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Tesis de doctorado

Identificación de un fenotipo astrocitario aberrante (células AbA) asociado a la neurodegeneración.

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Abreviaturas

AbA: Astrocitos aberrantes

AQP4: Acuaporina 4

Cx43: Conexina 43

EAAT2 o GLT-1: Transportador de aminoácidos excitatorios de tipo 2

ELA: Esclerosis Lateral Amiotrófica

FGF: Factor de crecimiento fibroblástico

GFAP: Proteína ácida fibrilar glial

GS: Glutamina sintasa

Iba-1: Ionized calcium binding adapter molecule 1

INFγ: Interferón γ

NG2: Neuroglicano 2

NGF: Factor de Crecimiento nervioso

NO: Oxido nítrico

S100β: isoforma β de la proteína de unión al calcio de la familia S100

SNC: Sistema nervioso central

SOD1: Superóxido dismutasa 1

TNFα: Factor de necrosis tumoral alfa

Resumen

La reactividad astrocitaria es un evento temprano en la Esclerosis Lateral Amiotrófica (ELA), una enfermedad neurodegenerativa que afecta a las motoneuronas corticales y espinales provocando una rápida degeneración muscular y parálisis progresiva que ocasiona la muerte entre 3 y 5 años tras el comienzo de los síntomas. La presencia de astrocitos reactivos rodeando a las motoneuronas espinales degenerantes se observa en etapas iniciales de la enfermedad, incluso antes de la fase sintomática. Una vez desencadenada, la reactividad astrocitaria se establece de forma progresiva y aumenta conforme lo hace la neurodegeneración, sugiriendo que los astrocitos podrían tener un papel relevante en la muerte neuronal ulterior.

Por otra parte, la característica "no autónoma celular" de la ELA han posicionado a las células gliales como principales actores en la fisiopatotología de dicha enfermedad. En los últimos años, la búsqueda de "la célula neurotóxica" y de los mecanismos implicados en la muerte neuronal ha acaparado la atención de los investigadores. La mayoría de los resultados obtenidos en modelos animales mostraron primeramente, y de forma clara, que los astrocitos reactivos eran las principales células neurotóxicas en la ELA. No obstante, recientemente algunos autores han demostrado que la microglia activada también puede ser responsable de la muerte de las motoneuronas espinales, por lo que el debate aún permanece abierto.

En el presente trabajo se reporta el aislamiento y la caracterización de las células *AbA*, un fenotipo celular astrocitario aberrante que es al menos diez veces más tóxico para las motoneuronas que todas las célula aisladas hasta el momento de modelos animales de la enfermedad. Además, se demostró que las células *AbA* derivan de un proceso celular de *trans*-diferenciación que podría tener lugar tanto a partir de la microglia residente así como de monocitos-macrófagos circulantes, cuestión que aún queda por resolver.

Los resultados presentados en esta tesis muestran como las células *AbA* aparecen en la médula espinal en un modelo animal de ELA a medida que la fase sintomática tiene lugar. El número de células *AbA* aumenta conforme lo hace la progresión de la enfermedad y éstas se localizan rodeando a las motoneuronas degenerantes, sugiriendo un papel relevante de las células *AbA* en la progresión de la ELA. La demostración de que las células *AbA* derivan de un proceso de trans-diferenciación a partir de células de estirpe mieloide, microglia/monocitos/macrófagos, podría explicar el motivo del dilema mencionado anteriormente sobre la identidad de la célula neurotóxica.

Los hallazgos reportados en esta tesis representan una contribución original al conocimiento de los mecanismos fisiopatológicos que ocurren en la ELA y sugieren un potencial blanco terapéutico novedoso para el tratamiento de dicha enfermedad. Además, las células *AbA* no solo constituyen una herramienta de enorme valor para avanzar en el conocimiento de los mecanismos implicados en la muerte de las motoneuronas, sino también para contribuir en la identificación de fármacos potencialmente efectivos para el tratamiento de tan agresiva enfermedad.

Introducción

Las células gliales en el Sistema Nervioso central (SNC)

El SNC es el órgano más complejo en el ser humano y en otros mamíferos superiores. Es una estructura dinámica capaz de adaptarse a los cambios ambientales debido a una extraordinaria plasticidad presente tanto en condiciones fisiológicas como en situaciones patológicas. Esta ductilidad puede explicarse por la enorme especialización de las células que componen el SNC, permitiendo la realización de funciones muy sofisticadas en un reducido volumen y porla existencia de un sistema capaz de regular la homeostasis de una manera altamente eficiente desde el punto de vista energético y temporal. Dicho sistema es la neuroglia, la cual puede ser considerada como el sistema de defensa intrínseco del SNC (Giaume et al. 2007, Kimelberg et al. 2010).

El término “neuroglia” fue acuñado por Rudolf Virchow en 1856 quien la definió como el tejido conectivo en el cual se encuentran embebidas las células nerviosas. Con el posterior desarrollo de la microscopia y de nuevas técnicas histológicas impulsado principalmente por Camilo Golgi, Santiago Ramón y Cajal y Pio del Rio Hortega se pudo revelar la morfología de las células gliales y su extraordinaria diversidad (Somjen et al. 1988, Kettenmann y Ransom 2004). La neuroglia constituye el tipo celular más abundante del Sistema Nervioso Central (SNC) de mamíferos representando más del 50% de las células que lo constituye (Verkhratsky y Butt 2005). Aunque durante muchos años las células gliales fueron consideradas un mero soporte pasivo para las neuronas, en las últimas décadas numerosos estudios han proporcionado evidencias sobre las diversas e imprescindibles funciones que éstas desempeñan para el SNC; durante el desarrollo, en el mantenimiento de la homeostasis y en la reparación frente a condiciones patológicas (Barres 2008, Fields et al. 2015).

La neuroglia esta constituida por una población celular heterogénea que involucra a los astrocitos, la microglia, los oligodendrocitos, las células ependimarias y las células positivas al neuroglicano 2 (NG2). De acuerdo a una clasificación morfológica y tomando en cuenta el origen de los diferentes tipos celulares se puede dividir a las células gliales en dos grupos; la microglia que está constituido por un único tipo celular que lleva el mismo nombre y la macroglia que engloba al resto de las células mencionadas anteriormente. Aunque cada uno de los grupos celulares mencionados tiene características comunes, dentro de cada grupo existe una marcada variabilidad funcional, que es posible observar en diferentes regiones del SNC y que se hace más evidente durante el desarrollo (Parpura y Verkhratsky 2012). A pesar de las marcadas diferencias dentro de las poblaciones celulares que componen la neuroglia, todas cumplen funciones relevantes en el mantenimiento de la integridad estructural y funcional del SNC (Sofroniew y Vinters 2010).

Las células que componen la microglia tienen origen mesodérmico y son generadas en el saco vitelino durante la embriogénesis, en el estadío embrionario 7.5 (E7.5) en el ratón (Aguzzi et al. 2013; Ginhoux y Prinz 2015; Casano y Peri 2015). Primero, las células madres hematopoyéticas en el saco vitelino se diferencian en macrófagos primitivos los que más tarde migran hacia el SNC en desarrollo para convertirse en la microglia residente. La microglia en condiciones basales es una célula altamente ramificada que se distribuye a lo largo de todo el parénquima. El SNC también posee macrófagos provenientes de la sangre, derivados de la médula ósea, y que no ocupan el parénquima, por lo que la microglia es única no solo por su localización sino también por su origen en el saco vitelino. Uno de los desafíos importantes es determinar que señales promueven el establecimiento de la microglia, su correcto funcionamiento y su renovación (Zuchero y Barres 2015)

Los astrocitos y los oligodendrocitos son las principales células que constituyen la macroglia y ambas derivan de células progenitoras

neuroepiteliales del tubo neural durante el desarrollo embrionario (Rowitch y Kriegstein 2010). Alrededor del día 9 embrionario (E9) del ratón, las células progenitoras neuroepiteliales se transforman en glia radial, las cuales pueden posteriormente dar origen tanto a neuronas como a la macroglia. Primero tiene lugar la generación de neuronas y una vez culminada esta etapa ocurre un cambio en el programa de desarrollo hacia la gliogénesis para dar lugar a la generación de astrocitos y precursores de oligodendrocitos (*OPC; oligodendrocytes precursors cells*) entre los que se encuentran las células gliales NG2. La gliogénesis es un procesos regulado por un importante número de señales que involucra factores secretados, como Sonic hedghog (Shh), el factor de crecimiento fibroblástico (FGF), Wnts, Notch/Delta, proteínas morfogenéticas ósea o BMPs *(bone morphogenetic proteins*) y citoquinas que actúan de forma espacialmente y temporalmente controlada para determinar el destino celular y el establecimiento de dominios celulares específicos (Rowitch y Kriegsteiny 2010; Zuchero y Barres 2013; Sloan y Barres 2014). La generación de un tipo celular u otro durante la gliogénesis es regulada por factores de transcripción específicos. Además, los astrocitos generados en diferentes regiones del SNC muestran importantes diferencias funcionales, las que están comenzando a ser reveladas en investigaciones actuales (Molofsky y Deneen 2015).

Biología de los astrocitos

Los astrocitos son las células especializadas de la macroglía no mielinizante responsables del mantenimiento de la homeostasis del SNC (Verkhratsky et al. 2012). Desde fines del siglo XIX los astrocitos fueron divididos en dos subtipos en base a sus diferencias morfológicas y su localización anatómica: astrocitos fibrosos y protoplasmáticos (Sofroniew et al. 2010; Ben Haim et al. 2015). Los astrocitos fibrosos se encuentran distribuidos a lo largo de la sustancia blanca y exhiben muchos procesos finos y largos que le proporcionan, como lo sugiere su nombre, el aspecto fibroso. Contrariamente, los astrocitos protoplasmáticos se encuentran localizados en la sustancia gris y, como lo mostraron las técnicas de

impregnación argéntica, exhiben un cuerpo celular más prominente que los astrocitos fibrosos, con procesos que son más numerosos, más cortos y más gruesos en la base afinándose hacia la punta lo que les confiere un aspecto de "esponja" (Ben Haim et al. 2015). Los estudios neuroanatómicos clásicos y modernos han mostrado que ambos tipos de astrocitos mantienen un contacto extensivo con los vasos sanguíneos. Los estudios de microscopía electrónica de mediados de siglo XX mostraron que los procesos de los astrocitos protoplasmáticos se encuentran principalmente envolviendo las sinapsis, mientras que los procesos de los astrocitos fibrosos hacen contracto con los nodos de Ranvier. Además se demostró que ambos tipos de astrocitos forman uniones gap en los procesos distales con astrocitos vecinos (Barres 2008)

La proteína ácida fibrilar glial o GFAP se convirtió en el primer marcador prototípico para la identificación de astrocitos mediante inmunohistoquímica. GFAP pertenece a la familia de proteínas que constituyen los filamentos intermedios del citoesqueleto dentro de las que también se encuentran las proteínas vimentina, nestina y sinemina, además de otras proteínas vinculadas al mantenimiento de la citoarquitectura. GFAP fue primero identificada como una proteína altamente concentrada en las placas desmielinizantes en pacientes con esclerosis múltiple y su expresión estuvo inicialmente asociada con los astrocitos reactivos presentes en dichas placas y en otros contextos patológicos del SNC (Eng et al. 2000, Sofroniew y Vinters 2010). Sin embargo, la expresión de GFAP puede ser detectada mediante inmunohistoquímica en condiciones fisiológicas en astrocitos no reactivos. Actualmente se sabe que en situaciones fisiológicas la expresión de GFAP es muy baja y está restringida a un porcentaje minoritario de los astrocitos. Además, la presencia de astrocitos GFAP positivos presenta variaciones regionales y locales dentro del SNC (Sofroniew 2009). En este sentido es importante reconocer el alcance y las limitaciones que representa el uso de GFAP como único marcador astrocitario. Por ejemplo, dado que la distribución de GFAP no es homogénea en toda la célula, la visualización de los astrocitos mediante inmunohistoquimica para GFAP induce a subestimar el

volumen celular total. GFAP no ocupa todo el citoplasma celular, se encuentra principalmente en los procesos más gruesos y no se detecta en los procesos finos. Esta característica llevó a grandes confusiones al inicio, cuando se intentó contrastar los resultados obtenidos con inmunohistoquímica para GFAP con las antiguas tinciones argénticas que marcan la totalidad del astrocito (Sofroniew y Vinters 2010). En resumen, GFAP es un buen marcador para identificar astrocitos reactivos usualmente asociados a situaciones patológicas, pero su expresión en condiciones fisiológicas se restringe a una población astrocitaria minoritaria.

En este sentido, estudios realizados en ratones transgénicos han mostrado que la expresión de GFAP no es esencial para las funciones astrocitarias en condiciones fisiológicas pero si lo es en la respuesta a un daño, durante la reactividad astrocitaria y para la formación de la cicatriz glial (ver mas adelante) (Pekny et al. 1995; 2004; Herrmann et al. 2008).

Otros marcadores que han sido utilizados para identificar astrocitos incluyen la proteína glutamina sintasa (GS) y la proteína de unión al calcio S100β, aunque no todas ellas sean exclusivamente expresadas en astrocitos (Goncalves et al. 2008). El análisis del transcriptoma de astrocitos murinos y humanos, ha permitido identificar un importante número de moléculas que se encuentran enriquecidas en astrocitos comparado con otras células neurales como neuronas y oligodendrocitos (Lovatt et al. 2007; Cahoy et al. 2008). Una de las proteínas sugerida a partir estos estudios y cuya expresión es altamente específica en astrocitos es una isoforma de la enzima aldehído deshidrogenasa 1, Aldh1L1. La expresión el gen reportero GFP (*green fluorescent protein*) bajo el promotor de la enzima Aldh1L1 ha permitido demostrar que se trata de un buen marcador astrocitario en condiciones fisiológicas ya que marca a toda la población astrocitaria (Yang et al. 2011).

En cuanto a su repertorio molecular, los astrocitos expresan además canales de potasio y sodio y pueden mostrar corrientes de entrada evocadas, aunque a

diferencia de una neurona, lo astrocitos no disparan o propagan potenciales de acción a lo largo de sus procesos (Nedergaard et al. 2003; Seifert et al. 2006). Sin embargo esto no significa que los astrocitos sean desde un punto de vista fisiológico células "silenciosas". Los astrocitos experimentan aumentos regulados de la concentración de calcio intracelular, $[Ca^{+2}]_i$, lo que representa una forma de excitabilidad astrocitaria (Cornell-Bell et al. 1990). Las evidencias actuales sugieren que dicho incremento en la $[Ca^{+2}]_i$ tienen un significado funcional relevante en la comunicación astrocito-astrocito y también astrocito-neurona (Agulhon et al. 2008). Como se discute más adelante, la participación de los astrocitos en la regulación de la actividad sináptica, a través de la modulación de los niveles de neurotransmisores en la hendidura sináptica, recae en gran medida en la capacidad de sufrir cambios a nivel de la $[Ca^{+2}]_i$. Además los astrocitos se encuentran acoplados entre si a través de uniones comunicantes o uniones GAP formadas por conexinas, principalmente por la conexina-43 (Cx43), lo que permite establecer una red multicelular (sincitio) que responde de forma sincronizada propagando un incremento puntual de la $[Ca^{+2}]_i$ a lo largo de toda la red astrocitaria. Este tipo de comunicación juega un papel muy importante tanto en condiciones fisiológicas como patológicas (Nedergaard et al. 2003; Seifert et al. 2006).

Los astrocitos en la fisiología del SNC

Los astrocitos constituyen la población glial más abundante y morfológicamente heterogénea del SNC. Debido a que desempeñan funciones esenciales para el correcto funcionamiento neuronal son consideradas las "células gliales homeostáticas" por excelencia (Verkhratsky et al. 2012). Los astrocitos participan en todas las funciones fisiológicas de soporte y en las últimas décadas se ha demostrado su participación en funciones superiores del SNC, por ejemplo en la regulación de la plasticidad sináptica asociada al aprendizaje y la formación de memoria (Zorec et al. 2015). A continuación se describe brevemente las principales funciones.

*1-**Desarrollo del SNC***

La generación de astrocitos durante el desarrollo embrionario (gliogénesis) tiene lugar después de la generación de las neuronas (neurogénesis) en casi todas las regiones del SNC. A pesar de ello, los astrocitos ejercen un importante número de funciones durante el desarrollo de las sustancias blanca y gris. Los límites moleculares que establecen los astrocitos son importantes para guiar a los axones en desarrollo y la migración de ciertos neuroblastos (Powell et al. 1999). Por otra parte, los astrocitos resultan esenciales para la formación y función de sinapsis en desarrollo a través de la liberación de moléculas de señalización como la proteína tromboposdina (Ullian et al. 2001; Christopherson et al. 2005; Barres 2008; Sloan y Barres 2014). Además, los astrocitos participan en un proceso clave durante el desarrollo del SNC que es la “poda sináptica” en español o “pruning” en inglés (ver más adelante) mediante la liberación de señales que inducen la expresión de proteínas del complemente como Cq1 en las sinapsis, marcando a las mismas para ser eliminadas por la microglia (Stevens et al. 2007; Barres 2008). En lo que respecta al desarrollo de la sustancia blanca, la pérdida o la disfunción de las uniones GAP entre los astrocitos compromete seriamente la mielinización, lo que impacta negativamente en el desarrollo del SNC (Lutz et al. 2009; Sloan y Barres 2014).

*2-**Regulación del flujo sanguíneo***

Los astrocitos mantienen contacto extensivo con las diferentes estructuras vasculares del SNC y recubren más del 99% de la superficie de los capilares cerebrales (Hawkin y Davis 2005). Estudios relativamente recientes muestran que los astrocitos regulan el flujo sanguíneo mediante la producción y liberación de mediadores como prostaglandinas (PGE), oxido nítrico (NO) y ácido araquidónico (AA) que pueden aumentar o disminuir el diámetro de los vasos y por ende el flujo sanguíneo de una forma finamente regulada (Gordon et al. 2007; Iadecola et al. 2007). Una de las funciones más especializadas de los astrocitos, es la inducción de aumentos locales en el flujo sanguíneo (hiperemia

cerebral) en regiones donde la actividad neuronal es alta. La disposición de los astrocitos con procesos perisinápticos y procesos en estrecho contacto con la vasculatura (Figura 1), le permiten censar cambios a nivel de la actividad neuronal y responder ante ello aumentando el flujo sanguíneo de forma local (Wolf y Kirchhoff et al. 2008; Koehler et al. 2009) en forma dependiente de la actividad.

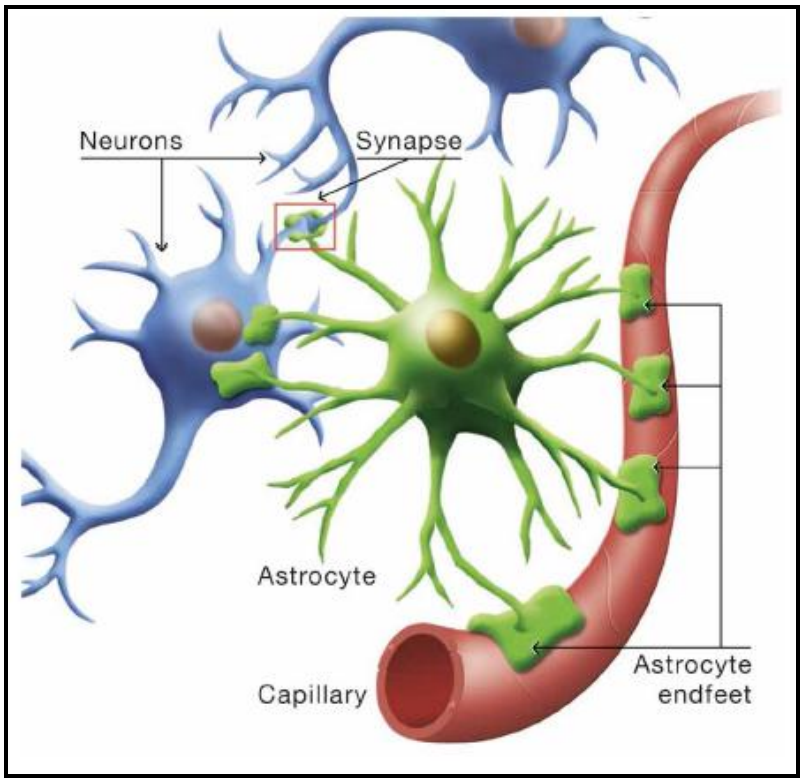

Fig. 1. Disposición espacial de los astrocitos en el SNC. La disposición de los astrocitos en el espacio le permite realizar funciones tan complejas e importantes como la regulación de la captación de metabolitos energéticos desde los capilares en respuesta a aumentos locales la de actividad sináptica, entre otras funciones. Figura de (*Lloyd et al. 2015*)

*3-**Homeostasis hídrica, iónica y regulación del pH***

Los astrocitarios ejercen funciones esenciales en el mantenimiento de la homeostasis de los fluidos, regulan el volumen de agua, el pH, la concentración iónica y los niveles de neurotransmisores en el intersticio sináptico. Los astrocitos expresan altos niveles de la proteína acuaporina 4 (AQP4), un canal

de agua que se encuentra mayoritariamente en los procesos astrocitarios que están en contacto con los vasos sanguíneos, manteniendo de esta forma la homeostasis hídrica (Seifert et al. 2006; Papadopoulos et al. 2013). Los astrocitos tienen diferentes formas de lanzar protones (H^+) a través de la membrana, ya que poseen una variedad de intercambiadores, como el de Na^+/H^+, transportador de bicarbonato, transportador de ácido monocarboxílico y una forma vacuolar de H^+/ATPasa (Obara et al. 2008). Por otra parte, en los procesos astrocitarios perisinápticos se concentra un alta densidad de transportadores para diferentes neurotransmisores, como glutamato, GABA y glicina, los que permiten mantener nivel de adecuados de los mismos en la hendidura sináptica para evitar fenómenos de excitotoxicidad (Sattler y Rothsteiny 2006; Seifert et al. 2006) por un lado o son empleados en las reacciones de detoxificación del amoníaco o para la síntesis de precursores de glutatión (Maragakis y Rothstein 2006).

Además, los neurotransmisores captados por los astrocitos son convertidos en sustrato para la síntesis de nuevos neurotransmisores por enzimas astrocitarias como la glutamina sintasa (GS), que convierte glutamato en glutamina. Dado que las neuronas no expresan la enzima GS, los astrocitos desempañan un papel clave en el reciclado del glutamato, primero transformándolo en glutamina y después liberándola a la hendidura sináptica para que la neurona pueda utilizarla para la síntesis de glutamato (Seifert et al. 2006). La disposición de los astrocitos formando una red multicelular a través de las uniones GAP, permite la difusión rápida de iones o moléculas pequeñas, como K^+ y glutamato, previniendo una acumulación local potencialmente deletérea (Sattler y Rothstein 2006).

*4-**Función Sináptica***

Una de las funciones recientemente descrita de los astrocitos se relaciona con la modulación de la transmisión sináptica mediante la liberación de gliotransmisores (algunos de ellos también neurotransmisores) como glutamato, ATP, adenosina, GABA y D-serina que regulan la señalización neuronal (Nedergaard et al. 2003; Perea et al. 2009; Heneka et al. 2010). La liberación de estas moléculas ocurre en respuesta a cambios en la actividad sináptica e involucra la "excitabilidad" astrocitaria mediada por un incremento en la concentración de calcio intracelular, $[Ca^{+2}]_i$. Estas evidencias dieron lugar a la hipótesis de la sinapsis tripartita, en la que se postula que los astrocitos tiene un papel directo e interactivo con las neuronas durante la actividad sináptica, y que dicha interacción resulta esencial en el procesamiento para el correcto procesamiento de la información en los circuitos neurales (Halassa et al. 2007; Perea et al. 2009). Además del efecto directo en la actividad sináptica a través de la liberación de gliotransmisores, los astrocitos pueden influir en la función sináptica a través de la liberación de factores de crecimiento y moléculas relacionadas que promueven el establecimiento, mantenimiento y función de la sinapsis. Una de la citoquinas involucradas en este proceso es el factor de necrosis tumoral alfa (TNFα), el cual potencia la comunicación sináptica induciendo la inserción en la membrana postsináptica de los receptores de glutamato de tipo AMPA (Stellwagen et al. 2006). Durante el desarrollo del SNC, los astrocitos (junto con la microglia) desempeñan una función relevante en el *pruning* o poda sináptica, mediante la cual se eliminan las sinapsis que no pudieron establecerse de forma correcta o son débiles (Christopherson et al. 2005; Stevensen et al. 2007; Barres 2008). Por otra parte, los astrocitos producen y liberan esteroides neuroactivos, como estradiol y progesterona que pueden tener un efecto en la actividad sináptica, principalmente a través del receptor de GABA tipo A ($GABA_A$) (Garcia-Segura and Melcangi 2006).

*5-**Metabolismo Energético***

Los astrocitos poseen una gran variedad de enzimas y rutas metabólicas lo que los convierte desde el punto de vista bioquímico en la célula más versátil del SNC. Debido a esta plasticidad metabólica, los astrocitos hacen una importante contribución al metabolismo del SNC en condiciones fisiológicas y más aún en situaciones patológicas (Magistretti 2006). La disposición de los astrocitos resulta clave para cumplir esta función, ya que por un lado tienen procesos en contacto con vasos sanguíneos y otros procesos contactan con la neurona a diferentes niveles, el soma, el axón y las sinapsis (Figura 1). Aunque desde hace algunos años se sabe que los astrocitos son la principal reserva de glucógeno en el SNC, y que la mayor acumulación de este ocurre en áreas de alta densidad sináptica (Brown et al. 2007; Bélanger et al. 2011), la contribución funcional de esta reserva de glucógeno se ha empezado a entender recientemente (Bélanger et al. 2011). Se ha demostrado que la utilización del glucógeno astrocitario es relevante para mantener la actividad neuronal en situaciones de hipoglicemia o durante largos períodos de actividad neuronal (Bélanger et al. 2011; Falkowska et al. 2015). En este sentido se observó que la reserva de glucógeno astrocitario es modulada por neurotransmisores como el glutamato (Brown et al. 2007) y que los metabolitos de glucosa pueden difundir a lo largo de la red astrocitaria a través uniones GAP, fenómeno que también es regulado por glutamato y la actividad neuronal (Falkowska et al. 2015). Además, durante situaciones de hipoglicemia el glucógeno puede ser degradado en los astrocitos hasta generar lactato, el cual es transferido a neuronas adyacentes como sustrato energético (Brown et al. 2004; Pellerin et al. 2007; Falkowska et al. 2015). Algunos estudios basados en modelos computacionales, sugieren que durante períodos de alta y prolongada actividad neuronal la inhibición de la enzima fosfofructoquinasa (*PFK*) conduce a un descenso o detenimiento de la glicólisis neuronal, haciendo que el lactato liberado por los astrocitos se convierte en el principal sustrato energético de las neuronas (Occhipinti et al. 2009). Esta interacción a nivel del metabolismo energético esta acoplada a la actividad neuronal a través de los transportadores de glutamato. Un aumento en

la captación de glutamato por parte de los astrocitos induce un incremento en la vía glicolítica teniendo como resultado una mayor producción de lactato. Este es liberado para que la neurona lo utilice como metabolito energético. Por otro lado, el glutamato captado por los astrocitos es convertido en glutamina por la enzima glutamina sintasa (GS) y también es liberado para que la neurona pueda sintetizar nuevamente glutamato (Figura 2) (Lloyd et al. 2015). El soporte metabólico que brinda el astrocito a la neurona es fundamental tanto en situaciones basales y más aún en situación de alta actividad neuronal. Algunos autores consideran que un desbalance en esta interacción subyace a muchas condiciones patológicas que afectan al SNC.

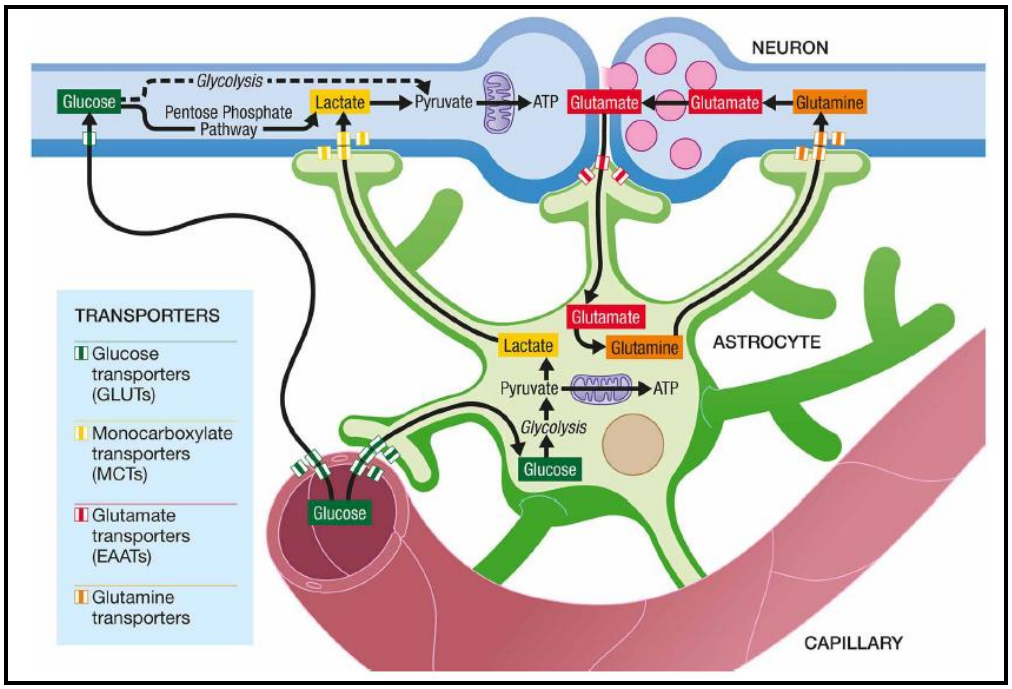

Fig. 2. **Interacción metabólica entre astrocito-neurona**. En una sinapsis excitatoria, el glutamato es liberado a la hendidura sináptica para actuar sobre la terminación post-sináptica. La activación termina cuando los astrocitos captan el glutamato remanente para transformarlo en glutamina. Esta es liberada nuevamente al espacio extracelular por los astrocitos para que sea captada por las neuronas y transformada nuevamente en glutamato. La internalización de glutamato por los astrocitos induce un aumento de la vía glicolítica aumentando la captación de glucosa desde los capilares. El lactato producido por los astrocitos a través de la glicólisis puede ser liberado al espacio extracelular. Las neuronas pueden captar y utilizar lactato para la generación de ATP y de esta forma hacer frente a la demanda energética que provoca un aumento en la actividad sináptica. Figura de (*Lloyd et al. 2015*)

*6-**Barrera Hematoencefálica***

La barrera hematoencefálica (BHE) es una estructura altamente especializada y selectiva, que regula la entrada al SNC de moléculas pequeñas, macromoléculas e incluso células (Ballabh y Nedergaard 2004; Abbott et al. 2006). Aunque el principal componente estructural y funcional de la BHE son las células endoteliales, en los últimos años el papel de los pericitos y los astrocitos en el mantenimiento y función de esta estructura está adquiriendo mayor relevancia (Ballabh y Nedergaard 2004; Abbott et al. 2006). Los astrocitos pueden inducir ciertas propiedades de barrera en células endoteliales tanto cerebrales como periféricas y en células epiteliales relacionadas lo que sugiere un papel directo en la inducción de la BHE (Alvarez et al. 2013). Sin embargo, como ciertas propiedades de la BHE empiezan a ser funcionales antes de la aparición de los astrocitos (Saunders et al. 2008) y los progenitores neurales embrionarios también inducen propiedades de barrera en células endoteliales (Weidenfeller et al. 2007), la evidencia reciente indica que los astrocitos desempeñan un papel clave en el mantenimiento y correcto funcionamiento de la BHE. A modo de ejemplo, los astrocitos captan el K+ extracelular en los sitios de sinapsis y lo eliminan a nivel de los podocitos realizando un mecanismo de control espacial de la concentración de potasio (Kofuji et al. 2004). A nivel de los podocitos astrocitarios también se elimina el agua captada por ósmosis hacia el líquido intersticial (Amiry-Moghaddam et al. 2003). Cuando se afecta a los astrocitos, por ejemplo mediante algunas toxinas, o se impide la expresión de ciertas proteínas involucradas en el mantenimiento de la BHE, como proteínas de la familia BMP (*Bone Morphogenetic Protein*), la integridad de la BHE se compromete (Araya et al. 2008).

Los astrocitos en la patología del SNC

1- **Reactividad astrocitaria**

Los astrocitos responden a todas las formas de daño y enfermedades que afectan al SNC a través de un proceso llamado astrogliosis (Figura 3) (Sofroniew y Vinters 2010). Los estudios realizados en los últimos 20 años han proporcionado evidencias concluyentes acerca de que la reactividad astrocitaria no es un fenómeno simple y único en su manifestación, sino que se trata de un proceso que presenta diferentes grados que van desde alteraciones reversibles en la expresión génica con hipertrofia celular, hasta la formación de cicatrices con remodelación permanente del tejido (Pekny y Pekna 2014). Se ha demostrado además que la magnitud de los cambios estructurales y funcionales que acompañan a la reactividad astroglial ocurre en relación y proporcionalmente al contexto en la que ésta tiene lugar y es regulada por diferentes tipos de señales. Estas observaciones no solo han puesto de manifiesto la heterogeneidad de la reactividad astroglial como respuesta integrada sino además la diversidad a nivel celular y molecular de los astrocitos reactivos que participan de la respuesta (Anderson et al. 2014).

Dado que en condiciones fisiológicas los astrocitos también pueden sufrir cambios morfológicos es importante dejar claro algunos conceptos. Los términos “reactividad astroglial” o “astrocitos reactivos” denotan la respuesta de los astrocitos frente a cualquier tipo de daño o enfermedad que afecta al SNC. Como se mencionó previamente es una respuesta heterogénea y diversa que implica diferentes grados de complejidad. Por otra parte, los términos “activación astrocitaria” o “astrocitos activados” no solo hacen referencia a cambios en condiciones patológicas, sino que también implican los cambios desencadenados en situaciones fisiológicas normales en respuesta a la actividad normal del SNC. Uno de los cambios involucra, entre otras cosas, aumento de los niveles intracelular de calcio ($[Ca^{+2}]i$) como respuesta por ejemplo a la actividad sináptica y a modificaciones del flujo sanguíneo cerebral.

(Attwell et al. 2010; Tong et al. 2013). Por lo tanto el término “activación astrocitaria” hace referencia a un conjunto más amplio de cambios celulares y moleculares que pueden encontrarse en un rango mayor de condiciones, tanto fisiológico como patológico, y que contribuyen al normal funcionamiento del SNC.

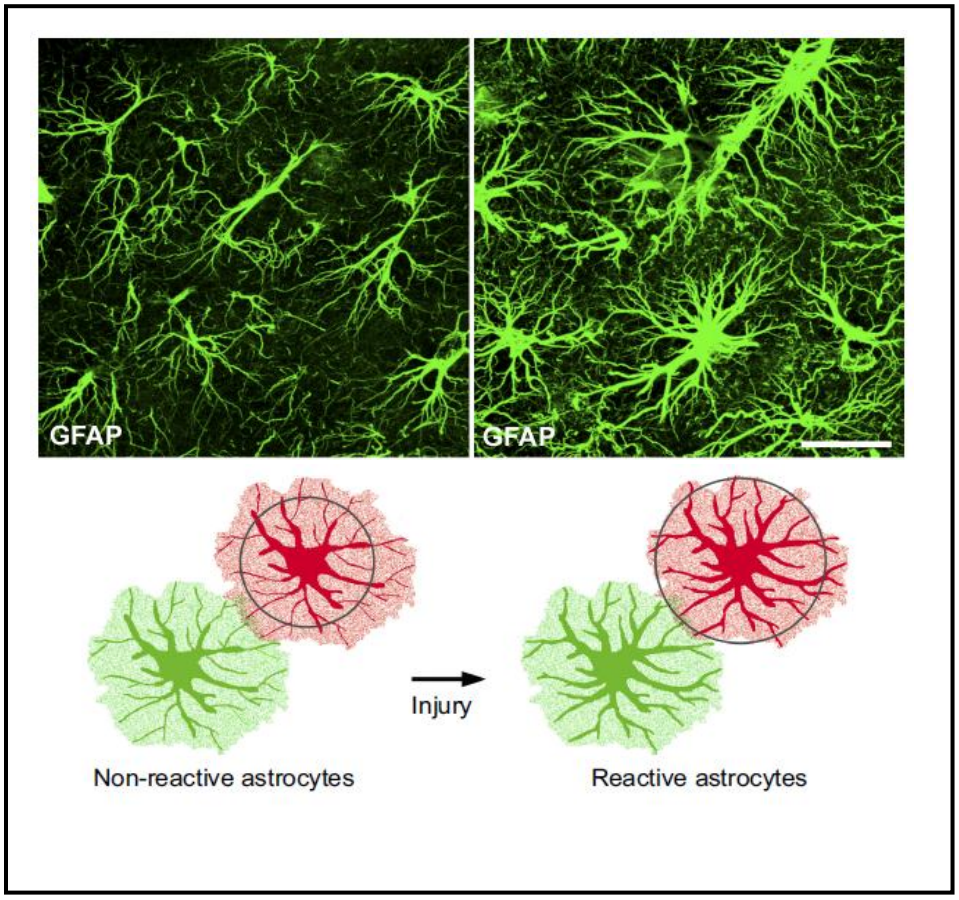

Figura 3. **Astrogliosis**. Los astrocitos responden a diferentes tipos de injuria en el SNC cambiando de forma y aumentando la expresión de la proteína GFAP. Figura modificada de (*Pekny et al 2014)*

Como se mencionó previamente, los astrocitos constituyen un grupo celular heterogéneo dependiendo de su localización y la función que desempeña dentro del SNC. Esto implica que la reactividad astrocitaria frente al daño también es un fenómeno heterogéneo, que depende además del tipo de daño, de la intensidad del mismo y de la localización y el contexto en el que tiene lugar. Esta

heterogeneidad en la respuesta astrocitaria se manifiesta a múltiples niveles, desde diferencias en los cambios en la expresión génica, bioquímicos y morfológicos hasta diferencia en la duración y forma en la que se resuelve la respuesta (Figura 3) (Tsai et al. 2012; Zhang y Barres 2010).

Observaciones realizadas en modelos animales y en muestras de tejido humano en condiciones patológicas, permitieron a los investigadores proponer una definición de reactividad astrocitaria que incluye varios grados de severidad y manifestación, los que pueden ser corroborados no solo de forma experimental sino que también en la clínica, a través de estudios anatomopatológicos con fines diagnóstico (Sofroniew 2009; Sofroniew y Vinters 2010). Esta definición comprende cuatros enunciados principales: i) la astrogliosis reactiva es un espectro de cambios funcionales, celulares y moleculares que sufre el astrocito en respuesta a diversos insultos o enfermedades que afectan al SNC, ii) los cambios que experimentan los astrocitos reactivos varían dependiendo de la severidad del insulto de forma gradual y continua, iii) los cambios que tienen lugar durante la astrogliosis reactiva son regulados de forma específica por el contexto en el que se da la respuesta e involucra señalizaciones inter- e intracelulares y iv) los cambios mencionados en los puntos anteriores tienen el potencial de alterar las actividades astrocitarias conduciendo tanto a la pérdida como a la ganancia de funciones específicas de los astrocitos (Sofroniew 2009; Sofroniew y Vinters 2010).

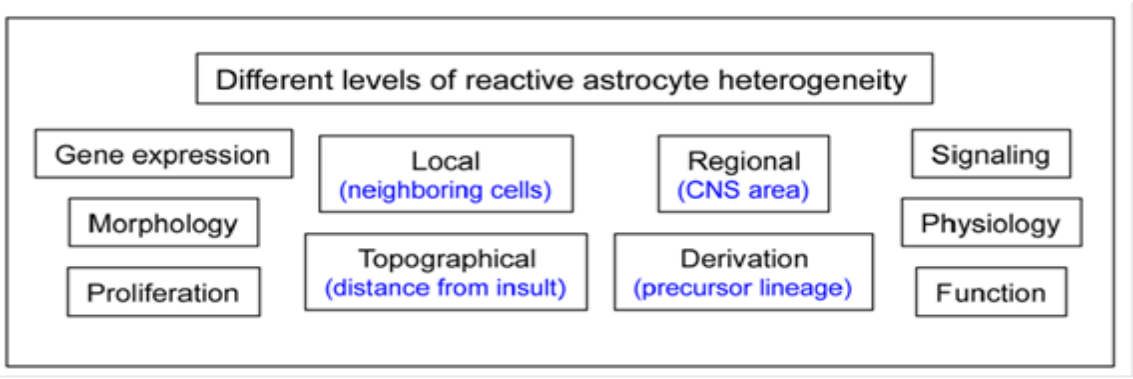

Fig 4. Heterogeneidad de la reactividad astrocitaria a diversos niveles. En el texto se describe la mayoría de ellas. Figura de (*Anderson et al. 2014)*

*1.1-**Cambios morfológicos***

La hipertrofia del cuerpo celular y de los procesos es una característica preservada de los astrocitos reactivos (Sofroniew y Vinters 2010). Sin embargo este cambio de forma no es una respuesta estereotipada y uniforme. Se puede observar diferente grado de hipertrofia celular y una amplia variabilidad dependiendo principalmente de la severidad del insulto y de la proximidad del astrocito a la región injuriada (Figura 4). Es así que puede observase un continuo de diferente magnitud en el cambio morfológico astrocitario cuando se examina un tejido dañado tendiente a disminuir en intensidad a medida que nos alejamos del epicentro del daño (Wilhelmsson et al. 2006) Las implicancias funcionales de esta heterogeneidad morfológica aún se desconocen. Otro grado de heterogeneidad morfológica se produce en los procesos astrocitarios, ya sea en la longitud y volumen de los mismos, como en el nivel de interdigitación entre los procesos de astrocitos próximos. En el tejido sano, los procesos astrocitarios individuales se encuentran ocupando dominios territoriales que no se solapan y que persisten incluso durante reactividades astrocitarias leves o moderadas (Wilhelmsson et al. 2006). Sin embargo, en la reactividad astrocitaria severa, que involucra proliferación celular, se observa un claro solapamiento de los procesos astrocitarios, particularmente en los bordes de la cicatriz que se forma rodeando el sitio de la lesión. Esto pone en evidencia una clara diversidad en la reactividad astrocitaria que se manifiesta a través de diferentes grados de hipertrofia celular y de interacción e interdigitación de los procesos celulares (Figura 5) (Sofroniew y Vinters 2010)

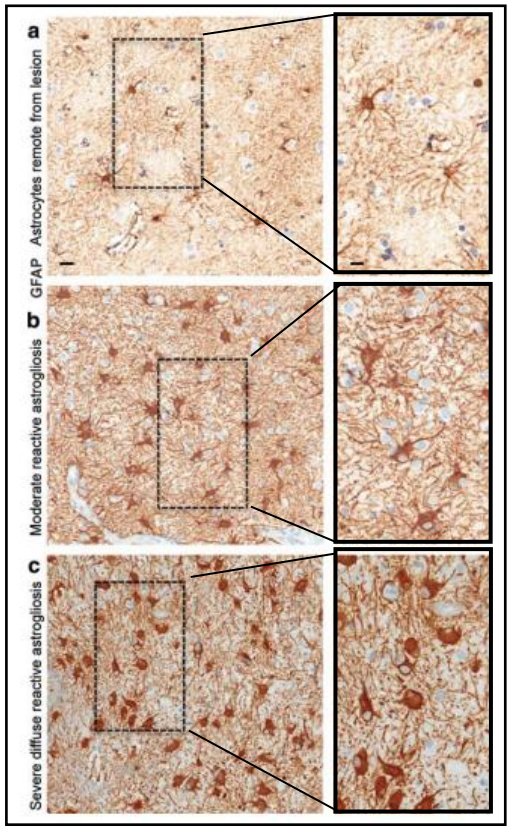

Fig. 5. **Diferentes grados de reactividad astrocitaria.** Inmunohistoquímica para GFAP **a)** Astrocitos en un tejido sano, no lesionado. Pocos astrocitos expresan niveles detectables de GFAP y estos están alejados entre si. El territorio de los astrocitos no se solapa. **b)** Reactividad astrocitaria moderada con un aumento de astrocitos que expresan GFAP. Se observa una leve hipertrófia celular pero aún se preserva el territorio individual de los astrocitos sin observarse solapamiento. **c)** Reactividad astrocitaria severa y difusa mostrando un significativo aumento de la expresión de GFAP, de la hipertrófia celular, de la proliferación y presencia de solapamiento de los procesos astrocitarios. Figura de (Sofroniew 2010)

*1.2-**Proliferación celular***

El aumento de proliferación de los astrocitos frente al daño del SNC es un fenómeno que se identificó hace ya varios años pero que no ha sido bien caracterizado en el contexto de la reactividad astrocitaria. Trabajos recientes muestran que existe una relación directa entre la severidad de la reactividad astrocitaria y la presencia de proliferación celular (Wanner et al. 2013). En respuestas leves y moderadas puede no observarse cambios en la proliferación astrocitaria, sin embargo, es una característica imprescindible para la formación de la cicatriz glial que se forma rodeando el sitio de daño que es típicamente observada después de una lesión por compresión o contusión en la médula espinal (Faulkner et al. 2004; Wanner et al. 2013). Esto implica que la formación

de la cicatriz que se desencadena inmediatamente después de la lesión y que rodea al tejido inflamado y dañado esta principalmente constituida por astrocitos proliferantes que interdigitan sus procesos. La inhibición de la proliferación astrocitaria impidió no solo la formación de la cicatriz glial sino también las respuestas reparativas causando deficiencias motoras significativas (Faulkner et al. 2004).

Las investigaciones en modelos experimentales de daño en médula espinal que muestran una reactividad astrocitaria típica muestran que i) existe una gradiente en la tasa de proliferación astrocitaria que disminuye a medida que nos alejamos del sitio de lesión, ii) la densidad de astrocitos encontrada en una cicatriz glial es el doble que la encontrada en condiciones basales, iii) la cicatriz es una estructura compacta de varias micras de espesor que aísla al tejido dañado y iv) acompañando al gradiente de proliferación celular que se observa a medida que nos alejamos del sitio de lesión, existe un gradiente de células astrocitaria morfológicamente heterogéneas que denotan los diferentes grados de la reactividad astrocitaria (Sofroniew y Vinters 2010; Wanner et al. 2013). Es interesante destacar que los nuevos astrocitos que se generan como resultado de la proliferación tienen aspectos morfológicos y un perfil molecular similar a los progenitores que comprenden la glia radial GFAP positiva, aunque solo mantienen el potencial de generar nuevas células gliales pero no pueden actuar como progenitores neuronales (Imura et al 2006; Wanner et al. 2013). Por otra parte, los astrocitos que proliferan en las regiones más alejadas al sitio de la lesión en la médula, tienen el aspecto estrellado típico de astrocitos maduros que también puede observarse en el tejido sano, particularmente en las regiones perivasculares (Bardehle et al. 2013).

Estas observaciones enfatizan las diferencias en la proliferación de los astrocitos reactivos frente a los diversos tipos de estímulos y evidencia la generación de una gama de fenotipos celulares que se extiende desde células con cierto potencial progenitor hasta astrocitos maduros. Se desconoce frente a

que tipo de estímulos y en que situaciones se originan estas células inmaduras o indiferenciadas, pero pone de manifiesto la plasticidad de la respuesta astrocitaria para hacer frente al daño en el SNC.

*1.3-**Cambios en la expresión génica***

Los astrocitos sufren cambios significativos en la expresión génica cuando se vuelven reactivos frente a diversos insultos al SNC. Estudios a nivel transcripcional han demostrado que la expresión de un número importante de genes se modifica durante la reactividad astrocitaria, tanto en más como en menos. Comprender los cambios a nivel de la expresión génica, es decir identificar cuales genes comienzan a expresarse más y cuales se expresan menos, es clave para entender las consecuencias de la respuesta astrocitaria. Estos cambios se observan tanto *in vivo* como *in vitro*, cuando los astrocitos son expuestos a diferentes agentes que disparan la reactividad astroglial (Cahoy et al. 2008; Zamanian et al. 2012). La expresión de la proteína GFAP aumenta en la gran mayoría de las formas de reactividad astrocitaria y esta característica es ampliamente utilizada para identificar la respuesta tanto a nivel experimental como en los estudios clínicos (Sofroniew 2009). Otra proteína cuya expresión aumenta en la reactividad astrocitaria desencadenada por diversos estímulos es la lipocalina 2 (Lcn2; Lee et al. 2008) y se ha propuesto que también puede ser útil como un marcador general de diferentes tipos de astrogliosis. Por otra parte, se han identificado genes cuya expresión está selectivamente aumentada en la reactividad astrocitaria desencadenada por estímulos específicos. Los estudios mediante microarreglos (*microarrays*) han posibilitado la generación de una base de datos de perfiles de expresión génica (transcriptoma) que ponen de manifiesto la heterogeneidad de la reactividad astrocitaria a nivel transcripcional. Por ejemplo existen diferencias sustantivas en el perfil transcripcional entre astrocitos reactivos aislados de un tejido sometido a isquemia o tras la inyección sistémica de lipopolisacárido (LPS; Zamanian et al. 2012) o frente a

hiperamonemia (Lichter-Konecki et al. 2008) o los astrocitos reactivos que se observan en el envejecimiento normal (Orre et al. 2013).

Estudios relacionados en astrocitos en cultivo han mostrado las diferencias en el perfil transcripcional desencadenada por diversos inductores de reactividad astrocitaria, como LPS, IL1β, TNFα, INFγ o TGFβ tanto individualmente como combinados (Meeuwsen et al. 2003; Hamby et al. 2012; Zamanian et al. 2012). Estos resultados son consistentes con la visión de que lo cambios que experimentan los astrocitos durante la reactividad astrocitaria son dependientes del contexto y están dirigidos por mecanismos de señalización específicos (Sofroniew 2009). Por ejemplo, entre los aspectos que comúnmente cambian durante la reactividad astrocitaria inducida por citoquinas y mediadores inflamatorios se encuentran los mecanismos moleculares y celulares que regulan la morfología celular y la proliferación, dando como resultado la hipertrofia celular y un aumento en el número de astrocitos reactivos, dos características que definen la reactividad astrocitaria y que se observan en respuesta al daño del SNC en modelos *in vivo* (Hamby et al. 2012; Zamanian et al. 2012). Tales cambios probablemente intenten reorientar las funciones astrocitarias hacia la interacción con células del sistema inmune para desencadenar una respuesta inflamatoria controlada y acorde al estimulo desencadenante. Por otra parte, frente a un daño traumático que involucre daño del tejido, los cambios en la expresión génica que sufren los astrocitos reactivos, parecen estar orientados hacia la protección frente a la entrada de patógenos, la reparación y la preservación de las funciones celulares elementales, particularmente en las neuronas circundantes (Zamanian et al. 2012).

*1.4-**Regulación***

Mediante estudios de silenciamiento génico se ha demostrado un rol importante de las vías de señalización que involucran a los factores de transcripción STAT3 (del inglés; *signal transducer and activator of transcription*

3), SOCS3 (del inglés; *suppressor of cytokine signaling 3*) y NFκB (del inglés; *nuclear factor KB*) en la regulación de la reactividad astrocitaria en diferentes modelos de daño en el SNC (Okada et al. 2006; Herrmann et al. 2008; Brambilla et al. 2009). Por ejemplo, la deleción de STAT3 o su receptor de membrana GP130 atenúan de forma pronunciada ciertas manifestaciones de la astrogliosis reactiva, como la hipertrofia celular, el aumento de immunoreactividad de la proteína GFAP y la formación de la cicatriz glial. Además, se observa un aumento en la extensión de la neuroinflamación, del tamaño de la lesión y mayor dificultad en la recuperación funcional después de un daño en la médula espinal, sugiriendo un rol antiinflamatorio de los mecanismos de señalización que involucran a STAT3 en los astrocitos, regulando la inflamación a nivel global (Okada et al. 2006; Herrmann et al. 2008). Por otro lado, la deleción de SOCS3 o NFκB también atenúa ciertos aspectos de la astrogliosis reactiva tales como la hipertrofia celular y el aumento de GFAP, pero la extensión de la inflamación y el tamaño de la lesión se reducen, dejando en evidencia el rol pro-inflamatorio de estas vías de señalización en los astrocitos y particularmente durante la respuesta astrocitaria (Okada et al. 2006; Brambilla et al. 2009). Por lo tanto, diferentes vías de señalización que desencadenan cambios comparables en la morfología astrocitaria y/o en la expresión de GFAP, pueden estar llevando adelante tanto una respuesta pro o anti inflamatoria, con resultados totalmente diferentes.

Dado que existen evidencias tanto del efecto beneficioso como deletéreo de los astrocitos reactivos en el SNC, es importante identificar y definir cuales son las señales que están liderando uno u otro efecto, teniendo en cuenta el contexto en el cual la reactividad astrocitaria esta teniendo lugar. Por ejemplo, la respuesta de ciertos astrocitos reactivos puede ser beneficiosa en un contexto, como por ejemplo frente a una infección bacteriana, pero puede ser deletérea en otros, como por ejemplo un daño isquémico o traumático aséptico (Failli et al. 2012). Sin embargo es importante hacer notar que aún cuando los astrocitos estén respondiendo a un daño traumático aséptico, pueden ser influenciados por

una infección periférica que estimula la producción de citoquinas y mediadores inflamatorios circulantes (Biesmans et al. 2015). Esta interferencia puede provocar la transición de los astrocitos reactivos hacia un fenotipo "antimicrobiano" que no debería estar presente en una respuesta frente a un daño aséptico. Este cambio en el fenotipo de los astrocitos reactivos, fuera de contexto, puede resultar deletéreo para la resolución del daño que desencadenó la respuesta, exacerbar los procesos inflamatorios y por ende el sufrimiento y muerte neuronal (Hernández-Romero et al. 2012). En este sentido, existen datos provenientes de la epidemiología clínica que muestran claramente el impacto negativo que tiene una infección periférica en las consecuencias neurológicas tras una lesión de médula espinal (Failli et al. 2012).

De acuerdo a estos resultados no es posible asumir que todos los astrocitos reactivos que muestran una hipertrofia celular y un incremento en la expresión de GFAP comparables, tengan desde el punto de vista funcional el mismo cometido. Esta heterogeneidad en la reactividad astrocitaria, impone la necesidad de comprender mejor las vías de señalización, los factores de transcripción y el perfil de expresión génica que presentan los astrocitos reactivos en un determinado contexto para entender la función que están desempeñando.

La Esclerosis Lateral Amiotrófica

La Esclerosis Lateral Amiotrófica (ELA) es una enfermedad neurodegenerativa fatal que tiene una prevalencia de 2-3 por cada 100.000 personas. Se caracteriza por la pérdida de las motoneuronas ubicadas en el tronco cerebral, la médula espinal y la corteza motora lo que conduce a debilidad muscular inicialmente y posteriormente parálisis progresiva hasta la muerte en pocos años desde el inicio de los síntomas. La ELA fue reportada inicialmente por el neurólogo francés Jean-Martin Charcot en 1869 (Kumar et al. 2011). La enfermedad captó la atención a nivel internacional cuando Lou Gehring, un

beisbolista que jugaba en los New York Yankees tuvo que abandonar el deporte tras ser diagnosticado con ELA en 1939. Por este motivo también se conoce a la ELA como enfermedad de Lou Gehrig. Se ha reportado que los veteranos de guerra, en particular los que participaron en la guerra del Golfo (1990-1991) tienen un riego mayor, aproximadamente el doble, de desarrollar ELA (Schmidt et al. 2008). Datos recientes muestran que la incidencia de ELA está en aumento a nivel mundial y que es razonable pensar que esta tendencia continuará en los próximos años.

La enorme mayoría de los casos de esta enfermedad se conocen como esporádicos ya que se desconoce la causa, pero entre un 5 y 10% de los casos están asociados a mutaciones genéticas transmisibles por lo que son referidos como casos familiares. Hasta hace pocos años aproximadamente un 90% de los casos de ELA familiar se asociaron a mutaciones en el gen de la enzima superoxido dismutasa 1 (SOD1) (Rosen et al. 1993; Bruijn et al. 1997; Cleveland y Rothstein 2001; Rowland y Shneider 2001). Otros genes también han sido relacionados con el desarrollo de la enfermedad, entre ellos FUS/TLS y TDP-43 (Sreedharan et al. 2008; Kwiatkowski et al 2009). Recientemente se identificó que la expansión de un repetido de hexanucleótidos en el gen C9orf72 podría explicar la gran mayoría de los casos de ELA familiar, sugiriendo que los casos ligados a la genética podrían representar un porcentaje mayor de lo reportado hasta el momento (De Jesus-Hernandez et al. 2011; Ling et al. 2013)

Dado que diferentes mutaciones en el gen de una importante enzima antioxidante como SOD1 conducen al desarrollo de ELA, la hipótesis de que el estrés oxidativo podría desempeñar un papel clave en la patología ha prevalecido por años. Datos anatomopatológicos obtenidos de tejidos *post-morten* tanto de caso familiares como esporádicos de ELA, así como de las investigaciones realizadas en modelos murinos que sobre-expresan una forma mutada de la SOD1 humana (SOD1G93A), han demostrado la presencia de un daño oxidativo importante en esta enfermedad (Beckman et al. 2001; Barber

y Shaw 2010). Una de las principales hipótesis que aún persiste sobre las causas de la ELA, implica una desregulación de la señalización excitatoria mediada por el neurotransmisor glutamato, fenómeno conocido como excitotoxicidad mediada por glutamato, que podría conducir a la muerte de la motoneurona (Rothstein 2009). Además, una combinación de alteraciones a nivel celular, como pérdida en la homeostasis del calcio, disfunción mitocondrial, agregación de proteínas, disrupción del citoesqueleto y neuroinflamación crónica podrían explicar el daño y posterior muerte de las motoneuronas (Cassina et al. 2008; Ilieva et al. 2009; Leal et al. 2013; Manfredi y Kawamata 2016).

El rol de las células gliales y la neuroinflamación en la ELA.

Una de las principales características patológicas de la ELA es la generación y migración de nuevas células gliales, particularmente astrocitos, a la región afectada de la médula espinal (Barbeito et al. 2004). Sin embargo las investigaciones en modelos animales han demostrado que no solos los astrocitos, sino también la microglia y los oligodendrocitos están implicados de una u otra forma en diferentes etapas del procesos neurodegenerativo en la ELA (Figura 7) (Souza et al. 2016). Debido a la complejidad intrínseca en la respuesta de cada uno de los tipos gliales frente al daño, sumado a las dificultad que implica su estudio in vivo, resulta clave establecer cuales de las alteraciones observadas podría ser causa y no consecuencia del proceso neurodegenerativo subyacente.

La reactividad astrocitaria y la activación de la microglia (microgliosis) son fenómenos que se observan tempranamente en la médula espinal de modelos animales y paciente con ELA y que están implicados en el establecimiento y mantenimiento de la neuroinflamación crónica que acompaña a la neurodegeneración (Komine et al. 2015). El estudio de pacientes con ELA por tomografía de emisión de positrones (PET) muestra una correlación entre la intensidad de la microgliosis y la severidad de la patología, sugiriendo un

estrecha relación entre la muerte neuronal progresiva y la neuroinflamación (Turner et al. 2004). Dicho proceso inflamatorio ha sido bien caracterizado en modelos animales ligados a la mutación de la SOD1. Altos niveles de moléculas proinflamatorias como TNF-α, IL-1β, (*interleuquina 1β)*, IL-12 (*interleuquina 12*) e INF-γ, entre otras, sumado a la presencia especies reactivas de oxígeno (O_2^-, NO y $ONOO^-$) y diferentes citoquinas se observa en las primeras etapas de la fase sintomática (Hensley et al. 2003; Elliott et al. 2001; Yoshihara et al. 2002). Por otra parte, la expresión de factores tróficos como GM-CSF (factor estimulante de colonias de granulocitos y macrófagos), citoquinas antiinflamatorios como TGF-β (factor de crecimiento transformante-β) y factores neurotróficos como IGF-1 (factor de crecimiento tipo-insulina 1) por parte de la microglia sugiere la existencia de un componente neuroprotector en la respuesta neuroinflamatoria (Henkel et al, 2009; Komine et al 2015).

Al igual que los macrófagos a nivel periférico, la activación de la microglia puede dar origen a dos estados celulares conocidos como M1 y M2, con características moleculares muy diferentes y funcionalmente opuestos. La microglia M1 se considera citotóxica ya que produce y libera moléculas proinflamatorias como TNFα, IL-1β, IL-12 y NO, entre otras, y presenta menor actividad fagocítica, mientras que la microglia M2 libera moléculas antiinflamatorias como IL-4 (interleuquina-4), IL-10 (interleuquina-10) y TGF-β, factores tróficos como IGF-1 y presenta mayor actividad fagocítica por lo que se considera citoprotectora (Murray et al. 2011). Se ha reportado un cambio en el fenotipo microglial durante la progresión de la enfermedad en ratones $SOD1^{G93A}$, siendo el fenotipo M2 predominante en las primeras etapas de la fase sintomática y el fenotipo M1 sobre el final (Henkel et al. 2009). La transición de un fenotipo microglial a otro ha sido reportado en otros procesos neurodegenerativos, y aunque para algunos autores es un mecanismo muy implicado en la fisiopatología de los procesos neurodegenerativos, para otros microglial esta transición en el fenotipo microglial pueda estar relacionada a la fisiopatológico de la ELA, evidencias recientes muestran que la activación

microglial es un proceso mucho más complejo que involucra fenotipos intermedios entre los estados M1 y M2 (Komine et al. 2015)

A pesar de que los astrocitos no son células del sistema inmune, la regulación que estos ejercen sobre la microglia los convierte en células clave durante la respuesta inflamatoria en el SNC. Se ha demostrado que tanto en pacientes como en los modelos animales de ELA los astrocitos adoptan un fenotipo inflamatorio que implica la liberación de citoquinas proinflamatorias, quimioquinas, factores del complemento y especies reactivas del oxígeno y nitrógeno (Haidet-Phillips et al. 2011). Por otra parte, la expresión de TGF-β1 esta aumentada en astrocitos SOD1G93A y la sobreexpresión de esta citoquina específicamente en astrocitos (bajo el promotor de GFAP) acelera la progresión de los síntomas a través de un mecanismo que involucra un descenso en la expresión de IGF-1 y un aumento de IFN-γ (Endo et al. 2015).

Se ha demostrado que tanto los astrocitos como la microglia pueden provocar la muerte de motoneuronas espinales *in vitro* a través de diferentes mecanismos. A pesar de ello, ni la eliminación selectiva de los astrocitos proliferantes (Lepore et al. 2008) ni de la microglia proliferante (Gowing et al. 2008) tuvo gran impacto en el desarrollo de la patología en el modelo transgénico $SOD1^{G93A}$. En este sentido, la respuesta neuroinflamatoria global, más que la participación individual de los diferentes tipos celulares, parece resultar relevante en la fisiopatología de la ELA. Una mayor comprensión de la interacción astrocito-microglia, de las moléculas y vías de señalización implicadas y de la respuesta neuroinflamatoria resultante, podría ser relevante para el desarrollo de nuevas estrategias terapéuticas para el tratamiento de enfermedades neurodegenerativas.

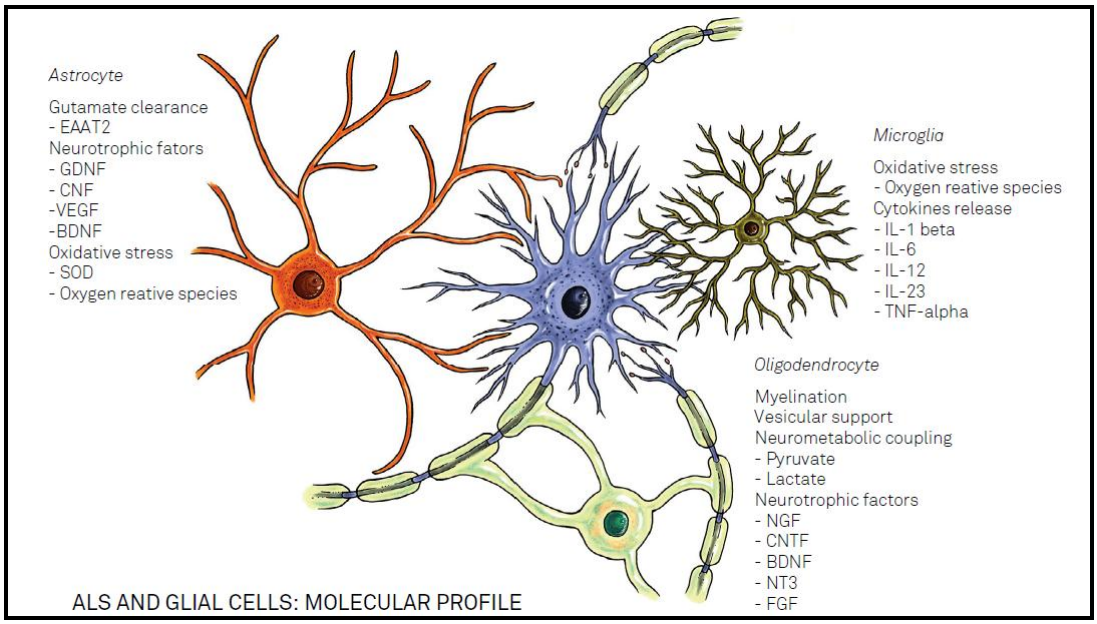

Figura 6. Implicancia de las células gliales en la ELA. Se ha reportado que las células gliales mayoritarias en el SNC están implicadas en la fisiopatología de la ELA. En la figura se muestra el perfil molecular implicado en la interacción entre los diferentes tipos celulares y la motoneurona. Alteraciones en la expresión de factores tróficos y citoquinas asociados con aumentos en los niveles de estrés oxidativo ha sido demostrado en astrocitos, microglia y oligodendrocitos. Figura de (*Souza et al. 2016*)

El rol de los astrocitos en la ELA

El estrés celular inducido por diferentes señales de daño hace que los astrocitos respondan proliferando y adoptando un fenotipo reactivo caracterizado por un pronunciado cambio morfológico y un aumento de la inmunoreactividad para la proteína GFAP. Estos cambios pueden reproducirse en cultivos primarios de astrocitos espinales cuando se exponen a estrés oxidativo, sugiriendo que esté podría ser uno de los mecanismos implicados en la fisiopatología de la ELA (Barbeito et al. 2004). Cambios profundos en los astrocitos podrían ser inducidos por alteraciones a nivel epigenético mediados por la propia enzima SOD1 mutada y por otros tipos de estrés celular, originando una transformación fenotípica y la aparición de astrocitos neurotóxicos. En este contexto, aún no resulta claro si la muerte de las motoneuronas que tienen lugar en la ELA a través de un mecanismo no autónomo celular, se debe a un mecanismo pasivo

mediado por la pérdida de astrocitos normales, que brindan soporte trófico a la neurona o por un mecanismo activo a través de la aparición de astrocitos neurotóxicos que provocan la muerte neuronal (Lee et al. 2016). Muchos trabajos se han dirigido a responder esta interrogante utilizando básicamente una estrategia experimental similar: co-cultivos astrocitos-motoneuronas (Vargas et al. 2006; Di Giorgio et al. 2007). La monocapa de astrocitos que sobre-expresan la mutación de la SOD1 ligada a la ELA, SOD1G93A, o incluso astrocitos normales expuestos al medio condicionado de astrocitos SOD1G93A inducen la muerte de motoneuronas primarias o motoneuronas derivadas de células madre embrionarias jerarquizando el papel determinante de los astrocitos en la progresión de la enfermedad (Vargas et al. 2006; Di Giorgio et al. 2007). Estos resultados fueron confirmados *in vivo* utilizando ratones modificados genéticamente para producir la deleción específica del gen de la SOD1 mutada en astrocitos. Este abordaje permitió demostrar que la presencia de astrocitos mutantes es clave durante la progresión de la enfermedad ya que le deleción de la SOD1 mutada provocó un enlentecimiento de la progresión sin modificar el inicio de la sintomatología. (Yamanaka et al. 2008). Posteriormente se demostró que los astrocitos SOD1G93A inducen la muerte de motoneuronas en cultivo y a través de la liberación de un factor tóxico aún no identificado (Nagai et al. 2007). Sin embargo, y a pesar de que se han publicado muchos trabajos al respecto, el mecanismo exacto por el cual los astrocitos inducen la muerte de la motoneurona en el contexto de la ELA no ha podido ser explicado cabalmente.

Uno de los mecanismos propuestos implica la nitración del factor de crecimiento neural NGF mediada por peroxinitrito, lo que provoca la generación de agregados de NGF de alto peso molecular que inducen la muerte de motoneuronas en cultivo a través del receptor p75 (Pehar et al. 2004). Otros autores han propuesto que los agregados de la SOD1 mutada podrían ser responsables de la muerte neuronal en la ELA (Urushitani et al. 2007). Sin embargo, solo algunas mutaciones de la SOD1, como la SOD1G37R, estarían

comprendidas dentro de esta hipótesis ya que la inmunización contra agregados de la SOD1 produjo un aumento en la sobrevida en el modelos animal de ELA SOD1G37R pero no en el SOD1G93A (Urushitani et al. 2007). Recientemente se reportó que los astrocitos SOD1G93A inducen la muerte de las motoneuronas mediante la liberación de interferón-γ (IFNγ) activando la vía de muerte LIGHT-LT-βR en la motoneurona. Además, mostraron que la deleción del receptor LIGTH en un modelo animal de ELA aumenta la sobrevida de los animales por un enlentecimiento de la progresión de la enfermedad pero no cambia el inicio de la misma (Aebischer et al. 2011).

Como se mencionó previamente, la excitotoxicidad por glutamato es uno de los mecanismos más estudiados en relación a la muerte neuronal en la ELA. Los astrocitos expresan altos niveles del transportador de aminoácidos excitatorios de tipo 2 (EAAT2) a través del cual se capta el glutamato y el aspartato de la hendidura sináptica (Karki et al. 2015). Se estima que el 90% del glutamato captado por los astrocitos es realizado por el transportador EAAT2 en humanos, GLT1 en roedores. En condiciones fisiológicas, el glutamato captado por los astrocitos es convertido en glutamina por la enzima glutamina sintasa la que es devuelta a la neurona para la síntesis de glutamato mediante la acción de la enzima neuronal glutaminasa. Sin embargo, cuando los astrocitos se vuelven reactivos la expresión génica del transportador EAAT2 disminuye generando un incremento en la cantidad de glutamato en la hendidura sináptica, a niveles que resultan tóxicos. La reducción en la expresión de EAAT2 se ha observado en muestras de corteza motora y de médula espinal obtenidas de individuos fallecidos por ELA (Rothstein et al. 1995). Esto también ha sido evidenciado en modelos animales de ELA (Pardo et al. 2006) donde se ha observado una disminución de la expresión de GLT1, el homólogo en roedores del transportador humano. En este sentido, la inducción farmacológica de la expresión de EAAT2 no solo aumentó la sobrevida neuronal en un modelo *in vitro* de excitoxicidad aguda por glutamato, sino que además provocó un aumento en la sobrevida en animales de un modelo de ELA (Rothstein et al. 2005; Ganel et al. 2006). La

generación de ratones transgénicos SOD1G93A que sobre-expresan el transportador EAAT2, mostró que el aumento en la captación de glutamato provoca una mejora los síntomas motores asociados a esta patología pero no modifica ni el comienzo de los síntomas ni parálisis ulterior (Guo et al. 2003). Estos resultados muestran que la excitotoxicidad es un mecanismo presente en la ELA, pero aún se desconoce su relevancia en comparación con otros mecanismos de daño propuestos.

En resumen, diferentes mecanismos patológicos, como estrés oxidativo, disfunción mitocondrial, excitotoxicidad por glutamato, neuroinflamación, entre otros, han sido propuestos para explicar la muerte neuronal que ocurre en la ELA. Sin embargo la relevancia de cada uno de estos mecanismo aislados parece ser poco significativa para explicar el proceso fisiopatológico global. Teniendo en cuenta que la mayoría de las alteraciones mencionadas involucra a las células gliales, la interrogante que aún persiste entorno a la ELA es si realmente es una enfermedad “de la motoneurona” o si se trata de una enfermedad que primariamente afecta a las células gliales y que tiene como consecuencia secundaría la muerte neuronal.

Hipótesis.

La muerte de motoneuronas espinales que tienen lugar en la ELA desencadena una cascada patológica caracterizada por un ambiente inflamatorio crónico, altos niveles de estrés oxidativo, pérdida de la homeostasis y acumulación de detritos celulares y agregados proteicos. La combinación de estos fenómenos induce una pronunciada respuesta astrocitaria y microglial que al principio intenta recuperar la homeostasis para frenar la muerte neuronal subyacente. Debido a que este intento no solo es insuficiente sino que además el microambiente inflamatorio y los fenómenos acompañantes son cada vez más acentuados y crónicos, las células gliales experimentan niveles elevados de estrés celular.

Creemos que la capacidad de los astrocitos de soportar diferentes tipos y niveles de estrés celular debido a la enorme plasticidad adaptativa podría generar alteraciones a nivel epigenético capaces de inducir una profunda transformación fenotípica. En este contexto**, proponemos que el microambiente inflamatorio que acompaña a un proceso neurodegenerativo crónico estaría propiciando la aparición de astrocitos altamente neurotóxicos que agravarían la situación. Creemos que estos astrocitos proliferantes podrían ser identificados y aislados en cultivo y que su estudio sería de gran utilidad para comprender los procesos fisiopatológicos que tienen lugar en la ELA.**

Objetivos

Objetivo general

Este estudio pretende aportar evidencias sobre la generación de astrocitos altamente neurotóxicos en el contexto neurodegenerativo que tiene lugar en la médula espinal durante la progresión de la ELA. Se evaluará la posibilidad de obtener estos astrocitos en cultivo celular para su caracterización. Se intentará establecer una relación, si es que la hay, entre la aparición de estos astrocitos en la médula espinal de animales SOD1G93A y la muerte neuronal que ocurre durante la progresión de la enfermedad. Finalmente, se buscará determinar el origen de los astrocitos que proliferan en la médula espinal en fase sintomática de ratas SOD1G93A, con el objetivo de aportar al conocimiento de la respuesta glial frente a los procesos neurodegenerativos crónicos.

Objetivos específicos

1. *Aislar y cultivar astrocitos espinales de un modelo animal de ELA en la fase sintomática de la enfermedad.*

2*. Caracterizar la/s población/es celular/es obtenidas mediante el análisis de expresión de marcadores gliales prototípicos y determinar su toxicidad para motoneuronas*

3*. Identificar una relación temporal entre la aparición de los astrocitos proliferantes en la medula espinal y el avance de la neurodegeneración*

4. *Estudiar el posible origen de los astrocitos proliferantes en la médula espinal en el contexto neurodegenerativo.*

Abordaje metodológico

El diseño experimental y las metodologías utilizadas se describen detalladamente en los artículos correspondientes a cada uno de los objetivos. Brevemente se menciona las técnicas utilizadas.

1. *Cultivo de astrocitos a partir de médula espinal de animales SOD1G93A en fase sintomática.* Se intentará aislar y cultivar los astrocitos presentes en la médula espinal degenerante con el objetivo de estudiar su fenotipo y compararlo con cultivos de astrocitos neonatales.

2. *Caracterización molecular de los astrocitos obtenidos en el objetivo 1 y evaluación de su toxicidad para motoneuronas*. Utilizando cultivo de astrocitos neonatales como control, se analizará la expresión de marcadores astrocitarios prototípicos mediante western botting, inmunocitoquímica y microscopía confocal. Para evaluar su toxicidad, por un lado se utilizará un sistema de co-cultivo astrocito-motoneurona en el cual las motoneuronas embrionaria son sembradas sobre monocapa de astrocitos. Para evaluar la toxicidad de factores liberados se utilizará cultivos puros de motoneuronas que serán tratados con los medios condicionados de los diferentes tipos astrocitarios.

3. *Estudio de la relación entre la aparición de astrocitos proliferantes en la médula espinal y el avance de la neurodegeneración*. Se obtendrá muestras de médula espinal de animales transgénicos a diferentes etapas mediante fijación por perfusión intracardiaca. Los cortes serán analizados mediante inmunohistoquímica y microscopía confocal para determinar la aparición de astrocitos proliferantes. El análisis de las imágenes permitirá establecer si existe alguna relación entre la aparición de astrocitos proliferación en la medula espinal y la fase sintomática de la ELA. De esta

forma también se podrá determinar la distribución y el comportamiento de los astrocitos a medida que la sintomatología avanza.

4. *Estudio del origen de las células aisladas en el objetivo 1*. Las células aisladas a partir de la medula espinal en fase sintomática serán analizadas y separadas por citometría de flujo (*cell sorting*) mediante el uso de anticuerpos específicos de tipo celular. Las células separadas por citometría serán cultivadas nuevamente para caracterizar el fenotipo obtenido utilizando los mismos marcadores que se utilizaron para el objetivo 1.

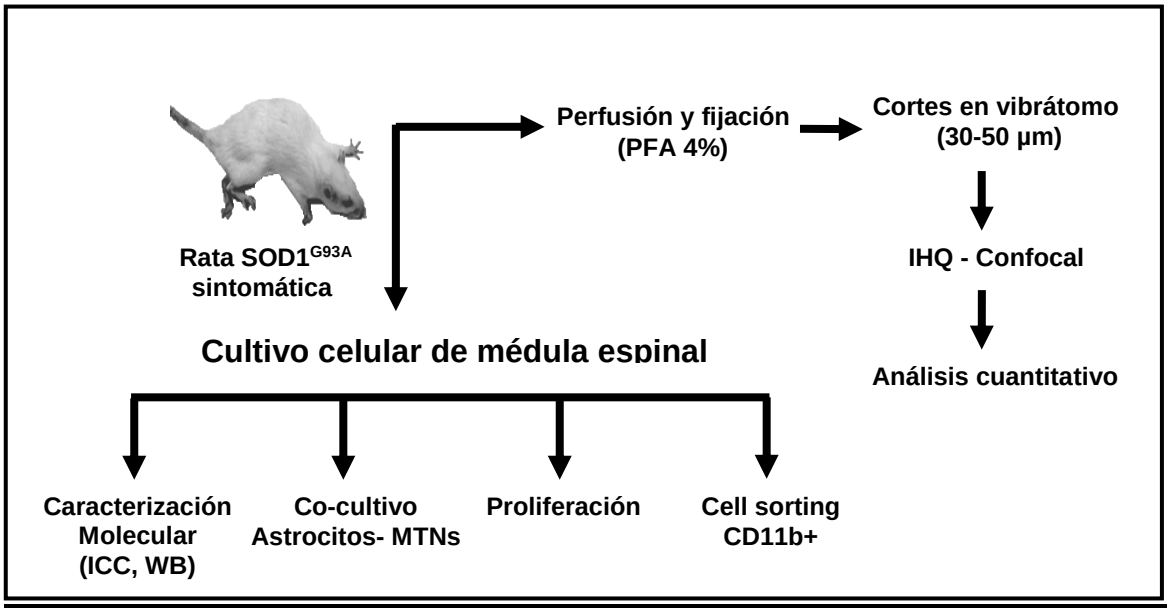

Figura 7. **Esquema del abordaje metodológico**. ICC= inmunocitoquímica, IHC= inmunohistoquímica; WB= western blotting.

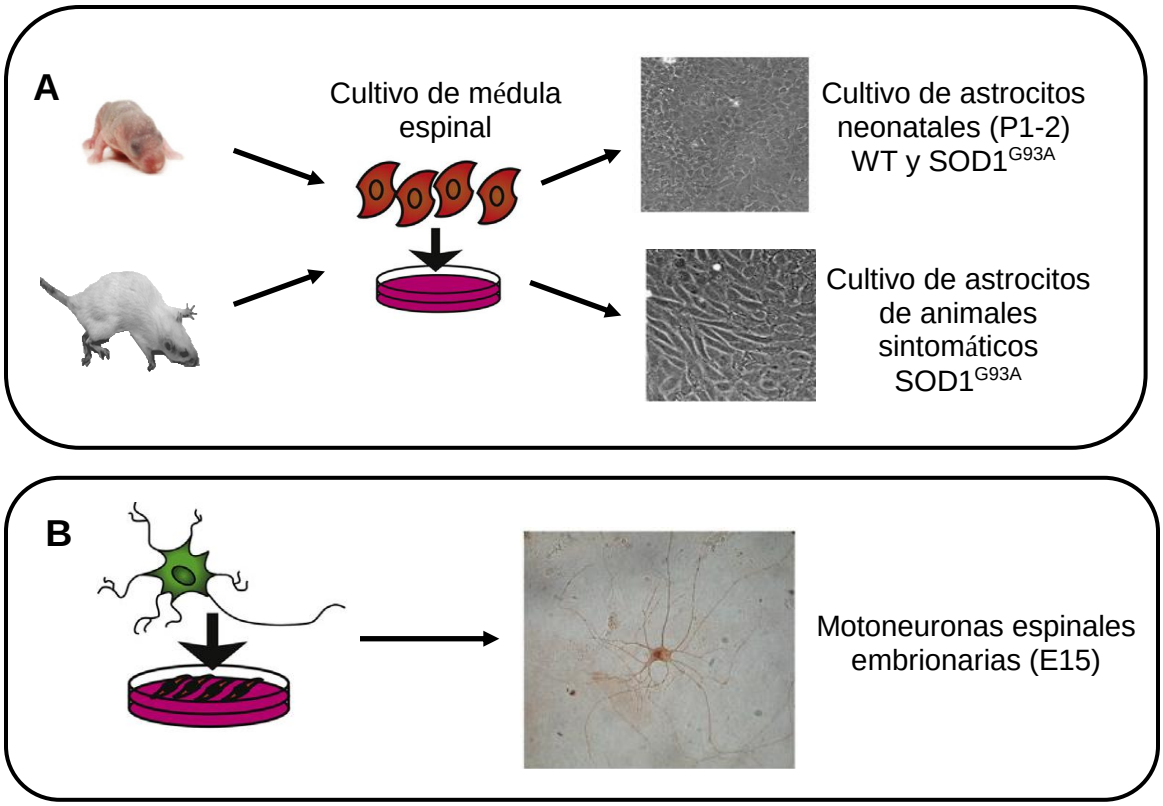

Figura 8. Cultivos celulares utilizados en la tesis. A) Cultivos astrocitarios. Los cultivos de astrocitos fueron realizados a partir de ratas recién nacidas P1-2 (*día post natal 1 o 2*) tanto no transgénicas como transgénicas SOD1^{G93A}. Para ello se cruza un animal portador de la mutación con otro animal no transgénico. Dado que el transgén se hereda de forma mendeliana se espera que el 50% de la camada sea portador del transgen lo que implica un paso de genotipado por PCR para identificar los portadores. **B**) Cultivo de motoneuronas. Se trata de cultivos de motoneuronas espinales embrionarias (E15) a partir de ratas salvajes.

Resultados

Los resultados de este trabajo se encuentran dentro de artículos científicos publicados en revistas internacionales arbitradas. Los mismos estarán precedidos por una breve descripción de los resultados obtenidos y algunos comentarios sobre su relación con los objetivos planteados.

Objetivos 1, 2 y 3. Cultivo de astrocitos a partir de animales $SOD1^{G93A}$ sintomáticos, caracterización y evaluación de su toxicidad y estudio de la aparición de estos astrocitos en la médula espinal degenerante.

Los resultados obtenidos en el contexto de los objetivos específicos 1, 2 y 3 se encuentran en del artículo 1.

Pablo Díaz-Amarilla, Silvia Olivera-Bravo, Emiliano Trias, Andrea Patricia Cassina, Joseph Beckman, and Luis Barbeito. *Phenotypically aberrant astrocytes that promote motoneuron damage in a model of inherited amyotrophic lateral sclerosis.* Proc Natl Acad Sci U S A. 2011 Nov 1;108(44).

En este trabajo se abordaron los 3 primeros objetivos planteados en la tesis. Basados en la hipótesis sobre la posibilidad de que durantes el proceso neurodegenerativo que tiene lugar en el contexto de la ELA los astrocitos podría sufrir profundas alteraciones fenotípicas, nos propusimos realizar cultivo de médula espinal en la fase sintomáticas de ratas SOD1G93A con el objetivo de aislar dichos astrocitos. Hasta ese momento no había reporte de este tipo de cultivos, si se había reportado el aislamiento de astrocitos corticales de animales adultos pero no en el contexto de una enfermedad neurodegenerativa. Para nuestra sorpresa, se pudo obtener un número de células muy alto a partir del cultivo de la médula espinal degenerante. Además las células mostraron una tasa proliferativa un poco más elevada que los cultivos astrocitarios neonatales, pero significativamente inferior a la tasa proliferativa de la línea tumoral glial C6 (una línea celular proveniente de glioma de rata) indicando que no se trataba de células transformadas. Particularmente las células mantuvieron una tasa proliferativa constante después de un importante número de pasajes en cultivo

(entre 15-20 pasajes) y mostraron una inhibición por contacto parcial, ya que la tasa de proliferación celular disminuía cuando la monocapa alcanzaba la confluencia pero no se volvía nula. Este comportamiento en cultivo nos hizo sospechar que estábamos frente a un fenotipo astrocitario diferente al que conocíamos.

La caracterización molecular posterior confirmo en gran medida nuestra sospecha. El análisis comparativo de la expresión de los marcadores astrocitarios prototípicos mediante inmunocitoquímica y *western blotting* demostró que los astrocitos aislados de animales sintomáticos tenían un perfil de expresión muy particular y significativamente diferente a los astrocitos neonatales tanto salvajes como $SOD1^{G93A}$. Para resaltar, se observó una baja expresión de la proteína GFAP, el marcador prototípico astrocitario, y con un patrón globular perinuclear a diferencia del patrón fibrilar característico que se observa en astrocitos neonatales. Contrariamente, la expresión de la proteína S100β otro de los marcadores astrocitarios estaba incrementada al igual que la expresión de la proteina Cx43, otra proteina que es característicamente expresada por los astrocitos. Finalmente, no se detectó la expresión del transportador de glutamato GLT-1 en los astrocitos adultos, siendo esta una de las características más notables que vincula a las células aisladas con la excitotoxicidad por glutamato, uno de los mecanismos fisiopatológico mas estudiados en la ELA.

Desde el punto de vista fenotípico no se ha reportado diferencias significativas entre los astrocitos neonatales salvajes y $SOD1^{G93A}$. Sin embargo los astrocitos neonatales $SOD1^{G93A}$ brindan menor soporte tróficos a las motoneuronas en co-cultivos y además liberan factores solubles que causan la muerte de motoneuronas aisladas. Por lo tanto, la principal pregunta que se planteaba a continuación era que comportamiento tendrían las nuevas células aisladas en relación a la sobrevida de las motoneuronas. Mediante un sistema de co-cultivo astrocitos-motoneurona se demostró que las células aisladas de

animales adultos sintomáticos muestran un nivel de toxicidad para la motoneurona sin precedentes. Como se había reportado previamente, los astrocitos neonatales SOD1^{G93A} inducen la muerte de aproximadamente un 50% de las motoneuronas en comparación con astrocitos neonatales salvajes. Sorprendentemente, aproximadamente solo un 20% de las motoneuronas sembradas sobre astrocitos aislados de animales adultos sintomáticos sobrevivió. Además estás neuronas mostraron signos de sufrimiento cuando se analizaron mediante inmunohistoquímica. Igual de interesante resultó la evaluación del efecto de los factores liberados por los astrocitos sobre cultivos de motoneuronas aisladas. La utilización de diluciones seriadas de los medios condicionados de los diferentes tipos de astrocitos permitió demostrar que el medio condicionado de astrocitos aislados de animales adultos es 10 veces más tóxico para la motoneurona que el medio de los astrocitos neonatales SOD1^{G93A}. De esta forma, los astrocitos obtenidos de animales adultos en fase sintomática se convertían en las células más tóxicas para la motoneurona reportadas hasta el momento.

Por las particulares características proliferativa, fenotípicas y de toxicidad denominamos astrocitos aberrantes o células AbA (del inglés *Aberrant astrocytes*) a las nuevas células aisladas.

En el tercer objetivo nos planteamos estudiar la existencia de una posible relación entre la aparición de los astrocitos aberrantes o células AbA en la médula espinal de animales SOD1^{G93A} y la progresión de la fase sintomática. Utilizando anticuerpos para los marcadores que se encontraron diferencialmente expresados en las células AbA, analizamos cortes de médula espinal en diferentes estadios de la enfermedad mediante inmunohistoquímica. Mediante un análisis cuantitativo se pudo establecer que las células AbA aparecen en la médula espinal poco antes del comienzo de la fase sintomática y que su número aumenta rápidamente conforme avanza la progresión de la enfermedad. De forma llamativa, las células AbA se identificaron cerca de los cuerpos de la motoneuronas o rodeando los vasos lo que sugiere un papel protagónico de estas células en el proceso neurodegenerativo.

Phenotypically aberrant astrocytes that promote motoneuron damage in a model of inherited amyotrophic lateral sclerosis

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Motoneuron loss and reactive astrocytosis are pathological hallmarks of amyotrophic lateral sclerosis (ALS), a paralytic neurodegenerative disease that can be triggered by mutations in Cu-Zn superoxide dismutase (SOD1). Dysfunctional astrocytes contribute to ALS pathogenesis, inducing motoneuron damage and accelerating disease progression. However, it is unknown whether ALS progression is associated with the appearance of a specific astrocytic phenotype with neurotoxic potential. Here, we report the isolation of astrocytes with aberrant phenotype (referred as "AbA cells") from primary spinal cord cultures of symptomatic rats expressing the SOD1^{G93A} mutation. Isolation was based on AbA cells' marked proliferative capacity and lack of replicative senescence, which allowed oligoclonal cell expansion for 1 y. AbA cells displayed astrocytic markers including glial fibrillary acidic protein, S100β protein, glutamine synthase, and connexin 43 but lacked glutamate transporter 1 and the glial progenitor marker NG2 glycoprotein. Notably, AbA cells secreted soluble factors that induced motoneuron death with a 10-fold higher potency than neonatal SOD1^{G93A} astrocytes. AbA-like aberrant astrocytes expressing S100β and connexin 43 but lacking NG2 were identified in nearby motoneurons, and their number increased sharply after disease onset. Thus, AbA cells appear to be an as-yet unknown astrocyte population arising during ALS progression with unprecedented proliferative and neurotoxic capacity and may be potential cellular targets for slowing ALS progression.

neurodegeneration | glial cells

Amyotrophic lateral sclerosis (ALS) is a paradigmatic disease of upper and lower motoneurons leading to progressive paralysis and death. Mice and rats expressing human Cu-Zn superoxide dismutase (SOD1) mutations develop a motor syndrome with symptoms and pathological features of the human disease (1–3). Current evidence indicates that mutant SOD1 leads to motoneuron loss because of a toxic gain of function secondary to misfolding of the protein. Mutant SOD1 activates several pathogenic mechanisms including altered redox chemistry, protein aggregation, endoplasmic reticulum stress, mitochondria dysfunction, defective axonal transport, and inflammation (4, 5). Motoneuron degeneration and prominent astrogliosis are pathological hallmarks of ALS both in patients and in animal models (6). In rats expressing the SOD1^{G93A} mutation, astrogliosis coincides with the disappearance of ventral motoneurons and a striking loss of the astrocytic glutamate transporter 1 (GLT1), also known as "excitatory amino acid transporter 2" (EAAT2) (3, 4).

Mutant SOD1 toxicity involves a non–cell-autonomous mechanism mediated at least in part by disrupted communication between motoneurons and surrounding glial cells (5, 7–13). Although astrocytes and microglia become activated in the spinal cord of symptomatic ALS animal models, the selective ablation of either proliferating GFAP-expressing astrocytes (14) or microglia (15) failed to modify disease progression, suggesting the involvement of a different glial cell type specifically contributing to motoneuron pathology. In addition, the pathogenic role of NG2 oligodendrocyte precursors that proliferate at the end-stage of ALS models (16, 17) remains unknown.

Although the expression of mutant SOD1 restricted to astrocytes is not sufficient to induce motoneuron degeneration in transgenic (Tg) mice (18), genetic excision of mutant SOD1 in astrocytes extended survival and decreased microglia activation, with no effect on disease onset (13). In addition, astrocytes expressing mutant SOD1 exert direct and selective toxicity to motoneurons by secreting soluble factors (12, 19, 20). Human astrocytes derived from the spinal cord of persons with sporadic and familial ALS also can kill motoneurons in culture (21), indicating that astrocytic toxicity is not restricted to animal models expressing mutant SOD1. However, it remains unknown whether all astrocytes are intrinsically neurotoxic for motoneurons or whether instead toxicity is restricted to a specific subclass of astrocytes. Because astrocytes expressing mutant SOD1 are more prone to enter an activated inflammatory and metastable state (22), we hypothesized that a subpopulation of astrocytes might follow a phenotypic transition deleterious to motoneuron survival. The aim of this study was to determine whether such pathogenic subtype of astrocytes occurs in a model of ALS in rats and might be isolated.

Results

Establishment of Astrocyte Cultures from Symptomatic SOD1^{G93A} Rats. To isolate glial cell populations occurring during the symptomatic stage of ALS, we established primary cultures from spinal cord of symptomatic 175-d-old Tg SOD1^{G93A} rats. Identical cultures from non-Tg littermates prepared in the same way yielded only a few cells at day 2 in vitro that proliferated slowly in the following days but failed to reach confluence or survive subsequent passages (Fig. 1, *Insets*). In contrast, cultures from Tg rats yielded numerous cells that proliferated rapidly at day 7 in vitro (Fig. 1) and formed clusters of elongated flat cells resembling astrocytes and often associated with numerous ionized calcium-binding adaptor molecule 1 (Iba1)-positive microglial cells (arrows in Fig. 1, and Fig. S1*C*). At day 10 in vitro, a 3- to 4-mm layer of agarose was polymerized on top of the cell layer.

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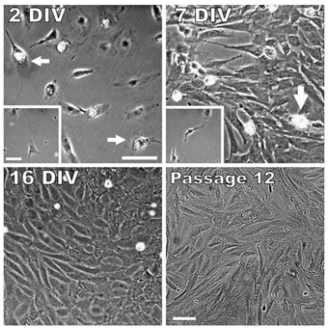

Fig. 1. Establishment of AbA cell cultures. Representative phase-contrast microphotographs of the establishment of primary spinal cord cultures prepared from symptomatic SOD1^{G93A} rats or non-Tg littermates (*Insets*). The cells were plated in a culture flask and observed under a phase-contrast microscope at 2, 7, and 16 days in vitro (DIV) and after replating at passage 12. Note the increased cell number in the culture of the symptomatic rat spinal cord compared with the culture from the non-Tg littermate. Microglial cells (arrows) disappeared progressively with successive passages. (Scale bars: 50 μm.)

Although agarose did not attach to the cultured cells, it further stimulated the intrinsic proliferating potential of Tg astrocytes but failed to stimulate proliferation of non-Tg astrocytes. After 6 d under the agarose layer (day 16 in vitro), the population of cells with astrocytic phenotype in the Tg cultures increased sharply and organized into a monolayer bearing sparse microglial cells (Fig. S1*C*). In contrast, only a few viable cells were found in non-Tg cultures (Fig. 1). After withdrawal of agarose, Tg cells continued proliferating vigorously, yielding highly homogeneous monolayers of flat, fusiform to polygonal cells devoid of microglia (Fig. 1). These cells were passaged successfully and propagated for 1 y without undergoing replicative senescence. Because of these distinctive features, we referred to these cells as "aberrant astrocytes" (AbA cells).

When grown in defined medium to oligodendrocytes (23), cultures from Tg rats yielded NG2 cells but also abundant S100β^{+} astrocytes (Fig. S1*A*). These results show that AbA cells do not arise from the NG2 oligodendrocyte precursors also known to proliferate in the spinal cord of symptomatic ALS mice (16, 17).

AbA cells were generated systematically from more than 10 independent cultures prepared from different symptomatic Tg rats, suggesting that they represent a common cell type resident in the degenerating spinal cord during the symptomatic phase of the disease.

Expression of Astrocytic Markers in AbA Cells. We next assessed the expression of astrocytic markers in AbA cell cultures from passages 8–15. Because we were unable to grow and passage astrocytes from non-Tg adult rats, the phenotypic features of AbA cells were compared with primary spinal cord astrocytes prepared from neonatal SOD1^{G93A} rats and non-Tg littermates. Among the antigenic markers listed in Table S1, we found that both AbA cells and neonatal astrocytes expressed most of the typical astrocytic markers such as GFAP, vimentin, S100β, connexin 43 (Cx43), and glutamine synthase, but GLT1 protein was not detected in AbA cells. NG2 glycoprotein was not expressed in AbA cells immediately after culture establishment, but it increased gradually, beginning after passage 4 (Fig. S1*B*). AbA cells were negative for A2B5, oligodendrocyte transcription factor 2, and CD68 antigen clone (ED1), which are expressed by glial precursors, oligodendrocyte progenitor cells, and phagocytic microglia, respectively (Table S1).

AbA cells displayed weak and diffuse perinuclear GFAP labeling and intense staining for S100β, in contrast with the filamentous GFAP and low S100β expression in neonatal astrocytes (Fig. 2*A*). GFAP levels in AbA cells were decreased by 60% in comparison with neonatal astrocytes, as estimated by Western blotting (Fig. 2*B*). AbA cells displayed processes and increased GFAP staining when challenged with forskolin (10 μM; Fig. S1*D*), a recognized stimulus that promotes astrocyte differentiation in vitro (24). AbA cells expressed Cx43 in a cytoplasmic and patchy surface distribution (Fig. 2*A*) at levels increased fivefold in comparison with control astrocytes (Fig. 2*B*). The expression of glutamine synthase (Fig. 2*B* and Fig. S1*D*) and vimentin (Fig. S1*D*) in AbA cells was comparable to that found in neonatal SOD^{G93A} astrocytes.

AbA Cell Proliferation. The growth of AbA cells through passage 10 was faster than that of Tg and non-Tg neonatal astrocytes (Fig. 2*C*). The doubling times, calculated during the linear phase of the growth curve, were 30 h for AbA cells, 52 h for non-Tg astrocytes, and 49 h for Tg astrocytes. AbA cell numbers con-

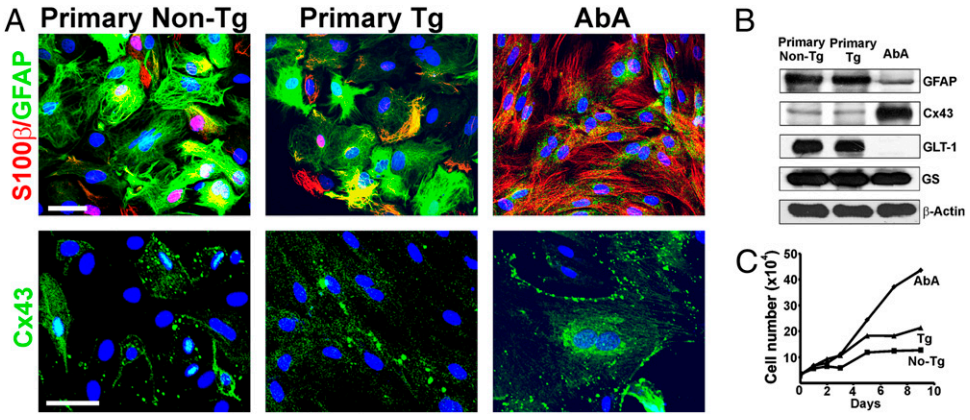

Fig. 2. Expression of astrocyte markers and proliferation in AbA cells. Cultures of AbA cells were compared with non-Tg or SOD1^{G93A} Tg primary neonatal spinal cord astrocytes. (*A*) Confocal imaging of cells immunostained against GFAP (green) and S100β (red) (*Upper*) and Cx43 (green) (*Lower*). In upper panels, yellow staining indicates cells expressing both GFAP and S100β. (Scale bars: 50 μm.) Note AbA cells' low GFAP immunoreactivity but increased S100β and Cx43 immunoreactivity compared with neonatal astrocytes. (*B*) Western blotting analysis of astrocytic markers in AbA cells compared with primary astrocytes. AbA cells' differential protein expression pattern is evidenced by decreased GFAP, absent GLT1, and augmented Cx43. (*C*) Growth of AbA cells (passage 10) and primary neonatal astrocytes was assessed over 9 d. The growth rate of AbA cells was almost twice that of primary astrocytes.

tinued increasing after reaching confluence at day 5 in vitro, suggesting a defect in contact inhibition.

AbA Cells Specifically Induced Motoneuron Death. Because astrocytes carrying the $SOD1^{G93A}$ mutation have been shown specifically to induce motoneuron death (10–12), we assessed the neurotoxic potential of AbA cells by plating embryonic motoneurons (25) on top of confluent AbA or neonatal astrocyte monolayers. The survival rate after 2 d was <10% for motoneurons in cocultures with AbA cells but was 60% for neurons cocultured with Tg cells and was 100% for neurons cultured with non-Tg neonatal astrocytes (Fig. 3*A*). These data suggest that AbA cells exert a marked nonpermissive environment for motoneuron growth and differentiation.

We next investigated whether AbA cell toxicity was mediated by soluble factors secreted to the conditioned media (CM), as previously described for mutant primary astrocytes (12). Increasing dilutions of CM from AbA cells or neonatal astrocyte cultures having similar cell number were added to embryonic motoneuron cultures maintained with glial cell-derived neurotrophic factor (GDNF) as the trophic factor. As expected, CM from neonatal non-Tg astrocytes did not cause motoneuron death (100% survival in Fig. 3*B*). In contrast, after exposure to AbA CM in a range of dilutions from 1:10 to 1:1,000, the number of motoneurons was significantly lower than the number surviving after addition of CM from neonatal Tg astrocytes, which reduced motoneuron survival significantly only at dilutions up to 1:100. Thus, the neurotoxic potential of AbA CM is at least 10-fold greater than that of Tg astrocyte CM (Fig. 3*B*). Notably, the neurotoxicity of AbA CM was specific to motoneurons, because even a 1:10 dilution failed to kill primary cultures of embryonic hippocampal neurons (Fig. 3*C*).

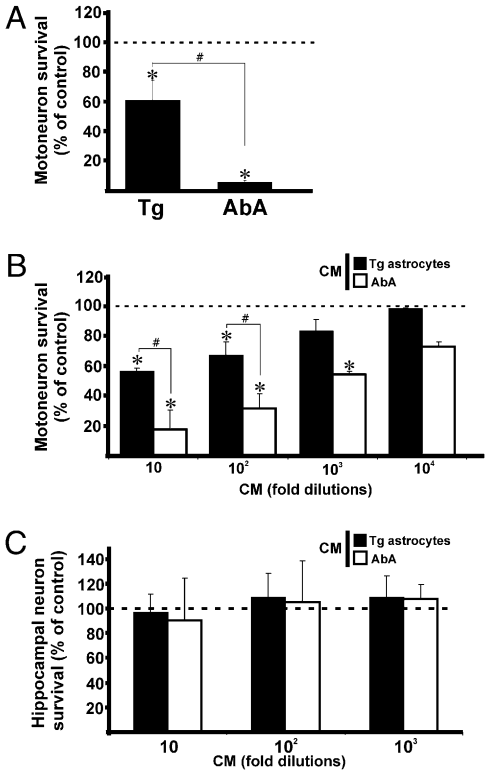

Fig. 3. AbA cells specifically induced motoneuron death. (*A*) Embryonic motoneurons were seeded on top of confluent feeder layers of neonatal astrocytes or AbA cells, and survival was assessed 48 h later. Note that the survival rate of motoneurons maintained on top of AbA cells was <10% but was 100% for non-Tg (100%, dotted line) and 60% for Tg neonatal astrocytes. (*B*) CM from neonatal astrocytes or AbA cells was added to pure motoneurons 24 h after plating. The final fold dilution is indicated in each condition. CM from non-Tg astrocytes did not induce motoneuron death; thus it was taken as a control (100%, dotted line). Note that CM from AbA cells exerted significant motoneuron loss at dilutions up to 1:1,000, whereas Tg astrocyte CM was neurotoxic at dilutions up to 1:100. (*C*) Lack of neurotoxic activity of increasing dilutions of AbA CM in primary cultures of hippocampal neurons. Data are shown as mean ± SD; in *A* and *B*, $^{\#}P < 0.01$ and $^{*}P < 0.05$ with respect to CM from non-Tg astrocytes.

Identification of AbA-Like Cells in the Degenerating Spinal Cord. We used immunohistochemistry to S100β/Cx43 to determine whether AbA-like cells were present in the degenerating spinal cord of $SOD1^{G93A}$ rats. S100β staining was low or moderate in non-Tg and Tg asymptomatic rats. In contrast, S100β increased dramatically during the symptomatic stage of Tg rats, being localized in the nuclei and cytoplasm in a population of hypertrophic astrocytes that expressed GFAP restricted to cell bodies and proximal processes. Typically, these cells were observed in the ventral spinal cord, near damaged motoneurons, and at the boundary between gray and white matter. Cx43 staining also increased dramatically in symptomatic rats and colocalized with S100β hypertrophic astrocytes (Fig. 4*A*). Such astrocytes appeared at the time of disease onset, and their number increased sharply at disease end stage (Fig. 4*B*). In the ventral horn of symptomatic rats, S100β did not colocalize with NG2 oligodendrocyte precursors previously described in ALS mice (Fig. 4*C*; Manders coefficient ≤ 0.02), suggesting that S100β cells constitute a different cell population. Furthermore, $S100\beta^{+}$ or $Cx43^{+}$ AbA-like cells surrounding motoneurons were labeled with the proliferation markers Ki67 or BrdU in animals systemically injected with the nucleotide (Fig. S2*C*). The number of $Ki67^{+}$ proliferating AbA-like cells in the ventral cord region enriched in motoneurons represented 20 ± 5% of cells, compared with 33 ± 8% proliferating NG2 cells and 43 ± 10% microglia (Fig. S2*B*).

Discussion

Neuronal degeneration in ALS begins as a focal process that spreads contiguously through the upper and lower motoneurons (26), suggesting an acquired pathogenic mechanism in which motoneuron pathology and inflammation actively propagate in the CNS. Here we report the isolation of a type of astrocyte with aberrant phenotypic features (AbA cells) and an unprecedented toxicity to motoneurons in vitro from symptomatic $SOD1^{G93A}$ rats. Notably, AbA cells exhibit a distinctive pattern of astrocytic markers with an increased proliferation rate and a lack of replicative senescence. Proliferating AbA-like astrocytes were localized near motoneurons in the spinal cord of symptomatic $SOD1^{G93A}$ rats, suggesting a link between the appearance of pathogenic AbA cells and the rapid progression of paralysis characteristic of the $SOD1^{G93A}$ rat model.

The proliferative potential of AbA cells after isolation from the Tg spinal cord was strong enough to allow their oligoclonal expansion in conditions where age-matched non-Tg rats yielded few cells with limited growth potential. The AbA proliferation rate was almost twice that of the neonatal astrocytes but still was far below that of the C6 astrocyte cell line (27). Thus, AbA cells do not appear to be fully transformed cells, even though they do not follow replicative senescence. Cultured AbA cells are almost undistinguishable morphologically from primary neonatal astrocytes and exhibit a set of distinctive antigenic markers of undifferentiated astrocytes including high S100β and Cx43 expression and low levels of nonfilamentous GFAP. As a prototypic subunit of the calcium-binding S100 proteins, S100β is known to exert paracrine effects in astrocytes that contribute to proliferation, migration, differentiation, and neurotoxicity (28–30). Intracellular S100β can interact with GFAP monomers to prevent their assembly into filaments (31), possibly contributing to the

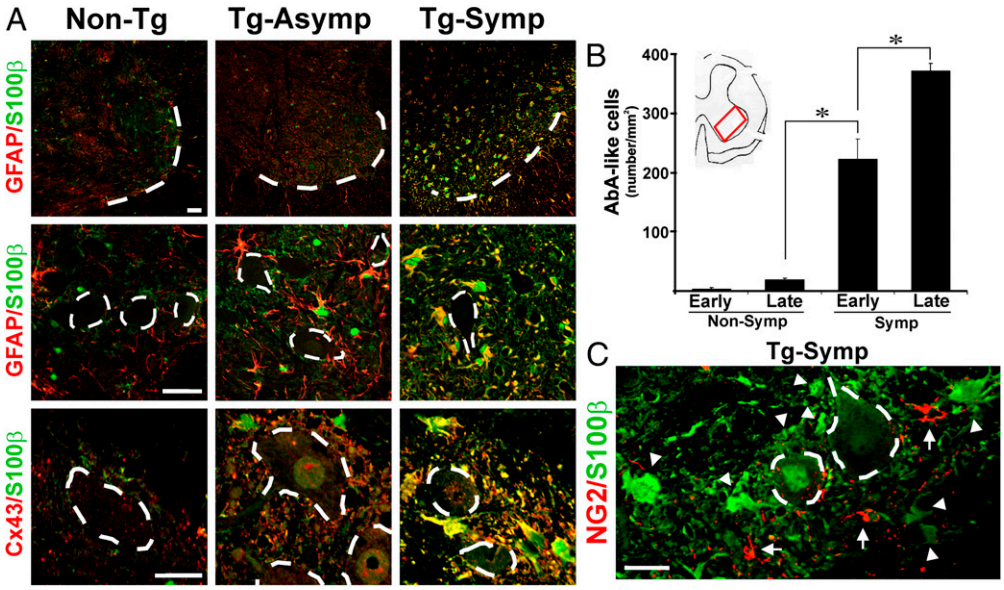

Fig. 4. Identification of AbA-like cells in the degenerating spinal cord. (*A*) Representative microphotographs of GFAP (red), S100β (green), and Cx43 (red) immunostaining in lumbar spinal cord sections from non-Tg, asymptomatic (Tg-Asymp), and symptomatic (Tg-symp) $SOD1^{G93A}$ rats. Dotted lines in the top row indicate the border between gray and white matter in low-magnification representative microphotographs. The perimeter of large motoneurons has been drawn in the middle and bottom rows. Note that S100β was up-regulated in the spinal cord of symptomatic rats and especially was expressed in a population of hypertrophic cells with astrocyte morphology. Most of these cells displayed colocalization of S100β and GFAP (yellow). Cx43 immunoreactivity also was increased in Tg-symp spinal cords, being colocalized with S100β in most hypertrophic AbA-like cells (yellow in bottom row). (Scale bars: 50 μm in GFAP/S100β and 20 μm in Cx43/S100β.) (*B*) Increased number of AbA-like cells in the ventral horn of Tg rats during the progression of the disease. *Inset* shows the ventral horn area analyzed. Data are shown as mean ± SD; $*P < 0.05$. (*C*) Representative confocal immunostaining against NG2 and S100β in the ventral horn of a Tg-symp rat showing that AbA cells are not stained for NG2. (Scale bar: 20 μm.)

diffuse GFAP distribution in AbA cells. Forskolin, which induces astrocytic process growth and differentiation (24), also increased process growth and GFAP expression in AbA cells, further indicating their astrocytic phenotype.

Another distinctive marker of AbA cells is the high expression of the gap junction protein Cx43, which also is found in cultured neonatal astrocytes (32) and is known to modulate their proliferation, migration, and differentiation (33). Cx43 also can form hemichannels opened to the extracellular space in inflammatory astrocytes (34, 35), which can release extracellular ATP (34, 36). Thus, the high levels of Cx43 in AbA cells may explain their potential to trigger glial activation and excitotoxic degeneration of motoneurons (37). The blunted levels of astroglial glutamate transporter GLT1 protein in AbA cells suggest that these cells recapitulate a defect in GLT1 expression previously described in ALS patients (38) and $SOD1^{G93A}$ rats (3). Because GLT1 is expressed in differentiated astrocyte endings to uptake synaptic glutamate, accumulation of AbA cells around motoneurons may promote further excitotoxic damage in vivo.

Furthermore, AbA cells do not appear to be derived from NG2 oligodendrocyte progenitors that recently have been reported to proliferate in the ALS spinal cord after disease onset (16, 17). AbA-like cells in the spinal cord of symptomatic rats are $NG2^-$ and display a morphology and location distinct from that of typical NG2 cells. In addition, AbA cells were $NG2^-$ when first established in culture, and the glycoprotein was expressed only after more than four passages. Characteristic AbA cells coexisted with and were easily distinguishable from $NG2^+$ cells when primary cultures of the spinal cord were prepared in a defined medium that favors oligodendrocyte differentiation.

AbA cells represent a population of glial cells with undifferentiated features. Thus, their generation in the spinal cord could be associated with the overt inflammatory microenvironment that accompanies motoneuron loss, particularly in the $SOD1^{G93A}$ animal models (39). Such a milieu may promote the recruitment and phenotypic transition of glial cells or precursors (40), leading to the generation of AbA cells. A similar proliferative and immature phenotype of astrocytes can be found following defective function in paired box 6 (Pax6) transcription factor (41) and in pathological conditions leading to aberrant specification of glial precursors (42).

Previous studies have shown that astrocytes bearing the $SOD1^{G93A}$ mutation induce apoptosis of motoneurons both in coculture conditions and through soluble factors found in the culture media (10–12, 19, 20), whereas non-Tg astrocytes provided excellent trophic support. However, we show here that AbA cells display an unprecedented toxicity to motoneurons that greatly exceeds that of neonatal Tg astrocytes expressing mutant SOD1. The complete failure of motoneurons to survive when plated on a confluent layer of AbA cells suggests that AbA cells create a nonpermissive microenvironment for motoneuron growth. This effect could be mediated at least in part by the accumulation of specific extracellular matrix proteins that are known to be up-regulated in ALS rats (43). In addition, the AbA cell CM were >10-fold more potent than neonatal Tg astrocytes in inducing motoneuron death. Because AbA cell CM were applied in the presence of GDNF, which supports motoneuron survival, it is unlikely the toxicity is caused solely by decreased trophic activity. Rather, AbA cells appear to be a subclass of astrocytes producing active soluble factors that kill motoneurons. Although the mechanism of AbA neurotoxicity is under active investigation, the possibility exists that AbA cells produce cytokines, excitotoxins, or trophic factors such as nerve-growth factor that may kill motoneurons specifically (8).

Hypertrophic AbA-like cells strongly expressing S100β and Cx43 were identified in the degenerating spinal cord, suggesting a common phenotype with AbA cells isolated in culture. Moreover, $BrdU^+$ and $Ki67^+$ AbA-like cells were found systematically in close contact with degenerating motoneurons, suggesting that they proliferate in the ventral horn as the rat becomes paralytic. Evidence for the occurrence of astrocytic-like $BrdU^+$ nuclei

coexisting with dividing NG2 cells was provided previously by Kang et al. (17) in SOD1^{G93A} mouse spinal cord. Although AbA-like cells represented only about 20% of the proliferating glial cells in the ventral cord gray matter, they were more closely associated with degenerating motoneurons than were NG2^{+} cells and frequently were associated with proliferating microglia. Perineuronal S100β^{+} astrocytes were described previously in ALS patients (44), suggesting that AbA cells also may occur in human terminal disease. S100β can form heterodimers with S100A6, which is up-regulated specifically in reactive astrocytes occurring in ALS patients and mutant SOD1 Tg animals (45). The increased expression of Cx43 in AbA cells is intriguing. Although increased astrocytic expression of Cx43 has not been reported in ALS, neurogenic activation of spinal cord astrocytes is known to induce Cx43 (46) following axotomy and spinal cord injury (47). The fact that blocking Cx43 in spinal cord injury improved recovery (48) anticipates a pathogenic role of Cx43 up-regulation in ALS.

In conclusion, the present study addressed one key question about ALS pathogenesis: the identification and isolation of an astrocyte population with the potential to mediate motoneuron disease. Such astrocytes were abundant in the symptomatic phase of disease and typically localized close to motoneurons, suggesting a link between the emergence of pathogenic AbA cells and the rapidly progressing neurodegeneration characteristic of the SOD1^{G93A} rat model. Thus, AbA cells represent an intriguing cell target for further understanding the pathogenesis of neurodegenerative diseases.

Materials and Methods

Details for materials and methods used in this study are provided in *SI Materials and Methods*.

Animals. Male hemizygous NTac:SD-TgN(SOD1^{G93A})L26H rats (Taconic), originally developed by Howland, et al. (3), were bred locally as outbred Sprague-Dawley background. The onset of symptomatic disease (~160 d) and lifespan (180 d) in our colony were delayed considerably compared with the original report (3).

Establishment of Aba Cell Cultures. AbA cells were obtained from adult spinal cord of symptomatic SOD1^{G93A} rats (175 d) according to the procedures described by Saneto and De Vellis (49) with minor modifications (25). Adult age-matched non-Tg rats were used as controls. Briefly, animals were killed by deeply anesthesia, and spinal cord was dissected on ice. After the meninges were removed carefully, spinal cord was chopped finely and dissociated with 0.25% trypsin in calcium-free buffer for 5 min at 37 °C. Trypsinization was stopped by adding DMEM/10% (vol/vol) FBS in the presence of 50 μg/mL DNaseI and mechanical disaggregation by repeated pipetting. The resulting extract was passed through an 80-μm mesh to eliminate tissue debris and then was spun. The pellet was resuspended in culture medium [DMEM/10% (vol/vol) FBS, Hepes (3.6 g/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL)] and then was plated in a 25-cm^2 tissue culture flask. Because large amounts of fat hindered cell counting, the cells isolated from individual spinal cords were plated in individual culture flasks. Culture medium was removed after 24 h and then was replaced every 48 h. At day 10 in vitro, 3 mL of a 1% agarose solution maintained at 37 °C was layered on top of the cells according to procedures described by Hassell, et al. (50). Although the agarose layer did not attach to the cell layer, it facilitated astrocyte proliferation. At day 20 in vitro, agarose was withdrawn as an entire piece, and AbA cells were passaged every week. To do so, the monolayer was incubated at 37 °C with 0.25% trypsin without calcium. After 5 min, the cells were harvested in DMEM/10% (vol/vol) FBS and spun at 250 × *g* for 10 min. The resultant pellet was resuspended in DMEM/10% (vol/vol) FBS and plated at a density of 1 × 10^6 cells per 25-cm^2 culture flask. After confluence, each flask yielded up to 2 × 10^6 cells, and 1 wk after seeding each bottle was divided in two bottles. AbA cells were cultured in the same medium during the entire procedure.

In some experiments, primary cultures from spinal cord of symptomatic Tg rats were plated on 0.1 mg/mL polylysine-covered plates and maintained in oligodendrocyte-defined medium (Neurobasal medium supplemented with B-27) for 2 d (23).

Primary Cell Cultures. Heterozygous Tg and non-Tg astrocytes were prepared from spinal cords of 1-d-old pups according the methods described by Saneto and De Vellis (49) with minor modifications (25). Motoneuron cultures were prepared from embryonic day 15 wild-type rat spinal cords and purified by immunopanning (25). Motoneurons were seeded on polyornithine-laminin–coated substrate and maintained in Neurobasal medium supplemented with GDNF (1 ng/mL) (Sigma). After 24 h in vitro, motoneurons were treated with dilutions of CM. Survival was assessed after 48 h. For preparation of CM, confluent neonatal astrocyte and AbA monolayers were incubated in complete L15 medium for 24 h. Respective supernatants were centrifuged at 1,000 × *g* for 15 min and were applied immediately to motoneuron cultures. For coculture experiments, motoneurons were plated on confluent AbA or neonatal astrocyte monolayers and were maintained for 48 h in complete L15 medium supplemented as previously described (25). Hippocampal neuronal cultures were obtained from embryonic day18 embryos as described in *SI Materials and Methods*.

Western Blots. Protocols for Western blotting are described in *SI Materials and Methods*.

Assessment of Cell Proliferation. Cell proliferation was assessed as described in *SI Materials and Methods* by counting the number of viable nuclei.

Immunolabeling. Methanol-fixed cultured cells or paraformaldehyde perfusion-fixed free-floating spinal cord sections were processed for immunocytochemistry or immunohistochemistry as described in *SI Materials and Methods* and Table S1. Quantification of AbA-like cells in the ventral horn of spinal cord sections was done by direct counting of hypertrophic cells that surround motoneurons and exhibit cytoplasmic S100β.

Statistical Snalysis. Statistical studies were performed using statistical tools of Origin 8.0. Descriptive statistics were used for each group, and one-way ANOVA, followed by Scheffé post hoc comparison if necessary, was used among groups. All experiments were performed in duplicate or triplicate and were replicated at least three times. All results are presented as mean ± SD. $P < 0.05$ was considered significant.

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Supporting Information

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SI Materials and Methods

Materials. Media (DMEM, Neurobasal, Invitrogen, and L15) and chemicals for cell culture (horse serum, FBS, penicillin/streptomycin, trypsin, and polylysine, laminin) were from Invitrogen. Commercial antibodies were from Dako, Abcam, Sigma, Covance, Cell Signaling Technology, and Invitrogen. All other chemicals of analytical grade were obtained from Sigma. Reagents used in Western blotting were from Pierce.

Animals. All procedures using laboratory animals were performed in accordance with the international guidelines for the use of live animals and were approved by the Institutional Animal Committee. Male hemizygous NTac:SD-TgN(SOD1^{G93A})L26H rats (Taconic), originally developed by Howland et al. (1), were bred locally by crossing with wild-type Sprague-Dawley female rats. Male SOD1^{G93A} progenies were used for further breeding to maintain the line. Rats were housed in a centralized animal facility with a 12-h light-dark cycle with ad libitum access to food and water. Symptomatic disease onset was determined by periodic clinical examination for abnormal gait, typically expressed as subtle limping or dragging of one hind limb. Rats were killed when they reached the end stage of the disease. Both the onset of symptomatic disease (160–170 d) and lifespan (180–195 d) in our colony were delayed considerably compared with earlier reports (1).

Primary Cultures of Neonatal Astrocytes. Heterozygous transgenic (Tg) and non-Tg astrocytes were prepared from spinal cords of 1-d-old pups according to the procedures of Saneto and De Vellis (2) with minor modifications (3). Astrocytes were plated at a density of 2×10^4 cells/cm^2 in 35-mm Petri dishes or 24-well plates and were maintained in DMEM, 10% FBS, Hepes (3.6 g/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL). Astrocyte monolayers were >98% pure as determined by GFAP immunoreactivity and were devoid of OX42$^+$ microglial cells.

Hippocampal Neurons. Hippocampi from embryonic day 18 (E18) embryos were dissected, stored in Neurobasal medium containing 2% B-27 and 1 mM glutamine, mechanically dissociated, and passed through an 80-μm mesh. The isolated cells (300,000 cells/mL) were seeded onto plates covered with 0.1 mg/mL poly-D–lysine, and half of the medium was replaced 3 d after plating according to procedures given in ref. 4 with minor modifications. At day 5 in vitro, cells were treated with conditioned medium collected from astrocytes with aberrant phenotype (AbA cells) and from primary non-Tg and Tg astrocytes. After 24 h, viable cells were counted using a phase-contrast microscope.

Motoneurons. Motoneuron cultures were prepared from day E15 wild-type rat spinal cord by a combination of metrizamide-gradient centrifugation and immunopanning with the monoclonal antibody IgG192 against p75^{NTR} (5, 6). Purified motoneurons were seeded on polyornithine-laminin–coated substrate or onto astrocyte monolayers, depending on the experimental protocols used.

Assessment of Motoneuron Survival. Motoneurons were plated at a density of 350 cells/cm^2 on Lab-Tek or Nunclon four-well multidishes precoated with polyornithine-laminin (5). Cultures were maintained in Neurobasal medium supplemented with 2% horse serum, 25 mM L-glutamate, 25 μM 2-mercaptoethanol, 0.5 mM L-glutamine, and 2% B-27 supplement (Gibco-Invitrogen). Glial cell line-derived neurotrophic factor (GDNF) (1 ng/mL; Sigma) was added to the culture medium to maintain the trophic support of motoneurons. Twenty-four hours after plating, motoneurons were treated with increasing dilutions of conditioned medium from AbA cells, non-Tg astrocytes, or Tg astrocytes. Survival was assessed after 48 h by direct counting of cells displaying intact neurites longer than the diameter of four cell bodies.

For coculture experiments, motoneurons were plated on rat astrocyte (Tg and non-Tg) or AbA cell monolayers at a density of 300 cells/cm^2 and maintained for 48 h in complete L15 medium supplemented as previously described (3). Motoneurons were immunolabeled with anti-p75^{NTR} antibodies followed by HRP-conjugated secondary antibody (6). Counts were performed over an area of 0.90 cm^2 in 24-well plates (3). The mean density of motor neurons in control cocultures was 84 ± 3 cells/cm^2.

Analysis of Cell Proliferation. AbA cells or primary neonatal astrocytes were plated in 35-mm dishes at 1.2×10^4 cells/cm^2. Six hours after plating, cells were harvested and counted to obtain the cell number at day zero. Counting continued for 9 d as indicated in Fig. 2*C*. Cells were lysed, and intact nuclei were counted with a hemacytometer as described previously (7, 8). Only healthy, bright-phase nuclei with clearly defined limiting membranes were considered for quantification. Cell counts were performed in duplicate.

Western Blot Analysis. For cellular protein extraction, astrocytes and AbA confluent monolayers were washed with PBS, and whole-cell extracts were prepared in lysis buffer [50 mM Hepes (pH 7.5), 50 mM NaCl, 1% Triton X-100, and complete protease inhibitor mixture] (Sigma) and then were sonicated three times for 3 s. Protein concentration was measured with a Bicinchoninic Acid (BCA) kit (Sigma). Then protein extracts were placed in loading buffer containing 15% SDS, 0.3 M Tris (pH 6.8), 25% glycerol, 1.5 M β-mercaptoethanol, and 0.01% bromophenol blue. Protein samples (40 μg) were resolved on 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Amersham). Membranes were blocked for 1 h in Tris-buffered saline (TBS), 0.1% Tween-20, and 5% nonfat dry milk, followed by overnight incubation with the corresponding primary antibody diluted in the same buffer. After washing with 0.1% Tween in TBS, the membrane was incubated with peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h and washed and developed using the ECL chemiluminescent detection system (Amersham) (9). Primary antibodies were used at dilutions recommended by the manufacturers. For the time-course analysis of NG2 expression, AbA protein extracts were obtained from passages 1–15.

Immunocytochemistry. Cultured cells were fixed with absolute methanol at −20 °C for 5 min on ice and then were washed three times with 10 mM PBS (pH 7.4). Nonspecific binding was blocked by incubating fixed cells with 5% BSA in PBS for 1 h at room temperature. Corresponding primary antibodies (Table S1) were diluted in blocking solution and incubated overnight at 4 °C in a wet, closed chamber. After several washes and 2 h of incubation with corresponding secondary antibodies conjugated to fluorescent probes (1:1,000; Invitrogen), cells were mounted and imaged in an Olympus FV300 laser scanning confocal microscope. Antigen retrieval to recognize BrdU in cells was performed according to the manufacturer's instructions.

Immunohistochemistry. Animals were deeply anesthetized, and transcardial perfusion was performed with 0.9% saline and 10% paraformaldehyde in 0.1 M PBS (pH 7.2–7.4) at a constant flow of 1 mL/min. Fixed spinal cord was removed, postfixed by immersion for 24 h, and then transverse sectioned serially (30–50 μm) on a vibrating microtome. Serial sections were collected in 100 mM PBS for immunohistochemistry. Free-floating sections were permeabilized for 15 min at room temperature with 0.1% Triton X-100 in PBS, passed through washing buffered solutions, blocked with 5% BSA:PBS for 30 min at room temperature, and incubated overnight at 4 °C in a solution of 0.1% Triton X-100 and PBS containing the primary antibodies (Table S1). After washing, sections were incubated in 1:1,000-diluted secondary antibodies conjugated to Alexa Fluor 488 and/or Alexa Fluor 546 (Invitrogen). Antibodies were detected by confocal microscopy using a confocal Olympus FV300 microscope. The same protocol was followed for the recognition of all antigens studied except Ki67, for which antigen retrieval was performed according to the manufacturer's instructions (Table S1). To label mitotic cells in vivo, symptomatic $SOD1^{G93A}$ rats received 100 mg/kg bodyweight of BrdU (Sigma) i.p. for a period of 2 d (10). Three days after the last BrdU injection, animals were processed for immunohistochemistry as described above.

Quantitative Analysis of Proliferating Glial Cells in the Degenerating Spinal Cord. The number of proliferating cells labeled with Ki67 and also stained for the glia markers S100β, NG2, or ionized calcium-binding adaptor molecule 1 (IbA1) was assessed by counting the respective double-positive cells in the gray matter of the lumbar cord of symptomatic $SOD1^{G93A}$ Tg mice. Quantification was performed only in the ventral horn, comparing the cell numbers in Rexed laminae VII and IX, which display low and high density of large motoneurons, respectively. The analysis was performed manually in five sections per animal ($n = 2$) using the cell counter tool of the Image J software. Values were expressed as a percentage (± SD) of the total number of $Ki67^{+}$ nuclei.

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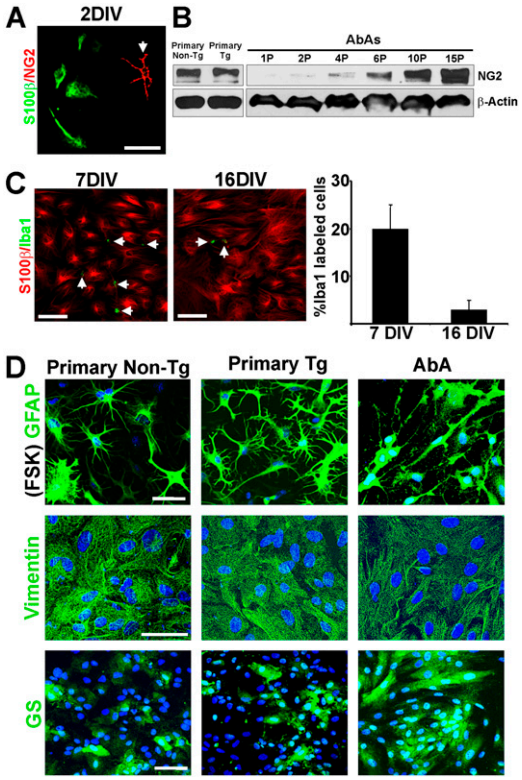

Fig. S1. Establishment and characterization of AbA cells. (*A*) Confocal immunofluorescence of a representative primary spinal cord culture (day 2 in vitro, DIV2) from a symptomatic Tg rat showing the coexistence of $S100^{+}$ astrocytes and $NG2^{+}$ oligodendrocyte-like cells (arrow). (Scale bar: 50 μm.) Cultures were maintained in Neurobasal medium supplemented with B-27 to promote oligodendrocyte differentiation. (*B*) Western blotting analysis of NG2 protein in 40 μg of cell homogenates. Note that NG2 was expressed in neonatal astrocytes but was expressed gradually in AbA cells after passage 4 (4P). (*C*) Representative views (*Left*) and quantitative analysis (*Right*) of $Iba1^{+}$ cells (*Right*, arrows) in primary spinal cord cultures from a symptomatic $SOD1^{G93A}$ rat at days 7 and 16 in vitro showing the progressive decrease in the number of microglia. (Scale bar: 50 μm.) (*D*) Cultures of AbA cells at passage 12 were compared with non-Tg or Tg neonatal astrocytes. Confocal imaging of cells immunostained against GFAP, vimentin, and glutamine synthase (GS). Note that 6-h pretreatment with 10 μM forskolin (FSK) increased GFAP staining and process growth in both AbA and primary astrocytes as compared with controls shown in Fig. 2*A*. (Scale bars: 50 μm.)

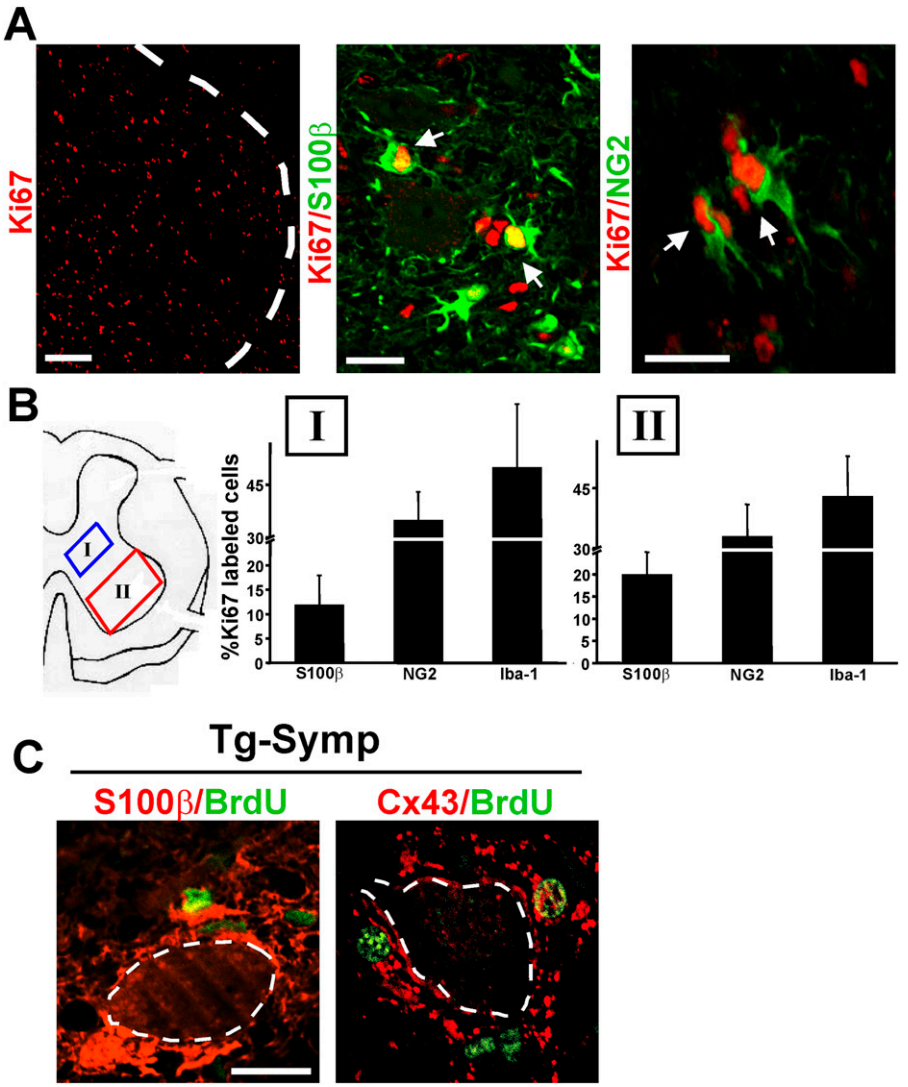

Fig. S2. Proliferating AbA-like cells in the degenerating spinal cord. (*A*) (*Left*) Ki67 immunostaining showing the distribution of proliferating cells in the lumbar ventral horn of the spinal cord of symptomatic SOD1^{G93A} rats. (*Center*) AbA-like Ki67^{+}/S100β^{+} cells displaying large nuclei mainly were found close to motoneurons. (*Right*) In comparison, Ki67^{+}/NG2^{+} cells formed clusters of many small cells with scarce and thin processes. (Scale bars: 100 μm for Ki67; 50 μm for Ki67/S100β and Ki67/NG2.) (*B*) Quantitative analysis (*Right*) and diagram showing distribution (*Left*) of proliferating glial cell subpopulations in the lateral part of the ventral horn with low density of motoneurons (Box I in VII Rexed laminae) compared with most ventral region enriched in large motoneurons (Box II in IX Rexed laminae). Note that proliferating AbA-like cells were associated mostly with degenerating motoneurons. (*C*) Double immunostaining against the proliferation marker BrdU and AbA cells [S100β and connexin 43 (Cx43)] in the spinal cord sections. Note the presence of BrdU/S100β/Cx43-positive cells surrounding motoneurons (indicated with dashed lines) in symptomatic SOD1^{G93A} rats, indicating that AbA-like cells proliferate in vivo. (Scale bars: 20 μm.)

Table S1. Comparative antigenic profile of AbA cells by immunocytochemistry

Antigen	Primary neonatal astrocytes (nontransgenic)	Primary neonatal astrocytes (transgenic)	AbA cells	Primary antibodies and dilutions
GFAP	XXX (filamentous pattern)	XX (filamentous pattern)	X (diffuse staining)	Sigma G9269 1:400, 1:4,000 (WB)
Cx43	X	X	XXX	Polyclonal (1) 1:500, 1:1,000 (WB)
S100β	X	X	XXX	Sigma S2532 1:400
Vimentin	X	X	XX	Dako M0725 1:250
GLT1	XX	XX	—	Cell Signaling 3838 1:1,000 (WB)
Glutamine synthase	XX	XX	XX	Abcam ab49873 1:500, 1:5,000(WB)
NG2	X	X	—	Millipore MAB5384 1:250
NG2	X	X	—	Millipore AB5320 1:250, 1:1,000 (WB)
Olig2	—	—	—	Abcam ab33427 1:400
A2B5	—	—	—	Abcam ab53521 1:400
ED1	—	—	—	Abcam ab31630 1:500
Iba1	—	—	—	Abcam ab5076 1:400
BrdU				Dako M0744 1:800
Ki67				Abcam ab16667 1:500

Neonatal astrocytes or AbA cell cultures were processed for immunocytochemistry as described in *SI Materials and Methods* using the indicated primary antibody dilutions. The relative intensity of the immunoreactivity is expressed semiquantitatively: —, no apparent immunoreactivity; X, low intensity; XXX, high intensity. The qualitative differences in GFAP staining are indicated in parentheses. BrdU and Ki67 antibodies were used only in vivo, and thus quantitation is not indicated in cell cultures. Cx43, connexin 43; Glt1, glutamate transporter 1; Olig2, oligodendrocyte transcription factor 2; WB, dilutions used for Western blotting experiments.

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Objetivo 4. Estudio del origen de las células AbA.

Los resultados obtenidos en el contexto del objetivo 4 se encuentran en el artículo 2.

Trias E, Díaz-Amarilla P, Olivera-Bravo S, Isasi E, Drechsel DA, Lopez N, Bradford CS, Ireton KE, Beckman JS, Barbeito L. *Phenotypic transition of microglia into astrocyte-like cells associated with disease onset in a model of inherited ALS*. Front Cell Neurosci. 2013 Dec 24;7:274

Diversos trabajos habían estudiado la población celular proliferante en la médula espinal de los modelos animales de ELA durante la fase sintomática. Los resultados reportados coinciden en que las dos poblaciones celulares con mayor tasa de proliferación son las células positivas para NG2 y la microglia. Mediante estudios que utilizan la incorporación de bromo deoxiuridina (BrdU) un análogo de base nucleotídica que se incorpora en el ADN y el co-marcado con marcadores específicos de estirpe celular, se demostró que la tasa de proliferación astrocitaria, GFAP positivos, era muy inferior a la de las dos poblaciones celulares mencionadas previamente.

Dado que las células AbA mostraban una tasa proliferativa elevada y sostenida en comparación con los cultivos de astrocitos neonatales, y teniendo en cuenta que la tasa proliferativa de los astrocitos GFAP positivos en la médula espinal era poco significativa, nos preguntamos cual sería el origen de las células AbA. Como se menciona en el artículo correspondiente a este objetivo, la observación del cultivo en las primeras 24 horas nos llevó a sospechar que el fenotipo aislado inicialmente podría corresponder a una célula proveniente de la estirpe mieloide, microglia/monocito/macrófago que después de algunos días en cultivo adquiría un comportamiento similar al de una monocapa de astrocitos.

Bajo la hipótesis, de que las células AbA podrían resultar de un proceso de transdiferenciación a partir de células de estirpe mieloide, microglia residente o monocitos/macrófagos circulantes realizamos un análisis mediante citometría de

flujo del cultivo obtenido en las primeras 24-48 horas utilizando anticuerpos para CD11b, una proteína de membrana de la estirpe mieloide. Las células CD11b+ fueron separadas mediante *cell sorting* y fueron cultivadas nuevamente para seguir su comportamiento. En análisis del perfil de expresión de los mismos marcadores utilizados en el objetivo 1, permitió demostrar que las células CD11b+ aisladas sufrían un proceso de transdiferenciación que daba lugar a la aparición de un fenotipo celular con características similares a las células AbA. El comportamiento de las células AbA obtenidas a partir de la población CD11b+ mostró el mismo comportamiento en cultivo que las células AbA obtenidas directamente.

La pregunta que nos planteamos seguidamente, fue si este proceso de transdiferenciación que daba origen a las células AbA tenía lugar y podía ser observado *in vivo.* Mediante el análisis por inmunohistoquímica de cortes de médula espinal de animales en fase sintomática se pudo determinar la presencia de células que expresaban simultaneamente el marcador astrocitario GFAP y el marcador microglial Iba-1 (Ionized calcium binding adapter molecule 1) sugiriendo la existencia de un fenotipo celular con características hibridas. Estas células además resultaron positivas para el marcador de proliferación Ki-67 y se localizaban muy cerca de los cuerpos de las motoneuronas.

Como se discute en el artículo a continuación el fenómeno de transdiferenciación a partir de microglia, ya ha sido reportado, de hecho se ha demostrado que la microglia podrían dar origen a nuevas neuronas. El mismo fenómeno se ha reportado para monocitos y macrófagos periféricos, por lo que aún resta estudiar si el proceso de transdiferenciación que reportamos en este trabajo se origina a partir de la microglia residente en la médula espinal o si las células de estirpe mieloide circulantes que ingresan al SNC en condiciones patológicas también están implicadas en este proceso

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Phenotypic transition of microglia into astrocyte-like cells associated with disease onset in a model of inherited ALS

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Microglia and reactive astrocytes accumulate in the spinal cord of rats expressing the Amyotrophic lateral sclerosis (ALS)-linked SOD1^{G93A} mutation. We previously reported that the rapid progression of paralysis in ALS rats is associated with the appearance of proliferative astrocyte-like cells that surround motor neurons. These cells, designated as Aberrant Astrocytes (AbA cells) because of their atypical astrocytic phenotype, exhibit high toxicity to motor neurons. However, the cellular origin of AbA cells remains unknown. Because AbA cells are labeled with the proliferation marker Ki67, we analyzed the phenotypic makers of proliferating glial cells that surround motor neurons by immunohistochemistry. The number of Ki67^{+}AbA cells sharply increased in symptomatic rats, displaying large cell bodies with processes embracing motor neurons. Most were co-labeled with astrocytic marker GFAP concurrently with the microglial markers Iba1 and CD163. Cultures of spinal cord prepared from symptomatic SOD1^{G93A} rats yielded large numbers of microglia expressing Iba1, CD11b, and CD68. Cells sorted for CD11b expression by flow cytometry transformed into AbA cells within two weeks. During these two weeks, the expression of microglial markers largely disappeared, while GFAP and S100β expression increased. The phenotypic transition to AbA cells was stimulated by forskolin. These findings provide evidence for a subpopulation of proliferating microglial cells in SOD1^{G93A} rats that undergo a phenotypic transition into AbA cells after onset of paralysis that may promote the fulminant disease progression. These cells could be a therapeutic target for slowing paralysis progression in ALS.

Keywords: microglia, astrocytes, AbA cells, ALS, phenotypic transformation, neurodegeneration

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) may be considered as a paradigm of neurodegeneration involving the progressive death of upper and lower motor neurons (Cleveland and Rothstein, 2001; Boillee et al., 2006a). A consistent neuropathological feature of ALS is the extensive inflammation around motor neurons and axonal degeneration, evidenced by the accumulation of reactive astrocytes, activated microglia and lymphocytes (Engelhardt et al., 1993; Barbeito et al., 2004; Ilieva et al., 2009; Graber et al., 2010). Neuroinflammation is evident in rodent models of inherited ALS overexpressing mutant Cu/Zn superoxide dismutase (SOD1) and in ALS human patients (Gurney et al., 1994; Bruijn et al., 1997; Howland et al., 2002; McGeer and McGeer, 2002; Appel, 2009). Several studies suggest that glial cells, including astrocytes and microglia, play a pathogenic role in ALS through promoting motor neuron death and spreading paralysis after disease onset (Hall et al., 1998; Barbeito et al., 2004; Sargsyan et al., 2005; Boillee et al., 2006b; Papadeas et al., 2011). These observations suggest that therapeutics targeting the inflammatory response of glial cells could slow ALS progression.

We have recently reported the isolation of astrocytes-like glial cells with an aberrant phenotype (AbA cells) from primary spinal cord cultures of symptomatic transgenic rats expressing the SOD1^{G93A} mutation (Diaz-Amarilla et al., 2011). Isolation was based on AbA cell's marked proliferative capacity and lack of replicative senescence. These cells secrete soluble factors that induce motor neuron death with a higher potency than neonatal transgenic astrocytes. Aberrant astrocytes only appear after disease onset in SOD1^{G93A} rats and are localized adjacent to motor neurons, suggesting a link between generation of AbA cells and the progression of paralysis. However, the origin of AbA cells remains unknown. Because AbA cells actively proliferate in the degenerating spinal cord, we hypothesized they could originate from glial progenitors with a high proliferative potential. Previous studies have identified phagocytic microglia as well as NG2^{+} glial progenitors that proliferate during the active phase of motor neuron degeneration in ALS mice and rats (Magnus et al., 2008; Kang et al., 2010; Sanagi et al., 2010). Because we have previously shown that AbA cells do not express NG2 (Diaz-Amarilla et al., 2011), we examined whether AbA cells might be derived from

microglia proliferating adjacent to motoneurons. In this study, we have characterized both *in vivo* and *ex vivo* the phenotype of proliferating glial cells in symptomatic ALS rats and found evidence that neurotoxic AbA cells result from a phenotypic transition from activated microglial cells.

MATERIALS AND METHODS

ANIMALS

All procedures using laboratory animals were performed in accordance with the international guidelines for the use of live animals and were approved by the Institutional Animal Committee. Male hemizygous NTac:SD-TgN(SOD1^{G93A})L26H rats (Taconic), originally developed by Howland et al. (2002), were bred locally by crossing with wild-type Sprague–Dawley female rats. Male SOD1^{G93A} progenies were used for further breeding to maintain the line. Rats were housed in a centralized animal facility with a 12-h light-dark cycle with ad libitum access to food and water. Symptomatic disease onset was determined by periodic clinical examination for abnormal gait, typically expressed as subtle limping or dragging of one hind limb. Rats were killed when they reached the end stage of the disease. Both the onset of symptomatic disease (160–170 d) and lifespan (180–195 d) in our colony were delayed considerably compared with earlier reports (Howland et al., 2002). This study was carried out in strict accordance with the IIBCE Bioethics Committee's requirements and under the current ethical regulations of the Uruguayan Law N° 18.611 for animal experimentation that follows the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). All surgery was performed under 90% ketamine – 10% xylazine anesthesia, and all efforts were made to minimize suffering, discomfort or stress.

CELL CULTURE FROM END-STAGE SYMPTOMATIC SOD1^{G93A} RATS

Microglia cells were obtained from adult spinal cord of symptomatic SOD1^{G93A} rats (175 d) according to the procedures described by Diaz-Amarilla et al. (2011) with minor modifications. Adult age-matched non-Tg rats were used as controls. Briefly, animals were killed by deeply anesthesia, and spinal cord was dissected on ice. After the meninges were removed carefully, spinal cord was chopped finely and dissociated with 0.25% trypsin in calcium-free buffer for 5 min at 37°C. Trypsin treatment was stopped by adding DMEM/10% (vol/vol) FBS in the presence of 50 μg/mL DNaseI and mechanical disaggregation by repeated pipetting. The resulting extract was passed through an 80-μm mesh to eliminate tissue debris and then was spun. The pellet was resuspended in culture medium [DMEM/10% (vol/vol) FBS, Hepes (3.6 g/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL)] and then was plated in a 25-cm^2 tissue culture flask. Because large amounts of fat hindered cell counting, the cells isolated from individual spinal cords were plated in individual culture flasks. Culture medium was removed after 24 h and then was replaced every 48 h.

LEUCINE-METHYL ESTER TREATMENT

Leucine-Methyl Ester (Leu-OMe, Sigma) was prepared in DMEM, pH adjusted to 7.4. Cultures from transgenic symptomatic rats were treated 3 days after plated with 25 mM of Leu-OMe during 1 h. Then, the cells were washed three times with PBS and fixed with cold methanol during 5 min (n = 3).

IMMUNOCYTOCHEMICAL STAINING OF CULTURED CELLS

Cultured cells were fixed with absolute methanol at -20°C for 5 min on ice and then were washed three times with 10 mM PBS (pH 7.4). Non-specific binding was blocked by incubating fixed cells with 5% BSA in PBS for 1 h at room temperature. Corresponding primary antibodies were diluted in blocking solution and incubated overnight at 4°C in a wet closed chamber. The primary antibodies for microglia recognition were rabbit anti-Iba1 (1:200, Abcam), rabbit anti-CD11b (1:200, Abcam), and mouse anti-CD68 (1:300, Abcam). The antibodies used for astrocyte recognition were mouse anti-GFAP (1:500, Sigma), rabbit anti-GFAP (1:500, Sigma), mouse anti-S100β(1:400, Sigma). After washing, sections were incubated in a 1,000-fold dilution of secondary antibodies conjugated to Alexa Fluor 488 and/or Alexa Fluor 546 (1:1000, Invitrogen). Antibodies were detected by confocal microscopy using a confocal Olympus FV300 microscope.

ANALYSIS OF MICROGLIAL MARKERS EXPRESSION

After isolation of the symptomatic spinal cord, cells were plated in 35-mm dishes at 1.2 × 10^4 cells/cm^2. 7 days after plating, cells were fixed and stained with microglia specific markers as described above. The analysis was performed manually in using the cell counter tool of the Image J software. Values were expressed as a percentage (± SD) of the total number of DAPI$^+$ nuclei. Only healthy nuclei with clearly defined limiting membranes were counted. Cell counts were performed in duplicate.

FORSKOLIN TREATMENT

After 20 days *in vitro*, 35 mm dishes were treated with 10 μM of forskolin (FSK; Sigma) during 3 h. Then, the cells were fixed using cold methanol and stained as described above.

FLOW CYTOMETRIC ISOLATION

After 7 days *in vitro* the cells were incubated at 37°C with 0.25% trypsin without calcium. After 5 min, the cells were harvested in DMEM/10% (vol/vol) FBS and spun at 250 × *g* for 10 min. The resultant pellet was washed three times in PBS at 37°C. After that, the cells were re-suspended in blocking solution (PBS; 5% FBS, and 1% BSA). The cells were labeled at 4°C for 15 min with mouse anti-CD11b-FITC (1:100, Abcam) and sorted using the MoFlo^{TM} XDP–Beckman Coulter. After sorting, the cells were re-plated in a 25 cm^2 bottle with DMEM/10% FBS.

IMMUNOHISTOCHEMICAL STAINING OF RAT SPINAL CORDS

Animals were deeply anesthetized and transcardial perfusion was performed with 0.9% saline and 4% paraformaldehyde in 0.1 M PBS (pH 7.2–7.4) at a constant flow of 1 mL/min. Fixed spinal cord was removed, post-fixed by immersion for 24 h, and then transverse sectioned serially (30–50 μm) on a vibrating microtome. Serial sections were collected in 100 mM PBS for immunohistochemistry. After citrate antigen retrieval, free-floating sections were permeabilized for 15 min at room temperature with 0.1% Triton X-100 in PBS, passed through washing buffered solutions, blocked with 5% BSA:PBS for 1 h at room temperature, and incubated overnight at 4°C in a solution of 0.1% Triton X-100

and PBS containing the primary antibodies, mouse anti-Iba1 (1:200, Abcam), mouse anti CD163 (1:100, Serotec) for microglia recognition, and rabbit anti-GFAP (1:500, Sigma) for astrocyte recognition. A rabbit anti-Ki67 (1:400, Abcam) was used as a proliferation marker. The expression of nitrotyrosine was recognized with a mouse anti-NO_2-Tyr (1:300, Millipore) antibody. The immunoreactivity was completely blocked by pre-incubation of the primary antibody with free nitrotyrosine (10 mM). No antigen retrieval was needed to detect nitrotyrosine. After washing, sections were incubated in 1:1,000-diluted secondary antibodies conjugated to Alexa Fluor 488 and/or Alexa Fluor 546 (Invitrogen). Antibodies were detected by confocal microscopy using a confocal Olympus FV300 microscope.

QUANTITATIVE ANALYSIS OF ABA CELLS IN THE DEGENERATING SPINAL CORD

The number of proliferating cells labeled with Ki67 and also stained for the astrocytic marker GFAP or microglial marker Iba1 was assessed by counting the respective double-positive cells in the gray matter of the lumbar cord of symptomatic or asymptomatic $SOD1^{G93A}$ rats. Quantification was performed only in the ventral horn, comparing the cell numbers in Rexed laminae VII and IX, which display low and high density of large motor neurons, respectively. Double-positive cells were counted in a perimeter of 100 μm, surrounding motor neurons. The analysis was performed manually in 10 histological sections per animal (two different rats for each condition) using the cell counter tool of the Image J software. Values were expressed as a ratio of double-positive cells per motor neuron. The number of double-positive cells labeled with Iba1 or CD163 and GFAP assessed by counting the respective double-positive cells in the gray matter of the lumbar cord of asymptomatic and symptomatic $SOD1^{G93A}$ rats. Quantification was performed only in the ventral horn, comparing the cell numbers in Rexed laminae VII and IX, which display low and high density of large motor neurons, respectively. Values were expressed as the number of double-positive cells per mm^2.

RESULTS

GLIAL PROLIFERATION ADJACENT TO DEGENERATING MOTOR NEURONS

The number of proliferating glia as identified by $Ki67^+$ nuclei sharply increased in the ventral horn of $SOD1^{G93A}$ symptomatic rats and accumulated near surviving motor neurons as well as at sites of apparent motor neuron loss (**Figure 1**). This population of $Ki67^+$ cells had large cell bodies (30–50 μm) with processes embracing motor neurons and expressed GFAP and Iba1 (**Figure 1A**, upper panels). Both Ki67/GFAP and Ki67/Iba1-positive cells displayed morphological features of AbA cells in culture (Diaz-Amarilla et al., 2011) and could be easily differentiated from astrocytes and microglia from non-transgenic or Tg asymptomatic rat's spinal cord. These large GFAP/Ki67 or Iba1/Ki67 cells were rarely observed in asymptomatic or non-transgenic rats (**Figure 1A**, lower panels). Due to the antigen retrieval procedure, motor neuron cell bodies were non-specifically labeled with Ki67 in all experimental conditions. The ratio of GFAP/Ki67 cells and Iba1/Ki67 cells to motor neurons in symptomatic rats was 2.7 and 2.9 respectively, whereas the ratio was < 0.3 in asymptomatic animals for both markers (**Figure 1B**).

CO-EXPRESSION OF MICROGLIAL AND ASTROCYTIC MARKERS IN ABA CELLS

While astrocytes and microglial cells constituted two separate cell populations in asymptomatic rats, being typically detected by GFAP and Iba1 respectively, most AbA cells surrounding the motor neurons in symptomatic rats were surprisingly co-labeled with both markers as well as CD163 (**Figure 2A**). 70% of the GFAP-positive AbA cells in the ventral horn exhibited microglial markers, as compared as <1% in asymptomatic rats. The number of cells labeled with Iba1/GFAP or CD163/GFAP was similar (∼100 cells per mm^2) in symptomatic rats, suggesting the same cells expressed both microglial markers (**Figure 2B**). Detection of the microglial markers required the use of strong antigen retrieval methods, which made motor neuron to artifactually stained with CD163.

Both peri-neuronal AbA cells as well as motor neurons were strongly stained for nitrotyrosine in symptomatic rats, consistent with the production of peroxynitrite (**Figure 2C**). This immunoreactivity was completely blocked by preincubation of the primary antibody with free nitrotyrosine (data not shown).

CHARACTERIZATION OF FIRST STAGES OF ABA CELLS *IN VITRO*

Because AbA cells can be cultured from symptomatic rats (Diaz-Amarilla et al., 2011), we determined the time course of microglial and astrocytic marker expression. Soon after primary cultures of spinal cord were established (DIV2–DIV7), most of the cells displayed the morphology of phagocytic microglia and were fluorescently labeled with the microglia markers CD11b, CD68, and Iba1 (**Figure 3A**). The detailed morphology of cultured cells closely corresponded to the typical features of microglial cells previously reported (Kreutzberg, 1996; **Figure 3B**). No immunoreactivity for GFAP or S100β was detected in cultures since the establishment of cultures and until 10–12 DIV (data not shown). Microglial cells were also analyzed by flow cytometry (FACS) using FITC-labeled CD11b antibodies. FACS analysis showed that > 99% of cells of the primary spinal cord culture of symptomatic rats belonged to the microglia lineage. The purity of this culture was also determined by counting $CD68^+$ and $Iba1^+$ cells and found to be > 98% (**Figure 3C**).

PHENOTYPIC TRANSFORMATION OF MICROGLIA INTO ABA CELLS *IN VITRO*

To confirm that AbA cells were derived from microglia cell progenitors, $CD11b^+$ expressing cells were FACS-sorted and re-established in culture until DIV15. Sorted cells displayed the typical microglia morphology and phenotypic marker until DIV10. This population of cells progressively transitioned into astrocyte-like cells forming monolayers between DIV10 and DIV15, while losing the morphology and phenotypic markers of microglia (**Figure 4A**). At this time, transition zones in the border separating the microglial and the astrocyte-like cells expressed both microglial (Iba1 and CD11b) and astrocytic (GFAP) markers (**Figure 4B**). These transition zones were transient because most cells expressed S100β without microglial markers by DIV15. The treatment of

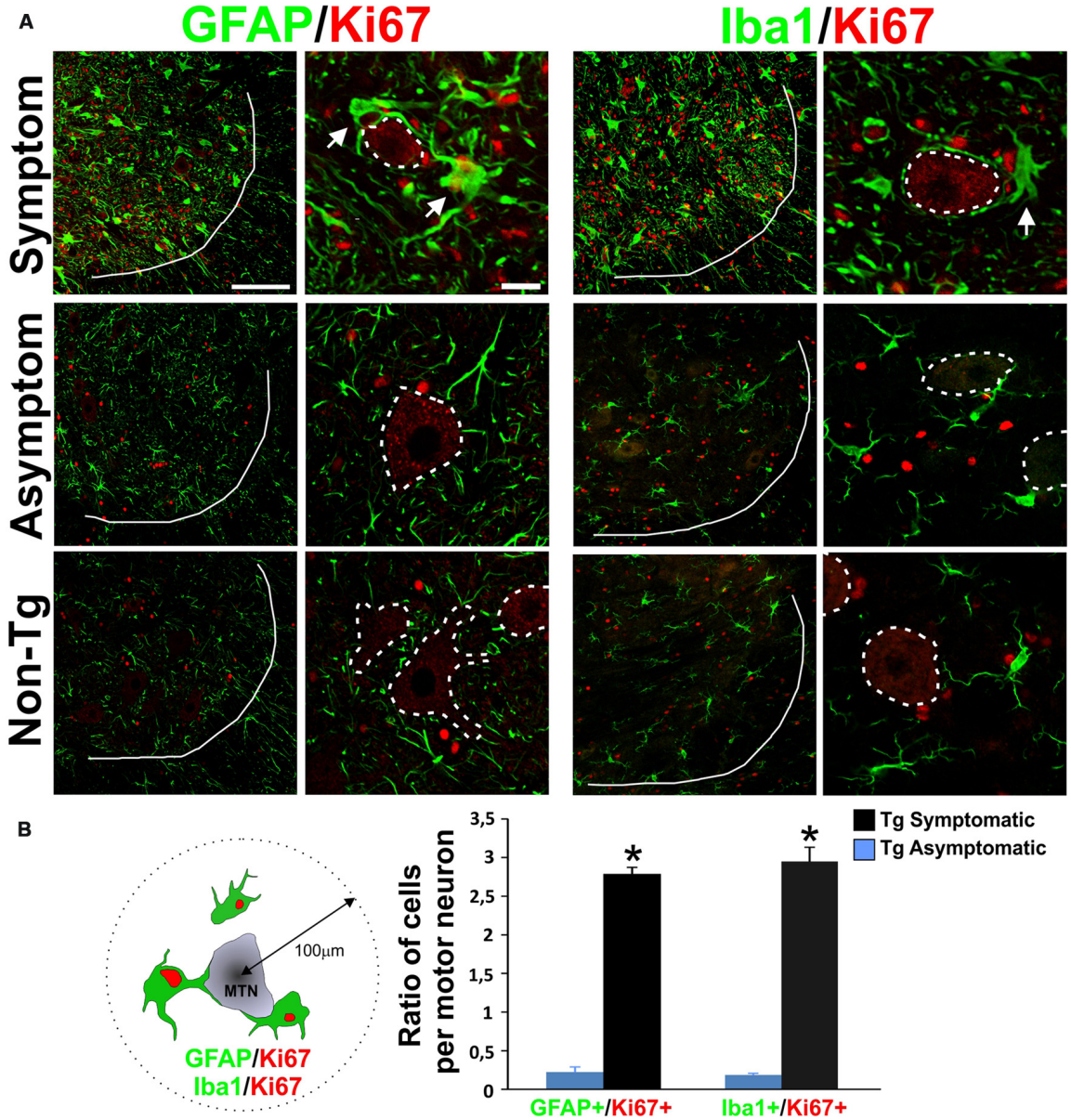

FIGURE 1 | Characterization of proliferative astrocytes and microglia in the degenerating lumbar spinal cord. **(A)** Photomicrographs showing GFAP/Ki67 and Iba1/Ki67 stained lumbar spinal cord sections measured in symptomatic (upper panels), asymptomatic (middle panels) SOD1^{G93A} rats, and non-transgenic rats (lower panels). Low magnification panels show the notorious increase in the number of Ki67^{+} red nuclei and the appearance of large GFAP^{+} and Iba1^{+} cells (green) in the symptomatic rats, as compared to low cell proliferation in asymptomatic or non-Tg rats. A white line indicates the border between white and grey matter. The high magnification images show that GFAP/Ki67 and Iba1/Ki67 cells are typically located around the motor neurons (indicated as dotted lines) and their processes closely embrace the neuronal cell body. The arrows indicate double-labeled cells. Note the unspecific binding of Ki67 antibody to motor neuron cell bodies. **(B)** Ratio of GFAP/Ki67 and Iba1/Ki67-positive cells per motor neuron in symptomatic and asymptomatic rats. The scheme at the left shows the methods used to count the cells in a perimeter of 100 μm around motor neurons. The counting was performed in 10 histological sections per animal. Two different rats were used for each condition. Data are shown as mean ± SD; *$P < 0.05$. Scale bars: 100 μm for low magnification panels; 20 μm for high magnification panels.

the cultures with 10 μM FSK, which is known to induce astrocytic processes growth and differentiation (Abe and Saito, 1997), accelerated the transition from microglia to AbA cells, while down-regulating the expression of Iba1 and promoting the growth of processes stained with GFAP (**Figure 4C**). To further confirm the microglia to astrocyte phenotypic switch, we treated the cultures with Leu-OMe, a compound used to selectively deplete microglia from primary cell cultures (Uliasz et al., 2012). **Figure 4D** shows that microglia from ALS rats were completely killed by 25 mM of Leu-OMe at DIV3, whereas there was no toxicity at DIV20 after the cells had undergone the phenotypic transition to an AbA morphology.

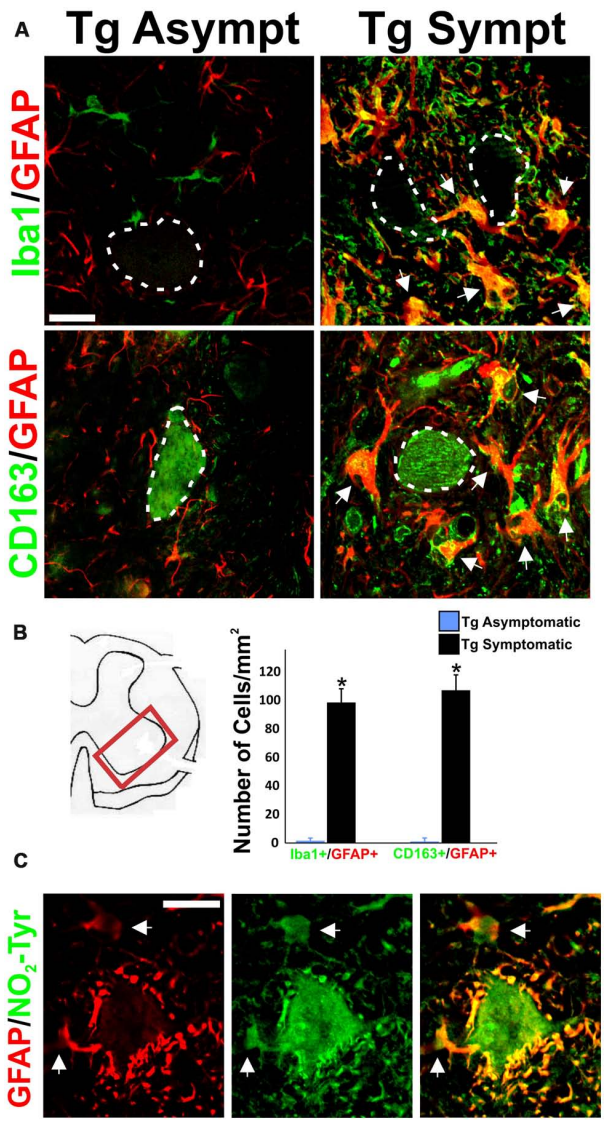

FIGURE 2 | Concurrent expression of microglia and astrocytic markers in AbA cells.(A) Representative image of GFAP (red), Iba1 (green), and CD163 (green) in lumbar spinal cord sections from transgenic asymptomatic (Tg-asymp) and symptomatic (Tg-sympt) $SOD1^{G93A}$ rats. Dotted lines indicate motor neuron cells bodies, which artifactually stained for CD163. Note that microglia and astrocytes in asymptomatic rat expressed GFAP and Iba1 in well-segregated cell populations. In comparison, most of the $Iba1^{+}$ and $CD163^{+}$ cells also expressed GFAP in symptomatic animals (arrows). **(B)** Quantification of cells expressing Iba1/GFAP and CD163/GFAP in the lumbar spinal cord. The counting was performed in 10 histological sections per animal. Two different rats were used for each condition. Data are shown as mean ± SD; $*P < 0.05$. **(C)** Representative confocal immunostaining against GFAP (red) and nitrotyrosine (NO_2–Tyr, green) in the ventral horn of a symptomatic rat showing the intense nitration of Perineuronal AbA cells (arrows). Scale bars: 20 μm.

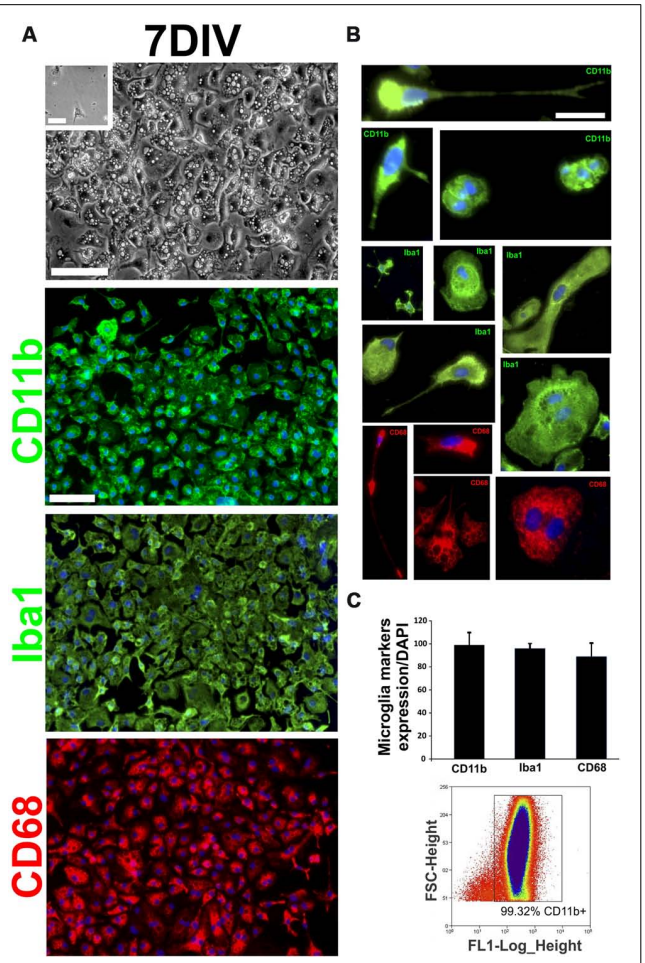

FIGURE 3 | Characterization of first stages of AbA cells *in vitro*. Primary cultures of the spinal cord of symptomatic rats were established as in (Diaz-Amarilla et al., 2011) and the cell phenotypes were analyzed on DIV7. **(A)** The upper panel shows a representative phase-contrast image of the culture at DIV7. The inset shows the same culture obtained from non-Tg littermates where no cells could be grown. The lower panels show confocal images of cells immunostained for typical microglia markers such as CD11b, CD68, and Iba1 at 7 DIV. Scale bars: 50 μm. **(B)** Characterization of different typical microglia phenotypes present at 7 DIV, cultured from Tg symptomatic rats. Scale bar: 25 μm. **(C)** Quantification of the microglia markers expression (CD11b, CD68, and Iba1) in culture at 7 DIV, counting cell positive for microglia marker respect to the total number of nuclei labeled with DAPI (upper panel). Typical data from flow cytometry showing that 99% of cells were $CD11b^{+}$. The data are representative of three independent experiments.

DISCUSSION

Glial cells expressing mutant SOD1 are now well established to be toxic to motor neurons in rodent models as well as in ALS patients (Barbeito et al., 2004; Nagai et al., 2007; Yamanaka et al., 2008; Haidet-Phillips et al., 2011). AbA cells are the most toxic cells yet identified to motor neurons (Diaz-Amarilla et al., 2011). These distinctive glial cells are directly associated with motor neuron disease, because they actively proliferate after the onset of progressive paralysis and make intimate contact with degenerating motor neurons. By analyzing the population of proliferating $Ki67^{+}$ glial cells in the ventral horn of symptomatic SOD1 rats, we found that AbA cells most likely originate from microglia. Notably, purified microglia isolated from the spinal cord of symptomatic rats spontaneously transformed into AbA cells.

The extremely rapid progression of paralysis in $SOD1^{G93A}$ rats is characterized by prominent neuroinflammation associated with

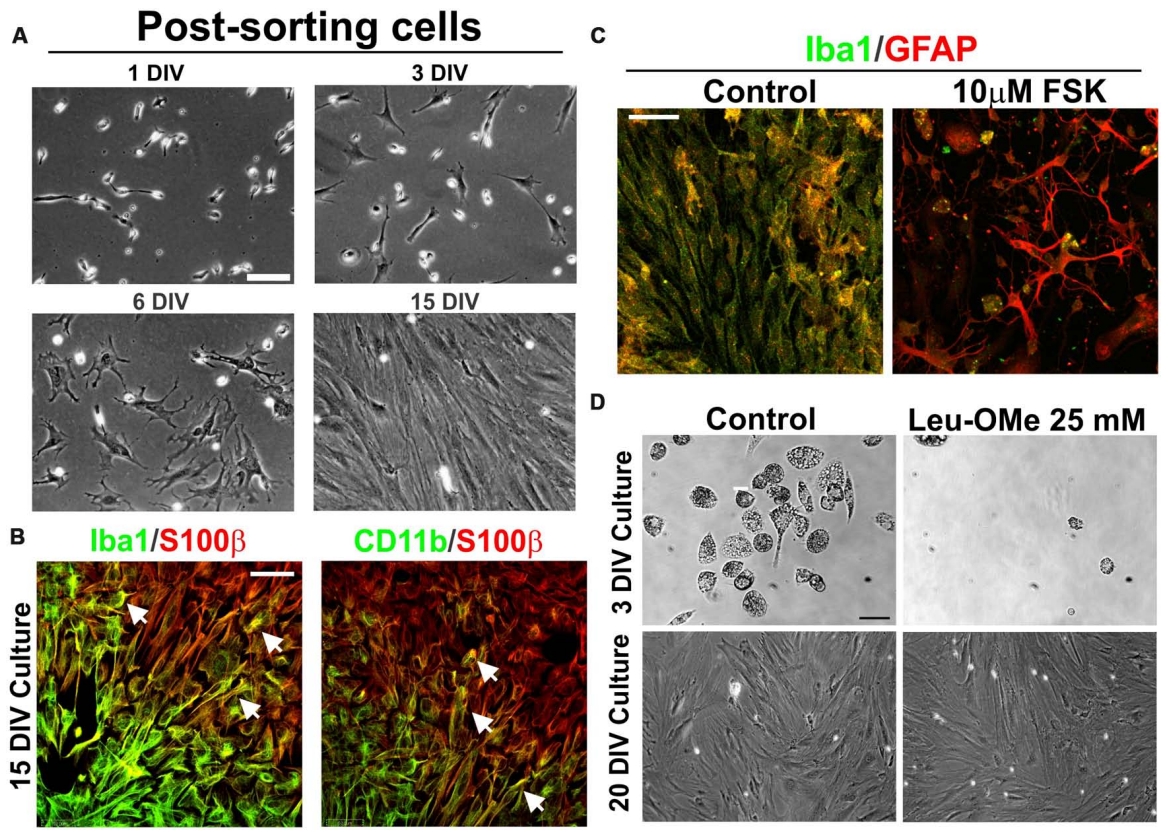

FIGURE 4 | Phenotypic transition of microglia into AbA cells in culture. **(A)** Microglia cell cultures (7 DIV) from symptomatic spinal cord were dissociated and stained with FITC-labeled CD11b. After FACS sorting, cells were re-plated and analyzed for phenotypic transition at DIV15. Scale bar: 50 μm. **(B)** Confocal image showing transition zones observed at 15 DIV. Note the segregation of astrocytic S100β staining with the microglia Iba1 or CD11b markers, which coincides with the morphological change of cells. The co-localization of astrocytic and microglia markers was found in a few cells in the border between these zones. Scale bar: 50 μm. **(C)** Treatment of cultures at DIV15 with foskolin (10 μM, 3 h) down regulated Iba1 expression and stimulated GFAP expression and growth of processes. Scale bars: 50 μm. **(D)** Differential effect of 25 μM of Leucine-Methyl Ester (Leu-OMe) before and after the phenotypic transition. Treatment with Leu-OMe was applied to cultures at DIV3 and DIV20 to assess the toxicity. Toxicity of Leu-OMe was restricted to the microglia phenotype. This experiment was repeated with three independent isolations of AbA cells. Scale bars: 20 μm

microglia activation (Sanagi et al., 2010; Philips and Robberecht, 2011). This inflammatory response is consistent with the sharp increase in cell proliferation others and we have observed with Ki67-staining in symptomatic animals (Schaefer et al., 2005; Pun et al., 2006). Previously, BrdU incorporation was used to demonstrate increased cell proliferation in ALS rats of NG2-positive glial progenitor cells (Magnus et al., 2008), which can potentially differentiate into astrocytes. Our results indicate that microglia more likely gave rise to AbA cells expressing astrocytic markers in regions adjacent to motor neurons.

The finding that spinal AbA cells co-express astrocytic GFAP with two different microglia markers Iba1 or CD163 was surprising because such a mixed phenotype is rarely observed. Co-expression of both markers has been observed in neoplastic glioblastoma multiforme cells (Huysentruyt et al., 2011; Persson and Englund, 2012). These human astroglial tumor cells seem to acquire phagocytic properties as a consequence of the dramatic inflammatory conditions occurring in tumors (Persson and Englund, 2012). Similarly, spinal AbA cells may originate from a phenotypic transition of inflammatory microglia into astrocytes-like cells in the degenerating cellular environment of the ventral horn. Other reports have shown aberrant features of microglial cells in symptomatic $SOD1^{G93A}$ rats, including formation of microglia clusters (Howland et al., 2002) and multi-nucleated giant cells (Fendrick et al., 2007). Based on the morphology, localization, high proliferation rate, and other phenotypic features, spinal AbA cells are distinct from previously described M1 or M2 microglia (Kigerl et al., 2009; Durafourt et al., 2012; Liao et al., 2012). It is uncertain whether the phenotypic transition is specific for mutant SOD1 microglia or might also be observed in other CNS insults where phagocytic microglia accumulate around dying neurons (Beyer et al., 2000; Sanagi et al., 2010; Neher et al., 2012). For example, ameboid microglia-like cells expressing markers of oligodendrocyte and monocyte lineages have been described in hippocampus following acute neuronal damage (Fiedorowicz et al., 2008). AbA cells represent a novel pathological phenotype of microglia/macrophages derived from their prominent plasticity following activation (Schwartz et al., 2006; Luo and Chen, 2012). This phenotypic transformation of microglia may explain why the ablation of dividing

astrocytes did not alter astrogliosis in SOD1 mice (Lepore et al., 2008).

Further evidence that AbA cells derive from activated microglia was provided in cell culture experiments showing that purified endogenous microglia can transition to astrocyte-like cells. Microglia expressing CD11b, Iba1, and CD68 represented more than 98% of cells isolated from the spinal cord of symptomatic $SOD1^{G93A}$ rats. Moreover, FACS sorting of these cells using CD11b antibodies resulted in typical microglia cultures that also transitioned to AbA cells after 2 weeks, showing not only that AbA cells are originated from microglia but also the phenotypic change occurs *in vitro*.

Because the phenotypic switch is associated to sustained cell proliferation and a critical cell density, we suggest that inflammatory mediators secreted by the activated microglia induced the transformation. Previous reports have shown the ability of microglia to be progenitors for different neural cell types, including astrocytes *in vitro* (Yokoyama et al., 2004). This property in turn may be related to their hematopoietic origin (Yokoyama et al., 2006). Compared to AbA cells growing in the degenerating spinal cord, the concurrent expression of astrocytic and microglia markers by cultured AbA cells is only transiently in restricted to the borders of the transition zones. Therefore, it appears that activated microglial cells in degenerating spinal cord are prone to transition to astrocyte-like phenotype both in culture conditions as well as *in vivo*, in the cellular niche surrounding the motor neurons. These cells may play a role in the killing and subsequent phagocytosis of motor neurons.

Spinal AbA cells also show a number of aberrant features including high levels of S100β and Cx43 expression (Diaz-Amarilla et al., 2011) that may be relevant for neuronal toxicity through secreted S100 proteins as well as extracellular ATP released through connexin hemichannels. Activation of the extracellular ATP receptor/channel P2X7 has recently been shown to induce motor neuron death and to induce the neurotoxic phenotypes of astrocytes in culture (Gandelman et al., 2010, 2013). Furthermore, endogenous nitration of tyrosine near the ATP binding pocket of HSP90 activates P2X7, which induces motor neuron apoptosis (Franco et al., 2013).

We also showed that spinal AbA cells are strongly stained for nitrotyrosine especially in the distal perineuronal processes, consistent with the production of peroxynitrite. Microglia bearing mutant SOD1 has been shown to damage motor neurons through the production of peroxynitrite (Thonhoff et al., 2012).Because their microglia origin, spinal AbA cells may have primed to generate superoxide and hence peroxynitrite on the exterior face of the plasma membrane in close proximity to motor neurons (Beckman et al., 2001).

CONCLUSION

Taken together, the present work supports the concept that aberrant astrocyte-like cells in the degenerating spinal cord are derived from activated microglia that proliferate around damaged motor neurons. The present study provides evidence that microglia isolated from the spinal cord of ALS–SOD rats developing paralysis have the potential to transition into an astrocyte-like phenotype. The proliferating spinal AbA cells concurrently express markers of both microglia and astrocytes lineages. Because the appearance of AbA cells is closely associated to the progression of paralysis in $SOD1^{G93A}$ rats, a better understanding of the mediators inducing the phenotypic transition may provide another avenue of intervention to slow the progressive spread of disease in ALS patients.

AUTHOR CONTRIBUTIONS

Emiliano Trias, Pablo Díaz-Amarilla, Silvia Olivera-Bravo, Joseph S. Beckman, and Luis Barbeito designed research; Emiliano Trias, Pablo Díaz-Amarilla, Silvia Olivera-Bravo, Eugenia Isasi, Derek A. Drechsel, Nathan Lopez, C. Samuel Bradford, and Kyle E. Ireton performed research; Emiliano Trias, Pablo Díaz-Amarilla, C. Samuel Bradford, Joseph S. Beckman, and Luis Barbeito analyzed data; and Emiliano Trias, Joseph S. Beckman, and Luis Barbeito wrote the paper.

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Estudios relacionados en ELA

Durante la realización de esta tesis participé en dos trabajos, en uno como autor y en otro como co-autor, relacionados al estudio del potencial terapéutico de diferentes moléculas en el contexto de la ELA. Uno de estos trabajos implicó el estudio del potencial terapéutico de masitinib, un inhibidor de *tirosina kinasas* ligadas a receptores de factores de crecimiento, en ratas $SOD1^{G93A}$. El segundo trabajo tuvo como objetivo evaluar el potencial terapéutico *in vitro* de un grupo de lípidos nitrados mediante la inducción de la vía Nrf2/ARE, una vía antioxidante celular, específicamente en astrocitos y su impacto sobre la sobrevida de motoneuronas.

A pesar de muchos años de investigación básica y preclínica, y de la gran cantidad de fármacos evaluados en los modelos animales de ELA, actualmente se dispone de un solo fármaco en la clínica llamado Riluzol, cuya eficacia se limita a un cierto porcentaje de pacientes. Es por ello que la evaluación del potencial terapéutico de nuevas moléculas sigue siendo un campo muy activo en las investigaciones relacionadas a la ELA.

A continuación se adjuntan las dos publicaciones que resultaron de estos estudios, precedidas por una breve descripción y discusión de los principales hallazgos.

Estudio 1: Evaluación del potencial terapéutico de masitinib en un modelo animal de ELA.

Artículo 3

Trias E, Ibarburu S, Barreto-Núñez R, Babdor J, Maciel TT, Guillo M, Gros L, Dubreuil P, Díaz-Amarilla P, Cassina P, Martínez-Palma L, Moura IC, Beckman JS, Hermine O, Barbeito L. Post-paralysis tyrosine kinase inhibition with masitinib abrogates neuroinflammation and slows disease progression in inherited amyotrophic lateral sclerosis. *J Neuroinflammation. 2016 Jul 11;13(1):177.*

Una de las características más enigmáticas de la ELA es la rápida progresión de los síntomas, provocando el deceso de los pacientes en pocos años tras el diagnostico de la enfermedad. A nivel fisiopatológico, la principal característica es el establecimiento de un proceso neuroinflamatorio que aumenta en intensidad conforme avanza la neurodegeneración. La participación de las células gliales en este proceso ha sido demostrada y caracterizada en modelos animales de ELA (Barbeito eta l, 2004; Ilieva et al. 2009). Recientemente nuestro grupo reportó la identificación de la células AbA, un fenotipo astrocitario aberrante con un alto potencial toxico para la motoneuronas (Díaz-Amarilla et al. 2011). Debido a la correlación observada entre la aparición de la células AbA y el avance de los síntomas, nos preguntamos si la regulación farmacológica de este fenotipo celular podría impactar en la progresión de la ELA.

Bajo la hipótesis de que la proliferación de las células AbA podría ser controlada por inhibidores de *tirosina kinasas* involucradas en la señalización de receptores para factores de crecimiento tipo III, como PDGF-R, c-Kit, FLT3 y CSF-1R, se evaluó el potencial terapéutico de masitinib (AB1010) un inhibidor altamente selectivo kinasas (Dubreuil et al. 2009).

Trabajos previos mostraron que el tratamiento con masitinib redujo significativamente la neuroinflamación en patología del SNC como Esclerosis Múltiple (Vermersch et al. 2012), el daño isquémico (Kocic et al. 2015) y la enfermedad de Alzheimer (Piette et al. 2011). Estos resultados motivaron le caracterización del efecto de masitinib sobre las células AbA en cultivo y determinar su potencial terapéutico suministrado oralmente en ratas $SOD1^{G93A}$. Teniendo en cuenta que las células AbA aparecen en la médula espinal poco después del comienzo de la parálisis, el tratamiento se realizó durante la fase sintomática, reproduciendo la situación real que tiene lugar a nivel clínico.

La administración oral de masitinib en ratas $SOD1^{G93A}$ comenzando 7 días después de constatar el inicio de la parálisis, prolongó la duración de la fase sintomática en un 40% comparado con el grupo control. Se observó una reducción significativa en el número de células AbA en la médula espinal durante el tratamiento acompañado por una significativa reducción de la microgliosis y de la presencia de marcadores de daño neuronal. Se observó que el tratamiento con masitinib inhibe la proliferación de la microglia aislada de médula espinal de animales SOD1G93A en fase sintomática a través de la inhibición del receptor para CSF-1 en concentraciones nanomolares. Además, impidió la migración de dichas células en cultivo y la expresión de mediadores inflamatorios. El conjunto de resultados obtenidos en este trabajo, demuestra que el tratamiento de ratas $SOD1^{G93A}$ con masitinib inhibe significativamente la activación de las células gliales y reduce la neuroinflamación subyacente. El significativo aumento en la sobrevida reportado en este trabajo, indica que el tratamiento con masitinib constituye una prometedora estrategia terapéutica para el tratamiento de la ELA.

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Post-paralysis tyrosine kinase inhibition with masitinib abrogates neuroinflammation and slows disease progression in inherited amyotrophic lateral sclerosis

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Abstract

Background: In the SOD1^{G93A} mutant rat model of amyotrophic lateral sclerosis (ALS), neuronal death and rapid paralysis progression are associated with the emergence of activated aberrant glial cells that proliferate in the degenerating spinal cord. Whether pharmacological downregulation of such aberrant glial cells will decrease motor neuron death and prolong survival is unknown. We hypothesized that proliferation of aberrant glial cells is dependent on kinase receptor activation, and therefore, the tyrosine kinase inhibitor masitinib (AB1010) could potentially control neuroinflammation in the rat model of ALS.

Methods: The cellular effects of pharmacological inhibition of tyrosine kinases with masitinib were analyzed in cell cultures of microglia isolated from aged symptomatic SOD1^{G93A} rats. To determine whether masitinib prevented the appearance of aberrant glial cells or modified post-paralysis survival, the drug was orally administered at 30 mg/kg/day starting after paralysis onset.

Results: We found that masitinib selectively inhibited the tyrosine kinase receptor colony-stimulating factor 1R (CSF-1R) at nanomolar concentrations. In microglia cultures from symptomatic SOD1^{G93A} spinal cords, masitinib prevented CSF-induced proliferation, cell migration, and the expression of inflammatory mediators. Oral administration of masitinib to SOD1^{G93A} rats starting after paralysis onset decreased the number of aberrant glial cells, microgliosis, and motor neuron pathology in the degenerating spinal cord, relative to vehicle-treated rats. Masitinib treatment initiated 7 days after paralysis onset prolonged post-paralysis survival by 40 %.

Conclusions: These data show that masitinib is capable of controlling microgliosis and the emergence/expansion of aberrant glial cells, thus providing a strong biological rationale for its use to control neuroinflammation in ALS. Remarkably, masitinib significantly prolonged survival when delivered after paralysis onset, an unprecedented effect in preclinical models of ALS, and therefore appears well-suited for treating ALS.

Keywords: ALS, Aberrant glial cells, Neurodegeneration, Masitinib, M-CSF

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Background

Amyotrophic lateral sclerosis (ALS) is a paralytic neurodegenerative disease characterized by the progressive degeneration of upper and lower motor neurons. Survival after diagnosis varies between 1 and 5 years or more, largely determined on the rate of spread of motor neuron pathology. Paralysis progression in rodent models of ALS appears to be modulated by glial cells that proliferate and express inflammatory mediators in the degenerating spinal cord [1–4]. In the SOD1^{G93A} mutant rat model of ALS, a rapid spread of paralysis is associated with marked glial cell activation and the emergence of aberrant glial cells that actively proliferate around degenerating motor neurons [4, 5]. Furthermore, aberrant glial cells display a marked neurotoxic potential on cultured motor neurons [4], suggesting that they might directly contribute to the rapid spread of paralysis of ALS rats. It remains unknown, however, whether pharmacologically downregulation of aberrant glial cells could slow paralysis progression in the rat model of ALS.

We have theorized that aberrant glial cells proliferating in the ALS spinal cord could be sensitive to tyrosine kinase inhibitors that target the family of type III growth factor receptors including PDGF-R, c-Kit, FLT3, and CSF-1R. These receptors synergistically signal cell proliferation and the migration of cancer and hematopoietic cells, including macrophages [6]. In particular, while M-CSF/CSF-1R signaling is critical for the mononuclear phagocytic system [7, 8], a recent report indicates that damaged motor neurons induce the expansion of spinal cord microglia by expressing M-CSF [9]. Thus, we have explored whether the inhibition of CSF-1R and related kinase receptors could modulate neuroinflammation and slow disease spreading in an inherited rat model of ALS.

Among candidate tyrosine kinase inhibitors, masitinib (AB1010) was found to be a highly selective kinase inhibitor [10, 11] and shown to prevent central nervous system (CNS) neuroinflammation in multiple sclerosis [12], stroke [13], and Alzheimer's disease [14]. Thus, we aimed to characterize the effects of masitinib on cultured aberrant glial cells and determine its therapeutic potential after oral administration to SOD1^{G93A} rats. Because aberrant glial cells emerge only after paralysis onset [4], the drug treatment was initiated following overt disease onset to better simulate the clinical condition of ALS patients. We found that masitinib inhibited glial cell activation in SOD1^{G93A} rats and prolonged survival, indicating a promising therapeutic approach to ALS.

Methods

Animals

Male SOD1^{G93A} progeny were used for further breeding to maintain the line. Rats were housed in a centralized animal facility with a 12-h light-dark cycle with ad libitum access to food and water. Perfusion with fixatives was performed under 90 % ketamine—10 % xylazine anesthesia and all efforts were made to minimize animal suffering, discomfort, or stress. All procedures using laboratory animals were performed in accordance with the national and international guidelines and were approved by the Institutional Animal Committee for animal experimentation. Male hemizygous NTac:SD-TgN (SOD1-^{G93A})L26H rats (Taconic), originally developed by Howland et al. [15], were bred locally by crossing with wild-type nontransgenic Sprague-Dawley female rats.

Determination of disease onset and end-stage

All rats were weighed and evaluated for motor activity daily. Disease onset was determined for each animal when pronounced muscle atrophy was accompanied by abnormal gait, typically expressed as subtle limping or dragging of one hind limb. End-stage was defined by a lack of righting reflexes or the inability to reach food and water.

Masitinib post-paralysis survival trial

Only transgenic rats showing weakness and gait alterations in hind limbs as first clinical sign were selected for masitinib treatment studies. Male and female rats were divided randomly into the masitinib or vehicle-treated groups. Masitinib mesylate (AB1010) freshly prepared in drinking sterilized water was administrated daily at a dose of 30 mg/kg using a curved stainless steel gavage needle with 3-mm ball tip. Dosing was defined in accordance to previous studies in a rat model of stroke that was shown to be safe for chronic treatments [13]. Rats were treated from day 1 or day 7 post-paralysis during 20 days or until end-stage, when they were euthanized.

Immunohistochemical staining of rat spinal cords

After 20 days of treatment using 30 mg/kg/day of masitinib, starting after paralysis onset, animals were deeply anesthetized and transcardial perfusion was performed with 0.9 % saline and 4 % paraformaldehyde in 0.1 M PBS (pH 7.2–7.4). Fixed spinal cord was removed, post-fixed by immersion for 24 h, and then transverse sectioned (30 μm) in a Leica cryostat. Serial sections were collected in 100 mM PBS for immunohistochemistry. Free-floating sections were permeabilized for 30 min at room temperature with 0.3 % Triton X-100 in PBS, passed through washing buffered solutions, blocked with 5 % BSA:PBS for 1 h at room temperature, and incubated overnight at 4 °C in a solution of 0.3 % Triton X-100 and PBS containing the primary antibodies, rabbit anti-GFAP (1:500, Sigma), mouse anti-S100β (1:400, Sigma), rabbit anti-Iba1 (1:300, abcam), rabbit anti-

CD206 (1:300 abcam), mouse anti-CD68 (1:200, abcam), mouse anti-ChAT (choline acetyltransferase) (1:300, Millipore). After washing, sections were incubated in 1:1000-diluted secondary antibodies conjugated to Alexa Fluor 488 and/or Alexa Fluor 633 (1:1000, Invitrogen). Antibodies were detected by confocal microscopy using a confocal LEICA TCS-SP5-DMI6000 or a confocal ZEISS LSM 780.

Analysis of glial cells in the lumbar spinal cord of hind limb symptomatic rats

The number of aberrant glial cells co-expressing the astrocytic markers GFAP and S100β or the microglia markers Iba-1, CD206, and CD68 were assessed by counting the respective positive cells for the different markers in gray matter from the lumbar cord of $SOD1^{G93A}$ asymptomatic or symptomatic rats that had been treated with either vehicle or masitinib. The analysis was performed manually in at least 20 histological sections per animal (four different rats for each condition) using the cell counter tool of the ImageJ software. For aberrant glial cell counting, values were expressed as the number of GFAP+/S100β+ cells in each ventral horn of the spinal cord in masitinib-treated rats relative to vehicle-treated rats. For microgliosis analysis, the number of Iba1+, CD206+, or CD68+ cells was assessed via manual counting using the ImageJ software tools. Only microglia cells present in the grey matter of the ventral horn of the spinal cord were taken into consideration. Statistical studies were performed using statistical tools of the free Software PAST3. Descriptive statistics were used for each group, and Kruskal-Wallis analysis or one-way ANOVA, followed by Scheffé post hoc comparison if necessary, was used among groups. All results are presented as mean ± SEM, with $p < 0.01$ considered significant.

Analysis of microgliosis spreading along the degenerating spinal cord of $SOD1^{G93A}$ rats

The spinal cord of hind limb symptomatic rats was dissected at thoracic and cervical levels. Three masitinib-treated rats were compared with three vehicle-treated rats. As previously described, immunohistochemistry was assessed to determine the levels of microgliosis by Iba1 detection. At least ten different 30 μm sections of each rat were visualized using a LEICA TCS-SP5-DMI6000 confocal microscope.

Analysis of motor neuron number and size

The number of motor neurons expressing ChAT was assessed by counting the positive cells in the gray matter of the lumbar spinal cord of non-transgenic compared with symptomatic $SOD1^{G93A}$, vehicle-, and masitinib-treated rats. Motor neuron counting was based on a stereological approach as previously reported [16]. Briefly, ChAT positive cells were quantified on five 30 μm sections taken 300 μm apart from the ventral horn, comparing the cell numbers in Rexed laminae VII and IX, which display low and high density of large motor neurons, respectively. Results are presented as mean ± SEM, with $p < 0.01$ considered significant. The longest axis (length) of each soma was taken into consideration to quantify the mean size of motor neuron soma. The analysis was performed manually in at least 25 histological sections per animal (four different rats for each condition) using the cell counter tool of the ImageJ software. Results are presented as median ± SD, with $p < 0.01$ considered significant. Statistical studies were performed using statistical tools of the free Software PAST3. Descriptive statistics were used for each group, and Kruskal-Wallis analysis or one-way ANOVA, followed by Scheffé post hoc comparison if necessary, was used among groups.

Microglia cell cultures from symptomatic $SOD1^{G93A}$ rats

Microglial cells were obtained from a primary culture adult spinal cord of symptomatic $SOD1^{G93A}$ rats according to the procedures described by Trias et al. [5] with minor modifications. Briefly, animals were euthanized by administering an overdose of ketamine/xylazine, and the spinal cord was dissected on ice. After the meninges were removed, the spinal cord was chopped finely and dissociated with 0.25 % trypsin in calcium-free buffer for 5 min at 37 °C. Trypsin treatment was stopped by adding DMEM/10 % (vol/vol) FBS in the presence of 50 μg/mL DNaseI and mechanical disaggregation by repeated pipetting. The resulting extract was passed through an 80-μm mesh to eliminate tissue debris and was then spun. The pellet was resuspended in culture medium [DMEM/10 % (vol/vol) FBS, Hepes (3.6 g/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL)] and was then plated in a 24-multiwell culture dish. Culture medium was removed after 24 h and subsequently replaced every 24 or 48 h depending on the procedure.

Microglia migratory capacity assay

Primary cultures were plated in high-density 4-multiwell plates during 4 days. After that, and when cells already reached confluence, media was changed to DMEM-0.5 % FBS, thereby, significantly reducing the proliferation rate. A scratch was then made in the monolayer using a 1000 μL tip, and cells were treated with increasing doses of masitinib in DMSO (used as vehicle in control-treated cells). Post-scratch pictures were taken at 24 h using a bright field NIKON microscope attached to a Canon HD camera, and cells that invaded the scratch were counted manually in at least 12 different pictures (three different rats were cultured for this

experiment, $n = 3$) using the cell counter tool of ImageJ software.

Proliferation assay induced by macrophages-colony stimulating factor (M-CSF)

Cells were isolated as described above from the three different symptomatic rat spinal cords and plated in 24-multiwell dishes during 24 h in low serum, DMEM-0.5 % FBS. Cells were then treated with 30 ng/mL of rat M-CSF in PBS-0.1 % BSA (vehicle-treated cells were treated with the same amount of PBS-0.1 % BSA). To determine the inhibitory capacity of masitinib against the tyrosine kinase receptor CSF-1R, cells were treated with increasing doses of the drug (0.01–1 μM) in the presence of M-CSF and compared with vehicle-treated cells for which masitinib was substituted with DMSO. In total, three experimental groups were analyzed: control cells (in DMEM-0.5 % FBS + PBS-0.1 % BSA), vehicle-treated cells (DMEM-0.5 % FBS + M-CSF + DMSO), and masitinib-treated cells (DMEM-0.5 % FBS + M-CSF + masitinib). All wells were treated at the same time with 10 μM of BrdU (Sigma). After 24 h, cells were fixed and immunocytochemistry using anti-BrdU antibody was followed. Briefly, cells were washed and fixed with cold methanol during 5 min at 4 °C, then washed with PBS and treated with 2 M of HCl for 30 min. Cells were blocked using 5 % of BSA in PBS for 1 h, and rat anti-BrdU was incubated for 24 h at 4 °C. After that, the primary antibody was removed, washed with PBS three times for 10 min, and goat anti-rat antibody was incubated for 1 h at room temperature. After washing away the secondary antibody, cells were covered in glycerol mounting medium with 1/2000 DAPI dilution and a cover slip (Sigma). Cells were visualized in an epifluorescence microscope Olympus IX81. BrdU+ nuclei were counted and ratio of DAPI to BrdU labeling was compared among groups. Data were analyzed using analyzing tools of ImageJ software and shown as mean ± SEM, with $p < 0.01$ considered significant.

Cultured microglia treated with masitinib

Microglial cells were plated in a 24-multiwell dish during 24 h and floating fat was removed. Masitinib treatment was started at that time and was repeated every 48 h chronically with each media change. Three doses of masitinib diluted in DMSO were tested, 0.1, 0.5, and 1.0 μM. Vehicle cells were treated with the same amount of DMSO as a control. Microphotographs were taken using a phase contrast microscope equipped with a Canon HD camera. Cells were treated during 15 days until cell transformation into monolayers of aberrant astrocyte cells. Quantitative analysis of the cell number every 48 h was assessed using the "cell counter" of the ImageJ Software. The number of cells in the masitinib-treated plates was compared to vehicle-treated ones. Data are shown as mean ± SEM, with $p < 0.01$ considered significant.

Cell cultures from symptomatic SOD1^{G93A} masitinib-treated rats

After 20 days of treatment with 30 mg/kg/day of masitinib, spinal cords were cultured in p35 dishes (three different treated animals were cultured as described previously). Spinal cords from vehicle-treated animals were cultured as controls. After 24 h, floating fat was removed and pictures were taken every 48 h after every change of culture media, using a phase contrast microscope and a Canon HD camera. Pictures were taken during 10 days. Cells were counted using the tool "cell counter" from ImageJ Software. Data is shown as the number of microglia cells/mm^2 in the masitinib-treated rats and compared to vehicle. Kruskal-Wallis analysis was used among groups. Data are shown as mean ± SEM, with $p < 0.01$ considered significant.

Real-time PCR analysis in microglia cell cultures

Three different end-stage symptomatic rat spinal cords were cultured to obtained microglia as described previously [5]. Cells were plated in p60 dishes during 5 days and treated during 72 h with different doses of masitinib (0.5–1 μM) in DMEM-10 % FBS. An estimated 200,000 cells were processed for each mRNA extraction using RNeasy Micro kit (QIAGEN) according to the manufacturer's instructions. mRNA yields were measured on Nanodrop device (Thermo Scientific) and cDNA were obtained from 0.5 μg of RNA (−80 °C), 4 μL of iScript reverse transcription Supermix for RT-qPCR (BIORAD, −20 °C) in a final volume of 20 μL filled with nuclease free water. The Thermo cycler was set as follows: priming 5 min at 25 °C followed by 30 min at 42 °C for reverse transcription and 5′ at 85 °C for RT inactivation. RT-qPCR was performed on reverse transcribed cDNA using SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD) on a CFX96 Touch™ real-time PCR detection system. For each well, 5 μL of diluted DNA was added to 20 μL of mix (containing 1 μL of each primer, 12.5 μL of SsoAdvanced™ Universal SYBR® Green Supermix, 5.5 μL of nuclease free water). Each sample was run in duplicate. The cycling parameters were as follows: 30 s at 95 °C then 40 cycles at 95 °C for 10 and 30 s at 60 °C. Cq values were obtained for every cycle. Primers were designed on NCBI Primer-BLAST following the best guidelines to exclude genomic DNA amplification. The analysis was done using BioRad CFX manager 3.1 with a threshold set at 650 RFU corresponding to the amplification curves linear portion. Variations between samples were normalized using two housekeeping genes PGK1 and HPRT. All primers were validated for

specificity and efficiency. Primers were designed on PrimerBlast. All primers were validated with differentiated bone marrow-derived rat macrophages (BMDM) in vitro and selected for specificity and quantitativity before being tested on primary microglia from $SOD1^{G93A}$ rats (only primers achieving quantitatively up to a dilution factor of 500 were kept). The following primers were used: monocyte chemoattractant protein-1 (**MCP-1**) forward 5′-TGT CTC AGC CAG ATG CAG TTA AT-3′; reverse 5′-TCC AGC CGA CTC ATT GGG AT-3′; Interleukin-6 (**IL-6**) forward 5′-TTC TCT CCG CAA GAG ACT TCC-3′; reverse 5′-TCT CCT CTC CGG ACT TGT GAA-3′; tumor necrosis factor alpha (**TNFα**) forward 5′-ATC CGA GAT GTG GAA CTG GC-3′; reverse 5′-TGG GAA CTT CTC CTC CTT GTT G-3′; inducible nitric oxide synthase (**iNOS**) forward 5′-AGC CTA GTC AAC TAC AAG CCC C-3′; reverse 5′-CAT CCT GTG TTG TTG GGC TG-3′; interleukin-1 beta (**IL-1β**) forward 5′-TAG CAG CTT TCG ACA GTG AGG-3′; reverse 5′-CTC CAC GGG CAA GAC ATA GG-3′; cyclooxygenase-2 (**Cox2**) forward 5′-TGT ACT ACG CCT GAG TTT CTG AC-3′; reverse 5′-GGG ATC CGG GAT GAA CTC TC-3′; ionized calcium-binding adaptor molecule 1 (**Iba1**) forward 5′-CAA GGA TTT GCA GGG AGG AAA A-3′; reverse 5′-TTG AAG GCC TCC AGT TTG GAC-3′; transcription factor Spi-1/PU.1 **(PU.1)** forward 5′-GGA GAC AGG CAG CAA GAA GAA G-3′; reverse 5′-CCT TCA TGT CTC CGC TAC GC-3′; hypoxanthine-guanine phosphoribosyl transferase (**HPRT**) forward 5′-GTC ATG TCG ACC CTC AGT CC-3′; reverse 5′-GCA AGT CTT TCA GTC CTG TCC-3′; phosphoglycerate kinase 1 (**PGK1**) forward 5′-GTC GTG ATG AGG GTG GAC TT-3′; reverse 5′-AAC CGA CTT GGC TCC ATT GT-3′.

Kinase inhibition assay

CSF-1R kinase domain (AA 538–972) was expressed as a *N*-terminus 6HN-tagged protein in Sf21 cells using the BacPAK6 baculovirus expression system (Clontech, Mountain View, CA 94043, USA) and purified by Ni^{2+} affinity chromatography. The analysis of the effect of masitinib on CSF-1R kinase activity was assessed with the *HTRF® KinEASE assay* (Cisbio International, Bagnols-sur-Cèze, France) using a biotinylated poly(-Glu_4Tyr) peptide (1 μM) as substrate. Kinase assays were performed at an ATP concentration of 100 μM (CSF-1R Km_{ATP} = 52 μM) in kinase buffer (50 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 0.01 % Brij-35) for 30 min at room temperature in the presence of various masitinib concentrations (0 to 10 μM). Reactions were stopped by addition of EDTA, and samples were incubated for 1 h with an anti-phospho peptide-Eu^{3+} antibody (emission 620 nm) and streptavidin XL-665 (emission 665 nm) according to manufacturer's instructions. After incubation, the obtained signal is proportional to the concentration of phosphorylated peptide in the sample. All measurements were performed on a BMG Labtech Pherastar FS apparatus. Results are expressed in delta fluorescence (DF) unit defined as follow DF % = [(ratio – ratio blank) / (ratio blank)] × 100, where ratio = (665 nm/620 nm) × 10^4. Each experiment was performed in duplicate and repeated three times.

Statistics analysis and survival curves

Survival curves were compared by Kaplan-Meier analysis with the log-rank test using PAST3 software. Quantitative data were expressed as mean ± SEM and Student's *t* test or ANOVA followed by Scheffé post hoc comparison if necessary were used for statistical analysis, with $p < 0.01$ considered significant.

Results

Masitinib prevents M-CSF-induced proliferation in cultured microglia

To determine the effect of tyrosine kinase inhibition with masitinib, we used microglia isolated from the primary spinal cord cultures of symptomatic $SOD1^{G93A}$ rats before their transformation into astrocyte-like cells [5]. Microglia appeared as hypertrophic phagocytic cells that actively proliferate in the presence of fetal bovine serum (FBS) or M-CSF (Fig. 1a). Treating cell cultures using pharmacological concentrations of masitinib (0.1–1 μM) dose-dependently abrogated the morphology of hypertrophic phagocytic microglia and also inhibited M-CSF-induced proliferation as measured by BrdU uptake (Fig. 1b). In accordance, masitinib potently inhibited the kinase activity of recombinant CSF-1R with an IC_{50} of 90 ± 35 nM (Fig. 1c), a concentration that is reachable in vivo.

Masitinib prevents $SOD1^{G93A}$ microglia cells inflammatory profile

As predicted by the hypertrophic and phagocytic morphology, primary cultured microglia from symptomatic $SOD1^{G93A}$ spinal cords displayed a robust transcriptional activity for inflammatory genes. After exposure to masitinib during 72 h, the transcription of several genes highly involved in neuroinflammation decreased more than 50 %. In particular, relevant inflammatory transcripts such as IL1β, IL6, Iba1, and Cox2 were downregulated by approximately 80 % (Fig. 2a). In addition, Fig. 2b shows that masitinib inhibited by more than 50 %, the ability of microglia to migrate across a scratch made in the culture dish in low FBS conditions.

Previously, we have reported that hypertrophic microglia from ALS rats follow a phenotypic transition after 12–15 days in culture turning to flat, astrocyte-like cells characterized by being highly toxic for cultured motor

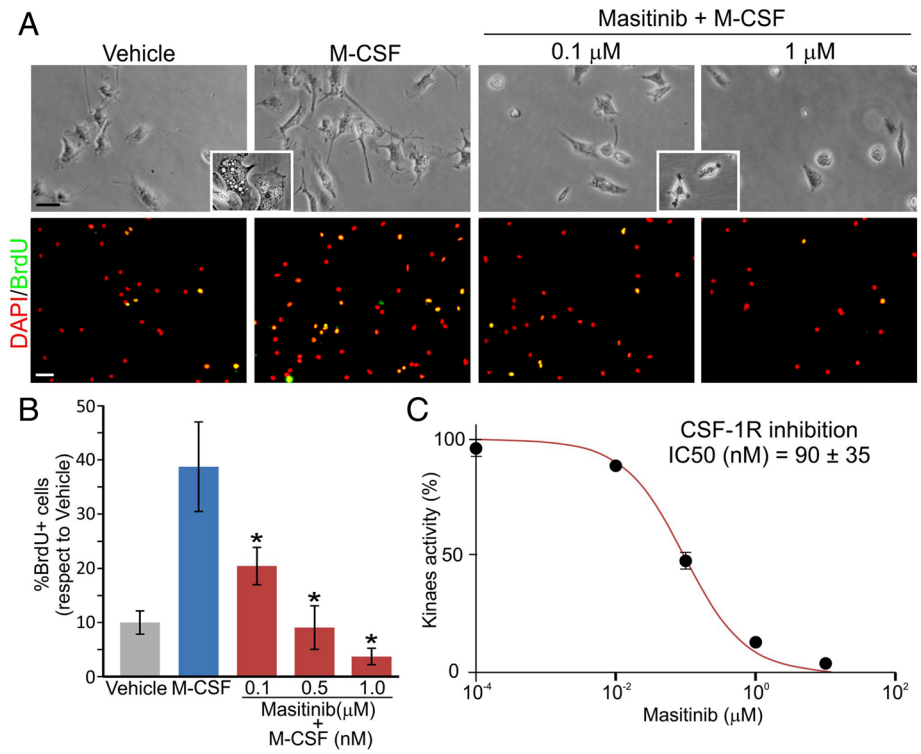

Fig. 1 Masitinib prevented microglia proliferation by inhibiting CSF-1R. **a** Microglia cultured from symptomatic $SOD1^{G93A}$ rat spinal cord in low FBS conditions (0.5 %) adding 30 ng/mL of M-CSF with indicated masitinib concentrations. The *insets* show the hypertrophic vacuolated cells in vehicle and M-CSF conditions and the small rounded cells after masitinib treatment (*scale bars*: contrast 20 μm and DAPI/BrdU 50 μm). **b** The *graph* shows the quantitative BrdU analysis where positive cells were counted and expressed as percentage of total cells stained with DAPI. M-CSF treatment produced a significant increase in microglial proliferation that was blocked by masitinib. **c** Kinase inhibition assay showing that masitinib inhibited CSF-1R with an $IC_{50} = 90 \pm 35$ nM. All data are expressed as mean ± SEM $^*p < 0.01$

neurons [4, 5]. Figure 2c shows that masitinib (0.1–1 μM) potently prevented this phenotypic transformation by more than 50 %, thus preventing the emergence of the aberrant glial cell phenotype in culture.

Post-paralysis masitinib treatment reduces the number of aberrant glial cells and neuroinflammation

We then explored whether chronic treatment with masitinib could reduce the number of aberrant glial cells in the degenerating spinal cord, which were identifiable as large GFAP/S100β-positive cells located around motor neurons as described previously [4]. Rats were orally treated with masitinib (30 mg/kg/day), starting right after paralysis onset and during the next 20 days, corresponding to the average post-paralysis survival in untreated rats (Fig. 5). Only rats that initiated paralysis in the hind limbs were used in the experiments in order to reduce experimental variables. As compared with rats treated with vehicle, masitinib significantly reduced the number of aberrant glial cells in the lumbar spinal cord by 40 % (Fig. 3a).

In accordance, masitinib treatment to symptomatic rats prevented the isolation and subsequent proliferation of microglia in primary cell cultures of the degenerating spinal cord (Fig. 3b). This sharply contrasted with a large number of hypertrophic and phagocytic cells obtained in cultures from vehicle-treated rats, indicating that masitinib treatment reduces inflammatory and proliferative potential of endogenous glial cells.

Post-paralysis masitinib treatment also significantly reduced microgliosis as assessed by the number of cells expressing Iba1+, CD206+, or CD68+ cells in the ventral horn of the lumbar spinal cord, when compared with vehicle-treated animals (Fig. 4a, see Additional file 1: Figure S1A). Remarkably, there was a reduction of hypertrophic Iba1+ microglia cells surrounding motor neurons at thoracic and cervical levels of the degenerating spinal cords, suggesting that masitinib may prevented the spread of neuroinflammation along the neuraxis (see Additional file 1: Figure S1B).

Masitinib ameliorates motor neuron pathology

Because motor neuron death is the main pathological feature of symptomatic rodent models and human ALS, we used the same experimental setting to determine if motor neuron pathology was influenced by masitinib

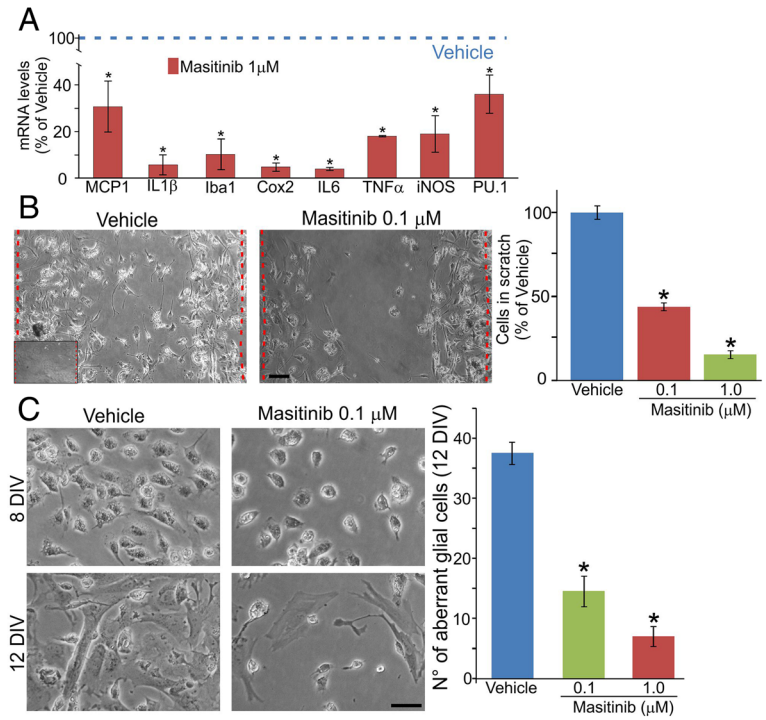

Fig. 2 Masitinib inhibited microglia proinflammatory phenotype and prevented microglia migration and transformation into aberrant glial cells. **a** Real-time PCR analysis showed that the treatment with pharmacological concentration (1 μM) of masitinib during 72 h is sufficient to significantly reduce the expression of several genes involved in inflammatory processes. **b** A confluent monolayer was scratched to determine the migratory capacity of aberrant microglia. After 24 h, cells located between the *dashed lines* were counted. Vehicle-treated microglia covered most of the scratch after 24 h, while masitinib-treated cells showed significantly less migratory capacity. The *inset* shows the open space in the monolayer immediately after making the scratch (*scale bar* 20 μm). The graph to the *right* shows the quantitative analysis of migration. **c** Masitinib prevented microglia transformation into aberrant glial cells in a dose-dependent manner when compared with vehicle-treated cultures. Note how after 12 days in vitro, microglia transition to a flat astrocyte-like cell that reach confluence. Masitinib significantly prevented this transformation and few microglia cells transitioned into aberrant glial cells (*scale bar* 10 μm). The graph to the *right* represents the quantitative analysis showing the number of aberrant glial cells after 12 days in vitro (12DIV). All data are expressed as mean ± SEM *$p < 0.01$

treatment. As shown in Fig. 4b, in vehicle-treated $SOD1^{G93A}$ rat, the number of ventral horn ChAT+ motor neurons decreased by 60 % when measured 20 days after paralysis onset. In comparison, masitinib significantly reduced motor neuron loss to 40 % when administered after paralysis onset. We next analyzed the effect of masitinib treatment on the motor neuron soma diameter in surviving motor neurons. When compared with non-transgenic animals (soma diameter 35.8 ± 8.7 μm), there was a significant reduction in the soma diameter of end-stage symptomatic rats (23.4 ± 5.8 μm). This neuron atrophy was significantly prevented by masitinib treatment (30 ± 10.3 μm) (Fig. 4b).

Masitinib prolongs post-paralysis survival in $SOD1^{G93A}$ rats

Next, we designed two randomized trials using masitinib to determine how the drug affected the survival of $SOD1^{G93A}$ rats with hind limb onset. As shown in Table 1, our rat colony develops disease with a delayed onset (187 ± 15 days for vehicle rats) if compared with that originally described by Howland et al. [15]. In our colony, the post-paralysis survival with hind limb onset has been highly reproducible in non-treated animals (20 ± 3.8 days, Fig. 5). Table 1 shows that there were no significant differences in the pre-treatment parameters analyzed such as age and weight between treated and non-treated animal groups (see Additional file 1: Figure S2C, D).

Masitinib (30 mg/kg/day) was administrated immediately upon abnormal gait onset (day 1) or after day 7. The effect of masitinib treatment was compared against age, weight, and gender-matched control groups treated with vehicle (Fig. 5a, b, see Additional file 1: Figure S2A–D). Without masitinib, the ALS rats died within 3 weeks of paralysis onset (Fig. 5a). Rats in the masitinib day 1 cohort ($n = 14$) had a statistically significant ($p < 0.0006$) difference in the probability of survival when compared with vehicle-treated animals (Kaplan-Meier curves, Fig. 5a).

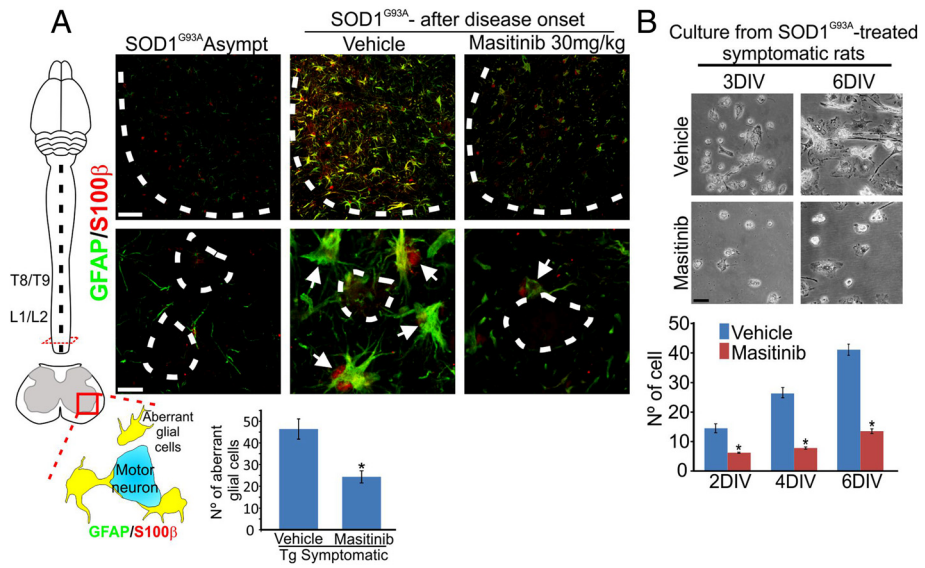

Fig. 3 Masitinib treatment reduced the number of aberrant glial cells in the degenerating spinal cord. **a** Aberrant glial cells expressing GFAP (*green*) and S100β (*red*) in the ventral horn of the spinal cord. *Dotted white lines* mark the border between white and grey matter in the upper images and outline motor neurons in high magnification panels. In the vehicle-treated rats aberrant glial cells (*white arrows*) surround motor neurons as compared with asymptomatic (Tg Asympt) where glial cells express low S100β. Masitinib (30 mg/kg/day) prevented the appearance of aberrant glial cells in the degenerating spinal cord after 20 days treatment. The scheme represents the level of spinal cord segments and Rexed laminae VII and IX where aberrant glial cells were counted (*scale bars* 50 μm in low magnification and 10 μm in high magnification). **b** Spinal cord culture from masitinib-treated rats compared with vehicle-treated rats. Relatively few cells were obtained from the degenerating spinal cord after masitinib treatment when compared with vehicle-treated animals (*scale bar* 15 μm). All data are expressed as mean ± SEM *$p < 0.01$

Remarkably, animals in the masitinib day 7 cohort ($n = 9$) also had a statistically significant ($p < 0.0001$) difference in the probability of survival with respect to controls.

Comparing each cohort's mean survival time also evidenced a survival benefit for masitinib. The mean post-paralysis survival time was respectively 30 ± 8 days and 27 ± 4.3 days for day 1 and day 7 masitinib cohorts, which were both significantly longer than the 20 ± 3.8 days observed for vehicle-treated rats (Fig. 5b, $p < 0.0016$ and 0.0003, respectively). The protective effect of masitinib was equally observed in both female and male rats (see Additional file 1: Figure S2A, B).

Discussion

Tyrosine kinase inhibitors are a well-established class of drugs typically used to suppress or decrease cancer cell proliferation and modulate the associated tumor microenvironment [17, 18]. Here, we show evidence that the tyrosine kinase inhibitor masitinib therapeutically modulates the neuroinflammation associated with the ALS progression. Masitinib reduced microgliosis and the subsequent emergence of aberrant glial cells in the degenerating spinal cord, which is consistent with a potent effect in microglial cell cultures, downregulating proliferation, migration, and inflammatory transcriptional profile. Remarkably, treatment of already paralytic rats with masitinib resulted in an unprecedented increase (~40 %) in post-paralysis survival in both genders. While other drugs modulating glial cell inflammation can prolong survival in ALS models [19], our study is the first one showing a protective effect when the drug is delivered post-paralysis. Such a therapeutic approach is appealing in the clinical setting of ALS where drug treatment is initiated only after overt motor symptoms.

Tyrosine kinase inhibition with masitinib sharply decreased the number microglia cells expressing Iba1, CD206, and CD68 and the appearance of aberrant glial cells in the lumbar spinal cord, thus supporting the concept that preventing the emergence of aberrant glial cells moderates the accelerated paralysis progression characteristic of SOD1^{G93A} rats. Our results also anticipate that tyrosine kinase inhibition could be also protective in other ALS models involving overt glial activation [20, 21]. Indeed, the SOD1^{G93A} rat appears as a useful model to study drugs with post-paralysis effects through the modulation of CNS neuroinflammation, contrasting with SOD1^{G93A} mice where distal axonopathy appears as a more important determinant of post-paralysis survival [15, 22].

The inhibition of receptor and non-receptor tyrosine kinases controlling inflammation and exaggerated glial cell activation appears as the most plausible mechanism of action of masitinib. Compared to previous studies in ALS murine models based on ablation of proliferating

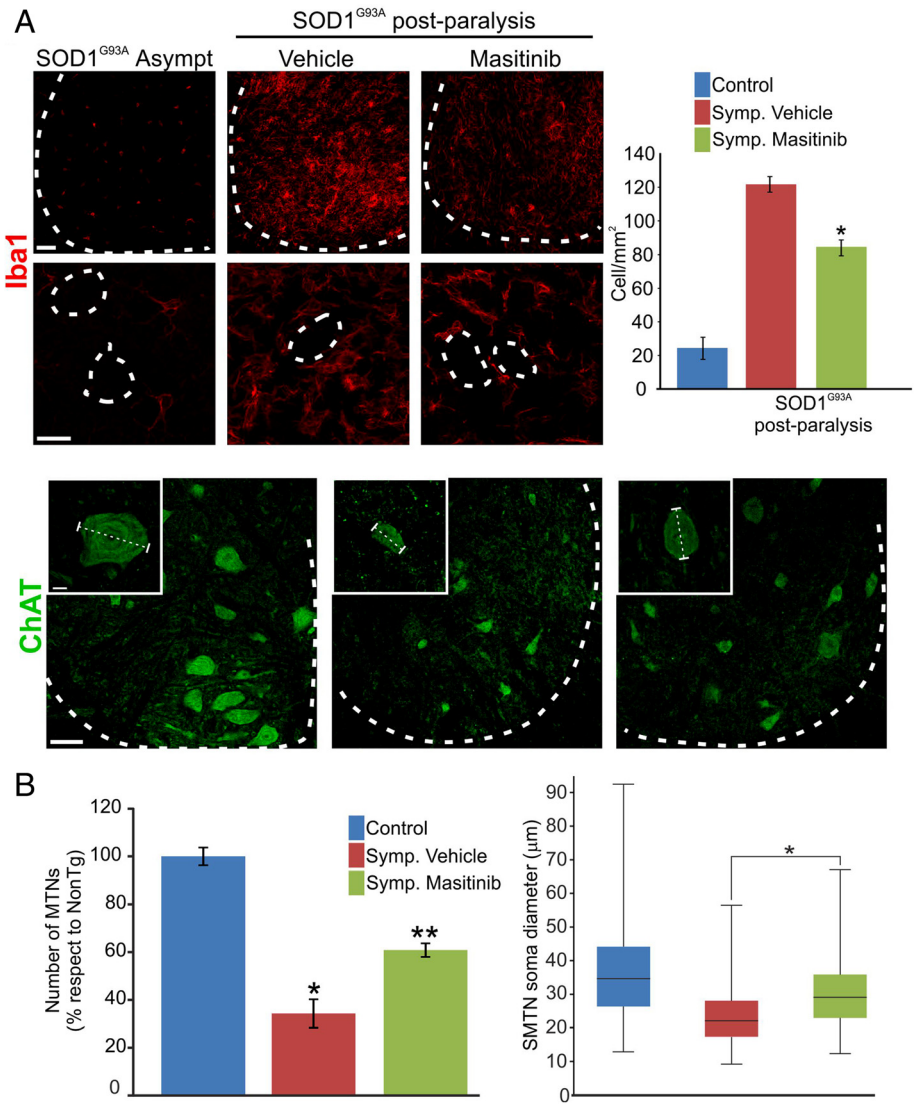

Fig. 4 Masitinib ameliorates microgliosis and motor neuron pathology. **a** Microglia marker Iba1 confocal images. Note the significant reduction of microglial cells in masitinib-treated rat spinal cords when compared with vehicle-treated ones. High magnification panels show a significant reduction in the number of microglial cells that surround motor neurons after masitinib chronic treatment (*scale bars* 50 µm low magnification and 20 µm high magnification). **b** Confocal image of ChAT in the lumbar spinal cord (*dotted line*) indicates the border between white and grey matter (*scale bar* 50 µm). The *graph* below to the *left* represents the quantitative analysis showing the number of motor neurons in the ventral horn in each condition. The *graph* to the *right* represents the quantitation of the motor neuron soma diameter showing the decreased diameter of surviving motor neurons in vehicle-treated rats and the protective effect of masitinib (*insets* in a) (*scale bar* 10 µm). All data are expressed as mean ± SEM $*p < 0.01$, $**p < 0.01$

Table 1 Characteristics of SOD1^{G93A} rats used in the post-paralysis masitinib trial

	Vehicle	Masitinib (>day 1)	Masitinib (>day 7)
Age at onset (days)	187 ± 15	182 ± 25	198 ± 14
Weight at onset (g)	315 ± 56	306 ± 16	323 ± 71
Weight at end-stage (g)	235 ± 13	214 ± 50	211 ± 50
Survival range (days)	174–234	177–249	201–246

The table shows characteristics of the consolidated studies for rats treated with vehicle, masitinib starting 1 (>day 1) and 7 (>day 7) days after paralysis onset. Age and weight values are expressed as mean ± SD. Survival range indicates the age of rats at the time animals reached end-stage of paralysis

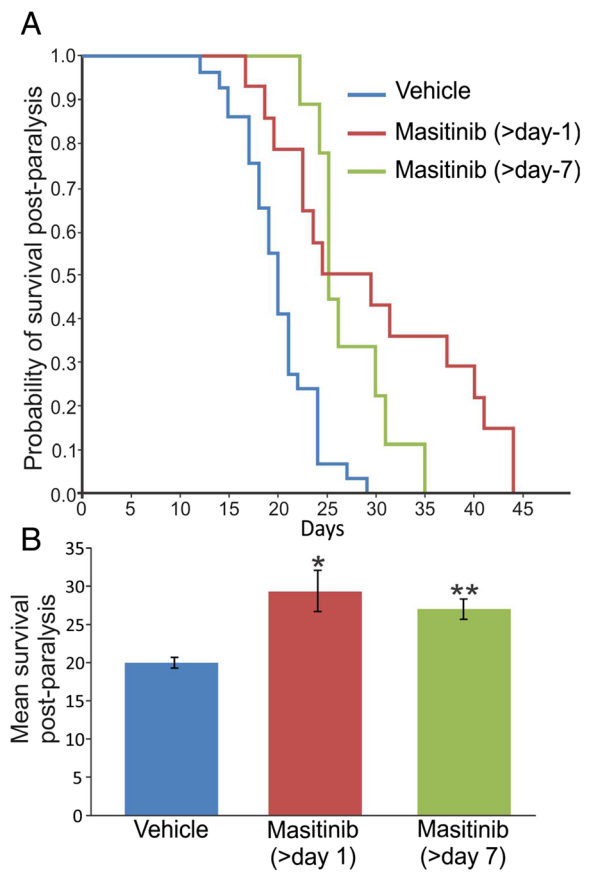

Fig. 5 Masitinib treatment after paralysis onset increased survival of SOD1^{G93A} transgenic rats. **a** Kaplan-Meier survival curves from masitinib-treated and vehicle-treated SOD1^{G93A} rats. SOD1 ^{G93A} transgenic rats were treated with masitinib (30 mg/kg/day) or vehicle (water, $n = 29$, *blue line*) immediately after observation of paralysis onset of one limb (day 1; $n = 14$, *red line*) or starting 7 days after paralysis onset (day 7, $n = 9$, *green line*). There was a statistically significant difference in the probability of survival for both masitinib-treated groups when compared with vehicle-treated group, according to the log-rank test of the Kaplan-Meier analysis ($p < 0.0006$ for masitinib—gait onset vs. vehicle and $p < 0.00025$ for masitinib—7 days onset vs. vehicle). **b** The *graph* shows the mean survival of the three different groups. All data are expressed as mean ± SEM. $p < 0.01$ was considered significant

microglial cells [23–25], treatment with masitinib does not eliminate the proliferating microglia. Rather, it more likely modulates the proliferation and inflammatory signaling underlying the emergence of aberrant glial cells, representing a new pharmacological approach to control detrimental neuroinflammation.

In particular, we found that masitinib inhibits purified recombinant CSF-1R kinase activity at nanomolar concentrations and reduces M-CSF-induced microglia proliferation and migration ability in vitro, suggesting that it regulates a key inflammatory pathway, thus promoting microglia expansion and invasive behavior. Previous reports have shown that activation of CSF-1R by the agonist M-CSF or interleukin 34 potently regulates macrophage/microglia number and inflammatory phenotype in animal models [26, 27]. Recent reports have shown that motor neurons express M-CSF upon damage causing the expansion of surrounding spinal microglial cells [9, 28], thus prompting a pathogenic pathway where motor neuron pathology exacerbates deleterious microgliosis. In accordance, M-CSF levels are elevated in ALS patients as well as in ALS mouse models and may represent a key pathway exacerbating microgliosis and ALS progression [29–31]. Moreover, a recent report has shown that CSF-1R blockade with the drug GW2580 administered to ALS mice several weeks before paralysis onset decreased both microgliosis and slowed disease progression [32].

Although masitinib is a relatively selective kinase inhibitor [33] for CSF-1R, it also targets a few other tyrosine kinases such as PDGF-R, c-Kit, Lyn, and Fyn [10, 11], whose activation may also be associated with the modulation of the neurodegenerative microenvironment. A number of tyrosine kinase inhibitors targeting specific receptors have been approved over the last 5 years for many different types of cancer. However, there are few reports of central nervous system adverse effects or direct neuronal damage. The effect of masitinib, as well as other tyrosine kinase inhibitors, is not selective for a specific cell type, because it blocks several kinases expressed in many cell types. However, masitinib is one of the most selective kinase inhibitors currently in development and as such, potentially exerts a low toxicity profile [11]. In addition, the effects of inhibition of kinase targeted by masitinib are more involved in cell proliferation (c-Kit, PDGF-R, and MCSF/CSF-1R) and immune activation (Lyn, Fyn) than apoptosis. Thus, post-mitotic cells and particularly neurons and resting astrocytes are generally less vulnerable to pharmacological tyrosine kinase inhibition by masitinib as reported previously [13, 34]. Furthermore, inhibition of the non-receptor c-Abl kinase by imatinib has been shown to prevent astrocyte-induced motor neuron death in cell cultures [34], further suggesting an alternative neuroprotective pathway unrelated to neuroinflammation. Such a unique combination of molecular effects could explain our results showing the potent effect of masitinib downregulating the expression of inflammatory mediators, characteristic of deleterious aberrant glial cell phenotype. Thus, further work will be required to decipher the precise tyrosine kinases deregulated in ALS and their pharmacological targeting.

The present study does not establish whether masitinib targets inflammatory cells outside the CNS also known to influence motor neuron degeneration [35, 36]. In particular, masitinib may target the peripheral monocyte/macrophage system, which appears affected in ALS animal models. Immunological downregulation of Ly6C^{hi}

monocytes that infiltrate the degenerative spinal cord attenuates motor neuron loss and delays disease progression in mutant SOD1 mice [37]. Masitinib could also target macrophages that infiltrate and promote degeneration of peripheral motor axons [38]. Moreover, macrophage activation and microgliosis are known to be influenced by mast cells located inside or outside the blood-brain barrier [39]. Because masitinib potently prevents mast cell differentiation and activation [10], it could also indirectly regulate neuroinflammation by targeting mast cells through the inhibition of c-Kit, Lyn, and Fyn. Future studies in patients and animal models are needed to determine alternative mechanisms of action of tyrosine kinase inhibitors and masitinib in ALS.

There are currently no effective treatments for ALS. Riluzole, an anti-glutamatergic drug, is the sole authorized product used in ALS, providing a modest improvement in survival (~3 months) [40]. Over the past 10 years, a number of drugs were identified as providing survival benefits in rodent preclinical trials [19]. However, none of them proved to be clinically better than riluzole in ALS patients. Such incongruence could be explained, at least in part, by the fact that most animal studies that were translated to clinical trials have been started before paralysis onset. Therefore, tyrosine kinase inhibition with masitinib appears unique among other ALS-developmental drugs because it exerts neuroprotection when administrated post-paralysis. A randomized phase III clinical trial testing the effect of masitinib in ALS patients is currently running (Clinicaltrial.gov NCT02588677).

Conclusions

The present study shows that tyrosine kinase inhibition with masitinib is capable of controlling microgliosis, neuroinflammation, and the emergence/expansion of aberrant glial cells in $SOD1^{G93A}$ rats. Remarkably, masitinib significantly prolonged survival when delivered after paralysis onset, an unprecedented effect in preclinical models of ALS, and therefore appears well-suited for treating ALS where drug treatment is initiated only after diagnosis based on overt motor symptoms.

Additional file

Additional file 1: Figures S1. Masitinib inhibited microgliosis along the degenerating spinal cord. **Figure S2.** Masitinib treatment after paralysis onset increased survival of $SOD1^{G93A}$ female and male rats. (DOCX 700 kb)

Abbreviations

ALS, amyotrophic lateral sclerosis; BrdU, bromodeoxyuridine; ChAT, choline acetyltransferase; c-Kit, stem cell growth factor receptor (SCFR); CNS, central nervous system; Cox2, cyclooxygenase-2; CSF-1R, colony-stimulating factor 1 receptor; FBS, fetal bovine serum; FLT3, fms-related tyrosine kinase 3; Iba1, ionized calcium binding adaptor molecule 1; IL1β, interleukin 1 beta; IL6, interleukin 6; M-CSF, macrophages colony-stimulating factor; PDGF-R, platelet-derived growth factor receptor; SOD1, superoxide dismutase 1

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Availability of data and materials

There is no data, software, databases, application/tool available apart from the reported in the present study. Data supporting the conclusions are presented in the manuscript. For other information please contact corresponding author.

Authors' contributions

ET, TTM, ICM, JB, OH, and LB designed the study. ET, SI, RBN, JB, MG, LG, PD, PDA, PC, and LMP performed the experiments. ET, SI, RBN, TTM, LG, and LB analyzed the data. ET, SI, RBN, JB, OH, and LB prepare the manuscript. All authors contributed to discussion of the results and edited and approved the final version.

Competing interests

OH and PD are cofounder and shareholder of AB Science, LG is an employee of AB Science, JB received salary from AB Science, and others declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All procedures using laboratory animals were performed in accordance with the national and international guidelines and were approved by the Institutional Animal Committee for animal experimentation. This study was carried out in strict accordance with the Instituto de Investigaciones Biológicas Clemente Estable Bioethics Committee's requirements and under the current ethical regulations of the Uruguayan Law No. 18.611 for animal experimentation that follows the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA).

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Estudio 2: Evaluación del potencial neuroprotector de nitrolípidos a través de la regulación de la vía antioxidante Nrf2/ARE en astrocitos

Artículo 4

Diaz-Amarilla P, Miquel E, Trostchansky A, Trias E, Ferreira AM, Freeman BA, Cassina P, Barbeito L, Vargas MR, Rubbo H. Electrophilic nitro-fatty acids prevent astrocyte-mediated toxicity to motor neurons in a cell model of familial amyotrophic lateral sclerosis via nuclear factor erythroid 2-related factor activation. *Free Radic Biol Med. 2016 Jun;95:112-20*

El rol del estrés oxidativo en la muerte neuronal que tiene lugar en la ELA ha sido estudiado durante décadas y ha sido el blanco de un importante número de estudios preclínicos (Vargas et al. 2010). En este sentido, la inducción de la vía antioxidante y citoprotectora regulada por el factor de transcripción Nrf2 (del inglés; *Nuclear factor-erythroid 2-related factor 2*) es una de las estrategias más usadas para limitar el daño causado por el incremento del estrés oxidativo a nivel celular. Nrf2 regula la expresión de una batería de enzimas y proteínas antioxidantes mediante su unión a una secuencia ubicada en el promotor de estos genes conocida como ARE (*Antioxidant Response Element)* (Zhang et al. 2006). En condición normales, Nrf2 se encuentra unido Keap1 (*del inglés; Kelch-like ECH-associated protein 1*) una proteína que se ancla al citoesqueleto de actina y promueve la degradación de Nrf2 a través del proteosoma. En presencia de estrés oxidativo, Nrf2 se libera del Keap1 se transloca en el núcleo donde induce la expresión de los genes que poseen una secuencia ARE en su promotor (Kensler et al. 2007). Se ha demostrado que la inducción de la vía Nrf2/ARE impacta significativamente en la capacidad de la célula para soportar y sobrevivir en condiciones inflamatorias crónicas y el estrés agudo generado por diversas condiciones metabólicas (Zhang et al. 2006).

Diversos estudios han demostrado que la activación de la vía Nrf2/ARE en astrocitos aumenta su capacidad antioxidante y previene la muerte de

motoneuronas inducida por astrocitos $SOD1^{G93A}$ (Vargas et al. 2006; Pehar et al. 2007). En el mismo sentido, la sobre-expresión de Nrf2 específicamente en astrocitos produjo un significativo retraso en la aparición de los síntomas en ratones $SOD1^{G93A}$ (Vargas et al. 2008). Muchas moléculas exógenos y endógenas tienen la capacidad de activar la vía Nrf2/ARE ya que inducen la liberación de Nrf2 de Keap1 y su translocación al núcleo. Por ejemplo, el tratamiento de astrocitos $SOD1^{G93A}$ con terbutil hidroquinona (TBHQ), un inductor prototípico de Nrf2, indujo un aumento en la síntesis de glutatión y previno la muerte de motoneuronas en cultivo (Vargas et al. 2006). Es por ello que la búsqueda de nuevos inductores de la vía Nrf2/ARE para el tratamiento de diferentes patologías con involucran estrés oxidativo constituye una línea de investigación muy activa.

En este trabajo evaluamos la capacidad inductora de la vía Nrf2/ARE de dos nitrolípidos, nitro-araquidónico ($AA\text{-}NO_2$) y nitro-oleico ($OA\text{-}NO_2$), en astrocitos y su efecto neuroprotector para motoneuronas. El tratamiento de astrocitos no transgénicos y $SOD1^{G93A}$ con $AA\text{-}NO_2$ u $OA\text{-}NO_2$ indujo la translocación de Nrf2 al núcleo y un aumento significativo en la expresión de genes antioxidantes. El pre-tratamiento de astrocitos $SOD1^{G93A}$ con $AA\text{-}NO_2$ u $OA\text{-}NO_2$ previno la muerte de motoneuronas espinales en co-cultivo. El efecto neuroprotector, desapareció cuando se silencio la expresión de Nrf2 en astrocitos, indicando que la vía Nrf2/ARE astrocitaria es clave en la capacidad neuroprotectora inducida el tratamiento con nitrolípidos. Dado que tanto $AA\text{-}NO_2$ u $OA\text{-}NO_2$ pueden generarse endógenamente en condiciones inflamatorias, el potencial terapéutico de estas moléculas genera un interés particular. Teniendo en cuenta que el SNC es un tejido altamente enriquecido en ácidos grasos como acido araquidónico y acido oleico, el tratamiento de patología neurodegenerativas con estas moléculas nitradas resulta una estrategia terapéutica muy atractiva. Los resultados obtenidos en este trabajo sugieren que el uso de los nitrolípidos $AA\text{-}NO_2$ u $OA\text{-}NO_2$ podría resultar efectivo en el tratamiento de la ELA a través de la inducción de la vía Nrf2/ARE.

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Original Contribution

Electrophilic nitro-fatty acids prevent astrocyte-mediated toxicity to motor neurons in a cell model of familial amyotrophic lateral sclerosis via nuclear factor erythroid 2-related factor activation

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ABSTRACT

Nitro-fatty acids (NO_2-FA) are electrophilic signaling mediators formed in tissues during inflammation, which are able to induce pleiotropic cytoprotective and antioxidant pathways including up regulation of Nuclear factor erythroid 2-related factor 2 (Nrf2) responsive genes. Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by the loss of motor neurons associated to an inflammatory process that usually aggravates the disease progression. In ALS animal models, the activation of the transcription factor Nrf2 in astrocytes confers protection to neighboring neurons. It is currently unknown whether NO_2-FA can exert protective activity in ALS through Nrf2 activation. Herein we demonstrate that nitro-arachidonic acid (NO_2-AA) or nitro-oleic acid (NO_2-OA) administrated to astrocytes expressing the ALS-linked $hSOD1^{G93A}$ induce antioxidant phase II enzyme expression through Nrf2 activation concomitant with increasing intracellular glutathione levels. Furthermore, treatment of $hSOD1^{G93A}$-expressing astrocytes with NO_2-FA prevented their toxicity to motor neurons. Transfection of siRNA targeted to Nrf2 mRNA supported the involvement of Nrf2 activation in NO_2-FA-mediated protective effects. Our results show for the first time that NO_2-FA induce a potent Nrf2-dependent antioxidant response in astrocytes capable of preventing motor neurons death in a culture model of ALS.

1. Introduction

Nitro-fatty acids (NO_2-FA, nitroalkenes) are electrophilic products formed by the nitration of unsaturated fatty acids [1]. These species trigger signaling cascades *via* covalent and reversible post-translational modifications of susceptible nucleophilic amino acids in transcriptional regulatory proteins and enzymes. Moreover, NO_2-FA can activate heat shock [2] as well as antioxidant response pathways [3]. As electrophiles, nitroalkenes activate the nuclear factor-erythroid 2-related factor 2 (Nrf2) [2,4]. Nrf2, a member of the cap "n" collar transcription factor family, represents a master regulator of the Antioxidant Response Element (ARE)-regulated genes. The binding of Nrf2 to the *cis*-acting DNA promoter sequence ARE allows transactivation of a group of cytoprotective genes that encode proteins known as phase-II enzymes. In normal conditions Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1), and through a two-site interaction, the transcription factor is ubiquitinated by Cul3/Rbx1 and targeted for degradation [5,6]. During oxidative stress the two-site interaction between Nrf2 and Keap1 is disrupted, allowing Nrf2 to evade Keap1-

Abbreviations: NO_2-FA, Nitro-fatty acids; SOD1, Cu/Zn superoxide dismutase; ALS, Amyotrophic Lateral Sclerosis; NO_2-OA, Nitro-oleic acid; NO_2-AA, Nitro-arachidonic acid; Nrf2, Nuclear factor-erythroid 2-related factor 2; ARE, Antioxidant Response Element; Keap1, Kelch-like ECH-associated protein 1; HO-1, Hemoxygenase-1; tBHQ, *tert*-butylhydroquinone; AA, Arachidonic acid; OA, Oleic acid; NQO1, NAD(P)H:quinone oxidoreductase 1; Srnx1, Sulfiredoxin 1; GCL, Glutamate-cysteine ligase

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mediated ubiquitination and accumulate in the nucleus where it activates genes containing an ARE sequence within their promoters, leading to an induction of antioxidant response. Upregulation of the ARE-driven gene battery has a significant impact on the ability of the cell to withstand and survive inflammatory and metabolic stress [6]. Among phase-II enzymes, hemoxygenase-1 (HO-1) has attracted special attention because of its therapeutic effects in neurodegenerative disease models [7]. Hemoxygenase-1 oxidatively cleaves heme to biliverdin, forms CO and releases the chelated $Fe2^{+}$. Bilirubin (a reduction product of biliverdin) also serves as a potent radical scavenger and protects neuronal cells against oxidative stress at nanomolar concentrations [8]. Two isoforms of heme oxygenase have been characterized: a constitutive isoform, HO-2, and the inducible enzyme, HO-1 [9]. The induction of HO-1 counteracts oxidative damage and confers cytoprotection [10,11]. In the nervous system, HO-1 can be highly induced in glia by its substrate heme and by a variety of pro-oxidant, inflammatory stimuli and trophic factors [12–15]. In accordance, increased HO-1 expression is observe in neurodegenerative diseases involving glial activation, such as Alzheimer's and Parkinson's diseases and Amyotrophic Lateral Sclerosis (ALS) [7,16].

Amyotrophic Lateral Sclerosis is the most common adult-onset motor neuron disease caused by the progressive degeneration of motor neurons in the spinal cord, brain stem and motor cortex [17]. Approximately 10–20% of familial ALS is cause by a toxic gain-of-function induced by mutations of the Cu/Zn-superoxide dismutase (SOD1) [18]. Rodents over-expressing mutated forms of hSOD1 generally develop an ALS-like phenotype [19,20]. Although the molecular mechanism underlying this toxic gain-of-function remains unknown, toxicity to motor neurons requires mutant SOD1 expression in non-neuronal cells as well as in motor neurons [21]. In ALS patients and rodent models, a strong glial reaction typically surrounds degenerating motor neurons [22]. Astrocytes isolated from $hSOD1^{G93A}$ rats [23] or mice [24] are toxic to co-cultured motor neurons, suggesting a role of glial cells in motor neuron degeneration [22,25–27].

Increased oxidative stress has been implicated in the pathogenesis of ALS and a variety of antioxidants have been tested [28]. Several studies have demonstrated that Nrf2 activation in astrocytes can improve antioxidant defenses and prevent the motor neuron loss induced by $SOD1^{G93A}$ astrocytes [23,29]. Consistently, it has been shown that ALS-mice with specific Nrf2 overexpression in astrocytes developed the onset of the disease later, increasing survival and exhibiting lower glial reactivity [30]. However, signaling by the Nrf2-ARE pathway may be initiated by several mechanisms not necessarily involving Nrf2 overexpression. In fact, there may be multiple approaches for targeting Nrf2 as therapeutic tool where exogenous and endogenous molecules have demonstrated an ability to activate Nrf2 and induce cytoprotective genes [31]. Furthermore, Nrf2 and HO-1 levels are increased and co-localized with reactive astrocytes in the degenerating lumbar spinal cord of $hSOD1^{G93A}$ rats, suggesting this pathway counteracts accelerated disease progression [15]. On the other hand, astrocytic induction of Nrf2 by *tert*-butylhydroquinone (tBHQ), a prototypic inducer of Nrf2 activation, has also prevented motor neuron death induced by $SOD1^{G93A}$ astrocytes *in vitro* through an increase in glutathione biosynthesis [23]. Considering that prophylactic activation of Nrf2 in astrocytes has shown to be a plausible strategy to ameliorate neuronal dysfunction and death, herein we explored the use of nitro-arachidonic acid (NO_2-AA) and nitro-oleic acid (NO_2-OA) [32,33] as potential Nrf2 activators in astrocytes and their effects on motor neuron survival.

2. Material and methods

2.1. Materials

Culture media and serum were obtained from Life Technologies, Inc. Primers were obtained from Integrated DNA Technologies, Inc. Antibody to HO-1 was from StressGen Biotech, antibodies to β-actin was from Sigma, antibodies against carboxyl and amino termini of Nrf2 and histone H1 were from Santa Cruz Biotechnology. Arachidonic acid (AA) and oleic acid (OA) were purchased from Nu-Check Prep (Elysian, MN). All other reagents were from Sigma unless otherwise specified.

2.2. Nitro-fatty acids

Synthesis and quantitation of NO_2-AA and NO_2-OA were performed as previously and analyzed for purity by 1H NMR and HPLC-MS [32,33]. A mixture of NO_2-AA isomers was obtained: 12- and 15-NO_2-AA (23%), 9-NO_2-AA (55%) and 14-NO_2-AA (22%). No differences between batches were observed. Nitro-oleic acid is an equimolar mixture of 9- and 10-nitro-octadec-9-enoic acid.

2.3. Cell cultures

Primary astrocytes cultures were prepared from non-Tg or hemizygous rats and mice as indicated. Animals: Transgenic ALS Sprague-Dawley rats carrying the G93A mutated human SOD1, strain NTac:SD-TgN(SOD1G93A)L26H, were obtained from Taconic (Hudson, NY; [20]) and were bred locally by crossing hemizygous male carriers to wild-type Sprague-Dawley female rats. Transgenic $SOD1^{G93A}$ ALS mice, strain B6SJL-TgN(SOD1-G93A)1Gur [19], were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were bred locally by crossing hemizygous male carriers to B6SJLF1 female hybrids. The offspring was genotyped as previously described [34]. The animals were housed under controlled conditions with free access to food and drinking water.

2.4. Ethics statement

Procedures using laboratory animals were in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for the International Organizations of Medical Sciences and were approved by the Institutional Animal Committee resolution no. 66 (Exp. no. 071140-001465-10); Comisión honoraria de experimentación animal de la Universidad de la República (CHEA; http://www.chea.udelar.edu.uy). The offspring was genotyped at birth as described previously [35]. The non-transgenic littermates were used as controls. Primary cortical or spinal astrocyte cultures were prepared from 1–2 day-old rat pups according to the procedure of Saneto and De Vellis with minor modifications [36]. *Rat cortical astrocytes* were used for cell yield purposes and compared with findings in spinal cord rat o mice astrocytes. Previous reports indicate that astrocyte mediated-motor neuron toxicity is detected in astrocytes from spinal cord and cerebral cortex [34] and also from rats and mice $SOD1^{G93A}$ transgenic rodent models of ALS [37]. Cells were plated at a density of 2×10^4 cells/cm^2 in 35-mm Petri dishes or 24-well plates and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, HEPES (3.6 g/l), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Astrocyte monolayers were >98% pure as determined by GFAP immunoreactivity and were devoid of OX42-positive microglial cells. ***Motor neuron cultures*** were prepared from embryonic day 15 (E15) wild-type rat spinal cord by a combination of OptiPrep™ gradient centrifugation and immunopanning with the monoclonal antibody Ig192 against p75 neurotrophin receptor, as previously

described [38]. Neurons were directly plated on astrocyte monolayers at a density of 300 cells/cm^2 and maintained for 72 h in L15 medium supplemented as previously described [36].

2.5. Cell treatment and transient transfection

Confluent astrocyte monolayers from cortex and spinal cord were changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum prior to treatment. Astrocytes were treated for 24h with vehicle (MetOH, 1:400), AA, OA, NO_2-AA or NO_2-OA, homogenized and then proteins analyzed by Western blot. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Astrocytes were transfected with mammalian expression control vector (pEF) or dominant-negative mutant Nrf2 (Nrf2-DNM), kindly provided by Dr. Jawed Alam (Alton Ochsner Clinic Foundation, New Orleans, LA) [39]. Post-transfection astrocytes were treated with nitroalkenes as before and protein analyzed by Western blot.

2.6. siRNA transfection

Confluent spinal cord astrocyte monolayers were changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum prior to treatment. siRNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Astrocytes were transfected with 40 nM of predesigned dicer-substrate siRNAs (DsiRNAs) targeting Nrf2 mRNA (ID#RNC.RNAI.N031789.12.1, RNC.RNAI.N031789.12.2, RNC.RNAI.N031789.12.3, IDT) or negative control DsiRNA (DS NC1, IDT) 24 h before either total RNA isolation using TRIzol reagent (Invitrogen) for quantitative PCR, or treatment with vehicle or nitrated fatty acids for co-culture experiments.

2.7. Whole cell and nuclear extracts

After treatment, astrocytes were washed with cold PBS and whole cell extracts were prepared in 50 mM HEPES, pH 7.5, 50 mM NaCl, 1% Triton X-100 and Complete protease inhibitor mixture (Roche) and sonicated 3 times for 3 s. Protein concentration was measured by the bicinchoninic acid method (Pierce). Nuclear cell extracts were prepared as described by Schreiber and cols. [40]. Briefly, cells were resuspended in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, and 1 g/ml leupeptin). The cells were allowed to swell on ice for 15 min, after which 0.5% Nonidet P-40 was added and the tube was vortex mixed for 10 s. The homogenate was centrifuged for 30 s and nuclear pellet was resuspended in ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM each of dithiothreitol, EDTA, EGTA, and phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, and 1 g/ml leupeptin). The tube was mixed thoroughly and vigorously rocked at 4 °C for 15 min. The nuclear extract was centrifuged at 11,000*g* for 5 min at 4 °C and the supernatant containing nuclear proteins was removed, quantified and stored in loading buffer for western blot.

2.8. Real-time PCR

Confluent astrocyte monolayers were changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum prior to treatment. Astrocytes were treated with vehicle, nitrated or non-nitrated fatty acids for 12 h. Total RNA was isolated using TRIzol reagent (Invitrogen). 2 μg of RNA were randomly reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCRs were carried out in a 20 μl reaction with 1 × SYBR Green PCR Master Mix Applied Biosystems containing 1 μl of cDNA and 20 pmoles of each specific primer in aStepOnePlus™ Real-Time PCR System (Life technologies). The cycling parameters were as follows: 95 °C, 10 s; 55 °C, 10 s; 72 °C, 15 s. Specific primers for Nrf2, HO-1, NQO1, GCLC, GCLM and β-Actin were used [30]. Primers for Sulfiredoxin-1 were:

Srxn1/5′GGCTTGGTTACTCTTGTTGCCTCT
Srxn1/3′GGGTGCTTTGCTCGAATGTGTTG.

2.9. Glutathione measurement

Confluent astrocyte monolayers were changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum prior to treatment. Astrocytes were treated with vehicle as control or different concentrations of AA, NO_2-AA, OA or NO_2-OA for 24 h. Cells were lysed with ice-cold 3% perchloric acid and total glutathione levels (GSH and GSSG) determined using the Tietze method as previously described [23]. Glutathione content was corrected by protein concentration determined as explained previously.

2.10. Co-culture experiments

Non-transgenic as well as SOD1^{G93A} spinal cord astrocyte monolayers were changed to L15 medium supplemented with 0.63 mg/ml sodium bicarbonate, 5 μg/ml insulin, 0.1 mg/ml conalbumin, 0.1 mM putrescine, 30 nM sodium selenite, 20 nM progesterone, 20 mM glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2% horse serum. Astrocytes were treated for 24 h with different concentrations of nitroalkenes. After washing twice with phosphate buffered saline (PBS) wild-type motor neurons were plated on top at a density of 350 cells/cm^2. Co-cultures were maintained in L15 medium supplemented with 0.63 mg/ml sodium bicarbonate, 5 μg/ml insulin, 0.1 mg/ml conalbumin, 0.1 mM putrescine, 30 nM sodium selenite, 20 nM progesterone, 20 mM glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2% horse serum for 48 h. Motor neuron survival was assessed after fixing the cells and immunostained for p75NTR. Counts were performed over an area of 0.9 cm^2 in 24-well plates and by counting all cells displaying intact neurites longer than 4 cells in diameter [26].

2.11. Statistics

All statistics were performed using Sigmaplot 12 (Systat software, San Jose, CA, USA), GraphPad Prism 5.0 or GraphPad InStat software, version 3.06.

3. Results

3.1. NO_2-FA induce Nrf2 activation-dependent HO-1 expression in astrocytes

Cultured non-transgenic (non-Tg) astrocytes were exposed to nitro-arachidonic acid (NO_2-AA) or arachidonic acid (AA) 5 μM for 24 h. In these experimental conditions, NO_2-AA-but not AA-induced a significant accumulation of Nrf2 in the nucleus (Fig. 1A). As a positive control *tert*-butyl hydroquinone (tBHQ), an electrophilic activator of Nrf2, was included (Fig. 1A). In addition, this treatment determined a potent increase in hemoxygenase-1 (HO-1) protein levels (Fig. 1B). As expected, HO-1 protein levels were not detected in untreated or non-nitrated fatty acid-treated cells. To further support the role of Nrf2 in HO-1 expression, transfection of astrocytes with a dominant negative Nrf2 plasmid

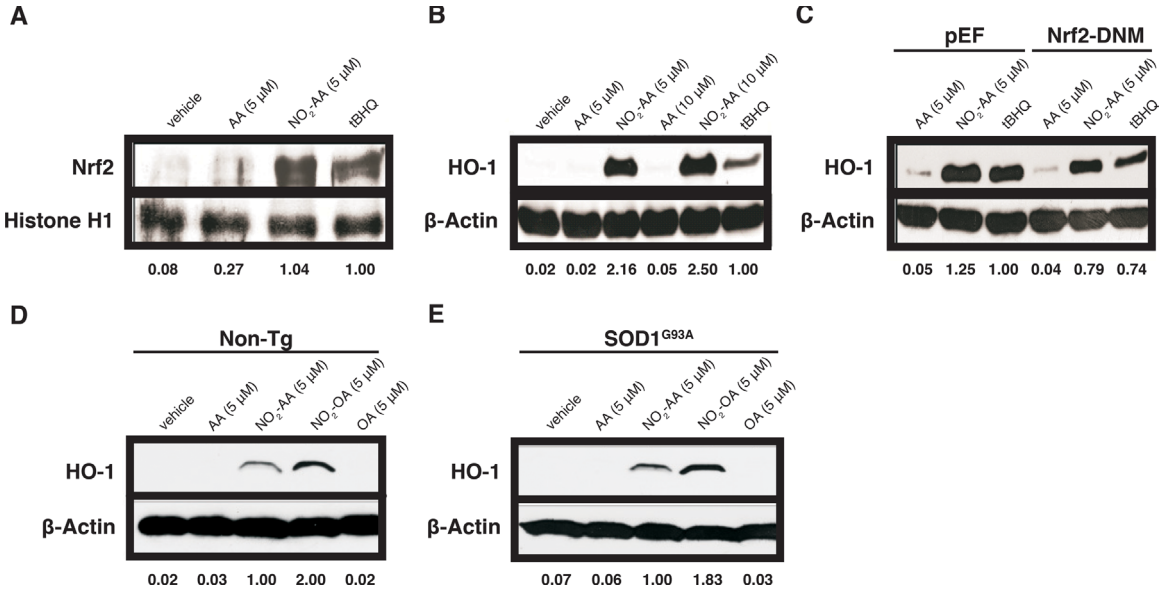

Fig. 1. NO_2-FA induce HO-1 expression in astrocytes *via* Nrf2. (A) Nrf2 activation was determined by western blot of a nuclear extract from astrocytes treated with NO_2-AA for 6h. Controls with AA or tBHQ were included. (B) Confluent astrocyte monolayers were treated with MetOH (vehicle), AA (5 and 10 μM), NO_2-AA (5 and 10 μM) or tBHQ (40 μM). After 24 h, HO-1 protein levels were analyzed by western blot. (C) The Nrf2-dependent HO-1 expression was determined by treatment with AA (5 μM), NO_2-AA (5 μM) or tBHQ (40 μM) of astrocytes transfected with a dominant-negative mutant Nrf2 form (Nrf2-DNM). As in figure (B), after 24 h HO-1 expression was analyzed by western blot. Controls were performed by transfecting the cells with an empty vector (pEF). Band intensities were determined and related to the condition with NO_2-AA. (D) Non-transgenic (non-Tg) and (E) $SOD1^{G93A}$ astrocyte monolayers were treated with MetOH (vehicle), AA (5 μM), NO_2-AA (5 μM), OA (5 μM), NO_2-OA (5 μM) as in figure (A). After 24 h, HO-1 protein level was determined by western blot. Band intensities were determined and related to the response elicited by tBHQ (A, B, C) or NO_2-AA (D, E). Results shown are representative of at least three independent experiments.

partially abrogated NO_2-AA and tBHQ-induced HO-1 expression (Fig. 1C). Furthermore, we compared this effect on $SOD1^{G93A}$-expressing astrocytes. As expected, NO_2-AA induced HO-1 expression in both non-Tg (Fig. 1D) and $SOD1^{G93A}$-expressing ones (Fig. 1E). This effect was not selective for NO_2-AA since the use of nitro-oleic acid (NO_2-OA) instead NO_2-AA produced similar induction of HO-1.

3.2. NO_2-FA induce Nrf2/ARE-related genes expression in non-transgenic and $SOD1^{G93A}$ astrocytes

To confirm that the changes observed were not due to increased Nrf2 mRNA levels we performed real-time PCR on astrocytes cultures. Neither NO_2-AA nor NO_2-OA induced changes in the mRNA levels of Nrf2 in either non-Tg or $SOD1^{G93A}$ astrocytes (Fig. 2A). Since HO-1 expression depends almost exclusively on *de novo* gene transcription, we analyzed NO_2-FA effects on HO-1 mRNA levels. A 6-fold increase in HO-1 mRNA was observed after treatment with either NO_2-AA or NO_2-OA as compared with vehicle and non-nitrated fatty acids conditions (Fig. 2B). Similar results were observed when analyzing other Nrf2/ARE-related gene expression: NAD(P)H:quinone oxidoreductase 1 (NQO1) and Sulfiredoxin 1 (Srnx1, Fig. 2C and D).

3.3. NO_2-FA increase glutathione biosynthesis through the glutamate-cysteine ligase modulatory subunit

The increase in intracellular GSH levels is indicative of the activation of the Nrf2/ARE pathway. Transgenic $SOD1^{G93A}$ astrocytes incubated with either NO_2-AA or NO_2-OA exhibited greater levels of GSH+GSSG compared to control AA and oleic acid (OA) treatments (Fig. 3A). This seems to be due to increased expression of the modulatory subunit of glutamate-cysteine ligase (GCLM, Fig. 3B), which is the rate-controlling enzyme in GSH synthesis. No effects on the expression of the catalytic subunit (GCLC, Fig. 3C) were observed. Similar results were observed in non-Tg cells (Fig. 3).

3.4. NO_2-FA inhibit $SOD1^{G93A}$ astrocyte-mediated motor neurons death

A feeder layer of $SOD1^{G93A}$ astrocytes decreased the survival of non-Tg motor neurons by 50% compared with a feeder layer of non-Tg astrocytes (Fig. 4A) as previously described [23]. Neuronal survival on top of a feeder layer of non-Tg astrocytes was considered 100% (dotted line in Fig. 4A). Pre-treatment of $SOD1^{G93A}$ astrocytes with either NO_2-AA or NO_2-OA before motor neuron plating, prevented motor neuron death induced by $SOD1^{G93A}$ astrocytes (Fig. 4A). Control studies showed no effects of NO_2-AA on motor neurons survival when added in the absence of astrocytes (not shown). To find out whether the protective effects of NO_2-FA on $SOD1^{G93A}$ astrocyte-mediated motor neuron death were Nrf2-dependent, we used synthetic siRNAs to induce Nrf2 RNA interference (Fig. 4B and C). Transfection of the astrocyte monolayers with Nrf2-siRNA prior to NO_2-FA treatment abolished the beneficial effect of the treatment on motor neuron survival (Fig. 4C).

4. Discussion

Nitro-fatty acids exert pleiotropic anti-inflammatory and adaptive signaling actions, including activation of HO-1 expression in activated macrophages as well as down regulation of nitric oxide synthase 2 (NOS2) expression and inhibition of pro-inflammatory cytokines secretion [32,41,42]. Significant inhibition of NADPH oxidase assembly and superoxide production by activated macrophages also occurs [43]. Moreover, NO_2-AA is a non-competitive inhibitor of inducible prostaglandin endoperoxide H synthase (PGHS-2) which, in addition to the suppression of NOS2

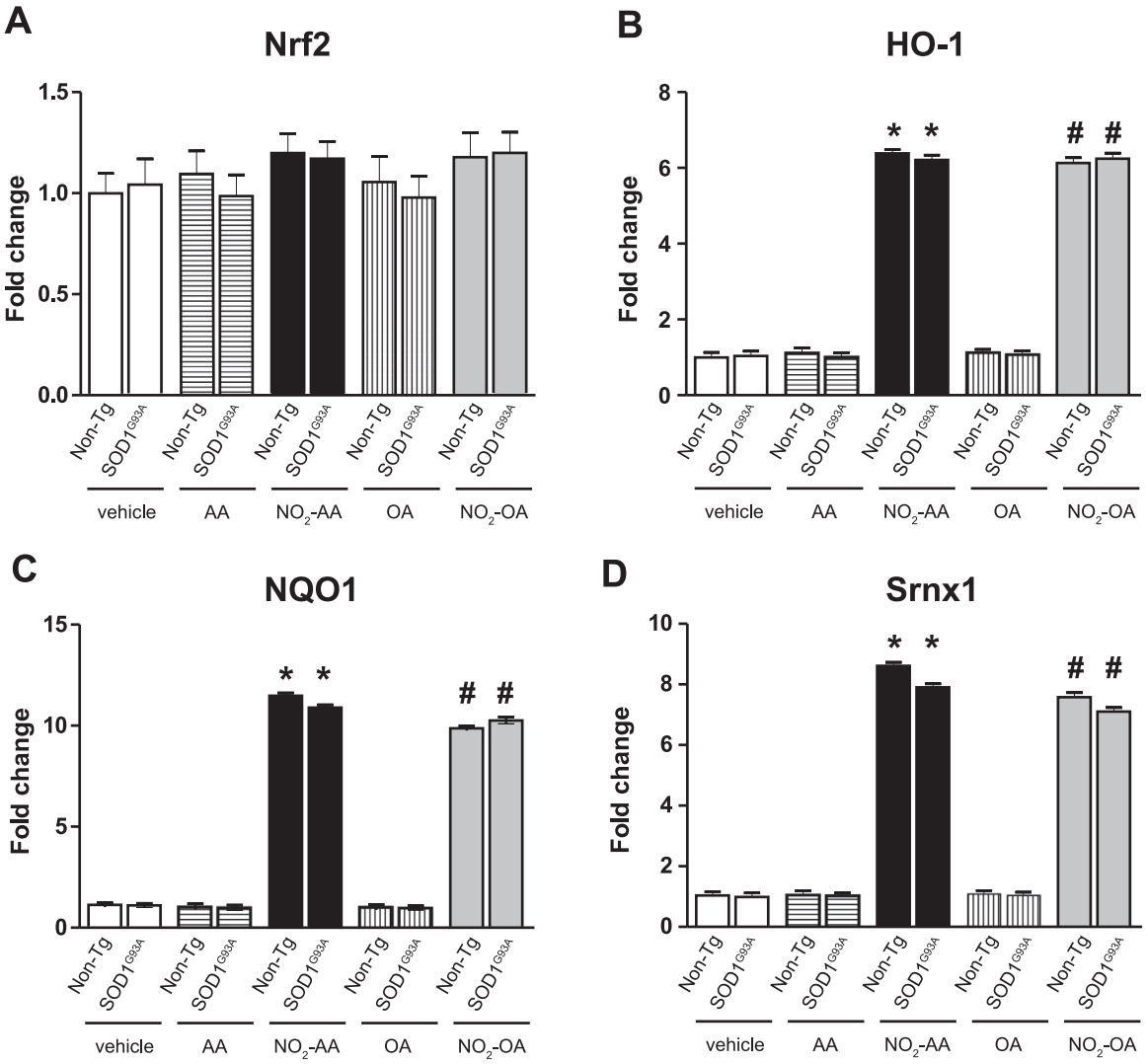

Fig. 2. Phase II antioxidant enzymes are induced by NO_2-FA in $SOD1^{G93A}$ astrocytes. (A) Nrf2 mRNA levels in both non-Tg and $SOD1^{G93A}$ astrocytes were determined by real-time PCR, in the absence or presence of 5 μM NO_2-AA or NO_2-OA. Controls with the non-nitrated AA or OA were included. (B–D) Activation of the Nrf2 pathway by nitro-fatty acids was analyzed by real-time PCR and the mRNA levels of HO-1 (A), NQO1 (B) and Srnx1 (C) are shown. Non-Tg and $SOD1^{G93A}$ cells were incubated with the 5 μM nitrated or non-nitrated fatty acids, as in (A). Results are expressed as the mean ± SD of the fold increase of mRNA respect to vehicle condition, $n=5$. *Significantly different from AA and vehicle ($p < 0.05$); #significantly different from OA and vehicle ($p < 0.05$).

expression after inflammatory stimulus can also contribute to the limitation of inflammatory responses [44,45]. The mechanisms of NO_2-FA incorporation into cells are currently unknown, but once into cells, NO_2-FA could activate Nrf2 through electrophilic-mediated reversible nitroalkylation reactions. Nrf2 activity is principally governed by Kelch-like ECH-associating protein 1 (Keap1) a protein with elevated cysteine content, which renders it highly reactive to electrophiles. Villacorta et al. demonstrated a direct reaction of NO_2-FA with Keap1 impairing Keap1-mediated inhibition of Nrf2/ARE signaling [3].

It is currently unknown whether NO_2-FA exert actions in neurodegenerative diseases. Herein, we demonstrate a potent protective role of NO_2-FA on astrocytes expressing the ALS-linked $SOD1^{G93A}$ mutation-mediated toxicity to motor neurons. The effects of NO_2-FA on motor neuron degeneration induced by astrocytes revealed that NO_2-FA administration to cultured astrocytes caused (a) Nrf2 activation and antioxidant phase II enzymes induction and (b) an increase in total glutathione levels. These effects were independent of changes in Nrf2 mRNA levels. Previous work has shown that NO_2-OA exerted potent antioxidant and anti-inflammatory potential through Nrf2 activation [2–4,42,46]. Herein, the involvement of Nrf2 in astrocyte activation was demonstrated by the observed increase in Nrf2 translocation to the nucleus in the presence of NO_2-FA as well as by a decrease in HO-1 expression when cells were transfected with a negative dominant plasmid and then exposed to NO_2-AA or NO_2-OA.

Approximately 10–20% of familial ALS is caused by a toxic gain-of-function induced by mutations of SOD1 [18]. Over-expression of mutated forms of hSOD1 in rodents resulted in animal models of the disease, e.g. $hSOD1^{G93A}$ rats [20] or mice [19]. Toxicity to motor neurons requires mutant SOD1 expression in non-neuronal cells as well as in motor neurons [21]. Increased motor neurons HO-1 expression occurs in the spinal cord from ALS patients [47]. We have previously shown that both Nrf2 and HO-1 levels were increased and co-localized with reactive astrocytes in the degenerating lumbar spinal cord of $hSOD1^{G93A}$ rats [15]. Herein, we show that HO-1 expression in isolated cultured astrocytes from both transgenic and non-transgenic cells increased following

exposure to NO_2-FA. NQO1 and Srnx1, two other Nrf2-driven genes, were also induced by nitroalkenes in cultured astrocytes. Primary spinal cord astrocyte monolayers support the survival of purified embryonic motor neurons in the absence of added trophic factors [36], where ~50% of motor neurons die when co-cultured with transgenic astrocytes [36]. Thus, the influence of NO_2-AA or NO_2-OA applied to astrocytes bearing the $SOD1^{G93A}$ mutation on astrocyte-mediated motor neurons death in co-culture conditions was explored. Pre-treatment of $SOD1^{G93A}$ astrocytes with either NO_2-AA or NO_2-OA significantly reduced motor neurons loss at low micromolar levels. This effect was prevented by transfecting astrocytes with a Nrf2-siRNA before NO_2-FA treatment, further supporting that Nrf2 activation is mediating the protective effect of NO_2-FA. Herein, we also demonstrate that an increase in GSH levels in astrocytes may account for the observed protection of motor neurons death. In fact, when $SOD1^{G93A}$ astrocytes were incubated with either NO_2-AA or NO_2-OA, GSH + GSSG levels increased concomitant with an induction of the modulatory subunit of the glutamate-cysteine ligase which catalyzes GSH synthesis.

Besides providing structural and functional support to neurons, neighboring astrocytes also collaborate during the progression of neurological disease displaying high antioxidant capacity [48]. These properties might be due in part to the metabolic interaction between astrocytes and neurons affecting glutathione metabolism. In fact, *de novo* neuronal biosynthesis of GSH depends on the supply of GSH precursors from astrocytes [48]. Increased production and secretion of glutathione by astrocytes is known to protect cocultured neurons from oxidative insults [49]. Moreover, astrocytes are enriched with antioxidant enzymes, such as the ARE-regulated gene HO-1 [50], whose upregulation could protect surrounding neuronal cells from oxidative stress. In addition, enhancing mitochondrial antioxidants defenses in $SOD1^{G93A}$ astrocytes reverts astrocyte-mediated toxicity [34]. Thus the increase in antioxidant defenses induced by the NO_2-FA treatment could potentially improve mitochondrial function in astrocytes and be partially responsible for the protection observed. These mechanisms may play an important role in NO_2-FA-triggered astrocyte-mediated increase in motor neuron survival.

Although the molecular mechanism underlying the selective death of motor neurons in ALS remains unknown, there is strong evidence that the mechanism is non-cell-autonomous, as the expression of mutant SOD1 in neurons affects disease onset, but glial cells, and in particular astrocytes, play a fundamental role in modulating disease progression [51,52]. Herein, we show that NO_2-FA induces ARE-driven gene expression as well as astrocytic GSH production, and has a significant protective effect against astrocyte-mediated motor neuron death. However, direct stimulatory effects of NO_2-FA on Nrf2 signaling in motor neurons cannot be discarded with the data presented here and needs further experimentation.

Our results show for the first time that NO_2-FA induce a potent antioxidant response in astrocytes which is dependent on Nrf2 activation and prevents motor neurons death in a culture model of ALS. Overall, our data not only propose NO_2-FA as potential novel therapeutic agents in ALS but also support the role of astrocyte antioxidant defenses in determining motor neuron fate. Considering that the central nervous system is abundant in

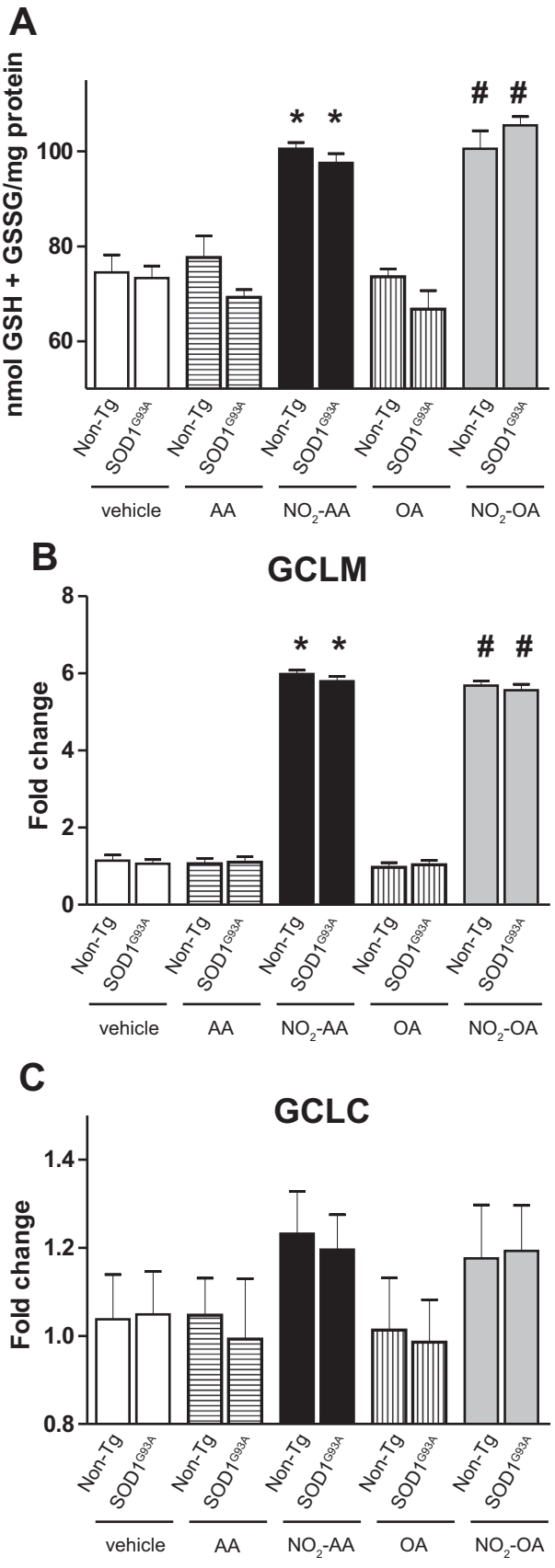

Fig. 3. NO_2-FA increase glutathione content in $SOD1^{G93A}$ astrocytes by increasing the Glutamate-cysteine ligase (GCL) modulatory subunit. Non-Tg and $SOD1^{G93A}$ astrocytes were exposed to NO_2-FA as before. (A) GSH+GSSG content in the cells after 24 h of incubation were analyzed. The mRNA levels of both the modulatory (B) and catalytic (C) subunits of the GCL were analyzed by RT-PCR. Vehicle and non-nitrated fatty acids were included as controls. In all cases, data are expressed as the mean ± SD, $n=5$. *Significantly different from AA and vehicle ($p<0.05$); #significantly different from OA and vehicle ($p<0.05$).

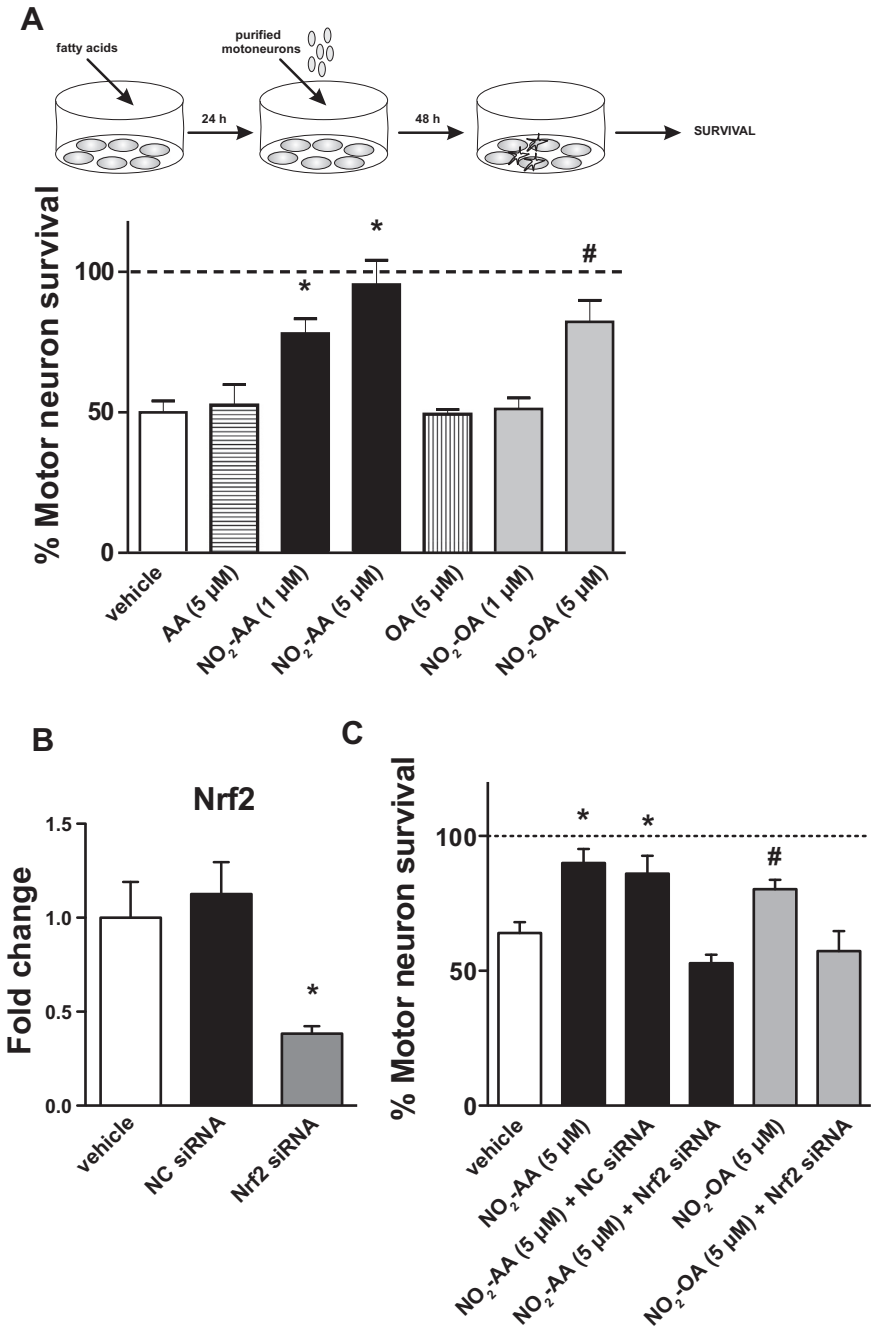

Fig. 4. NO_2-FA prevent motor neurons death. (A) Purified Non-Tg motor neurons were plated on top of non-Tg or $SOD1^{G93A}$ astrocytes pre-treated as described above. Treatment of non-Tg astrocytes with NO_2-AA, NO_2-OA or tBHQ had no significant effects on motor neuron survival (100%, dotted line). As expected, $SOD1^{G93A}$ astrocytes induced a 50% reduction in motor neuron death respect to non-Tg ones in basal conditions. Motor neuron loss observed in co-culture with $SOD1^{G93A}$ astrocytes was significantly reduced when astrocytes were treated with NO_2-AA or NO_2-OA for 24 h before motor neuron plating. Controls with the non-nitrated fatty acids were included without any effect in the survival of the motor neuron cells. *Significantly different from vehicle and AA ($p < 0.05$); #significantly different from vehicle and OA ($p < 0.05$). (B) Nrf2 mRNA levels in astrocytes treated with Nrf2-siRNA were determined by real-time PCR. A siRNA that does not target any sequence in the transcriptome was included as negative control (NC siRNA). *Significantly different from vehicle and NC siRNA ($p < 0.05$). (C) Motor neuron survival on a feeder layer of $SOD1^{G93A}$ astrocytes treated with NO_2-FA following a pre-treatment with Nrf2-siRNA or NC-siRNA (negative control). Nrf2-siRNA pre-treatment abolished the beneficial effects of NO_2-FA on motor neuron survival. *Significantly different from vehicle and NO_2-AA + Nrf2-siRNA ($p < 0.05$); #significantly different from vehicle and NO_2-OA + Nrf2-siRNA ($p < 0.05$). Data are expressed as the mean ± SEM of at least three independent experiments.

polyunsaturated fatty acids, it is possible that NO_2-FA being generate as adaptive response during inflammatory conditions to protect motor neurons by the mechanisms reported here. Current work is focusing on demonstrate the neuroprotective role of NO_2-FA by their ability to cross the brain blood barrier and extend the survival of ALS-linked mutant $SOD1^{G93A}$ mice.

Acknowledgments

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Discusión

La degeneración neuronal que tiene lugar en la médula espinal en el contexto de la ELA, se origina de forma focal, pero rápidamente se expande a lo largo de la médula afectando de forma progresiva al resto de las motoneuronas (Ravits et al. 2009). Se ha demostrado una relación directa entre el grado de avance de la enfermedad, la proliferación de células gliales y la intensidad del proceso inflamatorio (Philips and Robberecht, 2011, Schaefer et al., 2005). Trabajos previos mostraron un aumento en la proliferación de progenitores gliales NG2 positivos en la medula espinal degenerante de ratas $SOD1^{G93A}$, los que podrían diferenciarse en astrocitos (Mangus et al. 2008). También se observa un rápido aumento en la proliferación y activación de la microglia concomitantemente con el avance de la progresión de la fase sintomática (Brites et al. 2014). El establecimiento un fuerte componente neuroinflamatorio que involucra principalmente a astrocitos y microglia al inicio de la fase sintomática es una característica fisiopatología que tienen en común muchas enfermedades neurodegenerativas, incluyendo la ELA (komine et al. 2015)

Se ha demostrado que tanto los astrocitos (Vargas et al. 2006; Nagai et al. 2007) como la microglia (Frakes et al. 2014) obtenidos de pacientes y de modelos animales de ELA inducen la muerte de motoneuronas en co-cultivo. Los astrocitos producen y liberan algún factor tóxico que ha demostrado ser selectivamente tóxico para motoneuronas (Nagai et al. 2007). Además, la implantación de astrocitos $SOD1^{G93A}$ en la médula espinal de ratas normales, indujo una fuerte reactividad astrocitaria, reducción local en la expresión del transportador de glutamato GLT-1 y la muerte de motoneuronas (Papedeas et al. 2011). El rol de los astrocitos en la muerte neuronal que tiene lugar en la ELA ha sido bien demostrado y por eso es considerada una célula clave en la fisiopatología de esta enfermedad.

En este trabajo reportamos es aislamiento de un fenotipo astrocitario con características aberrantes, que denominamos células AbA *(del inglés **Ab**errant **A**strocytes)*, a partir de la médula espinal de ratas SOD1G93A en fase sintomáticas. Los cultivos de células AbA muestran una tasa de proliferación más alta que los cultivos de astrocitos neonatales, un patrón distintivo de la expresión de marcadores astrocitarios y una elevada toxicidad para motoneuronas *in vitro*, la mayor reportada hasta el momento. La ausencia de senescencia replicativa de las células AbA, permitió su expansión en cultivo, sin sufrir cambios significativos en un rango de 15-20 pasajes. Si bien la tasa proliferativa de las células AbA en cultivo es casi el doble que la de los astrocitos neonatales, es muy a la tasa proliferativa de la línea celular C6 aislada de un glioma de rata. Esto sugiere que las células AbA no representan un fenotipo celular completamente trasformado a pesar de la ausencia de senescencia replicativa.

En cultivo la células AbA son morfológicamente casi indistinguibles de los astrocitos neonatales pero tienen un patrón de expresión distintivo de los marcadores astrocitarios prototípicos. El alto nivel de expresión de las proteínas S100β y Cx43, sumando al bajo nivel de expresión de la proteína GFAP es característico de un fenotipo astrocitario indiferenciado. Además este patrón de expresión sugiere que las células AbA podría estar participar en el procesos neurodegenerativo que ocurre en la ELA. La proteína S100β actúa de forma parácrina sobre los astrocitos contribuyendo en la proliferación, migración, diferenciación y neurotoxicidad de estas células (Raponi et al. 2007; Donato et al. 2009). Cx43 también es una proteína relevante para el normal funcionamiento de los astrocitos, ya que permite la formación de uniones gap formando un gran sincitio que permite transferir información de un punto a otro rápidamente (Homkajorn et al. 2010). En condiciones patológicas se ha demostrado que las uniones *gap* entre astrocitos disminuyen y la proteína Cx43 puede formar hemicanales a través de los cuales pueden liberarse al espacio extracelular moléculas pro-inflamatorias como ATP, entre otras (Sáez et al. 2005; Orellana et al. 2009). En este sentido la elevada expresión de Cx43 encontrada en la células

AbA podría desempeñar un papel muy importante en el mantenimiento del estado inflamatorio crónico que acompaña a la neurodegeneración, así como participar en la muerte de las motoneuronas (Gandelman et al. 2010). Finalmente, quizás la características molecular más sorprendentes de las células AbA es la ausencia del transportador de glutamato GLT-1. Este transportador, expresado mayoritariamente en astrocitos, es relevante para la función de las neuronas glutamatérgicas y para prevenir la excitoxicidad generada por la acumulación de glutamato en la hendidura sináptica. Este mecanismo patogénico ha sido profundamente estudiado en la ELA y se ha demostrado una reducción significativa en la expresión de este transportador en la médula espinal de modelos animales de ELA (Howland et al. 2002) y en pacientes (Lin et al. 1998).

Otra de las características remarcables de las células AbA es su elevada toxicidad para la motoneurona. Trabajos previos han demostrado que los astrocitos neonatales portadores de la mutación $SOD1^{G93A}$ inducen la muerte de motoneuronas en co-cultivo y a través de la liberación de factores soluble (Vargas et al. 2006, Di Giorgio et al. 2008). Sin embargo en este trabajo mostramos que la células AbA muestran un toxicidad para la motoneurona sin precedente, tanto en co-cultivo como a través de la liberación de factores tóxicos. El porcentaje de motoneuronas que logra sobrevivir sobre la monocapa de células AbA no alcanza al 20%, significativamente menor que el 60% de sobrevida reportado para los astrocitos neonatales $SOD1^{G93A}$. Estos resultados podría sugerir alteraciones importantes en las células AbA referente a la expresión de ciertas proteínas de matriz extracelular necesaria para la adhesión neuronal. Además la evaluación comparativa del efecto de los medios condicionados (MC) de los diferentes tipos astrocitarios sobre cultivos de motoneuronas aisladas, mostró que el MC de las células AbA es 10 veces más tóxico que el MC de los astrocitos neonatales $SOD1^{G93A}$, sugiriendo que las células AbA constituyen una nueva subclase de astrocitos con un grado de toxicidad que no había sido reportado hasta el momento.

La presencia de células con características similares a las células AbA, astrocitos hipertróficos positivos para S100β y CX43, fue observada cerca de las motoneuronas en la médula espinal de animales SOD1^{G93A} en fase sintomática. La aparición de estas células en la médula espinal ocurre en los estadios previos al comienzo de la fase sintomática, y su número aumenta significativamente conforme la enfermedad avanza. La elevada toxicidad de la células AbA para la motoneurona, sumado a la simultaneidad entre aparición dichas células en la médula espinal y el comienzo de la sintomatología, sugiere un papel relevante de las células AbA principalmente en la progresión tan rápida que caracteriza a la ELA.

El estudio sobre el origen de la células AbA mostró que éstas derivan de un proceso de transdiferenciación a partir de células que expresan el marcador de estirpe mieloide CD11b. Este hallazgo resultó muy sorprendente ya que es muy bajo el número de reportes acerca de células que co-expresen marcadores astrocitarios y microgliales. Un tipo de patología donde este fenómeno ha sido observado es en un tipo de glioma llamado glioblastoma multiforme (Huysentruyt et al., 2011; Persson and Englund, 2012). Las células gliales tumorales parecen adoptar propiedades fagocíticas como consecuencia de la drásticas condiciones inflamatorias a la que están sometidas (Persson and Englund, 2012). En este sentido, las células AbA podrían resultar de un proceso de transición de la microglia que da lugar a la generación de un fenotipo con características astrocitarias. Se ha reportado la observación de comportamientos aberrante en la microglia e animales sintomáticos SOD1G93A, incluyendo la formación de acumulas o “clusters” celulares (Howland et al., 2002) o la formación de célula gigantes multinucleadas (Fendrick et al., 2007). No está claro si la transición fenotípica observada es un propiedad específica de la microglia SOD1G93A o si puede ser observada en otras patologías del SNC donde la microglia se acumula en regiones de activa muerte neuronal.

Como se mencionó previamente, la microglia $SOD1^{G93A}$ induce la muerte de motoneuronas a través de un mecanismo que involucra la activación del factor NF-kβ. Sin embargo, la eliminación selectiva de la población CD11b proliferante no tuvo un efecto significativo en ratones $SOD1^{G93A}$ (Gowing et al. 2008). La transición de la microglia hacia un fenotipo astrocitario que reportamos en este trabajo, podría explicar el resultado inesperado obtenido por Gowing y cols. En tal sentido, el diseño de estrategias terapéuticas centradas en la células gliales para el tratamiento de la neurodegeneración deben tener en cuenta los fenómenos de transformación, transición y fusión celular que pueden tener lugar en un ambiente neuroinflamatorio crónico y que ha sido descritos en otras condiciones patológicas. Una mayor comprensión de estas alteraciones podría resultar clave para la búsqueda y desarrollo de novedosos fármacos.

Conclusiones.

La participación de las células gliales en los procesos neurodegenerativos comenzó a acaparar la atención de los investigadores hace ya más más de tres décadas. La activación de los astrocitos y la microglia que acompaña a la neurodegeneración condujo a cambiar la mirada obstinada en la neurona y comenzar a mirar a su entorno. Desde entonces años de investigación han aportado un sin fin de evidencias acerca de la complejidad que subyace a los procesos neurodegenerativos y la importancia de las células gliales en dicho proceso. Sin embargo, las importantes alteraciones homeostáticas, moleculares y bioquímicas que tienen lugar en condiciones patológicas, han agregado un poco más de dificultad en el estudio de la implicancia de las células gliales en diversas patología del SNC.

En este trabajo de tesis se contribuye al conocimiento de la biología de las células gliales en la patología neurodegenerativa Esclerosis Lateral Amiotrofia. Los resultados obtenidos aportan importantes evidencias acerca de la generación de fenotipos gliales con características aberrantes, que denominamos células AbA, durante el proceso neurodegenerativo que tiene lugar en la medula espinal de animales $SOD1^{G93A}$. El aislamiento de estas células y su caracterización permitió demostrar que derivan de un proceso de transición a partir de células de la estirpe mieloide CD11b positivas (microglia/monocitos/macrófagos) lo que se manifiesta a través de un particular patrón de expresión de marcadores moleculares.

Las células AbA aparecen en la médula espinal en la fase sintomática de ratas $SOD1^{G93A}$ lo que sugiere que la participación de estas en la muerte neuronal merece ser estudiada de forma exhaustiva.

Las células AbA constituyen el fenotipo celular más neurotóxico aislado hasta el momento, lo que representa una importante oportunidad para el estudio y

búsqueda de novedosas estrategias terapéuticas. En tal sentido, cabe destacar que la identificación de este fenotipo celular motivó la redacción de una patente para su uso en la búsqueda de nuevos fármacos para el tratamiento de la ELA.

ISOLATION AND USE OF A NEW TYPE OF GLIAL CELL WITH NEUROTOXIC POTENTIAL. International Patent Application No. PCT/IB2011/000997. May 11, 2011

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