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Caracterización del proceso degenerativo de las motoneuronas espinales por excitotoxicidad in vivo.

T E S I S

Que para optar por el grado de

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

P R E S E N T A:

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Índice

I. Resumen

La esclerosis lateral amiotrófica (ALS) es una enfermedad neurodegenerativa caracterizada por la muerte selectiva y progresiva de las motoneuronas (MNs) de la corteza motora, el tallo y la médula espinal, causando un déficit motor progresivo de las regiones afectadas. Existen dos tipos de ALS, la familiar y la esporádica. Algunas de las mutaciones encontradas en pacientes con ALS familiar han sido usadas para el diseño de modelos transgénicos *in vivo*, tal es el caso de la superóxido dismutasa-1 y el TDP-43. Diversos mecanismos han sido involucrados con la fisiopatología de la ALS, entre ellos, se ha propuesto que degeneración de las MNs puede ser causada por estrés oxidativo, alteraciones mitocondriales y falla energética, deficiencias del transporte axonal, incremento de la actividad neuronal o excitotoxicidad mediada por glutamato. Sin embargo, el proceso de degeneración de las MNs aún no se determina, motivo por el cual la presente tesis se enfocó en caracterizar el proceso de degeneración de MNs en dos modelos *in vivo*. Con base en la excitotoxicidad hemos desarrollado dos modelos de degeneración de MNs lumbares *in vivo*. El tratamiento agudo con AMPA 6 mM (50 µL en 25 min; 0.0148 moles) causa la pérdida rápida de MNs desde las 3 h después del tratamiento, mientras que la aplicación crónica con AMPA 7.5 mM (6 µL por día; 0.00213 moles por día) provoca una pérdida gradual de MNs a lo largo de 5 días; en ambos modelos la pérdida de las MNs se asocia con un déficit motriz. En esta tesis se presentan evidencias que indican que el tratamiento agudo con AMPA causa un proceso de necrosis en las MNs, mientras que la aplicación crónica de AMPA desencadena un proceso complejo el cual presenta características apoptóticas tempranas y culmina con una necrosis. Además, el bloqueo farmacológico crónico de la inhibición GABAérgica es suficiente para desencadenar un proceso degenerativo de las MNs lumbares, causando su muerte y un déficit motriz. En conclusión nuestros resultados muestran que la deficiencia de la actividad GABAérgica induce un proceso degenerativo de las MNs, dependiente de la actividad de receptores tipo AMPA. También muestran que el proceso degenerativo

de MNs inducido por la administración crónica de AMPA en animales sanos, presenta semejanza con hallazgos histopatológicos en pacientes con ALS y con procesos descritos en modelos experimentales de esta enfermedad, lo cual es de importancia puesto que apoya a nuestros procedimientos como adecuados para el estudio de los mecanismos involucrados en la degeneración de las MNs espinales en la ALS.

Abstract

Amyotrophic lateral sclerosis (ALS) is a degenerative disease characterized by the selective and gradual death of motor neurons (MNs) in the motor cortex, brainstem and spinal cord, inducing progressive motor deficits according to the affected area. ALS has been classified as familial or sporadic. Mutations found in familial ALS-patients have been used to generate *in vivo* models of familial ALS, for example: mutations in the superoxide dismutase-1 and TDP-43. Several pathophysiological mechanisms have been associated with MN degeneration, for instance: oxidative stress, mitochondrial alterations and energetic failure, axonal transport alterations, increase of the neuronal activity or glutamate mediated excitotoxicity. However, the process of MN-degeneration still unknown, therefore this thesis was focus on characterized the degenerative process of MNs in two models *in vivo*. Based on the excitotoxic hypothesis, we have designed two experimental models of MN-degeneration *in vivo*. The acute administration of AMPA 6 mM (50 µL in 25 min) causes rapid MN loss, 3 h after the treatment, while the chronic treatment (AMPA 7.5 mM, 6 µL per day) induces a progressive MN loss along five days, with both models causing motor deficits. This thesis presents experimental evidences showing that the acute treatment with AMPA induces MN loss through necrosis, while the chronic treatment triggers a more complex process, involving early apoptotic characteristics and late necrotic appearance. On the other hand, this work also shows that the pharmacological and chronic GABAergic blockade causes MN degeneration and motor deficits. Taking together, our results show that GABAergic blockade in

the lumbar spinal cord is enough to induce degeneration and MN loss with motor deficits by activation of AMPA receptors. Also, that MN degenerative process by chronic AMPA treatment in healthy animals presents close similarity with clinic evidences and with alterations in experimental models of ALS, which is important, because support our procedures like a models for the study of MN degeneration in ALS.

II. Lista de abreviaturas.

ALS: amyotrophic lateral sclerosis, usado para referirse a la esclerosis lateral amiotrófica

AMPA: ácido α-amino-3-hidroxil-5-metil-4-isoxazol- propiónico

GABA: ácido γ-amino butírico

MN: motoneurona

NMDA: N-Metil D-aspartato

PGE: Paw grip endurance test

III. Organización de la tesis

El presente trabajo se encuentra dividido en ocho secciones: Introducción, Antecedentes, Planteamiento del problema, Objetivos, Métodos, Resultados, Discusión y Conclusión.

La Introducción abarca un marco teórico enfocado en las generalidades de la ALS y la médula espinal que es la estructura principalmente afectada, y a que contiene a las MNs cuya pérdida da origen a la enfermedad.

Debido a que la presente investigación se enfocó en caracterizar el proceso degenerativo de las MNs durante una condición de excitotoxicidad, incluimos en la sección de antecedentes el capítulo "*Role of Mitochondrial Dysfunction in Motor Neuron Degeneration in ALS*" del cual soy coautor, y abarca los principales mecanismos involucrados en la degeneración de estas células en la ALS. Como parte de los antecedentes y dado que este proyecto también se enfocó en el estudio de los circuitos inhibitorios y su posible participación en la degeneración de las MNs, incluimos la revisión "*Spinal inhibitory circuits and their role in motor neuron degeneration*", de la cual soy primer autor y describe las alteraciones de las redes neuronales dentro del contexto de la degeneración de MNs. Enseguida se presenta el planteamiento del problema y se enlistan los objetivos del estudio. A continuación se describen los métodos empleados para el desarrollo del proyecto.

En la primera parte de resultados se presenta el artículo "*Neuropathological characterization of spinal motor neuron degeneration processes induced by acute and chronic excitotoxic stimulus in vivo*" en el que describimos detalladamente los cambios ultraestructurales y bioquímicos la degeneración de las MNs en nuestros modelos de excitotoxicidad espinal. En la segunda sección de resultados presentamos el artículo (en revisión) "*Chronic GABAergic blockade in the spinal cord in vivo induces motor alterations and neurodegeneration*", en el cual describimos el efecto del bloqueo agudo y crónico de los sistemas de inhibición de la médula espinal, así como su efecto al combinarse con la excitotoxicidad mediada por la activación de receptores AMPA.

En la discusión se hace una integración de los hallazgos del presente trabajo, centrándonos en la importancia del proceso de muerte celular, para el diseño de nuevas estrategias farmacológicas para la ALS, así como en la relevancia de considerar el estudio de la parte inhibitoria en la degeneración de las MNs. Para terminar, en la sección de Conclusión recalco la importancia de considerar a la ALS como una enfermedad multifactorial y la relevancia de conocer los diferentes componentes involucrados para poder generar estrategias integrales que sirvan para el tratamiento de esta enfermedad.

IV. Introducción

La médula espinal

La médula espinal es la parte del sistema nervioso central que se encuentra alojada en el conducto raquídeo desde el agujero magno, en la parte media arquial del atlas hasta la primera o segunda vértebra lumbar. Se encarga de comunicar al encéfalo con el cuerpo mediante impulsos nerviosos. Entre sus funciones también se encuentran el control de los movimientos inmediatos y vegetativos, como el arco reflejo y las funciones de los sistemas simpático y parasimpático. Su longitud varía ontogenéticamente, en el embrión llega hasta la base del coxis, en el recién nacido llega a la cuarta vértebra lumbar, dos vértebras por debajo que en el adulto. En la etapa adulta, desarrollada completamente, puede alcanzar una longitud de 43 cm en mujeres y 45 cm en hombres (Watson et al., 2009).

Anatomía y función

Longitudinalmente la columna vertebral, que aloja a la médula, se divide en: cervical, torácica, lumbar, sacra y coxígea. En la rata está compuesta por 34 segmentos: 8 cervicales, 13 torácicos, 6 lumbares, 4 sacros y 3 coxales; en humanos la columna difiere un poco, ya que presenta 7 cervicales, 12 torácicos, 5 lumbares, 5 sacros y uno coxígeo, en total 33 segmentos. El grosor de la médula varía dependiendo la cantidad de fibras que lleven sus tractos. Básicamente la médula presenta dos ensanchamientos, uno cervical y otro lumbar. A nivel cervical, la médula se encuentra notablemente aplanada en sentido anteroposterior formando el ensanchamiento cervical, en roedores se extiende desde C5-T1, las raíces que surgen de este engrosamiento están formadas por los nervios que transmiten la sensibilidad y la activación de los miembros superiores (brazos, antebrazos y manos). A nivel lumbar, la médula se vuelve a engrosar desde L2 a L6 en roedores y de L2 a S2 en humanos, este ensanchamiento contiene los nervios que transmiten la sensibilidad e impulsos motores desde y hacia las extremidades posteriores (muslos, piernas y pies). En su parte superior la médula espinal se encuentra en continuidad con el

bulbo raquíde o, en ta nto que la parte caud al se adelgaza para f ormar el cono medular, la s raíces de los seg mentos l umbares, sacros y coxígeo f orman la ca uda equina, hasta unirse al periostio del dorso del coxis (Watson et al., 2009).

Al igual que el e ncéfalo, la médula espi nal se enc uentra cubierta por tr es meninges (Figura 1A): la pia madre, la ara cnoides y la duramadre. L a dura madre es la meninge mas externa y dura, la arac noides es la membrana intermedia, delgada y permeable, y la pia madre es la m embrana m as interna. E ntre l a pia madre y la aracnoides se encuentra el espacio s ubaracnoideo, que e s el lu gar en que se encuentra el líquido cefalorraquídeo (Guyton, 1994, Watson et al., 2009).

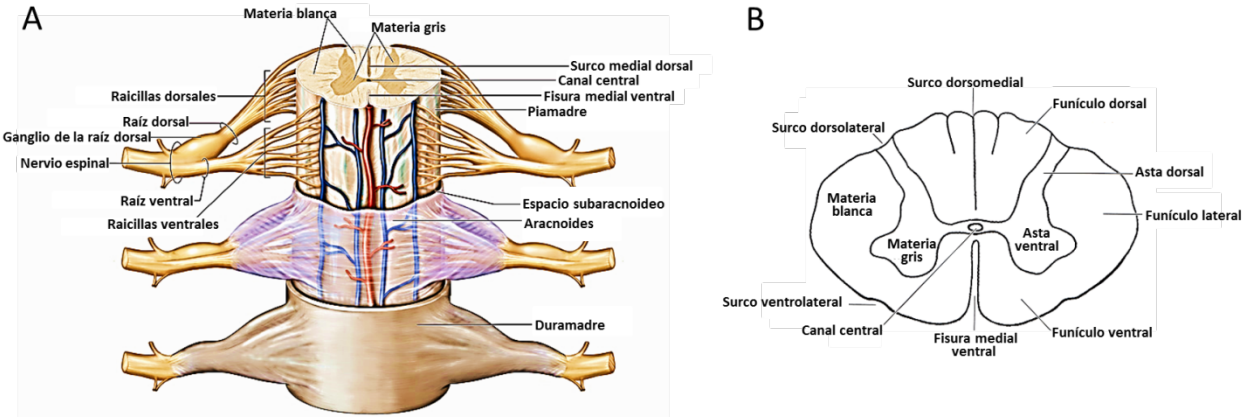


Figura 1. Anatomía de la médula espinal. A) Esquema de la médula espinal en que se aprecian las meninges que la cubren, así como las raíces dorsales y ventrales. En B se ilustra un corte transversal de la médula espinal indicando los principales surcos, funículos y áreas que la conforman.

Observando transversalmente un corte de la médula se pueden apreciar cuatro regiones, una ventral, dos laterales y una dorsal. La ventral presenta la fisura media ventral y limita lateralmente por los surc os ventrolaterales, Así mismo, la superficie dorsal en la par te media presenta el s urco medial dorsal. E n las pa rtes laterales se encuentran los funículos que limitan con los surcos ventrolaterales y dorsolaterales. Transversalmente es posible distinguir la materia blanca en la parte m as externa y la materia gris en la parte mas interna en un arre glo en f orma de mariposa o letra “H” (dependiendo el segmento observado). La materia gris de la barra horizontal de la H es l lamada materia gri s comisural y rode a al canal central, las pro yecciones de los brazos de la H forman las llamadas astas ventrales y dorsales (Figura 1B).

El flujo sanguíneo de la médula espinal es suministrado por una sola arteria ventral y dos dorsales. La arteria espinal ventral es originada de la arteria vertebral y desciende dentro de la fisura medial ventral. Ambas arterias espinales dorsales se originan de la arteria ventral y descienden por el surco dorsolateral.

Las motoneuronas (MNs) se encuentran ubicadas en el asta ventral de la sustancia gris a lo largo de toda la médula espinal. Funcionalmente, podemos encontrar dos tipos de MNs: las somáticas y las viscerales, las primeras inervan a músculos esqueléticos y las segundas a vísceras. Con base en su tamaño las MNs somáticas pueden clasificarse como alfa, gama y beta, cada una da origen a un tipo de fibra nerviosa llamadas alfa, beta o gama, según la MN que las origine. Las alfa inervan principalmente a las fibras musculares extrafusales del músculo esquelético, las gama a fibras musculares intrafusulares dentro de los husos musculares y las beta inervan principalmente a músculos distales de las extremidades. Un grupo muy particular de MNs somáticas que no son afectadas en la ALS (Celio, 1990, Ince et al., 1993, Siklos et al., 1998) son las que conforman el núcleo de Onuf, que se encuentran en la región caudal lumbar y rostral sacra, controlan los músculos perineales a los esfínteres anal, uretral y al cremaster. Otro aspecto importante de la organización de la médula espinal es la existencia de interneuronas que sirven como conexión entre las fibras sensoriales y las motoras, permitiendo las respuestas reflejas, pero también pueden estar conectadas con las MNs formando redes neuronales intraespinales; en la sustancia gris de la médula las interneuronas son 60 veces mas abundantes que las MNs (Watson et al., 2009).

Circuitería de la médula espinal.

La actividad de la médula espinal está determinada por la acción de diversos neurotransmisores, dentro de los cuales para la parte excitadora el de mayor importancia es el glutamato, y para la parte inhibidora son el GABA (ácido gama amino butírico) y la glicina. El balance de estos dos sistemas de neurotransmisión es de suma importancia, ya que un incremento de la parte excitadora o bien un

decremento de la inhibidora puede desencadenar un evento de hiperexcitabilidad que lleve a procesos deletéreos para las neuronas.

Tanto el GABA como la glicina activan a un receptor canal que forma parte de la superfamilia de receptores pentaméricos, dentro de los cuales se encuentran los receptores tipo 3 para serotonina, los receptores para GABA tipo C, los receptores de unión a Zinc y los receptores para cationes de unión a GABA (Beg y Jorgensen, 2003, Davies et al., 2003, Lynch, 2004, Lynch, 2009), sin embargo tanto los receptores para GABA tipo A y los receptores para glicina permiten el flujo de cloro. El principal receptor postsináptico para GABA es el de tipo A o GABAA (Foster y Kemp, 2006), mientras que el de glicina solamente es conocido como receptor para glicina. Ambos receptores están presentes en la densidad sináptica de las MNs lumbares y cervicales y en menor porcentaje en sitios extrasinápticos (Sur et al., 1995, Murphy et al., 1996).

El receptor GABAA es un complejo molecular integrado como heteropentámero de entre 275 a 400 kDa (Macdonald y Olsen, 1994, Hevers y Luddens, 1998). Puede estar formado por combinaciones de los subtipos de las subunidades alfa (α 1-6), beta (β 1-6), gama, delta, épsilon, rho y theta. La combinación de las diversas subunidades puede modular la actividad del receptor. En la médula espinal los receptores GABAA están formados principalmente por las combinaciones $\alpha2\gamma2$ o $\alpha2\beta3\gamma2$ (De Blas, 1996, Foster y Kemp, 2006). Los receptores para glicina están formados por cinco subunidades, que pueden ser α1-4 y β. Este receptor puede estar asociado a la gefirina que es una proteína de anclaje para estos receptores. Se sabe que en adultos la mayor asociación entre subunidades corresponde a la combinación $\alpha1\beta$. En las MNs espinales, los transcritos para las subunidades $\alpha1$ y $\alpha2$ se encuentran en el soma y las dendritas, mientras que los transcritos para la subunidad β y gefirina, se restringen al soma (Rekling et al., 2000, Alvarez, 2016).

Los receptores para glicina y los GABAA se acumulan en los sitios postsinápticos para permitir una neurotransmisión inhibidora rápida y eficaz. Su agregación y estabilización requiere de su interacción con un complejo multiproteíco para anclarse al citoesqueleto (Legendre, 2001). Estos receptores pueden mostrar diferentes propiedades funcionales dependiendo de su distribución subcelular. La acumulación de estos receptores se ve delimitada por su capacidad para unirse a la gefirina (Tretter et al., 2008), que es una proteína periférica de membrana. La cantidad de gefirina ha sido correlacionada con grandes corrientes postsinápticas inhibitorias (Oleskevich et al., 1999, Lim et al., 2000, Geiman et al., 2002, Alvarez, 2016). El hecho de que la gefirina estabiliza y une a los receptores de GABA y de glicina, sugiere que el GABA y la glicina pueden estar siendo coliberados como cotransmisores. En el tronco cerebral se ha demostrado la coliberación de GABA y glicina desde la misma terminal. En la médula espinal GABA y glicina se encuentran continuamente colocalizados en terminales de interneuronas inhibidoras (Ornung et al., 1994, Lahjouji et al., 1996, Dumba et al., 1998), en donde son ingresados a las vesículas presinápticas por el mismo transportador (Burger et al., 1991). Interesantemente en edades postnatales tempranas (P5), estudios electrofisiológicos en sinapsis mixtas para GABA y glicina en MNs del núcleo hipoglosal, demuestran que las corrientes postsinápticas mediadas por los receptores el receptor GABAA presentan mayor facilitación y sumación, comparadas con las corrientes mediadas por el receptor para glicina, las cuales presentan menor facilitación (Donato y Nistri, 2001), sugiriendo un papel funcional diferente para los dos neurotransmisores.

La proporción de sinapsis GABAergicas y glicinérgicas en la médula espinal varía dependiendo del estado de madurez del organismo. En MNs embrionarias (E17-E18), 60% son únicamente para GABA, 20% para glicina y 20% sinapsis mixtas; después de nacer en etapas postnatales (P1-P3) los porcentajes son: 60% glicinérgicas, 20% GABAérgicas y 20% sinapsis mixtas (Singer y Berger, 2000, Gao et al., 2001). Se estima que de los eventos cuánticos regitrados en MNs de ratas

en el día po stnatal 5 -10, aproxi madamente el 44% corre sponde a un co mponente dual de GABA-glicina, el 41% solo a glicina, y el 17% a GABA (Jonas et al., 1998).

El papel f uncional que desempeñan estos neurotransmisores en l a médula espinal ha sid o un te ma controversia l. Actual mente no exist e un co nsenso para est e punto, pero s e han des arrollado trabajos que permiten hacer aproxi maciones sobre este te ma. Se ha de mostrado que d urante el desarrollo embrionario, el GABA y la glicina f uncionan co mo neurotrans misores e xcitadores, ju gando un papel de gran relevancia para la maduración de las redes neuronales que conf orman el sistema nervioso (Ben-Ari et al., 2007). En organis mos adultos , el GABA y la glicina median la inhibici ón de la actividad de las MNs, contribu yen a la generación de patrones rítmicos de actividad, incluyendo la r espiración (Merrill y Fedorko, 1984), la locomoción (Cazalets et al., 1996), masticación (Inoue et al., 1994) y la deglución (Bieger, 1991). En estos patron es, la activid ad de las MNs se ca racteriza por f ases secuenciales de exci tabilidad e inhibici ón. El control inhibit orio durante la f ase activa puede afectar el tiempo de inicio y final de los trenes o ráf agas de potenciales de acción, afectando el inicio de la contracción y la relajación muscular, los patrones de disparo y el or den de recluta miento (Parkis et al., 1 999, Rekling et al., 20 00). Registros de la ac tividad eléctrica espinal en ratones (E 11 -12.5), indican qu e la transmisión glicinér gica f avorece la propaga ción episódic a a trav é s de seg mentos espinales con tiguos, mientras que l a gener ación de la activi dad sináptica a niv el local se bas a en r edes neuronales formadas p or MNs e inter neuronas GABAérg icas (Hanson y La ndmesser, 2003, Moody y Bosma, 2005). Dentro de l as inter neuronas inhibidoras, la más estudiada es la lla mada c élula de Renshaw, ide ntificada por vez primera en la médula espin al d el gato adulto por Re nshaw. Esta s interneur onas reciben af erencias acetil colinérgi cas de las MNs (Mentis et al., 2 006) y rea lizan predominantemente pr oyecciones glici nérgicas hacia las MNs (Goulding et al. , 2002). S e ha s ugerido que la inhibici ón ap ortada por esta s inter neuronas se da después de su maduración, la cual ocurr e postnatalmente, desarro llando grande s agregados de gefirina (Gonzalez-Forero y Alvarez, 2005).

Por otra parte, la excitabilidad de la médula espinal está controlada por el neurotransmisor glutamato, cuya acción rápida depende de tres receptores ionotrópicos postsinápticos: los receptores tipo AMPA (ácido α-amino-3-hidroxil-5-metil-4-isoxazol- propiónico), los tipo kainato y los tipo NMDA (N-Metil D-aspartato). Estos receptores son tetrámeros conformados por diversas subunidades: GluR 1-4 para los AMPA, subunidades GluR5-7, KA1 y KA2 para los kainato, y las subunidades NMDAR1, NMDAR2A-D y NMDAR3A para los NMDA (Schoepfer et al., 1994, Bettler y Mulle, 1995, Michaelis, 1998). La formación y degradación del glutamato son parte del metabolismo energético, ya que este aminoácido se sintetiza principalmente a partir de la glucosa. El metabolismo del glutamato en el sistema nervioso ocurre en ciclos coordinados entre las neuronas y las células gliales. Una de vía para la obtención de este neurotransmisor es la conversión del α-cetoglutarato a glutamato por transaminación, una alternativa a la vía anterior es la conversión de glutamina a glutamato mediante la acción de la glutaminasa. La glutamina se sintetiza en la glía teniendo como precursor al glutamato recapturado, este glutamato es convertido a glutamina por amidación mediante la glutamina sintetasa; posteriormente, la glutamina es liberada y capturada por las neuronas, quienes la convierten en glutamato, constituyendo el ciclo de glutamato-glutamina (Zhou y Danbolt, 2014).

La acción del glutamato se ha relacionado con diversas funciones tales como: la cognición, memoria y aprendizaje, así como en la sinaptogénesis, organización de los circuitos neuronales, plasticidad sináptica, migración y diferenciación celular. Sin embargo, es bien sabido que la sobre estimulación de la neurotransmisión excitadora de glutamato puede inducir procesos degenerativos, ya que genera una hiperexcitabilidad neuronal, que provoca un influjo masivo de calcio en las neuronas, desencadenando procesos deletéreos que culminan con la muerte de las neuronas (Arundine y Tymianski, 2003). Este fenómeno es denominado excitotoxicidad mediada por glutamato y ha sido involucrado en varias patologías neuronales, por ejemplo: el accidente cerebrovascular, los traumatismos y la

epilepsia; de igual manera se ha relacionado con diversos trastornos neurodegenerativos, como en la enfermedad de Parkinson, la enfermedad de Alzheimer y la ALS (Lipton y Rosenberg, 1994, Meldrum, 2000, King et al., 2016).

Los tres tipos de receptores inotrópicos para glutamato han sido descritos en las MNs. Sin embargo, su abundancia relativa no es la misma: los AMPA son los más abundantes, después los NMDA y finalmente los kainato. La entrada de calcio en las MNs espinales es mediada por los receptores AMPA, particularmente se sabe la permeabilidad para este catión está determinada por la sub unidad GluR2, ya que si los receptores AMPA no contienen o presentan la GluR2 sin editar, serán permeables a calcio, pero si la GluR2 es modificada postranscripcionalmente en un residuo de glutamina (ubicada en el segundo dominio transmembranal) sustituyéndola por una arginina, el receptor será impermeable a calcio (Burnashev et al., 1992, Corona y Tapia, 2007, Corona et al., 2007).

Las señales motoras se comunican a las MNs espinales desde la corteza motora, a través del tracto corticoespinal. Aunque todos los mamíferos poseen esta estructura, existen variaciones considerables en la posición del tracto en la médula espinal. En roedores, se ubica en la columna dorsal, en los primates en la columna lateral. En los primates la mayoría de las fibras del tracto provienen directamente de la corteza motora. Las fibras que originan al tracto corticoespinal en mamíferos adultos se derivan de las neuronas piramidales de la capa 5 de las áreas motora, premotora y somatosensorial (Watson et al., 2009).

La inervación del tracto corticoespinal sobre las MNs se ha demostrado de diversos modos, por ejemplo, *in vitro* se ha probado la participación de neurotransmisores excitadores en el inicio de la actividad semejante a la locomoción (Jordan et al., 2008), y se ha demostrado que la administración intratecal de antagonistas glutamatérgicos bloquea la locomoción inducida por la estimulación eléctrica de la región locomotora mescencefálica del cerebro medio del gato (Douglas et al., 1993). De la misma manera, la administración de NMDA induce el inicio de la locomoción en gatos (Chau et al., 2002). En la rata también se ha

demostrado que la estimulación eléctrica del funículo dorsal, que contiene axones corticoespinales descendentes, desencadena potenciales postsinápticos excitadores, que son bloqueados por CNQX, un antagonistas de receptores glutamatérgicos de tipo AMPA (Hori et al., 2002). Además de las inervaciones del tracto corticoespinal y de las fibras sensitivas, cuya acción no involucra necesariamente el paso por el cerebro, existen interneuronas glutamatérgicas que se encuentran en estrecha relación con las MNs, asociándose en la formación y control de la actividad de circuitos premotores (Hagglund et al., 2010).

No obstante, a pesar de no ser descritas a detalle por fines prácticos, es importante señalar que la actividad de las MNs no solo es controlada estrictamente por GABA/glicina y glutamato, sino que en realidad la integración que estas células realizan es mucho mas compleja, ya que también pueden recibir aferencias de otros neurotransmisores y moléculas como la serotonina, la norepinefrina, la hormona liberadora de tirotropina, adenosina, neurocininas, vasopresina e inclusive la acetil colina (Zagoraiou et al., 2009, Ramirez-Jarquin et al., 2014).

Esclerosis Lateral Amiotrófica

La médula espinal es susceptible a desarrollar enfermedades, en este caso las más frecuentes son la poliomielitis, el síndrome de la cola de caballo, la siringomielina y la ALS. Para el presente proyecto, la patología de mayor interés es la ALS, por ello describiremos brevemente las principales características de esta enfermedad.

La ALS también conocida como enfermedad de Lou Gehrig, fue descrita en 1869 por Jean Martin Charcot. Es caracterizada por la muerte selectiva y progresiva de las MNs superiores e inferiores, produciendo una parálisis según la región afectada. Se conocen dos tipos de ALS, la de tipo familiar que abarca solo 10% de los casos clínicos. Esta se asocia con diversas mutaciones, entre las más conocidas mutaciones de la superoxido dismutasa 1 y de la TDP 43. Por otra parte la ALS de tipo esporádico comprende el 90% de los casos clínicos y para la cual no se ha identificado un factor que determine su aparición. En ambos tipos de ALS los

síntomas son práctic amente lo s mismos, la progres iva muerte de MNs genera espasticidad, f asciculaciones y pará lisis. A dif erencia de otras enf ermedades neurodegenerativas co mo el Alzh eimer y la enf ermedad de Huntingto n, los pacientes con ALS n o presentan deter ioro del proceso cognitiv o, sin em bargo, algunos pa cientes pueden llegar a presentar c ambios de persona lidad e irritabi lidad (Phukan et al., 200 7). El diagn ostico de es ta enfermedad se re aliza de acuerd o con los criter ios d e "El S corial" y de "The Int ernational Classif ication of Diseases ". Generalmente los paci entes diag nosticados con ALS mueren de tr es a cinco años después del inicio de l os sínto mas, debido a una f alla respiratoria . Actual mente el único tratamiento aprobado para esta enfermedad es el riluzol, cuyo uso prolonga la supervivencia de las p ersonas por un peri odo de tres a s eis meses. La prevalenc ia mundial de A LS es de 2 -8 en 100 000, y su aparición suel e asociar se con la edad, pero no es u na regla ya q ue puede comenzar en edades te mpranas (Chancellor y Warlow, 1992 , Corona et al. , 200 7, Al-Chalabi et al., 2016). Existen d iversos mecanismos asociados a la dege neración de las MNs, los cuales serán tratados en la siguiente sección de Antecedentes.

V. Antecedentes

Excitotoxicidad y mecanismos de degeneración de las motoneuronas por sobreactivación de receptores AMPA

Los mecanismos que causan la ALS no son completamente conocidos. Sin embargo, algunos pro cesos cel ulares y moleculares qu e conducen a l a dege neración de la s MNs han sido i dentiicados, por eje mplo: el estrés ox idativo, la pérdida de la homeostasis del calcio, la agregación de pr oteínas, la excitotoxicidad, la necrosis y la apoptosis.

El estrés oxidante pue de ser generado por una producción incre mentada de especies r eactivas de o xígeno y radicales l ibres o b ien p or u na dis minución de l os mecanismos antioxidantes de las células (Davies, 2000). En la ALS este mecanismo es de i mportancia deb ido a q ue den tro de las mutaciones mas est udiadas está n las

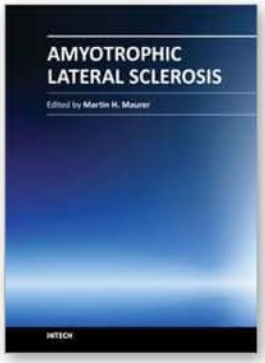
descritas para la superoxido dismutasa tipo 1, cuya función normal es la catalizar la conversión de aniones superoxido a peróxido de hidrógeno. Se han propuesto diversas alteraciones para la función de esta enzima, como es una deficiencia en su capacidad antioxidante, también que en la SOD1 mal plegada el peroxinitrito puede acceder a su sitio catalítico conduciendo a la nitración de residuos de tirosina, en ambos casos estos posibles cambios en su actividad catalítica pueden resultar tóxicos para las MNs (Beckman et al., 1993, Brown, 1995, Wiedau-Pazos et al., 1996, Julien, 2001). El estrés oxidante puede dañar diversas moléculas como ácidos nucleicos, lípidos y proteínas (Valko et al., 2007) y puede involucrarse con la inducción de procesos de apoptosis, autofagia y necrosis.

La homeostasis del calcio es preservada mediante el control de su entrada y salida de la célula, su amortiguamiento y de su almacenaje intracelular. Debido a que este catión controla diversas vías intracelulares, el control de su concentración citosólica debe ser muy estricto. Un exceso de su concentración conlleva a la activación inadecuada de procesos que pueden alterar el metabolismo celular y eventualmente desencadenar procesos de muerte celular (Arundine y Tymianski, 2003). Dentro de las proteínas activadas por calcio se encuentran las nucleasas, proteasas, lipasas y fosfatasas dependientes de calcio (Bano y Nicotera, 2007), el excesivo almacenamiento de este catión en las mitocondrias puede generar su hinchamiento, causando la pérdida del potencial de la membrana mitocondrial y consecuentemente la apertura del poro de transición mitocondrial, lo cual puede llevar a la activación de mecanismos de muerte celular.

La agregación de proteínas mal plegadas es un fenómeno común en las enfermedades neurodegenerativas, que puede ocurrir por mutaciones de las proteínas agregadas o por modificaciones postraduccionales. Aun cuando no se sabe con certeza si la agregación de proteínas induce la muerte neuronal, o por el contrario participa en mecanismos de protección, se asocian dos posibles componentes celulares del control de la calidad de las proteínas: la actividad de las chaperonas y de la vía de la ubiquitina-proteosoma. (Berke y Paulson, 2003). En la ALS, se ha

encontrado la acumulación de neurofilamentos en axones, lo cual se relaciona con la degeneración de las MNs causada por la estrangulación del transporte axonal. En modelos transgénicos de la ALS, se ha demostrado que la reducción de la expresión de neurofilamentos pesados y ligeros aumenta la esperanza de vida de ratones que expresan la SOD 1 mutada (Julien, 2001).

La excitotoxicidad involucrada como mecanismo de degeneración de las MNs ha sido ampliamente estudiada, debido a la evidencia que se ha encontrado en pacientes con ALS y en modelos transgénicos de esta enfermedad. Esta excitotoxicidad es debida al incremento del glutamato extracelular. A continuación se incluye el capítulo "*Role of Mitochondrial Dysfunction in Motor Neuron Degeneration in ALS*" publicado en el libro "Amyotrophic Lateral Sclerosis", ISBN 978-953-307-806-9, de la editorial InTech, en el cual se discuten con mayor detalle los mecanismos involucrados en la degeneración de las MNs en la ALS.



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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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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).

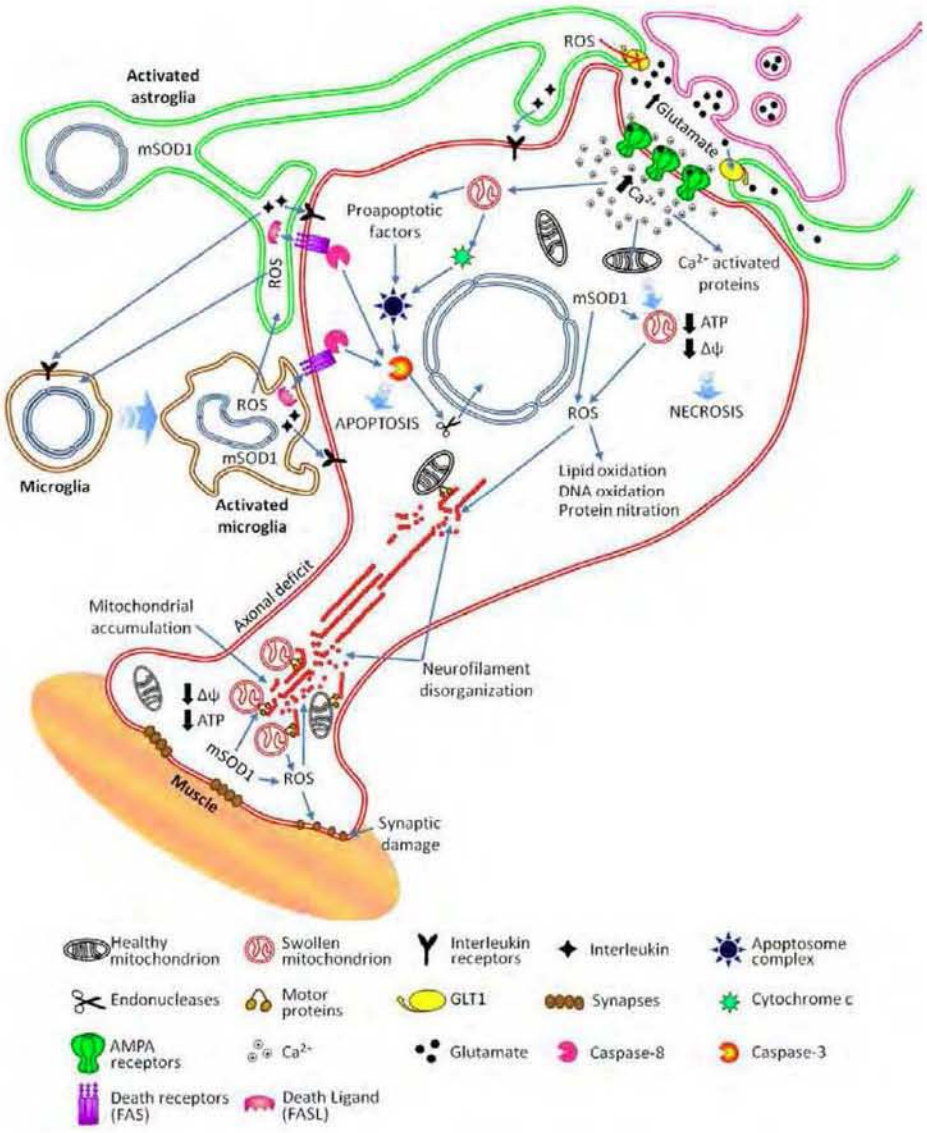

Fig. 1. Scheme of the main proposed mechanisms involved in motor neuron death. Description in the text.

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.

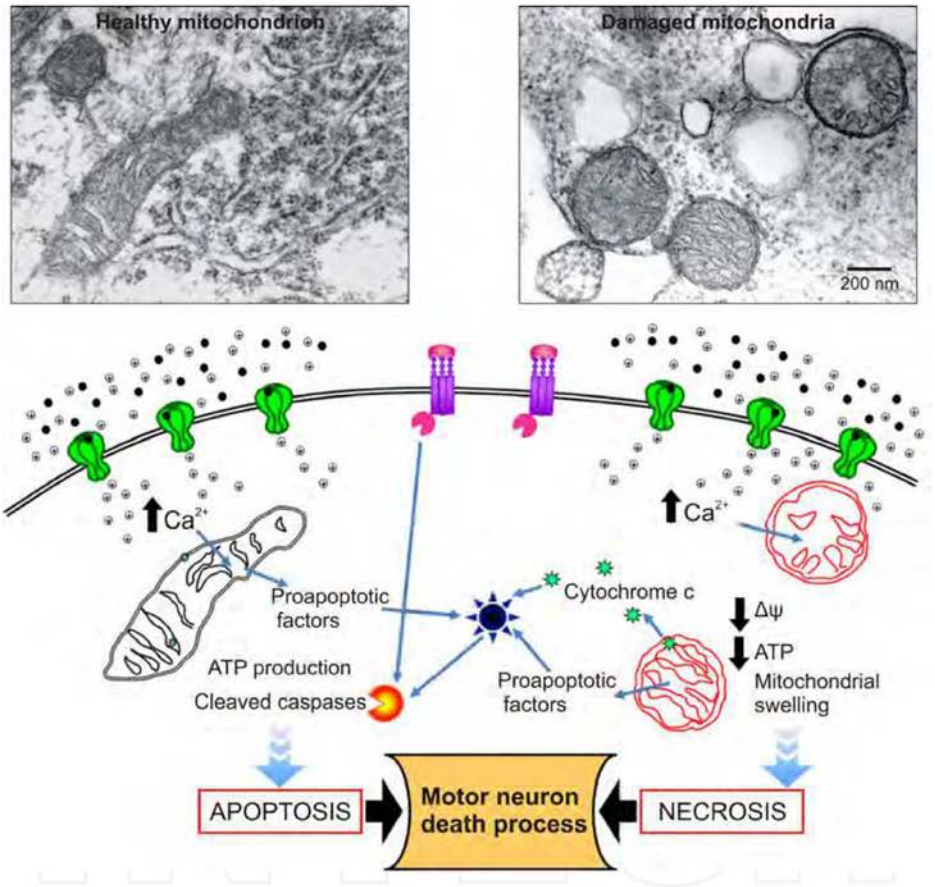

Fig. 2. Role of mitochondrial damage in motor neuron excitotoxicity. The electron-micrographs show normal mitochondria and endoplasmic reticulum in a spinal motor neuron of a control rat (left), and swollen mitochondria with altered cristae observed 2 h after perfusion of AMPA by microdialysis (right) (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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INTECH

Retomando a la excitotoxicidad como un mecanismo que causa la degeneración de las MNs, en el laboratorio se han desarrollado dos modelos de degeneración espinal lumbar, los cuales causan la muerte de las MNs y subsecuentemente, parálisis por la sobreactivación de los receptores AMPA. Las principales diferencias entre ambos modelos son: el método de administración del AMPA y el tiempo en el que se presentan los déficits motrices. En el modelo agudo la microdiálisis del AMPA 6 mM (50 µl en 25 min) en la médula espinal, induce, en tan solo tres horas, la muerte de las MNs y la parálisis de la extremidad inervada por estas (Corona y Tapia, 2004). En el modelo crónico, se emplean minibombas osmóticas como vía de administración de AMPA 7.5 mM. La muerte de las MNs y sus consecuencias ocurren de manera progresiva a lo largo de varios días (Tovar-y-Romo et al., 2007).

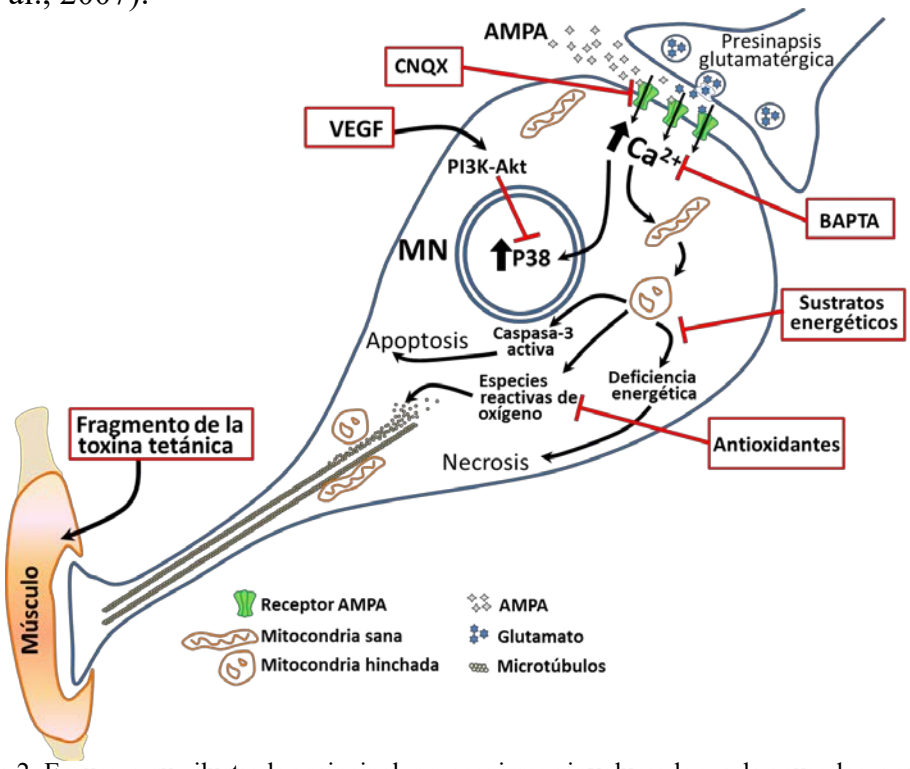

Figura 2. Esquema que ilustra los principales mecanismos involucrados en la neurodegeneración de las MNs, señalando con recuadros las estrategias de neuroprotección que se han utilizado en nuestro laboratorio.

Utilizando nuestros modelos para el estudio de los mecanismos de degeneración de las MNs se han desarrollado estrategias de neuroprotección, enfocadas en combatir alguno de los mecanismos descritos en el capítulo presentado

(Figura 2) . Estas estr ategias han usado agentes antioxidan tes como m edio de protección, sustratos energéticos (Corona y Tapia, 2004, Netzahualcoyotzi y Tapia, 2014, Santa-Cruz y Tapia, 2014, Santa-Cruz et al., 2016), también factores tróficos como el VEGF, el cual protegió incluso después de iniciado el proceso excitotóxico (Tovar-y-Romo et al., 2007, Tovar-y-Romo y Tapia, 2010, 2012).

Alteraciones de los circuitos inhibitorios en la ALS

El presente estudio también se enfocó en el papel de los circuitos inhibitorios en la degeneración de las MNs espinales, por ello como parte final de la sección de antecedentes incluimos la revisión "*Spinal inhibitory circuits and their role in motor neuron degeneration*", publicado en la revista *Neuropharmacology*, 2014, (82): 101-107, la cual se enfoca en el estudio y descripción de las redes neuronales espinales y sus alteracion es en la ALS , alteracio nes de scritas en modelos ex perimentales y también en la clínica, pero que han sido poco estudiadas.

Resumen de este trabajo

En la m édula espinal la actividad neuronal es controlada por el b alance entre las neurotransmisiones ex citadoras e inh ibidoras, mediadas p rincipalmente por los neurotransmisores glu tamato y GABA/glicina, respect ivamente. Cuando este equilibrio se afecta, ya sea por un incremento de la activi dad glutamatérgica o bie n por un decre mento de la actividad GABA érgica, el resultado es el incre mento de la excitabilidad neuronal, el cual puede llega r a desencadenar degener ación mediante procesos de exc itotoxicidad, causan do muerte neur onal y daño f uncional según el área af ectada. Altera ciones de este equ ilibrio han sido a sociadas con la hiperexcitabilidad de l as MNs espi nales y s u degeneració n en la ALS. En esta revisión se aba rcan las generalidades de las r edes neuronal es de las astas ventrales , así co mo l a posib le i mplicación de los cir cuitos inhibid ores en la de generación de las MNs en ambos tipos de ALS. Uno de los principales componentes de los circuitos espinales son las interneuronas, las cuales según la expresión de factores de transcripción se clasifican como: dI1 a dI6, V 0 a V3, VM W, muchas de estas clases

son inter neuronas exc itadoras y solo las c lases V2 b, V0 $_{C/G}$, V 0_D y V 0_V son inhibidoras. Dentro d e la médula, interneuronas y n euronas de pro yección se organizan en r edes locales lla madas centros organizadores de patro nes, los cuale s controlan d iversos patrones motores co mo la loco moción, la masticación y la respiración. Un componente i mportante d e estos circuit os son la células de Renshaw, las cuale s median la i nhibición rec urrente de las MNs, que en conjunto con las d emás i nterneuronas controlan l a exci tabilidad de las MNs. Recientemente se ha dem ostrado e xperimentalmente e histopatol ógicamente que durante el progreso de la ALS existe un decremento de la actividad de los circuitos inhibidores, el cual se asocia principalmente con la pérdida de las células de Renshaw, o bien con una reducción en s u acción inhibido ra ocasionada por un d ecremento de su excitabilidad mediada por interneuro nas colinérgicas. Finalmente, una falla inhibidora por cualquier mecanismo p uede c onducir a la de generación de l as MNs, sugiriendo a l os cir cuitos inh ibitorios y las células de Renshaw co mo blanco s farmacológicos para el tratamiento de la ALS.

means of the control of the amplitude of locomotor activity generated by motor neuron output (Iwagaki and Miles, 2011; Taccola et al., 2004a, 2004b).

Other spinal neuromodulators are classified into several classes according their chemical nature. The first group, biogenic amines, arises from cerebral structures. Serotoninergic pathways originate in the raphe nuclei and reach spinal motor neurons (Alvarez et al., 1998; Maxwell et al., 2000), Renshaw cells (Carr et al., 1999), commissural interneurons (Hammar et al., 2004), V0c interneurons (Zagoraiou et al., 2009), V2-derived interneurons (Al-Mosawie et al., 2007), Hb9-interneurons (Wilson et al., 2005) and neurons in laminae VII and VIII. Noradrenergic pathways originate from locus coeruleus and brainstem (Commissiong et al., 1978; Westlund et al., 1983), and make synaptic contacts on motor neurons (Gladden et al., 2000; Rajaofetra et al., 1992), commissural interneurons (Hammar et al., 2004) and interneurons of laminae VII (Maxwell et al., 2000). Axons from dopaminergic neurons from hypothalamic A11 region are distributed throughout the ventral horn, where all subtypes of dopamine receptors are expressed. It has been shown in preparations in vitro that dopamine slows locomotor-like rhythmic activity, while serotonin increases rhythm frequency (Barbeau and Rossignol, 1990; Grillner and Jessell, 2009; Kiehn and Kjaerulff, 1996).

The second category of modulators is peptides. Experimental application of oxytocin, vasopressin, bombesin and TRH can trigger tonic or loosely coordinated rhythmic activity when applied in in vitro preparations, whereas other peptides like proctolin and neurotensin increase the frequency of locomotion, and substance P, met-enkephalin and oxytocin decrease it (Barriere et al., 2005; Barthe and Clarac, 1997; Pearson et al., 2003; Rekling et al., 2000). Table 1 summarizes the neurotransmitters and modulators of the spinal cord circuitry.

3. Role of inhibitory neurotransmission in experimental motor neuron death

Electrophysiological experiments carried out in vitro, in spinal cord organotypic cultures (Kuo et al., 2004) and in cortical and brainstem slices (van Zundert et al., 2008) of presymptomatic transgenic SOD1 ALS mice, have shown the appearance of an intrinsic hyperexcitability caused by the generation of aberrant action potentials, which might be related to the occurrence of neurodegeneration. Although the authors did not propose an explanation, it seems possible that such hyperexcitability might be due to a decrease in inhibitory neurotransmission, because in gliosomes isolated from the spinal cord of presymptomatic ALS mice the GABA transporter GAT1 (which is located mainly on presynaptic neurons and on distal astrocytic processes in close proximity to axon terminals (Borden, 1996; Conti et al., 1998)), was found to be increased and GABA release was reduced (Milanese et al., 2010). Furthermore, in cultured motor neurons from ALS mice a $GABA_AR$ desensitization, but only of receptors with $\alpha1$ subunit, was described (Carunchio et al., 2008), and in motor cortex slices of the wobbler mouse a decrease in tonic GABAergic inhibition, related to a reduction in the vesicular GABA transporter, was found (Nieto-Gonzalez et al., 2011). In addition, in mixed and dissociated spinal cord cultures of ALS mice a reduction of glycinergic miniature inhibitory postsynaptic currents and of the density of GlyRs on large-sized motor neurons (but not GABA receptors) was reported (Chang and Martin, 2011a, 2011b).

An increase in inhibitory neurotransmission has also been postulated as a compensatory mechanism of hyperexcitability in the transgenic SOD1 mice, on the basis of an increase in the population in cortical parvalbumin interneurons, found in motor and somatosensory cortex (Minciacchi et al., 2009). However, there are contradictory results, such as ubiquitination (Martin et al., 2007) and loss of putative interneurons in the spinal cord of symptomatic mice (Morrison et al., 1998). Also, Renshaw cells are lost during disease progression, and glycinergic but not GABAergic synapses around the motor neuron are lost since presymptomatic age, before motor deficit and motor neuron loss occur (Chang and Martin, 2009; Martin et al., 2007). The vulnerability of specific motor neurons pools in the transgenic ALS mice has been correlated with the density of subunit $\alpha1$ in GlyRs (Lorenzo et al., 2006), whereas high-resolution magnetic resonance spectroscopy has shown a decrease of $GABA_AR$ since early and presymptomatic age in transgenic SOD1 mice (Niessen et al., 2007). Although mutations in glycinergic receptors have been associated with some motor disorders characterized by spinal cord hyperexcitability, such as hyperekplexia (Dutertre et al., 2012), there is no motor neuron degeneration in these disorders.

Spinal motor neuron increased excitability may be due to a decreased amount of the excitatory of amino acid transporter 2 (EAAT2), although it is not clear whether this decrease is due to degeneration itself or played a causative role (Ganel et al., 2006; Rothstein et al., 1992, 1995). A role of decreased glutamate transport in motor neuron hyperexcitation or degeneration is not supported by other studies showing that the pharmacologic acute (Corona and Tapia, 2004) and chronic (Tovar-y-Romo et al., 2009a, 2009b) in vivo blockade of EAAT1 and EAAT2 increased extracellular glutamate levels but did not produce motor neuron degeneration in the rat spinal cord or in the transgenic SOD1 mouse brain (Tovar-y-Romo and Tapia, 2006).

Using an acute (reverse microdialysis) and a chronic (miniosmotic pumps) experimental procedure for the infusion of drugs into the rat lumbar spinal cord in vivo (Corona and Tapia, 2004, 2008; Tovar-y-Romo et al., 2009a, 2009b, 2007; Tovar-y-Romo and Tapia, 2010), we have recently studied the effect of the blockade of $GABA_AR$ by the infusion of bicuculline, and of GlyR with strychnine. Various concentrations of strychnine were tested and no significant effects were observed. In contrast, both the acute and chronic infusion of bicuculline caused in a dose-dependent manner motor task deficits manifested by the occurrence of fasciculations and uncoordinated and involuntary muscular contractions of the ipsilateral hindlimb and deficits in motor tasks such as rotarod. From 3 days after the beginning of the chronic infusion of

Table 1
Neurotransmitters and modulators of the spinal cord circuitry.

Neurotransmitter	Effect	Source
Glutamate	Excitatory	Interneurons, descending pathways
Acetylcholine	Excitatory	Interneurons
GABA	Inhibitory	Interneuron Ia, Renshaw cell
Glycine	Inhibitory	Interneuron Ia, Renshaw cell
Noradrenaline	Modulatory	Locus coeruleus, brainstem, pons
Dopamine	Slows locomotor pattern	Hypothalamic A11 region
Serotonin	Increase frequency of locomotor pattern	Raphe nuclei
Peptides	Modulation of MN activity	Interneurons, glial and microglial cells
Oxytocin Vasopressin Bombesin TRH	Trigger tonic or loosely coordinated rhythmic activity	
Proctolin Neurotensin	Increase the frequency of locomotion	
Substance P Met-enkephalin Oxytocin	Decrease the frequency of locomotion	

bicuculline a discrete motor neuron loss of about 20% was observed. The acute bicuculline perfusion caused a transitory effect on motor behavior, which lasted about 40–60 min, but no histological alterations were observed. These results show that inhibitory GABAergic blockade can cause hyperexcitability of the intraspinal neuronal networks and motor neuron degeneration (Ramírez-Jarquín and Tapia, in preparation).

4. Alterations of inhibitory motor neuronal pathways in ALS

Some components of inhibitory circuits seem to be altered in ALS patients. GABA levels in serum did not change in ALS patients during several stages of the disease, but glycine concentration was diminished (Malessa et al., 1991). No changes in CSF GABA have been found, whereas an increase (Kostera-Pruszczyk et al., 2002) or a decrease (Malessa et al., 1991) of glycine has been described. Cortical hyperexcitability in ALS patients has been shown to occur in early stages of the disease by transcranial magnetic stimulation recorded with superficial electrodes (Vucic et al., 2009), and this has been proposed as an early diagnostic marker (Eisen and Weber, 2000). Post-mortem analyses of cortex and spinal cord of ALS patients have shown a decrease in $\alpha 1$ subunit of $GABA_AR$, an upregulation of glutamate decarboxylase expression (Petri et al., 2006), and a decrease in glycine binding sites in the ventral horn (Hayashi et al., 1981; Whitehouse et al., 1983).

Several enhancers of GABAergic transmission have been tested in clinical trials in ALS patients. Intrathecal infusion of the $GABA_B$ agonist baclofen via a percutaneous pump has been used for spasticity-related pain treatment, without clear beneficial effects (McClelland et al., 2008). In one study, gabapentin (a GABA analog with multiple mechanisms of action) reduced fasciculations (Romano, 1996), but later phase II and phase III trials with magnetic resonance spectroscopy analysis revealed no beneficial effects (Kalra et al., 2003; Miller et al., 2001). Nowadays, the only therapy approved for ALS treatment is riluzole, but the benefits produced by this drug are limited (Miller et al., 2003). The exact mechanism of action of riluzole is unknown, but it has multiple properties including inhibition of sodium, calcium, potassium and glutamate currents (Gibson and Bromberg, 2012).

5. The role of Renshaw cells

As previously mentioned, the Renshaw cell is the only interneuron that receives afferents directly from motor neurons and mediates recurrent inhibition to them. Inhibitory terminals of Renshaw cells are widely distributed over the dendrites of motor neurons (Burke et al., 1971; Fyffe, 1991) and their activity is directly associated with the activation and modulation of motor neurons, which in turn is important to determine the level of excitation in spinal cord circuits and motor output. The Renshaw cell inhibitory action seems to be mediated by both GABA and glycinergic synapses, but not all their efferents to motor neurons have both inhibitory neurotransmitters. Shigenaga et al. (2005) showed that synaptic boutons immunoreactive to glycine alone are more numerous than boutons double-labeled for GABA and glycine, which in turn occurred more frequently than boutons immunoreactive to GABA alone. Renshaw cells can be identified by its size (mostly medium to large), biochemical markers such as glycine transporter GlyT2, calcium buffering proteins calbindin and parvalbumin, large gephyrin clusters, location, and electrophysiologic properties such as a high postsynaptic sensibility to acetylcholine and large glycine- and GABA-evoked currents (Alvarez and Fyffe, 2007). The Renshaw cell to motor neuron ratio is estimated to be 1:5 (Mentis et al., 2006).

In order to understand the mechanisms involved in neuronal hyperexcitability in ALS, one of the most attractive circuits is the recurrent inhibition formed by motor neurons and Renshaw cells. Its precise role is still unknown, but there are two hypotheses which try to explain how Renshaw-cell alterations may lead a hyperexcitability state and eventually motor neuron degeneration (Fig. 1). The first hypothesis postulates that the hyperexcitability is caused by the loss of the recurrent Renshaw cells-mediated inhibition. Experimental models of FALS have shown that a progressive loss of glycinergic boutons occurs throughout the soma of the motor neurons, which starts in the early symptomatic stage before motor neuron degeneration. Whereas GABAergic terminals are affected only until the final stage, these changes have been associated with Renshaw cell loss, which also has been observed in early and late symptomatic stages (Chang and Martin, 2009). Taking the inhibitory loss as a cause of motor neuron degeneration, some groups have developed pharmacological strategies for the preservation of these cells. One treatment used to rescue Renshaw cells is the administration of lithium, which prevented Renshaw loss and delayed the onset of symptoms, although mice eventually developed the disease (Chang and Martin, 2009; Fornai et al., 2008; Pasquali et al., 2009) (Fig. 1B).

The second hypothesis is based on evidence which suggests that Renshaw cell loss is not an initial causal event of motor neuron hyperexcitability and neurodegeneration, and proposes that the recurrent inhibitory circuit is altered previously to motor neuron degeneration and the onset of the symptoms but is not a consequence of Renshaw cell loss. First, Mazzocchio and Rossi (2010) shown that activation of Renshaw cells has a poor effect on the motor neuron soma activity; second, in both experimental models in the symptomatic stage and in human tissue samples interneurons are preserved, indicating that progress of motor neuron degeneration is independent of the Renshaw cells loss (Wootz et al., 2013). In agreement, in transgenic ALS mice, inhibitory neurons degeneration occurs after motor neuron loss (Hossaini et al., 2011). What is then the role of Renshaw cells in motor neuron hyperexcitability and degeneration?

Recently, other two significant components have been involved in the control and modulation of the motor neuron activity: cholinergic interneurons named V0c (Zagoraiou et al., 2009), which innervate the soma of motor neurons and inhibitory interneurons (Nagy et al., 1993; Zagoraiou et al., 2009); and, second, a kind of inhibitory interneuron named Ia (Ia), which can inhibit motor neurons through the release of GABA and glycine (Siembab et al., 2010). So, the recurrent and reciprocal inhibition of motor neurons is mediated by both Renshaw cells and Ia, and both interneurons can be excited by V0c cholinergic action.

Although Pullen and Athanasiou (2009), using electron microscopy and morphometric analysis, found that presumed cholinergic C terminals on motor neurons are preserved at presymptomatic stages in a transgenic model of ALS (without determining the origin of these terminals), in the same model Casas et al. (2013) showed an early reduction of choline acetyltransferase (ChAT) content in the presynaptic boutons of V0c on motor neuron somas, as well as on Renshaw cells, also at a presymptomatic stage. Similarly, Wootz et al. (2013) demonstrated that the cholinergic afferent to Renshaw cells from motor neurons are lost at 20–30 days of age, by retraction of the motor neuron collateral. However, the inhibitory boutons from Renshaw cells on motor neurons and the number of Renshaw cells were not affected at this time. In both studies, these changes occurred long before the markers of motor neuron degeneration appeared. Therefore, according to these findings cholinergic dysfunction can be the trigger of a hyperexcitation and neurodegeneration process of the spinal circuits through a decreased excitatory action on inhibitory neurons (Fig. 1C).

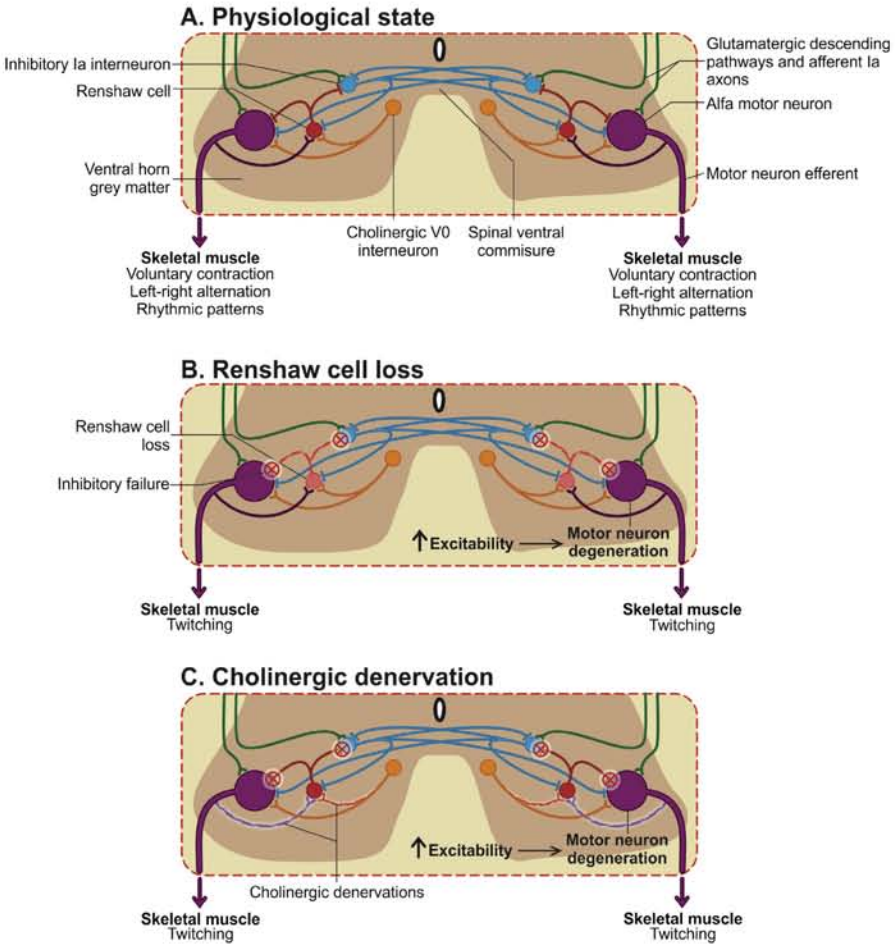

Fig. 1. **A**, schematic representation of the local spinal circuitry controlling motor neuron excitation under physiological conditions, showing the inhibitory Ia interneurons connecting both ventral horns, Renshaw cells and the excitatory V0c cholinergic interneurons. **B** and **C**, increased motor neuron excitability that may lead to degeneration due to Renshaw cell loss (pink dotted neurons in **B**) or cholinergic denervation of V0c and motor neuron axon collaterals (orange and purple discontinuous lines, respectively, in **C**). As discussed in the text, both pathological situations lead to inhibitory synaptic deficits of Renshaw cells (red X), resulting in increased motor neuron excitability and delayed degeneration. Based on Chang and Martin (2009, 2011a); Casas et al. (2013), and Wootz et al. (2013).

These findings in animal models agree with reports that in both SALS and FALS there is a reduction in ChAT activity in the ventral horn of the spinal cord. Also in patients, morphologic studies demonstrated a loss of ChAT mRNA (Virgo et al., 1992), suggesting that the low expression of ChAT in the spinal cord may be an early alteration in ALS (Oda et al., 1995).

6. Conclusion

The knowledge of the spinal ventral horn circuitry shows great structural and functional complexity and therefore several possible mechanisms that may lead to dysregulation of motor excitability and to motor neuron degeneration. We have focused on inhibitory networks alterations, because the inhibitory control is extremely important for the physiological control of motor activity. Growing evidence shows that, besides an increased excitation intrinsic of motor neurons, or excitotoxicity mediated by overactivation of glutamate receptors, a loss of action of inhibitory neurons, mainly Renshaw cells, may be an important factor leading to motor neuron death. A decreased inhibitory control on motor neurons may be due to degeneration of inhibitory interneurons or to a loss of cholinergic excitatory action on them, which may have implications for the understanding of the mechanisms of motor neuron degeneration in ALS. Therefore, these findings suggest new pharmacological strategies for the treatment of the disease, for example to preserve Renshaw cells, restore inhibition failure and study the relationship between GABAergic, glycinergic, glutamatergic and cholinergic neurotransmission in the spinal cord, even when these alterations have been shown mainly in experimental models of motor neuron degeneration and data obtained in human tissue from ALS patients are still scarce.

Conflicts of interest

The authors declare that they do not have conflicts of interest.

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VI. Planteamiento del problema

A pesar de los avances alcanzados en el entendimiento de los mecanismos involucrados en la degeneración las MNs en la ALS y particularmente en condiciones de excitotoxicidad, actualmente el proceso de muerte involucrado aún continúa sin determinarse. Algunos trabajos postulan un proceso apoptótico caracterizado principalmente por la presencia de la caspasa-3, mientras que otros estudios proponen un proceso no apoptótico con características necróticas. Un aspecto que ha complicado el entendimiento de este mecanismo, es que estas dos vertientes se han estudiado de manera independiente, una enfocada en el estudio morfológico mediante microscopía electrónica y la otra mediante bioquímica usando para ello técnicas de inmunocitoquímica, sin que se integren en una sola propuesta o mecanismo que conjunte ambos grupos de evidencias. Aunado a lo anterior, la mayoría de estos estudios se han realizado en modelos transgénicos de la ALS de tipo familiar, por lo que solo representan una minoría del total de los casos clínicos (10%).

En nuestro laboratorio se han desarrollado dos modelos de degeneración espinal basados en la excitotoxicidad mediada por receptores AMPA, estos modelos reproducen la muerte de MNs, parálisis y astrogliosis, permitiendo el estudio de los mecanismos de degeneración de las MNs. A pesar de que en nuestros procedimientos, la pérdida de MNs y la parálisis ocurre a lo largo de varios días y no durante meses o años como en la enfermedad, nuestros modelos al no depender de un factor genético, podrían ser empleados para comprender los mecanismos que participan en degeneración de las MNs en ALS esporádica, que representan la gran mayoría de los casos clínicos (90%). Sin embargo, al igual que en la ALS, en nuestros modelos aún se desconoce el proceso mediante el cual degeneran las MNs, lo cual es de suma importancia ya que al conocerlo podríamos decir la real similitud o diferencia entre la degeneración de las MN en nuestros modelos y la degeneración de las MNs en los modelos transgénicos de ALS.

Como se menciona en los antecedentes, la acción del GABA y la glicina en los circuitos espinales es de suma importancia para el control de la excitabilidad de las MNs. Sin embargo, ha sido poco estudiada como mecanismo de degeneración de MNs, por ello en esta tesis planteamos su estudio mediante el bloqueo farmacológico de cada neurotransmisión.

Estamos convencidos que al conocer el proceso degenerativo de las MNs, y el papel del bloqueo de los circuitos inhibidores en nuestros modelos, podríamos ir en busca de nuevas alternativas dirigidas a la prevención y atenuación de los diferentes componentes degenerativos que caracterice mos, con miras a diseñar estrategias farmacológicas para el tratamiento de la ALS. Por ello, el principal objetivo de la presente tesis fue caracterizar el proceso de degeneración de las MNs en nuestros modelos de excitotoxicidad por sobreactivación de receptores AMPA *in vivo*, y determinar el efecto del bloqueo de los circuitos inhibidores espinales, y su efecto en conjunto con la excitotoxicidad mediada por AMPA.

VII. Hipótesis

Basados en la literatura revisada y en los tiempos de progreso de la parálisis observados para cada uno de nuestros procedimientos, proponemos que el proceso degenerativo de las MNs lumbares es diferente para cada modelo: para el modelo agudo proponemos una degeneración mediante necrosis, y para el tratamiento crónico un proceso apoptótico. Así mismo, teniendo como premisa que un evento de hiperexcitabilidad puede causar excitotoxicidad y con ello inducir degeneración de las MNs, planteamos que el bloqueo farmacológico de los circuitos inhibitorios espinales puede desencadenar la degeneración de MNs, y potenciar el efecto excitotóxico de la administración de AMPA.

VIII. Objetivo

Con el fin de comprobar nuestra hipótesis el objetivos general de este estudio fue caracterizar el proceso degenerativo de las MNs espinales inducido por

excitotoxicidad mediada por la sobreactivación de receptores AMPA en ambos modelos, agudo y crónico. Así como determinar el efecto del bloqueo farmacológico de los circuitos inhibitorios de GABA y glicina en la médula espinal lumbar de rata.

Objetivos específicos.

1. Usando microscopía electrónica de transmisión determinar los cambios ultraestructurales de las MNs lumbares durante su degeneración inducida por excitotoxicidad *in vivo*.
2. Empleando técnicas de inmunocitoquímica determinar si la caspasa-3 activa participa en la degeneración de las MNs en ambos modelos.
3. Utilizando como vía de administración la técnica de microdiálisis y las minibombas osmóticas, realizaremos tratamientos con los fármacos que bloquean los circuitos inhibidores de la médula espinal lumbar.
4. Evaluar la actividad motriz y sus posibles alteraciones mediante las pruebas de rotarod, paw grip endurance (PGE) test y el registro de la zancada.

IX. Métodos

En este apartado se amplía la información incluida y descrita en los métodos y procedimientos utilizados en cada uno de los artículos. Utilizamos ratas macho cepa Wistar (270-300 g de peso), las cuales se mantuvieron en un ambiente de laboratorio con ciclo de luz/oscuridad de 12h, con alimento y agua *ad libitum*, todos los experimentos se realizaron con la aprobación del comité local de cuidados animales (Número de aprobación RT121-14).

Previo a la cirugía los animales se entrenaron por siete días en las pruebas de rotarod, PGE y el registro de la zancada. Posteriormente, solo los animales que realizaron correctamente las pruebas fueron seleccionados para la cirugía estereotáxica (microdiálisis o implante de minibombas osmótica). Después de la cirugía, los animales fueron evaluados rutinariamente (según el protocolo a seguir)

hasta terminar el experimento, para finalmente ser perfundidos según el objetivo del experimento (detallado en cada artículo) para la obtención de sus médulas.

Pruebas conductuales

La actividad motriz fue evaluada mediante el rotarod, que consiste en un cilindro que comienza girando a una velocidad de 10 r.p.m. con una aceleración constante de 0.2 r.p.m/seg. Las ratas se entrenan para permanecer caminando en el rodillo durante un periodo de 2 minutos. Cada evaluación del rotarod consistió en una serie de tres intentos, quedando como registro el tiempo promedio de los tres intentos. El PGE sirve para medir el impulso de los cuartos traseros de las ratas, las cuales son colocadas sobre una reja horizontal que es lentamente girada hasta una posición vertical, la rata tiene que escalar hasta llegar a la parte superior de la reja, se cuantifica el tiempo de escalada, o bien cuando existe déficit motriz y las ratas no pueden subir, se cuantifica el tiempo en caer. También se realizan tres intentos y se registra su promedio. El registro de la zancada consiste en pintar con tinta china no tóxica los cuartos traseros de las ratas y ponerlas a caminar sobre un papel a lo largo de un corredor (10 cm x 70 cm), registrando sus huellas en papel. Mediante esta prueba es posible identificar alteraciones en las huellas o zancadas de las ratas, en esta prueba cuantificamos los pasos necesarios para recorrer 60 cm, así como los centímetros recorridos en 5 pasos.

Cirugía estereotáxica

Para el procedimiento a través de la microdiálisis, las ratas fueron anestesiadas con isofluorano al 5% disuelto en carbógeno (O_2 95%, CO_2 5%), posteriormente se colocaron en una unidad estereotáxica para médula espinal y la anestesia se reajustó al 1.5 - 2%. Se rasuró la espalda de las ratas y se hizo una incisión en la región lumbar, posteriormente se realizaron dos cortes laterales a la columna vertebral a la altura de la región lumbar, se colocó a la columna sobre soportes especiales para medula espinal y se estimuló entre la primera y segunda lumbar para corroborar que

fuera la región que inerva a los cuartos traseros. Se removieron los músculos que cubrían el proceso espino de L2 y se rebajó el proceso hasta la base del mismo, del lado derecho se realizó un hoyo hasta descubrir a la médula espinal, posteriormente se rasgaron cuidadosamente las meninges y se introdujo una cánula de diálisis (CMA7, ~1 mm de profundidad). Una vez colocada la cánula, se inició el protocolo a seguir, que consistió en pasar solución Ringer por 60 minutos, y posteriormente el protocolo farmacológico. Al finalizar el procedimiento, las ratas fueron suturadas y colocadas en cajas individuales para posteriormente ser evaluadas.

Para el implante de las minibombas osmóticas (ALZET, modelo 2004; flujo de 0.25 µL/h), el procedimiento comienza igual hasta el paso donde se rebaja el proceso espinoso, donde posterior a ello del lado izquierdo se fijó un tornillo que sirvió como medio de anclaje del implante, y del lado derecho se realizó una laminectomía hasta descubrir la superficie de la médula espinal, cuidadosamente se rompieron las meninges y se insertó una cánula (~1 mm de profundidad) que estaba conectada a un tubo con el otro extremo libre. Se aplicó acrílico dental para fijar todo el implante, posteriormente se colocó subcutáneamente la minibomba osmótica en la espada de la rata y se unió al extremo libre de la cánula y se selló con cianocrilato. Al terminar el procedimiento, la incisión fue cerrada con grapas quirúrgicas, al despertar de la anestesia, las ratas recibieron una aplicación de antibiótico y se colocaron en cajas individuales para ser evaluadas según el protocolo a seguir. Previo a su implante, las minibombas fueron llenadas con los tratamientos a probar y puestas a incubar por lo menos durante 48 h a 37° C para estabilizar su flujo (según las indicaciones de la casa comercial ALZET).

Inmunocitoquímica

Una vez terminado el experimento, las ratas se perfundieron con paraformaldehído al 4%, se obtuvieron las médulas y se trataron para realizar conteos por violeta de cresilo e inmunocitoquímicas para los diferentes anticuerpos utilizados. Los detalles

de los protocolos, los anticuerpos primarios y secundarios se encuentran en los artículos.

Microscopía electrónica de transmisión

Las ratas utilizadas para el estudio de la ultraestructura fueron perfundidas con paraformaldehído al 4 % adicionado con gluteraldehído al 2.5%, para posteriormente tratar la médula para ser observada en el microscopio electrónico de transmisión. Los detalles del protocolo se encuentran el artículo "*Neuropathological characterization of spinal motor neuron degeneration processes induced by acute and chronic excitotoxic stimulus in vivo*".

X. Resultados

Sección I. Caracterización del proceso degenerativo de las MNs inducido por excitotoxicidad in vivo

Los resultados de esta sección se incluyen el artículo titulado "*Neuropathological characterization of spinal motor neuron degeneration processes induced by acute and chronic excitotoxic stimulus in vivo*", publicado en *Neuroscience* 331: 78-90, 2016, el cual describe los cambios ultraestructurales y bioquímicos que se desencadenan por la sobreactivación aguda y crónica de los receptores para glutamato tipo AMPA en la médula espinal lumbar de rata *in vivo*.

Resumen

A continuación se presenta el resumen del artículo "*Neuropathological characterization of spinal motor neuron degeneration processes induced by acute and chronic excitotoxic stimulus in vivo*".

Las enfermedades asociadas con alteraciones de las MNs son caracterizadas por una degeneración progresiva, en ellas la excitotoxicidad ha sido postulada como un factor causal. En nuestro laboratorio hemos desarrollados dos procedimientos

experimentales para inducir degeneración de las MNs espinales in vivo por excitotoxicidad, mediante la sobreactivación aguda y crónica de receptores para glutamato tipo AMPA. En este trabajo caracterizamos el curso temporal de los cambios neuropatológicos. El análisis de la ultraestructura mediante microscopía electrónica de transmisión mostró que la perfusión aguda con AMPA por microdiálisis causó hinchamiento de las MNs después de 1.5 h de la cirugía y lisis con ruptura de membranas a las 3 h, sin embargo durante este proceso la activación de caspasa-3 no fue detectada por inmunocitoquímica. La infusión crónica de AMPA mediante minibombas osmóticas indujo un proceso degenerativo lento a lo largo de cinco días, caracterizado por cambios progresivos de las MNs: hinchamiento del retículo endoplásmico, vacuolización del citoplasma, fusión de vacuolas y ruptura de membranas. La cuantificación de estas alteraciones ultraestructurales mostró que el incremento del área vacuolizada fue a expensas del área nuclear. En este procedimiento, la activación de la caspasa -3 fue observada desde el primer día de la infusión de AMPA, sin embargo al analizar el número de MNs con caspasa 3 activada con la actividad motora no encontramos relación entre ambos parámetros. Concluimos que la excitotoxicidad aguda inducida por AMPA provoca la pérdida de las MNs a través de necrosis, mientras el progreso de la degeneración inducida por la infusión crónica es lenta, comenzando con un proceso apoptótico temprano, seguido por necrosis. En ambos procedimientos, agudo y crónico, se puede establecer una correlación entre el número de MNs perdidas por necrosis, pero no con apoptosis ligada a la caspasa -3, y la severidad de los déficits motores, así como con la parálisis de los cuartos traseros. Nuestros hallazgos son relevantes para el entendimiento de los mecanismos de la muerte neuronal en enfermedades degenerativas y por ello para el diseño de estrategias terapéuticas farmacológicas.

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NEUROPATHOLOGICAL CHARACTERIZATION OF SPINAL MOTOR NEURON DEGENERATION PROCESSES INDUCED BY ACUTE AND CHRONIC EXCITOTOXIC STIMULUS IN VIVO

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Abstract—Motor neuron (MN) diseases are characterized by progressive cell degeneration, and excitotoxicity has been postulated as a causal factor. Using two experimental procedures for inducing excitotoxic spinal MN degeneration in vivo, by acute and chronic overactivation of α-amino-3-hydroxy-5-methyl-4-isoxazoleacetic acid (AMPA) receptors, we characterized the time course of the neuropathological changes. Electron transmission microscopy showed that acute AMPA perfusion by microdialysis caused MN swelling 1.5 h after surgery and lysis with membrane rupture as early as 3 h; no cleaved caspase 3 was detected by immunochemistry. Chronic AMPA infusion by osmotic minipumps induced a slow degeneration process along 5 days, characterized by progressive changes: endoplasmic reticulum swelling, vacuolization of cytoplasm, vacuole fusion and cell membrane rupture. Quantification of these ultrastructural alterations showed that the increase of vacuolated area was at the expense of the nuclear area. Caspase 3 cleavage was observed since the first day of AMPA infusion. We conclude that acute AMPA-induced excitotoxicity induces MN loss by necrosis, while the progress of degeneration induced by chronic infusion is slow, starting with an early apoptotic process followed by necrosis. In both the acute and chronic procedures a correlation could be established between the loss of MN by necrosis, but not by caspase 3-linked apoptosis, and severe motor deficits and hindlimb paralysis. Our findings are relevant for understanding the mechanisms of neuron death in degenerative diseases and thus for the design of pharmacological therapeutic strategies. **© 2016 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: spinal cord, motor neuron, apoptosis, necrosis, caspase, excitotoxicity.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by selective and progressive loss of upper and lower motor neurons (MNs), which causes a progressive paralysis and finally death by respiratory failure. The minority (about 10%) of ALS cases is of familial type (fALS), which is associated with several mutations in hundreds of genes, and the remainder ~90% are sporadic (sALS), whose cause is mainly unknown (Robberecht and Philips, 2013). However, several factors have been implicated in the mechanisms of MN death in both familial and sporadic ALS, such as: oxidative stress, mitochondrial dysfunction, energy failure, axonal transport deficits, inflammatory processes and non-autonomous cellular death. Among them, one of the most studied in view of considerable experimental and clinical evidence is glutamate-mediated excitotoxicity (Corona et al., 2007). A reduction of the glutamate transporter EAAT2 was found in post-mortem analysis in the cortex and spinal cord of ALS patients (Rothstein et al., 1992, 1995), and elevated concentrations of glutamate were detected in cerebrospinal fluid of about 40% of ALS patients (Shaw et al., 1995; Spreux-Varoquaux et al., 2002).

In spite of these findings, we have shown that acute (Corona and Tapia, 2004) and chronic (Tovar-Y-Romo et al., 2009) increase of extracellular glutamate levels induced by glutamate transport inhibition is innocuous for lumbar spinal MN in vivo. Nevertheless, we have also demonstrated that selective and pharmacological overactivation of α-amino-3-hydroxy-5-methyl-4-isoxazoleacetic acid (AMPA) receptors by AMPA itself induces remarkable MN loss in the rat spinal cord and consequent progressive and irreversible paralysis. This was shown using two experimental procedures, the acute administration of AMPA by means of microdialysis and the chronic continuous infusion of AMPA using osmotic minipumps, directly in the lumbar spinal cord. The acute procedure results in MN death in few hours after the experiment and is due to overactivation of the Ca^{2+}-permeable AMPA receptors, because specific antagonists and Ca^{2+} chelators prevent the effects of AMPA (Corona and Tapia, 2004, 2007, 2008). In contrast, the continuous AMPA infusion induces progressive MN loss and bilateral paralysis along several days, and can be partially prevented by vascular endothelial growth factor (Tovar-y-Romo et al., 2007; Tovar-y-Romo and Tapia,

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Abbreviations: ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleacetic acid; ccasp3, cleaved caspase 3; ChAT, choline acetyltransferase; GFAP, glial fibrillary acidic protein; MN, motor neuron; PB, phosphate buffer; PFA, paraformaldehyde; PGE, paw grip endurance.

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2010, 2012). Although in both procedures AMPA-induced excitotoxicity is the cause of MN death, the ample differences in the temporal progress offers the possibility of studying comparatively the mechanisms of MN degeneration under these different conditions, such as apoptosis and necrosis, which have been the subject of considerable discussion to explain MN death in both ALS (Gurney et al., 1994; Ripps et al., 1995; Wong et al., 1995; Ilzecka et al., 2001; Martin et al., 2009; Ilzecka, 2011, 2012; Hart and Gitler, 2012) and in other experimental models, mainly transgenic rodents (Li et al., 2000; Pasinelli et al., 2000; Bendotti et al., 2001; Tokuda et al., 2007; Ohta et al., 2008; Rossi et al., 2008; Martin et al., 2009) with mutant SOD (Gurney et al., 1994; Ripps et al., 1995) or, more recently other proteins, and this is the aim of the present work. Therefore, we have carried out a morphologic, ultrastructural and immunohistochemical characterization of the MN changes during the degeneration processes in our two experimental models of excitotoxicity in vivo, correlating the cellular findings with the motor deficits produced by the MN loss during the time periods studied. The relevance of our results is that they shed light for the understanding of the cellular mechanisms that determine the degeneration, thus opening the possibilities of new approaches to prevent the MN death progression in ALS and other neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

Adult male Wistar rats (270–300 g) were used in all experiments. Animals were housed in a laboratory environment with a 12-h light/dark cycle with food and water ad libitum. All procedures were performed in accordance with of the Institutional Committee for the Care and Use of Laboratory Animals (Approval No. RTI21-14). All efforts were made to minimize suffering of the animals.

For the acute procedure, AMPA (Tocris, Ellisville, MO, USA) was perfused by microdialysis as previously described (Corona and Tapia, 2004). Rats were anesthetized with 5% isoflurane in a 95% O_2/5% CO_2 mixture and placed in a stereotaxic spinal unit. Anesthesia was lowered and maintained to 1-2% isoflurane during surgery. A longitudinal incision of back skin was made at the lumbar region and muscles surrounding lumbar vertebrae were cut and retracted. The second lumbar vertebra was exposed and a 2-mm hole was drilled on the right side. After carefully cutting the meninges a microdialysis probe (CMA7) was lowered down into the right dorsal horn of the spinal cord. The probe was perfused continuously with Krebs–Ringer solution containing (in mM concentrations) 118 NaCl, 4.5 KCl, 2.5 $MgSO_4$, 4.0 $Na_2H_2PO_4$, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 10 glucose, pH 7.4, at flux rate of 2 μL/min, using a microinjection pump (CMA/100, Carnegie, Sweden). After 1 h of stabilization, medium containing 6 mM AMPA was perfused for 25 min (total volume perfused = 50 μL). This concentration was chosen on the basis of previous results (Corona and Tapia, 2004). After the experiment the microdialysis probe was gently removed, the skin incision was sutured and after recovery from anesthesia rats were placed in individual cages and divided in four groups for the different times studied, 1.5, 3, 6 and 24 h after surgery. At these times animals were subjected to the motor tests described below and then intracardially fixed for the histological, immunochemical or ultrastructural studies.

Chronic infusion of AMPA was made through osmotic minipumps (ALZET model 2004), which were filled with filtered 0.1 M phosphate buffer (PB) or PB containing 7.5 mM AMPA, at least 48 h before the implantation, and incubated in sterile saline at 37 °C for stabilization. The flow rate of the pumps is 0.25 μL/h, so that the volume continuously infused was 6 μL per day. Surgery was carried out exactly as previously described (Tovar-y-Romo et al., 2007), and on this basis we used 7.5 mM AMPA concentration in the osmotic pumps. Briefly, rats were anesthetized and placed in a stereotaxic spinal unit as described above. A longitudinal incision of back skin was made at the lumbar region and muscles surrounding lumbar vertebrae were cut and retracted. On the second lumbar vertebra, the spinous process was removed and a one mm-diameter hole was drilled on the left side of the lamina in which a stainless-steel screw (1 mm diameter; 3.7 mm long) was inserted to anchor the implant. A 2 mm laminectomy was made in the right side of the lamina of the same vertebra and after cutting the meninges the cannula was inserted into the dorsal parenchyma (1 mm deep). Dental acrylic cement was poured over the screw and the external end of the cannula was connected to the minipump tube. The pumps were implanted subcutaneously in the back of the rats. Union between the minipumps and cannula was sealed with instant glue and the incision was closed with surgical stainless-steel clips. After recovery from anesthesia rats received an i.m. dose of penicillin and were placed in individual cages and divided in groups for the different times studied: 1, 2, 3, 4 and 5 days after surgery. At these times animals were subjected to motor tests and, as described above, fixed for histological, immunochemical or ultrastructural studies.

Evaluation of motor function

Motor activity was evaluated as described previously (Tovar-y-Romo et al., 2007). Rats were trained for a week prior the surgery on two motor tests: the rotarod (Columbus Instruments, Columbus, OH, USA) and a variation of the paw grip endurance (PGE) task. Animals were evaluated in each test routinely until fixation. For the rotarod test, rats walked individually on an accelerating (0.2 rev/min per s) rod, starting from 10 rpm with a cut-off of 120 s, and the time on the rod was scored. For the PGE test, rats were trained to climb to the top of a vertical grip. Rats were placed individually on a horizontal placed grid (40 × 25 cm), the grid was gently turned until reaching a vertical position. The time taken by rats for climbing to the top of the gird and reaching a stable position or the latency to fall from the gird when they were unable to climb was scored with a cut-off of 40 s. In addition to the motor tests, the overall stride pattern of the hind footprints was quantitatively analyzed; for this purpose, the hindpaws of treated

rats were inked with non-toxic Chinese ink before animals walked along a paper runway delimited by two walls forming a corridor (10 × 100 cm). For quantitative analysis the number of steps required to cross 60 cm were counted, and the distance (cm) in five steps.

Histology and immunohistochemistry

For histological and immunohistochemical analyses, rats subjected to AMPA microdialysis or chronic infusion were fixed at the times indicated above for each procedure. For fixation, animals were anesthetized with sodium pentobarbital and perfused transcardially with 250 ml of ice-cold 0.9% saline, followed by 250 ml of ice-cold 4% paraformaldehyde (PFA) in 0.1 M PB, pH 7.4. The spinal cord was removed, post-fixed in 4% PFA at 4 °C for a week, and successively cryoprotected in sucrose gradients (up to 30%). Transverse sections (40-μm-thick) of the lumbar region, where the infusion or microdialysis cannula was implanted, were obtained in a cryostat. Alternated sections were stained with cresyl violet or immunostained for choline acetyltransferase (ChAT), glial fibrillary acidic protein (GFAP) and cleaved caspase 3 (ccasp3).

For ChAT, GFAP and ccasp3 immunohistochemistry, free-floating sections were blocked with 5% of bovine serum albumin in PB 0.1 M and Triton X-100 (0.3%) for 2 h, followed by incubation with goat polyclonal anti-ChAT (1:200; Chemicon, Temecula, CA, USA), mouse anti-GFAP (1:1000; Sigma) and rabbit anti-ccasp3 antibodies (Abcam), for 48 h at 4 °C. Sections were washed three times for 15 min in PB-Triton and incubated with biotinyl-conjugated mouse anti-goat IgG (1:200; Vector, Burlingame, CA, USA) for 1.5 h. After three washes, sections were incubated for 2 h with avidin-Texas Red conjugate (1:200, pH 8.2; Vector) for ChAT, fluorescein-conjugated anti-mouse antibody (1:250; Zymed, Carlsbad, CA, USA) for GFAP, and Cy5 anti-rabbit (Invitrogen) for ccasp3. Finally, sections were washed and mounted on silane-covered slides (c-methe cryloxypropyltrimethoxysilane; Sigma) and coverslipped with fluorescent mounting medium (DAKO, Carpinteria, CA, USA). Sections were visualized by confocal microscopy (FV-1000, Olympus IX81); merged images are the overlay of five laser sections in the Z plane, using the Olympus 1.6 Fluoview.

Morphologically undamaged MNs in the Nissl preparations (with a soma diameter >25 μm and distinguishable nucleus) were counted in a 10× microscopic field. The number of cells was determined in sections where the trace of the infusion cannula was evident; seven sections per rat were analyzed and the values were averaged.

Transmission electron microscopy was used in order to characterize the ultrastructural alterations of MNs, also at the times after surgery mentioned above for each experimental procedure. Rats were deeply anesthetized with pentobarbital and perfused transcardially with 250 ml of ice-cold 0.9% saline, followed by 250 ml of ice-cold 4% PFA and glutaraldehyde (2.5%) in 0.1 M PB pH 7.4. Spinal cord was removed and post-fixed at 4 °C over night in the same solution used for perfusion. Infusion site of AMPA was delimited and the anterior horns were excised. Tissue was osmicated (1% osmium tetroxide in PBS), washed with PB, dehydrated and embedded in epoxy resin. Several semithin sections (500 nm) were obtained in an ultramicrotome (Reichert-Jung) and stained with Toluidine Blue for light microscopic observation, to identify the area of interest. Ultrathin sections were obtained and stained with Reynolds mixture (2% lead citrate and 2% uranyl acetate), and observed in a Jeol 1200EXII transmission electron microscope.

Quantitative analysis of cellular area alterations

Electron micrographs were scanned and digitized to analyze morphologic changes in MNs with the software Image J. According to the program, each micrograph was scaled to pixels, and total cell area was delimitated by drawing the outline of each MN and this measure was taken as 100% percent. Nuclear area was calculated outlining the nucleus of each MN, and its percent was determined with respect to the total cell area. Vacuolated and swollen areas were quantified by drawing the edge of all vacuoles and swollen zones for each MN, and again their percentages were determined with respect to the cell area. Only MNs with visible nucleolus were considered. According to the number of MN identified, 10–20 ultrathin sections were observed for each rat, and 5 rats for group were analyzed. For details see the Table inserted in Fig. 6C.

Statistical analysis

Comparisons regarding the number of MNs and cellular and nuclear areas were made using ANOVA followed by a Fisher's *post hoc* test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Acute and chronic AMPA treatment cause the death of lumbar MNs and paralysis with different time course

In order to characterize the mechanisms of MN death in our acute and chronic models, we first established the time course of the degeneration and the consequent motor alterations, including some additional parameters not previously studied, such as the occurrence of astrogliosis and the stride pattern.

Similarly to previous results (Corona and Tapia, 2004), acute infusion of 6 mM AMPA induced ipsilateral loss of lumbar MNs, assessed by Nissl staining and ChAT immunochemistry, since 3 h after treatment and increased during the following 24 h, accompanied by a progressive increase in astrogliosis, detected by GFAP immunoreactions; loss of MNs and glial reaction were not observed in the contralateral side (Fig. 1). This loss was associated with the paralysis of ipsilateral hindpaw, manifested by a notable decrease in the time to fall in the rotarod test (Fig. 1C).

In contrast to the rapid effects of acute AMPA treatment, as shown in Fig. 2 the chronic infusion of

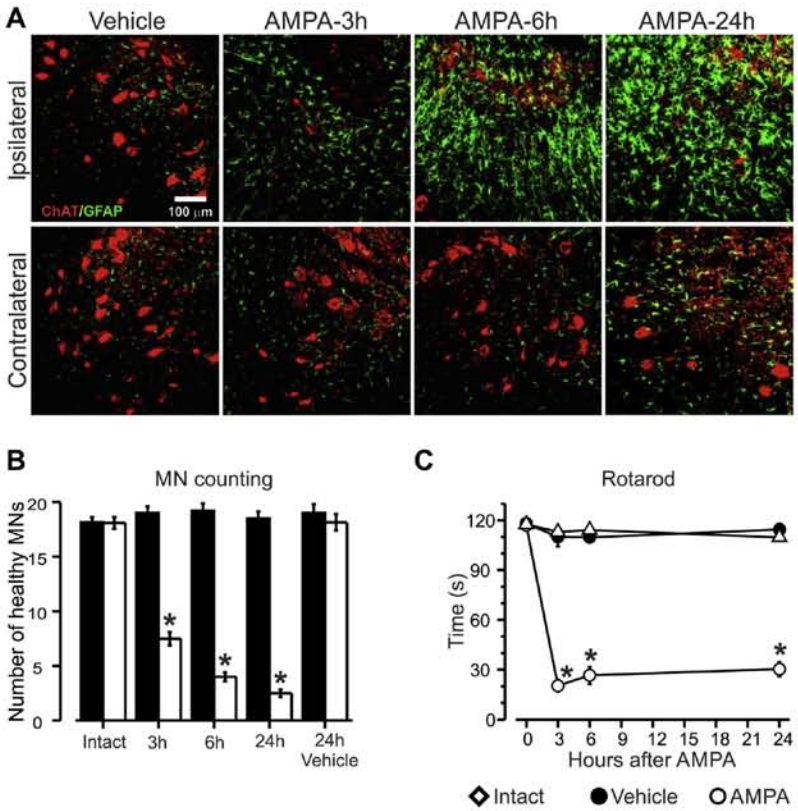

Fig. 1. Microdialysis perfusion of AMPA causes progressive MN loss, astroglial reaction and motor deficits within 24 h. (A) Representative immunohistochemistry of the ventral horns of rats at the indicated times after surgery. MNs are identified by ChAT (red) and astroglial reaction by GFAP (green). Note progressive MN loss and astroglial reaction in the ipsilateral side, starting as early as 3 h after the experiment, whereas the contralateral side is only slightly affected at 24 h. Control refers to animals 24 h after perfusion with vehicle. (B) Quantification of MN loss. (C) Rotarod performance of control and AMPA-perfused rats at the times indicated. In B and C, values are mean ± SEM for 10 AMPA-treated rats, six intact control rats, and seven vehicle control rats (SEM was smaller than the size of the symbol in some cases). $^{*}p \leq 0.0001$ as compared with control groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AMPA caused a slowly progressing MN loss in ipsilateral and contralateral ventral horns along 5 days, starting in the ipsilateral horn at day one and reaching an almost total loss at day 5 in both horns; these changes were accompanied by intense astroglial reaction in both horns at day 5, shown by GFAP inmunolabeling (Fig. 2A, B). This progressive MN loss was associated with gradual bilateral motor deficits of hindlimbs, which started by flexion of the ipsilateral hindpaws at days 2–3, manifested in the lack of fingerprints in the stride pattern, which progressed to bilateral rigid extension at day 5 (Fig. 2E). These motor alterations progressed gradually to complete bilateral paralysis, which produced a rapid fall from the rotarod and in the grid of the PGE test (Fig. 2C, D). Quantitative analysis of stride pattern showed that since day one the number of steps needed to cross 60 cm increased with time and the distance crossed in 5 steps decreased (Fig. 2F); the distance between left and right steps and the length of the steps were also quantified but the differences were not significant (data not shown). With the exception of the newly described stride pattern alterations, these results are similar to those previously described (Tovar-y-Romo et al., 2007).

After this characterization of the acute and chronic AMPA treatments, we focused on the mechanisms of MN degeneration by means of ultrastructural alterations and casp3 immunolabeling occurring in each experimental condition.

Acute AMPA treatment causes MN ultrastructural and chemical alterations characteristic of necrosis

As illustrated in Fig. 3 (top row), no alterations in MN ultrastructure were observed in the control vehicle-treated group. Neurons show a well-defined soma and nuclear membrane, homogenous electrodensity in the cytoplasm, well preserved mitochondrial internal membrane and crest, and normal endoplasmic reticulum with attached ribosomes. This ultrastructural morphology was similar to that observed in intact rats (not shown).

MNs in the ipsilateral horn of AMPA-treated rats showed some degenerative features since 1.5 h after AMPA, such as the appearance of small round empty

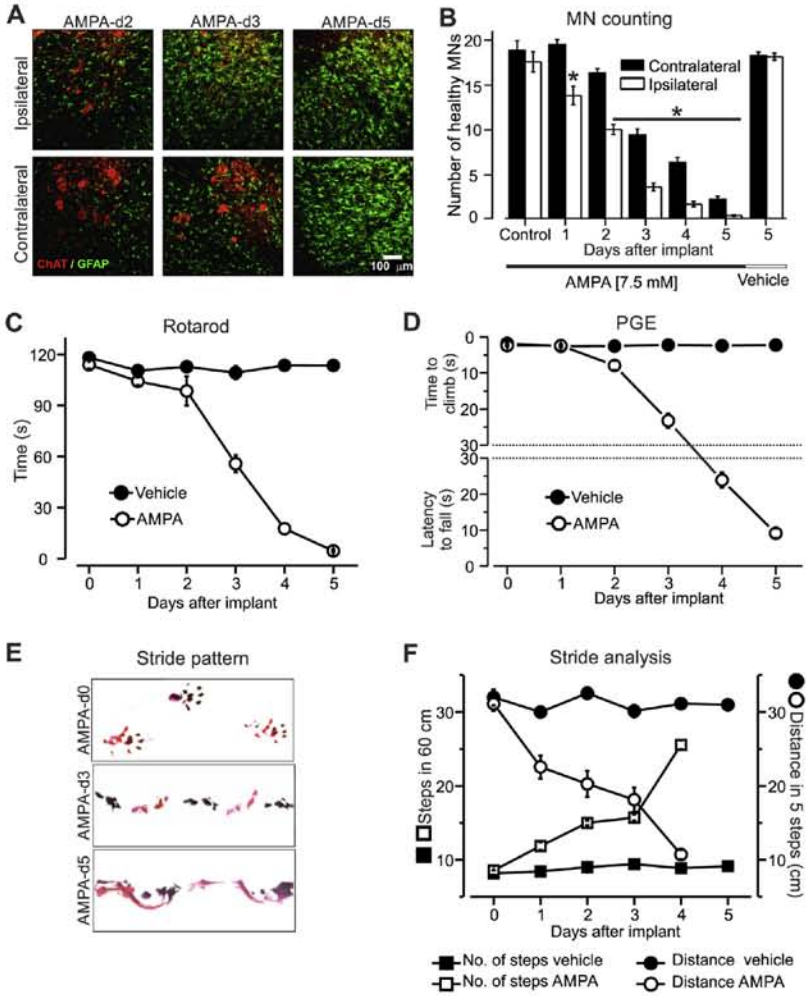

Fig. 2. Chronic infusion of AMPA with osmotic pumps induces progressive MN loss and motor deficits along several days. (A) Representative immunohistochemistry of the ventral horns of rats at the indicated times after surgery. MNs are identified by ChAT (red) and astroglial reaction by GFAP (green). Note progressive MN loss and astroglial reaction along days in the ipsilateral side, starting at 1–2 days after the experiment, whereas the damage in the contralateral side progresses more slowly. (B) Quantification of MN loss (Control value represents the initial day of AMPA treatment, 15–30 min after surgery). (C) Rotarod and (D) PGE performance of control and AMPA-perfused rats at the indicated times. (E) Stride pattern alterations (ipsilateral hindpaw-red, contralateral hindpaw-black) at the times indicated. (F) Quantitative analysis for stride pattern, showing the increase in the number of steps to walk a distance of 60 cm (squares), and the decrease in the covered distance for five steps for AMPA-treated rats (circles), compared to vehicle group. Values are mean ± SEM for seven animals per group, $^{*}p \leqslant 0.0001$ as compared with control group (SEM was smaller than the size of the symbol in some cases). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

spaces in the cytoplasm (* in Fig. 3). After 3 h, where MN loss is about 50% (Fig. 1C), the surviving MNs were swollen, and present rupture of cytoplasmic, mitochondrial, endoplasmic reticulum and nuclear membranes. At 24 h practically all MN were lost and only cell debris were observed, next to the appearance of new small cells with morphological features similar to macrophages, such as: kidney-shaped nucleus, dense bodies in the cytoplasm, and cytoplasmic extensions forming filopodia-like structures (Fig. 3, AMPA-24 h column C). The very few surviving MNs at this time showed severe morphologic alterations, such as distortion of nucleus and mitochondria, but no lysis. No alterations were found in the contralateral horn at any time (not shown).

In order to determine the possible involvement of apoptosis, we searched by immunolabeling the expression of ccasp3 in MNs at 1.5 h, 3 h and 6 h after AMPA treatment. Confocal examination at these times showed that no casp3 cleavage occurred, discarding an apoptotic process at these early times. However, at 24 h ccasp3 labeling was found in all the surviving MNs in the ipsilateral horn (Fig. 4). Caspase cleavage was absent in the control rats (not shown).

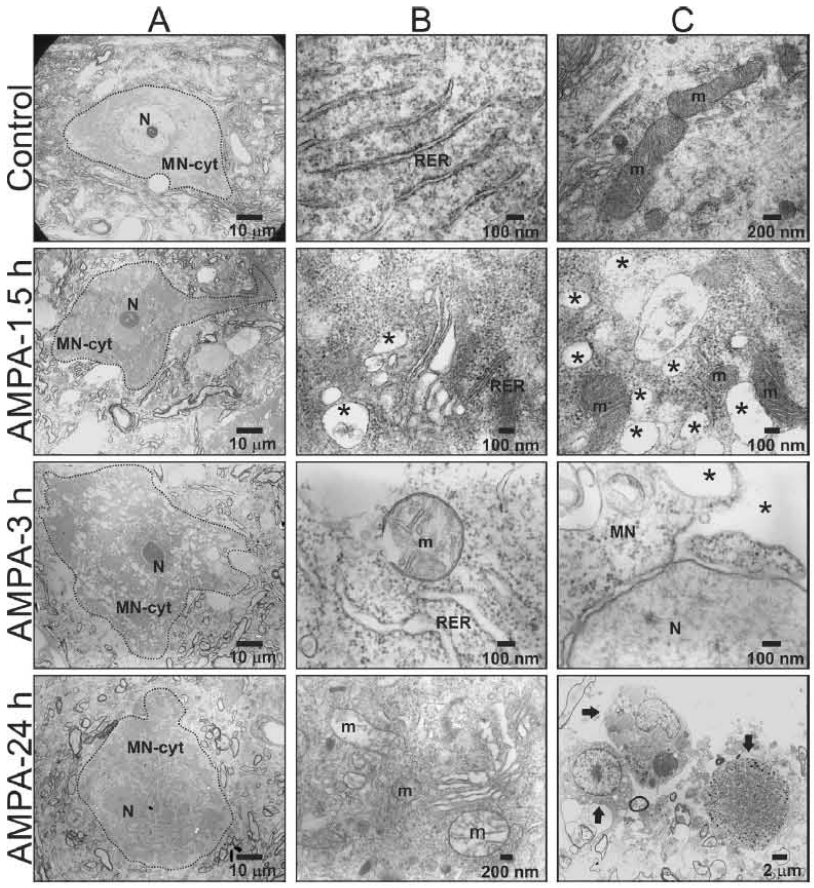

Fig. 3. Acute infusion of AMPA causes death of MNs by necrosis. Representative electron-micrographs of MNs in control rats (intact, $n = 3$, vehicle, $n = 4$, they showed no alterations) and AMPA-treated rats, at the indicated times after surgery ($n = 5$–6 per group). Column A shows low magnification micrographs; one MN per field is shown, with the membrane outline marked with a dotted line. Columns B and C, high magnification of intracellular regions of the corresponding MN in column A (except bottom right micrograph), showing mitochondria (m), rough endoplasmic reticulum (RER) and nucleus (N). Note that since early times (1.5 h), MNs perfused with AMPA begins a classic necrotic process characterized by empty spaces ( ) in MN cytoplasm (MN-cyt), and that three hours after AMPA necrosis progresses to RER swelling, mitochondrial swelling and lysis, and distortion of the nuclear envelope. At 24 h only a few MNs survive (see Fig. 1B), and they present altered shape and swollen mitochondria and RER. Bottom right micrograph shows MN debris and the appearance of macrophage-like cells (arrows). Scales are indicated in each micrograph.

Chronic infusion of AMPA causes ultrastructural and chemical alterations characteristic of early apoptosis and late necrosis

In contrast to the acute AMPA perfusion, the ultrastructural alterations observed during chronic infusion of AMPA produced consisted of both apoptosis-necrosis characteristics, according to the progress of MN degeneration that we have described as four sequential stages (stages 1–4 in Fig. 5). These four degenerative stages could be present simultaneously in the same tissue section, in different MN and different days, so that we could not correlate the timing of the changes with the days after surgery. Similar stages were observed in the contralateral horn but with a delayed appearance, for example stage 1 is usually present in day one and stages 2–4 in days two–three in ipsilateral horn, whereas they appear at days two and three-four, respectively, in the contralateral. Five days after surgery practically there are no MNs on both sides.

Stage 1 occurred since the first day after surgery, and is characterized by slight RER swelling and discrete reduction of nuclear area, without changes in MN shape. During stage 2 RER swelling was more intense, even forming vacuoles in the cytoplasm, nuclear area continues to decline and some mitochondria are swollen. During stage 3, vacuoles increase in size and form large empty areas usually located near the cytoplasmic membrane (pools, P in stage 3 in Fig. 5), which appear to occur by fusion of vacuolar membranes and contain probable cell debris. Nevertheless, pools do not seem to be autophagosomes because high magnification showed that are surrounded just by a single membrane and not for a double membrane, which is a characteristic of autophagosomes. Finally, in

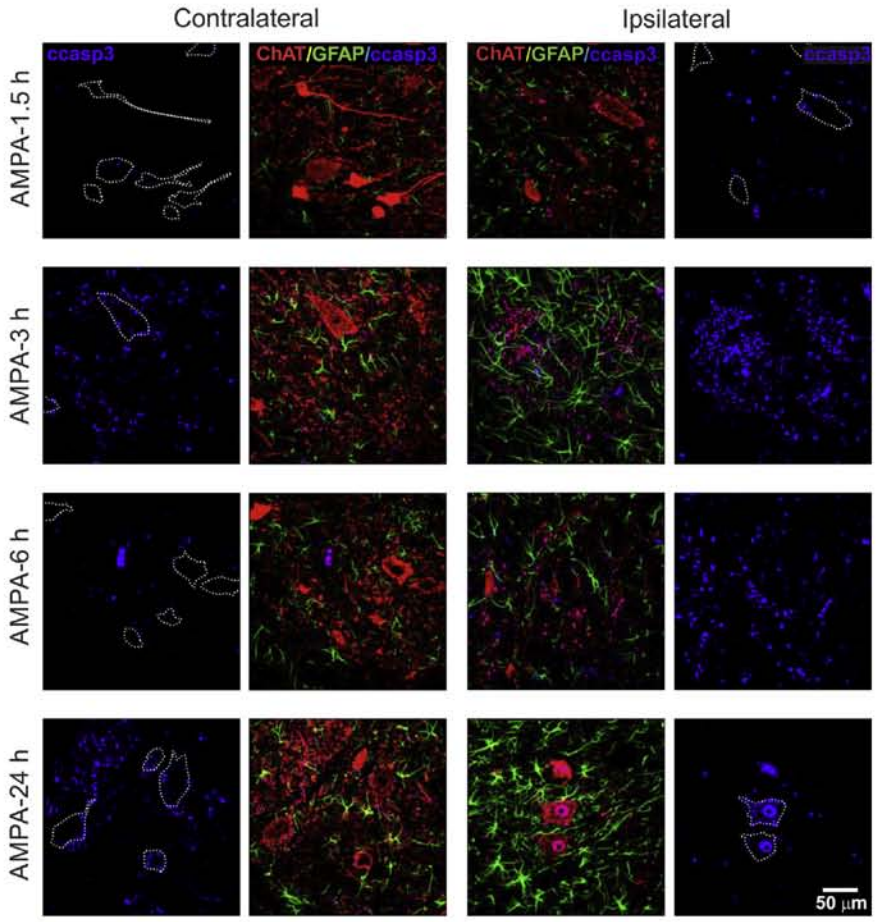

Fig. 4. Death of MNs induced by acute infusion of AMPA is independent of caspase-3 cleavage. ChAT (red), GFAP (green) and ccasp3 (blue) immunolabeling in the ipsilateral contralateral and ventral horns, at the indicated times after surgery. Micrographs are representative of seven rats for 1.5 and 24 h, and eight rats for 3 and 6 h groups. No changes were observed at 1.5 h. In the surviving MNs at 3 and 6 h no ccasp3 labeling occurred, whereas at 24 h all the few surviving MNs were ccasp3-positive in the ipsilateral horn. Shapes of MNs are indicated by white dotted lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

stage 4 MNs are completely disrupted and their debris appear to be phagocytosed by macrophage-like cells that at this stage are frequent (Fig. 5).

In order to further characterize the four stages described, we carried out a quantitative analysis of the ultrastructural changes for each stage (Fig. 6). Total cellular area is not significantly affected in stage 1, is considerable reduced in stage 2 and increases in stage 3 due to swelling of the RER and the appearance of vacuoles. In fact, nuclear area presents a progressive decrease in stages 1–3 and the area occupied by the vacuoles and pools notably increase in stage 3 (Fig. 6B). In order to test whether the decrease in nuclear size was an artifact caused by the decrease in total cell area, we standardized to 100% the total cell area to each analyzed MN and determined the corresponding percent for nuclear and vacuolated area (Fig. 6C); this analysis shows that decrease in nuclear area was consistent. Stage 4 was not analyzed because MNs are lysed.

The results of the immunohistochemical labeling of ccasp3 during the chronic AMPA treatment are shown in Fig. 7. Differently from the results with the acute perfusion, cleavage of casp3 is initially detected in the ipsilateral horn since day one, although because MN loss occurs so quickly further description was not possible. In the contralateral horn changes occurred more slowly and the strongest signal of ccasp3 in MNs was observed during day 3, but at day 5 basically all MNs were lost. MN degeneration and cleavage of casp3 was accompanied by a progressive intense astroglial reaction (Figs. 7 and 8A–C).

In order to explore in detail the cleavage of ccasp3 as related to MNs, we carried out double immunocytochemistry for ChAT and ccasp3 (Fig. 8, red and green, respectively), at different times during the progress of paralysis, and quantified the number of both ccasp3-positive (+ccasp3) and ccasp3-negative (−ccasp3) MNs. We found that the number of MNs positive only for ChAT is higher than MNs labeled with

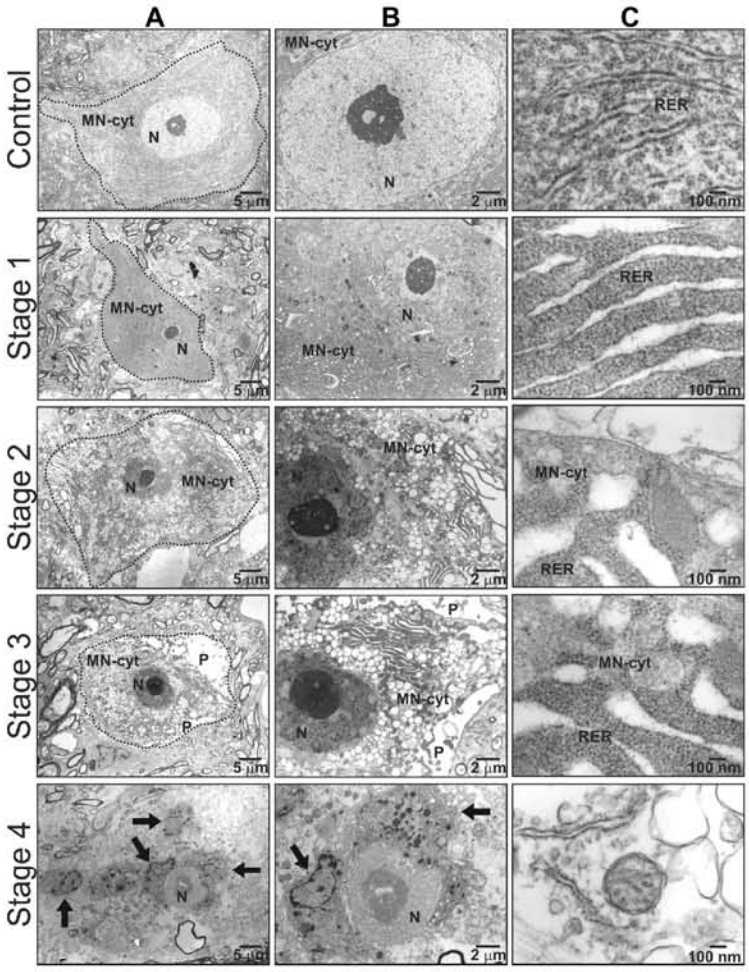

Fig. 5. Chronic infusion of AMPA induces MN death by early apoptosis and late necrosis, shown here as four stages of the degenerative process. Column A shows representative electron micrographs of whole MNs for each neurodegenerative stage; MNs shape is marked by a black dotted line. Columns B and C, high magnification of intracellular regions of the corresponding MN in column A. Stage 1 occurs at about 24 h after surgery and is characterized by slight RER swelling and some reduction of nuclear area. Stages 2–4 occur in different neurons at various times, between 48 and 96 h after surgery, and present more evident RER swelling and cytoplasmic vacuoles (stage 2); remarkable RER swelling and cytoplasmic large empty areas (pools, P) and further reduction in nuclear area (stage 3); Stage 4 is characterized by dissolution of cytoplasmic membrane, cell lysis, and the appearance of cells with phagocytic features (arrows in A and B), surrounded MN-debris, and very reduced nucleus. Abbreviations as in Fig. 3. See Fig. 6 for quantitative data of the changes described.

both ChAT and ccasp3, because +ccasp3-MNs were never over 50% of the population analyzed. The number (Fig. 8B) and percentage (Fig. 8C) of +ccasp3 and −ccasp3 changed differently with time after the implant: at day one 34.7 ± 3% of MNs are +ccasp3 in the ipsilateral horn and 13.4 ± 1% in the contralateral horn, and the following days these figures of +ccasp3-MNs changed in both horns but in opposite direction, as follows (Fig. 8C): day two, 43.5 ± 1% (ipsilateral) and 30.5 ± 2% (contralateral); day three, 38.3 ± 2% (ipsilateral) and 31.3 ± 2% (contralateral); day four, 17.5 ± 4% (ipsilateral) and 43.3 ± 1% (contralateral); and day five, 11.9 ± 4% (ipsilateral) and 27.9 ± 3% (contralateral).

In order to determine if the expression of ccasp3 may be related to the degeneration process, we determined the correlation by linear fit for the association between the number of +ccasp3-MNs per day and the MN loss quantified the following day, in each horn (Fig. 8D). The correlation values obtained clearly suggest that +ccasp3-MNs correspond to the MN loss for the next day (R^2 values 0.89 and 0.94 for the ipsilateral and contralateral horns, respectively).

Then we analyzed the correlation between the progress of motor deficits, expressed as the rotarod performance values, and the number of healthy MNs (Fig. 8E) as well as the number of damaged MNs identified by ccasp3 labeling (Fig. 8F). This analysis

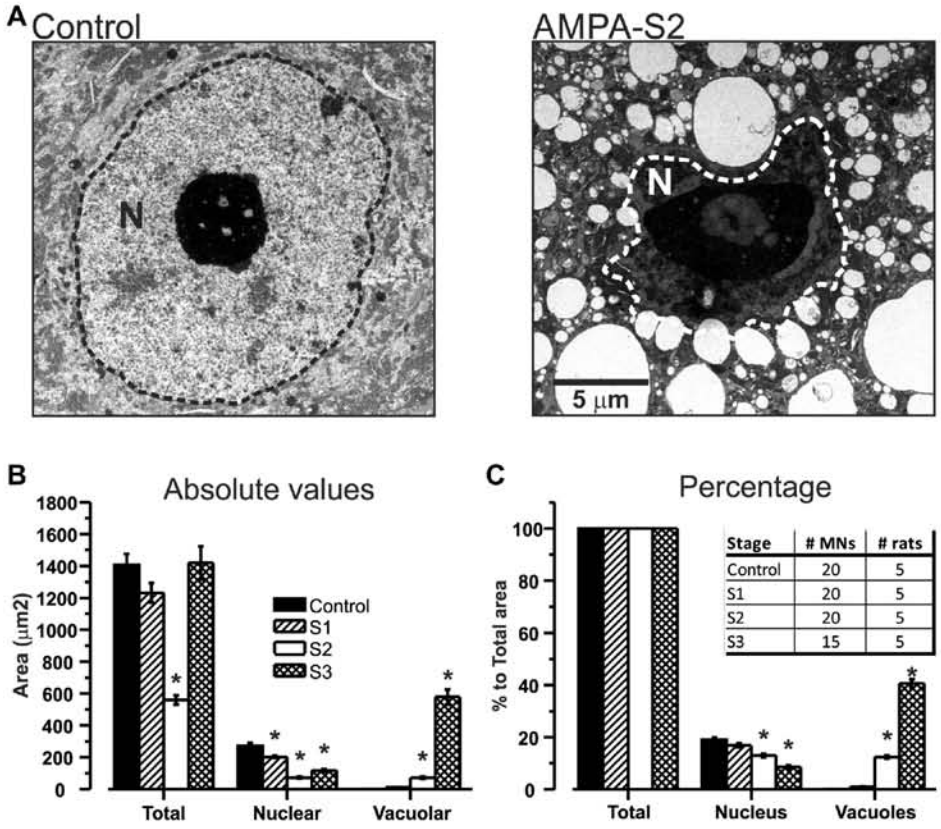

Fig. 6. Quantitative analysis of the ultrastructural alterations showed during MN degeneration induced by chronic AMPA infusion. (A) Representative electron micrographs for healthy (control) and degenerated (stage 2) MN-nucleus (N); nuclear area is delimited by a dotted line (black in control and white in degenerative stage 2). (B) Absolute values ± SEM for cell, nuclear and vacuolated areas of MNs, at the four stages described in Fig. 5. (C) Percentage of total area, nuclear and vacuolated areas, calculated with respect to the total area per MN. Note consistent significance in the reduction of nuclear area and in the increase in the vacuolated area in B and C. Scale bar is the same for both micrographs in A. The inserted table indicates the number of MNs and rats analyzed per group and condition. $^{*}p \leqslant 0.0001$ with respect to control.

showed that the motor deficits are strongly correlated with the total number of MNs (R^2 values 0.94 for contralateral, and 0.89 for ipsilateral horns) while +ccasp3-MNs bears no relation with the paralysis progress (R^2 values 0.09 for contralateral and 0.02 for ipsilateral side).

DISCUSSION

In the present work we took advantage of the remarkable differences in the duration of the degenerative process leading to MN death and consequent paralysis, depending on the method of AMPA administration. Because there is no genetic factor involved in these excitotoxicity experiments, our results may be relevant for understanding MN death in sALS as compared to that occurring in models of fALS.

Autophagy, apoptosis or necrosis are the more common recognized processes of cell death. Although each one has been characterized by molecular, biochemical and morphological analyses, these processes are not mutually exclusive and can occur simultaneously or in succession. Which of these processes are involved in MN death in ALS is unclear. Some data support the participation of apoptotic pathways, such as an increase in granzymes A y B levels (Ilzecka, 2011), enzymes involved in apoptosis activation pathway, as well as an increase in the cleavage of caspase-9 (Ilzecka, 2012) and caspase-1 (Ilzecka et al., 2001) in the serum, and caspase-3 in MNs of spinal and cortical tissue of ALS patients (Hart and Gitler, 2012; Ilzecka, 2012). However, if activation of apoptotic pathways occur as an initial mechanism or as a consequence of the MN degeneration is difficult to establish. In spinal MNs of transgenic SOD1 mutant mice, sequential activation of caspase-1, caspase-3 and caspase-9 has been described since presymptomatic stages (Li et al., 2000; Pasinelli et al., 2000; Ohta et al., 2008; Rossi et al., 2008), but one of the most important morphological features of apoptosis, apoptotic bodies (Yamazaki et al., 2005), have not been reported, even at final stages. On the other hand, morphologic observations of MNs during the progress of the symptoms in these mice have shown massive vacuolization of cytoplasm accompanied by mitochondrial swelling (Bendotti et al., 2001; Martin et al., 2009) suggesting a necrotic process. Thus, these necrotic and apoptotic characteristics described in ALS

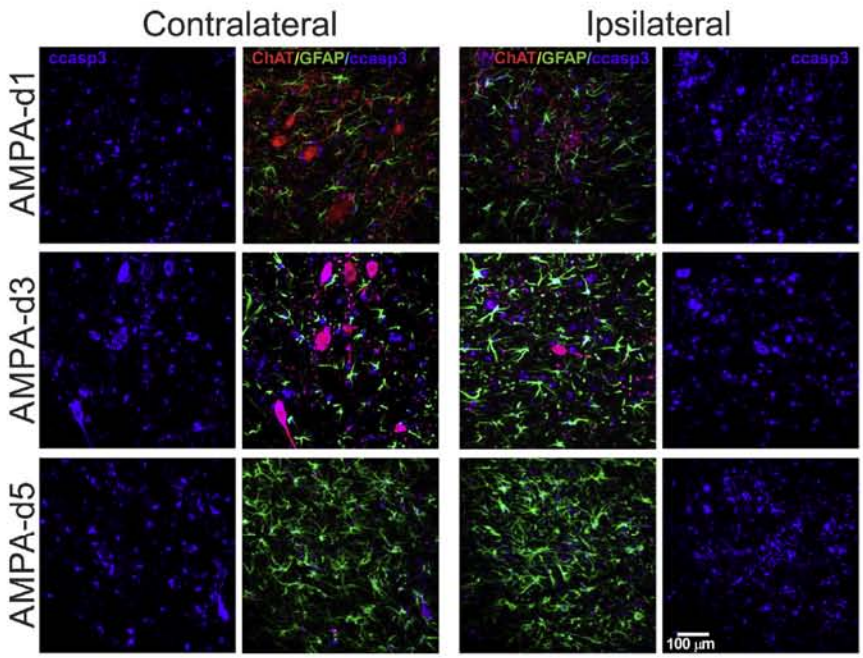

Fig. 7. Caspase 3 is cleaved during the degenerative process induced by chronic infusion of AMPA. Representative micrographs (10 rats per group) at the indicated times after surgery for ChAT (red), GFAP (green) and ccasp3 (blue) immunnolabeling in the ipsilateral and contralateral ventral horns. Note that positive mark to ccasp3 in MNs (pink color in central columns) begins in ipsilateral horn and progresses to the contralateral side, associated with a glial reaction. Scale bar is the same for all micrographs. See Fig. 8 for quantitative data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transgenic models and in tissues from ALS patients are similar to the MN degeneration processes described here, although the alterations occur faster.

In this work we show that the MN death process depends on the severity of excitotoxic stimulus. MN swelling and lysis without caspase cleavage observed in the ipsilateral horn 3 h after the acute perfusion of AMPA suggests a rapid classic necrotic process. Although at 24 h ccasp3 was observed in the surviving MNs, the ultrastructural alterations and the appearance of macrophage-like cells are in agreement with a necrotic process. It is worth noting that the early necrotic process observed at 3 h is correlated with a remarkable MN loss resulting in a complete ipsilateral paralysis and thus a rapid falling in the rotarod test.

Chronic perfusion of AMPA caused a progressive MN loss which started in the ipsilateral side and extended progressively to the contralateral side. The slight decrease in MN observed at days 1 and 2 were not enough to cause rotarod or PGE deficits, a fact that can be ascribed to the apparent existence of a threshold value, of ~50% of MN population, necessary to produce paralysis (Santa-Cruz and Tapia, 2014). However, at this time we observed stride pattern alterations, which could be explained by changes in the excitable properties of MN circuits that affect the recruitment of MNs. In fact, progressive disorders in the MN synaptic inputs occur during the course of the disease in SOD1 mouse model of ALS, even before MNs loss and paralysis (Jiang et al., 2009), and deficient H reflex probably due to segmental MN dysfunction have been described in ALS patients (Vucic et al., 2009; Simon et al., 2015).

The ultrastructural changes found during the chronic AMPA infusion suggest a mixed process of MN degeneration with early apoptotic features, such as decreased nuclear and cellular areas, and subsequent necrotic features characterized by a progressive vacuolization of MNs and finally cell lysis. In fact, biochemical and morphologic characteristics of MNs at the first and second neurodegenerative stages are typical of an apoptotic process (Orrenius et al., 2003; Kroemer et al., 2009), and the temporal correspondence between cleavage of casp3 and these stages also suggest an initial apoptotic mechanism. However, at the late stages increased cellular area, mitochondrial swelling and lack of caspase cleavage, resembles a necrotic process. Thus, the chronic excitotoxic process seems to occur in two phases, the first one with apoptotic characteristics and the second with necrotic features. Interestingly, both processes have been described in models of fALS (Nagy et al., 1994; Pasinelli et al., 2000; Bendotti et al., 2001; Locatelli et al., 2007; Ohta et al., 2008; Rossi et al., 2008) and in tissue (Hart and Gitler, 2012) and serum (Ilzecka et al., 2001; Ilzecka, 2011, 2012) of ALS patients. An apoptosis-necrosis continuum similar to our findings with the chronic model has been described to occur in a 24 h period after the acute administration of excitotoxins in the developing and adult rat brain (Portera-Cailliau et al., 1997a,b), as well as in the spinal cord of chick

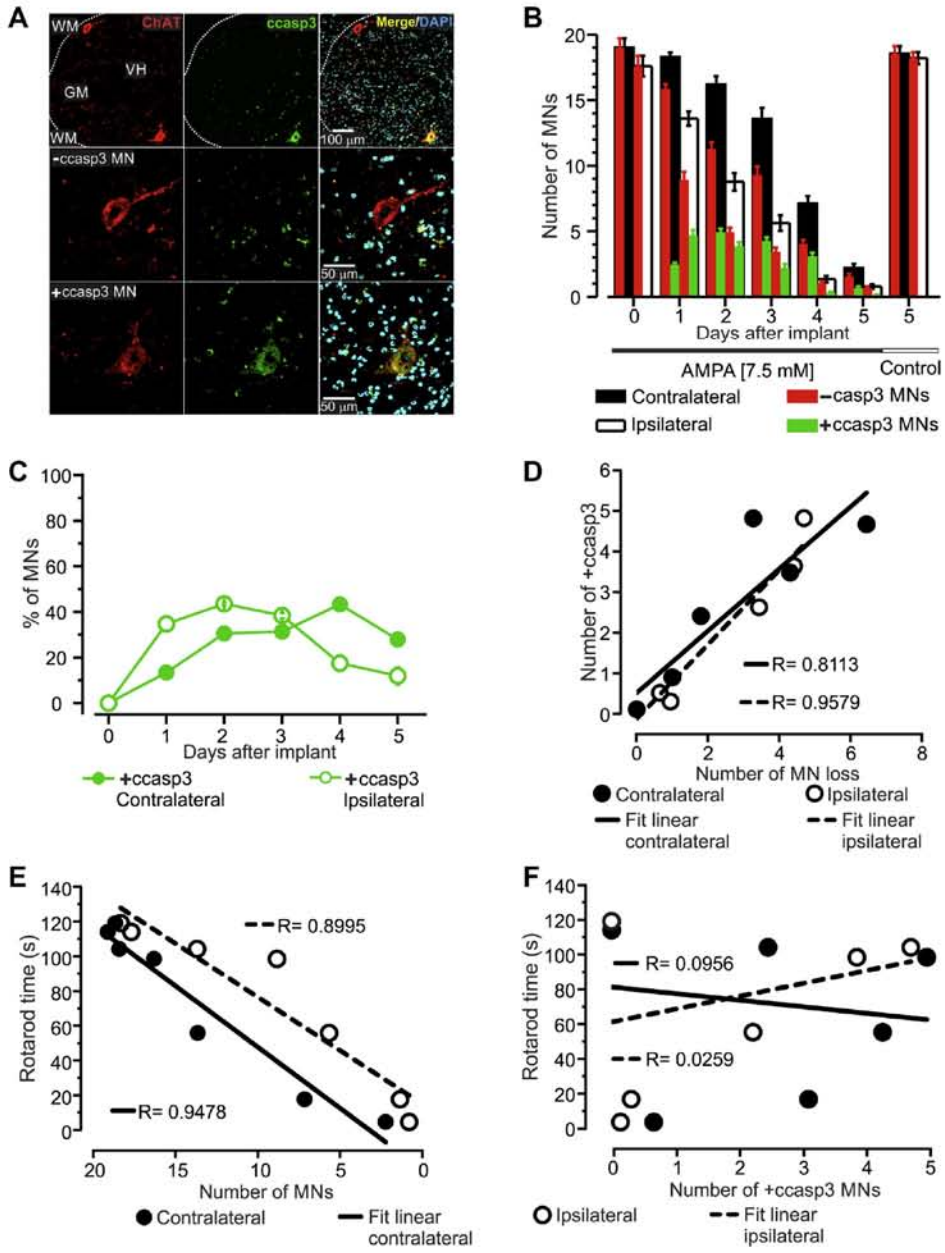

Fig. 8. Quantitative analysis of casp3 cleavage during MN degeneration induced by chronic AMPA infusion, and its correlation with motor alterations and MN loss. (A) Representative micrographs showing one positive ccasp3 MN (+ccasp3 MN, green) and one negative (−ccasp3 MN, red) in the same ventral horn, 3 days after surgery. (B) Quantification of number of +ccasp3 MNs (green bars) and −ccasp3 MNs (red bars) in the contralateral (black bars) and ipsilateral (white bars) horns at the days indicated. Graph C shows that along MN loss during AMPA treatment, the percentage of +ccasp3 never exceeds fifty percent of MNs. (D) Correlation between the number of +ccasp3 MNs with MN loss. Graphs E and F show that motor deficit is strongly correlated with MN loss (E), but not with the number of +ccasp3 MNs (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

embryos (Caldero et al., 1997), but no correlation with behavioral changes were described.

The analysis of the relationship between the expression of +ccasp3, MN loss and motor deficits show some interesting points: (1) the number and proportion of MNs labeled with both ChAT and ccasp3, was never higher than the number of MNs with only ChAT; (2) the latest MNs to be lost were negative for ccasp3, suggesting that at the end of MN degenerative process casp3 is not necessary involved. This poses

the question of whether +ccasp3 MNs are related to the motor deficits. As shown in Fig. 8, there is no correlation between these parameters, in spite of the fact that casp3 cleavage is a mark of MN damage because the number of +ccasp3 MNs each day correspond with the MN loss quantified the next day. This is probably one of the reasons that could explain why treatments focused on preventing the cleavage of caspases only delayed but did not prevent the MN death and paralysis in transgenic mouse model of ALS (Li et al., 2000; Pasinelli et al., 2000; Ohta et al., 2008).

The appearance of macrophage-like cells at the late stages of MN degeneration after both acute and chronic treatment suggests that these cells are involved in the phagocytosis of the lysed MNs debris. Participation of phagocytic microglia has been observed in mutant SOD1 mice since presymtomatic stages, before morphological alterations of MNs, suggesting that these cells may be involved in the mechanism for MN degeneration (Sanagi et al., 2010). However, our results show that these cells appear only after MN lysis has occurred.

The astroglial reaction observed since 3 h after AMPA microdialysis, previous to the ccasp3 detection, has not been previously described, whereas that after chronic treatment was previously showed (Tovar-y-Romo et al., 2007). Astroglial reaction is considered a histopathological feature of ALS, because it has been observed in cerebral cortex (Kushner et al., 1991; Nagy et al., 1994) and spinal cord (Schiffer et al., 1996; Schiffer and Fiano, 2004) of ALS patients, and in spinal tissue of transgenic mice (Alexianu et al., 1994; Hall et al., 1998; Feeney et al., 2001; Boillee et al., 2006; Neymotin et al., 2009; Guo et al., 2010). Both astroglia and microglia activation has been associated with the release of proapoptotic factors (Alexianu et al., 2001; Raoul et al., 2002; Locatelli et al., 2007) and reactive oxygen species (Sofroniew, 2005). However, efforts to avoid glia or microglia activation have been unsuccessful to prevent paralysis in SOD1 mutant models (Neymotin et al., 2009; Keller et al., 2011).

CONCLUSION

In conclusion, in this study we show that an acute excitotoxic insult induces rapid necrotic death while chronic excitotoxicity triggers a slow progressive degenerative process, involving apoptotic and necrotic features. The delayed cleavage of casp3 occurring after the astroglial reaction suggests a role of non-neuronal cells in both neurodegenerative processes. Although excitotoxicity has not been clearly demonstrated to be a causal factor in ALS, the findings described in the present work, using experimental models independent of genetic factors, show remarkable similarity with the MN degeneration processes described in transgenic fALS mice and with several pathological observations in tissues from ALS patients, and thus are relevant for understanding the pathophysiology of MN diseases.

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Sección II. Papel del bloqueo farmacológico de los circuitos inhibitorios en la degeneración de las MNs espinales lumbares.

Para esta sección de resultados incluimos el manuscrito "*Chronic GABAergic blockade in the spinal cord in vivo induces motor alterations and neurodegeneration*", el cual se ha enviado a publicación y se encuentra en revisión; contiene los resultados obtenidos del bloqueo agudo y crónico de los sistemas de inhibición GABAérgica y glicinérgica de la médula lumbar. A continuación se hace un breve resumen del artículo y posteriormente se incluye el escrito en revisión.

Resumen del artículo "*Chronic GABAergic blockade in the spinal cord in vivo induces motor alterations and neurodegeneration*".

La actividad de los circuitos neuronales de la médula espinal es controlada principalmente por las neurotransmisiones inhibidoras de GABA y glicina, las cuales regulan la excitabilidad mediada por glutamato. Alteraciones de los circuitos inhibidores y excitadores han sido involucradas en la patofisiologia de diversas enfermedades como epilepsia, esquizofrenia autismo y ALS. En nuestro laboratorio hemos demostrado que el uso agudo y crónico de agonistas glutamatérgicos, como el AMPA induce la muerte de las MNs lumbares por excitotoxicidad, pero poco es conocido acerca del efecto de una falla en la neurotransmisión inhibidora de GABA y glicina. En este trabajo estudiamos las consecuencias del bloqueo agudo (mediante microdiálisis), y crónico (usando minibombas osmóticas) de los circuitos inhibidores en la medula espinal lumbar de rata in vivo, mediante el uso de los antagonistas: estricnina para glicinia y bicuculina para GABA.

Demostramos que la falla aguda de los sistemas glicinérgicos y GABAérgicos no provoca consecuencias significativas en la actividad motora ni en la supervivencia de las MNs. Sin embargo, el bloqueo crónico de la actividad GABAérgica, pero no de glicina, indujo alteraciones en la marcha, pérdida significativa de motoneuronas y flacidez de los dedos en la extremidad que recibió

el tratamiento, estas alteraciones fueron prevenidas por el bloqueo farmacológico del receptor AMPA con CNQX, pero no por el bloqueo de los receptores NMDA. Finalmente determinamos que la falla GABAérgica crónica incrementa el efecto excitotóxico del tratamiento crónico con AMPA, causando una parálisis bilateral mas rápida y severa de los cuartos traseros.

En conclusión, demostramos que el bloqueo del sistemas inhibitorio GABAérgico causa un desbalance del equilibrio entre inhibición/excitación en la médula espinal, causando déficits motores transitorios y muerte de MNs, efectos mediados principalmente por la sobreactivación secundaria de receptores AMPA, causando hiperexcitabilidad de las MNs. Este mecanismo de degeneración neuronal, involucrando alteraciones primarias de los circuitos inhibitorio, parecen ser de relevancia para el entendimiento de la patofisiología de diversas enfermedades de las MNs y para el diseño de nuevas estrategias para su tratamiento.

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Manuscript Draft

Manuscript Number:

Title: Chronic GABAergic blockade in the spinal cord in vivo induces motor alterations and neurodegeneration

Article Type: Research Paper

Keywords: AMPA receptor, excitotoxicity, GABA, glycine, motor neuron, spinal cord

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Abstract: Inhibitory GABAergic and glycinergic neurotransmission in the spinal cord play a central role in the regulation of neuronal excitability, by maintaining a balance with the glutamate-mediated excitatory transmission. Glutamatergic agonists infusion in the spinal cord induce motor neuron death by excitotoxicity, leading to motor deficits and paralysis, but little is known on the effect of the blockade of inhibitory transmission. In this work we studied the effects of GABAergic and glycinergic blockade, by means of microdialysis perfusion (acute administration) and osmotic minipumps infusion (chronic administration) of GABA and glycine receptors antagonists directly in the lumbar spinal cord. We show that acute glycinergic blockade with strychnine or GABAergic blockade with bicuculline had no significant effects on motor activity and on motor neuron survival. However, chronic bicuculline infusion, but not strychnine, induced ipsilateral gait alterations, phalange flaccidity and significant motor neuron loss, and these effects were prevented by AMPA receptor blockade with CNQX but not by NMDA receptor blockade with MK801. In addition, we demonstrate that the chronic infusion of bicuculline enhanced the excitotoxic effect of AMPA, causing faster bilateral paralysis and increasing motor neuron loss. These findings indicate a relevant role of GABAergic inhibitory circuits in the regulation of motor neuron excitability and suggest that their alterations may be involved in the neurodegeneration processes characteristic of motor neuron diseases such as amyotrophic lateral sclerosis.

Keywords

AMPA receptor, excitotoxicity, GABA, glycine, motor neuron, spinal cord

1. Introduction

Activity of neuronal circuits is controlled by the action of excitatory or inhibitory neurotransmitters. Dysregulation of this synaptic control has been associated with several neurological and psychiatric diseases that have as a common endpoint hyperexcitability, which may be consequence of a decrease in inhibitory neurotransmission or an increase in excitatory neurotransmission. Overactivation of excitatory neuronal networks can trigger neurodegeneration by excitotoxicity, causing neuronal death and irreversible functional damage according to the affected area. Most experimental studies on excitotoxicity have been focused on excessive glutamatergic transmission, using glutamate receptor agonists, despite the fact that alterations of inhibitory circuits have been shown to be involved in the pathophysiology of several diseases, such as epilepsy, schizophrenia, autism and amyotrophic lateral sclerosis (ALS).

ALS is characterized by the loss of motor neurons (MNs) in cortical areas, brainstem and spinal cord, in both familial and sporadic forms, and the hyperexcitation observed has been associated with alterations of the function of spinal circuitries, controlled by neuronal networks formed by several kinds of cells. The most important components that regulate excitability are the abundant inhibitory interneurons V2b, V0C/G, V0D and V0V, as well as the Renshaw cells, that receive afferents from the MNs and directly inhibit them through the release of GABA and glycine (Ramírez-Jarquín et al., 2014; Schneider and Fyffe, 1992; Todd and Sullivan, 1990). Whereas the glutamate-mediated excitotoxicity has been amply studied, little is known on the effect of the blockade of GABAergic and glycinergic inhibitory transmission in vivo, and this is the purpose of the present work.

For this purpose, we studied the effects of the direct administration of GABAergic and glycinergic antagonists in the lumbar spinal cord, as well as their interaction with glutamate receptor agonists and antagonists, to know whether the alteration of excitatory-inhibitory balance could result in MN hyperexcitation and degeneration. We administered the drugs acutely, by means of reverse microdialysis, and chronically using osmotic minipumps. Using these procedures, we have previously shown that acute AMPA perfusion produces MN loss and paralysis in 3-12 h (Corona and Tapia, 2004), whereas its chronic infusion results in progressive MN degeneration and gradual paralysis along several days (Tovar-y-Romo et al., 2007).

2. Material and methods

2.1. Animals

All the experiments were made using adult Wistar male rats (270-300 g), handled in accordance with the Rules for Research and Health Matters (Mexico) and with international standards of research animal welfare (including ARRIVE guidelines), and with approval of the local Animal Care Committee (Approval No. RTI21-14). Animals were housed in a controlled laboratory environment: 12 h light/dark cycle, ad libitum access to regular animal chow and water. All surgical procedures were performed under general anesthesia. All efforts were made to minimize suffering of the animals.

2.2. Drugs

AMPA, CNQX and MK801 were purchased from Tocris Bioscience, and bicuculline methbromide (Bic) and strychnine (Stry) from Sigma Aldrich. For acute treatment (microdialysis) Bic and Stry (1 or 5 mM each) were dissolved in Krebs-Ringer solution containing (in mM) 118 NaCl, 4.5 KCl, 2.5 $MgSO_4$, 4.0 $Na_2H_2PO_4$, 2.5 $CaCl_2$, 25 $NaHCO_3$ and 10 glucose, pH 7.4, in all cases osmolarity was maintained by reducing the NaCl concentration proportionally For chronic treatment, osmotic minipumps (Alzet model 2004, volume ~250 µL, flow rate 6 µL/day) were filled with one of the following solutions: saline or phosphate buffer (PB) for control groups; Bic 5, 10 or 17.5 mM; Stry 20 mM; Bic 10 mM + CNQX 1 mM; Bic 10 mM + MK801 14 mM; AMPA 3.8 mM; and AMPA 3.8 mM + Bic 10 mM. Pumps were incubated for 48 h in filtered saline solution at 37°C for stabilization. These concentrations were chosen on the basis of previously published results and on preliminary experiments (Corona and Tapia, 2004; Lazo-Gómez and Tapia, 2016; Tovar-y-Romo et al., 2007). In the case of AMPA, we reduced the previously used 7.5 mM concentration to 3.8 mM in order to diminish the severity of the excitotoxic effect.

2.3. Surgical procedures

Surgery for the microdialysis procedure was performed essentially as previously described (Corona and Tapia, 2004). Rats were anesthetized with 5.0% isoflurane in carbogen (95% O_2/5% CO_2 mixture) and placed in a stereotaxic spinal unit; isofluorane concentration was gradually diminished to 1.5 - 2.0 % during the surgery. A median sagittal incision (3.5 - 4

cm long) was made in the back (skin was shaved, cleaned and disinfected previously) and the underlying fascia and muscle tissue were dissected. The spinous process was removed with a drill, and a ~2 mm diameter hole was drilled in the right lamina of the third lumbar. The meninges were carefully removed with a metallic hook and a microdialysis probe (CMA7, Carnegie, Sweden) was lowered into the right dorsal horn of the spinal cord. The probe was perfused at a flux rate of 2 µL/min with Krebs medium during one h for stabilization, and then perfused for 25 min (50 µL) with one of the media indicated above, using a microinjection pump (CMA/100, Carnegie, Sweden). After the experiment, the microdialysis probe was gently removed and the skin incision was sutured, and after recovered from the anesthesia animals were placed in individual cages and subjected to the motor tests at the times indicated in Results, and finally sacrificed for histological studies.

Surgery for the chronic procedure was performed as previously described (Tovar-y-Romo et al., 2007), slightly modified. The initial tissue dissection was made as described above, and after the spinous process was removed a stainless-steel screw (3.7 mm long, 1 mm diameter) was fixed in the base of the process. A cannula (1 mm long, 50 µm internal diameter and 80 µm external diameter, VitroCom Inc.) was carefully advanced down into the dorsal horn; this probe was attached to the catheter of the osmotic minipumps; union was sealed with cyanoacrylate and the implant was fixed with dental cement. Osmotic minipumps were subcutaneously implanted in the back of the animal. Finally, the skin incision was closed with surgical stainless-steel clips, anesthesia was withdrawn and animals received a single intraperitoneal antibiotic shot. They were kept in individual cages with food and water ad libitum during the period of the motor tests.

2.4. Motor behavior evaluation

Seven days prior to surgery, rats were trained in rotarod (Columbus Instruments, Columbus, OH, USA) and a variation of the paw grip endurance (PGE) task. After surgery animals were evaluated in each test daily until fixation. For rotarod test, rats walked on an accelerating (0.2 rev/min per s) rod, starting from 10 rpm with a cut-off of 120 s, the average time for three attempts was scored. For the PGE, rats were placed on a horizontal placed grid (40 x 25 cm) that was gently turned until reaching a vertical position. Average time of three attempts to climb to the top of the grid or the latency to fall when they were

unable to climb was scored with a cut-off of 40 s. In addition, stride pattern of the hind footprints was recording by inking the hindpaws with non-toxic Chinese and make the animals walk along a paper 10 x 100 cm runway.

2.5. Histology and immunofluorescence

At the end of each motor test period, rats were perfused and fixed for histological and immunohistological analyses as previously described (Corona and Tapia, 2004; Tovar-y-Romo et al., 2007). Animals were deeply anesthetized with an intraperitoneal injection of pentobarbital and perfused transcardially with 250 mL of ice-cold 0.9 % saline, followed by 250 mL of ice-cold 4% paraformaldehyde in 0.1 M PB, pH 7.4. Spinal cord was removed, post-fixed in 4% paraformaldehyde at 4°C for a week, and successively dehydrated in sucrose gradients (up to 30%). Twenty transverse sections (40 µm thick) of the lumbar region, at the site of the cannula, were obtained in a cryostat. Alternate sections were stained with cresyl violet or immunostained for choline acetyltransferase (ChAT) and glial fibrillary acidic protein (GFAP).

Immunofluorescence for ChAT and GFAP was performed on floating slices which were blocked with 5% of bovine serum albumin in PB 0.1 M with Triton X-100 (0.3 %) for 2 h and after exposed to goat polyclonal anti-ChAT (ChAT, 1:200; Chemicon, Temecula, CA, USA) and mouse polyclonal anti-GFAP (1:1000, Sigma Aldrich) as primary antibodies for 48 h at 4°C. Sections were washed three times for 15 min in PB-Triton and incubated with biotynil-conjugated mouse anti-goat IgG (1:200; Vector, Burlingame, CA, USA) for 1.5 h. After three washes, sections were incubated for 2 h with biotin-conjugated horse anti-goat IgG (1:200, Vector Labs) and after with avidin-Texas Red conjugate (1:200, pH 8.2; Vector) and FITC-conjugated anti-mouse antibody (1:250; Zymed, Carlsbad, CA, USA) as secondary antibodies. Slices were mounted on silane (Sigma Aldrich) treated glass slides and coverslipped with fluorescent mounting medium (DAKO, Carpinteria, CA, USA). Sections were visualized under a confocal Olympus FV10i microscope, manually adjusting the parameters of laser intensity and sensitivity for each channel. Merged images are the overlay of five laser sections in the Z plane, using the Olympus 1.6 Fluoview.

Morphologically undamaged MNs in the Nissl preparations (with a soma diameter >25 µm and distinguishable nucleus) were counted in a 10X microscopic field. The number

of cells was determined in sections where the trace of the infusion cannula was evident. Ten histological sections per rat were counted and the values were averaged.

3. Results

The microdialysis perfusion of Stry at 1 or 5 mM concentration did not induce any significant behavioral or histological alteration: rats performed similarly to controls in the rotarod test for seven days and histological and immunochemical observations with ChAT and GFAP did not show MN loss or glial reaction (Fig. 1). Animals receiving Bic (1 or 5 mM) fell from the rotarod in 80-90 s 6 h after the experiments and recovered slowly during the next hours (Fig. 1A), and those treated with 5 mM Bic showed an increased sensitivity to the contact in the ipsilateral hindquarter, observed by the contraction of ipsilateral phalanges induced by a discreet mechanical stimulation of the ipsilateral footpad. After recovery from anesthesia these animals showed transitory contractions of the ipsilateral hind paw and occasional wet-rat shakes; these symptoms of hyperexcitability disappeared after 40-50 min. No MN damage or glial reaction was observed in these animals at the end of the 7-days observation period (Fig. 1B,C).

In contrast to the minor effects of the acute microdialysis perfusion, chronic infusion of Bic at 5, 10 and 17.5 mM concentrations caused concentration-dependent alterations of motor behavior along several days, whereas chronic 20 mM Stry was innocuous (Fig. 2). Bic 10 and 17.5 mM induced transitory effects characterized by grooming, hypersensitivity, focal fasciculations, scratching and biting of the ipsilateral limb, and myoclonus of hindpaw. These behavioral alterations were most intense with 17.5 mM, included also frequent wet-rat shakes, and 5 of the 10 rats treated died in generalized tonic seizure within the first 24 h after the implant. The latency to the first symptom and the duration of these effects, as indicated in Fig. 2B (white arrows), was 4-6 days, 2 days and 1 day for 5, 10 and 17.5 mM, respectively. The alterations of the hind limb movements were manifested in the stride pattern as the absence of the ipsilateral footprint, because uncoordinated muscle contraction of this limb caused that rats walked with the paw raised, and consequently this paw was not recorded in the stride pattern (Fig. 2A). Footprint records also show a permanent effect, that started at the times indicated in Fig. 2B (red arrows), characterized by enduring phalanges flaccidity that keeps them tightly joint,

differently from the healthy footprint which show separated fingers (Fig. 2A). All the animals treated with 10 mM and 17.5 mM presented these permanent alterations but with 5 mM only 50% showed them.

As shown also in Fig. 2B, the motor alterations, as detected by the rotarod test, started simultaneously with the first behavioral symptom. Remarkably, with the three concentrations of Bic the maximal effect was to fall at ~50-60 s and in all cases this effect was transitory, but with notable time course differences: with 17.5 mM this score was attained at the first day and gradually recovered by day 4; with 10 mM it was reached at days 5-6 and the recovery at day 14; and with 5 mM it was observed at days 8-10 and recovery at day 16. In contrast, no significant alterations were detected in the PGE test at any Bic concentration (Fig. 2C).

Histological and ChAT immunocytochemical observations at day 16 after pump implant revealed no significant changes with 20 mM Stry or with 5 mM Bic, whereas 10 and 17.5 mM Bic induced ~33% loss of MNs in the ipsilateral ventral horn, but not in the contralateral side (Fig 3). This MN degeneration was accompanied by an astroglial reaction, as demonstrated by intense GFAP immunolabeling. Some GFAP immunofluorescence was also observed with 5 mM Bic and with Stry, probably due to the mechanical damage produced by the cannula.

Based on the previous results, we selected the concentration of Bic 10 mM for the next experiments, aimed at studying if the hyperexcitability induced by Bic was mediated by glutamatergic pathways.

Blockade of NMDA receptors by MK801 decreased the intensity of the transitory behavioral effects of Bic, reducing grooming and hypersensitivity of the ipsilateral hind limb and preventing the fasciculations and myoclonus. This protective effect was manifested in a better performance in the rotarod test (Fig. 4A). However, the permanent effect detected by the footprints was not prevented by MK801 (Fig. 4C). Interestingly, the blockade of AMPA-receptors by CNQX had opposite effects, since neither the transitory action of Bic nor the rotarod score were modified, as compared to Bic alone, whereas the permanent changes were prevented (Fig. 4A, C). The PGE performance showed no alterations in any of the groups (Fig. 4B). There was also a different effect of MK801 and

CNQX regarding MN degeneration: the former was ineffective while CNQX totally prevented the MN loss caused by Bic (Fig. 4D).

In other experiments to test whether glutamate receptors are involved in the mechanism of the hyperexcitability induced by Bic, we studied whether the previously demonstrated excitotoxic action of AMPA (Corona and Tapia, 2004; Ramírez-Jarquín and Tapia, 2016; Tovar-y-Romo et al., 2007) was affected by Bic infusion. As shown in Fig. 5, at the concentration used AMPA induced slowly progressing ipsilateral motor deficits and paralysis along 10 days, manifested as fall from the rotarod at 90 s at day 4, that reached ~50-60 s the following days; differently from the results with Bic, this effect was permanent until day 10 (Fig. 5A). However, AMPA did not alter the PGE performance, most probably because, as shown by the stride pattern, the contralateral limb was not affected and this was enough to climb the grid; only the ipsilateral limb presented paralysis, manifested by the dragging of the paw (Fig. 5C). These effects of AMPA were clearly potentiated by Bic, since the animals co-infused with both compounds fell in ~100 s at day 2 and the deficit rapidly progressed to reach ~20-30 s at day 5 and did not recover (Fig. 5A). This potentiation was also remarkable in the PGE test and in the stride pattern, since from day 2 the time to climb increased ~15 s and by day 4 the rats started to fall, at about ~20-25 s, instead of climbing (Fig. 5B). These deficits can be correlated with the alterations in the stride pattern that indicated that both limbs were affected from day 4 and by day 8 the animals presented bilateral paralysis (Fig. 5C).

The potentiation of the motor effects of AMPA by Bic was clearly correlated with the histological and ChAT and GFAP immunochemical observations. AMPA induced ~80% MN loss in the ipsilateral horn and 20% in the contralateral horn, as well as intense astroglial reaction. Remarkably, AMPA + Bic co-infusion increased the MN loss to nearly 100% in the ipsilateral horn and to ~80% in the contralateral one, and further increased GFAP labeling (Fig. 5D, E).

4. Discussion

Inhibition in the spinal cord is mediated by the action of the GABA and glycine, but they seem to play different roles in the control and modulation of the spinal cord neuronal activity. GABAergic circuits have been associated with the modulation of the activity of

local networks (intrasegmental communication), while glycine neurotransmission is involved mainly in the control of the communication between different segments along the spinal cord (intersegmental communication) (Hanson and Landmesser, 2003; Moody and Bosma, 2005). This differential role in the spinal network activity may explain the lack of effects of Stry and the alterations of motor behavior induced by Bic. In fact, Stry did not evoke motor alterations, even when infused during several days, because its effect would require the blockade of the intersegmental glycinergic circuits, which apparently did not occur. In contrast, both acute and chronic treatments of Bic evoked hyperexcitation of the ipsilateral hind limb that can be ascribed to deficient control by the local GABAergic neuronal circuits in the lumbar segment infused. The fact that the immediate acute effect by microdialysis perfusion was weak and of short duration is probably due to its rapid diffusion after the short time period (25 min) of administration.

We used three different concentration of Bic for its chronic infusion and, interestingly, the duration of the initial transitory symptoms of hyperexcitation was inversely proportional to the concentration used, and the hyperexcitation was manifested later as a loss of motor coordination that made the animals fall from the rotarod (Fig. 1A,B). The beginning of this effect was concentration-dependent but, notably, the recovery along the following days was much more rapid with the highest concentrations than with 5 mM. Because in all cases the recovery was complete, these effects cannot be due to MN degeneration, as confirmed by the slight loss of MN (about 30%) that was observed at the time of complete recovery with 10 mM and 17.5 mM concentrations. This is in agreement with previous results showing that there is a correlation between MN death and paralysis (Ramírez-Jarquín and Tapia, 2016), as well as with the finding of a threshold number of about 50% healthy MN, below which paralysis is complete and irreversible (Santa-Cruz and Tapia, 2014). Therefore, the motor deficits caused by Bic could be explained by a temporal disinhibition of MN activity leading to motor incoordination. A probable explanation of why this effect disappears even when Bic is being continuously infused is that the chronic GABAergic blockade elicits homeostatic mechanisms to compensate for the inhibition/excitation imbalance. This has been shown to occur as a result of Bic-induced hyperexcitation in mouse neocortical cultures, which causes a down scaling of GluR1 and

GluR2 subunits of surface AMPA receptors (Reimers et al., 2014; Sun and Wolf, 2009) as well as reduction of synaptic AMPA receptors in rat nucleus accumbens (Ibata et al., 2008).

This possible compensatory mechanism could occur if the effects of Bic were due to disinhibition of the excitatory neuronal circuits involving AMPA receptors, which is in accord with our findings that Bic clearly potentiated the effect of AMPA when both drugs were co-infused, and with the total prevention of the Bic-induced MN degeneration by CNQX, although this antagonist did not significantly improved the performance in the rotarod test. Such protection was not observed with the NMDA receptor antagonist MK801, which is consistent with our previous findings showing that AMPA infusion induces MN loss through the activation of Ca^{2+}-permeable receptors, while NMDA is innocuous (Corona and Tapia, 2004, 2007). Therefore, we conclude that chronic deficient GABAergic neurotransmission is sufficient to induce motor alterations and MN degeneration by secondary overactivation of AMPA receptors due to endogenous glutamate.

In the mutant SOD1-G93A mouse model of familial ALS, neurophysiological studies of neuronal activity in spinal cord organotypic cultures (Kuo et al., 2004) and in cortical slices (van Zundert et al., 2008) have demonstrated MN hyperexcitability during presymptomatic stages. In these transgenic mice, a reduction of GABA release and an increase of the GABA transporter, measured in gliosomes isolated from the spinal cord (Milanese et al., 2010), as well as desensitization of $GABA_A$ receptors observed in cultured MNs (Carunchio et al., 2008), indicate deficient GABAergic inhibitory neurotransmission. Changes suggesting this type of alterations have been described also in ALS patients, such as decreased levels of glycine in spinal cord tissue (Malessa et al., 1991), although no changes of GABA in CSF and spinal cord were detected (Kostera-Pruszczyk et al., 2002; Malessa et al., 1991). Reduction of GABA levels was shown by magnetic resonance spectroscopy in the motor cortex of ALS patients (Foerster et al., 2013), as well as decreased alpha-1 subunit of $GABA_A$ receptors and upregulation of glutamate decarboxylase expression (Petri et al., 2006). Regarding glutamate neurotransmission, the lack of RNA editing of the Q/R site in the GluR2 subunit of AMPA receptors, which results in Ca^{2+}-permeable receptors (Hume et al., 1991), has been considered a possible cause of ALS (Kawahara et al., 2003; Kwak et al., 2010). These findings, taken together, suggest the

involvement of inhibitory transmission failure and Ca^{2+}-permeable AMPA receptors in ALS.

5. Conclusion

In conclusion, our results demonstrate that an imbalance inhibition/excitation in the spinal cord, induced by blockade of inhibitory GABAergic neurotransmission, causes transitory motor deficits and MN death, effects that are mediated mainly by secondary overactivation of AMPA receptors leading to MN hyperexcitation. This mechanism of neuronal degeneration, involving primary alterations of spinal cord inhibitory circuitries, seems relevant for understanding the pathophysiology of MN diseases and for the design of new strategies for their treatment.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

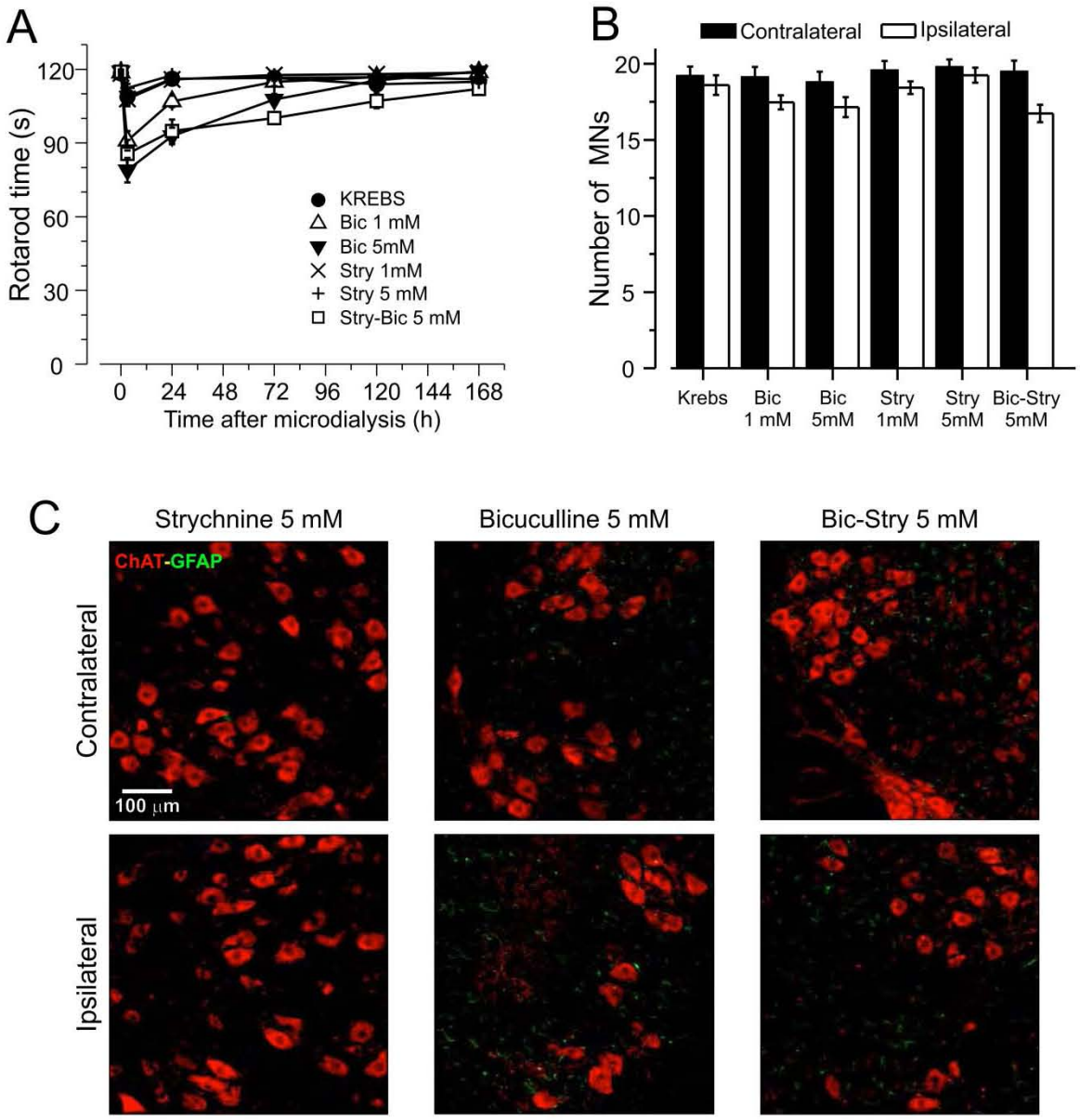
Fig. 1. Acute perfusion of Stry and Bic did not induce any significant effect. **A**) Rotarod test after acute treatments with Bic (1 mM and 5 mM), Stry (1 mM and 5 mM), mixture (Bic-Stry 5 mM) and control group (Krebs medium). **B**) Quantification of healthy cresyl violet stained MNs, 7 days after microdialysis. Mean ± SEM for 10 rats per group, differences were not statistically significant. **C**) Representative immunocytochemistry for ChAT (red) and GFAP (green) in each group. Note the preserved MNs and the absence of glial reaction.

Fig. 2. Chronic infusion of Bic, but not of Stry, induced motor alterations. **A**) Representative stride patterns for control group, showing the transitory and permanent effects of Bic and the lack of effect of Stry. The photographs show the position of the limb during the transitory and permanent effects of Bic (white and red arrows, respectively). **B**) Rotarod performance of rats treated as indicated. The white arrows show the duration of the transitory effects of each concentration of Bic, and red arrows indicate the duration of the phalange flaccidity, showed in **A** as permanent Bic-effect. All values below 100 s were significantly different from the control group ($P < 0.01$). **C**) PGE performance; the differences were not significant. Mean ± SEM for 10 rats per group.

Fig. 3. Chronic infusion of Bic, but not of Stry, induced MN loss and astrogliosis. Representative micrographs of ChAT (red) and GFAP (green) immunocytochemistry, 16 days after the implant. Note the glial reaction and the decrease of MNs in the ipsilateral side after 10 mM and 17.5 mM Bic, shown quantitatively in the graph. Mean values ± SEM for 10 rats per group. $^{*}P < 0.05$ vs control value.

Fig. 4. Effects of CNQX and MK801 on the motor alterations and neurodegeneration induced by Bic. **A**) Rotarod performance of rats treated as indicated. **B**) PGE performance; the differences were not significant. **C**) Representative stride patterns, showing that CNQX, but not MK801, prevented the phalange flaccidity induced by Bic. **D**) Quantification of healthy cresyl violet stained MNs, 16 days after the implant. Mean values ± SEM for 10 animals. $^{*}P < 0.05$ vs contralateral side.

Fig. 5. Bic enhanced AMPA-induced excitotoxicity. **A**) Rotarod performance of rats treated as indicated. **B**) PGE performance. **C**) Representative stride patterns for AMPA and AMPA + Bic infusion, showing the ipsilateral paralysis induced by AMPA at day 10 and the bilateral paralysis induced by AMPA + Bic at days 3 and 8 after implant. **D**) Representative ChAT (red) and GFAP (green) immunocytochemistry. Co-infusion of Bic enhanced MN loss and gliosis induced by AMPA. **E**) Quantification of the healthy cresyl violet stained MNs. Mean values ± SEM for 10 rats per group. $^{*}P < 0.05$ vs control values.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Time after microdialysis (h)
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Bic 5mM
Stry 1mM
Stry 5 mM
Stry-Bic 5 mM
B
Contralateral
Ipsilateral
Number of MNs
20
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10
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0
Krebs
Bic 1 mM
Bic 5mM
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Stry 5mM
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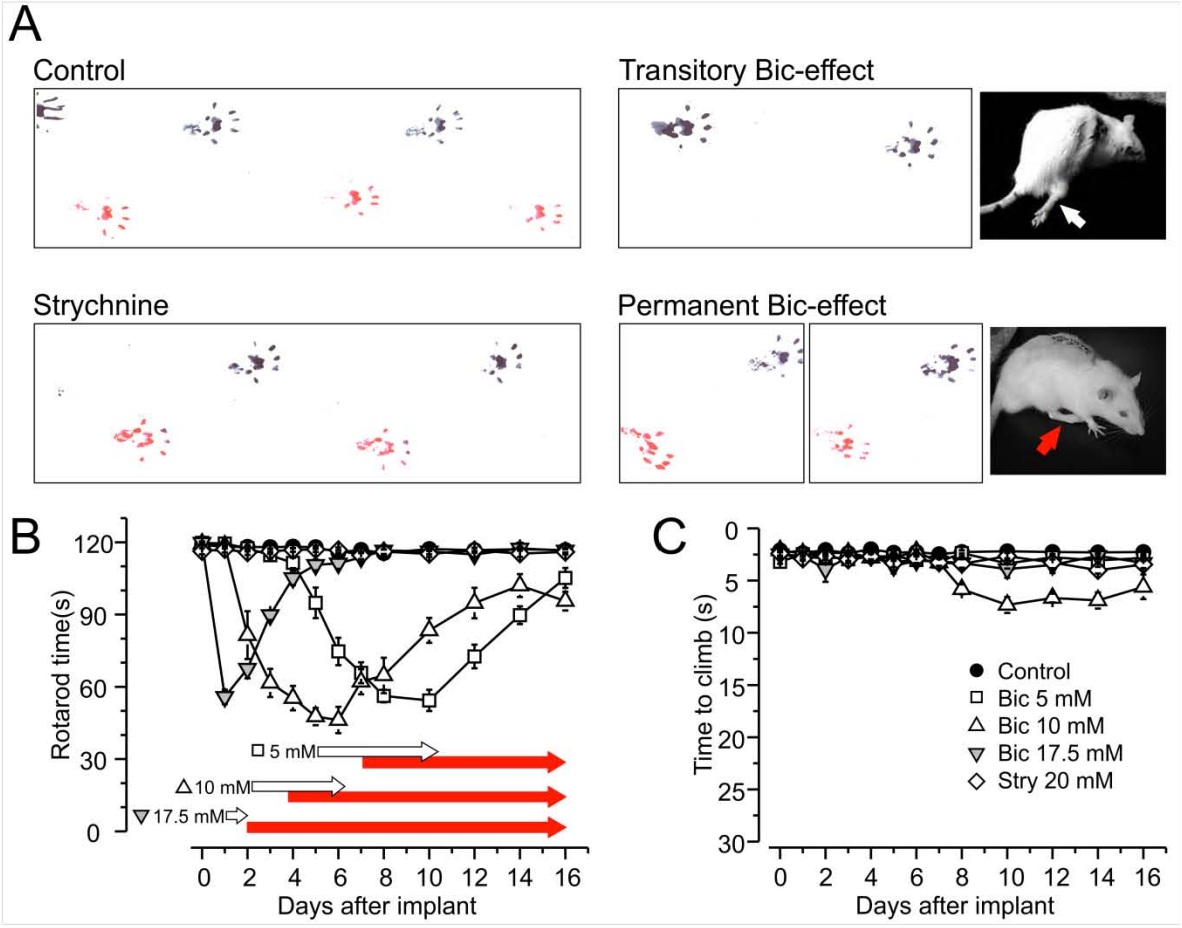
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Control
Transitory Bic-effect
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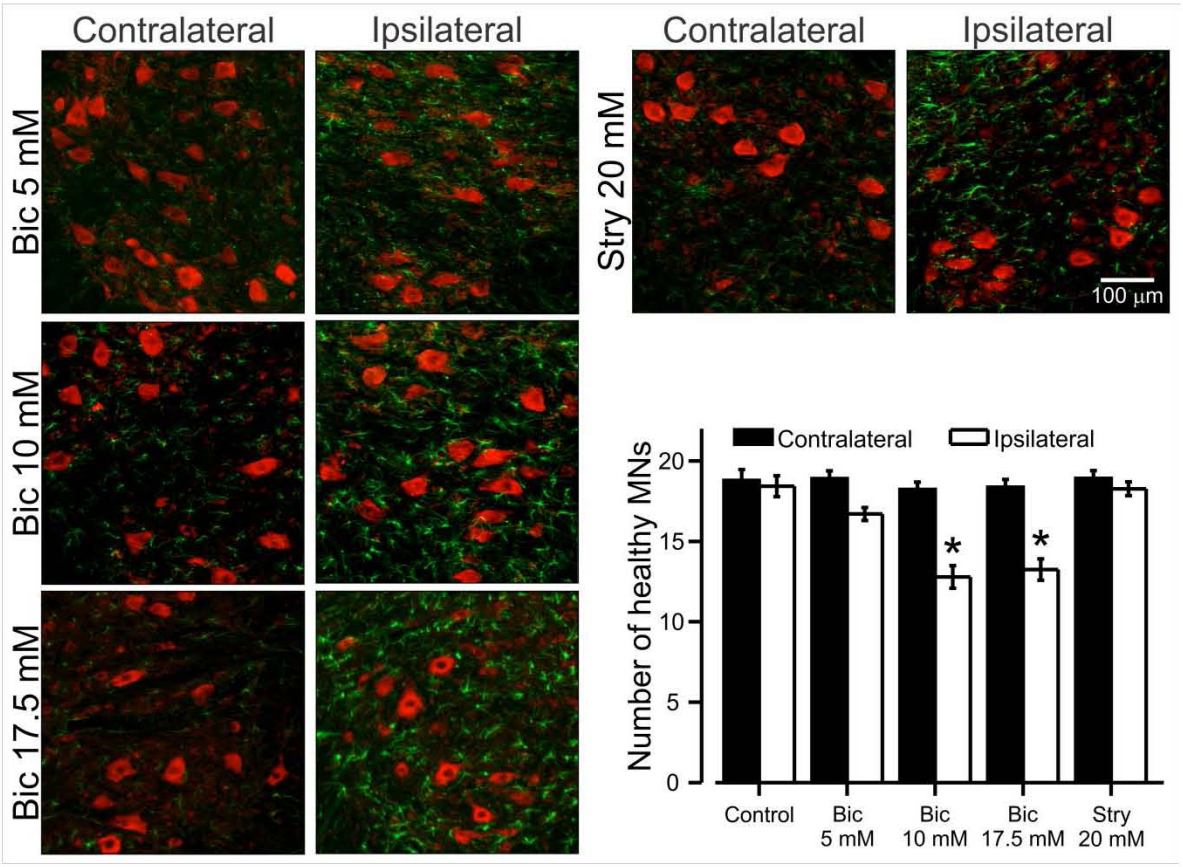
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Number of healthy MNs
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Bic 5 mM
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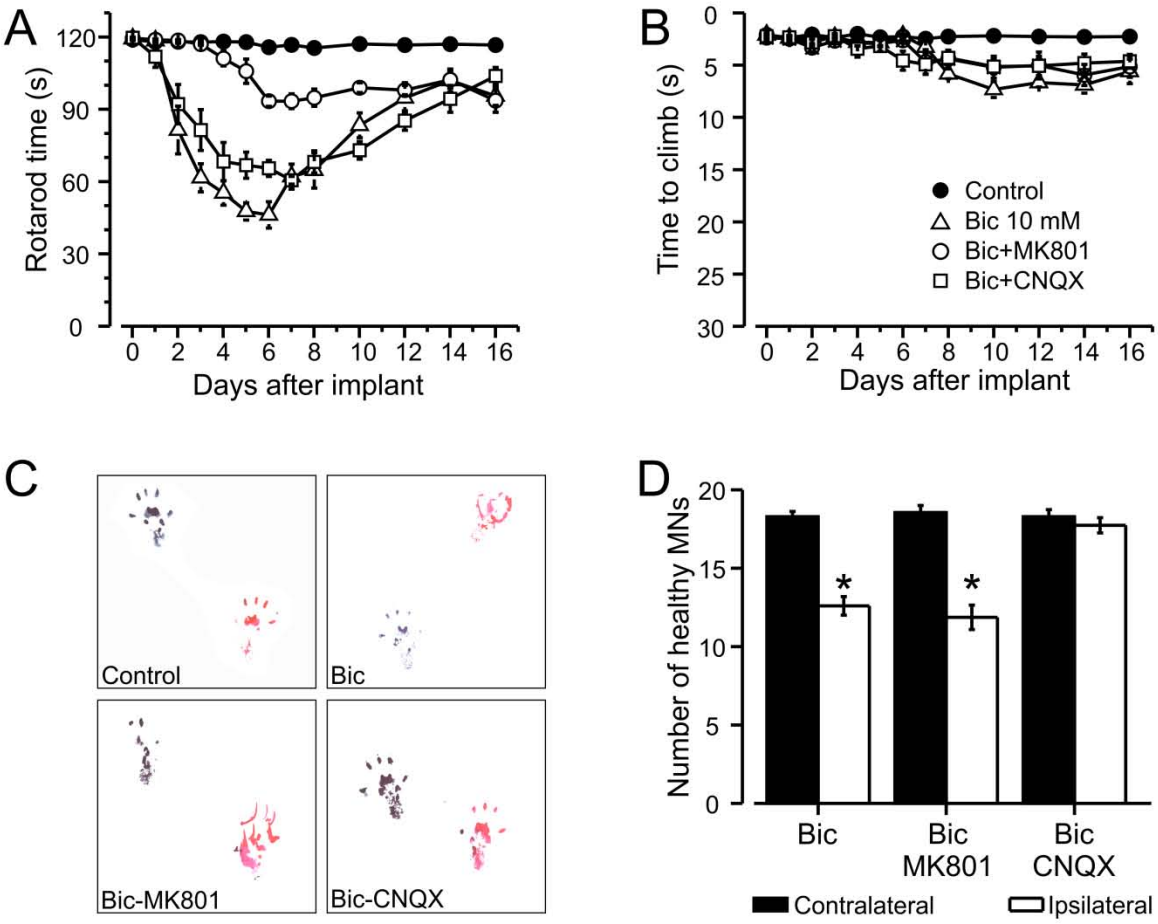
Bic 17.5 mM
Stry 20 mM


A
Rotarod time (s)
120
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0 2 4 6 8 10 12 14 16
Days after implant
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Time to climb (s)
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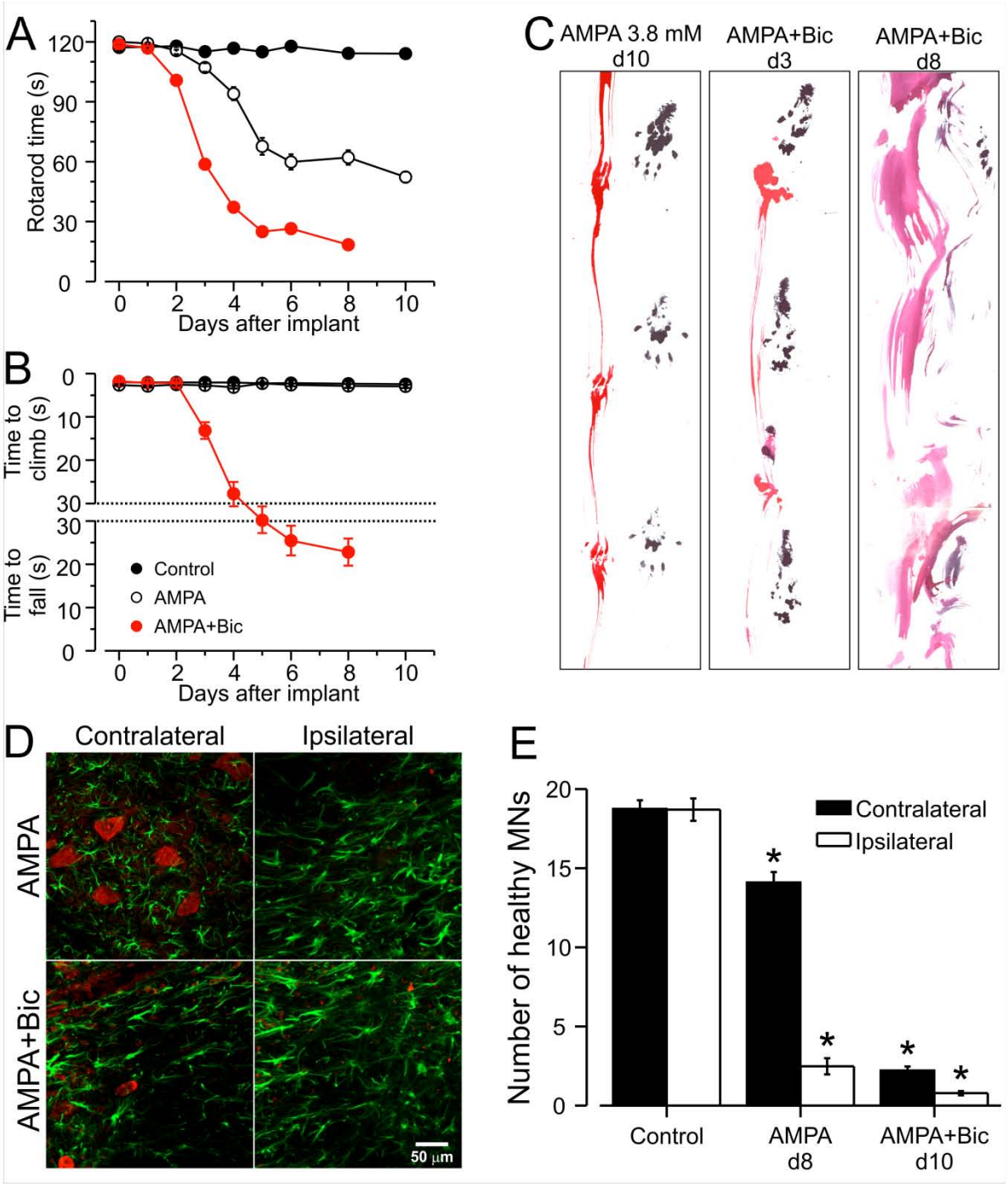
30
Control
Bic 10 mM
Bic+MK801
Bic+CNQX
0 2 4 6 8 10 12 14 16
Days after implant
C
Control
Bic
Bic-MK801
Bic-CNQX
D
Number of healthy MNs
20
15
10
5
0
*
*
Bic
Bic MK801
Bic CNQX
Contralateral
Ipsilateral


A
Rotarod time (s)
120
90
60
30
0
0
2
4
6
8
10
Days after implant
B
Time to climb (s)
Time to fall (s)
Control
AMPA
AMPA+Bic
Days after implant
C
AMPA 3.8 mM d10
AMPA+Bic d3
AMPA+Bic d8
D
Contralateral
Ipsilateral
AMPA
AMPA+Bic
50 μm
E
Number of healthy MNs
Contralateral
Ipsilateral
Control
AMPA d8
AMPA+Bic d10

XI. Discusión

Excitotoxicidad y degeneración de MNs: ¿apoptosis o necrosis?

Las patologías originadas por los procesos de la muerte neuronal manifiestan sus síntomas según el tipo celular y el área afectada. Particularmente las enfermedades relacionadas con alteraciones de las MNs, como la ALS, presentan déficits motores a diferencia de otras enfermedades neurodegenerativas que afectan a los procesos cognitivos. A pesar de décadas de investigación aún no se conocen las razones de la selectiva vulnerabilidad de las MNs. Se ha sugerido que las MNs son altamente sensibles a la estimulación de receptores AMPA permeables a calcio (Carriedo et al., 1996, Van Den Bosch et al., 2000, Van Damme et al., 2002, Corona y Tapia, 2007), probablemente debido a que presentan un mal amortiguamiento de este catión (Ince et al., 1993, Alexianu et al., 1994, Siklos et al., 1998), ya que interesantemente las MNs del núcleo de Onuf, que no se afectan en la ALS, presentan una elevada concentración de las proteínas amortiguadoras de calcio, la calbindina-D28K y la parvalbúmina, a diferencia de las MNs que sí son afectadas por la ALS (Alexianu et al., 1994); respecto a este tema, se ha demostrado que la sobreexpresión de la proteínas con capacidad de amortiguar los cambios de calcio, como la parvalbúmina, causa una neuroprotección de las MNs en modelos transgénicos de ALS (Beers et al., 2001, Van Den Bosch et al., 2002, Das et al., 2013). Otros factores asociados podrían ser la gran cantidad de neurofilamentos y la alta vulnerabilidad al mal funcionamiento mitocondrial (Bergmann y Keller, 2004).

Es importante hacer notar que al final todos los mecanismos de degeneración se interconectan, probablemente como consecuencia uno de otro, o bien como mecanismos independientes que culminan con la muerte de las MNs. Justamente este último evento fue el tema principal del presente estudio y como demuestran nuestros resultados, los procesos mediante los cuales degeneran las MNs ante una situación de excitotoxicidad son variables, y bajo nuestras condiciones básicamente dependen de los estímulos, ya que un evento muy severo como la administración

aguda del AMPA degenera rápidamente por necrosis a las MNs, mientras que un evento crónico de menor intensidad, desencadena un proceso mas complejo, que presenta estadios tempranos con características apoptóticas y culmina con la muerte de las MNs por necrosis (Ramirez-Jarquin y Tapia, 2016).

¿Apoptosis o necrosis? es una pregunta bastante frecuente en cuestiones de degeneración celular, y la ALS no es la excepción, y sugerimos que el conocer la respuesta a esta pregunta será de gran ayuda para el tratamiento y entendimiento de esta enfermedad. Esta idea se basa en el supuesto que si conocemos las características del proceso degenerativo será posible diseñar estrategias para combatirlo, detenerlo o prevenirlo. Sin embargo, en la práctica resulta no ser sencillo. Respecto a la ALS, aún no se conoce con certeza qué proceso de muerte celular participa en la degeneración de las MNs, ya que los resultados encontrados resultan complicados y por de más variables (Pasinelli et al., 2000, Bendotti et al., 2001, Ilzecka et al., 2001, Raoul et al., 2002, Martin et al., 2007, Martin et al., 2009, Ilzecka, 2011, 2012).

En modelos transgénicos se han descrito diferentes componentes de procesos apoptóticos que sugieren la participan de diferentes vías de activación de la apoptosis, tanto la intrínseca como la extrínsecas (Li et al., 2000, Raoul et al., 2002, Locatelli et al., 2007, Ohta et al., 2008). Referente a la vía intrínseca, se ha demostrado que desde etapas tempranas de la enfermedad, previo a la parálisis, ocurre la apertura del poro de transición mitocondrial, evento que se ha asociado con la liberación de moléculas proapoptóticas como el citocromo c, lo cual conlleva a la formación del apoptosoma y la activación de esta vía intrínseca.

Asimismo, en modelos transgénicos, se ha identificado la participación de componentes de la vía extrínseca dependiente de la liberación de factores apoptóticos desde células vecinas (Raoul et al., 2002, Locatelli et al., 2007). Inclusive en tejido postmortem de pacientes se ha demostrado la presencia de caspasas activadas (Hart y Gitler, 2012). Bajo este contexto, la cuestión sería, si ya se conoce el proceso degenerativo de las MNs ¿por qué no se puede detener en

modelos transgénicos? Por ejemplo en modelos experimentales de ALS la expresión de proteínas antiapoptóticas como Bcl-2 ó bien la inhibición farmacológica de la activación de las caspasas por el agente z-VAD, demuestran que prevenir las vías apoptóticas no es suficiente, ya que estas estrategias antiapoptóticas han fallado en su efecto protector, puesto que todas han tenido un efecto poco significativo y lo único que han conseguido es retrasar discretamente el inicio de la parálisis o su progreso (Li et al., 2000, Ohta et al., 2008).

En busca de una explicación del por qué prevenir la apoptosis no es suficiente, otros grupos han mostrado que existen evidencias de un proceso diferente a la apoptosis, el cual presenta características mas semejantes a la necrosis (Bendotti et al., 2001, Martin et al., 2007, Martin et al., 2009). Resulta necesario resaltar que en los estudios anteriormente descritos, los autores no discuten la posibilidad de conjuntar las alteraciones ultraestructurales (vacuolización e hinchamiento de las MNs), con las evidencias bioquímicas (activación de caspasas) durante la degeneración de las MNs, en un mecanismo que conjunte los datos descritos en un solo proceso.

A pesar de que la excitotoxicidad tampoco se ha demostrado que sea directamente la responsable de provocar la muerte de las MNs en la ALS, nuestros modelos la utilizan como método para provocar la degeneración de MNs a través de la sobreactivación de receptores para glutamato tipo AMPA. En este trabajo demostramos que la excitotoxicidad mediada por AMPA crónico desencadena un proceso degenerativo muy semejante al observado en modelos transgénicos de la ALS, caracterizado por la activación de la caspasa 3, una vacuolización gradual e hinchamiento de las MNs, a pesar de que en nuestros procedimientos la degeneración de las MNs ocurre independiente de factores genéticos en animales sanos.

Nuestros resultados sugieren que la degeneración de las MNs ocurre en dos etapas sucesivas: la primera con características apoptóticas que al progresar a la segunda etapa se convierte en proceso con características necróticas, mostrando

como ambos tipos alteraciones (ultraestructural y bioqu ímico), pueden ocurrir en respuesta a un evento excitotóxico, permitiendo conjuntar las evidencias encontradas en los modelos transgénicos de la ALS y en pacientes, ya que nuestro estudio abarca tanto el proceso ap optótico mediante la acti vación de caspas as, c omo el proceso necrótico a trav és del estudio de la ul traestructura. La sucesión en tre apoptosis y necrosis es un proce so que anter iormente se ha descrito que o curre en etapas embrionarias, señalan do que el progre so d el proceso dege nerativo depende del estado de maduración del sistema nervioso (Caldero et al., 1997, Portera-Cailliau et al., 1997a, b).

También en adulto se ha dem ostrado que e l proceso de muerte p uede ser determinado por el t ipo de rec eptor estimulado, ya que al estimular receptores tipo NMDA, se induce un proceso semejante a la necrosis, y al estimular receptores no-NMDA se pr ovoca u n proceso se mejante a la apoptosis (Portera-Cailliau et al. , 1997b). Pese a que est os resulta dos de muestran que un event o exci totóxico p uede desencadenar diversos tipos de muerte, queda claro que el proceso a seguir depende del receptor estimulado.

Nuestro est udio muestra que en el modelo crónico, se genera un proceso continuo, que ba sados en la te mporalidad d e las obs ervaciones sugerimos inicia como apoptosis y culmina con una necrosis. Basados en trabajos previos en el modelo agudo, sabemos que las alt eraciones causadas por el A MPA se asocian con un incremento del influjo de calcio, ya que el uso de un agente q uelante de este ion, previene las pr oceso d egenerativo de las MN s, de igual manera, el bloqueo de lo s receptores AM PA p ermeables a calcio por la n-acetil esper mina, evita la degeneración de las MNs inducida por AMPA (Corona y Tapia, 2007).

El principal in dicador bioquímico de la ap optosis es la act ividad de las caspasas, por ello es c omún pensar que una célula que co ntiene la caspasa-3 activa podría ser una célula en degeneración que pi erde su función, por encontrarse en un proceso degenerat ivo. Sin em bargo, nuestros resultados no demuestran una correlación entre la s MNs con caspasa-3 a ctiva y la afectación de la activ idad

motriz. La actividad motora no se vió relacionada con la cantidad de MNs con caspasa-3 activa, pero sí con la pérdida de MNs, sugiriendo que las MNs con caspasa-3 continúan funcionando.

De esta manera, nuestro trabajo brinda las evidencias que explican por qué los tratamientos antiapoptoticos no son capaces de prevenir la muerte de las MNs en los modelos transgénicos de la ALS, ya que estos tratamientos dejan de lado la degeneración necrótica, que según nuestros resultados es el proceso que termina por matar a las MNs, es decir, aun cuando se prevenga la apoptosis habrá otras rutas que desencadenen en la degeneración de las MNs.

Detección temprana del proceso degenerativo de las MNs

Detectar el inicio de la ALS es un serio problema en la clínica, debido a varios factores los cuales frecuentemente están relacionados con el hecho de que las alteraciones iniciales de esta enfermedad pueden fácilmente ser confundidas con otras patologías, o bien pasar desapercibidas.

En este estudio justamente pudimos demostrar que el estudio de la zancada es una prueba importante para detectar el inicio de las alteraciones causadas por la ALS, ya que a diferencia de las pruebas del rotarod y del PGE (las cuales son de utilidad para medir resistencia y coordinación en el caso del rotarod, y fuerza en el caso del PGE, resultando poco efectivas para detectar cambios discretos), el registro de la zancada detectó mas tempranamente las alteraciones motrices inducidas por el tratamiento con AMPA. Probablemente debido a que en el rotarod no importa si los pasos son grandes o chicos, basta con que sean lo suficientemente frecuentes para mantenerse en el rodillo girando y el caso del PGE basta con que se genere un solo impulso para que la rata escale la rejilla; en cambio en el registro de la zancada las alteraciones discretas como la distancia recorrida en cierta cantidad de pasos, así como la cantidad de pasos usados para recorrer un a distancia, son fácilmente detectables y cuantificables, lo cual podría ser de relevancia para la detección temprana de las alteraciones motrices. Alteraciones de la marcha o del andar han

sido discretas en pacientes de la ASL (Goldfarb y Simon, 1984, Wu y Ng, 2010, Radovanovic et al., 2014), dejando demostrado que pueden servir como medio de detección de esta enfermedad. Sin embargo, en humanos el diagnostico de esta enfermedad se da al cubrirse diversos tipos de alteraciones, no solo las motoras, también electrofisiológicas, biopsias, entre otras. Aunado a lo anterior en humanos, la ALS puede tener un origen lumbar, pero también bulbar, causando un progreso diferente para cada tipo de inicio (Radovanovic et al., 2014). Por ello pensar en implementar el estudio de la zancada en humanos es bueno, puesto que se han demostrado alteraciones en pacientes. Sin embargo sería solo otro dato que sugiera el diagnóstico de la enfermedad y tendría que ser necesariamente complementado por otras pruebas como las histológicas y electrofisiológicas, además de considerar el tipo de inicio que presente cada caso clínico.

Sobreexcitación de los circuitos motores lumbares por un mecanismo diferente a la activación de vías glutamatérgicas mediante agonistas glutamatérgicos exógenos

Como se ha mencionado, una de las características más frecuentes en las enfermedades del sistema nervioso es el incremento de la excitabilidad de las redes neuronales, que de forma general se ha estudiado más extensamente desde el punto de vista del incremento de la neurotransmisión glutamatérgica. Sin embargo, existe otro origen para la hiperexcitabilidad, que es el decremento de la inhibición, el cual se ha descrito en diversas patologías, incluida la ALS (Malessa et al., 1991, Petri et al., 2006, Carunchio et al., 2008, Vucic et al., 2009, Nieto-Gonzalez et al., 2011, Ramirez-Jarquin et al., 2014), pero se ha explorado poco como mecanismo de neurodegeneración de MNs.

En este trabajo mostramos que el bloqueo de la inhibición GABAérgica causa hiperexcitabilidad y alteraciones motrices, además cuantificamos que este incremento de la actividad neuronal provoca la muerte de MNs lumbares. Identificamos que tanto la hiperexcitabilidad, como la degeneración de MNs dependen de vías glutamatérgicas, ya que observamos un papel diferencial para los

receptores para gluta mato tipo NMDA y AMPA, debido a que al bloquear a los receptores NMDA se disminuyen las con ductas provocada por los agon istas glutamatérgicos, pero no la degenerac ión de MNs. E n ca mbio, al bloquear a los receptores AMPA se previene la muerte de MNs pero las conductas provocadas los agonistas gl utamatérgicos, co mo las co ntracciones de la extre midad posterio r ipsilateral y las sacudidas de rata mojada.

Éstos resultado s son sumamente intere santes, puesto que estas alteraciones ocurren al bloquear la neurotrans misión GA BAérgica, dejando actu ar al glutamato presente en los circuitos motores lumbares, sin nece sidad de us ar un ag onista exógeno. Lo cual es de interés, puesto que anteriormente se había demostrado que el incremento crónico de gluta mato inducido farmacológicamente por l a aplicación de un bloqueador del tra nsportador de este ne urotransmisor no caus ó alteraciones ni muerte de MNs (Tovar-y-Romo et al., 2009).

Nuestros resul tados c oncuerdan con estudios previos que de muestran que solamente la activación selectiva de los receptores AMPA causa muerte de las MNs. En conjunto e sta se rie de res ultados nos per mite sugerir q ue no se requiere de un incremento de glutamato extracelular, sino de un incremento de la a ctividad de las redes neuronales de la médula para inducir un proceso deg enerativo de las MNs por sobreactividad sináptica, resaltando el papel d el control de los circuitos inhibitorios en la fisiología de la médula espinal.

Actualmente, el estud io de las redes ne uronales es un ca mpo q ue se ha comenzado a e xplorar mediante estudios de imagenología de calcio en circuitos del cerebro en animales neonatos, no mayores a 14 días postnatales (Carrillo-Reid et al., 2011, Stetter et al., 2012). Sin embargo, aún no se han realizado estudios de este tipo en la médula espinal, posiblemente debido a que estos circuitos neuronales son mas susceptibles al daño durante la preparación de las muestras, particularmente las MNs son sumamente sensibles a eventos de hipoxia.

Recientemente se des arrolló un modelo tra nsgénico en rat ón, qu e per mite observar la actividad de las MNs mediante uso del microscopio de dos fotones, esto

debido a que estos animales utilizan la proteína GCaMP6f (un indicador de calcio) como reportero de la actividad neuronal (Hinckley et al., 2015). Sin embargo, aún no se han realizado estudios asociados con la degeneración de las MNs en la ALS.

Componente no neuronal en la degeneración de las MNs

El proceso conocido como muerte celular no autónoma ha sido ampliamente estudiado en los últimos años. Básicamente hace referencia al proceso degenerativo que una célula inicia por acción de un componente extracelular proveniente de otra célula vecina y no propiamente de la célula misma. En este contexto, el papel de la glía ha tomado gran importancia en la patogénesis de la ALS, proponiéndola como posible responsable de iniciar la degeneración de las MNs (Kushner et al., 1991, Nagy et al., 1994, Schiffer et al., 1996, Hall et al., 1998, Schiffer y Fiano, 2004, Nagai et al., 2007, Guo et al., 2010, Sanagi et al., 2010).

Una característica del proceso degenerativo de las MNs es la aparición de glía reactiva, la cual se asocia con mecanismos que dañan a las MNs, por ejemplo: pueden alterar la recaptura de neurotransmisores y liberar factores que provoquen la degeneración de las MNs, como ligandos FAS, que provocan la activación de la vía extrínseca de la apoptosis o especies reactivas de oxígeno (Alexianu et al., 2001, Raoul et al., 2002, Sofroniew, 2005, Locatelli et al., 2007), el papel de los ligandos de muerte se ha estudiado mediante la medición del RNAm de FAS, así como con el uso de sondas de interferencia del ligando FAS (Raoul et al., 2002, Locatelli et al., 2007).

Recientemente también se ha demostrado que los astrocitos portadores de la SOD1 mutada liberan una molécula (aún no identificada) que provoca la apertura de una corriente de sodio persistente, provocando un estado de hiperexcitabilidad que favorece la degeneración de las MNs (Fritz et al., 2013). En este trabajo describimos la astrogliosis como un proceso asociado a la degeneración de las MNs tanto en la excitotoxicidad por AMPA, como en el bloqueo de la actividad GABAérgica. La excitotoxicidad del tratamiento agudo con AMPA provocó la activación tardía de la

caspasa-3 en las MNs (24 h después del tratamiento), probablemente debido a la liberación de ligandos de muerte desde los astrocitos activos. Sin embargo, a pesar de la evidente asociación entre la degeneración de las MNs y la astrogliosis, en este trabajo no exploramos a detalle el papel que la activación glial desempeñan en la muerte de las MNs. De igual manera nuestros resultados son insuficientes para determinar si la activación de la astroglia ocurre como un mecanismo de iniciación que contribuye a la degeneración de las MNs o bien como un mecanismo secundario al daño de las MNs, aunado a que en algunas áreas del sistema nervioso central como el tálamo, hipocampo y corteza, se demostrado que los astrocitos expresan receptores GABAA y AMPA (Hoft et al., 2014). Lo que sí sabemos es que la degeneración de las MNs está siempre asociada con la activación de la astroglia.

La ALS y la excitotoxicidad como procesos multifactoriales

Actualmente no existe un mecanismo concreto que explique el origen de la muerte selectiva de las MNs en la ALS. Sin embargo, se han descrito diversos mecanismos involucrados en la degeneración de las MNs, por lo que podríamos considerar que la ALS no es necesariamente el resultado de un solo mecanismo, sino o muy posiblemente es el resultado de la suma de varios componentes que causan la enfermedad, definiendo a la ALS esporádica (que representan el 90 % de los casos) y la degeneración de las MNs como patologías de origen multifactorial. Nosotros demostramos que la suma del bloqueo de la actividad GABAérgica, con la excitotoxicidad mediada por AMPA, es capaz de inducir un efecto sinérgico de ambos tratamientos, por ello nuestros resultados indican que la degeneración de las MNs puede ser afectada o inducida por la combinación de varios factores, enfatizando la necesidad de conocer a fondo los procesos de degeneración involucrados en dichas alteraciones.

XII. Conclusión

En el presente trabajo demostramos que el proceso degenerativo de las MNs lumbares es determinado por el estímulo que lo provoque: un estímulo agudo desencadena una degeneración rápida, en tanto que la excitotoxicidad crónica induce alteraciones graduales más severas, lo cual aplica tanto para excitotoxicidad mediada por los receptores tipo AMPA para glutamato, como para la degeneración inducida por el bloqueo de los receptores GABAA.

Aún queda mucho por investigar con respecto a la degeneración de las MNs. Sin embargo, este trabajo muestra datos relevantes a considerar para investigaciones futuras, ya que demostramos cómo diversos procesos degenerativos pueden ocurrir simultáneamente o de manera sucesiva, lo cual es de relevancia para el diseño de estrategias farmacológicas, para cuyo diseño sugerimos que se considere no solo a un proceso degenerativo.

También demostramos que el bloqueo de la actividad inhibidora causa la contracción descontrolada de los músculos de la extremidad ipsilateral al sitio del tratamiento farmacológico, sugiriendo la activación de los circuitos espinales, y la subsecuente degeneración de las MNs de esta área, mediante vías glutamatérgicas, que utilizan al glutamato de los estos circuitos y la falta de inhibición mediada por el receptor GABAA.

En resumen, concluimos que el proceso de degeneración de las MNs lumbares inducido por excitotoxicidad depende de la intensidad y continuidad del estímulo, y que los circuitos inhibitorios tienen un papel de gran relevancia en la degeneración de las MNs lumbares.

XIII. Referencias

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