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Central Nervous System Involvement in the Animal Model of Myodystrophy

Clarissa M. Comim · Bruna P. Mendonça · Diogo Dominguini · Andreza L. Cipriano · Amanda V. Steckert · Giselli Scaini · Mariz Vainzof · Emílio L. Streck · Felipe Dal-Pizzol · João Quevedo

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Abstract Congenital muscular dystrophies present mutated gene in the LARGE mice model and it is characterized by an abnormal glycosylation of α-dystroglycan (α-DG), strongly implicated as having a causative role in the development of central nervous system abnormalities such as cognitive impairment seen in patients. However, the pathophysiology of the brain involvement remains unclear. Therefore, the objective of this study is to evaluate the oxidative damage and energetic metabolism in the brain tissue as well as cognitive involvement in the LARGE$^{(myd)}$ mice model of muscular dystrophy. With this aim, we used adult homozygous, heterozygous, and wild-type mice that were divided into two groups: behavior and biochemical analyses. In summary, it was observed that homozygous mice presented impairment to the habituation and avoidance memory tasks; low levels of brain-derived neurotrophic factor (BDNF) in the prefrontal cortex, hippocampus, cortex and cerebellum; increased lipid peroxidation in the prefrontal cortex, hippocampus, striatum, and cerebellum; an increase of protein peroxidation in the prefrontal cortex, hippocampus, striatum, cerebellum, and cortex; a decrease of complex I activity in the prefrontal cortex and cerebellum; a decrease of complex II activity in the prefrontal cortex and cerebellum; a decrease of complex IV activity in the prefrontal cortex and cerebellum; an increase in the cortex; and an increase of creatine kinase activity in the striatum and cerebellum. This study shows the first evidence that abnormal glycosylation of α-DG may be affecting BDNF levels, oxidative particles, and energetic metabolism thus contributing to the memory storage and restoring process.

Keywords LARGE · Mice · BDNF levels · Behavior · Memory · Oxidative stress · Energetic metabolism · Brain · Central nervous system

C. M. Comim (✉)
Laboratory of Experimental Neurosciences,
University of Southern Santa Catarina, 88137-270,
Palhoca, Santa Catarina, Brazil
e-mail: clarissamc@terra.com.br

B. P. Mendonça · D. Dominguini · A. L. Cipriano · A. V. Steckert
Laboratory of Neurosciences and National Institute
for Translational Medicine (INCT-TM), Health Sciences Unit,
University of Southern Santa Catarina, 88806-000, Criciúma,
Santa Catarina, Brazil

G. Scaini · E. L. Streck · F. Dal-Pizzol · J. Quevedo
Laboratory of Experimental Pathophysiology and National
Institute for Translational Medicine (INCT-TM), Health Sciences
Unit, University of Southern Santa Catarina, 88806-000, Criciúma,
Santa Catarina, Brazil

M. Vainzof
Human Genome Research Center, Biosciences Institute,
University of São Paulo, São Paulo, São Paulo, Brazil

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Introduction

Congenital muscular dystrophies (CMDs) with central nervous system (CNS) manifestations are a group of heterogeneous genetic diseases including Fukuyama CMD, muscle–eye–brain disease, Walker–Warburg syndrome, and CMD 1D [1–3]. This last type of CMD presents mutated gene in the LARGE mice (encoding like-glycosyltransferase) breed and it is characterized by an abnormal glycosylation of α-DG (α-dystroglycan), which is strongly implicated as having a causative role in the development of CNS abnormalities such as severe mental retardation. This model of CMD1D was identified in 1963 at the Jackson Laboratory, USA. The animals have an abnormal shuffling gait, a diffuse

and progressive myopathy, a shortened life span, and it can be recognized at 12–15 days by their small size and abnormal posturing of their hind limbs [4]. The histology analyses of the muscle demonstrates a myopathy with focal areas of acute necrosis and clusters of degenerating and regenerating fibers in affected homozygotes, while heterozygotes show no pathological changes in muscle [5]. Following the identification of the mutation, additional aspects of the LARGE^{myd} phenotype were shown to include cardiac and CNS alterations [6, 7].

Brain magnetic resonance imaging shows extensive white matter abnormalities and subtle structural changes indicative of a neuronal migration defect in patients [8, 9]. The animal model of CMD1D also presents CNS abnormalities such as disturbed cortical lamination throughout the cerebrum and cerebellum, with neuronal heterotopia throughout the outer layer, the molecular layer of the cortex. There is abnormal migration of granule cells in the cerebellum and hippocampus, and clusters of heterotopic neurons are present in the cerebellar white matter. Severe focal disruptions are also seen in the glia limitans of the cerebellum [6, 7]. There is also abnormal migration of granule cells in the abnormal dentate gyrus morphology [6]; increased expression of GFAP by the astrocytes; abnormality in neuronal layers of the hippocampus; and severely blunted hippocampal long-term potentiation with electrophysiologic characterization, indicating that dystroglycan might have a postsynaptic role in learning and memory processes [8–10].

The hippocampal long-term potentiation is a form of plasticity widely believed to be critical for memory formation. During the process of learning and memory formation, there is an important peptide involved: the brain-derived neurotrophic factor (BDNF)—a member of the neurotrophin family and the most widespread growth factor in the brain. BDNF is highly expressed in the hippocampus and it has diverse functions in the adult brain as a regulator of neuronal survival, fast synaptic transmission and activity dependent synaptic plasticity [11]. Recently, it was demonstrated that in adult rats, the hippocampal BDNF levels were positively correlated with the ability to learn [12].

In this context, it is known that the brain is particularly vulnerable to reactive oxygen species (ROS) production, since it metabolizes 20 % of total body oxygen and possesses a limited amount of antioxidant capacity. In situations where the generation of free radicals exceeds the capacity of antioxidant defense, oxidative stress may lead to membrane degradation, cellular dysfunction, and apoptosis. Oxidative stress can result from increased production of ROS, decreased antioxidant defense, or failure to repair oxidative damage. ROS are free radicals or reactive anions/molecules containing oxygen atoms such as hydroxyl radical, superoxide, hydrogen peroxide, and peroxynitrite. They can cause cell damage by enzyme inactivation, lipid peroxidation, and DNA modification [for review see: 13]. Oxidative stress is well-known to contribute to neuronal degeneration in the CNS, in the process of aging as well as in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) [14]; Alzheimer's dementia [15]; Parkinson's disease [16]; major depression [17, 18]; and, recently, in the brain of adult mdx mice [19].

Additionally, the mitochondria represent the main cellular source of ROS and key regulators of cell death. The impairment in energy production caused by mitochondrial dysfunction has been found in some neurodegenerative diseases such as dementia, Alzheimer's, and Parkinson's disease, all culminating in some level of cognitive impairment. The mitochondrial respiratory chain is responsible for oxidative phosphorylation leading to the production of adenosine triphosphate (ATP). Tissues with high-energy demands, such as, the brain, contain a large number of mitochondria and are therefore more susceptible to the effects of reduced aerobic energy metabolism. Alteration in mitochondrial function could decrease ATP production and elevate mitochondrial creatine kinase (CK) activity, which works as an effective buffering system of cellular ATP levels [20, 21].

It is known that the CNS is involved in the CMD, and the spectrum of alterations in different brain regions is not yet clear. Thus, the objective of this study is to understand the CNS involvement in the animal model of CMD1D through the alteration of both oxidative parameters and energetic metabolism in the brain tissue in the adult animals as well as the occurrence of cognitive impairment.

Material and Methods

Animals

Adult homozygous (KO) (n=20) and heterozygous (HT) LARGE^{myd} mice (n=20) as well as wild-type (WT) littermate mice (n=20) used in this study were genotyped using PCR tail DNA following the protocol described previously [22]. Once the genotype was determined, the mice were divided into two groups: behavior analyses and biochemical analyses. We used only male mice weighing 25 and 30 g. They were housed five to a cage with food and water available ad libitum and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). All procedures were approved by the Animal Care and Experimentation Committee.

Behavioral Tests

The animals separately underwent two behavioral tasks: habituation to an open field and step-down inhibitory avoidance. Thus, using this design we do not assess time-dependent memory but assess memory over time (with

new training at each test session). All behavioral procedures were conducted between 1300 and 1600 hours in a sound-isolated room, and a single animal performed only one behavior test in only one time point after surgery. All behavioral tests were recorded by the same person who was blind to the animal group.

Habituation to the Open-Field Task This task evaluates motor performance in the training section and non-associative memory in the retention test session. Habituation to an open field was carried out in a 20×30-cm open field surrounded by 30-cm high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear quadrant and left to explore the arena for 5 min (training session). Immediately following this, the animals were taken back to their home cage and 24 h later submitted again to a similar open-field session (test session). Crossing of the black lines and rearing performed in both sessions were counted. The decrease in the number of crossings and rearings between the two sessions was taken as a measure of the retention of habituation.

Step-Down Inhibitory Avoidance Task This task evaluates aversive memory. The apparatus and procedures have been described in previous reports. Briefly, the training apparatus was a 25×12×12-cm acrylic box whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm-wide, 2.5-cm-high platform was placed on the floor of the box against the left wall. In the training trial, the animals were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Immediately after stepping down on the grid, the animals received a 0.2-mA, 2.0-s foot shock and returned to their home cage. A retention test trial was performed 24 h after training (long-term memory). The retention test trial was procedurally identical to training, except that no foot shock was presented. The retention test step-down latency (maximum, 180 s) was used as a measure of inhibitory avoidance retention.

Biochemical Assays

BDNF Measurement The BDNF levels in the prefrontal cortex, hippocampus, striatum, and cortex were measured by sandwich-ELISA, according to the manufacturer's instructions (Chemicon, USA for BDNF and Millipore). Briefly, the rat prefrontal cortex, hippocampus, striatum, and cortex were homogenized in phosphate buffer solution (PBS) with protease inhibitor cocktail (Sigma). Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluent and the standard curve ranged from 7.8 to 500 pg/ml of BNDF. The plates were then washed four times with sample diluent and a monoclonal anti-BNDF rabbit antibody (diluted 1:1000 in sample diluent) was added to each well and incubated for 3 h at room temperature. After washing, a peroxidase conjugated anti-rabbit antibody (diluted 1:1000) was added to each well and incubated at room temperature for 1 h. After the addition of the streptavidin-enzyme, substrate and stop solutions, the amount of each neurotrophin was determined by absorbance in 450 nm. The standard curve demonstrates a direct relationship between Optical Density (OD) and the concentration.

Lipid Peroxidation Lipid peroxidation was measured by formation of thiobarbituric acid (TBA) reactive substances (TBARS) [23]. After brain dissection, brain structures were washed with PBS, harvested, and lysed. TBA 0.67 % was added to each tube and vortexed. The optical density of each solution was measured in a spectrophotometer at 535 nm. Data were expressed as nanomoles of TBARS equivalents per milligram of protein.

Protein Carbonyl Formation Protein carbonyl content was measured in brain homogenates using 2,4-dinitrophenylhydrazine (DNPH) in a spectrophotometric assay [24]. Absorbance was recorded in a spectrophotometer at 370 nm for both DNPH-treated and HCl-treated samples. Protein carbonyl levels were expressed as nanomoles of carbonyl per milligram of protein.

Mitochondrial Respiratory Chain Enzyme Activities Brain structures were homogenized (1:10, *w*/*v*) in SETH buffer (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/mL heparin, pH7.4) for determination of mitochondrial respiratory chain enzyme activities (complexes I, II, II–III, and IV). NADH dehydrogenase (complex I) was evaluated according to the method [25] by the rate of NADH-dependent ferricyanide reduction at 420 nm. The activities of succinate: DCIP oxidoreductase (complex II) and succinate: cytochrome c oxidoreductase (complex II–III) were determined according to the method [26]. Complex II activity was measured by following the decrease in absorbance due to the reduction of 2,6-DCIP) at 600 nm. Complex II–III activity was measured by cytochrome c reduction from succinate. The activity of cytochrome c oxidase (complex IV) was assayed by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c at 550 nm [27]. The activities of the mitochondrial respiratory chain complexes were expressed as nanomoles per minute milligram protein.

CK Activity CK activity was measured in brain homogenates pretreated with 0.625 mM lauryl maltoside. The

reaction mixture consisted of 60 mM Tris–HCl, pH7.5, containing 7 mM phosphocreatine, 9 mM MgSO4, and approximately 0.4–1.2 lg protein in a final volume of 100 lL. After 15 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 lmol of ADP plus 0.08 lmol of reduced glutathione. The reaction was stopped after 10 min by the addition of 1 lmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method [28]. The color was developed by the addition of 100 lL 2 % a-naphtol and 100 lL 0.05 % diacetyl in a final volume of 1 mL and read spectrophotometrically after 20 min at 540 nm. Results were expressed as units per minute milligram protein.

All the results were normalized by protein concentration measured by the Lowry assay [29]

Statistical Analysis

Data from the biochemical analyses and the inhibitory avoidance task are reported as median and interquartile ranges, and comparisons among groups were performed using Mann–Whitney *U* tests. The within-individual groups were analyzed by the Wilcoxon tests. The data from the habituation to the open field are reported as mean and S.E.M., and comparisons among groups were performed using the ANOVA post hoc Tukey and the within-individual groups were analyzed by paired *t* test. In all comparisons, $p<0.05$ indicated statistical significance.

Results

Behavior Tests

Habituation to the Open-Field Task

In the open-field task, there were no differences in the number of crossings and rearings between groups in the habituation to the open-field training session ($p>0.05$), demonstrating no difference in motor and exploratory activity between groups. In the test session, a significant reduction in both crossings and rearings of the KO mice was not observed when comparing training and test sessions, suggesting memory impairment (Fig. 1a).

The Step-Down Inhibitory Avoidance

A significant decrease of the latency time in the KO mice in the step-down latency of the inhibitory avoidance task was not observed, when comparing the test with training (Fig. 1b), suggesting impaired aversive memory.

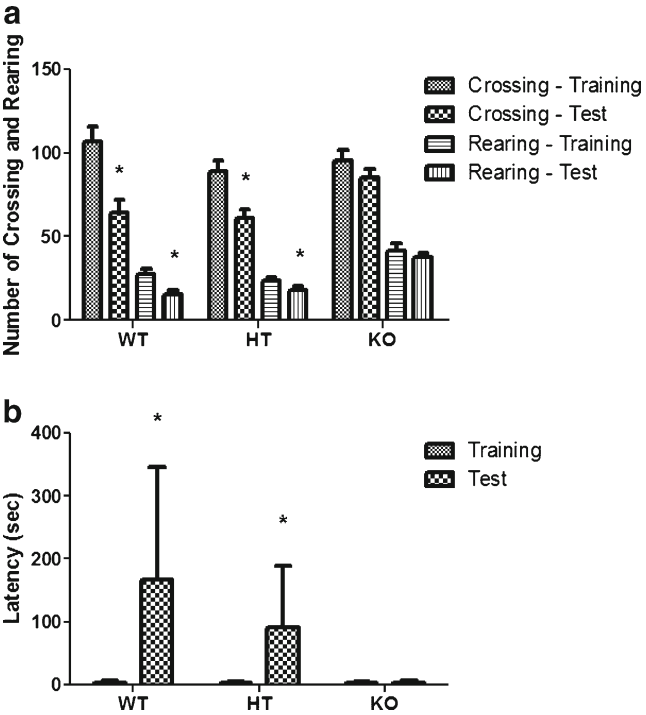

Fig. 1 Behavior tests. The animals underwent two behavioral tasks: habituation to an open field (**a**) and step-down inhibitory avoidance (**b**). Data from the habituation to an open field are presented as mean ± SEM and data from step-down inhibitory avoidance are presented as median ± interquartile ranges, $n=10$ rats per group. *$p<0.05$ versus training

BDNF Levels

As depicted in Fig. 2, KO mice display a decrease in the availability of BDNF levels in the prefrontal cortex, hippocampus, and cerebellum when compared with WT mice. However, no statistical significance was observed in the BDNF levels between WT and HT mice in all structures analyzed.

Oxidative Damage

Figure 3 shows that oxidative damage, assessed by TBARS (Fig. 3a) and the protein carbonyl (Fig. 3b) assays, occurred

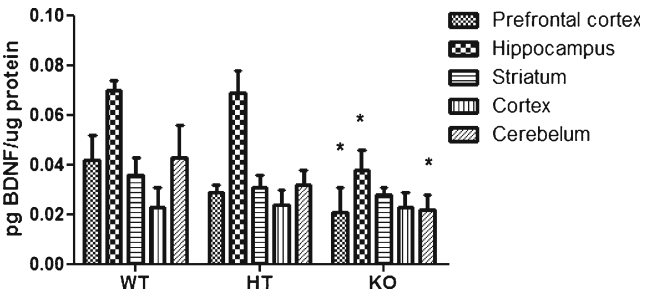

Fig. 2 BDNF protein levels. Data are presented as mean ± SEM, $n=10$ rats per group. *$p<0.05$ versus WT

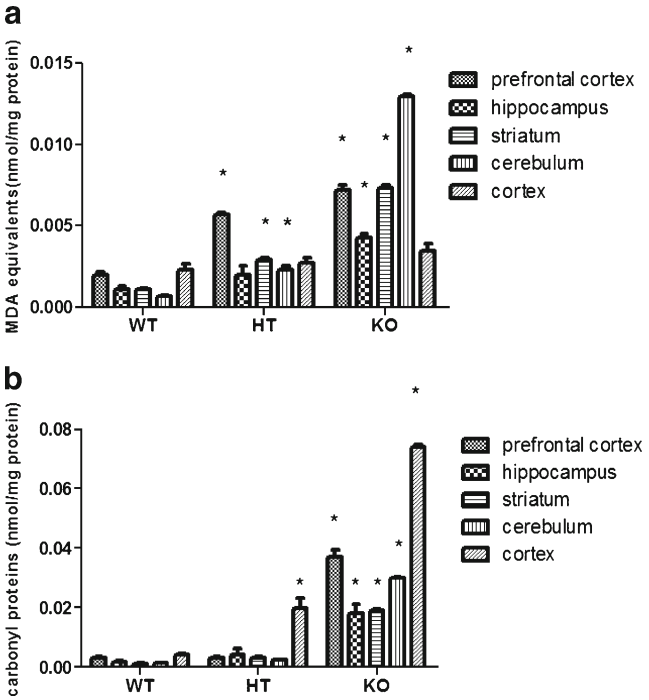

Fig. 3 Oxidative parameters. Data from the lipid peroxidation (**a**) and protein peroxidation (**b**) are presented as mean ± SEM, $n=10$ rats per group. *$p<0.05$ versus WT

in several brain regions of both KO and HT mice. The TBARS levels were increased in the prefrontal cortex, hippocampus, striatum, and cerebellum in the KO mice, and the HT mice presented an increase of TBARS levels in the prefrontal cortex, striatum, and cerebellum. Carbonyl levels were increased in the cortex of the HT mice and in the prefrontal cortex, hippocampus, striatum, cerebellum, and cortex of the KO mice. All results were compared with WT mice.

Energetic Metabolism

Figure 4 presents the mitochondrial respiratory chain (complex I, II, III, and IV) activity in the brain. Complex I (Fig. 4a) decreased its activity in the prefrontal cortex and cerebellum in the KO mice; increased activity of complex II (Fig. 4b) in the hippocampus of HT mice; decreased complex II activity in the prefrontal cortex and cerebellum in KO mice; increased complex III (Fig. 4c) activity in the hippocampus in HT mice; and decreased complex IV (Fig. 4d) activity in the prefrontal cortex and cerebellum and increased in the cortex. All results were compared with WT mice. In CK activity (Fig. 4e), there was increased activity in the striatum and cerebellum in the KO mice when compared with WT mice.

Discussion

Brain dystroglycans have been localized as neuronal elements in several locations, including the hippocampus and cerebellar cortex, where it may form a structural element of certain synapses [30]. The hippocampus is a brain area required for many forms of long-term memory in both

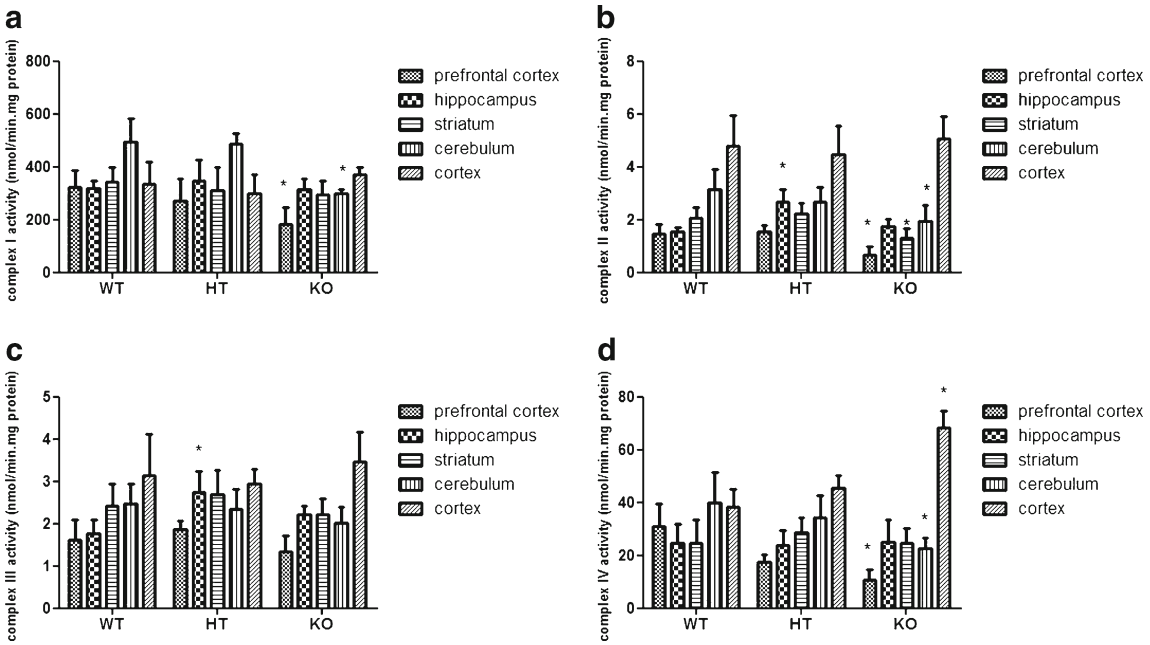

Fig. 4 Energetic metabolism. Data from the activity of complex I (**a**), complex II (**b**), complex III (**c**), complex IV (**d**), and creatine kinase (**e**) are presented as mean ± SEM, $n=10$ rats per group. *$p<0.05$ versus WT

humans and animals. During the process of learning and memory formation, the BDNF acts in the diverse functions in the adult brain as in learning and memory formation. In our study, it was observed that the homozygous mice presented cognitive damage in habituation and avoidance memory with decreased BDNF protein levels in the prefrontal cortex, hippocampus, and cerebellum. In this context, patients with CMD may present severe mental retardation and neuronal migration defect [4, 5]. Others types of muscular dystrophies such as Duchenne muscular dystrophy and miotonic dystrophy presented cognitive impairment in both animal models and patients [31, 32]. It is known that neuronal migration can be seen as very important in the formation of the mammalian brain during development, and defects in neuronal migration lead to disorganization of cortical lamination and architecture, which causes mental retardation, epilepsy, and severe learning disabilities. The role of different proteins involved in neuronal migration and cerebral organization during development remains unclear. Thus, it seems that the disruption of the basal lamina, caused by loss of interaction between hypoglycosylated α-DG and its ligand, plays a part in the pathogenesis of CNS involvement.

However, the enzymatic activity of LARGE has yet to be defined and its role in α-DG glycosylation is unclear in the brain. Alternatively, LARGE may act indirectly on α-DG processing via interaction with other proteins. Oxidative damage and alterations in the energetic metabolism in brain tissue also have been found in the various animal model of muscular dystrophy such as mdx mice [19, 33]. In this study, we demonstrated that there was increased lipid peroxidation in the prefrontal cortex, hippocampus, striatum, and cerebellum and increased protein peroxidation in the prefrontal cortex, hippocampus, striatum, cerebellum, and cortex. Free radicals are known to be responsible for chemical and molecular damage of DNA, nucleotides, proteins, lipids, carbohydrates, and cell membrane structure. The brain is particularly sensitive to oxidative damage due to its relatively high content of peroxidizable fatty acids and limited antioxidant capacity. Several enzymes generate free radicals, including xanthine, urate, coproporphyrinogen III, glucose, lysyl, monoamine, D-amino acid oxidases, and superoxide dismutase [34]. Because ROS have exceedingly short half-lives and are likely to be formed at a low rate in a chronic disorder such as muscular dystrophy, most studies of this issue have utilized 'indirect' measures of antioxidant enzyme protein and/or activity, rather than the more direct spin trap methodology.

In this regard, a number of neurological diseases are associated with neurodegeneration and neuronal death associated with cognitive impairment [35] caused primarily by abnormal brain energy metabolism and mitochondrial dysfunction [36]. We demonstrated mitochondrial dysfunction in the LARGE^{myd} mouse brain caused by a decrease in complex I activity in the prefrontal and cerebellum; decrease in complex II activity in the prefrontal cortex, striatum, and cerebellum; and a decrease in complex IV activity in the prefrontal cortex and cerebellum and an increase in the cortex. Alterations in mitochondrial function could decrease ATP production [21]. We demonstrated an increase in CK activity in the striatum and cerebellum in LARGE^{myd} mouse brain. Furthermore, CK plays a central role in the metabolism of high-energy-consuming tissues such as the brain. It catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, regenerating ATP [37]. Therefore, the increased ATP-regenerating capacity via the CK reaction might be related to a delay in ATP depletion and, thereby, protect the brain from damage. The decrease in CK activity is associated with a neurodegenerative pathway that results in neuronal loss [38, 39]. On the other hand, the increase in lipid peroxidation and protein peroxidation described above may be involved in the alteration of energy metabolism in the LARGE^{myd} mouse brain.

In conclusion, we demonstrated that LARGE^{myd} mouse presented cognitive impairment, low levels of BDNF, oxidative damage, and energetic metabolism impairment in the brain. This study shows the first evidence that abnormal glycosylation of α-DG may be affecting BDNF levels, oxidative particles and energetic metabolism, and may be contributing to the memory storage and restoring process. Furthermore, understanding the pathophysiology of CNS commitment is important to facilitate the search for pharmacological interventions that augment LARGE glycosyltransferase activity and may be used as treatment for these diseases.

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Conflict of interest None of the authors or funding sources has conflict of interest.

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