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Levels of membrane fluidity in the spinal cord and the brain in an animal model of amyotrophic lateral sclerosis

Francisco Javier Miana-Mena · Eduardo Piedrafita · Cristina González-Mingot · Pilar Larrodé · María Jesús Muñoz · Enrique Martínez-Ballarín · Russel J. Reiter · Rosario Osta · Joaquín J. García

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Abstract A mutant form of the copper/zinc superoxide dismutase (SOD1) protein is found in some patients with amyotrophic lateral sclerosis (ALS). Alteration of the activity of this antioxidant enzyme leads to an oxidative stress imbalance, which damages the structure of lipids and proteins in the CNS. Using fluorescence spectroscopy, we monitored membrane fluidity in the spinal cord and the brain in a widely used animal model of ALS, the SOD^{G93A} mouse, which develops symptoms similar to ALS with an accelerated course. Our results show that the membrane fluidity of the spinal cord in this animal model significantly decreased in symptomatic animals compared with age-matched littermate controls. To the best of our knowledge, this is the first report showing that membrane fluidity is affected in the spinal cord of a SOD^{G93A} animal model of ALS. Changes in membrane fluidity likely contribute substantially to alterations in cell membrane functions in the nervous tissue from SOD^{G93A} mice.

Keywords Amyotrophic lateral sclerosis · Membrane fluidity · G93A · Oxidative stress · Spinal cord · Brain · SOD

F. J. Miana-Mena · E. Piedrafita · C. González-Mingot · M. J. Muñoz · E. Martínez-Ballarín · J. J. García
Department of Pharmacology and Physiology,
Universidad de Zaragoza,
Zaragoza, Spain

C. González-Mingot · P. Larrodé
Neurology Service,
Universitary Hospital Lozano Blesa of Zaragoza,
Zaragoza, Spain

R. Osta
LAGENBIO, Universidad de Zaragoza,
Zaragoza, Spain

R. J. Reiter
Department of Cellular and Structural Biology,
University of Texas Health Science Center,
San Antonio, USA

J. J. García (✉)
Departamento de Farmacología y Fisiología,
Facultad de Medicina de Zaragoza,
C/Domingo Miral s/n,
50009 Zaragoza, Spain
e-mail: jjgarcia@unizar.es

Introduction

Amyotrophic lateral sclerosis (ALS), the most common form of motor neuron disease with an incidence of 1.2 to 1.8 per 100,000 individuals, is an age-dependent progressive disorder resulting from degeneration of motor neurons in the ventral horns of the spinal cord, brainstem, to motor cortex and an associated degeneration of the corticospinal tract. This neurodegenerative disorder, which involves both upper and lower motor neurons, is clinically characterized by progressive muscle weakness leading to the loss of the ability to move and speak; it causes death in virtually all patients within 3 to 5 years of diagnosis (Orrell 2007).

Despite extensive studies in patients and animal models, the etiopathogenesis of this disease remains unclear. Around 10% of ALS cases have a genetic cause (familial ALS or FALS). Approximately 20% of FALS patients have mutations in copper/zinc superoxide dismutase (*SOD1*), a crucial antioxidant enzyme (Deng et al. 1993; Rosen et al. 1993). Several transgenic mouse models overexpressing various human FALS mutations in SOD1 have been generated to study ALS. Mice that carry the $Gly^{93} \rightarrow Ala$ in the SOD1 enzyme are the most

widely used (Gurney et al. 1994; Dal Canto and Gurney 1995; Wong et al. 1995; Bruijn et al. 1997). This animal model, known as the SOD^{G93A} mouse, is interesting because of the many clinical similarities between the SOD^{G93A} mouse model and human patients. Disease progression in this mouse is characterized by asymmetric hind-limb weakness, spasticity, and atrophy beginning at 90–100 days of age that progresses to complete paralysis and death within 130–140 days of age (Chiu et al. 1995). Thus, it is generally well-accepted that the SOD^{G93A} mouse model is a valuable tool for studying the pathophysiology of ALS.

A biological membrane is a two-dimensional fluid of orientated lipids and proteins. Membranes are dynamic fluid structures that include molecules that are able to move through the surface in all directions. The lipid dynamics of the bilayer modulate numerous essential cell functions, including the regulation of the densities and binding affinities of neurotransmitters and neurohormones, cell growth, solute transport, and membrane-associated enzyme activities (Heron et al. 1980; Levi et al. 1990; Sunshine and McNamee 1994; Reddy and Yao 1996; Emmerson et al. 1999; Baenziger et al. 2000; Oghalai et al. 2000).

Phospholipids and cholesterol are major constituents of neural membranes; they provide structural integrity and essential functional properties (Farooqui and Horrocks 1991). The fluidity of the lipid bilayer depends on the relative composition of fatty acids and cholesterol (Holloway and Garfield 1981). Even slight modifications in the composition of the lipid bilayer alter fluidity levels of biological membrane. Although ALS is not a demyelinating disease, recent evidence suggests that altered membrane lipid composition may be a fundamental component of the neurodegeneration observed in ALS (Niebroj-Dobosz et al. 2007).

Disturbances in membrane fluidity levels have been described in several organs, including the brain of animals with physiological and accelerated aging (Viani et al. 1991; Yu et al. 1992; Choe et al. 1995; Lee et al. 1999; Reiter et al. 1999; Garcia et al. 2010) as well as in cell membranes obtained from patients with Alzheimer disease, epilepsy, to schizophrenia (Hitzemann et al. 1986; Nelson and Delgado-Escueta 1986; Pettegrew et al. 1991; Grimm et al. 2006; Clement et al. 2010). To our knowledge, however, no investigations have been carried out to evaluate fluidity changes in membranes isolated from the tissues of ALS patients or animal models of ALS. The purpose of this study was to measure the membrane fluidity of cell membranes isolated from the spinal cord to the whole brain of SOD^{G93A} transgenic mice at different clinical phases of the disease and to compare the fluidity levels obtained from sick animals to corresponding membranes isolated from healthy, age-matched, control mice.

Materials and methods

Transgenic mice

Animals were purchased from Jackson Laboratory (Bar Harbor, USA). We used a colony of SOD^{G93A} transgenic mice which were distributed into six groups depending on genotype and age. Gene carrier (positive) animals were aged 70, 100, and 130 days (n=20 in each group); age-matched littermate mice were used as controls (n=20 in each group). The number of males and females was similar in each group. Hemizygous mutants were used for all experiments (a mutant male mated with a wild-type female). Transgenic mice were identified by PCR amplification of DNA extracted from the tail, using the primer sequences for exon 4 described by Rosen et al. (1993). Animals were housed in the Unidad de Investigación Mixta of the University of Zaragoza, Spain. Food and water were available ad libitum. Beginning at 70 days of age, access to food and water was facilitated by placing food pellets on the floor of the cage for all transgenic animals. Environmental temperature was automatically regulated to 21–23 °C, relative humidity was 55%, and all cages were ventilated. Mice were maintained under a 12 h light, 12 h dark cycle (7 a.m. to 7 p.m.). Routine microbiological monitoring did not show any evidence of infection with common murine pathogens. All experiments and animal care were performed in accordance with the norms of the University of Zaragoza and the international guidelines for the use of laboratory animals. Animals were sacrificed at different ages. Brain and spinal cord were quickly removed, washed in saline, frozen, and stored at −80 °C until use.

Membrane isolation

Pooled membranes were isolated according to the protocol developed by Millan-Plano et al. (2003). Briefly, tissues were homogenized 1:10 (w/v) in 0.32 M sucrose then centrifuged at 1,000×g for 10 min at 4 °C. The supernatant was centrifuged at 30,000×g for 20 min at 4 °C. The pellet was re-suspended in ultrapure water and centrifuged at 8,000×g for 20 min at 4 °C. The supernatant and the buffy-coat were homogenized and centrifuged at 48,000×g for 20 min at 4 °C. The final pellet was resuspended 1:1 w/v in TRIS 50 mM and stored at −80 °C until assay.

Membrane fluidity

Fluidity of cell membranes was monitored using 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluene sulfonate (TMA-DPH) as a probe. The incorporation of TMA-DPH into the bilayer and membrane fluidity measurements were carried out according to the method of

Yu et al. (1992). Briefly, 0.5 mg membrane protein/mL was suspended in 50 mM TRIS (3 mL final volume) and TMA-DPH (66.7 nM) was added. After vigorous mixing, the membrane suspensions were incubated at 37 °C for 30 min. Fluorescence measurements (excitation λ = 360 nm; emission λ = 430 nm) were performed in a Perkin-Elmer LS-55 Luminescence Spectrometer equipped with a circulating water bath to maintain a cuvette temperature of 22±0.1 °C. The emission intensity of vertically polarized light was detected by an analyzer oriented parallel (IVv) or perpendicular (IV_H) to the excitation plane. A correction factor for the optical system, G was used. Polarization (P) was calculated according to the equation,

$$P = \frac{IV_V - GIV_H}{IV_V + GIV_H}$$

Because there is an inverse relationship between membrane fluidity and P (Yu et al. 1992), fluidity was expressed as 1/P and it was calculated from triplicate determinations.

Measurement of tissues protein

The protein concentrations were determined using the Bradford method (1976), in which bovine serum albumin served as standard.

Statistical analysis

Results are expressed as mean ± standard error (SE). Data were statistically analysed using an analysis of variance (ANOVA). Comparisons between groups were performed using a Scheffe test. Statistical significance was set at $p<0.05$.

Results

Animals were examined daily in their cages by a trained observer. Around the age of 90 days, the first clinical signs of an abnormal and slow gait appeared in mutant animals. Additionally, mice showed slight trembling of the hind limb when they were suspended by their tails during normal cage maintenance. These symptoms progressed quickly, and around the age of 130 days mice exhibit a total paralysis. At this terminal stage, animals were sacrified.

Figure 1 shows the membrane fluidity of the spinal cord cells from SOD^{G93A}-positive to healthy control mice at different ages. No significant changes were observed in healthy animals at 70, 100, and 130 days of age. However, membrane fluidity of this tissue from affected animals decreased with age. At day 70, during the preclinical period, SOD^{G93A}-positive and healthy animals showed similar membrane fluidity levels ($p>0.05$). At the onset of motor symptoms which occurs around day 100, affected SOD^{G93A}-positive animals showed a dramatic decrease in membrane fluidity (increased membrane rigidity) compared with the youngest SOD^{G93A}-positive animals ($p<0.05$). Moreover, membranes from SOD^{G93A}-positive animals exhibited a lower fluidity than those from age-matched controls at 100 days of age ($p<0.05$). The difference between symptomatic and control animals was even greater at day 130, when SOD^{G93A}-positive animals reached the terminal stage of the disease ($p<0.05$). Although membrane fluidity in spinal cord cells decreased between the preclinical period and the onset of symptoms in affected animals, no differences were found between this last period and at death.

In brain tissue, however, all measured values were equivalent for both healthy and affected animals over the time of the study (Fig. 2). The membrane fluidity values in the youngest mice were 3.49±0.11 for healthy animals vs. 3.44±0.07 (mean ± SEM) for the asymptomatic SOD^{G93A}-positive mice. At day 100, values in healthy animals were 3.38±0.09 whereas symptomatic mice had values of 3.36±0.06. Finally, terminal animals had a membrane fluidity of 3.53±0.04 compared with 3.49±0.16 in the age-matched healthy controls. No differences were detected among the groups.

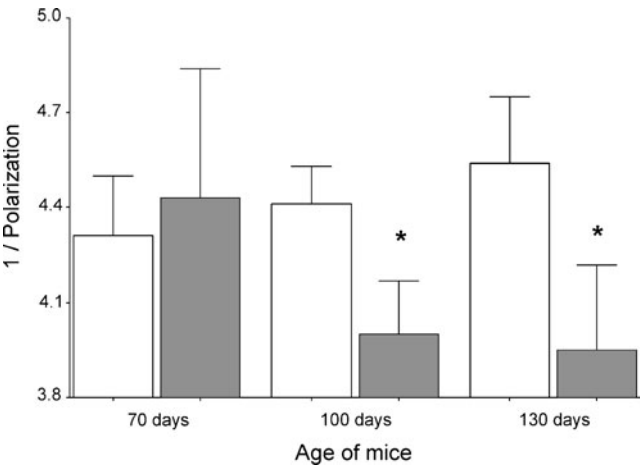

Fig. 1 Membrane fluidity in the spinal cord. Membrane fluidity of spinal cord was measured in SOD^{G93A}-positive and healthy mice at different ages. Membranes from cells of the spinal cord of SOD^{G93A}-positive animals show significant rigidity at 100 to 130 days (onset of clinical symptoms and terminal stage, respectively) compared with those of healthy age-matched littermate controls ($p<0.05$). At day 100, membranes from ill animals showed a lower fluidity (increased rigidity) than controls (4.00±0.17 vs. 4.41±0.12, respectively). At day 130, this difference increased (3.95±0.27 vs. 4.54±0.21). However, no differences were found in the youngest animals (4.43±0.41 vs. 4.31±0.19, respectively) where there were no symptoms of ALS. Values are expressed as mean ± SEM. (*white square* Control tissues; *black square* SOD^{G93A} tissues; * $p<0.05$ vs. control)

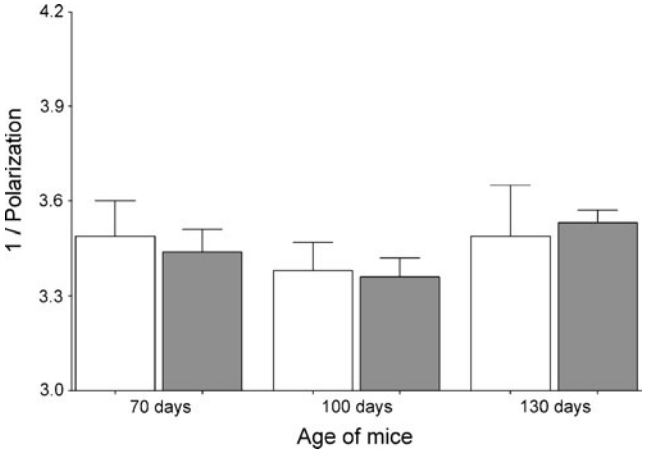

Fig. 2 Membrane fluidity in the brain. Brain cell membranes exhibited no change in the fluidity over time. Values are expressed as mean ± SEM. (*white square* Control tissues; *black square* SOD^{G93A} tissues; * $p<0.05$ vs. control)

Discussion

To the best of our knowledge, the present study is the first to report spinal cord cell membrane rigidity during the symptomatic period of SOD^{G93A} mice. Membrane fluidity was measured by fluorescence spectroscopy. The principle of this method relies on the intercalation of a fluorescent molecule, TMA-DPH, into the membrane which, when illuminated by polarized light, emits a fluorescent signal. The degree of polarization depends on the state of mobility of the TMA-DPH, which reflects motion in the membrane lipid environment (Reyes-Gonzales et al. 2009). TMA-DPH is a DPH derivative that incorporates a trimethylammonium substituent to improve its localization into the membrane. Therefore, TMA-DPH is intercalated parallel to the long molecular axis of the phospholipids with the cationic residue oriented at the surface (Prendergast et al. 1981).

The dynamic state or fluidity of a cell membrane is dependent on its composition. A recent study showed that total lipid content as well as phospholipid and cholesterol concentrations in the spinal cord from 120-day old SOD^{G93A} transgenic Sprague-Dawley rats with four-leg paralysis were markedly decreased compared with those from symptom-free transgenic rats at 60 days of age (Niebroj-Dobosz et al. 2007). Our investigation was also carried out in SOD^{G93A} transgenic animals, which have been extensively used for the in vivo development of novel experimental treatments for ALS since the SOD^{G93A} animals and the ALS patients have many clinical features in common. This transgenic model was generated by overexpression of a mutant allele of SOD1 carrying the Gly93 → Ala substitution (Gurney et al. 1994). The physiological role of SOD is to convert superoxide anions into hydrogen peroxide. Although initially it was proposed that ALS symptoms occurred as a result of a drop in free radical scavenging caused by the reduced activity of the mutated enzyme, it is now hypothesized as a gain in toxic function of the mutant SOD, since SOD1 knockout mice do not develop motor neuron disease (Reaume et al. 1996) and SOD^{G93A} mice develop FALS-like disease despite high SOD1 activity (Cleveland 1999). Taken together, these results suggested that the toxicity of the mutant SOD1 might arise from a gain-of-function rather than a loss-of-function.

The discovery of the SOD1 mutation in ALS led to several studies that suggested oxidative stress-mediated mechanisms play a central role in the pathogenesis of ALS. SOD1 mutations found in patients with FALS could result in free radical overproduction, which may cause systemic oxidative injury, thereby contributing to disease pathogenesis (Agar and Durham 2003). It is important to note that neural tissue is highly sensitive to free radical damage because of its poorly developed endogenous antioxidant defense system and its particular biochemical characteristics; e.g., in nervous tissue membranes the lipids are rich in polyunsaturated fatty acids that are particularly sensitive substrates in the lipid peroxidation reaction (Reiter 1998). This process is an auto-oxidative chain reaction initiated by a variety of free radicals that lead to the formation of peroxyl radicals, endoperoxides, and hydroperoxides (Curtis et al. 1984). Moreover, motor neurons in the CNS are particularly vulnerable to this stress in ALS (Halliwell and Gutteridge 1999).

The fact that oxidation is involved in ALS pathophysiology is supported by some oxidative structural injuries to lipids, proteins, and DNA obtained from the neural tissue of transgenic SOD^{G93A} mice (Andrus et al. 1998; Hall et al. 1998; Aguirre et al. 2005). Oxidative damage to lipids and proteins was detected in the cortical regions of patients with SALS and FALS (Ferrante et al. 1997) as well as in the spinal cord of patients with SALS (Shaw et al. 1995). Moreover, several studies which focused on ALS showed significantly elevated malondialdehyde concentrations, a widely used biochemical marker of the oxidative destruction of lipids, in the spinal cord of SOD^{G93A} mice at 90 and 120 days of age (Hall et al. 1997; Hall et al. 1998; Fang et al. 2010).

While these studies suggest structural modifications in the membranes caused by oxidative stress in ALS patients or SOD^{G93A} mice, abnormal hyperactivity in hypoglossal motor neurons as well as increased persistent Na^+ current and enhanced frequency of spontaneous excitatory and inhibitory transmission were detected in SOD^{G93A} mice compared with wild-type mice (van Zundert et al. 2008). Our study clearly demonstrates that the fluidity of membranes isolated from the spinal cords of SOD^{G93A} mice decrease significantly compared with control animals. It is well documented that lipid perox-

idation induces rigidity in a biological membrane (Garcia et al. 1997; Reiter et al. 1999; Garcia et al. 2001; Reyes-Gonzales et al. 2009). A possible explanation for the membrane rigidity is that lipid peroxidation modifies the lipid composition of the membranes. Oxidative stress causes a reduction in the polyunsaturated/saturated fatty acid ratio in the membrane, which could be explained by higher susceptibility to free radicals of the polyunsaturated fatty acids of membrane phospholipid molecules (Rice-Evans and Burdon 1993). In addition, oxidative stress induces lipid-lipid and lipid-protein moiety cross-linking (Chen and Yu 1994).

Of particular interest is that membrane fluidity decreased significantly in symptomatic animals compared with age-matched littermate controls. The association between membrane fluidity and clinical ALS progression may be consistent with the observations of some reports that show elevation in several oxidative stress indices at the onset of motor symptoms and during the clinical period in the spinal cord of the SOD^{G93A} mouse model (Hall et al. 1997, 1998; Fang et al. 2010). Accordingly, it seems reasonable to presume that oxidative damage in the motor neurons may involve cell membrane rigidity in the spinal cord in SOD^{G93A} animals.

In contrast to the membrane rigidity in the spinal cord, we found that membrane fluidity in the whole brain was unaltered when compared with cell membranes from healthy control animals. A possible explanation is that in this animal model, progressive death of motor neurons mainly occurs in the ventral horn of the lumbar spinal cord (Gurney et al. 1994). Moreover, the percentage of motor neurons in the brain is relatively low compared with that in the spinal cord.

In conclusion, we show that membrane fluidity in the spinal cord from $SOD1^{G93A}$ mice decreases at the onset of clinical symptoms and during the terminal stage of the disease. Based on these results and those from previous studies that showed oxidative injury in the spinal cord of ALS patients and in animal models (Hall et al. 1997, 1998; Fang et al. 2010), it is presumed that the membrane rigidity in the spinal cord from $SOD1^{G93A}$ mice is a phenomenological consequence, at least partially, of lipid peroxidation. Investigating the connections between membrane lipid composition, its oxidation, and membrane rigidity in the spinal cord of the $SOD1^{G93A}$ mouse may help to understand the pathogenic mechanisms involved in ALS.

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The authors have no conflicts of interest to declare.

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