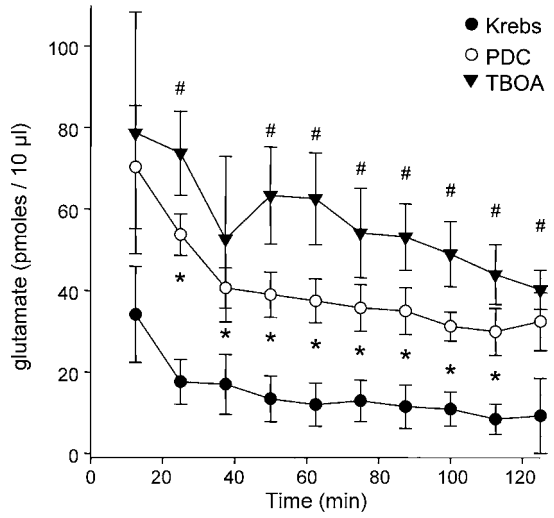

Fig. 2. Chronic blockade of glutamate transport augments the extracellular concentration of glutamate. Extracellular glutamate concentration was measured by microdialysis and HPLC as described in Section 2, 7–10 days after osmotic pump implantation and continuous Ringer–Krebs medium (control, $n = 4$), 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).

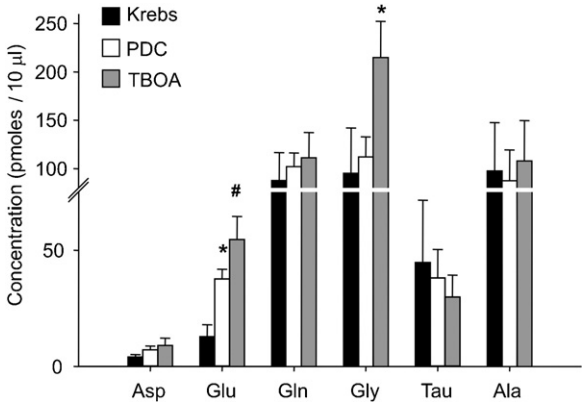

Fig. 3. Extracellular levels of different amino acids after chronic treatment with glutamate transport blockers. The extracellular concentration of aspartate, glutamate, glutamine, glycine, taurine and alanine were measured as described in Fig. 2. Each bar represents the mean ± S.E.M. of the averaged 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

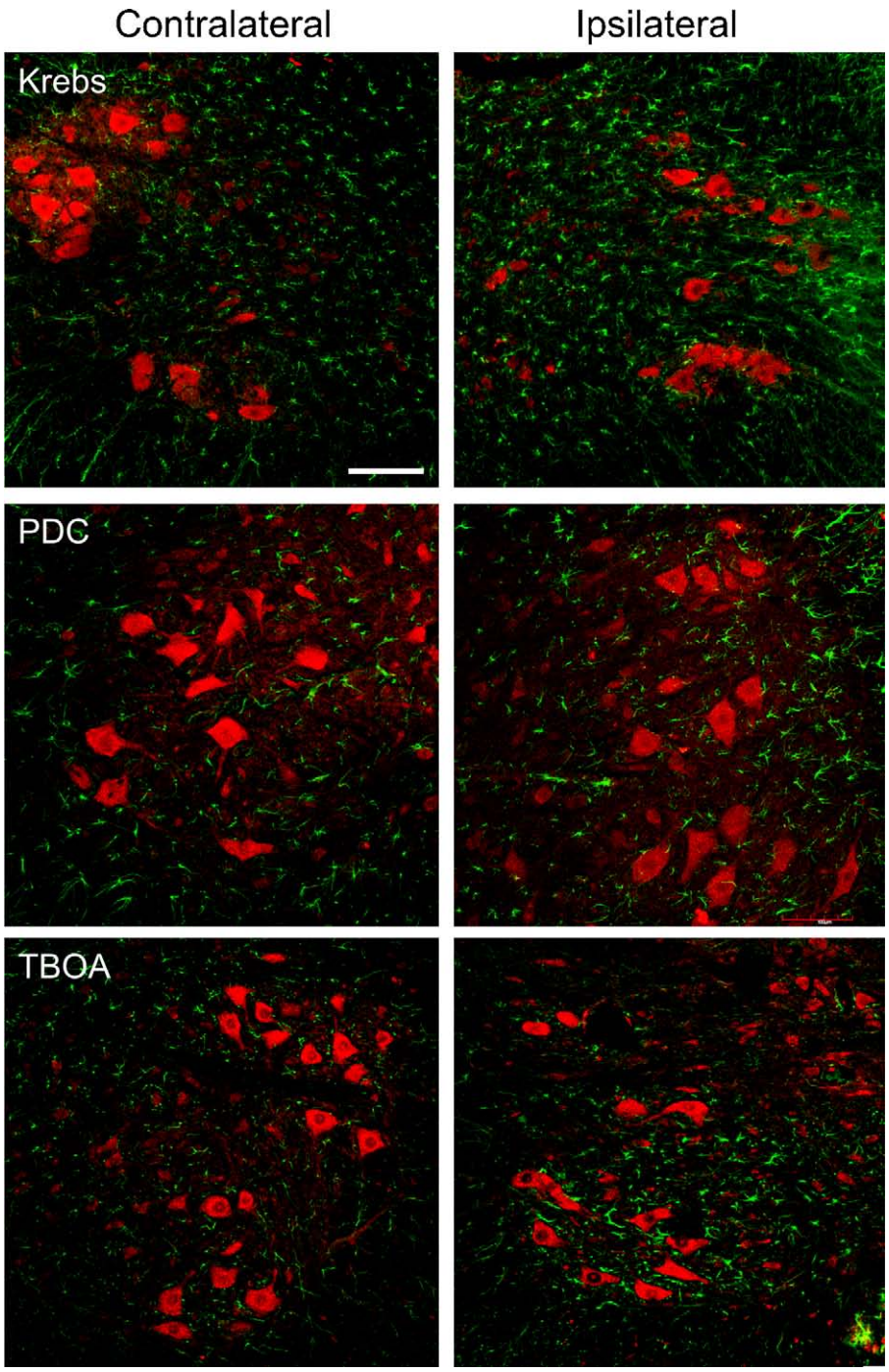

Fig. 4. Chronic blockade of glutamate transport does not induce motoneuron death. Representative micrographs of ChAT (red) and GFAP (green) immunohistochemistry in sections of the lumbar spinal cord, 7–10 days after pump implantation and continuous infusion with Krebs medium (control), PDC and TBOA. 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

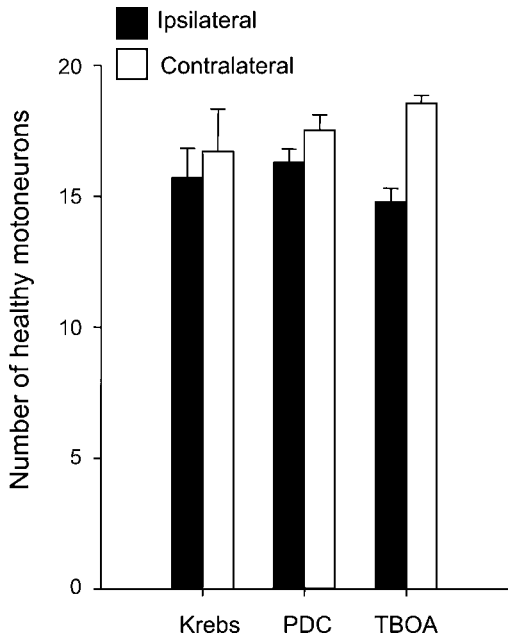

Fig. 5. Motoneuron number does not change after PDC or TBOA chronic administration. Number of healthy motoneurons in the ipsilateral and contralateral ventral horns of rats infused with Ringer–Krebs medium (control, $n = 4$), PDC ($n = 7$) and TBOA ($n = 4$). Five 40 μm sections from each rat were analyzed.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

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Aun así, no podemos descartar ni tampoco concluir si la excitotoxicidad glutamatérgica es el factor desencadenante que provoca la muerte selectiva de las motoneuronas en la ALS; no obstante, es muy probable que sí contribuya a esta degeneración pues también se han descrito niveles incrementados de glutamato en el líquido cefalorraquídeo y plasma de pacientes con ALS (Plaitakis y Caroscio 1987; Rothstein *et al.* 1990). Por otro lado, otros autores han reportado que este incremento solamente ocurre en ~40% de los pacientes (Shaw *et al.* 1995; Spreux-Varoquaux *et al.* 2002).

Las motoneuronas son particularmente vulnerables a la elevación intracelular de Ca^{2+} porque tienen baja capacidad de amortiguamiento debido a que sus mecanismos para mantener la homeostasis intracelular de Ca^{2+} son limitados (Grosskreutz *et al.* 2010). Las motoneuronas espinales de personas sanas y pacientes con ALS, de ratas y ratones, carecen de las 2 principales proteínas que unen calcio, la calbindina D-28k y la parvalbúmina. En cambio, otras motoneuronas que generalmente no se afectan en la ALS, como las de los núcleos oculomotor y de Onuf, sí expresan alguna de estas proteínas (Celio 1990; Ince *et al.* 1993; Alexianu *et al.* 1994; Palecek *et al.* 1999). Además, se ha demostrado *in vitro* que las motoneuronas no tienen la capacidad mitocondrial suficiente para amortiguar grandes elevaciones en las concentraciones de Ca^{2+} que se dan como consecuencia de la activación rápida y repetitiva de los receptores tipo AMPA por la exposición a kainato, debido en parte a una densidad mitocondrial por volumen menor comparada con neuronas no-motoras (Grosskreutz *et al.* 2007). En las motoneuronas espinales, el Ca^{2+} entra muy probablemente a través de los receptores tipo AMPA permeables a este catión. Los receptores tipo AMPA median la neurotransmisión sináptica excitadora rápida, y son homo- o hetero- tetrámeros compuestos por 4 subunidades, GluR1-GluR4, en varias combinaciones, que son codificados por genes separados y se expresan ubicua y abundantemente en todo el SNC (Hollmann y Heinemann 1994). La subunidad GluR2 determina la permeabilidad a Ca^{2+} de los receptores AMPA, dado que la presencia de al menos una de estas subunidades en la estructura de los receptores los hace impermeables a este catión (Hollmann *et al.* 1991; Hume *et al.* 1991). Sin embargo, no sólo la presencia de esa subunidad determina la permeabilidad de los receptores, sino también las modificaciones post-transcripcionales que editan el sitio Q/R de la subunidad substituyendo la glutamina (Q) sin carga por la arginina (R) cargada positivamente que impide el flujo de Ca^{2+} a través del poro (Burnashev *et al.* 1992). Por lo tanto, los receptores tipo AMPA que presentan una deficiencia en la edición post-transcripcional de la subunidad GluR2 o que carecen de ella son permeables a Ca^{2+}. Por consiguiente, la entrada de Ca^{2+} a las neuronas tras la activación

de este tipo de receptores, tiene consecuencias fisiológicas y patológicas importantes que pudieran estar ligadas a la ALS.

A pesar de que los resultados de los estudios sobre la expresión de la subunidad GluR2 en las motoneuronas espinales de rata y humano son controversiales (Tomiyama *et al.* 1996; Virgo *et al.* 1996; Williams *et al.* 1997; Bar-Peled *et al.* 1999; Kawahara *et al.* 2003), en motoneuronas normales de humano, no se detectó el mRNA para la subunidad GluR2, mientras que los mRNAs del resto de las subunidades estaban claramente presentes, sugiriendo que la mayoría de los receptores tipo AMPA en estas células son permeables a Ca^{2+} (Williams *et al.* 1997). También se encontró que los niveles de mRNA de GluR2 en motoneuronas de médula espinal individuales son menores en comparación con otras poblaciones neuronales. Sin embargo, no se encontraron diferencias entre los pacientes con ALS y otros pacientes neurológicos (Kawahara *et al.* 2003). A diferencia de esto, otro grupo encontró una disminución significativa del mRNA de la subunidad GluR2 en las médulas espinales de pacientes con ALS comparado con los controles (Virgo *et al.* 1996). Además, se encontró que la eficiencia de la edición del sitio Q/R de la subunidad GluR2 estaba disminuida en las astas ventrales de la médula espinal de pacientes con ALS (Takuma *et al.* 1999). Aunado a esto, también se encontró que la eficiencia en la edición de GluR2 no estaba completa en motoneuronas espinales individuales de algunos pacientes con ALS, mientras que estaba en un 100% completa en motoneuronas de pacientes control, y > 99% en las células cerebelares de Purkinje tanto en el grupo de pacientes con ALS como en el grupo control (Kawahara *et al.* 2004; Kwak y Kawahara 2005). Por otro lado, se encontró que la eficiencia de edición del RNA en el sitio Q/R de GluR2 era normal en ratas transgénicas con la SOD1 humana mutada (Kawahara *et al.* 2006), sugiriendo que los mecanismos que participan en la muerte de las motoneuronas en la FALS debida a las mutaciones en la SOD1, pudieran diferir de los de la SALS.

Se ha comprobado que las motoneuronas espinales son particularmente vulnerables a los agonistas de los receptores a glutamato tipo AMPA muy probablemente debido a la entrada de Ca^{2+} a través de este tipo de receptores permeables a este catión, tanto *in vitro* (Carriedo *et al.* 1995; Rothstein y Kuncl 1995; Carriedo *et al.* 1996; Greig *et al.* 2000; Van Den Bosch *et al.* 2000; Van Damme *et al.* 2002) como *in vivo* (Hugon *et al.* 1989; Corona y Tapia 2004; Corona y Tapia 2007). Por lo tanto, la sobreactivación de los receptores tipo AMPA permeables a Ca^{2+}, los cuáles son abundantes en las motoneuronas espinales, confiere a estas células una especial vulnerabilidad a la excitotoxicidad mediada por agonistas de este tipo receptores.

Además del bloqueo del transporte de glutamato, también se estudió en el laboratorio si la perfusión por microdiálisis de diferentes agonistas de los receptores a glutamato en la médula espinal lumbar de ratas provocaba algún daño *in vivo*, con el objetivo de abordar de manera más amplia la hipótesis de la excitotoxicidad. La perfusión de AMPA, a diferencia de la de NMDA o la de kainato, produjo parálisis permanente en la extremidad posterior ipsilateral y una pérdida notable de las motoneuronas espinales, que comenzó a las ~3-6 horas después del comienzo de la perfusión. Este efecto progresó hasta alcanzar el punto final a las 12 horas, cuando se da la pérdida de prácticamente todas las motoneuronas en el segmento espinal estudiado (Corona y Tapia 2004; Corona y Tapia 2008). Así, se desarrolló un modelo de degeneración excitotóxica de motoneuronas espinales *in vivo* mediante la sobreestimulación farmacológica de los receptores tipo AMPA. Este modelo se ha utilizado previamente en el laboratorio y es el que se utilizó en el presente trabajo para estudiar los mecanismos que participan en la degeneración de las motoneuronas espinales y para probar estrategias de neuroprotección. De entre los compuestos que evitan las deficiencias motoras, la parálisis de las ratas y la muerte de las motoneuronas, se encuentran el NBQX (2,3-dihidroxi-6-nitro-7-sulfamoil-benzo[f]quinoxalina) que es un antagonista de los receptores tipo AMPA, la NAS (1-naftil acetil espermina) que es un bloqueador selectivo de los receptores tipo AMPA que carecen de la subunidad GluR2, el BAPTA-AM (ácido 1,2-bis(2-aminofenoxi)etano-N,N,N´,N´-tetraacético tetrakis (acetoximetil ester)) que es un quelante de Ca^{2+} intracelular, y la leupeptina que es un inhibidor de calpaínas, las cuáles son proteasas activadas por Ca^{2+} (Corona y Tapia 2004; Corona y Tapia 2007; Corona y Tapia 2008). La protección ejercida por estos compuestos indica que las motoneuronas espinales de la rata poseen receptores tipo AMPA permeables a Ca^{2+} funcionales, y sugiere que el proceso celular que lleva a la muerte de las motoneuronas en este modelo *in vivo* es desencadenado por un incremento en la concentración intracelular de Ca^{2+} vía estos receptores.

La administración de piruvato en la médula espinal de las ratas también protegió contra la muerte de las motoneuronas y evitó la parálisis y cualquier daño motor (Corona y Tapia 2007); esto es de particular interés, pues sugiere que un incremento en la concentración intracelular de Ca^{2+} interfiere probablemente con el metabolismo energético mitocondrial.

En conjunto, todos los datos discutidos anteriormente sugieren que los receptores tipo AMPA permeables a Ca^{2+} pueden estar participando de manera importante en el desarrollo de la ALS.

A continuación, se presenta el artículo de revisión titulado "*Experimental models for the study of neurodegeneration in amyotrophic lateral sclerosis*" publicado en Molecular Neurodegeneration, en el cual se discute acerca de la formas espóradica y familiar de la ALS, algunas mutaciones que causan esta última, algunos de los factores que pudieran contribuir a la muerte de las motoneuronas - como son la excitotoxicidad, el estrés oxidativo, la agregación de proteínas - y los modelos experimentales tanto *in vitro* como *in vivo* que se han utilizado para estudiar los mecanismos patológicos celulares y moleculares de la enfermedad, su correlación con los síntomas (en los modelos *in vivo*), así como posibles estrategias terapéuticas. Se incluyen los modelos que se han desarrollado en nuestro laboratorio, y se discuten sus ventajas y desventajas.

Molecular Neurodegeneration

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Experimental models for the study of neurodegeneration in amyotrophic lateral sclerosis

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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.

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Disfunción mitocondrial y estrés oxidativo

Los hallazgos de anormalidades morfológicas y ultraestructurales en las mitocondrias (como son rearreglos en la membrana interna, vacuolización e hinchamiento mitocondrial), así como las deficiencias en las actividades de los complejos de la cadena respiratoria mitocondrial (Complejos I y IV) en músculo esquelético y médula espinal de pacientes con ALS, como también en los modelos experimentales de la enfermedad, dieron lugar a la hipótesis de que la disfunción mitocondrial participa importantemente en el proceso de la degeneración de las motoneuronas en la ALS.

También se ha propuesto que el estrés oxidativo contribuye a la muerte de las motoneuronas en la ALS dado que hay varias evidencias que demuestran esto, como son los incrementos en los marcadores de estrés oxidativo encontrados en tejidos postmortem, así como en plasma y líquido cefalorraquídeo de pacientes vivos con ALS, y en modelos experimentales de esta enfermedad.

Lo anterior se revisa en el capítulo que se presenta a continuación "*Role of mitochondrial dysfunction in motor neuron degeneration in ALS*" publicado en el libro "Amyotrophic Lateral Sclerosis", InTech, en el cual también se discuten algunos de los mecanismos involucrados en el proceso de la degeneración de las motoneuronas en la ALS, como son la excitotoxicidad, las deficiencias en el transporte axonal, el estrés oxidativo, los eventos inflamatorios, la falla energética y la disfunción mitocondrial. Ésta última es el principal enfoque de este capítulo, ya que la mitocondria es un punto convergente de todos estos mecanismos perjudiciales y es un organelo fundamental para la sobrevivencia de las neuronas, dado que su estatus bioenergético es crucial en el desarrollo de la susceptibilidad de las neuronas ante un estrés agudo o crónico, y también en la determinación de si la célula sobrevive o muere por necrosis o apoptosis. Así, se discuten ampliamente las funciones de este organelo, las evidencias de la participación de la disfunción mitocondrial en la ALS y modelos experimentales de degeneración de motoneuronas, su relación con la regulación de la homeostasis de Ca^{2+} y las consecuencias de la desregulación de la concentración intracelular de este catión, los déficits energéticos y el estrés oxidativo que se dan como consecuencia de la disfunción mitocondrial, la interrelación de todos estos mecanismos que crea un círculo vicioso letal, así como los daños estructurales relacionados con esta disfunción que se han observado tanto en pacientes como en modelos experimentales de la enfermedad.

8

Role of Mitochondrial Dysfunction in Motor Neuron Degeneration in ALS

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1. Introduction

Amyotrophic lateral sclerosis (ALS), which was described since 1869 by Jean Martin Charcot, is a devastating neurodegenerative disease characterized by the selective and progressive loss of upper and lower motor neurons of the cerebral cortex, brainstem and the spinal cord. Progressive motor neuron loss causes muscle weakness, spasticity and fasciculation, eventually paralysis and finally death by respiratory failure 3 to 5 years after diagnosis. ALS worldwide prevalence is about 2 to 8 people per 100,000, and presents two important differences with respect to other neurogenerative diseases: the cognitive process is not affected and is not merely the result of aging because may occur at young ages (Chancellor & Warlow, 1992; Huisman et al., 2011). Two forms of ALS are known, the familial type (FALS), associated with genetic mutations, mainly in the gene encoding superoxide dismutase 1 (SOD1, enzyme responsible for superoxide dismutation to oxygen and hydrogen peroxide), and the sporadic form (SALS), of unknown origin. FALS represents only about 5-10% of cases (Rosen et al., 1993; Rowland & Shneider, 2001), and SALS comprises the remaining 90%. Despite having different origins, both ALS types develop similar histopathological and clinical characteristics.

2. Mechanisms of motor neuron death in ALS

After one hundred fifty years since the first ALS description of the disease, the cause of motor neuron degeneration remains unknown, but progress in neuroscience and clinical research has identified several mechanisms that seem to be involved in the cell death process, such as glutamate-mediated excitotoxicity, inflammatory events, axonal transport deficits, oxidative stress, mitochondrial dysfunction and energy failure.

2.1 Excitotoxicity

Based on the reduction of glutamate transporter-1 (GLT1 in rodents and excitatory amino acid transporter 2 or EAAT2 in human) content detected post-mortem in motor cortex and spinal cord of ALS patients (Rothstein et al., 1992; Rothstein et al., 1995) and on the increase of glutamate concentration in the cerebrospinal fluid (CSF) of about 40% of ALS patients (Shaw et al., 1995b; Spreux-Varoquaux et al., 2002), one proposed mechanism to explain

motor neuron death in ALS is glutamate-mediated excitotoxicity. This hypothesis has been generally accepted, although some data from our laboratory do not support it because a chronic increase in extracellular glutamate due to glutamate transport inhibition in the spinal cord in vivo was innocuous for motor neurons (Tovar-y-Romo et al., 2009b). However, overactivation of glutamate ionotropic receptors by agonists leads to neuronal death by augmenting the influx of Ca^{2+} into motor neurons. Experimental models in vivo have shown that of three major glutamate ionotropic receptor types, NMDA (N-methyl-D-aspartate), kainate and AMPA (α-amino-3-hydroxy-5-isoxazolepropionate), the Ca^{2+}-permeable AMPA receptor seems to be particularly involved in motor neuron death, because the selective blockade of Ca^{2+}-permeable AMPA receptors or the chelation of intracellular Ca^{2+} prevents the motor neuron loss and the consequent paralysis induced by the infusion of AMPA into the rat lumbar spinal cord (Corona & Tapia, 2004, 2007; Tovar-y-Romo et al., 2009a). The Ca^{2+} permeability of this receptor is governed by the presence of the GluR2 subunit and its edition in the Q/R (glutamine/arginine) site of the second transmembrane domain (Burnashev et al., 1992; Corona & Tapia, 2007; Hollmann et al., 1991; Hume et al., 1991).

Increases in cytoplasmic Ca^{2+} concentration can be buffered by mitochondria, but when maintained for prolonged periods can cause mitochondrial swelling and dysfunction. These alterations are associated with deficits in mitochondrial ATP synthesis and energetic failure (this topic will be discussed later). The energetic deficits have been mainly associated with cell death process similar to necrosis (Kroemer et al., 2009; Martin, 2010). On the other hand, mitochondrial damage has also been linked to the release of proapoptotic factors such as cytochrome c and apoptosis-inducing factor (Martin et al., 2009). Cytochrome c involvement has been stressed because of its role in triggering the caspases pathway, which leads to apoptotic cellular death. In the cytoplasm cytochrome c promotes the formation of the apoptosome complex and activates caspase-3. The necrosis and apoptosis pathways are illustrated in Fig. 1.

2.2 Axonal transport deficits

Because of the structural and functional characteristics of motor neuron axons, the role of axonal transport is essential for the communication between the neuronal soma and the periphery, as well as for the anterograde and retrograde dispersive distribution of cargo intracellular structures such as vesicles or organelles. Changes in the speed of anterograde and retrograde transport (Breuer & Atkinson, 1988; Breuer et al., 1987; Sasaki & Iwata, 1996), as well as neurofilament disorganization and accumulation of mitochondria, vesicles and smooth endoplasmic reticulum have been described in peripheral nerves of ALS patients (Hirano et al., 1984a, b; Sasaki & Iwata, 1996). These alterations in axonal transport have been observed also in transgenic models of FALS, which have allowed the study of their progression and the molecular machinery involved (Bilsland et al., 2010; Brunet et al., 2009; Collard et al., 1995; De Vos et al., 2007; Ligon et al., 2005; Perlson et al., 2009; Pun et al., 2006; Tateno et al., 2009; Warita et al., 1999; Williamson & Cleveland, 1999). In mutant SOD1 (mSOD1) rodents, some motor proteins such as: dynein, dynactin, kinesin, myosin, actin, and microtubules and neurofilaments are affected by mSOD1 aggregates (Breuer & Atkinson, 1988; Breuer et al., 1987; Collard et al., 1995; Ligon et al., 2005; Sasaki & Iwata, 1996; Williamson & Cleveland, 1999; Zhang et al., 2007).

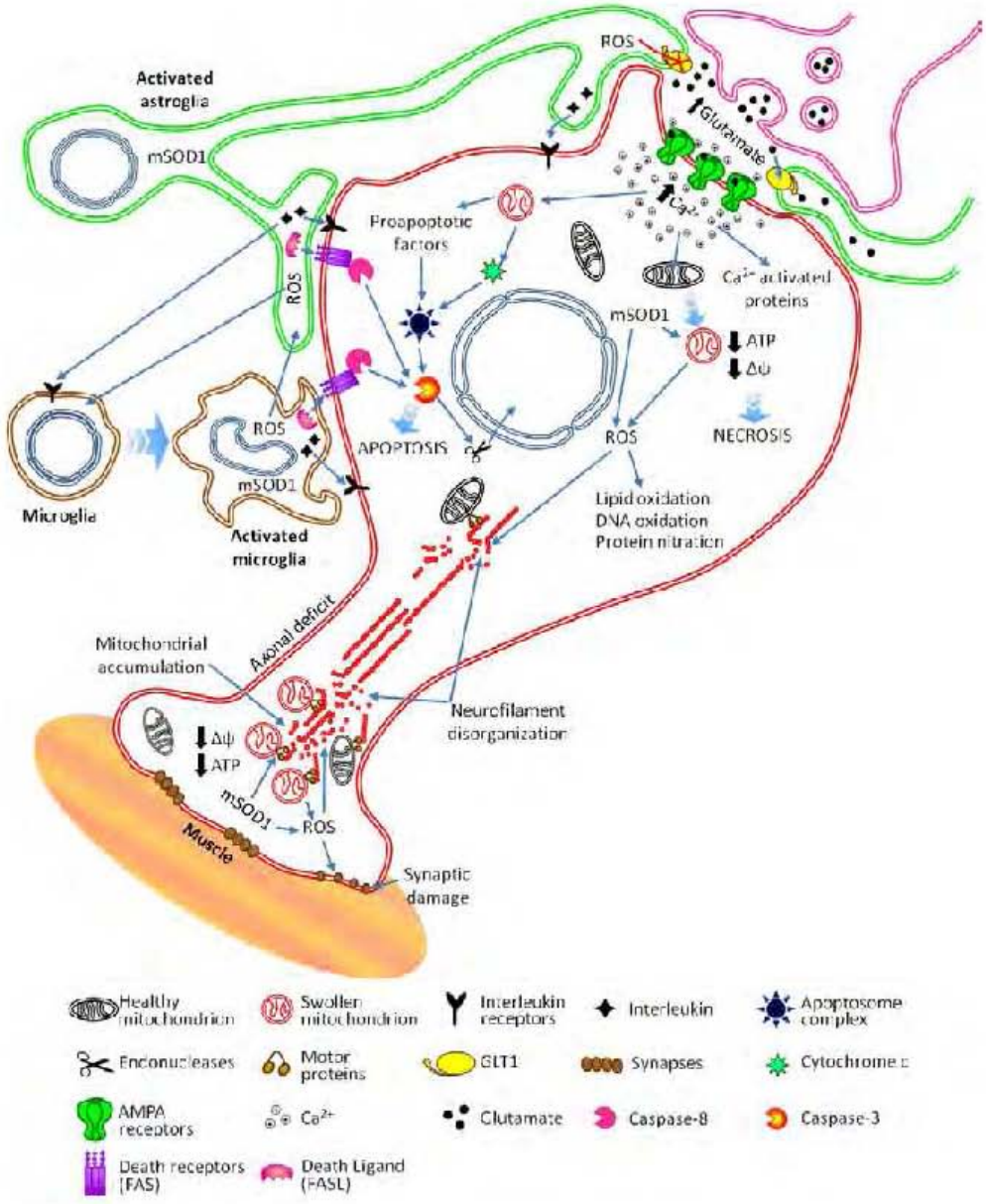

Fig. 1. Scheme of the main proposed mechanisms involved in motor neuron death. Description in the text.

These deficits may affect the renewal of organelles in the axon terminals of motor neurons, leading to accumulation of damaged mitochondria or autophagosomes, increased ROS production, disruption of microtubule formation and stability (Julien & Mushynski, 1998), as well as damage of presynaptic structure such as swelling of axon terminals (Komatsu et al., 2007). Accumulation of damaged mitochondria may result in energetic failure (Liu et al., 2004; Martin et al., 2009; Menzies et al., 2002a, b; Pasinelli et al., 2004; Wong et al., 1995; Zhu et al., 2002) and in the release of proapoptotic factors (Pasinelli et al., 2004) (Fig. 1, bottom left). These alterations may be involved in the distal neurophaty and impairment of muscular reinnervation observed in ALS.

2.3 Oxidative stress

Another mechanism implicated in motor neuron degeneration in ALS that involves both motor neurons and non-neuronal cells is oxidative stress. Reactive oxygen species (ROS) arise in cells as aerobic metabolism by-products, mostly due to the leakage of electrons from the mitochondrial respiratory chain, resulting in an incomplete reduction of molecular oxygen during the oxidative phosphorylation, generating the superoxide radical anion ($O_2^{\bullet -}$). The $O_2^{\bullet -}$ anion reacts quickly with the nitric oxide radical ($NO^{\bullet}$, produced by nitric oxide synthase, NOS) to form peroxynitrite ($ONOO^-$). Meanwhile, the product of $O_2^{\bullet -}$ dismutation, H_2O_2, slowly decomposes to form the highly reactive hydroxyl radical ($^{\bullet}OH$). Both $ONOO^-$ and $^{\bullet}OH$ are highly reactive and can damage proteins, membranes and DNA by oxidation. Cellular mechanisms to combat the constant production of free radicals are: 1) enzymes such as SOD, catalase and peroxidase, which catalytically remove reactive species; 2) reducing agents synthesized in vivo, such as glutathione, α-keto acids, lipoic acid and coenzyme Q, and compounds obtained from the diet, such as ascorbate (vitamin C) and α-tocopherol (vitamin E); and 3) chaperone heat shock proteins which remove or facilitate repair of damaged proteins. Oxidative stress arises from an imbalance between ROS production and its control mechanisms.

The involvement of oxidative stress in ALS pathogenesis is supported by abundant evidence that has been reported in both SALS and FALS patients, where several indicators of increased oxidative damage have been found: 1) In postmortem central nervous system (CNS) tissue samples (mainly spinal cord) these markers include oxidized DNA (Ferrante et al., 1997b; Fitzmaurice et al., 1996), lipid peroxidation (Siciliano et al., 2002), protein glycoxidation (Shibata et al., 2001), elevated protein carbonylation (Ferrante et al., 1997b; Shaw et al., 1995a), and increased protein tyrosine nitration; remarkably, nitrotyrosine immunoreactivity was more densely detected in motor neurons (Abe et al., 1995; Abe et al., 1997; Beal et al., 1997; Ferrante et al., 1997a). 2) Oxidation markers in CSF, plasma and blood from living ALS patients during the course of the disease have also been described. The most relevant are oxidized DNA (Bogdanov et al., 2000; Ihara et al., 2005), hydroxyl and ascorbate free radicals (Ihara et al., 2005), lipid peroxidation (Baillet et al., 2010; Bogdanov et al., 2000; Bonnefont-Rousselot et al., 2000; Ihara et al., 2005; Oteiza et al., 1997; Simpson et al., 2004; Smith et al., 1998), and a remarkable elevation of 3-nitrotyrosine levels in CSF (Tohgi et al., 1999). However, in other study, 3-nitroyrosine was not different between the CSF of ALS patients and control subjects (Ryberg et al., 2004). Increased oxidative damage to proteins, lipids and DNA has also been demonstrated in CNS tissue of transgenic mouse model of FALS expressing mSOD1 (Andrus et al., 1998; Casoni et al., 2005; Liu et al., 1999; Liu et al., 1998; Poon et al., 2005).

Mitochondria, ROS and glutamate-induced excitotoxicity are closely related and this is relevant in ALS, because the mitochondrion is the main oxygen consumer and it is also the main producer of ROS. These species can be produced in neurons and in non-neuronal cells and can cause failure in the glutamate uptake system of both motor neurons and astroglia (Rao et al., 2003; Trotti et al., 1996, 1998; Volterra et al., 1994; Zagami et al., 2009). This may contribute to an excitotoxic condition due to increased concentration of extracellular glutamate. ROS production and its effects on motor neurons and non-neuronal cells are illustrated in Fig. 1.

2.4 Inflammation

A mechanism of non-cell-autonomous death associated with motor neuron degeneration in both FALS and SALS is the participation of non-neuronal cells in inflammatory events (Boillee et al., 2006a; Boillee et al., 2006b; Hall et al., 1998; Yamanaka et al., 2008; Yang et al., 2011). The main histopathological feature of inflammation is the proliferation of reactive astrogliosis and of activated microglial cells, associated with alterations in their cellular functions, such as glutamate reuptake failure and release of proapoptotic and proinflammatory factors (Sanagi et al., 2010; Sargsyan et al., 2005; Sofroniew, 2005). Molecules associated with inflammatory process, such as interleukins 6, 12, 15, 17A, 23, C4d and C3d complement proteins, as well as tumor necrosis factor-alpha, have been found in blood and CSF from ALS patients (Almer et al., 2002; Fiala et al., 2010; Henkel et al., 2004; Kawamata et al., 1992; McGeer et al., 1991; Moreau et al., 2005; Rentzos et al., 2010a, b). The finding of increased levels of granzymes A, B in serum (Ilzecka, 2011) and decrease in cytochrome c levels in the CSF (Ilzecka, 2007), suggests an apoptotic process in human disease. The proliferation of activated non-neuronal cells has been associated with the disease severity (Clement et al., 2003). Nevertheless, alteration in their functions may be more important than their proliferation (Lepore et al., 2008). In experimental models of FALS (mSOD1) it has been attempted to prevent the motor neuron loss through the use of drugs with anti-inflammatory properties, such as minocycline (Keller et al., 2010; Kriz et al., 2002; Neymotin et al., 2009; Van Den Bosch et al., 2002; Zhu et al., 2002). This drug was effective in delaying the motor neurons loss when given prior to the symptoms onset, but when given at late stages it exaggerated the neuroinflammatory response and accelerated the progression of the symptoms (Keller et al., 2010). In this transgenic ALS model, apoptosis processes can be triggered by non-neuronal cells through the extrinsic apoptotic pathway, via the release from activated glial cells of several death ligands (for example FasL) that bind to their respective death receptor (Fas) and trigger the cleavage of caspase-8 (Locatelli et al., 2007; Petri et al., 2006; Raoul et al., 2006) (Fig. 1).

3. Mitochondrial dysfunction in ALS and in experimental motor neuron degeneration

A convergent point of the deleterious mechanisms discussed above is the mitochondrion. This organelle is the main energy producer in eukaryotic cells and plays a fundamental role in normal cell physiology. Among the functions mitochondria carry out, besides ATP synthesis, intracellular Ca^{2+}buffering has been recognized as another relevant factor for the protection against deleterious processes such as oxidative stress, excitotoxicity and necrotic and apoptotic death, thus playing a central role in neuronal survival.

Mitochondria are closely related to necrotic and apoptotic processes, which are the main cellular death mechanisms. During necrosis, mitochondria undergo rapid swelling and lysis. Although apoptosis is an energy-dependent active process, sometimes mitochondrial morphological alterations are associated with the intrinsic-apoptosis pathway. Furthermore, it is now recognized that apoptosis and necrosis are not two mutually exclusive processes, but they can occur simultaneously or one preceding the other (Kroemer et al., 2009; Martin, 2010; Martin et al., 2009; Shrivastava & Vivekanandhan, 2011).

As the organelle responsible for energy production in the cell, mitochondria possess the enzymatic machinery to catalyze the oxidation of various substrates generated inside and outside mitochondria, including pyruvate trough pyruvate dehydrogenase, fatty acids through β-oxidation, and carbon chains from amino acids. Energy is obtained by oxidation of all these biomolecules to finally CO_2 and H_2O through the tricarboxylic acid cycle and the respiratory chain. The tricarboxylic acid cycle is the converging point because the carbon skeletons of carbohydrates and fatty acids are metabolized to yield the acetyl group of acetyl-Coenzyme A, and many of the carbons of the amino acid skeleton also enter the cycle via its conversion to some cycle intermediates. The reducing equivalents generated in the tricarboxylic acid cycle reactions reduce pyridine and flavin nucleotides to NADH and $FADH_2$. These electron transporters enter the respiratory chain, where electron flux through various redox carriers and centers in the enzyme complexes located in the inner mitochondrial membrane finally reduces O_2 to H_2O; this flux is coupled to ATP synthesis through oxidative phosphorylation.

The energy released by the electron flux through respiratory chain complexes is used to pump protons through the inner mitochondrial membrane, producing an alkaline and negatively charged mitochondrial matrix, as compared to the intermembrane space, thus creating a proton gradient. This proton gradient generates an electrochemical potential called proton-motive force (Δp), which supplies the energy to ATP synthase for ATP synthesis from ADP and inorganic phosphate. The Δp depends mainly on the mitochondrial transmembrane potential ($\Delta\psi_m$), which is the electric potential (negative inside), but it also depends on the transmembrane pH gradient (ΔpH), which is the chemical potential (alkaline inside). Energy stored in the proton gradient can also transport solutes against concentration gradient across the membrane. The $\Delta\psi_m$ is a central parameter that controls three fundamental and highly relevant cellular processes for neuronal survival: ATP synthesis, mitochondrial Ca^{2+} sequestration, and mitochondrial ROS generation. On the other hand, $\Delta\psi_m$ is controlled by substrate availability, ATP demand, respiratory chain capacity, mitochondrial proton conductance, and mitochondrial Ca^{2+} sequestration (Nicholls & Budd, 2000). Therefore, mitochondrial bioenergetic status is crucial for controlling the susceptibility of neurons to chronic or acute stress and also in determining cellular fate (survival, apoptosis or necrosis).

Owing to the great relevance of mitochondria, their morphological, ultrastructural and functional characteristics have been studied in ALS patients. Deficits in respiratory chain complexes I and IV activities have been detected in the spinal cord and skeletal muscle (Borthwick et al., 1999; Crugnola et al., 2010; Vielhaber et al., 2000; Wiedemann et al., 2002; Wiedemann et al., 1998), and a temporal study of mitochondrial respiratory function in skeletal muscle in SALS demonstrated that respiratory complex IV activity is progressively altered as the disease develops (Echaniz-Laguna et al., 2006). Some cases of ALS have been described as a mitochondriopathy (Finsterer, 2002, 2003) including a mitochondrial DNA

mutation in the gene encoding subunit I of the mitochondrial respiratory chain complex IV (Comi et al., 1998). The electron transport chain proteins FAD synthetase, riboflavin kinase, cytochrome C1, and succinate dehydrogenase complex subunit B expression were significantly decreased in some ALS patients (Lin et al., 2009).

In the mSOD1 mice or cell culture familial ALS model, complexes I, II and IV of the electron transport chain exhibit decreased enzyme activities, even at early stages of the disease (Jung et al., 2002; Mattiazzi et al., 2002; Menzies et al., 2002a,b). In G93A-SOD1 mice the association of cytochrome c with the inner mitochondrial membrane was reduced and there was a significant decrease in respiratory chain complex IV (Kirkinezos et al., 2005). SOD-containing aggregates (Higgins et al., 2002; Jaarsma et al., 2001; Pasinelli et al., 2004) and decreased oxygen consumption, lack of ADP-dependent respiratory control, and decreased membrane potential (Cassina et al., 2008), were observed in mitochondria from spinal cord of transgenic mSOD1 rodents.

In neuronal cultures, glutamate-mediated excitotoxicity caused significant changes in mitochondrial function, such as decline in ATP levels, mitochondrial transmembrane potential collapse, decreased mitochondrial and cellular oxygen consumption, and oxidative phosphorylation uncoupling, all these events preceding cell death (Ankarcrona et al., 1995; Atlante et al., 1996; Maus et al., 1999; Monje et al., 2001). There is a link between excitotoxicity-induced intracellular Ca^{2+} overload and the collapse of $\Delta\psi_m$, since intracellular Ca^{2+} increase and its accumulation in mitochondria are sufficient to induce prominent and persistent depolarization, leading to mitochondrial dysfunction and to neuronal death in vitro (Schinder et al., 1996; White & Reynolds, 1996).

Few studies on excitotoxicity have been carried out in vivo. In our laboratory we have developed two experimental models of spinal motor neurons degeneration by overactivation of AMPA receptors, both by infusing AMPA directly in the lumbar spinal cord of rats. In the first one AMPA is administered through microdialysis cannulas during short time periods (Corona & Tapia, 2004) and in the other AMPA is infused chronically during several days, using osmotic minipumps (Tovar-y-Romo et al., 2007). These models reproduce the main histopathological features of ALS: loss of lumbar motor neurons, astroglial activation and motor deficits that progresses to complete paralysis of the rear limbs. The main difference between the two models is the time required for the occurrence of motor neuron degeneration and the development of the paralysis. AMPA perfusion by microdialysis causes a rapid loss of motor neuron and paralysis, occurring within the initial 12 hours, while chronic AMPA infusion with osmotic minipumps triggers a progressive motor neuron loss and motor deficits throughout three to four days. For these reasons, the microdialysis model is defined as an acute model and the minipumps model as a chronic model of spinal motor neuron degeneration by excitotoxicity (Tovar-y-Romo et al., 2009a). The most important feature of both models is that motor neuron loss occurs without the influence of a genetic factor and thus presumably can be used to study the mechanisms that may be involved in motor neuron loss occurring in SALS, which accounts for over 90% of ALS cases.

We have recently assessed mitochondrial function in our acute model of spinal excitotoxic motor neuron degeneration, by studying mitochondrial oxygen consumption and transmembrane potential in mitochondria isolated from the lumbar spinal cord of rats perfused with AMPA. The AMPA-treated group showed decreased oxygen consumption, ADP-dependent respiratory control and transmembrane potential, as compared to control

rats perfused only with Krebs-Ringer medium (Santa-Cruz and Tapia, in preparation). These results suggest that mitochondrial dysfunction plays a crucial role in spinal motoneuron degeneration induced by overactivation of AMPA receptors in vivo. These mechanisms could be involved in ALS motoneuron degeneration.

3.1 Ca^{2+}, mitochondria and motor neuron degeneration

Under physiological conditions, Ca^{2+} participates as intracellular messenger in many normal cellular functions, such as cell growth, differentiation, signal transduction, membrane excitability regulation, exocytosis and synaptic activity. Cytoplasmic Ca^{2+} concentration in resting neurons is maintained at low concentrations (~100 nM), 10,000 times lower than extracellular space concentration. To achieve this, neurons possess specialized homeostatic mechanisms, such as regulation of Ca^{2+} input and output, Ca^{2+} binding proteins, mitochondrial and endoplasmic reticulum storage, and Ca^{2+}-ATPases. Moreover, neurons not only control intracellular Ca^{2+} levels, but also its location in the cell by means of complex interactions among Ca^{2+} input, output, buffering and internal storage. Under physiological conditions, these processes maintain spatial and temporal location of Ca^{2+}, so that multiple Ca^{2+}-regulated signaling pathways can take place independently within the same cell.

Excessive intracellular Ca^{2+} concentration damages neurons through several mechanisms, including mitochondrial damage, energy metabolism deficit, toxic ROS generation, membrane depolarization, and activation of lytic enzymes such as proteases, lipases, phosphatases and endonucleases. Intracellular Ca^{2+} accumulation also stimulates ROS production through NOS activation and the conversion of xanthine dehydrogenase to xanthine oxidase through proteases activation. All these events eventually produce membrane destruction and neuronal death (Arundine & Tymianski, 2003; Shaw, 1999).

Intracellular Ca^{2+} regulation is an expensive process from the energy point of view. Ca^{2+} is extruded from the cell and sequestered into the endoplasmic reticulum through active transport using Ca^{2+}-ATPases, and it is also removed by secondary active transport using the Na^+/Ca^{2+} exchanger, which activates Na^+/K^+-ATPases to take out Na^+. Mitochondria also play a critical role in the regulation of cytosolic Ca^{2+} concentration, since they sequester this cation through a Ca^{2+} uniporter located in the inner mitochondrial membrane and driven by the electric potential (Nicholls, 1985). To prevent a potentially lethal Ca^{2+} accumulation in mitochondrial matrix, there is an output system that exchanges Na^+/Ca^{2+}, besides a mitochondrial Na^+/H^+ transporter that extrudes Na^+, so that ion flux under a constant Ca^{2+} entrance to mitochondria involves a sequential transfer of Ca^{2+}, Na^+ and H^+, the latter driven by the respiratory chain (Crompton & Heid, 1978; Nicholls & Budd, 2000). When Ca^{2+} concentration surpasses a certain critical point, under physiological phosphate concentration an osmotically inactive and rapidly dissociable Ca^{2+}-phosphate complex is formed in the mitochondrial matrix, so that mitochondria work as efficient buffers of extramitochondrial Ca^{2+} by accumulating this cation (Becker et al., 1980; Nicholls, 1978). Apparently, this organelle acts as a temporary Ca^{2+} store during high cytoplasmic concentrations peaks, as suggested by the kinetics of mitochondrial Ca^{2+} transport; because the Ca^{2+}-phosphate complex is rapidly dissociable, mitochondria can release Ca^{2+} back to the cytoplasm when its concentration decreases below the critical point. As long as mitochondria are polarized, cytosolic Ca^{2+} accumulates within the mitochondrial matrix through the Ca^{2+} uniporter. Mitochondrial Ca^{2+} uptake is driven by $\Delta\psi_m$, so it will compete with ATP synthase for proton gradient, in such a way that Ca^{2+} uptake could dominate due

to the fact that ATP synthesis requires a thermodynamic threshold for $\Delta\psi_m$, while Ca^{2+} transport can proceed at much lower $\Delta\psi_m$ and excessive Ca^{2+} concentrations reduce $\Delta\psi_m$ dramatically. When Ca^{2+} concentration does not recover below the critical point, excessive Ca^{2+} overload in the mitochondrial matrix can lead to mitochondrial swelling, loss of respiratory control, increased mitochondrial ROS generation, $\Delta\psi_m$ collapse (depolarization) diminished ATP synthesis, and Ca^{2+} release from the mitochondrial matrix caused by inner mitochondrial membrane permeabilization through the mitochondrial permeability transition pore (MPTP, a large protein complex forming a non-selective pore through the inner mitochondrial membrane) (Al-Nasser & Crompton, 1986; Nicholls & Budd, 2000; Peng & Jou, 2010). When mitochondrion depolarizes, accumulated Ca^{2+} goes back into the cytoplasm, either through the Ca^{2+} uniporter, the Na^+/Ca^{2+} exchanger, or through the MPTP. Since Δp depends mainly on $\Delta\psi_m$, its collapse causes Δp collapse, which results not only in halting ATP synthesis but also in a rapid cytoplasmic ATP hydrolysis because ATP synthase catalytic function reverses in an attempt to restore Δp.

In motor neurons, the damage produced by these alterations may be enhanced because they do not have sufficient mitochondrial Ca^{2+}-buffering capacity, due in part to a lower mitochondrial density per volume compared to non-motor neurons (Grosskreutz et al., 2007). In addition, other buffering mechanisms are deficient in spinal and cortical motor neurons because they lack the Ca^{2+}-binding proteins calbindin D-28K and parvalbumin. This may explain why other motor neurons that express these proteins, such as those located in oculomotor and Onuf's nuclei, are not usually affected in ALS (Alexianu et al., 1994; Celio, 1990; Ince et al., 1993; Palecek et al., 1999). For all these reasons, mitochondrial Ca^{2+} overload plays a key role in glutamatergic excitotoxicity (Nicholls et al., 2003), given that overactivation of Ca^{2+}-permeable AMPA receptors, which are abundant in spinal motor neurons, confers to these cells a special vulnerability to AMPA receptor-mediated excitotoxicity (Corona & Tapia, 2007; Grosskreutz et al., 2010). AMPA exposure to spinal motor neuron cultures results in an intracellular Ca^{2+} concentration increase that triggers mitochondrial Ca^{2+} overload, depolarization and ROS generation (Carriedo et al., 2000). So, there is abundant evidence that suggest that mitochondrial damage, probably related to Ca^{2+} homeostasis disturbances, is involved in SALS and FALS (Manfredi & Xu, 2005; Menzies et al., 2002a; Swerdlow et al., 1998; von Lewinski & Keller, 2005).

3.2 Energy deficits

Due to the large size of motor neurons and their long processes reaching muscles, they have an expensive energy cost and this renders them very vulnerable to energy deficits. Much of the ATP demand in neurons is used in the ion pumping through plasma membrane to maintain membrane potential. Thus, Na^+/K^+-ATPase is the most demanding ATP process in neurons (Scott & Nicholls, 1980) in order to expel Na^+ excess resulting from excitation. Intracellular Ca^{2+} regulation by Ca-ATPases is also highly energy consuming, as previously discussed.

There is abundant evidence both in vitro and in vivo that any restriction in the ability of the cell to generate ATP can exacerbate or even induce glutamatergic excitotoxicity. The energy-linked excitotoxic hypothesis (Beal et al., 1993; Greene & Greenamyre, 1996; Novelli et al., 1988) proposes that the correlation between excitotoxic damage and energy restriction is due to plasma membrane depolarization. Diminished ATP levels cause a decrease in Na^+/K^+-ATPase and Ca^{2+}-ATPase functions, lessening Na^+ and Ca^{2+} removal. This triggers plasma

membrane depolarization and as a consequence Ca^{2+} enters the cell through voltage-dependent Ca^{2+}channels and glutamate is released to the extracellular space by exocytosis. This in turn activates Ca^{2+} influx through the NMDA receptor, which is also voltage-dependent. Further, under energetic failure conditions, glutamate transporters operate in reverse because Na^+/K^+ electrochemical gradient collapse due to ATP decrease, resulting in diminished glutamate uptake and non-vesicular glutamate release into extracellular space (Jabaudon et al., 2000; Longuemare & Swanson, 1995).

The observation that inhibition of mitochondrial respiratory chain complexes activity can induce pathological changes similar to those observed in some neurodegenerative diseases in specific CNS regions has generated great interest. Association among glutamatergic excitotoxicity and bioenergetic limitation has been proposed for Alzheimer, Parkinson, Huntington's disease and ALS (Beal, 1998), and in many cases specific respiratory chain complexes are involved. In organotypic spinal cord cultures, motor neurons are selectively vulnerable to chronic mitochondrial blockade by inhibitors of mitochondrial respiratory chain complex II and complex IV and this motor neuron degeneration displays structural changes similar to those seen following excitotoxicity (Brunet et al., 2009; Kaal et al., 2000).

In our acute model of excitotoxic motor neuron degeneration previously described (Corona & Tapia, 2004, 2007) we have demonstrated the importance of Ca^{2+}-permeable AMPA receptors and of intracellular Ca^{2+} overload in motor neuron death process. Using this model, we aimed to study the importance of energy deficits and oxidative stress in AMPA-induced degeneration. With this purpose, we assessed the potential neuroprotection of various energy substrates and antioxidants at different concentrations, co-perfusing them with AMPA in the rat lumbar spinal cord. We observed protection at different degrees depending on the concentration of each compound, but in general antioxidants only partially protected, while various energy substrates prevented the AMPA-induced motor impairment and the spinal motor neuron loss (Santa-Cruz and Tapia, in preparation). These findings suggest that intracellular Ca^{2+} overload in vivo disrupts mitochondrial energy metabolism. On the other hand, energy substrates can directly prevent $\Delta\psi_m$ collapse and thus prevent mitochondrial dysfunction. Because one of the factors that control $\Delta\psi_m$ is substrate availability, excess mitochondrial substrates administered exogenously can stimulate respiratory chain and increase oxidative phosphorylation, maintaining the electrochemical proton gradient and thus preventing the collapse of ATP synthesis.

3.3 Oxidative stress

Since mitochondria are the organelles where oxidative phosphorylation is accomplished, they consume about 98 % of the cell oxygen requirement and constitute a major site for intracellular ROS production. Some steps along mitochondrial oxygen reduction pathway have the potential to produce, and indeed generate free radicals, due to the fact that electron flux along respiratory chain may have leakage of electrons to oxygen. The intermediate radical ubisemiquinone, involved in the transfer of electrons through respiratory complexes III and I, can grant an electron to oxygen, forming the superoxide radical $O_2^{\bullet-}$, a powerful oxidant and a very reactive intermediate (Turrens et al., 1985) that must be rapidly removed by antioxidant enzymes to avoid its lethal effects. About 0.1-4% of the O_2 used by actively respiring mitochondria is converted to $O_2^{\bullet-}$. Nevertheless, respiratory chain enzymes defects or other mitochondrial perturbations could be responsible of an excessive mitochondrial

ROS production, triggering or increasing cellular injure. Among them, mitochondrial Ca^{2+} overload resulting from NMDA, AMPA or kainate receptor overactivation (Carriedo et al., 1998; Carriedo et al., 2000; Dugan et al., 1995) increases ROS production (Dykens, 1994; Peng & Jou, 2010); thus, an initial excitotoxic event might also contribute to increased oxidative stress.

In addition, it is important to consider that mitochondria are not only ROS producers but also that they are a susceptible target of them. Thereby, in a pathologic situation where an increased ROS production occurs initially, oxidative damage to mitochondrial lipids, nucleic acids and proteins can reduce mitochondrial respiration, disturb normal function and seriously damage this organelle (Lenaz et al., 2002). Furthermore, mitochondrial DNA is more susceptible to oxidative damage than nuclear DNA, due to its close location next to an important ROS production site, to the lack of protective histones and to less effective repair mechanisms, as compared to the nuclear DNA (Richter et al., 1988). Mitochondrial redox status also influences the opening of the MPTP, since it is enhanced by oxidative stress in isolated mitochondria (Saxena et al., 1995).

4. Mitochondrial structural damage in ALS and experimental motor neuron degeneration

The death process involved in the motor neuron loss characteristic of ALS is not yet fully understood. Several functional alterations present in both human disease and experimental models have been reviewed in the previous sections, but several studies have shown also morphological and ultrastructural changes in motor neurons that may be associated with apoptosis and/or necrosis.

Postmortem examination of ALS patients tissues has revealed morphological and ultrastructural abnormalities in mitochondria. Atypical mitochondrial aggregates were found in skeletal muscle subsarcolemmal region and in intramuscular axons (Afifi et al., 1966; Atsumi, 1981), and morphological abnormalities were also detected in proximal axons, as well as dense clusters of mitochondria in the ventral horn of spinal cord SALS patients (Hirano et al., 1984a; b; Sasaki & Iwata, 1996). Giant mitochondria with intramitochondrial inclusions were observed in the liver of some ALS patients and these alterations were disease specific (Nakano et al., 1987). Further, mitochondria with increased volume and with high Ca^{2+} concentration were found in motor nerve terminals in muscle biopsies of alive ALS patients, which were not observed in patients with other neuropathies or in control subjects (Siklos et al., 1996). Ultrastructural damage of mitochondria, characterized by swelling and rounding, was recently described in platelets of ALS patients (Shrivastava & Vivekanandhan, 2011; Shrivastava et al., 2011a,b).

The main problem with pathological studies in human ALS is the difficulty in determining whether the alterations observed are a cause or a consequence of the disease. This highlights the importance of developing experimental models of motor neuron death to study the temporal progress of the morphological changes, including the alterations of mitochondrial structure. With this objective, we have recently studied the ultrastructural changes of mitochondria in both our acute and chronic models of spinal motor neuron death described above. In the acute model we observed motor neurons with mitochondrial swelling as soon as 2 h after AMPA perfusion, followed in a few hours by the rupture of mitochondrial, nuclear and plasma membranes, which led to total neuronal disruption. These ultrastructural alterations are characteristic of a necrotic process. In contrast, in the chronic

model we observed by day one swelling of the endoplasmic reticulum and only later progressive alterations in mitochondrial internal and external membranes that generated mitochondrial swelling. So, the initial mitochondrial integrity might indicate an apoptotic process, although motor neurons eventually probably die by a slow necrotic process (Fig. 2; Ramírez-Jarquín and Tapia, in preparation). The mitochondrial swelling observed in both models may be associated with energy failure, which as discussed above causes ATP depletion, oxidative stress and inflammatory events, leading to cell death.

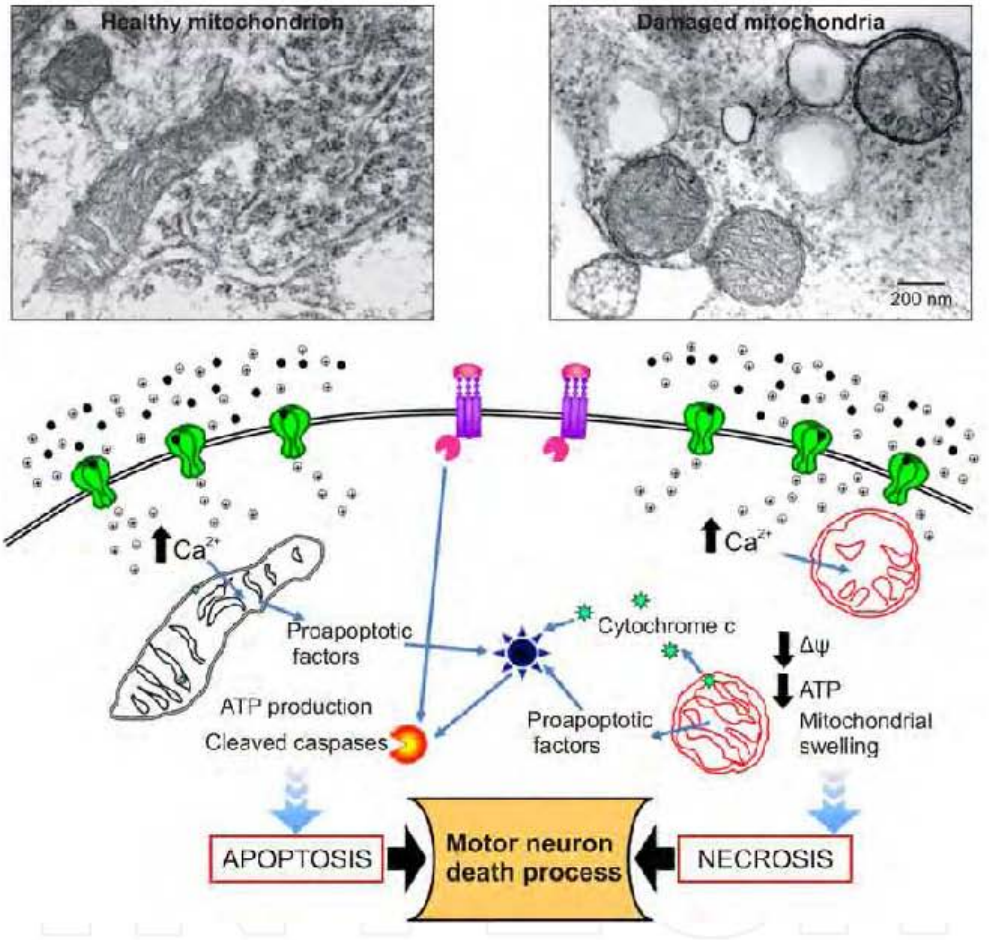

Fig. 2. Role of mitochondrial damage in motor neuron excitotoxicity. The electron-micrographs show normal mitochondria and endoplasmic reticulum in a spinal motor neuron of a control rat (left), and swollen mitochondria with altered cristae observed 2 h after perfusion of AMPA by microdialysis (right) (Ramírez-Jarquín and Tapia, unpublished). Bottom: proposal of the involvement of mitochondrial damage in the apoptosis and necrosis processes leading to motor neuron death. The symbols are the same as in Fig. 1. Description in the text.

The mitochondrial damage seen in our models is similar to those observed in the human disease and also in muscle and spinal cord of mSOD1 rodent models, namely mitochondrial fragmentation, enlargement, vacuolization, rearrangement of the cristae and swelling (Bendotti et al., 2001; Kong & Xu, 1998; Martin et al., 2009; Menzies et al., 2002b; Wong et al., 1995). The observed rearrangement of the inner membrane to form small vacuoles has been associated with an alteration in the MPTP permeability and also with the trigger of intrinsic-apoptosis pathway by release of proapoptotic factors, such as cytochrome c (Bendotti et al., 2001; Martin, 2010; Martin et al., 2009; Ohta et al., 2008) followed by the cleavage of caspases (Li et al., 2000; Pasinelli et al., 2000) Fig. 2 illustrates the ultrastructural mitochondrial damage and shows a schematic representation of the mechanisms associated with these alterations.

5. Conclusions

Altogether the foregoing data suggest that mitochondrial respiratory chain damage is a relevant event in ALS pathogenesis, although it is still unknown if mitochondrial abnormalities are the cause of the disease process or if they are consequence of neuronal degeneration, However, it is clear from the evidence reviewed here that mitochondria definitely play a central role in determining the fate of motor neurons and in their degeneration process. This evidence comes from studies in several tissues of ALS patients, both from biopsies or from postmortem observations, and from direct measurements of mitochondrial function in experimental models of motor neuron degeneration, both in vitro and in vivo. These experiments clearly point to energy deficits and disruption of Ca^{2+} homeostasis and axonal transport.

Integrity of the mitochondria morphology and structure is pivotal for their function and for cellular health. It is interesting that similar structural alterations have been observed in ALS tissues and in in vitro and in vivo models of motor neuron degeneration, including transgenic mSOD1 rodents and excitotoxicity. Clearly, this damage can be associated with the mitochondrial functional deficits, which trigger deleterious process resulting in cellular death by apoptosis, necrosis or a combination of these mechanisms. Although there is biochemical evidence of an apoptotic process involving the mitochondria, no ultrastructural evidence of classic apoptosis, such as apoptotic bodies, has been found. Rather, mitochondrial swelling and membrane disruption are frequently observed, suggesting the predominance of a necrotic process.

The evidence for a role of calcium homeostasis disruption in the induction of neuronal death is vast, and the involvement of mitochondria in this mechanism seems determinant. The advances in the elucidation of this process should help to understand the importance of the preservation of mitochondrial structure and function, which hopefully can lead to the design of preventive and therapeutic measures for ALS.

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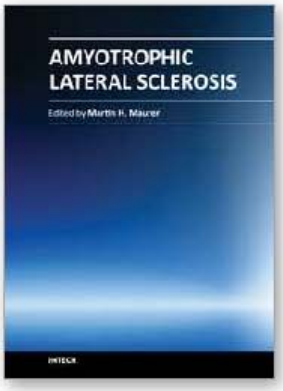

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Though considerable amount of research, both pre-clinical and clinical, has been conducted during recent years, Amyotrophic Lateral Sclerosis (ALS) remains one of the mysterious diseases of the 21st century. Great efforts have been made to develop pathophysiological models and to clarify the underlying pathology, and with novel instruments in genetics and transgenic techniques, the aim for finding a durable cure comes into scope. On the other hand, most pharmacological trials failed to show a benefit for ALS patients. In this book, the reader will find a compilation of state-of-the-art reviews about the etiology, epidemiology, and pathophysiology of ALS, the molecular basis of disease progression and clinical manifestations, the genetics familial ALS, as well as novel diagnostic criteria in the field of electrophysiology. An overview over all relevant pharmacological trials in ALS patients is also included, while the book concludes with a discussion on current advances and future trends in ALS research.

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III. PLANTEAMIENTO DEL PROBLEMA

Las evidencias que muestran alteraciones mitocondriales tanto morfológicas como funcionales, así como niveles elevados de marcadores de estrés oxidativo en los tejidos de los pacientes con ALS sugieren que estos mecanismos pueden contribuir de manera importante en la degeneración de las motoneuronas. Sin embargo, no se ha podido concluir si estas anormalidades reflejan la causa del proceso de la enfermedad o si son consecuencia del transcurso de la degeneración neuronal. La dificultad deriva de que los estudios en los tejidos humanos se han realizado después del comienzo de los síntomas o post-mortem. De ahí surge la necesidad de hacer más investigación para poder determinar el grado de implicación y estudiar a fondo los mecanismos que participan desde las etapas iniciales del proceso de degeneración de las motoneuronas en modelos *in vivo* que reproduzcan las principales características de la enfermedad. Para lograr lo anterior es ideal el modelo de degeneración de motoneuronas inducido por excitotoxicidad mediada por los receptores tipo AMPA, desarrollado en nuestro laboratorio.

Si bien no se tiene la certeza de que la excitotoxicidad sea la principal causante de la ALS, si es muy probable que esté participando. Por tanto es importante el estudio de agentes neuroprotectores dirigidos a los procesos involucrados en la muerte de las motoneuronas corriente abajo de los receptores a glutamato. El aumento en la concentración de Ca^{2+} puede elevar la producción de ROS y el estrés oxidativo es un mecanismo que se da durante la excitotoxicidad. Por otra parte, las mitocondrias juegan un papel fundamental en la homeostasis del Ca^{2+} intracelular y en la respuesta de las neuronas a la estimulación excitotóxica.

También es de gran importancia conocer la cronología y el grado de implicación de estos mecanismos en la degeneración de las motoneuronas. Esto es de gran utilidad para poder proponer agentes neuroprotectores dirigidos a evitar los mecanismos que contribuyen de manera sustancial y desde las etapas iniciales a la neurodegeneración.

IV. HIPÓTESIS

La deficiencia de la función mitocondrial, los trastornos del metabolismo energético, y el estrés oxidativo son mecanismos que participan de manera importante en la degeneración de motoneuronas espinales inducida por la administración de AMPA debido a que la función mitocondrial puede verse afectada y la producción de ROS incrementada ante un aumento en la concentración intracelular de Ca^{2+} tras su entrada masiva a través de los receptores tipo AMPA permeables a este catión.

Las estrategias terapéuticas dirigidas a preservar la función mitocondrial, así como a disminuir la producción de ROS y evitar el estrés oxidativo, pueden ejercer protección contra la neurodegeneración inducida por el AMPA, por lo que los sustratos energéticos y los antioxidantes pueden ser agentes neuroprotectores eficaces.

V. OBJETIVOS

Objetivo general

Conocer la importancia y cronología de la participación de la disfunción mitocondrial, las deficiencias del metabolismo energético, y del estrés oxidativo en el proceso de degeneración que conduce a la muerte selectiva de las motoneuronas espinales *in vivo* ocasionada por la sobreactivación farmacológica de los receptores AMPA.

Objetivos particulares

- ✓ Evaluar los efectos neuroprotectores contra la degeneración espinal de sustratos energéticos (lactato y creatina), sustratos energéticos con propiedades antioxidantes (α-cetobutirato y β-hidroxibutirato), y antioxidantes (ascorbato, glutatión (GSH) y etil éster de glutatión (GEE)), así como de la combinación de un sustrato energético con un antioxidante (piruvato + GEE). Todo esto para esclarecer el papel de las deficiencias del metabolismo energético y del estrés oxidativo en la muerte de las motoneuronas en el modelo *in vivo* inducido por la perfusión de AMPA por microdiálisis en la médula espinal de ratas y poder proponer agentes neuroprotectores.
- ✓ Realizar la correlación entre el número de motoneuronas espinales dañadas y las alteraciones en la función motora de las ratas en las diferentes condiciones estudiadas.

- ✓ Analizar durante las etapas iniciales del proceso de degeneración de motoneuronas (1.5–2 h después de la perfusión AMPA) distintos marcadores de estrés oxidativo para determinar el efecto del AMPA en la oxidación de proteínas y en la formación de ROS.
- ✓ Estudiar el grado de disfunción mitocondrial al inicio del proceso de degeneración de motoneuronas inducido por AMPA, evaluando las propiedades morfológicas y funcionales de mitocondrias aisladas de las astas ventrales (donde se localizan las motoneuronas) de la región lumbar de la médula espinal de las ratas. Esto mediante la observación y el análisis de las mitocondrias aisladas por microscopía electrónica, y examinando la capacidad de la cadena de transporte de electrones y de síntesis de ATP mediante la medición de la respiración mitocondrial, el cálculo del control respiratorio (RCR), la medición del potencial transmembranal mitocondrial ($\Delta\psi_m$), y de las actividades enzimáticas de los complejos de la cadena respiratoria.

- ✓ Explorar el papel del piruvato y los mecanismos de la neuroprotección proporcionada por él en la preservación de las motoneuronas espinales contra la muerte excitotóxica, dado que el piruvato es uno de los sustratos energéticos con propiedades antioxidantes que ejerció muy buena protección en nuestro modelo.

VI. RESULTADOS

Los resultados obtenidos en este trabajo fueron divididos en dos partes para su publicación. Los experimentos que se diseñaron para probar la hipótesis planteada y cumplir con los objetivos propuestos se presentan en la sección de materiales y métodos de cada uno de los artículos. De esta manera, tanto la metodología como los principales hallazgos se presentan en los siguientes artículos:

- ✓ Uno que ya ha sido publicado en ASN Neuro y lleva por título "*Role of energy metabolic deficits and oxidative stress in excitotoxic spinal motor neuron degeneration in vivo*". En éste se muestra que varios sustratos del metabolismo energético redujeron significativamente la muerte de las motoneuronas, preservaron la función motora y evitaron por completo la parálisis provocada por el AMPA al ser co-perfundidos con él, mientras que los antioxidantes protegieron en menor medida. También se muestra en este trabajo que, durante las primeras etapas del proceso degenerativo de las motoneuronas, el AMPA no modificó los marcadores de estrés oxidativo estudiados. Otra observación interesante es la correlación que encontramos entre las alteraciones de la función motora y el número de motoneuronas dañadas, que sugiere que existe un umbral de aproximadamente el 50% en el número de motoneuronas sanas necesario para preservar la función motora.
- ✓ Otro que se enviará próximamente para su publicación titulado "*Mitochondrial dysfunction during the early stages of excitotoxic spinal motor neuron degeneration in vivo*" en el cual estudiamos las propiedades morfológicas y funcionales de las mitocondrias aisladas de las astas ventrales de la región lumbar de las médulas espinales de las ratas, durante las etapas iniciales de la degeneración de las motoneuronas. Aquí mostramos que las mitocondrias de las ratas tratadas con AMPA estaban dañadas y presentaron anormalidades morfológicas como son hinchamiento, crestas desorganizadas y membranas rotas, así como disminuciones significativas en el consumo de oxígeno, el control respiratorio, el potencial transmembranal, y las actividades de los complejos I y IV de la cadena de transporte de electrones. También mostramos que la co-perfusión de piruvato evitó tanto las anormalidades morfológicas como las deficiencias funcionales mitocondriales.

ASN NEURO RESEARCH ARTICLE

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Role of energy metabolic deficits and oxidative stress in excitotoxic spinal motor neuron degeneration *in vivo*

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ABSTRACT

MN (motor neuron) death in amyotrophic lateral sclerosis may be mediated by glutamatergic excitotoxicity. Previously, our group showed that the microdialysis perfusion of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) in the rat lumbar spinal cord induced MN death and permanent paralysis within 12 h after the experiment. Here, we studied the involvement of energy metabolic deficiencies and of oxidative stress in this MN degeneration, by testing the neuroprotective effect of various energy metabolic substrates and antioxidants. Pyruvate, lactate, β-hydroxybutyrate, α-ketobutyrate and creatine reduced MN loss by 50–65%, preserved motor function and completely prevented the paralysis. Ascorbate, glutathione and glutathione ethyl ester weakly protected against motor deficits and reduced MN death by only 30–40%. Reactive oxygen species formation and 3-nitrotyrosine immunoreactivity were studied 1.5–2 h after AMPA perfusion, during the initial MN degenerating process, and no changes were observed. We conclude that mitochondrial energy deficiency plays a crucial role in this excitotoxic spinal MN degeneration, whereas oxidative stress seems a less relevant mechanism. Interestingly, we observed a clear correlation between the alterations of motor function and the number of damaged MNs, suggesting that there is a threshold of about 50% in the number of healthy MNs necessary to preserve motor function.

Key words: amyotrophic lateral sclerosis, antioxidant, mitochondrial energy substrate, motor neuron degeneration, oxidative stress, spinal cord.

INTRODUCTION

The neurodegenerative disease ALS (amyotrophic lateral sclerosis) is characterized by the selective and progressive degeneration of lower and upper MNs (motor neurons), leading to a progressive paralysis and finally death due to respiratory failure, usually 2–5 years after the symptoms onset. The cause of ALS is unknown and the several hypotheses that have been postulated to explain the selective MN death include glutamate-mediated excitotoxicity, mitochondrial dysfunction and oxidative stress (Tovar-y-Romo et al., 2009; Robberecht and Philips, 2013).

Excitotoxicity induced by overactivation of glutamate receptors may be involved in mechanisms of neurodegeneration in ALS, mainly because spinal MNs are particularly vulnerable to overactivation of the Ca^{2+}-permeable AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionate)-type receptors, both in cultures (Van Den Bosch et al., 2000; Van Damme et al., 2002) and *in vivo* (Corona and Tapia, 2007; Corona et al., 2007). An increase in intracellular Ca^{2+} entering through these receptors results in neuronal death due to activation of lytic enzymes, mitochondrial damage linked to energy metabolism disruption and generation of toxic ROS (reactive oxygen species) (Shaw, 1999; Arundine and Tymianski, 2003; Grosskreutz et al., 2010). Particularly, mitochondrial bioenergetic status is determinant for neuronal survival or death, and alterations in mitochondrial function occur after experimental excitotoxicity and in ALS (Nicholls and Budd, 2000; Duffy et al., 2011; Cozzolino and Carrì, 2012; Santa-Cruz et al., 2012).

Previously, our group developed an *in vivo* model of MN death by means of microdialysis perfusion of AMPA in the rat

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Abbreviations: ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; ChAT, choline acetyltransferase; GEE, GSH ethyl ester; MN, motor neuron; 3-NT, 3-nitrotyrosine; ROS, reactive oxygen species; SOD1, superoxide dismutase 1.

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lumbar spinal cord, which produced a remarkable progressive loss of spinal MNs, leading to permanent paralysis of the ipsilateral hindlimb (Corona and Tapia, 2004, 2008). In this model we found that pyruvate significantly protected against MN loss and paralysis (Corona and Tapia, 2007), probably acting as a supplemental energy substrate restoring mitochondrial function. However, abundant evidence supports the involvement of oxidative stress in ALS pathogenesis as well (Barber and Shaw, 2010; Santa-Cruz et al., 2012), so that the neuroprotective effect of pyruvate may be due also to its antioxidant properties (Desagher et al., 1997; Kim et al., 2005; Wang et al., 2007). Therefore the aim of the present work was to clarify the role of energy metabolic deficiencies and oxidative stress in the AMPA-induced spinal MN death *in vivo*. For this purpose, we assessed the neuroprotective effects of the energy substrates lactate and creatine, of α-ketobutyrate and β-hydroxybutyrate, which can supply energy and also have antioxidant properties, and of ascorbate, glutathione and glutathione ethyl ester, which are solely antioxidants. In addition, we determined the effect of AMPA on protein oxidation and ROS formation.

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 280–310 g were used in all the experiments and handled in accordance with the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care Committee. All efforts were made to minimize suffering of the animals. Animals were housed with a 12 h light/dark cycle and with food and water *ad libitum*.

Microdialysis procedure and drug administration

Microdialysis in the lumbar spinal cord was carried out essentially as previously described (Corona and Tapia, 2004, 2007), including the collection of perfused media fractions for the measurement of amino acids. Briefly, rats were anesthetized with 5% halothane or isoflurane in a 95% O_2/5% CO_2 mixture, placed in a stereotaxic spinal unit (Kopf), and maintained under low anesthesia (~0.8–1% halothane or isoflurane) during surgery. A ~1–2 mm diameter hole was drilled at the second–third lumbar laminae and a microdialysis probe (1 mm long and 0.24 mm diameter, CMA/7, Solna) was slowly lowered down into the right dorsal horn of the spinal cord. A Krebs–Ringer 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 continuously perfused at a flux rate of 2 μl/min, using a microsyringe mounted on a microinjection pump (CMA/100, Solna), during 160 min, as follows: 60 min equilibration period, followed by 37.5 min during which three 12.5 min fractions were collected for the measurement of the basal concentration of amino acids; then AMPA-containing medium (6 mM, Tocris) was perfused during 25 min, with collection of two 12.5 min fractions, and finally medium was perfused during 37.5 min, with collection of three 12.5 min recovery fractions. The energy substrates and antioxidants tested were always present during the 160 min of the microdialysis procedure, except in the controls perfused only with Krebs medium and in the rats perfused only with AMPA. Sodium pyruvate (20 mM), L-(+)-lactic acid (20, 50 and 100 mM), creatine monohydrate (20 mM), DL-β-hydroxybutyric acid sodium salt (20 and 50 mM), α-ketobutyric acid (20 mM), L-ascorbic acid (10, 20 and 50 mM), reduced L-glutathione (20 and 50 mM), reduced glutathione ethyl ester (20 and 50 mM), or the latter combined with sodium pyruvate (20 mM each) were dissolved in the Krebs–Ringer perfusion medium, adjusting the pH to 7.4 if necessary. All compounds were obtained from Sigma–Aldrich. Osmolarity was maintained by reducing the NaCl concentration proportionally. Initial concentrations of the compounds tested were chosen on the basis of previous results from this laboratory for pyruvate. At the end of the microdialysis procedure, the skin was sutured and anesthesia was discontinued. Rats were kept in individual cages with water and food *ad libitum*, and observed periodically during the next 24 h. At this time they were fixed for the histological analysis, as described below.

Glutamate and aspartate concentrations in the 25 μl dialysate fractions collected along the microdialysis procedure were measured by HPLC as previously described (Massieu et al., 1995; Corona and Tapia, 2004). No significant changes in the levels of these amino acids were observed under any of the experimental conditions used, and therefore no data are shown on amino acid quantification.

Assessment of motor function

Besides the observation of walking and general motor behavior, motor performance was evaluated 6 and 24 h after the experiment by the rotarod test (Columbus Instruments), as previously described (Corona and Tapia, 2007).

Histology and ChAT (choline acetyltransferase) immunohistochemistry

At 24 h after surgery, subsequent to the assessment in the rotarod test, the animals were fixed for histological and ChAT immunohistochemical analyses, exactly as previously described (Corona and Tapia, 2007). Briefly, transverse sections (50 μm thick) of the spinal cord segment where the cannula was introduced were obtained in a cryostat, and alternate sections were stained with Cresyl Violet or immunostained for ChAT, so that histological and immunohistochemical changes can be correlated. The morphologically undamaged MNs (i.e. large neurons, with a soma diameter

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>20 μm and a distinguishable nucleus, similar in appearance to those of the contralateral ventral horn and to those in control rats) were counted in a 10× microscopic field. Five to seven sections, 50 μm apart, where the trace of the cannula was evident, were counted in the ipsilateral and contralateral ventral horn of each rat, and the values were averaged. For ChAT immunohistochemistry, goat polyclonal anti-ChAT antibody (1:200; Chemicon), biotinylated-conjugated horse anti-goat IgG (1:200; Vector) and avidin-Texas Red conjugate (1:200, pH 8.2; Vector) were used. Sections were mounted on silane (Sigma)-covered slides and coverslipped with fluorescent mounting medium (DAKO); they were examined in a Nikon microscope equipped with an epifluorescence attachment. Parallel sections processed in the absence of the primary antibody showed no immunostaining.

3-NT (3-nitrotyrosine) and ChAT double immunohistochemistry

3-NT was assessed in order to study the effect on protein oxidation of AMPA, administered by microdialysis at a 6 mM concentration, as described above. Because MN loss started ∼3–6 h after AMPA perfusion (Corona and Tapia, 2008), in these experiments rats were transcardially fixed 2 h after as described above, so that oxidation could be measured at the beginning of the degeneration process rather than when MNs were already damaged. After fixation, spinal cords were post-fixed, transferred to sucrose and transverse sections 50 μm thick were obtained in a cryostat. For a positive control, nitration of tyrosine was carried out directly in spinal cord sections of intact rats by means of peroxynitrite generation *in situ*, accomplished by mixing 0.1 M sodium nitrite with 0.1 M hydrogen peroxide in acetate buffer, pH 5, for ∼20 min at room temperature (Viera et al., 1999).

Free-floating sections were blocked with 5% BSA in PBS-Triton X-100 (0.3%) for 2 h, and then incubated with goat polyclonal anti-ChAT (1:200) and mouse monoclonal anti-nitrotyrosine antibodies (1:50; Sigma–Aldrich), for 7 days at 4°C. Sections were washed three times for 10 min in PBS-Triton X-100 and incubated with biotinylated-conjugated horse anti-goat IgG (1:200) for 1–2 h. After three washes, sections were incubated for 2 h with avidin–Texas Red conjugate (1:200, pH 8.2), washed three times, and incubated with FITC-goat anti-mouse IgG (1:200; Zymed) for 2 h. Finally, sections were washed and mounted on silane-covered slides and coverslipped with fluorescent mounting medium. Sections were visualized under confocal microscopy (Olympus IX81). Merged images are the overlay of two laser sections in the Z plane, using the Olympus Fluoview laser scanning FV1000 Ver. 3.0 Viewer.

Measurement of ROS in spinal cord homogenates

The effect of 6 mM AMPA perfusion on ROS production was also studied at ∼2 h after the experiment, for the reasons mentioned above. In these experiments the microdialysis procedure was carried out as described above but bilaterally, using one microdialysis probe on each dorsal horn. The two ventral horns of the perfused lumbar segments were dissected out on ice and homogenized together in 1200 μl of buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM MOPS and 1 mM EGTA (pH 7.4). ROS production was measured by the oxidation of 2′,7′-dichlorodihydrofluorescein (Halliwell and Whiteman, 2004). Aliquots (10 μl) of the homogenates were mixed in triplicate with 190 μl of 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (Sigma) in the same medium with the addition of 2 mM phosphate, 20 mM KCl and 1 mM $MgCl_2$, and incubated at 37°C. Fluorescence signals (488 nm excitation and 525 nm emission wavelength respectively) were recorded every minute for 60 min in a Synergy HT Multi-Mode Microplate Reader (BioTek). The biuret method was used for the determination of protein concentration in the homogenates.

Statistical analysis

Comparisons regarding rotarod scores, number of MNs and ROS production were carried out using ANOVA followed by a Tukey's *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Figures 1 and 2 show representative micrographs of ventral horns of rats showing the clearest effects of the compounds tested on MN preservation, and Figures 3 and 4 show quantitative data of the results of the rotarod test and of MN counts respectively, grouped according to the effectiveness of the protective action, as explained in the Discussion.

Energy substrates prevent AMPA-induced paralysis and neurodegeneration

Control rats perfused only with the substrates or the antioxidants dissolved in the Krebs–Ringer medium did not show any behavioral alteration at any time. No histological alterations, other than the mechanical damage caused by the probe in the dorsal spinal horn, were observed in these control animals, 24 h after the experiment ($n \geqslant 3$).

We confirmed that 6 mM AMPA produces a dramatic MN loss (∼90%) in the ipsilateral ventral horn and consequently a total paralysis of the ipsilateral hindlimb 24 h after the experiment (Corona and Tapia, 2004, 2008). We also confirmed (Corona and Tapia, 2007), that 20 mM pyruvate exerts a remarkable neuroprotection, preventing paralysis and diminishing MN loss by >50% (Figures 1, 3 and 4). In contrast, when we tested 20 mM lactate no protective effect at all was observed (results not shown). However, 50 mM lactate

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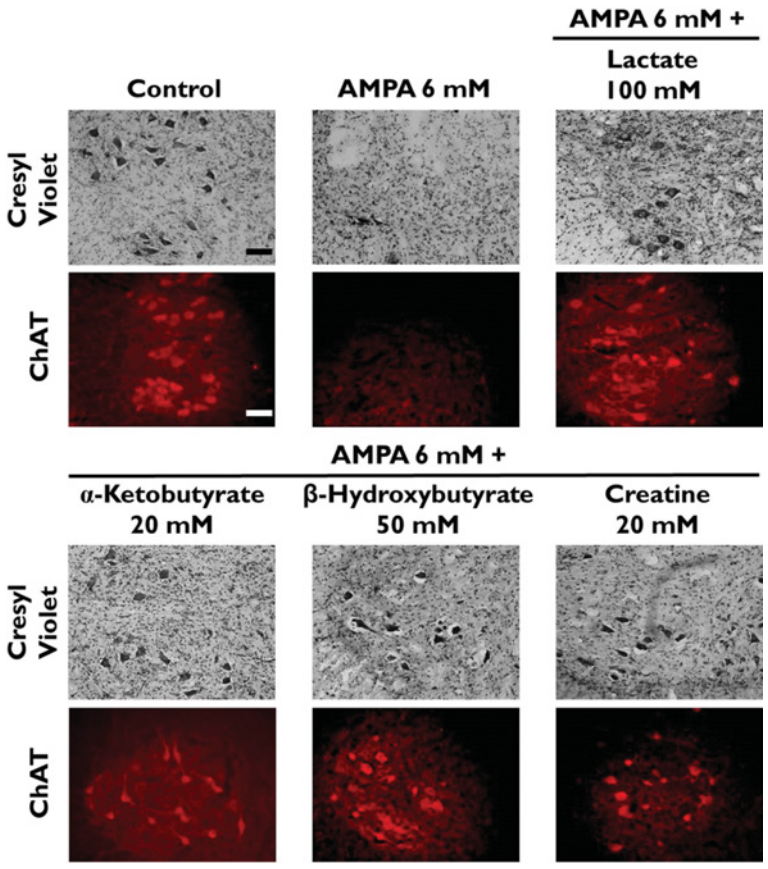

Figure 1 **Energy substrates protect MNs from AMPA-induced degeneration**
Cresyl Violet and ChAT immunocytochemistry representative micrographs of the ipsilateral ventral horn of rats treated as indicated, 24 h after the experiment. Note the severe MN loss caused by AMPA and the protective action of the energy substrates. No neuronal damage was observed in the contralateral ventral horn in any case. Scale bars = 100 μm. See Figure 4 for quantitative data.

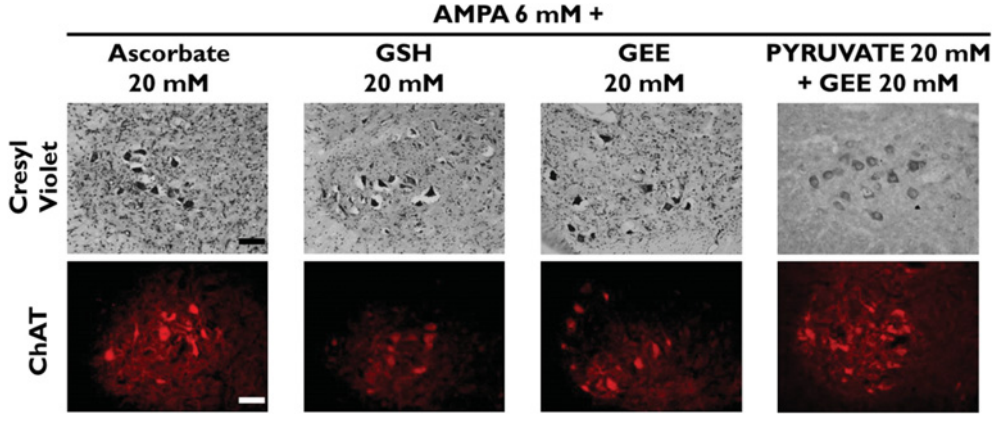

Figure 2 **Antioxidants partially protect MNs from AMPA-induced degeneration**
Cresyl Violet and ChAT immunocytochemistry representative micrographs of the ipsilateral ventral horn of rats treated as indicated, 24 h after the experiment. Note that co-perfusion of an antioxidant and an energy substrate does not have a synergic effect. Compare with control and with AMPA alone in Figure 1. Scale bars = 100 μm. See Figure 4 for quantitative data.

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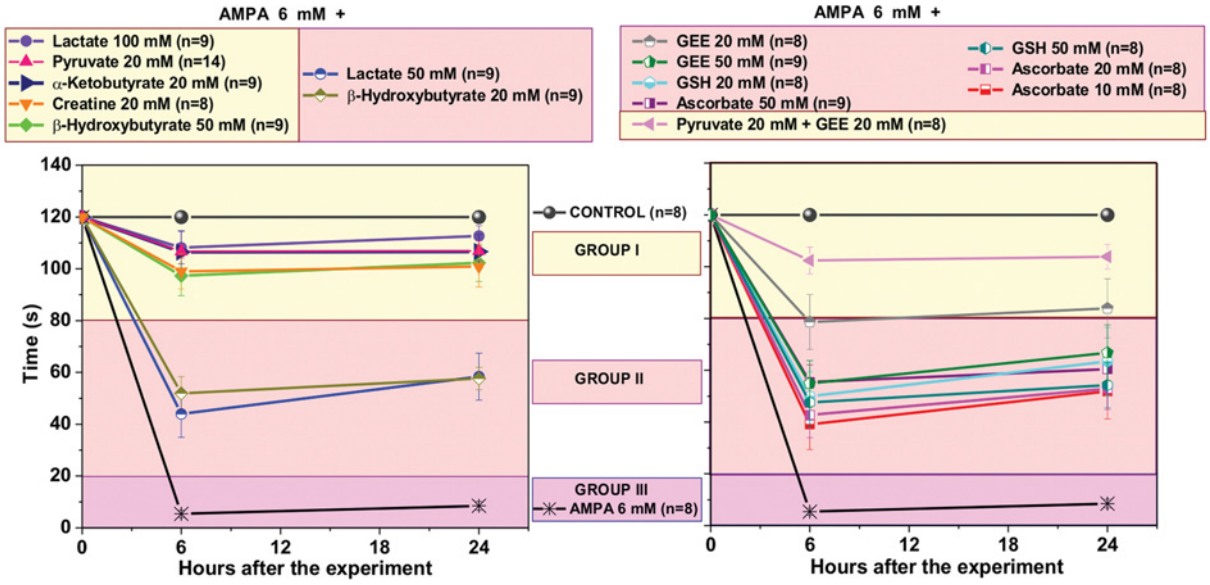

Figure 3 **Rotarod performance of rats treated with AMPA and AMPA + energy substrates or antioxidants**
Time remaining in the rotarod, with a cut-off of 120 s, 6 and 24 h after the experiment. Control rats (perfused with Krebs–Ringer medium) did not fall down before the cut-off time. Results are shown divided into three groups according to the animals performance: rats treated with AMPA alone (group III, purple background) presented a complete and permanent paralysis of the ipsilateral hind limb and fell down in less than 20 s, 6 h after the experiment. Group I (yellow background in graph and list) includes rats treated with AMPA + the compounds that at the concentrations indicated exerted remarkable protection, since none of these rats showed any sign of paralysis or other motor deficit and remained in the rotarod for at least 90 s. Group II (pink background in graph and list) includes rats treated with AMPA + the compounds that at the concentrations indicated exerted partial protection, because these animals showed partial paralysis and fell from the rotarod between 40 s and 70 s. Note that only energy substrates are in group I whereas all antioxidants are in group II. Data are means ± S.E.M. for the number of rats indicated in parentheses. All values of groups I and II differed significantly from group III ($P < 0.001$). Differences between control and group I values were not significant ($P > 0.05$), except in the case of creatine, β-hydroxybutyrate (50 mM), and pyruvate + GEE ($P < 0.05$); differences were significant between groups I and II ($P < 0.05$, except in the case of 20 mM GEE, in which four rats behaved as those of group I and four rats as those of group II).

partially prevented the motor impairment, assessed by the rotarod test (Figure 3), and MN loss diminished to ∼52 % (Figure 4). This protection was notably enhanced when lactate concentration was increased to 100 mM, since no paralysis was observed, the rotarod performance was only slightly affected (Figure 3) and MN loss was only ∼40 % (Figures 1 and 4). Notably, the protective effect of 100 mM lactate was equivalent to that of 20 mM pyruvate.

Creatine and α-ketobutyrate, both at a 20 mM concentration, also exerted a remarkable protection against AMPA-induced excitotoxicity, which was very similar to that of 100 mM lactate and of 20 mM pyruvate, in terms of motor behavior, rotarod performance and MN death (Figures 1, 3 and 4). The ketone body β-hydroxybutyrate was tested also at 20 and 50 mM concentrations, and its protective effect was similar to that of 50 and 100 mM lactate respectively, since the animals showed comparable rotarod performance and MN loss (Figures 1, 3 and 4).

Antioxidants only partially prevent AMPA-induced motor dysfunction and MN loss

Ascorbate was tested at 10, 20 and 50 mM concentrations. Although a significant protection against AMPA was observed, in both rotarod test and MN loss the protection was similar with all concentrations and was also similar to that exerted by 50 mM lactate (Figures 2, 3 and 4).

GSH, one of the major antioxidant defenses of the cell, was tested at 20 and 50 mM concentrations and, as shown in Figures 2, 3 and 4, the protection observed was strikingly similar to that produced by 20 and 50 mM ascorbate, in terms of both rotarod performance and MN loss. Since it has been reported that intracellular GSH levels are elevated more efficiently after the administration of GEE (GSH ethyl ester) than after GSH administration (Zeevalk et al., 2007), we also tested GEE as a protector against AMPA. Although the results with 20 mM GEE were more variable than with GSH (some rats behaved better than others in the rotarod test and showed >50 % MN protection), the effect exerted by both 20 mM and 50 mM concentrations did not differ significantly from that by GSH (Figures 2, 3 and 4).

Since in none of the above experimental conditions the protection of the healthy MNs exceded 65–70 %, we tested whether the combined perfusion of an energy substrate and an antioxidant resulted in a better protection. We chose 20 mM pyruvate + 20 mM GEE and the results obtained, both in the rotarod performance and MN number, were very

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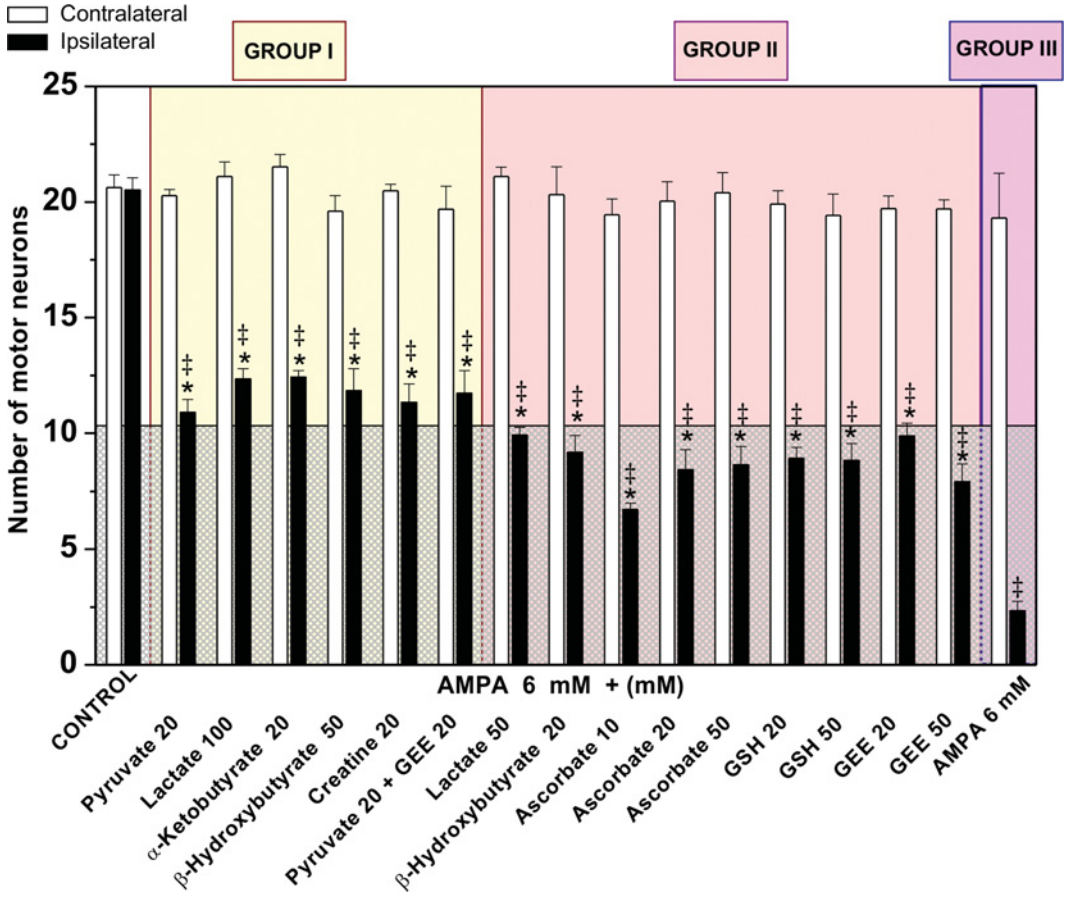

Figure 4 **Number of healthy MNs in control rats and rats treated with AMPA and AMPA + substrates and antioxidants**

Neurons of the ipsilateral and contralateral ventral horns were counted 24 h after the experiment and are classified in the three different groups described in Figure 3, using the same background colors. The horizontal line that divides the graph indicates 50% of the number of healthy MNs as compared with control rats. Note the remarkable (89%) loss of MNs in the ipsilateral horn in the AMPA group III, and that all compounds of group I protected by more than 50% whereas the protection by all compounds of group II was below 50% (shaded area). Data are means ± S.E.M. for the number of rats indicated in parentheses in Figure 3 for each group. *$P < 0.001$ vs group III. ‡$P < 0.001$ vs the corresponding contralateral ventral horn. All group II values, except lactate, β-hydroxybutyrate and 20 mM GEE, differed significantly from group I values ($P < 0.05$). Figure 6 shows a correlation between the data of this Figure and those of Figure 3.

similar to those with pyruvate alone, indicating that the effects were not additive (Figures 2, 3 and 4).

3-NT and ROS production were not affected by AMPA during the initial stages of the MN degeneration process

To further study the involvement of oxidative stress in the MN degenerating process, the oxidative stress markers 3-NT and ROS were measured. These determinations were carried out at 1.5–2 h after the microdialysis procedure, because we have observed previously that the MN degenerating process starts rapidly after AMPA perfusion and is observed as early as 3 h after AMPA microdialysis (Corona and Tapia, 2008).

As shown in Figure 5(A), although positive controls verified the detection of 3-NT in the spinal cord sections, in both control and AMPA-treated rats we observed only a very tenuous staining for 3-NT, which could not be related to the ChAT-labeled MNs. As shown in Figure 5(B), there was no difference in the ROS production rate in ventral horn homogenates between control and AMPA-perfused rats.

DISCUSSION

The study of the neuroprotective effects of various compounds with different properties can be useful to find out and understand which mechanisms are involved in selective degeneration of spinal MNs. We have used this approach to study the participation of mitochondrial energy deficiency and oxidative stress in the rapid neuronal death produced by AMPA receptor overactivation.

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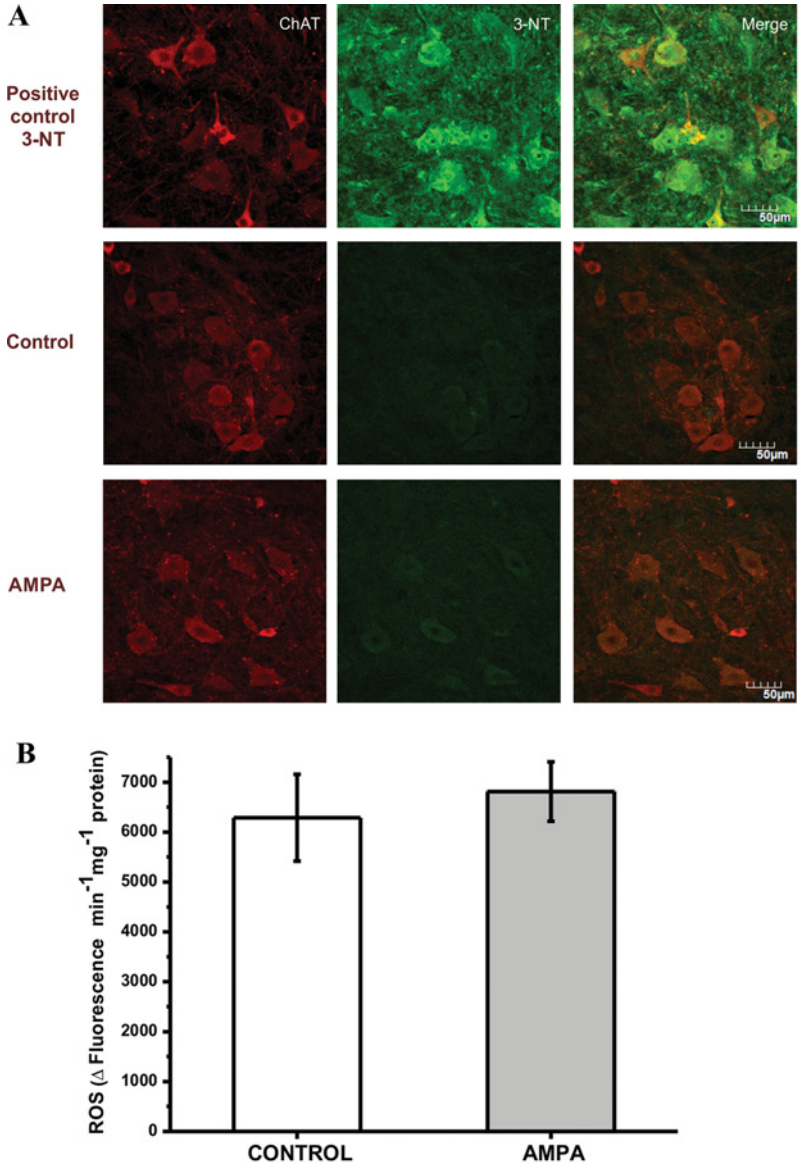

Figure 5 **Double immunohistochemistry for 3-NT and ChAT (A), and ROS production rate (B), during the first stages of the MN degeneration process**

(**A**) Representative micrographs ~2 h after the microdialysis procedure ($n = 8$). A positive control for 3-NT detection, as described in the Materials and Methods, is shown at the top line. Only a faint 3-NT labeling was detected in both control and AMPA-treated rats. (**B**) ROS production in the ventral horns is not affected by AMPA, ~2 h after the microdialysis procedure. Means ± S.E.M. ($n = 8$).

Of all compounds tested as protectors, pyruvate was one of the most effective since, as previously described (Corona and Tapia, 2007), at a 20 mM concentration it reduced MN death and completely prevented paralysis and motor impairment. In neurons, pyruvate is the most important mitochondrial substrate derived from glycolysis and, as discussed below, can also act as an antioxidant. When its protection was compared with that of lactate, an energy substrate that is not an antioxidant *per se*, we found a dose-dependent neuroprotection, but a 100 mM concentration was required to protect as compared with 20 mM pyruvate, suggesting that lactate is oxidized to pyruvate inside neurons by lactate dehydrogenase. Both lactate and pyruvate cross the blood–brain barrier, are transported to the glial cells and neurons by the monocarboxylate transporters, and can be used as energy substrates through the TCA (tricarboxylic acid) cycle (Nicholls and Budd, 2000; Smith et al., 2003; Aubert et al., 2005; Bouzier-Sore et al., 2006; O'Brien et al., 2007). In fact, astrocytes can protect neurons *in vitro* through the release of lactate, so that neighboring neurons can use it as an energy substrate, and this facilitates neuron recovery after a bioenergetic insult (Izumi et al., 1997; Schurr et al., 1997).

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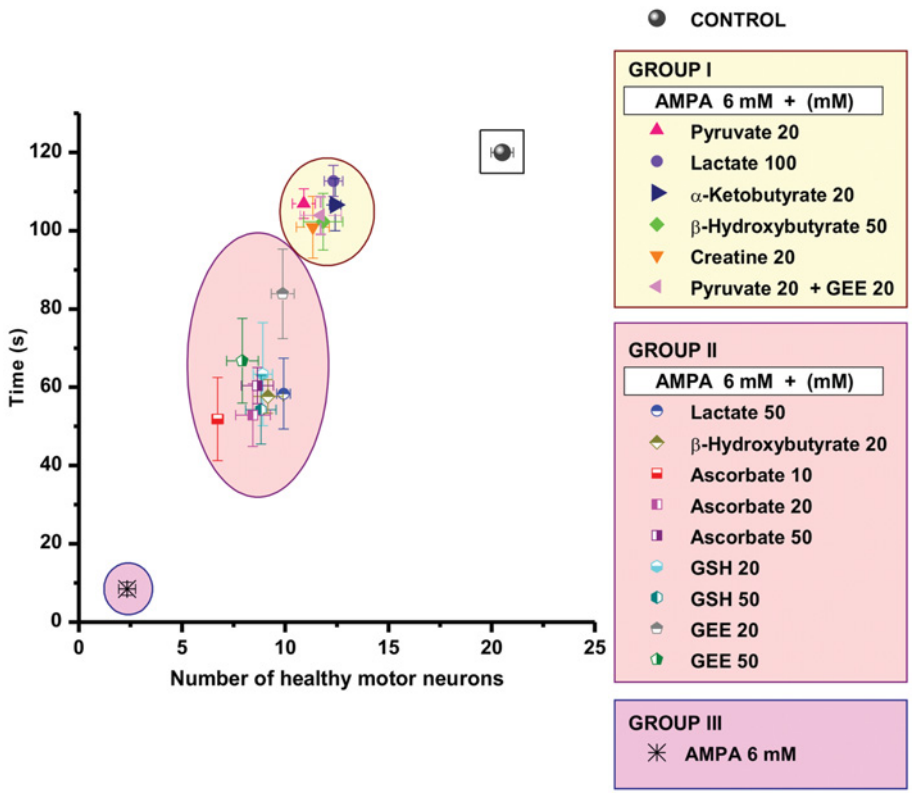

Figure 6 **Correlation between the number of healthy MNs and the rotarod performance of the animals**
This graph shows the number of healthy MNs in the ipsilateral ventral horns of the spinal cords of rats in each experimental condition tested plotted vs the time animals remained in the rotarod 24 h after the experiment. Plotted data, grouping of data and colors in the graph and the list are as described in Figures 3 and 4.

That lactate is used as a significant source of fuel for brain metabolism, predominantly in neurons, has been shown also *in vivo* under different energy requirement conditions, including lactate released from astrocytes or transported from blood (reviewed by Pellerin et al., 2007; Figley, 2011). Furthermore, it has been recently shown that the disruption of monocarboxylate transporter 1 present in oligodendroglia produces neuronal and axonal damage and that its expression is reduced in affected regions of ALS patients and mutant SOD1 (superoxide dismutase 1) mice (Lee et al., 2012).

However, neuroprotective effects of pyruvate have also been associated with its ability to exert antioxidant effects. Pyruvate and other α-ketoacids, including α-ketobutyrate, protected cultured striatal neurons against toxicity induced by H_2O_2, whereas lactate did not protect; this was attributed to the ability of α-ketoacids to react with H_2O_2 in a decarboxylating reaction (Desagher et al., 1997). Pyruvate protects also human neuroblastoma cells by means of its antioxidant actions in the mitochondria, since it suppressed mitochondrial superoxide production, mitigated mitochondrial transmembrane potential collapse induced by oxidative stress, and attenuated H_2O_2-induced ROS formation and cell death (Wang et al., 2007). When we tested α-ketobutyrate, another α-ketoacid that can react with H_2O_2 but can also be oxidized by α-ketobutyrate dehydrogenase, we observed a protective effect similar to that of pyruvate at equivalent (20 mM) concentrations, suggesting that they act by similar mechanisms.

It is well known that ketone bodies can be used as energy substrates in the CNS under some circumstances and they can exert protective effects in various pathological conditions (Maalouf et al., 2009). We tested the neuroprotective effect of β-hydroxybutyrate, a ketone body that can be oxidized to acetoacetate, which gives rise to two molecules of acetyl-CoA and therefore can promote mitochondrial energy production. β-Hydroxybutyrate exerted a dose-dependent protection, lessening motor impairment and MN loss. In agreement with these results, SOD1-G93A transgenic mice fed with a ketogenic diet preserved motor function longer, body weight loss was decreased and spinal MN loss was diminished; in addition, in these animals mitochondrial function assessed as ATP synthesis was increased, and *in vitro* β-hydroxybutyrate stimulated ATP synthesis and prevented MN loss induced by rotenone (Zhao et al., 2006). Further, treatment of SOD1-G93A mice with caprylic triglyderide (a medium-chain triglyceride that is metabolized into ketone

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bodies) attenuated progression of symptoms and promoted mitochondrial oxygen consumption rate measured *ex vivo* (Zhao et al., 2012).

We observed a good neuroprotective effect of 20 mM creatine, which helps to provide energy by stimulating ATP formation from the phosphocreatine reserve when an unexpected energy demand depletes ATP, which is what most likely occurs during AMPA-induced excitotoxicity. This neuroprotection is in agreement with results obtained in SOD1-G93A mice, showing that oral administration of creatine improved motor performance, extended survival and prevented MN loss (Klivenyi et al., 1999). However, at least two human clinical trials testing creatine in ALS patients showed no benefical effects (Groeneveld et al., 2003; Shefner et al., 2004). Ascorbate is a cofactor in many enzymatic reactions and a potent antioxidant. In the CNS its concentration is much higher than in plasma, suggesting that this compound plays a crucial role in ROS removal (Rice, 2000). Neuroprotective effects of ascorbate against damage induced by excitatory amino acids and ischemia have been shown *in vitro* and *in vivo* (Majewska and Bell, 1990; MacGregor et al., 1996; Stamford et al., 1999; Kim et al., 2008), and SOD1-G93A mice fed with a diet supplemented with ascorbate survived longer, but symptom onset was not delayed (Nagano et al., 2003). In our experiments we observed only partial protection with all of the concentrations tested, not better than 50 mM lactate.

When we tested GSH, the major antioxidant defense in the cell, 20 and 50 mM concentrations exerted only partial protection, similar to ascorbate. There is little information characterizing the cellular uptake of GSH, but since one study showed that administration of GEE can increase intracellular GSH concentration and provides neuroprotection against oxidative stress or chronic mitochondrial damage (Zeevalk et al., 2007), we tested this ester. The results were somewhat contradictory, since 50 mM GEE exerted less protection than 20 mM and in the latter group half of the rats were highly protected and reduced MN loss by more than 50%, whereas the rest of the rats were only partially protected. GSH plays an important role in detoxification of ROS in brain, reacting directly with superoxide, nitric oxide and its derivatives or the hydroxyl radical in non-enzymatic reactions, or indirectly by providing reducing capacity for several GSH-dependent enzymes (Dringen, 2000). Furthermore, GSH can be translocated to mitochondria where it contributes to defense against ROS-mediated damage, and against reactive nitrogen species (Heales and Bolaños, 2002). So, the results obtained with GSH and GEE are relevant and could be due to other neuroprotective antioxidants that are reduced by GSH, like vitamin E (Forman et al., 2009).

We then tested the hypothesis that neuroprotective properties of pyruvate as an energy substrate and GEE as an antioxidant would be additive, but the results show that there is no synergy effect, since the number of healthy MNs was not significantly higher than with pyruvate alone. This suggests that the main protection is due to an energy mechanism, whereas oxidative stress is perhaps a secondary event resulting from mitochondrial dysfunction.The findings that AMPA did not increase 3-NT staining and ROS production rate, at a time when, according to our previous findings (Corona and Tapia, 2008), MNs are in the initial stages of the degeneration progress, agree with this conclusion, although the participation of •NO-dependent oxidative stress in later stages of the degeneration cannot be excluded. So, the augmented reactive oxygen and nitrogen species could directly damage mitochondria as well, aggravating mitochondrial dysfunction and creating a vicious circle that ends up in MN death.

There is a threshold in the number of MNs necessary to prevent motor impairment and paralysis

We analyzed the relationship between the motor performance of the rats in the rotarod and the number of healthy MNs under all experimental conditions used and those of previous works in this acute model of excitotoxic MN degeneration (Corona and Tapia, 2004, 2007, 2008). As shown in Figures 3, 4 and 6, this correlation clearly shows, first, that a small difference in the number of MNs results in a remarkable difference in motor function, and second, that there is a threshold in the percentage of healthy MNs required to completely prevent motor function impairment. In fact, in all cases as long as more than 50% of MNs were preserved, paralysis and motor dysfunction were completely prevented (group I, yellow background in Figures 3, 4 and 6), whereas when MN preservation is slightly below this threshold value motor deficiencies leading to rotarod falling within 60–80 s occur (group II, pink background). A severe MN loss produces total and permanent paralysis of the ipsilateral hind limb and falling from the rotarod in a few seconds (group III, purple background). Figure 6 summarizes the data supporting this threshold hypothesis and the correlation between the number of healthy MNs and motor performance. It is noteworthy that besides these results, the findings with other compounds that have shown protective action in this acute model of excitotoxic MN degeneration also suggest that a ~50% MN loss correlates with motor deficits and none of them have protected 100%. The other compounds tested are the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (Corona and Tapia, 2004); the selective blocker of the Ca^{2+}-permeable AMPA receptors 1-naphthyl acetyl spermine and the intracellular Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (Corona and Tapia, 2007); and the calpain inhibitor leupeptin (Corona and Tapia, 2008).

It is noteworthy that all rats treated with antioxidants at any concentration tested belong to the partially protected group II, whereas all the well-protected rats of group I were treated with energy substrates. This supports our previous conclusion that oxidative stress is contributing, but is not

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the main triggering event, of AMPA-induced MN degeneration, whereas mitochondrial energy metabolism deficit seems crucial in the process of neurodegeneration.

As mentioned in the Introduction, the mechanism by which AMPA-induced excitotoxicity may generate mitochondrial energy dysfunction is probably mediated by an increase in intracellular Ca^{2+} due to the overactivation of Ca^{2+}-permeable AMPA receptors (Corona and Tapia, 2007). In fact, the maintenance of intracellular calcium homoeostasis, whether extruding it from the cell or sequestering it in intracellular stores, require high-energy expenditure, and on the other hand, mitochondrial damage related to Ca^{2+} homoeostasis alterations is involved in experimental MN degeneration, and in ALS (Carriedo et al., 2000; Nicholls and Budd, 2000; Grosskreutz et al., 2010; Santa-Cruz et al., 2012).

In conclusion, the mechanisms involved in *in vivo* excitotoxic spinal MN degeneration seem to be multifactorial, since none of the compounds tested in our model has completely prevented MN death. However, our results suggest that mitochondrial energy deficits are crucially involved in this degeneration, whereas oxidative stress seems a less relevant mechanism. Although it is not certain that excitotoxicity is the first cause of MN degeneration in ALS, it is very likely that it participates in neuronal death in this disease due to the selective vulnerability of MNs to AMPA, so these mechanisms could be involved in ALS MN degeneration. With the limitation that our experimental model is restricted to two or three lumbar segments of the spinal cord, we consider that the correlation showing that there seems to be a minimal threshold number of spinal MNs necessary to preserve hind limb movements is of interest regarding the analysis of the progress of ALS in humans and the potential therapeutic strategies for this devastating disease.

AUTHOR CONTRIBUTION

Luz Diana Santa-Cruz and Ricardo Tapia conceived and designed the study, contributed to the data analysis and drafted the manuscript. Luz Diana Santa-Cruz carried out the experiments and obtained the data. Both authors read and approved the final manuscript.

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MITOCHONDRIAL DYSFUNCTION DURING THE EARLY STAGES OF EXCITOTOXIC SPINAL MOTOR NEURON DEGENERATION *IN VIVO*

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ABSTRACT

Glutamate-mediated excitotoxicity and mitochondrial dysfunction are involved in motor neuron degeneration process in amyotrophic lateral sclerosis (ALS). We have previously shown that microdialysis perfusion of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) in the lumbar region of the rat spinal cord produces a permanent paralysis of the ipsilateral hindlimb and death of motor neurons by a Ca^{2+}-dependent mechanism, in an acute process that starts 2-3 h after AMPA perfusion. We have also shown that various energy metabolic substrates, mainly pyruvate, co-perfused with AMPA, prevented the paralysis and motor neuron degeneration induced by AMPA, suggesting that mitochondrial energetic deficiencies are importantly involved in this excitotoxic motor neuron death. To test this hypothesis, in the present work we studied the functional and ultrastructural characteristics of mitochondria isolated from the ventral horns of lumbar spinal cords of rats, at the beginning of the degeneration process, when motor neurons are still alive. Animals were divided in four groups: perfused with AMPA, AMPA + pyruvate, pyruvate alone, and Krebs-Ringer medium as controls. Mitochondria isolated from the AMPA-treated group showed decreased oxygen consumption, ADP-dependent respiratory control and transmembrane potential. Additionally, activities of the respiratory chain complexes I and IV were significantly decreased, and electron microscopy showed that mitochondria of this group of rats presented ultrastructural and morphological abnormalities such as swelling, disorganized cristae, and disrupted membranes. Remarkably, in the animals co-perfused with AMPA and pyruvate these functional deficits and ultrastructural abnormalities were prevented. We conclude that mitochondrial dysfunction plays a crucial role in spinal motor neuron degeneration induced by overactivation of AMPA receptors *in vivo*. These mechanisms could be involved in ALS motor neuron degeneration.

Introduction

Glutamate-mediated excitotoxicity, mitochondrial dysfunction and oxidative stress are mechanisms participating in motor neuron degeneration in the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS) which results in progressive paralysis (Corona *et al.* 2007; Tovar-y-Romo *et al.* 2009; Duffy *et al.* 2011; Santa-Cruz *et al.* 2012; Cozzolino and Carrì 2012). Formerly, our group developed an *in vivo* model of spinal motor neuron excitotoxic death by means of microdialysis perfusion of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) in the lumbar region of the rat spinal cord. This treatment produces a permanent paralysis of the ipsilateral hindlimb and death of motor neurons by a Ca^{2+}-dependent mechanism (Corona and Tapia 2004; 2007), so this increased intracellular Ca^{2+} may trigger other harmful processes. Previously, we studied the involvement of oxidative stress and mitochondrial dysfunction in this model by exploring the neuroprotective properties of various energy metabolic substrates and antioxidants co-perfused with AMPA. Energy substrates prevented motor neuron death induced by AMPA perfusion, and completely prevented paralysis or any motor impairment in the rats, while antioxidants only partially protected and oxidative stress markers were not increased during the first stages of motor neuron degeneration, suggesting that mitochondrial energy deficiencies play a crucial role in this excitotoxic motor neuron degeneration, whereas oxidative stress could be a consequence affecting afterwards (Santa-Cruz and Tapia 2014).

Mitochondrial morphological and ultra-structural abnormalities have been observed in ALS patients autopsy tissues and muscle biopsies (Hirano *et al.* 1984; Sasaki and Iwata 1996; 2007; Siklós *et al.* 1996). Mitochondrial functional studies have provided consistent evidence with morphological observations. Partially respiratory chain inhibited mitochondria and deficits in mitochondrial respiratory chain complexes I and IV activities have been identified in muscle and spinal cord of SALS patients (Wiedemann *et al.* 1998; Borthwick *et al.* 1999; Vielhaber *et al.* 2000; Wiedemann *et al.* 2002, Echaniz-Laguna *et al.* 2006; Crugnola *et al.* 2010). All these data suggest that mitochondrial impairment and damage to the mitochondrial respiratory chain are events of relevance in ALS etiology and pathogenesis, though it is unknown whether these mitochondrial abnormalities are the cause or a consequence of the neurodegenerative process.

In the present work, we aimed to study thoroughly and directly the extent of mitochondrial dysfunction in the motor neuron degeneration process by assessing whether mitochondrial respiration, electron transport chain and ATP synthesis capacities were altered in mitochondria of rats perfused with AMPA. Since pyruvate was one of the energy substrates that exerted better protection in our model (Corona and Tapia 2007; Santa-Cruz and Tapia 2014), we also explored its

role and mechanism in motor neuron preservation. For these purposes we studied the functional and ultrastructural properties of mitochondria isolated from the ventral horns, where motor neurons are located, of lumbar spinal cords of rats perfused bilaterally with AMPA, AMPA + pyruvate, Krebs-Ringer solution + pyruvate, and Krebs-Ringer medium solely as controls. We measured several parameters of mitochondrial function, including oxygen consumption, transmembrane potential, and the activities of the mitochondrial respiratory chain complexes I, II and IV. Additionally, we observed by electron microscopy the isolated mitochondria.

Materials and methods

Animals

Adult male Wistar rats weighing 280–310 g were used in all the experiments and handled in accordance with the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care Committee. All efforts were made to minimize suffering of the animals. Animals were housed with a 12 h light/dark cycle and with food and water *ad libitum*.

Microdialysis procedure and drug administration

Microdialysis in the rat lumbar spinal cord was carried out essentially as previously described (Corona and Tapia, 2004, 2007; Santa-Cruz and Tapia 2014), except that two microdialysis probes were used, so that the perfusion of the solutions is carried out bilaterally in order to have enough tissue for the isolation of mitochondria. This because we carried out preliminary assays of mitochondrial isolation in tissue perfused unilaterally and we obtained a very small amount. Briefly, rats were anesthetized with 5% isoflurane in a 95% O_2 / 5% CO_2 mixture, placed in a stereotaxic spinal unit (Kopf, Tujunga, CA, USA), and maintained under low anesthesia (~0.8-1 % isoflurane) during surgery. Two ~1-2 mm diameter holes were drilled at each side of the second-third lumbar laminae and two microdialysis probes (1 mm long and 0.24 mm diameter, CMA/7, Solna, Sweden) were slowly lowered down, one into the right and the other into the left dorsal horns of the lumbar spinal cord. All the perfusion solutions were dissolved in Krebs-Ringer 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). Most of the reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) and from J.T.Baker (Center Valley, PA, USA), unless otherwise indicated. The four experimental groups of rats were perfused with the following solutions: 1) Krebs-Ringer only or intact rats (control rats); 2) AMPA 6 mM (Tocris Bioscience, Ellisville, MO, USA); 3) pyruvate 20 mM + AMPA 6 mM; and 4) pyruvate 20 mM. Concentrations of the compounds tested were chosen on the

basis of previous results from this laboratory (Corona and Tapia, 2004, 2007; Santa-Cruz and Tapia 2014). Osmolarity was maintained by reducing the NaCl concentration proportionally. All media were continuously perfused at a flux rate of 2 μL/min, using a microsyringe mounted on a microinjection pump (CMA/100, Solna, Sweden), during 160 min. AMPA-containing medium, for groups 2 and 3, was perfused during 25 min after the first 97.5 min. At the end of the microdialysis procedure, the skin was sutured and anesthesia was discontinued. Rats were kept in individual cages for them to recover from surgery with water and food *ad libitum*. As already mentioned, previous results from our laboratory (Corona and Tapia, 2004, 2007; Santa-Cruz and Tapia 2014) have shown that AMPA perfusion produces ipsilateral permanent rear limb paralysis, and a remarkable loss of spinal motor neurons at 24 h, effects that were prevented by pyruvate, here we analyzed at 24 h spinals cords of some rats perfused with AMPA bilaterally, and confirmed the great loss of motor neurons in both ventral horns. In the present work rats were sacrificed before, so they were observed the following ~ 2 hours, rats perfused with AMPA presented muscular spasms and progressive weakness of the ipsilateral hindlimb, indicating that subsequent paralysis was inevitable, in accordance with previous observations that total paralysis is reached at about 4–6 h (Corona and Tapia 2008). Rats co-perfused with pyruvate did not present any motor alterations.

Isolation of spinal cord mitochondria

The isolation process was carried out about ~1:30 – 2 hours after the microdialysis procedure so that mitochondria could be studied at the beginning of the degeneration process rather than when motor neurons were already damaged, since we have previously observed that the motor neuron degenerating process begins rapidly after AMPA perfusion and motor neuron loss starts at ~3–6 h after it and progresses until reaching a maximum at 12–24 h, when practically all motor neurons in the spinal segment studied are lost (Corona and Tapia 2008). Rats were decapitated and the lumbar spinal cord was carefully and rapidly removed. Tissue dissection of the site where the microdialysis probes tracts were located was performed on ice. All the following steps were carried out at 0-4 °C. Dissected ventral horns of the spinal cord tissue was transferred to ice-cold isolation buffer (modified from Pérez-Vázquez *et al.* 2002) containing 220 mM mannitol, 70 mM sucrose, 2 mM MOPS (3-[N-morpholino]propanesulfonic acid), 1 mM EGTA, and 1 g/l bovine serum albumin (BSA) (pH=7.4) and homogenized using a 1 mL tissue grinder size 0 with a teflon pestle (Thomas Scientific, Swedesboro, NJ, USA). Mitochondria were obtained by differential centrifugation as follows: the homogenate was placed in a 1.5 mL microcentrifuge tube and centrifuged at 510 g at 4 °C for 10 min. The supernatant was carefully decanted, placed on another microcentrifuge tube and centrifuged at 7400 g at 4 °C for 10 min. The pellet formed after this centrifugation step consists of

2 parts that are clearly distinguishable, one on the top that is larger and white which contains mostly myelin, and the other one in the bottom that is smaller and brown which corresponds to the mitochondrial fraction. The supernatant and the white part of the pellet were slowly and carefully removed using a Pasteur pipette. Mitochondrial pellets were gently resuspended in ~30 µL of isolation buffer. Protein concentration was determined by the Lowry method using the D_C Protein Assay from BIO-RAD (Hercules, CA, USA) and with BSA as standard.

Oxygen uptake measurements

The rate of oxygen consumption was measured using an oxygen meter Strathkelvin model 782 (Warner/Strathkelvin Instruments, Scotland, UK) with a Clark type electrode in a 0.1 mL water-jacketed chamber (Mitocell MT200 100 µL) at 30 °C. The reaction mixture contained 220 mM mannitol, 70 mM sucrose, 2 mM MOPS, 20 mM KCl, 1 mM $MgCl_2$, 2 mM phosphate (pH = 7.4), and one of the following respiratory substrates to induce the resting respiration state 4: glutamate-malate (10 mM each) or succinate (10 mM), to quantify complex I- and II-dependent respiration, respectively. Mitochondria were added, oxygen consumption registered, and then state 3 of active respiration was induced by the addition of 100 or 200 µM ADP. Respiratory control ratios (RCRs) were calculated as the ratio of O_2 consumption rates during active phosphorylation in presence of ADP, and during resting rate before ADP addition or after ADP was consumed (RCR = State 3 / State 4).

Mitochondrial transmembrane potential (Δψ) measurements

Mitochondrial Δψ was monitored by following safranin O fluorescence (Akerman and Wikström 1976). Measurements were carried out using a SLM AMINCO fluorometer (SLM Instruments, Inc., Urbana, IL, USA) with 495/586 nm excitation/emission wavelengths, using 200 µl capacity quartz fluorometer cells (Starna Cells, Inc., Atascadero, CA, USA). Incubation medium was identical to the one used for respiratory assays but was supplemented with 10 µM safranin. Isolated mitochondria were added and the fluorescence response to the addition of glutamate-malate (10 mM each) or succinate (10 mM), and the uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 1.5 µM) to completely collapse Δψ, was monitored.

Mitochondrial electron transport chain complexes activity measurements

Mitochondrial complex I (NADH dehydrogenase) activity was measured by following the time-course of rotenone-sensitive NADH oxidation. This was achieved by monitoring the decrease in absorbance at λ 340 nm (ε = 6.3 $mM^{-1}cm^{-1}$) every 10 s during 10 min, with or without 2.5 µM

rotenone, in a Synergy HT Multi-Mode Microplate Reader (BioTek, VT, USA). Mitochondria were subjected to 3 freeze-thaw cycles to completely lose the permeability barrier for NADH. The reaction buffer contained 220 mM mannitol, 70 mM sucrose, 2 mM MOPS, 20 mM KCl, 1 mM $MgCl_2$, 2 mM phosphate, 200 µM NADH, 40 µM decylubiquinone (pH = 7.4), 0.1 mg/ml asolectin soybean phospholipid mixture (phosphatidylcholine), and mitochondria. Activity was reported as nmol NADH/min/mg of protein. Complex II (succinate dehydrogenase) activity was measured by monitoring the decrease in absorbance due to the reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) at λ 600 nm (ε = 21 $mM^{-1}cm^{-1}$) every 15 s during 10 min with or without 1 mM malonate in the microplate reader. The reaction buffer contained mitochondria, 220 mM mannitol, 70 mM sucrose, 2 mM MOPS, 20 mM KCl, 1 mM $MgCl_2$, 2 mM phosphate, 1 µM rotenone, 10 mM succinate, 66 µM DCPIP (pH = 7.4). Malonate-sensitive activity for complex II was reported as nanomoles/min/mg of protein. Complex IV (cytochrome *c* oxidase) activity was measured polarographically using a Clark-type electrode as described in the oxygen uptake measurements section. Oxygen consumption was measured in the presence of the reaction buffer that contained 220 mM mannitol, 70 mM sucrose, 2 mM MOPS, 20 mM KCl, 1 mM $MgCl_2$, 2 mM phosphate (pH = 7.4), mitochondria, and 10 µM antimycin A, then 0.2 mg/ml *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD), and 10 mM ascorbate were added. Finally, after observing constant oxygen consumption, 100 µM cyanide was added. Complex IV sensitive to cyanide-activity was reported as µmol O_2/min/mg of protein. Incubation temperature for these assays was 30 °C.

Electron microscopy

Mitochondrial pellets were fixed in a solution containing 3% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and phosphate buffer pH=7, for 2 h, at 4 °C, and then resuspended and rinsed in phosphate buffer. The samples were then post-fixed in 1% osmium tetraoxide (SPI-Chem™, SPI Supplies, West Chester, PA, USA) and rinsed with phosphate buffer. Osmicated pellets were dehydrated using a graded series of ethanol, then underwent propylene oxide clearing, and were embedded in epoxy resin (TED PELLA, INC., Redding, CA, USA) which was polymerized at 60 °C for 48 h. Ultrathin sections, 80-100 nm, were cut using a Reichert-Jung Ultracut Ultramicrotome and stained with 2% uranyl acetate (Merck, Darmstadt, Germany) followed by 2% lead citrate. Electron micrographs at 1000X, 3000X, 7500X, 15000X and 20000X were obtained using a JEOL 1200 EXII transmission electron microscope.

Statistical analysis

Data were analyzed using ANOVA followed by a Tukey's post hoc test. A value of $p<0.05$ was considered as a statistically significant difference.

Results

AMPA perfusion induced defective spinal cord mitochondrial respiration and decreased respiratory control ratios, and pyruvate co-perfusion prevented mitochondrial dysfunction

Oxygen consumption during resting respiration state 4 in the presence of glutamate-malate, to supply reducing equivalents through complex I, was similar in mitochondria from all experimental groups as shown in Figure 1A and 1B. To evaluate mitochondrial coupling, state 3 of active respiration was induced by ADP addition, this increases oxygen consumption in mitochondria that are functional and well coupled. Mitochondria from rats perfused with Krebs-Ringer solution or with Krebs-Ringer + pyruvate indeed increased oxygen consumption following ADP addition. However, mitochondria from rats perfused with AMPA failed to increase oxygen consumption. Notably, this deficiency did not occur in mitochondria from rats perfused with pyruvate + AMPA. On the other hand, when mitochondrial respiratory activity was registered in the presence of succinate, to supply reducing equivalents through complex II, state 4 oxygen consumption was significantly increased in mitochondria from rats perfused with AMPA, whereas mitochondria from rats co-perfused with pyruvate + AMPA presented similar values to mitochondria from control rats, as shown in Figures 1C and 1D. State 3 respiration was increased in mitochondria from the AMPA + pyruvate group, while mitochondria from the AMPA treated group exhibited only a very slight decrease in the state 3 respiration rate compared to mitochondria from control rats. Figure 1E shows RCRs of mitochondria from each group of rats stimulated with glutamate-malate or with succinate, note that in both cases the RCRs of mitochondria from AMPA treated rats were significantly decreased, and these defects were prevented by pyruvate co-perfusion.

AMPA perfusion induces mitochondrial $\Delta\psi$ collapse and pyruvate co-perfusion elicits increments in $\Delta\psi$

CCCP-sensitive mitochondrial $\Delta\psi$ was measured by following safranin O fluorescence. A decrease in fluorescence corresponds to an increase in mitochondrial $\Delta\psi$. The fluorescence response to mitochondrial $\Delta\psi$ formation upon addition of glutamate-malate or succinate, and collapse following CCCP addition is shown in representative traces in figure 2A and 2B. The difference in the fluorescence values measured just before and after CCCP addition (Δ fluorescence) is directly

proportional to mitochondrial Δψ. AMPA perfusion in rat spinal cords induced a significant drop in Δψ of mitochondria isolated from them, when stimulated with both glutamate-malate and succinate. This AMPA-mediated depletion of Δψ was not only prevented, but also Δψ was augmented with pyruvate co-perfusion. These changes were more pronounced in the presence of glutamate-malate than with succinate as shown in Figure 2C, which exhibits the Δ fluorescence / mg of protein of isolated mitochondria.

Measurements of mitochondrial electron transport chain complexes activities revealed different extent of damage in the mitochondrial respiratory chain sites

Because we observed different mitochondrial responses to glutamate-malate or succinate in states 3 and 4 oxygen consumption rates, we measured the activities of the mitochondrial electron transport chain complexes. As shown in figure 3, complex I is the most affected by AMPA perfusion, since its activity is diminished by ~ 46 % as compared to the control, while complex IV activity (Figure 5) is diminished only by ~ 24 %, and complex II activity shows a non-significant reduction compared to the control (Figure 4). Notably, all the impairments in the electron transport chain activities were completely prevented by pyruvate co-perfusion.

Ultrastructural observations of spinal cord mitochondria by electron microscopy revealed morphological alterations in mitochondria from rats perfused with AMPA

To examine whether the functional abnormalities observed correlate with ultrastructural alterations, we processed mitochondria isolated from the rats' spinal cords at 1:30 - 2 h after the microdyalisis procedure, for their observation during the degenerative process by electron microscopy. For each experimental condition, four separate experiments were conducted and from each individual experiment micrographs at 1000X, 3000X, 7500X, 15000X and 20000X were randomly taken. Representative micrographs are shown in Figure 6. Subfraction was enriched in mitochondria, with low myelin or other structures, indicating that the isolation procedure was adequate. The general mitochondrial populations of intact rats and control rats perfused with Krebs-Ringer solution were morphologically normal, with well-preserved membranes, normal matrix and cristae. Pyruvate perfusion with Krebs did not alter normal mitochondrial ultrastructure. On the other hand, most of the mitochondria of the rats perfused with AMPA were damaged and presented ultrastructural and morphological abnormalities such as swelling, disorganized cristae, and disrupted membranes. Pyruvate co-perfusion with AMPA prevented swelling and all these structural alterations in most of the mitochondria, preserving the typical mitochondrial morphology with normal cristae and matrix.

Discussion

In this work we have studied the functional and ultrastructural characteristics of mitochondria, in order to assess their involvement in the excitotoxic motor neuron degeneration induced by AMPA and the mechanisms of neuroprotection by pyruvate. The functional parameters studied were: oxygen consumption rates during states 4 and 3, RCR, $\Delta\psi$, and the activities of the electron transport chain complexes I, II and IV, and all these parameters were decreased by AMPA perfusion. The diminished mitochondrial functions clearly indicate that in the early stages of excitotoxic degeneration mitochondria are damaged, which suggests a causal relationship. Remarkably, pyruvate managed to overcome these deleterious effects.

In functional and well-coupled mitochondria, ADP addition increases their oxygen consumption because ATP synthesis is activated and oxidative phosphorylation is coupled to mitochondrial respiration, stimulating it. Therefore, the failure to increase oxygen consumption rate during state 3 presented by mitochondria from AMPA perfused rats in the presence of glutamate-malate, indicates uncoupling and impairment in their ability to synthesize ATP. Conversely, mitochondria from rats co-perfused with AMPA + pyruvate were coupled and able to synthesize ATP, just as the control and pyruvate alone groups. Similarly, in skeletal muscle fibers of SALS patients, a significantly lowered maximal glutamate + malate and pyruvate + malate supported respiration in the presence of ADP was detected (Wiedemann *et al.* 1998; Vielhaber *et al.* 2000). On the other hand, the increased state 4 oxygen consumption shown by mitochondria from AMPA rats in the presence of succinate, could be an attempt to cope with the abatement in transmembrame potential, which was also prevented by pyruvate. The RCR is a very sensitive indicator to evaluate mitochondrial coupling and integrity. Thus, the significantly decreased RCRs exhibited by mitochondria from AMPA perfused rats, with both glutamate-malate and succinate, also indicate mitochondrial dysfunction. Similar results were found in spinal cord mitochondria of transgenic mutant SOD1 rodents, including decreased oxygen consumption, lack of ADP-dependent RCR, and decreased $\Delta\psi$ (Cassina *et al.* 2008).

The differences observed in complex I- and complex II- dependent respiration rates during state 3 and state 4 suggests that complex I is more severely damaged than complex II. Indeed, in accordance with the results obtained in the oxygen consumption assays, complex I is the most damaged due to AMPA perfusion, while complex II activity showed a non-significant smaller decrease. Complex I activity, the electron entry to the chain, is greatly reduced (approximately half of the activity) in mitochondria from AMPA rats, and therefore neither state 4 nor state 3 glutamate-malate dependent respiration is accelerated. On the other hand, the increased oxygen consumption

during state 4 in the presence of succinate, indicates that complex II is still functional, therefore electrons entry to the chain is not so limited, and the slightly diminished activity in complex II may be somewhat limiting in state 3, but no in state 4. Actually, state 4 respiration is accelerated, probably because H^+/e^- ratio lowers, may be due to proton leak and/or a defect of complex IV (slip), resulting in incomplete coupling of oxidation and phosphorylation. Interestingly, very similar to our observations, in skeletal muscle of SALS patients, an approximately two-fold lower specific activity of NADH:CoQ oxidoreductase compared to controls was detected (Wiedemann *et al.* 1998). The observed diminished complex IV activity is in accordance with the decrease in mitochondrial enzyme cytochrome *c* oxidase activity detected in individual spinal cord motor neurons in patients with SALS (Borthwick *et al.* 1999). Similar observations have been obtained in skeletal muscle biopsies of SALS patients, where it was found that respiratory complex IV activity significantly decreased as the disease advanced (Echaniz-Laguna *et al.* 2006), and that cytochrome *c* oxidase-negative fibers is frequent in skeletal muscle from patients with SALS (Crugnola *et al.* 2010). Furthermore, SALS muscle biopsies had substantially decreased activities of NADH:cytochrome *c* oxidoreductase and cytochrome *c* oxidase, whereas citrate synthase and succinate:cytochrome *c* oxidoreductase activities were almost unchanged compared to controls (Vielhaber *et al.* 2000). In spinal cord post-mortem tissue, significantly higher levels of mutant mitochondrial DNA and lower amount of it in the spinal cords of ALS patients compared to controls were found, correlating this reduction with decreased activities of citrate synthase, and respiratory chain complexes I + III and IV, suggesting a loss of mitochondria in ALS spinal cords (Wiedemann *et al.* 2002), however it is not clear if this loss is a final consequence of the disease since these studies were made post-mortem. Nevertheless, in the mutant SOD1 mice or cell culture familial ALS model, complexes I, II and IV of the electron transport chain exhibit decreased enzyme activities, even at early stages of the disease (Jung *et al.* 2002; Mattiazzi *et al.* 2002; Menzies *et al.* 2002a,b). In addition, in brain and spinal cord tissue these mice showed a decreased mitochondrial respiration at the last step of the respiratory chain (complex IV), related to reduced association of cytochrome c with the inner mitochondrial membrane (Kirkinezos *et al.* 2005). Interestingly, chronic mitochondrial inhibition of respiratory chain complexes II and IV in organotypic spinal cord cultures is sufficient to induce selective motor neuron degeneration that displays structural changes similar to those seen following excitotoxicity (Brunet *et al.* 2009; Kaal *et al.* 2000).

Mitochondrial $\Delta\psi$ is controlled by substrate availability, ATP demand, respiratory chain capacity, mitochondrial proton conductance, and mitochondrial Ca^{2+} sequestration. (Nicholls and Budd 2000). Accordingly, we observed a significantly increased $\Delta\psi$ in mitochondria isolated from rats perfused with pyruvate which had plenty substrate availability, and a significantly decreased $\Delta\psi$ in

mitochondria isolated from rats perfused with AMPA, which had diminished respiratory chain capacity as observed in the significantly reduced complexes I and IV activities. Pyruvate also provides reducing equivalents through complex I, accordingly mitochondria from rats perfused with pyruvate + AMPA and also with pyruvate alone presented a more pronounced increase in $\Delta\psi$ when stimulated with glutamate-malate. On the other hand, mitochondrial $\Delta\psi$ (or the proton-motive force) controls 3 fundamental cellular processes: ATP synthesis, mitochondrial Ca^{2+} sequestration, and the generation of ROS. ATP synthesis and Ca^{2+} accumulation compete for the proton gradient, and Ca^{2+} transport can dominate because ATP synthesis has a thermodynamic threshold requirement for mitochondrial $\Delta\psi$, while Ca^{2+} accumulation can proceed at a much lower $\Delta\psi$ and this in turn lowers $\Delta\psi$ dramatically. Collapse in the proton-motive force (whose dominant component is the $\Delta\psi$) will alter oxidative phosphorylation efficacy leading to the cessation of mitochondrial ATP synthesis, and can also lead to a rapid hydrolysis of cytoplasmic ATP due to the ATP synthase reversal in an attempt to restore $\Delta\psi$ and the proton-motive force (Nicholls and Budd 2000). Thus, it is essential for the cell to have functional mitochondria and an important decline in their $\Delta\psi$, as the observed in mitochondria from AMPA-perfused rats, will impact these cellular processes. Furthermore, the diminished ATP will in turn affect the activity of ions pumps responsible for removing Ca^{2+} from the cytoplasm, causing slowdown or cessation of its ATP-dependent extrusion, resulting in Ca^{2+} deregulation, and as a consequence augmenting ROS production. In spinal motor neurons cultures, AMPA or kainate triggered mitochondrial Ca^{2+} overload, mitochondrial depolarization and ROS generation (Carriedo *et al.* 2000). Indeed, subsequent oxidative stress is involved in motor neuron degeneration in our model, since antioxidants partially protected from the deleterious effects of AMPA (Santa-Cruz and Tapia 2014). Oxidative damage to the cell can also lead to a failure of Ca^{2+} extrusion, exacerbating cytoplasmic Ca^{2+} deregulation.

All these is relevant since increased Ca^{2+} influx, altered Ca^{2+} homeostasis, and the mitochondrial damage and involvement related to it, play a key role in neuronal injury, indeed their participation in motor neuron degeneration models and in ALS is evident (Barrett *et al.* 2014; Carriedo *et al.* 2000; Grosskreutz *et al.* 2010; Santa-Cruz *et al.* 2012). In our model, it was previously demonstrated that Ca^{2+} overload is importantly involved in AMPA-induced motor neuron death, since the co-perfusion of an intracellular Ca^{2+} chelator, and of a selective blocker of the Ca^{2+}-permeable AMPA receptors, with AMPA significantly prevented this death (Corona and Tapia 2007). Mitochondria can buffer large amounts of Ca^{2+}, and when its concentration in the cytosol increases pathologically, as it occurs in an excitotoxic event, endogenous Ca^{2+} induces slight dissipation of respiratory energy, and a burst of it is observed upon pulsing mitochondria with this

ion (Carafoli 1987; 2012), consequently, activation of resting respiration after Ca^{2+} accumulation should be expected, as observed in mitochondria from AMPA rats, in which state 4 respiration was significantly increased in the presence of succinate. Mitochondria can deal with, maintain matrix free Ca^{2+} at a constant level, and accumulate large amounts of Ca^{2+} through the formation of insoluble, osmotically inactive, structurally amorphous and rapidly dissociable Ca^{2+}-phosphate rich precipitates in the matrix, acting as temporary stores of Ca^{2+} during cytoplasmic Ca^{2+} peaks, and releasing it back to the cytoplasm when its cytosolic concentration is recovered to normal levels. Remarkably, mitochondria appear to be relatively little disturbed, structurally and functionally with no apparent deterioration in their capacity to maintain a high proton-motive force or to generate ATP, by the accumulation of Ca^{2+} and phosphate, until very large levels of loading are reached. Under such circumstances Ca^{2+} overload induces mitochondrial swelling, loss of respiratory control, increased mitochondrial ROS generation and collapse of $\Delta\psi$, resulting in compromised Ca^{2+} sequestration and ATP synthesis. Hence, Ca^{2+} loading capacity is finite and typically limited by the opening of the mitochondrial permeability transition pore that is accompanied by rapid but incomplete release of accumulated matrix Ca^{2+} (Carafoli 1987; Nicholls and Budd 2000; Kristian *et al.* 2007; Carafoli 2012).

Regarding our observations in the electron microscope showing that mitochondria isolated of the spinals cords of AMPA-treated rats presented swelling (that might be a consequence of the mitochondrial permeability transition pore) and altered cristae, suggests that Ca^{2+} accumulation exceeded mitochondrial Ca^{2+} loading capacity, triggering Ca^{2+} homeostasis alterations and causing mitochondrial functional (diminished RCR and $\Delta\psi$) and ultrastructural damage. Interestingly, ultrastructural changes, such as marked accumulation of mitochondria in the somata, dendrites or proximal axons, stubby protrusions on the outer membrane, and swollen mitochondria with markedly increased cristae in the somata were reported in the anterior horns neurons of lumbar spinal cords of patients with SALS (Sasaki and Iwata 2007). Also, mitochondrial fragmentation, enlargement, vacuolization, rearrangement of the cristae and swelling were observed in muscle and spinal cord of mutant SOD1 rodent models (Bendotti *et al.* 2001; Higgins *et al.* 2003; Kong and Xu 1998; Martin *et al.* 2009; Menzies *et al.* 2002b; Wong *et al.* 1995). These morphological abnormalities appeared in mice before the onset of symptoms and motor neuron degeneration, so this also indicates that mitochondrial dysfunction is an early mechanism playing a key role in initiating motor neuron degeneration in ALS. Furthermore, direct evidence of increased neuronal Ca^{2+} in muscle biopsy specimens of ALS patients has been observed in the motor nerve terminals of neuromuscular junctions, and also higher density of synaptic vesicles, increases in mitochondrial volume and mitochondrial Ca^{2+} compared to disease control groups (Siklós *et al.* 1996). These

increased parameters might represent compensatory responses to excitotoxicity, the slight swelling of mitochondria secondary to Ca^{2+} uptake, and increased mitochondrial volume might be due also to increased energy demand in the nerve terminals. In our model augmented energy demand is also expected due to excitotoxicity, requiring more energy substrates and functional mitochondria for the motor neuron to survive as it occurs in the AMPA + pyruvate treated rats, whilst if this enhanced energy demand can not be fulfilled due to damaged mitochondria and energy metabolism disruption resulting from Ca^{2+} overload, motor neurons will inevitably die as it happens in the AMPA treated rats.

Indeed, we have observed here and in previous work (Santa-Cruz and Tapia 2014) that supplementation with pyruvate and other energy substrates prevent paralysis and motor neuron death. Mitochondria isolated of the spinal cords of the AMPA + pyruvate treated rats were structurally and functionally normal, similar to those isolated form control intact rats, Krebs-Ringer and pyruvate + Krebs perfused rats, i.e. pyruvate was able to prevent all the defects in the mitochondrial bioenergetic properties measured. Taken together, all our results suggest that neuroprotection exerted by pyruvate is primarily through the maintenance of healthy and functional mitochondria, by helping them to maintain $\Delta\psi$. Energy substrates can directly prevent $\Delta\psi$ collapse because one of the factors that control it is substrate availability, so excess mitochondrial substrates administered exogenously can stimulate respiratory chain and increase oxidative phosphorylation, maintaining the electrochemical proton gradient and thus preventing the collapse of ATP synthesis. So, as ATP synthesis can continue, the required energy production for the neuron to maintain intracellular Ca^{2+} homeostasis can be fulfilled, thus preventing mitochondrial dysfunction and the Ca^{2+}-dependent mitochondrial swelling and ultrastructural alterations. Besides, substrate concentration and pH are some of the factors that contribute to enzymatic activity regulation, pyruvate can help to augment NADH concentration and prevent transmembrane potential collapse, maintaining H^+ gradient and optimal ΔpH, thus helping to prevent respiratory complexes enzimatic activities reductions. All this is relevant since mitochondrial bioenergetic status is crucial for maintaining healthy neurons, for controlling their susceptibility to acute or chronic stress, and for determining survival, apoptosis or necrosis. Furthermore, it appears that an increase in mitochondrial Ca^{2+} loading capacity improves a cell´s resistance to insults, and P_i from a variety of sources, including ATP, can support precipitate formation and increase the Ca^{2+} loading capacity (Kristian *et al.* 2007). Ca^{2+} uptake, though supported by the activity of the respiratory chain, requires the presence of ATP, alongside with Ca^{2+} and phosphate, ATP/ADP are taken up by mitochondria to promote the formation of the dense granules and/or stabilize them (Carafoli 2012), and protection against the loss of Ca^{2+} is seen with ATP maintaining the intactness of mitochondria

during the phenomenon (Carafoli 1987). Thus, the augmented ATP production due to pyruvate co-perfusion could also help to increase Ca^{2+} loading capacity, preventing mitochondrial dysfunction and finally motor neuron death under these conditions where Ca^{2+} concentration is also increased due to AMPA overactivation. Moreover, three key metabolic enzymes of the tricarboxylic acid cycle are activated by increases in mitochondrial Ca^{2+}, pyruvate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase, thereby the increased flux in the cycle is reflected in an increased reduction state of NADH and a slight increase in the proton motive force when mitochondrial Ca^{2+} responds to an increased cytoplasmic Ca^{2+}, thus activating the respiratory chain and, ultimately the synthesis of ATP (Nicholls and Budd 2000; Carafoli 2012). This is in agreement with the augmented activation of the respiratory chain through a significantly elevated state-3 oxygen consumption in the presence of succinate, and the markedly increased transmembrane potential in the presence of both succinate and glutamate/malate presented by mitochondria from AMPA + pyruvate rats, indicating that substrate availability, in this case pyruvate, in conjunction with activation of the tricarboxylic acid cycle enzymes is helping to restore and even increase the energy production so necessary in this situation of high energy demand, thus helping to maintain functional mitochondria and leading neurons to survival.

It is important to bear in mind that even though we delimitated the tissue area in the spinal cords to the ventral horns and where the microdialyisis probes were introduced, we had diverse population of cells apart from motor neurons (such as astrocytes, interneurons), we could expect that mitochondria from motor neurons would be the most damaged given that these cells are more vulnerable to AMPA exposure and to increased intracellular Ca^{2+}, anyway it is important to consider that we have distinct subpopulations of functional and damaged mitochondria. Yet, we could detect significant decreases in RCR, $\Delta\psi$, and complexes I and IV activities in mitochondria from the AMPA perfused rats.

Taking together the results of the present and previous work (Santa-Cruz and Tapia 2014), we can conclude that mitochondrial bioenergetic deficiencies and the related ultrastructural abnormalities are crucial in the initial degeneration process, and not a consequence. Mitochondrial dysfunction in our model triggers impairment of energy metabolism and subsequently oxidative stress. This scenario may also be the case in ALS, since there is evidence of the participation of these mechanisms in the studies with patients, the early occurrence of mitochondrial dysfunction in models of the disease, and that it is a key mechanism in motor neurons degeneration.

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FIGURE LEGENDS

Figure 1. Mitochondrial respiration is impaired in AMPA-perfused rats and this impairment is prevented by pyruvate co-perfusion

A, C. Representative state-4 and state-3 oxygen consumption curves in the presence of (**A**) glutamate/malate (10 mM each) or (**C**) succinate (10 mM). ADP was added to induce state-3 respiration and evaluate mitochondrial coupling. Numbers under the traces represent nmol O_2 per

minute per mg of protein. **B, D.** Oxygen consumption rates of spinal cord mitochondria isolated from rats perfused with the indicated compounds, during the resting respiration state (State 4) and during active respiration state (State 3) induced by the addition of 100 or 200 μM ADP, in the presence of glutamate-malate (**B**) or succinate (**D**) as respiratory substrates to quantify complex I- and II-dependent respiration, respectively. Data are mean ± SEM from the number of rats specified in parenthesis for each group. *$p <$ 0.05, significantly different from the other groups of rats and &$p < 0.05$, significantly different from the group of control rats. **E.** Calculated respiratory control ratios (RCRs) for complex I (glutamate-malate) and for complex II (succinate) of spinal cord mitochondria from the indicated groups. Data are mean ± SEM from the same number of rats as in B and D. *$p <$ 0.05, significantly different from the other groups of rats.

Figure 2. Mitochondrial Δψ is diminished in spinal cord mitochondria from rats perfused with AMPA, and pyruvate co-perfusion increases it

A-B. Representative traces of mitochondrial Δψ formation and collapse in mitochondria isolated from ventral horns of rat lumbar spinal cords of control rats perfused with Krebs-Ringer solution, rats perfused with AMPA, AMPA + Pyruvate, and Pyruvate + Krebs. Mitochondria were added to the respiratory incubation medium supplemented with 10 μM safranin. Fluorescence response to the additions (indicated by arrows) of 10 mM glutamate-10 mM malate (**A**) or 10 mM succinate (**B**) and the uncoupler CCCP (1.5 μM) are shown. A decrease in fluorescence corresponds to an increase in mitochondrial Δψ. RFU are relative fluorescence units. **C.** Mitochondrial Δψ is directly proportional to the values of Δ fluorescence / mg of protein of isolated spinal cord mitochondria. Data represent mean values ± SEM of the number of rats indicated in parentheses. *$p < 0.05$ vs. values from the other groups of rats. &$p < 0.05$ vs. values from control rats.

Figure 3. Complex I activity is severely impaired in mitochondria from AMPA perfused rats and pyruvate prevents this impairment

A. Representative curves of spectrophotometric assays for NADH dehydrogenase activity showing the absorbance changes at λ 340 nm due to NADH oxidation against reaction times in seconds. **B.** Complex I activities obtained from the rotenone-sensitive NADH time-course oxidation by mitochondria from the rats of the indicated groups in the presence of 200 μM NADH and 40 μM decylubiquinone (DBQ) as artificial electron acceptor. NADH:DBQ oxidoreductase activities are reported as nmol NADH/min/mg of protein. Data represent mean values ± SEM of the number of rats indicated in parentheses. *$p < 0.05$ vs. values from the other groups of rats.

Figure 4. Complex II activity of spinal cord mitochondria from rats perfused with Krebs-Ringer solution, AMPA, AMPA + pyruvate, and Krebs + pyruvate

A. Representative curves of spectrophotometric assays for succinate dehydrogenase activity showing the decrease in absorbance at λ 600 nm due to the reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) against reaction times in seconds. **B.** Malonate-sensitive complex II activities of mitochondria from the rats of the indicated groups in the presence of 10 mM succinate, 66 µM DCPIP, and 1 µM rotenone. Malonate-sensitive succinate:DCPIP oxidoreductase activities are reported as nmol/min/mg of protein. Data represent mean values ± SEM of the number of rats indicated in parentheses. AMPA group was significantly different from the AMPA + pyruvate group, and the Krebs + pyruvate group at the 0.05 level, but not from the control Krebs group. No significant differences were detected in the other groups.

Figure 5. Complex IV activity is reduced in AMPA perfused rats, and this is prevented by pyruvate co-perfusion

A. Representative oxygen consumption curves of mitochondria from rats perfused with the indicated compounds in the presence of 10 µM antimycin A in the reaction buffer, 10 mM ascorbate + 0.2 mg/ml TMPD (Tetramethyl-*p*-phenylenediamine) were added, and, after observing constant oxygen consumption, 100 µM cyanide was added. Numbers under the traces represent nmol O_2 per minute. **B.** Cytochrome *c* oxidase activity measured polarographically by oxygen consumption. Complex IV sensitive to cyanide-activity is reported as µmol O_2/min/mg of protein. Data represent mean values ± SEM of the number of rats indicated in parentheses. *$p < 0.05$ vs. values from the other groups of rats.

Figure 6. Ultrastructural observation of spinal cord mitochondria

Representative digital transmission electron micrographs of mitochondria isolated from the spinal cords of control intact rats and rats perfused with Krebs-Ringer solution, AMPA, AMPA + Pyruvate, and Krebs + Pyruvate. Note that most of the mitochondria from AMPA-perfused rats are morphologically damaged, with disordered cristae and swollen, while most of the mitochondria from AMPA + pyruvate co-perfused rats are structurally and morphologically normal, with well-preserved membranes, normal cristae, homogeneous matrix, and unswollen, just as the mitochondria from the control intact rats, control Krebs, and pyruvate + Krebs perfused rats. Bars = 1 µm for 3000X and 200 nm for 7500X and 20000X.

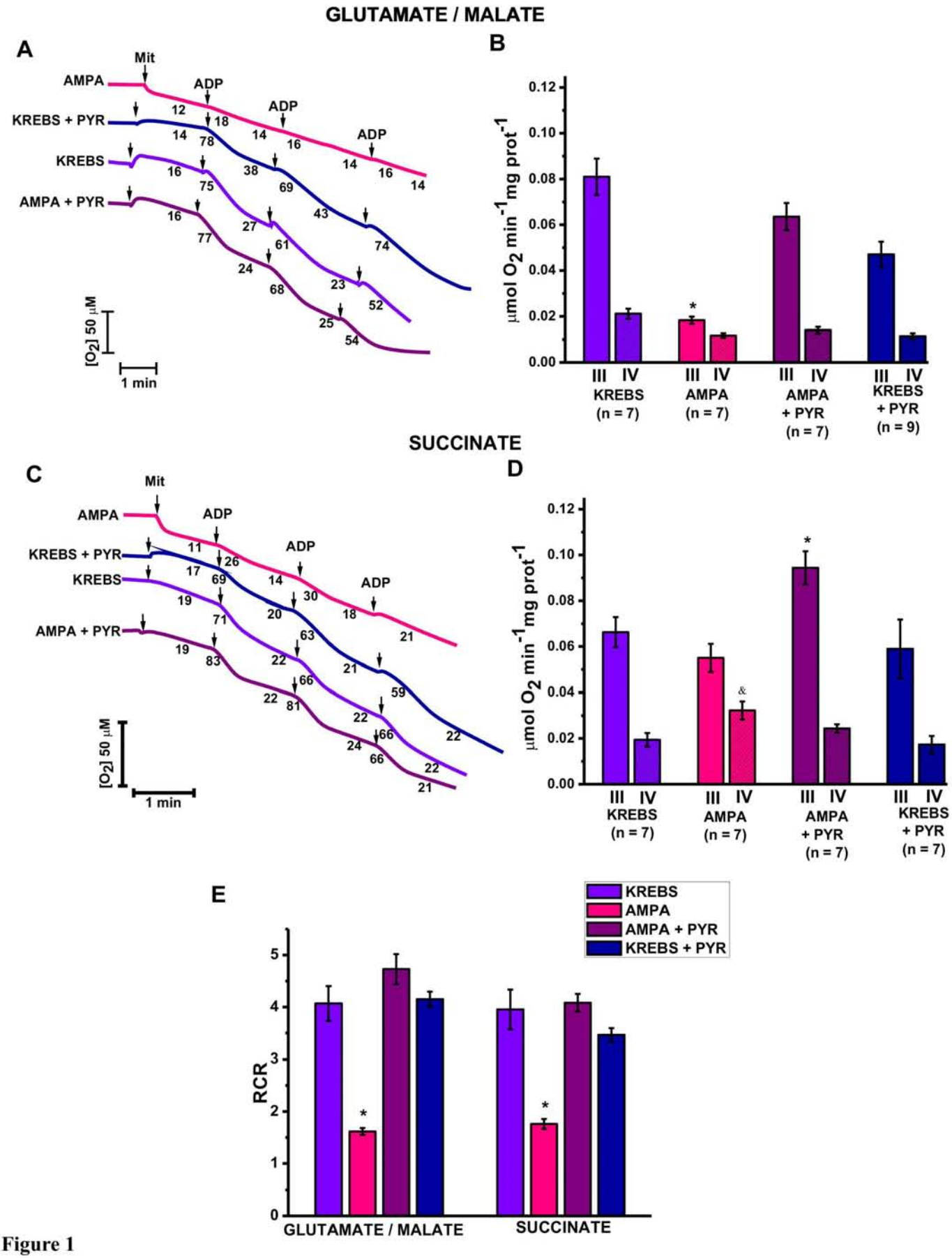

GLUTAMATE / MALATE
A
Mit
AMPA
ADP
KREBS + PYR
KREBS
AMPA + PYR
[O2] 50 µM
1 min
B
µmol O2 min-1 mg prot-1
III IV
KREBS (n = 7)
AMPA (n = 7)
AMPA + PYR (n = 7)
KREBS + PYR (n = 9)
SUCCINATE
C
D
KREBS (n = 7)
AMPA (n = 7)
AMPA + PYR (n = 7)
KREBS + PYR (n = 7)
E
RCR
GLUTAMATE / MALATE
SUCCINATE

Figure 1

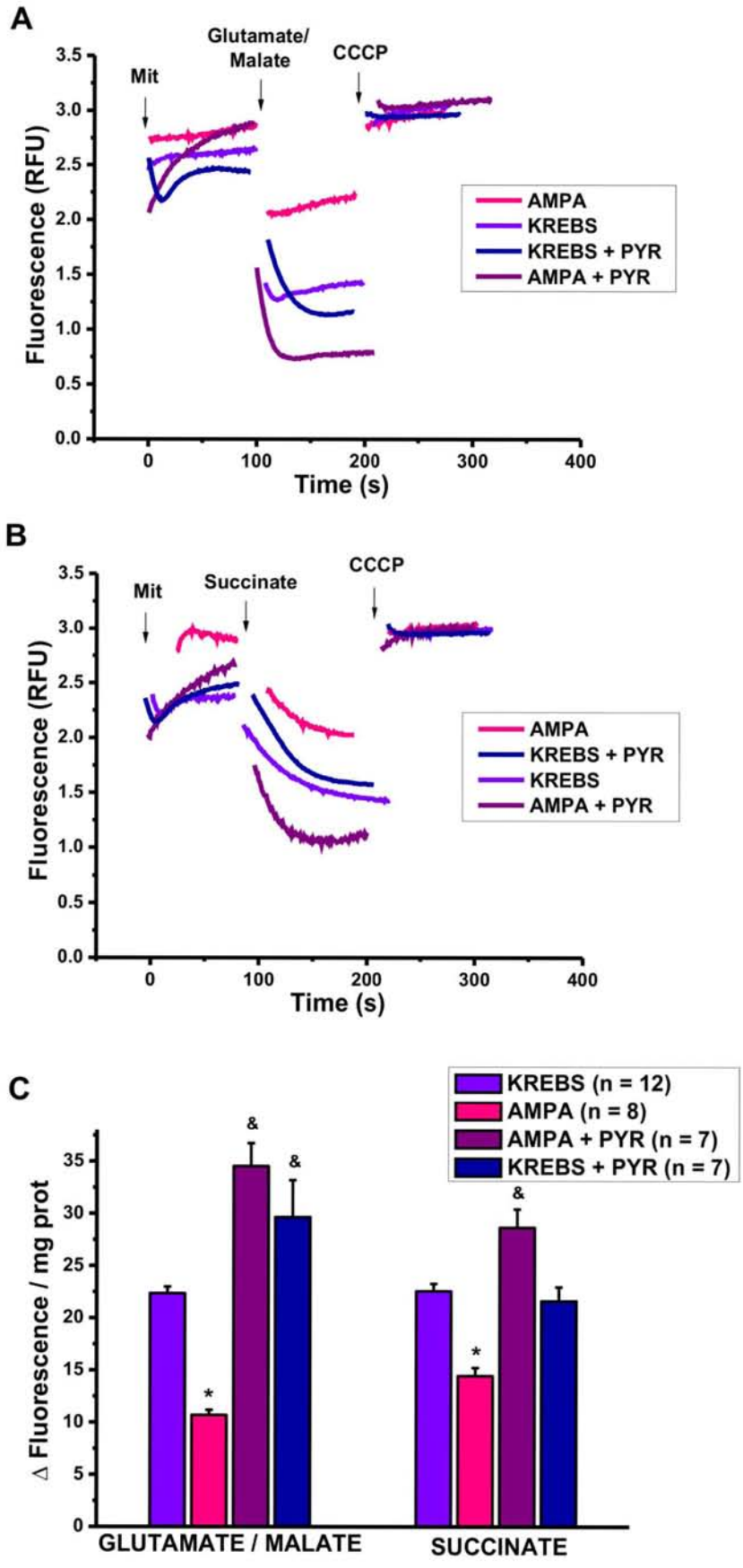

A
Glutamate/
Malate
CCCP
Mit
Fluorescence (RFU)
Time (s)
AMPA
KREBS
KREBS + PYR
AMPA + PYR
B
Mit
Succinate
CCCP
Fluorescence (RFU)
Time (s)
AMPA
KREBS + PYR
KREBS
AMPA + PYR
C
KREBS (n = 12)
AMPA (n = 8)
AMPA + PYR (n = 7)
KREBS + PYR (n = 7)
Δ Fluorescence / mg prot
GLUTAMATE / MALATE
SUCCINATE

Figure 2

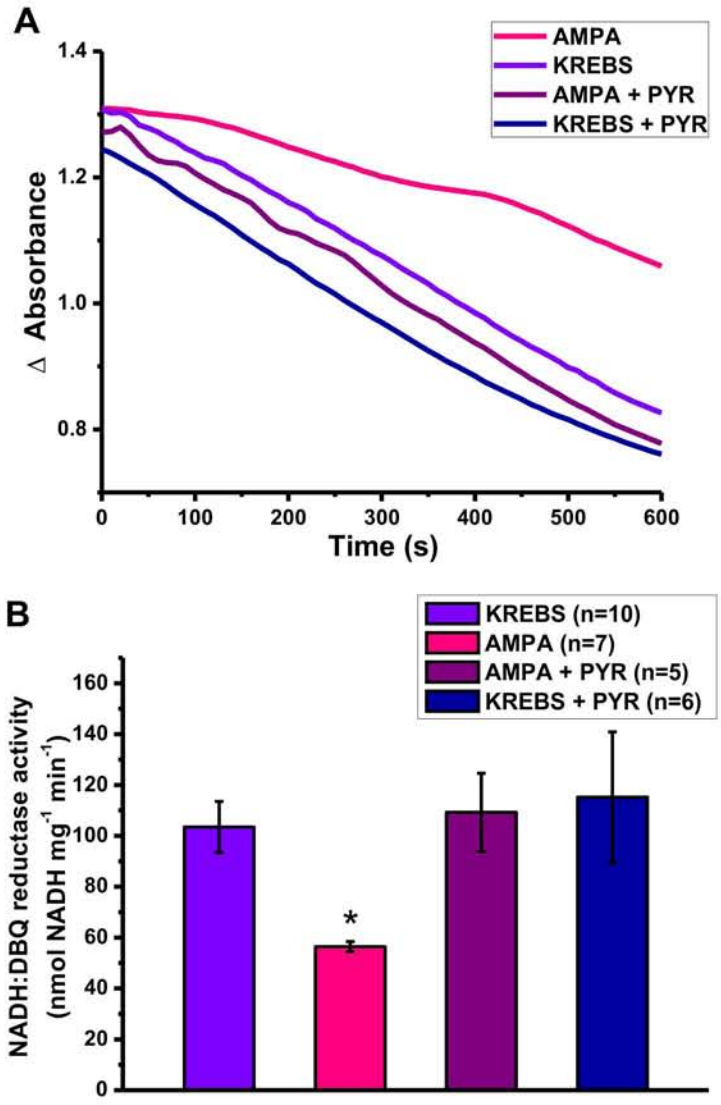

A
AMPA
KREBS
AMPA + PYR
KREBS + PYR
Δ Absorbance
1.4
1.2
1.0
0.8
0
100
200
300
400
500
600
Time (s)
B
KREBS (n=10)
AMPA (n=7)
AMPA + PYR (n=5)
KREBS + PYR (n=6)
NADH:DBQ reductase activity
(nmol NADH mg-1 min-1)
160
140
120
100
80
60
40
20
0
*

Figure 3

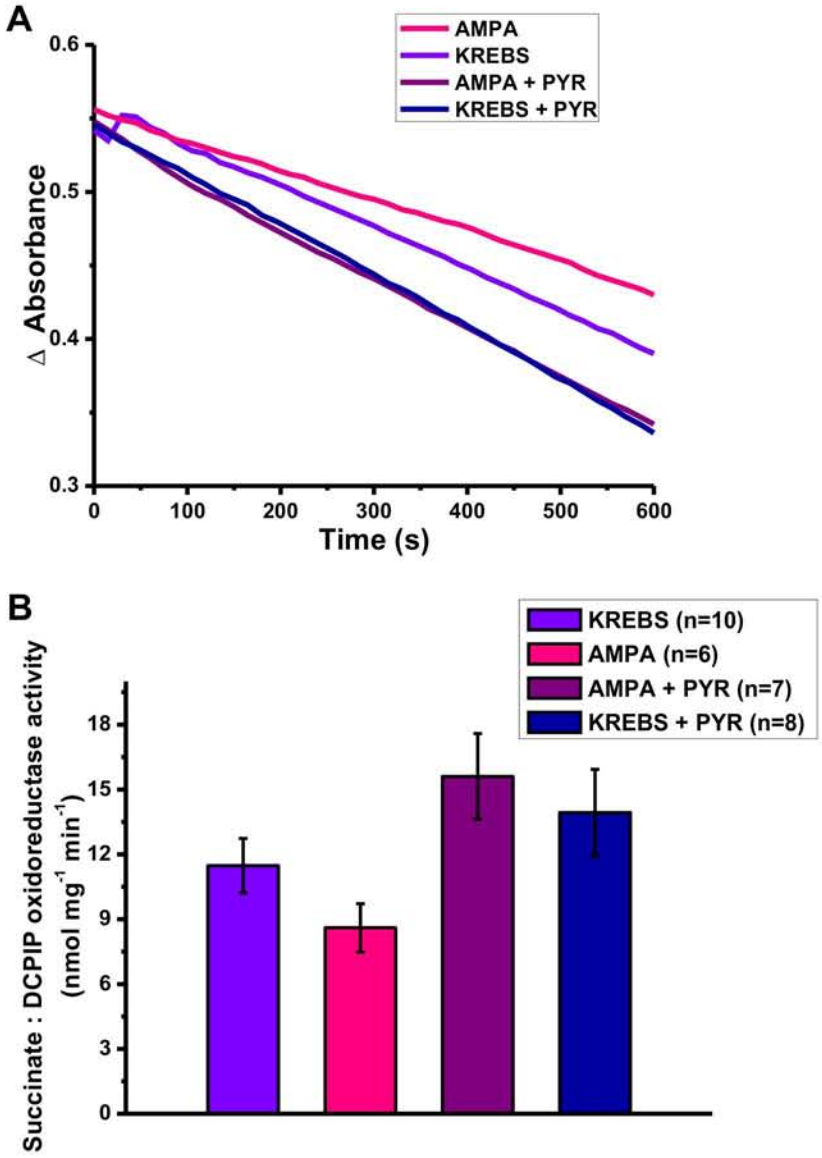

A
AMPA
KREBS
AMPA + PYR
KREBS + PYR
Δ Absorbance
0.6
0.5
0.4
0.3
0
100
200
300
400
500
600
Time (s)
B
KREBS (n=10)
AMPA (n=6)
AMPA + PYR (n=7)
KREBS + PYR (n=8)
Succinate : DCPIP oxidoreductase activity
(nmol mg-1 min-1)
18
15
12
9
6
3
0

Figure 4

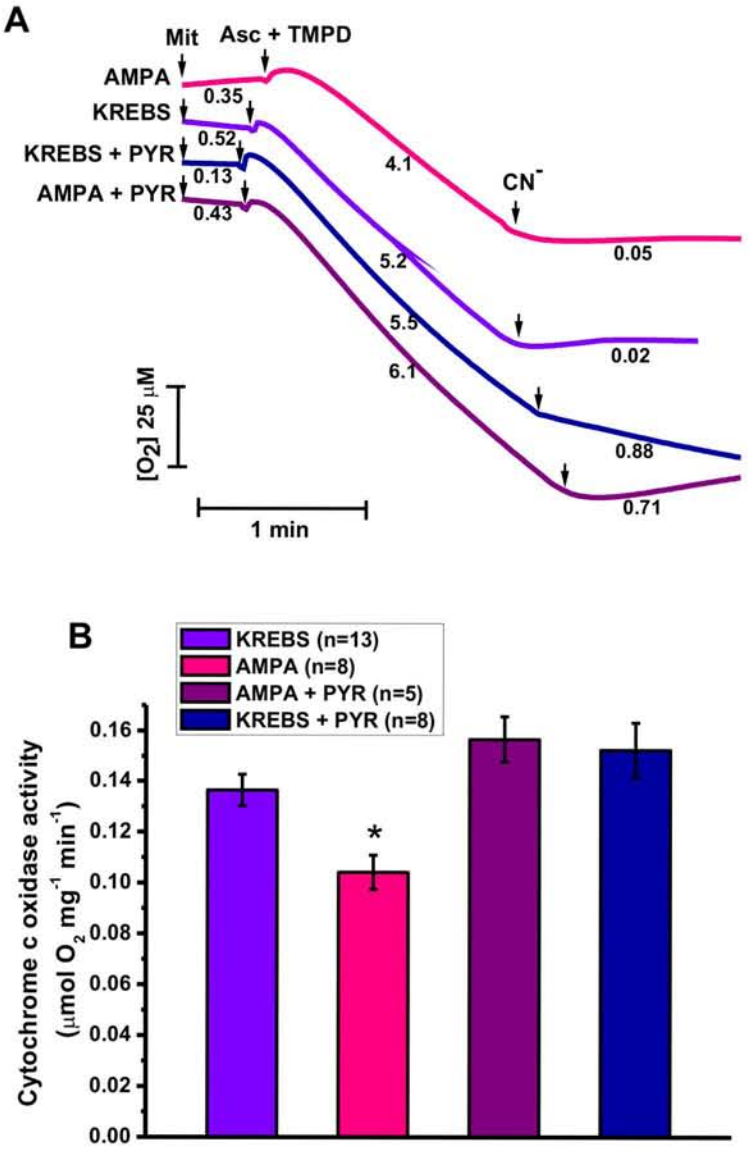
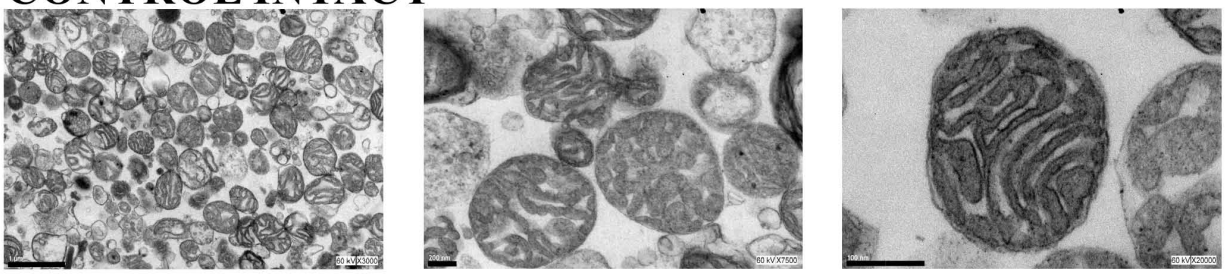

A
Mit
Asc + TMPD
AMPA
KREBS
KREBS + PYR
AMPA + PYR
0.35
0.52
0.13
0.43
4.1
5.2
5.5
6.1
CN-
0.05
0.02
0.88
0.71
[O2] 25 µM
1 min
B
KREBS (n=13)
AMPA (n=8)
AMPA + PYR (n=5)
KREBS + PYR (n=8)
Cytochrome c oxidase activity
(µmol O2 mg-1 min-1)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
*

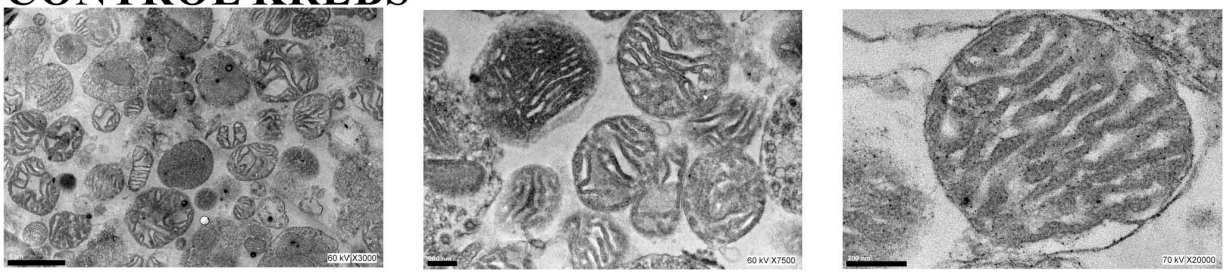
Figure 5

3000X 7500X 20000X

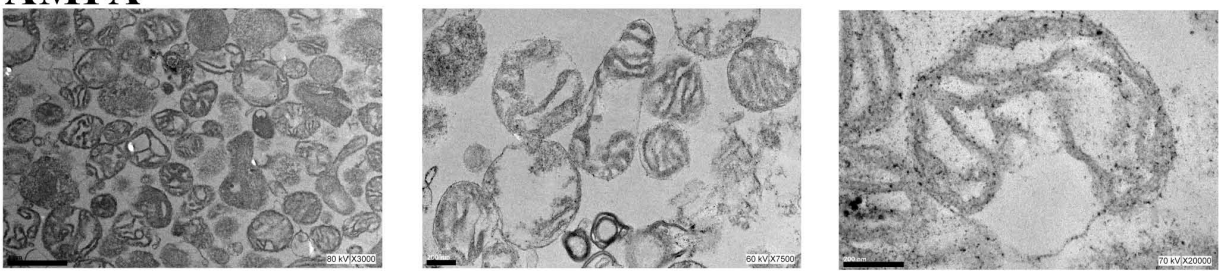
CONTROL INTACT



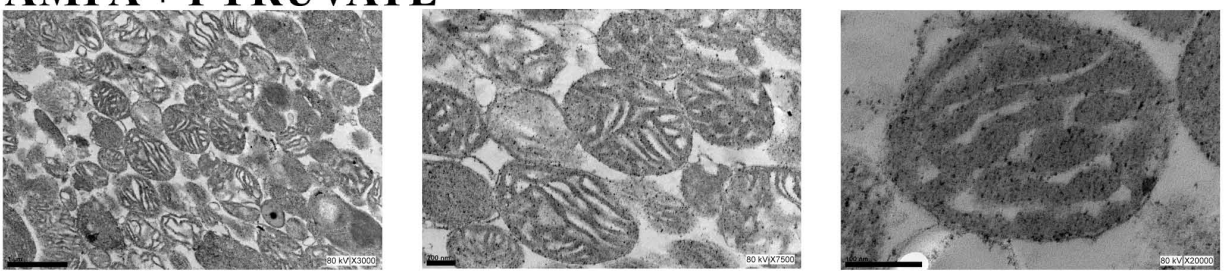
CONTROL KREBS



AMPA



AMPA + PYRUVATE



KREBS + PYRUVATE

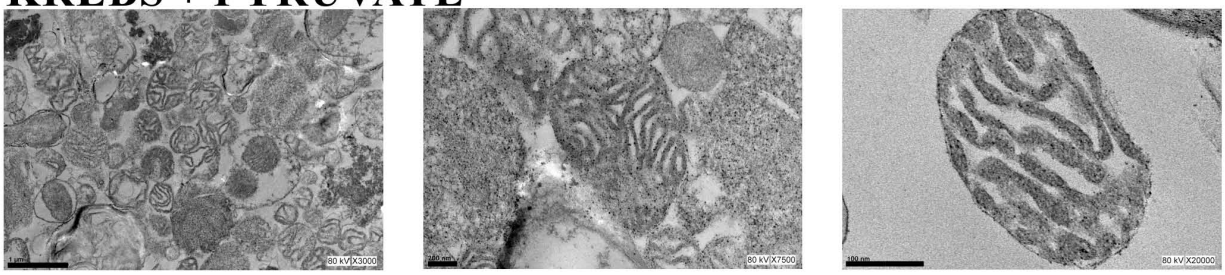


Figure 6

VII. DISCUSIÓN

Puesto que los artículos que se presentan en la parte de resultados tienen cada uno su propia discusión, aquí se trata de hacer una discusión integral y complementaria de estas discusiones para obtener una visión más amplia y unificada de la interpretación de todos los resultados obtenidos en este trabajo, haciendo énfasis en puntos importantes que vale la pena reiterar. Por esto, esta sección tiene muy pocas referencias.

Las anormalidades mitocondriales morfológicas y funcionales, y los niveles elevados de marcadores de estrés oxidativo que se han observado en los tejidos de los pacientes con ALS, sugieren que estos mecanismos pueden estar contribuyendo de manera importante en la degeneración de las motoneuronas en esta enfermedad. Por esta razón, en este trabajo nos enfocamos en investigar la importancia de la participación de la disfunción mitocondrial, la deficiencia en el metabolismo energético, y del estrés oxidativo mediante la exploración de las propiedades neuroprotectoras de diferentes sustratos energéticos y antioxidantes. También realizamos el estudio y la investigación directa de marcadores de estrés oxidativo, y de las propiedades funcionales y ultraestructurales de las mitocondrias desde las etapas iniciales del proceso neurodegenerativo, en el modelo agudo de degeneración de motoneuronas espinales por excitotoxicidad *in vivo*.

A pesar de que aún no hay evidencias que comprueben que la excitotoxicidad glutamatérgica es la causa primordial que desencadena la muerte de las motoneuronas en la ALS, la sobreactivación de los receptores tipo AMPA mediada por glutamato es muy probablemente un mecanismo que contribuye de manera importante a la degeneración selectiva de las motoneuronas en esta enfermedad, dada su particular vulnerabilidad a AMPA y a la sobrecarga de Ca^{2+} intracelular (Corona *et al.* 2007). Por esta razón, nuestro modelo de neurodegeneración excitotóxica perfundiendo AMPA en la médula espinal de ratas con la consecuente parálisis, puede ser un modelo útil para estudiar los mecanismos involucrados en el proceso de muerte selectiva de motoneuronas sin la participación de alteraciones genéticas, por lo que puede ser representativo de un mayor porcentaje de casos de ALS (Tovar-y-Romo *et al.* 2009a). De ahí la importancia de investigar los mecanismos degenerativos y probar compuestos neuroprotectores que puedan evitar los efectos deletéreos del proceso excitotóxico, lo cual nos ayuda a entender mejor los mecanismos implicados y a poder proponer agentes terapéuticos. Así, los datos obtenidos en este trabajo pueden contribuir al entendimiento de esta enfermedad que parece ser multifactorial.

Alteraciones mitocondriales funcionales y morfológicas

Nuestros resultados muestran que las mitocondrias definitivamente juegan un papel central en el proceso de degeneración de las motoneuronas. Las mitocondrias son fundamentales para el funcionamiento adecuado y la salud de las células. De entre las funciones que tiene la mitocondria la principal es la producción de energía por medio de la óxido-reducción de sustratos y de la síntesis de ATP; por esto, el estatus bioenergético mitocondrial es crucial para mantener a las neuronas sanas, determinante en el control de su susceptibilidad a estrés agudo o crónico y decisivo para la sobrevivencia de las neuronas o muerte por apoptosis o necrosis. Otra de las funciones mitocondriales importantes es la capacidad que tienen de amortiguar la concentración de Ca^{2+} intracelular contribuyendo así al mantenimiento de la homeostasis de la concentración citoplásmica de este catión. De esta manera, la habilidad que tengan las mitocondrias para manejar grandes cantidades de Ca^{2+} también puede ser determinante en cuanto a si las células dañadas sobreviven o no. Así, cuando la concentración citosólica de Ca^{2+} se incrementa patológicamente, como ocurre en un evento excitotóxico, las mitocondrias desempeñan un papel crucial para las células, ayudándolas a sobrevivir mediante el aumento en la producción de energía en respuesta a la alta demanda, y mediante la captura de Ca^{2+} en la matriz mitocondrial hasta que se reduce la gran acumulación de Ca^{2+} en el citoplasma. Sin embargo, si hay un incremento excesivo en el Ca^{2+} citoplásmico, esto provoca una sobrecarga de Ca^{2+} mitocondrial que induce hinchamiento mitocondrial, pérdida del control respiratorio, aumento en la producción de ROS mitocondrial y despolarización o colapso del $\Delta\psi_m$, lo que resulta en fallas en la captura de Ca^{2+} y la síntesis de ATP, causando disfunción mitocondrial y alteración del metabolismo energético. Esto es lo que observamos en las mitocondrias de las ratas perfundidas con AMPA, lo que sugiere que la acumulación de Ca^{2+} excedió la capacidad de carga de Ca^{2+} mitocondrial, provocando alteraciones en la homeostasis de Ca^{2+}, y causando disfunción mitocondrial reflejada en el RCR y el $\Delta\psi_m$ disminuidos, y en las alteraciones morfológicas, como son el hinchamiento mitocondrial y crestas dañadas y desordenas, observados por microscopía electrónica.

Las diferencias observadas en las velocidades de consumo de oxígeno dependientes de complejo I y complejo II durante los estados 3 y 4, sugirieron que el complejo I está más severamente dañado que el complejo II, y estas observaciones nos llevaron a medir directamente las actividades de los complejos de la cadena de transporte de electrones. Efectivamente, en conformidad con los resultados obtenidos en los ensayos de respiración, el complejo I es el más dañado como

consecuencia de la perfusión de AMPA, mientras que la actividad del complejo II mostró una disminución menor no significativa, y la actividad del complejo IV presentó una disminución significativa pero mucho menor que la presentada por el complejo I. Como se discute ampliamente en el segundo artículo de la sección de resultados, nuestras observaciones concuerdan con las encontradas en la médula espinal y en el músculo esquelético de pacientes y modelos de la enfermedad, en los que se han encontrado deficiencias en los complejos I y IV de la cadena respiratoria mitocondrial y cambios morfológicos y ultraestructurales en las mitocondrias. También se han encontrado estos defectos en otros tejidos de los pacientes. Así por ejemplo, en linfocitos de pacientes con ALS, se encontró que la actividad del complejo I estaba significativamente reducida y esto correlacionaba con el tiempo de progresión de la enfermedad, también la relación ATP/ADP estaba disminuida, sugiriendo que estos eventos están involucrados en el progreso y la patogénesis de la ALS (Ghiasi *et al.* 2012). Además, se ha encontrado una reducción en los niveles de expresión de proteínas de la cadena de transporte de electrones en muestras de sangre de pacientes con ALS, esto es de la flavina adenina dinucleótido sintetasa, la riboflavina quinasa, citocromo C1, y la subunidad B del complejo de la succinato deshidrogenasa. Estos hallazgos pueden tener un efecto en las rutas metabólicas dependientes de oxígeno y, consecuentemente, un metabolismo oxidativo sub-óptimo podría ser un aspecto relevante, puesto que las motoneuronas podrían ser particularmente susceptibles a daño bajo estas circunstancias (Lin *et al.* 2009). En las mitocondrias de plaquetas de pacientes con SALS se han observado cambios ultraestructurales como son falta de uniformidad en la matriz, crestas tenues, cuerpos lisosomales mayores, y gránulos intramitocondriales menores, sugiriendo una participación sistémica en la patofisiología de la ALS (Shrivastava *et al.* 2011). También, en mitocondrias de piel de pacientes con SALS, se encontraron anormalidades morfológicas como son cristólisis, ruptura de la membrana externa, y mitocondrias más pequeñas comparadas con los controles sanos (Rodríguez *et al.* 2012).

La disfunción mitocondrial y los daños morfológicos son observados desde las primeras etapas del proceso de degeneración de las motoneuronas en nuestro modelo, por lo que éstos y las consecuentes deficiencias en el metabolismo energético están contribuyendo de manera relevante desde las etapas iniciales, sugiriendo que estos mecanismos son primordiales y pueden desencadenar otros procesos perjudiciales, todo lo cual conduce a la muerte de estas neuronas.

Deficiencias energéticas

Las deficiencias energéticas provocadas por la disfunción mitocondrial afectan todas las funciones neuronales. Además, las motoneuronas son muy vulnerables a déficits energéticos porque requieren de un alto gasto energético debido a su gran tamaño y sus procesos largos para inervar los músculos.

Durante la excitotoxicidad, la entrada masiva de iones a la neurona provoca una alta demanda energética debido a que la hidrólisis de ATP se incrementa por la activación de la Na^+/K^+-ATPasa para extraer el exceso de Na^+ de la célula; además, la regulación intracelular de Ca^{2+} también es muy costosa. Nuestro grupo demostró previamente la importancia de la participación del incremento en la concentración intracelular de Ca^{2+} en la muerte de las motoneuronas en este modelo (Corona y Tapia 2007). Dado que los mecanismos que utiliza la célula para mantener la homeostasis del Ca^{2+} intracelular, ya sea secuestrándolo en las pozas intracelulares o extrayéndolo de la célula por medio de las vías impulsadas por ATP, requiere alto gasto de energía, la entrada aumentada de Ca^{2+} a las motoneuronas por la sobreactivación de los receptores AMPA permeables a este catión en esta situación excitotóxica, puede disminuir y hasta agotar el ATP. Esto se puede atribuir a que la síntesis mitocondrial de ATP también iría disminuyendo a medida que el Ca^{2+} se transporta del citoplasma a la matriz mitocondrial, lo que provocaría el colapso de $\Delta\psi_m$ y por lo tanto de Δp (fuerza protón-motriz), y la ATP sintasa puede invertir su función catalítica e hidrolizar ATP en un intento por restablecer el $\Delta\psi_m$. Como resultado de la deficiencia de la función mitocondrial, la producción de ATP también se verá disminuida, exacerbando la insuficiencia de energía. Las alteraciones en la síntesis de ATP afectan a su vez la actividad de las bombas de iones responsables de la remoción del Ca^{2+} del citoplasma, lo que junto con la salida posterior del Ca^{2+} de la matriz mitocondrial, daría lugar a la DCD que finalmente lleva a la muerte neuronal. Por tanto, durante la sobreactivación de la transmisión glutamatérgica, la función mitocondrial y la disponibilidad de ATP son críticas para la recuperación y sobrevivencia o muerte neuronal. Por consiguiente, es muy probable que la neuroprotección que observamos con los sustratos energéticos contra la muerte de las motoneuronas inducida por AMPA y la consecuente parálisis, se deba al restablecimiento o incremento en la producción de ATP que resulta de la utilización de los sustratos energéticos administrados, y que puede cubrir la alta demanda energética que tienen las motoneuronas en estas condiciones. Además, esta neuroprotección ejercida por los sustratos energéticos y los resultados que obtuvimos al observar directamente la

función y morfología mitocondriales, apoyan la idea de que la sobrecarga de Ca^{2+} intracelular *in vivo* altera el metabolismo energético y la función mitocondrial, y que estos déficits mitocondriales son cruciales en el proceso de neurodegeneración. El incremento en los niveles de ATP gracias a la administración de los sustratos energéticos puede también ayudar a la regulación de la concentración intracelular de Ca^{2+}, impidiendo el desencadenamiento de los mecanismos activados por Ca^{2+} que finalmente provocan la muerte neuronal. Nuestros resultados son relevantes puesto que hay muchas evidencias que sugieren que el daño mitocondrial posiblemente relacionado con alteraciones en la homeostasis de Ca^{2+} está implicado tanto en la SALS como en la FALS (Grosskreutz *et al.* 2010; Santa-Cruz *et al.* 2012). Concordando con esto, la exposición de motoneronas espinales a AMPA o kainato *in vitro*, provocó una sobrecarga de Ca^{2+} en las mitocondrias, despolarización mitocondrial y generación de ROS (Carriedo *et al.* 2000), sugiriendo que la absorción de Ca^{2+} y disfunción mitocondrial, y la consecuente generación de ROS son importantes en el proceso perjudicial.

Cuando estudiamos directamente las mitocondrias aisladas de la médula espinal, examinamos también el efecto del piruvato tanto en las propiedades bioenergéticas como en la morfología mitocondriales, porque el piruvato es uno de los sustratos que ejerció muy buena protección contra la muerte de las motoneuronas, evitando por completo la parálisis y cualquier alteración en la función motora. El piruvato no sólo es capaz de estimular el metabolismo energético y la producción de energía, sino que también tiene propiedades antioxidantes (Desagher *et al.* 1997; Kim *et al.* 2005; Wang *et al.* 2007), de ahí el gran interés en conocer los mecanismos mediante los cuáles está ejerciendo neuroprotección primordialmente. El piruvato evitó todos los defectos en las propiedades bioenergéticas provocados por el AMPA, pues las mitocondrias aisladas de las ratas tratadas con AMPA + piruvato eran estructural y funcionalmente normales, similares a las aisladas de las ratas control. Nuestros resultados sugieren que la neuroprotección ejercida por el piruvato es primordialmente como sustrato mitocondrial, ayudando a mantener mitocondrias sanas y funcionales. El piruvato es un intermediario oxidable del metabolismo energético que es convertido a acetil-CoA por la piruvato deshidrogenasa mitocondrial. La acetil-CoA entra directamente al ciclo de los ácidos tricarboxílicos (ciclo TCA), donde se da su oxidación completa a CO_2. La energía de oxidación se conserva en forma de GTP (Guanosín trifosfato) y, temporalmente, en forma de las coenzimas reducidas NADH y $FADH_2$, que son transportadores electrónicos que posteriormente entran a la cadena respiratoria, donde ocurre la transferencia de electrones al O_2 y la síntesis de ATP mediante la fosforilación oxidativa. En las neuronas, el sustrato mitocondrial

dominante es el piruvato derivado de la glucólisis. El piruvato y otros sustratos energéticos pueden ayudar a mantener el $\Delta\psi_m$ y evitar su colapso porque uno de los factores que controla al $\Delta\psi_m$ es la disponibilidad de sustratos, por lo que el exceso de sustratos mitocondriales administrados exógenamente puede estimular la cadena respiratoria e incrementar la fosforilación oxidativa, manteniendo el gradiente electroquímico de protones, evitando así la caída del $\Delta\psi_m$ y el colapso en la síntesis de ATP. Además, la falta de sustrato limita severamente la respiración mitocondrial exacerbando la alteración de la regulación de Ca^{2+}, y la administración de suficiente sustrato podría evitar esto, como pudimos observar en el grupo tratado con piruvato + AMPA. Así, al continuar y hasta aumentar la síntesis de ATP se puede satisfacer la producción de energía requerida para la homeostasis de Ca^{2+} neuronal, evitando la disfunción mitocondrial, el hinchamiento y otras alteraciones morfológicas dependientes de Ca^{2+}. El piruvato y el lactato (que es convertido intracelularmente a piruvato por medio de la lactato deshidrogenasa) son sustratos energéticos ventajosos para las neuronas porque la energía es obtenida como NADH sin el gasto de ATP. En 2 de las reacciones que se llevan a cabo en la glucólisis, se requiere el consumo de ATP, por lo que una célula que tenga niveles de ATP disminuidos y carencia de sustratos oxidables, aunque sólo sea temporalmente, podría sufrir un colapso irreversible mientras la glucólisis esté limitada, exacerbando así el decremento de ATP. Un mecanismo para reiniciar la glucólisis en una neurona cuyo ATP esté agotado es proveerla con lactato o piruvato (Nicholls y Budd 2000). El piruvato y el L-lactato pueden proteger a las neuronas *in vitro* de la toxicidad inducida por NMDA que es aumentada por la privación de glucosa, pues estos sustratos contrarrestan fuertemente la gran disminución en el contenido de ATP que resulta de estas condiciones (Maus *et al.* 1999). Además, los efectos neuroprotectores del piruvato bajo diferentes circunstancias han sido demostrados tanto *in vivo* (Massieu *et al.* 2000; Massieu *et al.* 2001), como *in vitro* (Eimerl y Schramm 1995; García y Massieu 2001; García y Massieu 2003). Estos trabajos sugieren que el efecto neuroprotector del piruvato está relacionado con su función como sustrato metabólico mitocondrial. En el ratón transgénico SOD1 G93A el piruvato también protegió, prolongando el promedio de esperanza de vida, ralentizando el progreso de la enfermedad y mejorando el desempeño motor (Park *et al.* 2007). Además, se ha propuesto que el piruvato (junto con el malato) ayuda a mejorar la función de los mecanismos homeostáticos de Ca^{2+}, en particular, aumentando la eficiencia de la extracción de Ca^{2+} al medio y de la acumulación de Ca^{2+} en las pozas intracelulares. Así, aumenta la contribución de la mitocondria para la homeostasis de Ca^{2+} neuronal, probablemente debido a que el incremento en la tasa respiratoria que se da en

presencia de piruvato y malato resulta en un $\Delta\psi_m$ aumentado, y por lo tanto mitiga la neurotoxicidad mediada por glutamato (Villalba *et al.* 1994; Ruiz *et al.* 1998). De la misma manera, nosotros observamos un incremento en la tasa respiratoria mitocondrial y en el $\Delta\psi_m$ en las ratas tratadas con piruvato + AMPA. Así, las mitocondrias en las neuronas utilizan el piruvato para generar Δp, sintetizar ATP y amortiguar el Ca^{2+} citosólico. Por lo tanto, los mecanismos protectores del piruvato, ya sea administrado exógenamente o producido endógenamente a partir del lactato, son probablemente múltiples, incluyendo la estimulación del ciclo TCA y de la producción de energía, por medio de su función antioxidante y ayudando a la homeostasis de Ca^{2+} intracelular.

El piruvato también evitó las disminuciones en las actividades enzimáticas de los complejos de la cadena respiratoria mitocondrial, lo que pudiera deberse a que de entre los factores que regulan la actividad enzimática se encuentran la concentración de sustrato; el piruvato ayuda a aumentar la concentración de NADH como sustrato del complejo I y de ahí puede continuar la cadena de transporte de electrones. Otro factor que regula la actividad enzimática es el pH; el piruvato evita el abatimiento del potencial transmembranal y así los cambios de pH que se pudieran dar como consecuencia de éste, manteniendo el ΔpH óptimo para el funcionamiento de los complejos enzimáticos.

El α-cetobutirato es un intermediario metabólico natural, producto de la degradación de los aminoácidos metionina y treonina, de modo que su oxidación completa pueda llevarse a cabo. El α-cetobutirato es metabolizado por la α-cetobutirato deshidrogenasa a propionil-CoA, que puede ser transformado a metilmalonil-CoA y subsecuentemente a succinil-CoA, un intermediario del ciclo TCA. Se ha demostrado que la α-cetobutirato deshidrogenasa activa está presente en el cerebro de rata (Steele *et al.* 1984). Los resultados tan similares que obtuvimos con el α-cetobutirato y el piruvato a la misma concentración y la similitud en sus estructuras químicas, sugieren que pueden proteger por mecanismos similares. El α-cetobutirato puede incrementar la producción de energía y también es un α-cetoácido que puede reaccionar directamente con el H_2O_2.

La creatina, fosfocreatina y la enzima creatina quinasa constituyen un sistema de amortiguamiento y transporte de energía en la célula, conectando los sitios de producción de energía (las mitocondrias) con los sitios de consumo de ésta (Hemmer y Wallimann 1993). La administración de creatina puede estimular la respiración mitocondrial y la síntesis de fosfocreatina (O'Gorman *et al.* 1996), la cual difunde hacia el citoplasma donde actúa como un

amortiguador temporal y espacial de energía para mantener los niveles de ATP donde se requiera la energía. La fosfocreatina sirve como una fuente de grupos fosforilo para sintetizar rápidamente ATP a partir de ADP por medio de la creatina quinasa quien cataliza esta reacción. Cuando una demanda repentina de energía agota el ATP, que es lo que muy probablemente esté sucediendo durante el evento excitotóxico tras la exposición a AMPA, la reserva de fosfocreatina se utiliza para reponer ATP a una velocidad considerablemente mayor que la velocidad de síntesis de ATP a través de las rutas catabólicas y la fosforilación oxidativa. Cuando disminuye la demanda de energía, el ATP producido por el catabolismo se utiliza para reponer la reserva de fosfocreatina por inversión de la reacción de la creatina quinasa. De esta manera la fosfocreatina, derivada de la creatina, constituye un importante amortiguador energético. Los factores que se han propuesto que probablemente contribuyen a la neuroprotección por la creatina son: la preservación de los niveles de ATP intracelulares, la protección de la función y estimulación de la respiración mitocondrial, así como la inhibición de la apertura del poro de la transición de la permeabilidad mitocondrial (MPTP) por Ca^{2+}, porque estabiliza la isoforma octamérica de la creatina quinasa (O'Gorman *et al.* 1997).

El SNC adulto es capaz de transportar y usar los cuerpos cetónicos como sustratos energéticos alternativos, aunque la glucosa sea el principal sustrato energético. Cuando la concentración de cuerpos cetónicos se incrementa (en situaciones como ayuno prolongado, como resultado de una dieta cetogénica o por su administración exógena), su oxidación en el cerebro aumenta. Así, los cuerpos cetónicos pueden promover la producción de energía mitocondrial. La enzima D-β-hidroxibutirato deshidrogenasa oxida el D-β-hidroxibutirato (el isómero fisiológicamente activo) a acetoacetato, el cuál se activa por la transferencia de CoA a partir de succinil-CoA formando su éster de coenzima A. El acetoacetil-CoA da lugar a dos moléculas de acetil-CoA por acción de la tiolasa, que a su vez son catabolizadas mediante el ciclo TCA donde son oxidadas por completo y producen energía sintetizando ATP por medio de la fosforilación oxidativa. Se han demostrado los efectos neuroprotectores del D-β-hidroxibutirato contra la toxicidad del glutamato; el tratamiento con este cuerpo cetónico reduce el daño neuronal y la lipoperoxidación provocados por la inyección intraestriatal de glutamato a ratas a las que se les ha inhibido la glucólisis (Mejía-Toiber *et al.* 2006). Por otro lado, también se ha demostrado la capacidad antioxidante de los cuerpos cetónicos incluyendo al acetoacetato y los dos isómeros D- y L- del β-hidroxibutirato; la administración de ambos evitó el aumento en la peroxidación de lípidos en el hipocampo de rata en un modelo de hipoglucemia *in vivo*, mientras que únicamente el D-β-hidroxibutirato y el

acetoacetato evitaron el decremento en el ATP neuronal *in vitro* (Haces *et al.* 2008). Puesto que nosotros perfundimos la mezcla racémica, los efectos neuroprotectores del DL-β-hidroxibutirato podrían ser tanto por la estimulación del metabolismo energético mitocondrial, como por su capacidad antioxidante.

Por lo tanto, la protección ejercida por los sustratos energéticos puede deberse a que la suplementación del metabolismo mitocondrial con ellos permite que la célula contrarreste la cascada de efectos neurotóxicos desencadenada por la sobreactivación de los receptores tipo AMPA. A través de estimular el consumo de O_2, estos sustratos energéticos favorecen el mantenimiento del $\Delta\psi_m$, y ayudan a incrementar la producción de energía aumentando la síntesis de ATP, y evitando que la integridad y función de las mitocondrias se dañen irreversiblemente.

Además, considerando que estos sustratos se perfundieron antes, durante y después (160 minutos) de la perfusión del AMPA (25 minutos), hay un aumento en la disponibilidad de sustrato que ayuda a que la célula cuente con una mayor reserva energética cuando es sobreexcitada por el AMPA. También es probable que luego de la administración de AMPA haya subpoblaciones de mitocondrias dañadas y mitocondrias sanas y funcionales dentro de la misma neurona (debido tal vez a su localización subcelular más lejana a los receptores o a los canales de Ca^{2+}) que estarían sintetizando una mayor cantidad de ATP, estimuladas por la alta demanda y la mayor cantidad de sustratos oxidables.

La ALS se asocia con varios defectos en el metabolismo energético. Los pacientes con ALS generalmente pierden peso y grasa corporal conforme avanza la enfermedad, la malnutrición proteico-energética es un hallazgo frecuente y la homeostasis energética está muy afectada. Esto es a causa de un mayor gasto que ingreso y producción de energía, debido a hipofagia y disfagia, hipermetabolismo, atrofia muscular, hipoxemia e hipercapnia. Otros factores que contribuyen a que los pacientes con ALS tengan el metabolismo energético severamente alterado son disfunción mitocondrial, estrés oxidativo e inflamación sistémica. Así, las reservas de energía están disminuídas, y además el bajo índice de masa corporal y la malnutrición afectan negativamente la evolución de la enfermedad y la sobrevivencia de los pacientes con ALS. La neurodegeneración es a menudo asociada con y potencialmente causada por un metabolismo energético defectuoso. Por consiguiente, las estrategias terapéuticas deberían ser dirigidas a corregir el metabolismo energético defectuoso y al establecimiento de un plan para el soporte metabólico y nutricional de los pacientes con ALS, que sea temprano (en el momento del diagnóstico de la enfermedad),

especializado y adecuado, incluyendo sustratos nutricionales específicos. Éstos se deben administrar en cantidades adecuadas que puedan influir positivamente en el progreso de la enfermedad y mejorar la calidad de vida y la respuesta a la poco efectiva terapia farmacológica disponible actualmente (Dupuis *et al.* 2011; Muscaritoli *et al.* 2012).

Los modelos animales de ALS muestran que el hipermetabolismo muscular y el deterioro del metabolismo energético son eventos tempranos, intrínsecos a la patogénesis de la ALS. Desde etapas tempranas en la fase asintomática de la enfermedad, los ratones transgénicos con la SOD1 mutada, tienen menor peso corporal, acumulación de tejido adiposo reducida, y un aumento del gasto energético e hipermetabolismo (Dupuis *et al.* 2004). La compensación de los déficits energéticos alimentando a los ratones transgénicos con la SOD1 mutada con una dieta altamente energética, mejoró el fenotipo alterado del metabolismo, retardó el comienzo de la enfermedad, ralentizó el progreso de la enfermedad, incrementó la esperanza de vida, redujo la denervación muscular, y hubo una mejora en la sobrevivencia de las motoneuronas (Dupuis *et al.* 2004; Mattson *et al.* 2007). Por otro lado, la restricción calórica apresuró el inicio clínico, aceleró el curso general de la enfermedad y acortó la esperanza de vida (Hamadeh y Tarnopolsky 2006; Patel *et al.* 2010). Las alteraciones metabólicas en el SNC de los modelos de ratones con ALS incluyen: utilización de glucosa reducida en componentes de los tractos motores cortico-espinal y bulbo-espinal, disminuciones significativas en el contenido de ATP cortical en los ratones G93A presintomáticos, e hipometabolismo en la médula espinal en la etapa sintomática (Browne *et al.* 2006). Por lo tanto, el metabolismo energético alterado es un defecto primario que subyace la disfunción y degeneración neuronal, por lo que el aumento en la producción de ATP puede ser benéfico.

Aun cuando la disfunción mitocondrial y la falla energética no fueran los eventos desencadenantes de la degeneración de las motoneuronas, es indudable que están participando y podrían también ser consecuencia de otros mecanismos propuestos, por lo que son un blanco terapéutico importante. Los sustratos energéticos probados en este trabajo podrían utilizarse como tratamiento potencial para la ALS y otras enfermedades neurodegenerativas, dada su baja toxicidad, la significativa neuroprotección ejercida por ellos y que algunos también tienen propiedades antioxidantes.

Estrés oxidativo

El hallazgo de que todos los antioxidantes a cualquier concentración protegieron sólo parcialmente, mientras que el grupo de ratas significativamente protegidas de los efectos perjudiciales del AMPA que no presentaron ningún déficit motor fueron co-perfundidas con sustratos energéticos, con y sin propiedades antioxidantes, indica que el estrés oxidativo está contribuyendo al proceso de degeneración. Sin embargo, no es un evento primario como lo pudimos comprobar al observar que en las primeras horas subsecuentes a la perfusión de AMPA no hay elevación en los marcadores de estrés oxidativo que medimos (ROS totales y nitración de proteínas). Además, cuando combinamos el GEE con el piruvato no hubo un efecto sinérgico y la neuroprotección que se obtuvo no fue mayor que la obtenida con la sola perfusión de piruvato. Por lo tanto, lo más probable, es que el estrés oxidativo sea un evento secundario, consecuencia del daño mitocondrial y de la desregulación en la homeostasis de Ca^{2+}.

Las mitocondrias normalmente producen una pequeña cantidad de ROS debido a la fuga de electrones de la cadena respiratoria al O_2, pero si existen defectos en los complejos de la cadena de transporte de electrones u otras perturbaciones mitocondriales, como las que observamos como consecuencia de la perfusión del AMPA, éstas pueden ser responsables de una producción excesiva de radicales libres, provocando daño celular o incrementándolo. La sobrecarga de Ca^{2+} en las mitocondrias incrementa la producción del radical superóxido $O_2^{\bullet -}$, y por lo tanto de ROS, provocando estrés oxidativo (Dykens 1994; Peng y Jou 2010). Es muy probable que esta carga de Ca^{2+} mitocondrial sea la responsable de la estimulación de la producción mitocondrial de ROS que se observa después de la sobreactivación de los receptores NMDA, AMPA y kainato (Dugan *et al.* 1995; Carriedo *et al.* 1998; Carriedo *et al.* 2000), por lo que un evento excitotóxico inicial puede contribuir a estrés oxidativo incrementado. El decremento en la respiración y síntesis de ATP, así como la elevación simultánea en la producción de ROS por la mitocondrias dañadas, ocurre como un proceso de autopropagación, donde se va agotando la energía celular almacenada, conduciendo al daño de ciertos mecanismos homeostáticos y protectores, dándose la transición de la permeabilidad mitocondrial y la alteración de la regulación del Ca^{2+} citosólico.

Las mitocondrias no sólo son capaces de producir ROS, sino que también son susceptibles al daño por estrés oxidativo, que puede provocar disfunción mitocondrial. El estado redox de los nucleótidos de nicotinamida intramitocondriales es crítico para mantener la poza de GSH reducido, y por lo tanto para la resistencia de las mitocondrias al estrés oxidativo, pues el GSH

(que se encuentra tanto en el citoplasma como en las mitocondrias) es el principal amortiguador redox celular contra el estrés oxidativo (Nicholls y Budd 2000; Atlante *et al.* 2001). Concordando con esto, el piruvato también disminuye la muerte celular de neuronas granulares cerebelares en la presencia de oligomicina (un inhibidor de la ATP sintasa) aunque no pueda contribuir a la producción de ATP, sugiriendo que la polarización mitocondrial puede proteger, probablemente porque mantiene los niveles de NADPH y GSH reducidos (Jekabsons y Nicholls 2004). El estrés oxidativo también induce el MPTP en mitocondrias aisladas (Saxena *et al.* 1995; Nicholls y Budd 2000). Además, la regulación a la alta de la respiración mitocondrial dependiente de Ca^{2+}, se ha relacionado a un aumento potencialmente perjudicial en la producción de ROS (Duan *et al.* 2007). Nosotros observamos un incremento en la respiración en estado 4 de las mitocondrias aisladas de las ratas perfundidas con AMPA cuando las estimulamos con succinato, lo que podría aumentar la producción de ROS causando estrés oxidativo como consecuencia, lo cual concuerda con nuestra conclusión de que el estrés oxidativo es un evento secundario que sí está participando dada la protección parcial de los antioxidantes.

El incremento del Ca^{2+} intracelular también puede elevar la producción de ROS, induciendo estrés oxidativo, por medio de la activación de la óxido nítrico sintasa (NOS) produciendo óxido nítrico ($NO^{\bullet}$), y de la conversión (por activación de proteasas) de xantina deshidrogenasa (XDH) a xantina oxidasa (XOD), que da lugar a la producción del radical superóxido ($O_2^{\bullet -}$). Estos radicales pueden reaccionar rápidamente con otros y estas reacciones en cadena producen radicales libres y especies altamente reactivas que provocan daño oxidativo a enzimas y otras proteínas, lípidos de membrana y ácidos nucléicos. A su vez, el daño oxidativo a la célula también puede conducir a fallas en los mecanismos para extraer Ca^{2+}, exacerbando la desregulación de Ca^{2+} citoplásmico.

Aun cuando el estrés oxidativo sea un evento secundario, este aumento en la producción de ROS crearía un desequilibrio en el balance normal redox de la célula superando la capacidad de sus defensas antioxidantes, dando lugar a un aumento generalizado de estrés oxidativo y desencadenando una serie de eventos destructivos al producir daño oxidativo a todos los componentes de la célula, siendo parte de la cascada de eventos que conducen al daño neuronal después de la activación excitotóxica de receptores a aminoácidos excitadores (Atlante *et al.* 2001). En estas circunstancias, la administración de los antioxidantes ayuda a desintoxicar los radicales libres altamente reactivos que están por encima de los niveles normales y a evitar el daño resultante, y por eso podemos observar esa protección a las neuronas que aún no se han

dañado por completo a causa de los mecanismos neurodegenerativos iniciales. Tampoco podemos descartar que las cualidades antioxidantes que tienen el piruvato (ya sea administrado directamente o producido intracelularmente tras la administración de lactato), el α-cetobutirato y el β-hidroxibutirato estén contribuyendo también en cierta medida a la protección que ejercen, además de que, como ya se ha discutido, pueden contribuir a la preservación de la homeostasis de calcio intracelular limitando así la producción de radicales libres.

El ascorbato es una vitamina (C) hidrosoluble que se encuentra en todo el cuerpo, es un cofactor en muchas reacciones enzimáticas y un excelente antioxidante. El ascorbato reacciona efectivamente con los radicales superóxido $O_2^{\bullet-}$ e hidroxilo $^{\bullet}OH$ donándoles un electrón, o bien puede regenerar la forma reducida del α-tocoferol. El ascorbato también puede evitar la pérdida de GSH reducido proporcionando protección antioxidante adicional indirecta. Los seres humanos, a diferencia de los roedores y otros animales, no podemos sintetizar la vitamina C endógenamente (Nishikimi *et al.* 1994), por lo que es indispensable consumirla en los alimentos o como suplemento. Las propiedades bioquímicas del ascorbato están vinculadas a papeles protectores críticos en estados normales y patológicos (Kok 1997). Se ha reportado que la vitamina C entra a las mitocondrias y las protege del daño oxidativo (Sagun *et al.* 2005). Concordando con la protección parcial que observamos con el ascorbato en nuestros experimentos, cuando los ratones transgénicos SOD1-G93A se alimentaron con una dieta suplementada con ascorbato desde antes del inicio de los síntomas, éstos sobrevivieron por más tiempo (Nagano *et al.* 2003). Sin embargo, la mejoría fue menor que con otros tratamientos probados en estos ratones; además, no se retrasó el inicio de los síntomas y la función motora se preservó por más tiempo solamente en los ratones tratados con ascorbato + trientina, un quelante de cobre. Es importante considerar que la eficiencia de la membrana de diálisis de la cánula para los compuestos de bajo peso molecular como los utilizados en este trabajo es del 7-11% (Massieu *et al.* 1995). De hecho, la eficiencia de las cánulas de microdiálisis para el AMPA fue previamente evaluada *in vitro* y se encontró que es de 9.5 ± 1.1% (Corona y Tapia 2007). Si consideramos que la eficiencia de la membrana de diálisis es ~10%, en nuestros experimentos las concentraciones administradas fueron aproximadamente 1, 2 y 5 mM, por lo que pensábamos que probablemente las concentraciones intracelulares alcanzadas no fueron lo suficientemente altas para ejercer la actividad antioxidante requerida, tal vez porque el transportador del ascorbato se satura (Spector 2009), y porque la reducción terapéutica de radicales libres puede ser llevada a cabo por el ácido ascórbico solamente a concentraciones fisiológicas muy altas (de al menos 10 mM; Jackson *et al.*

1998). Por esta razón, probamos el efecto neuroprotector del GSH, la principal defensa antioxidante en la célula, que también ejerció una protección parcial. Puesto que existe poca información que caracterice la captación celular de GSH, y como se ha demostrado que la administración exógena de GEE puede elevar la concentración intracelular de GSH, protegiendo a las neuronas contra el estrés oxidativo o el daño mitocondrial crónico (Zeevalk *et al.* 2007), probamos también el GEE. El hecho de que las ratas tratadas con 20 mM de GEE estuvieran mejor protegidas que las tratadas con 50 mM de GEE pudiera deberse a que este compuesto al entrar a la célula libera etanol, por lo que a concentraciones muy altas podría producir cierta toxicidad.

Es muy posible que el estrés oxidativo esté contribuyendo al daño neuronal en la ALS también dadas las evidencias de su participación y debido a que el SNC es particularmente vulnerable a daño oxidativo debido a la gran demanda de oxígeno por su metabolismo aeróbico altamente activo para cubrir su elevado gasto de energía, y especialmente las motoneuronas por su gran tamaño, como se discutió anteriormente. De cualquier manera, todavía es incierto si es causa o consecuencia del proceso degenerativo de las motoneuronas en la ALS, al igual que en otras condiciones neurodegenerativas (Andersen 2004).

Interrelación del daño mitocondrial, déficit energético, estrés oxidativo, excitotoxicidad y desregulación en la homeostasis de calcio, mecanismos que participan en la degeneración de las motoneuronas

Una de las razones por las que aún no está claro cuál o cuáles de los mecanismos involucrados en el proceso de degeneración de la motoneuronas en la ALS son causa y cuáles son consecuencia, es porque estos mecanismos están interrelacionados y unos pueden provocar o resultar de otros. Así, la disfunción mitocondrial, las deficiencias energéticas, el estrés oxidativo, la excitotoxicidad glutamatérgica y las alteraciones en la homeostasis de Ca^{2+} están estrechamente relacionados.

Como ya se mencionó, defectos en el metabolismo energético mitocondrial hacen más susceptibles a las neuronas a niveles normales de glutamato (Beal *et al.* 1993), y hay evidencia abundante *in vitro* e *in vivo* de que cualquier restricción en la capacidad de la célula para generar ATP, puede provocar desregulación en la homeostasis de Ca^{2+} y exacerbar o hasta inducir la excitotoxicidad glutamatérgica (excitotoxicidad indirecta).

Por otra parte, las producción incrementada de ROS tanto en células neuronales como en células no neuronales puede causar fallas en el sistema de recaptura de glutamato de las motoneuronas y de la astroglia (Volterra *et al.* 1994; Trotti *et al.* 1996; Trotti *et al.* 1998; Rao *et al.* 2003; Zagami *et al.* 2009), lo cual resultaría en concentración extracelular de glutamato incrementada, contribuyendo a una condición excitotóxica.

La disfunción mitocondrial puede resultar de o desencadenar otros mecanismos de neurodegeneración propuestos. Las mitocondrias participan en procesos asociados con daño y muerte celular, como son el estrés oxidativo, la excitotoxicidad y la muerte necrótica y apoptótica, desempeñando un papel central en la sobrevivencia y muerte de las neuronas.

La excitotoxicidad mediada por glutamato causa, por medio de la sobrecarga de Ca^{2+}, daño mitocondrial y modificación del estado bioenergético de las mitocondrias. Así, como pudimos observar en nuestro modelo *in vivo* con los resultados obtenidos en este trabajo, ésta provoca notables cambios en la función mitocondrial también *in vitro*, reducción en los niveles de ATP, colapso del $\Delta\psi_m$, una disminución del consumo de oxígeno mitocondrial y celular, y desacoplamiento de la fosforilación oxidativa que preceden a la muerte celular (Ankarcrona *et al.* 1995; Atlante *et al.* 1996; Maus *et al.* 1999). Uno de los principales mecanismos mediante los cuales la concentración intracelular incrementada de Ca^{2+} induce la muerte neuronal es la deficiencia en la función mitocondrial (Beal 1992; Schinder *et al.* 1996; Nicholls y Budd 2000; Manfredi y Xu 2005; von Lewinski y Keller 2005). De hecho, existe un vínculo entre la sobrecarga de Ca^{2+} intracelular inducida por excitotoxicicidad y el colapso de $\Delta\psi_m$, ya que este aumento de Ca^{2+} intracelular y su acumulación en el organelo es suficiente para inducir despolarización mitocondrial prominente y persistente, dando lugar a la disfunción mitocondrial y a la muerte de las neuronas *in vitro* (Schinder *et al.* 1996; White y Reynolds 1996).

El estrés oxidativo puede derivarse de alteraciones en otros mecanismos, así puede ser consecuencia del daño mitocondrial y también de la excitotoxidad. Varios estudios corroboran que hay mayor producción de ROS durante la excitotoxicidad y que el daño oxidativo resultante es un componente importante de la degeneración neuronal (Bondy y Lee 1993; Coyle y Puttfarcken 1993; Lafon-Cazal *et al.* 1993; Dugan *et al.* 1995; Atlante *et al.* 2001).

Por el contrario, el estrés oxidativo puede desencadenar o promover también otros mecanismos deletéreos, como el daño mitocondrial pues los componentes mitocondriales también pueden ser

afectados directamente por ROS, lo cual es relevante porque las mitocondrias son las principales consumidoras de oxígeno y también las principales productoras de ROS.

Además, el daño oxidativo a las mitocondrias y a las proteínas que participan en la extracción del Ca^{2+} de la neurona puede dar lugar a la DCD. La Ca^{2+}-ATPasa de la membrana plasmática es muy sensible a daño oxidativo (Pereira *et al.* 1996), provocando deficiencias en la extracción de Ca^{2+}. Hay evidencia que sugiere que el estrés oxidativo induce alteración de la regulación de Ca^{2+} citoplásmico y muerte neuronal (Coyle y Puttfarcken 1993; Beal 1996; Castilho y Nicholls 1999).

Otra razón por la que no se tiene la certeza de cuál es la causa primordial de la neurodegeneración es porque lo más probable es que no sea un solo mecanismo el que la provoque, sino que tenga que ser la concurrencia de varios de estos factores lo que conduce a la muerte de las motoneuronas.

Las interacciones recíprocas entre estos mecanismos pueden provocar una situación de retroalimentación mutua que va amplificando el daño, sin importar cuál de estos eventos se lleve a cabo primero, creando un círculo vicioso que perjudica a la célula más allá del daño inducido directamente por alguno de los mecanismos. De aquí, que en conjunto eventualmente termine en la muerte neuronal; por esta razón, las estrategias dirigidas a evitar uno o varios de estos mecanismos pueden ayudar a proteger en cierta medida.

No obstante, resulta claro que un punto convergente es la mitocondria, ya que varios de los mecanismos que participan en el proceso de degeneración involucran a las mitocondrias o están directa o indirectamente relacionados con ellas; como son las deficiencias energéticas, la desregulación de Ca^{2+} intracelular, las deficiencias en el transporte axonal de estos organelos, y el proceso de muerte apoptótica. Por lo tanto, las mitocondrias son un objetivo de tratamiento prometedor para combatir esta enfermedad.

Existe un número mínimo umbral de motoneuronas espinales que es capaz de evitar la parálisis y preservar la función motora de las ratas.

La correlación entre el número de motoneuronas sanas y el desempeño motor de los animales en todas las condiciones probadas en nuestro modelo de degeneración excitotóxica de motoneuronas espinales inducido por AMPA *in vivo*, muestra que:

1) Una pequeña diferencia en la pérdida de las motoneuronas resulta en una marcada diferencia en el desempeño de la función motora.

2) Al parecer, existe un umbral en el porcentaje de motoneuronas sanas necesario para prevenir por completo las alteraciones en la función motora del animal, preservar los movimientos de las extremidades posteriores de la rata y evitar su parálisis. En todos los casos en que se protegieron más del 50% de las motoneuronas, se evitaron por completo las alteraciones motoras y la parálisis, mientras que cuando la preservación de las motoneuronas está por debajo de este umbral de protección, el desempeño motor se ve afectado, y cuando la pérdida de motoneuronas es muy severa, se manifiesta con la parálisis total de la extremidad inervada por esa región de la médula espinal. A pesar de las limitaciones metodológicas y del modelo animal, que abarca sólo dos o tres segmentos lumbares de la médula espinal, nos parece justificada esta hipótesis del umbral, pues la hemos podido corroborar con todas las condiciones que hemos probado en este modelo.

VIII. CONCLUSIONES

- ✓ La disfunción mitocondrial y el déficit energético desempeñan un papel crucial en la degeneración de las motoneuronas espinales inducida por la sobreactivación de los receptores tipo AMPA *in vivo*, como lo indica la neuroprotección significativa ejercida por los sustratos energéticos oxidables administrados, y como lo indican también las evidencias que obtuvimos del estudio directo de las características de las mitocondrias aisladas. Aquí se encontraron alteraciones morfológicas, y deficiencias funcionales significativas en la respiración mitocondrial, el RCR, el $\Delta\psi_m$, y las actividades enzimáticas de los complejos I y IV de la cadena de transporte de electrones.

- ✓ Las mitocondrias se dañan desde etapas iniciales del proceso de degeneración excitotóxica de las motoneuronas, puesto que las alteraciones funcionales y deficiencias bioenergéticas, así como las anormalidades morfológicas y estructurales relacionadas, participan de manera crítica desde fases tempranas, lo cual sugiere una relación causal. Esta disfunción mitocondrial provoca deficiencia del metabolismo energético y, subsecuentemente, estrés oxidativo.

- ✓ El estrés oxidativo parece ser un mecanismo menos relevante que, sin embargo, sí contribuye a la muerte de las motoneuronas, dada la protección parcial ejercida por los antioxidantes. Puesto que no hubo incremento en los marcadores de estrés oxidativo en las etapas iniciales de la degeneración de las motoneuronas, éste pudiera ser consecuencia que afecta en etapas posteriores.

- ✓ El piruvato evitó los defectos mitocondriales incluyendo las alteraciones morfológicas - estructurales y bioenergéticas - funcionales provocados por la perfusión de AMPA, indicando que protege a las neuronas principalmente como sustrato mitocondrial estimulando la cadena respiratoria, aumentando la fosforilación oxidativa y la síntesis de ATP, gracias al mantenimiento (y hasta incremento) del $\Delta\psi_m$, preservando la función

mitocondrial. Sin embargo, no podemos descartar que sus propiedades antioxidantes también contribuyan a la neuroprotección.

- ✓ Un panorama similar puede estar ocurriendo en el caso de la ALS, ya que existen evidencias que demuestran la participación tanto del daño mitocondrial como del estrés oxidativo en estudios con pacientes, así como la aparición temprana del deterioro y disfunción mitocondrial también en otros modelos de la enfermedad. El daño mitocondrial y sus consecuencias participan de manera relevante en la degeneración de las motoneuronas, por lo que los sustratos probados podrían ser agentes terapéuticos viables.

- ✓ La correlación entre la sobrevivencia de las motoneuronas y la función motora observada en todas las condiciones probadas en este modelo, sugiere que existe un número umbral mínimo de motoneuronas espinales sanas (de aproximadamente el 50%) necesario para preservar por completo los movimientos de las extremidades posteriores y evitar la parálisis. Cuando no se supera este umbral de protección ocurre un déficit parcial en el desempeño motor, y cuando hay una pérdida severa de motoneuronas se produce inevitablemente la parálisis. A pesar de las limitaciones de nuestro modelo experimental, esto es de gran interés en cuanto al análisis del progreso de la ALS en los humanos y para las potenciales estrategias de tratamientos para esta enfermedad devastadora.

- ✓ Si bien aún se desconoce si la excitotoxicidad es la causa primaria de la degeneración selectiva de las motoneuronas en la ALS, es muy probable que participe en esta enfermedad debido a la especial vulnerabilidad de las motoneuronas a la sobreactivación de los receptores AMPA, a la elevación de Ca^{2+} intracelular, y al hecho de que este tipo de receptores se encuentran abundantemente en la médula espinal. Por lo tanto, los datos obtenidos en este trabajo pueden contribuir al entendimiento de los mecanismos que podrían estar involucrados en la degeneración de las motoneuronas en la ALS.

- ✓ La excitotoxicidad, la desregulación en la homeostasis de Ca^{2+}, la disfunción mitocondrial, las deficiencias energéticas y el estrés oxidativo están íntimamente relacionados y uno puede ser consecuencia o provocar el otro creando un círculo vicioso que conduce a la muerte neuronal. Por esto, y dado que ninguna de las estrategias que hemos probado en nuestro modelo ha evitado por completo la muerte de las motoneuronas, parece ser que los mecanismos que están involucrados en la degeneración excitotóxica de motoneuronas espinales *in vivo* son múltiples. De igual forma, lo más probable es que la causa y los mecanismos que participan en la degeneración de las motoneuronas en la ALS sean multifactoriales.

- ✓ A pesar de que aún no está claro si la disfunción mitocondrial es una causa primaria o secundaria de esta degeneración, no hay ninguna duda de que participa de manera decisiva determinando si las motoneuronas sobreviven o no, y por lo tanto las mitocondrias son un blanco terapéutico esperanzador para el tratamiento de la ALS.

IX. REFERENCIAS

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