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Neurocognitive Impairment in mdx Mice

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Abstract
Duchenne muscular dystrophy (DMD) is a neuromuscular disorder that affects muscles and also the brain, resulting in memory and behavioral problems. In the pathogenesis of DMD, inflammation is an important factor during the degenerative process. However, the involvement of the brain is still unclear. Therefore, the objective of this study is to evaluate the cognitive involvement, BDNF levels, cytokine levels through the levels of TNF-α and IL-1β, the myeloperoxidase (MPO) activity, and the expression of proteins postsynaptic density (PSD)-95 and synaptophysin in the brain of mdx mice. To this aim, we used adult mdx mice. It was observed that mdx mice presented deficits on the habituation, aversive, and object recognition memory. These animals also had a depression-like behavior and an anxiety-like behavior, a decrease of BDNF levels, an increase in the levels of TNF-α and IL-1β, an increase of MPO activity, and an overexpression of synaptophysin and PSD-95 in brain tissue. In conclusion, these data show that mdx mice possibly present a neuroinflammatory component and the involvement of synaptic proteins associated to memory storage and restoring process impairment as well as a depressive- and anxiety-like behavior.

Keywords Memory and learning · mdx mice · Duchenne muscular dystrophy · Cognition · Inflammation

Introduction

Duchenne muscular dystrophy (DMD) is a neuromuscular disease characterized by the absence of dystrophin protein. This protein is found in the skeletal muscle and is critical for cell integrity and function [1]. In the brain, dystrophin protein is normally expressed in several structures involved in memory process, such as the hippocampus, cerebellum, cerebral cortex, and amygdala [2]. These structures are also considered critical for synaptic transmission, plasticity, and memory process [3]. Moreover, the loss of brain dystrophin is associated to reduced neuronal density in the CA1 anterodorsal hippocampus [4], oxidative stress [5], energetic metabolism alterations [6], alterations of postsynaptic $GABA_A$ receptors [7], and reduction of acetylcholinesterase activity [8] and altered activity of Krebs cycle enzymes [9]. The findings are more prominent in the hippocampus and cortex. The hippocampus is the structure of the brain directly involved in the processes of learning and memory [3]. Besides, these alterations also are associated to a decrease of brain-derived neurotrophic factor (BDNF) levels in the striatum. The BDNF is a regulator of neuronal survival, fast synaptic transmission, and activity-dependent synaptic plasticity. Studies showed that BDNF levels are related to a cognitive impairment.

DMD patients exhibit non-progressing cognitive dysfunctions [3]. The expression of Dp71, a protein product produced through a mutation in the dystrophin gene and only expressed in the CNS, has been linked to intellectual disability [10]. Alterations in memory and attention, and deficits in language abilities and visuospatial learning are present in DMD patients [11, 12]. These patients also presented alterations in the intelligence quotients (IQ), characterized by one standard deviation below the normal range [13, 14]. Similarly, the mdx mice

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present cognitive impairment in the visuospatial [15] and avoidance memory [16]. However, the pathways involved in cognitive impairment in DMD are still unclear.

In the pathogenesis of DMD, inflammation is an important factor during the degenerative process. Studies showed that part of the secretion of pro-inflammatory immune mediators is from damaged dystrophin-deficient muscle fibers [17, 18]. DMD patients had a decrease of functionality associated to an increase in the IL-1β and TNF-α and a decrease of IL-10 levels in serum [19]. In the muscle biopsies from DMD patients, an increase of TNF-α [20, 21] and IL-1β [22] was verified. In the brain, cytokines are associated to neurogenesis and neuronal survival. However, chronically elevated levels of inflammatory cytokines can result in neuronal dysfunction [23]. It is known that activation of the immune system in the periphery greatly influences the normal function of the CNS [23]. Nico and colleagues [24, 25] showed that mdx mice present an increase of blood–brain barrier permeability associated to an increased matrix-metalloproteinase-2 and matrix-metalloproteinase-9 expression. These changes may facilitate the process of neuroinflammation in DMD, besides the absence of dystrophin in brain tissue.

In this context, the involvement of an inflammatory process in the brain in the pathophysiology of DMD, especially when associated with cognitive damage, is still not clear. However, it is known that inflammation plays an increasingly prominent role in the pathogenesis cognitive impairment [26]. In this line, there are evidences that excessive IL-1β and TNF-α affect long-term potentiation (LTP) and the synaptic process which underlies learning and memory [26]. In summary, to understand the relations between DMD genotype, brain function, and cognitive impairment is necessary in studying the relation between the cognitive alterations, pro-inflammatory parameters, and synaptic function. Thus, the objective of this study is to evaluate the cognitive function through the memory and learning tasks, depression and anxiety-like behaviors, BDNF levels, cytokines levels (TNF-α and IL-1β), myeloperoxidase (MPO) activity, and the expression of proteins postsynaptic density (PSD)-95 and synaptophysin in the brain of mdx mice.

Methods

Animals

To this aim, we used male dystrophin-deficient mdx mouse—this strain carries a spontaneous mutation that prevents expression of the longest isoform of the cytoskeletal protein dystrophin or normal control mice aged 60 days old, ceded by the Human Genome Research Center, Biosciences Institute, University of São Paulo, São Paulo, SP, Brazil. The animals were housed to a cage with food and water available ad libitum and were maintained on a 12-h light/dark cycle. All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All protocols performed were approved by the ethics committee at UNISUL (protocol number 16.033.4.01.IV).

Behavioral Tests

The animals were separated and submitted to five behavioral tasks (n = 10 per group and per task): habituation to an open-field, step-down inhibitory avoidance, continuous multiple trial step-down inhibitory avoidance task, object recognition, elevated plus-maze, and forced swimming test. 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. All behavioral tests were recorded by the same person who was blinded 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 40 × 60 cm open field surrounded by 50-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 [27].

Step-Down Inhibitory Avoidance Task This task evaluates aversive memory. The apparatus and procedures have been described in previous reports [28]. In the training trial, 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.

Object Recognition This task evaluates non-aversive, non-spatial memory. The apparatus and procedures for the object recognition task have been described elsewhere [29]. Briefly, the task took place

in a 40 × 50 cm open field surrounded by 50-cm-high walls made of plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. All animals were submitted to a habituation session where they were allowed to freely explore the open field for 5 min. No objects were placed in the box during the habituation trial. Crossings of the black lines and rearings performed in this session were evaluated as locomotors and exploratory activity, respectively. At different times after habituation, training was conducted by placing individual rats for 5 min in the field, in which two identical objects (objects A1 and A2, both being cubes) were positioned in two adjacent corners, 10 cm from the walls. In a short-term recognition memory test given 1.5 h after training, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B, a pyramid with a square-shaped base) object. All objects had similar textures (smooth), colors (blue), and sizes (weight 150–200 g), but distinctive shapes. A recognition index calculated for each animal is reported as the ratio TB/(TA + TB) (TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B). In a long-term recognition memory test given 24 h after training, the same rats were allowed to explore the field for 5 min in the presence of the familiar object A and a novel object C (a sphere with a square-shaped base). Recognition memory was evaluated as done for the short-term memory test. Exploration was defined as sniffing (exploring the object 3–5 cm away from it) or touching the object with the nose and/or forepaws.

Elevated Plus-Maze The apparatus used in animal models for anxiety has been described in detail elsewhere [30, 31]. Briefly, the apparatus consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm) arranged in such a way that the two arms of each type were opposite to each other, with a central platform (5 × 5 cm). The maze's height was 50 cm, and the tests were conducted under dim red light. Animals were exposed for 5 min to the red light in their own home cages before the testing procedure. Next, they were placed individually on the central platform of the plus-maze facing an open arm. During a 5-min test period, the following measurements were recorded by two observers: the number of entries, the time spent in the open and closed arms, and the total number of arm entries.

Forced Swimming Test The test was conducted according to previous reports [32, 33] and was used as a model for depressive behavior. Briefly, the test involves two exposures to a cylindrical water tank in which rats cannot touch the bottom or from which they cannot escape. The tank is made of transparent plexiglass (47 × 38 cm) and filled with water (22–23 °C) to a depth of 30 cm. Mice were placed and released into the water at the same time. Each mouse was given a single 7-min trial (training 2 min; test 5 min). Researchers manually recorded the total duration immobile (s). Immobility was defined as the period of time that the mouse was not swimming, when movement was only made in order to keep the body in balance or its head above water in the test session. Water was changed, and temperature was taken for the next trial.

BDNF Levels

BDNF levels were measured by anti-BDNF sandwich-ELISA, according to the manufacturer's instructions (Chemicon, USA). Briefly, the structures were homogenized in phosphate-buffered solution (PBS) with 1 mM phenylmethylsulfonyl fluoride and 1 mM EGTA. Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluent. The plates were then washed four times with sample diluent, and a monoclonal anti-BNDF rabbit antibody diluted to 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 addition of streptavidin enzyme, substrate, and stop solution, the amount of BDNF was determined by absorbance in 450 nm. A standard curve was produced, and it ranged from 7.8 to 500 pg/ml of BNDF. This curve was obtained from a direct relationship between optical density and BDNF concentration. The total protein was measured by Lowry's method using bovine serum albumin as a standard.

Cytokine Levels

The concentration of cytokines (IL-1β and TNF-α) was determined by ELISA (R&D Systems, Minneapolis, MN). All samples were assayed in duplicate. Briefly, the capture antibody (13 ml, contains 0.1% sodium azide) was diluted in phosphate-buffered saline (PBS), added to each well and left overnight at 4 °C. The plate was washed four times with PBS and 0.05% Tween 20 (Sigma, St. Louis, MO, USA). The plate was blocked with 1% bovine serum albumin and incubated for 1 h at room temperature before washing four times with PBS and 0.05% Tween 20. The samples and standards were added, and the plate was incubated overnight at 4 °C. After washing the plate, detection antibody (concentration provided by the manufacturer) diluted in PBS was added. The plate was incubated for 2 h at room temperature. After washing the plate, streptavidin (DuoSet R&D Systems, Minneapolis, MN, USA) was added and the plate was incubated for 30 min. At last, color reagent *o*-phenylenediamine (Sigma, St. Louis, MO, USA) was added to each well and the reaction was allowed to develop in the dark for 15 min. The reaction was stopped with the addition of 1 M sulfuric acid to each well. The absorbance was read on a plate reader at 492-nm wavelength (Emax, Molecular Devices, Minneapolis, MN,

USA). The total protein was measured using bovine serum albumin as a standard.

MPO Activity

Leukocyte infiltration in the brain was measured indirectly by quantifying MPO enzyme activity. Briefly, brain extracts were homogenized (500 mg/ml) in 0.5% hexadecyltrimethylammonium bromide and centrifuged at 4000 rpm for 15 min at 4 °C. MPO activity in the supernatant was measured spectrophotometrically as the change in optical density at 460 nm at 37 °C, using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM) as the substrates. The results were expressed relative to DO at 460 nm/mg protein.

Western Blotting Analysis

The tissue was homogenized and sonicated in ice-cold Tris–HCl buffer containing SDS (0.1%), and protease and phosphatase inhibitor cocktails (Complete, Roche, Indianapolis, IN, USA). The protein concentration was measured with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein electrophoresis was made in polyacrylamide 15% gels, and proteins were transferred to PVDF Immobillon-FL membranes (Millipore, MA, USA). Membranes were blocked with TBS-Tween-albumin and incubated overnight with a rabbit monoclonal antibody anti-synaptophysin (1:500, Abcam, Cambridge, MA, USA), mouse anti-PSD-95 (1:1000, Abcam, Cambridge, MA, USA), and mouse polyclonal anti-β-actin (1:10,000; Sigma-Aldrich, USA) as a loading control. After washing with PBS, membranes were incubated with IRDye secondary anti-mouse and anti-rabbit antibodies (Li-Cor Biosciences) for 30 min. Immunoreactivity was visualized with an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

Statistical Analysis

Data from the open-field task were analyzed with ANOVA followed by Tukey post hoc and paired Student's t test and expressed as mean ± SD. Data from the inhibitory avoidance task, the object recognition task, and the number of training trials from continuous multiple-trial step-down inhibitory avoidance 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 Wilcoxon tests. The data from the habituation to the open field, elevated plus-maze, forced swimming tests, and biochemical and molecular analyses are reported as means ± SD and were analyzed by the paired Student's t test. In all comparisons, $p < 0.05$ indicated statistical significance.

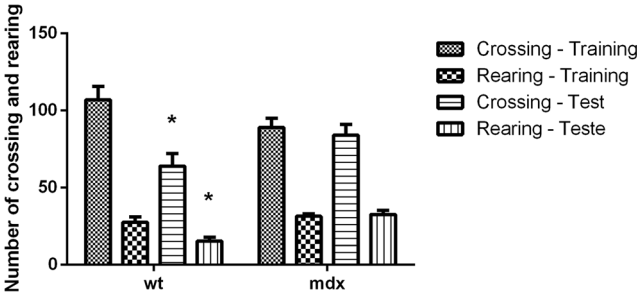

Fig. 1 Habituation on the open field. Numbers of crossing and rearing are presented as mean ± SD. *$p < 0.05$ versus training

Results

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, there was a significant reduction in both crossings and rearings of the wt group as compared to the mdx group (crossing $t = 9.765$, df = 9, $p = 0.0001$; rearings $t = 6.638$, df = 9, $p = 0.0001$), suggesting memory impairment (Fig. 1). Figure 2 shows the result of the step-down latency. In the test session, there was no significant difference between training and test in the mdx mice, suggesting impaired aversive memory ($Z = -1.311$, $p = 0.130$). Because DMD could affect sensory processing during training, such as the rats' reactivity to the foot shock, rather than memory, we evaluated the effects of DMD on foot shock sensitivity, and there were no significant differences between groups in the flinch or the jump nociceptive thresholds showing that DMD did not affect the animal's reactivity to the foot shock (data not shown). After object recognition test (Fig. 3), the mdx animals presented impairment of novel object recognition memory, i.e., they did not spend a significantly higher percentage of time exploring the novel object. The animals present memory impairment during short ($Z = -3.291$, $p = 0.622$) and long term ($Z = -0.611$, $p = 0.613$).

In the test of elevated plus-maze task (Fig. 4), there were no statistically significant differences in the number of entries ($t = -0.821$, df = 33, $p = 0.22$, Fig. 4a), but there were

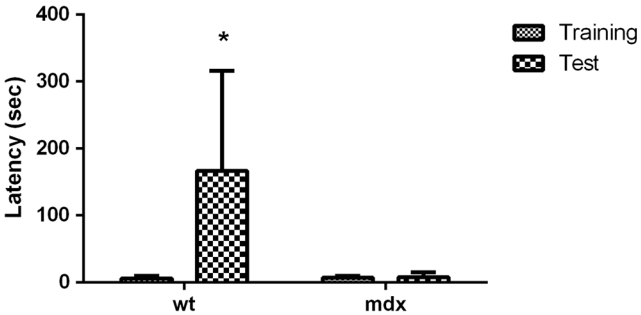

Fig. 2 The step-down inhibitory avoidance. Latency time is presented as median and interquartile ranges. *$p < 0.05$ versus training

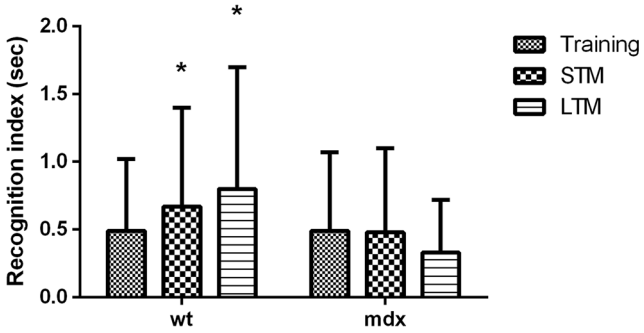

Fig. 3 Object recognition. Recognition index is presented as median and interquartile ranges. *$p < 0.05$ versus training

statistically significant differences in the time spent in the arms between groups in mdx mice ($t = -2.134$, df = 33, $p = 0.033$, Fig. 4b), suggesting that mdx mice present anxiety-like symptoms. Finally, Fig. 5 demonstrates the results of forced swimming test. In the test session (5 min), we observed a significant increase in the immobility time in the mdx mice as compared to the wt animals ($t = 3.739$, df = 28.350, $p = 0.001$), suggesting depressive-like behavior (Fig. 5).

The BDNF levels are demonstrated in the Fig. 6. There were observed decrease of BDNF levels in mdx brain tissue when compared to wt brain ($t = 3.333$, df = 27.312, $p = 0.001$). Figure 7 shows the results of cytokine levels. There were increase levels of IL-1β ($t = 3.234$, df = 29.344, $p = 0.001$) (Fig. 6a) and TNF-α ($t = 2.211$, df = 28.211, $p = 0.001$) (Fig. 6b) in the brain tissue of mdx mice when compared to wt mice. Figure 8 presents the results of MPO activity in brain tissue. It can be verified that MPO activity was elevated to

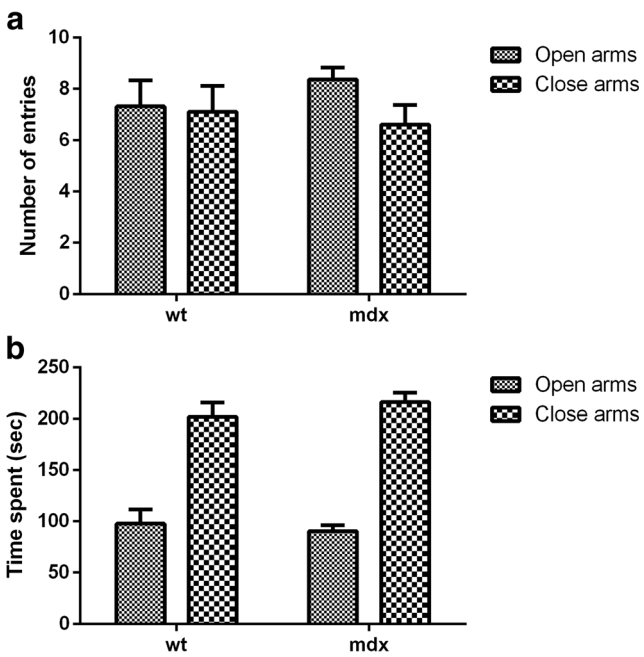

Fig. 4 Elevated plus-maze test. **a** Number of entries on the closed and open arms. **b** Time spent on the closed and open arms. Data are presented as mean ± SD. *$p < 0.05$ versus wt

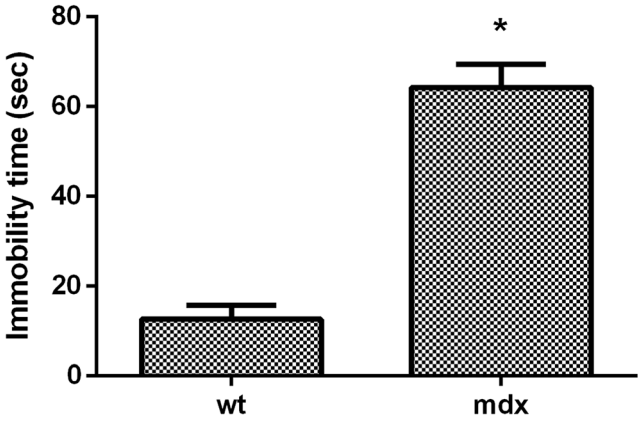

Fig. 5 The forced swimming test. They underwent the forced swimming test and the immobility time was recorded. Data are presented as mean ± SD, *$p < 0.05$ versus wt

mdx when compared to wt mice ($t = 3.239$, df = 28.978, $p = 0.001$). Finally, Fig. 9 demonstrates the PSD-95 and synaptophysin expression. There were increases of PSD-95 ($t = 2.359$, df = 27.121, $p = 0.001$) (Fig. 9a) and synaptophysin ($t = 3.111$, df = 28.288, $p = 0.001$) (Fig. 9b) expression in the brain of mdx mice when compared to wt mice.

Discussion

Our results demonstrate that mdx mice presented habituation, aversive, and recognition memory deficits associated to an anxiety-like and depressive-like behavior associated to low BDNF levels and an increase of pro-inflammatory cytokines and MPO activity and an overexpression of synaptic proteins—synaptophysin and PSD-95—in the brain of mdx mice. The protein dystrophin is expressed in neurons within specific brain regions, including the prefrontal cortex and hippocampus, structures associated with learning and memory formation [3, 11, 34]. In the DMD patients, deficits in verbal, working, and visuospatial memory are consistently reported [34–36].

Similarly, to DMD patients, the mdx mice lack dystrophin in muscle and brain tissue and alteration is present in some types of memory such as visuospatial [15] and avoidance [16]. In mice, only nonlinguistic behavioral characteristics are evaluated

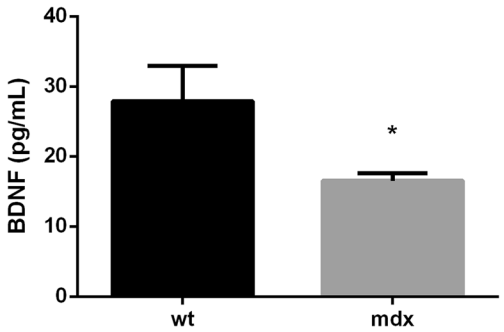

Fig. 6 BDNF levels. Data are presented as mean ± SD. *$p < 0.05$ vs. wt

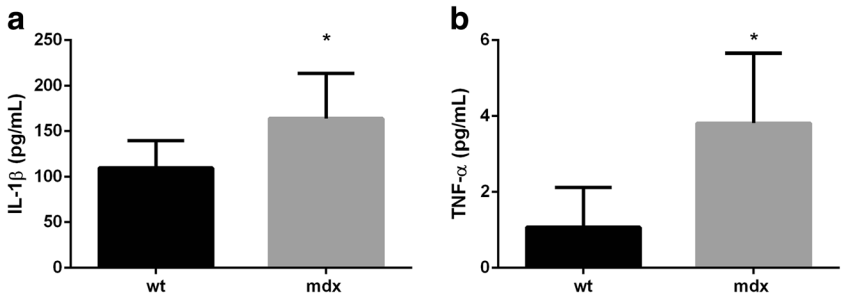

Fig. 7 Pro-inflammatory cytokine levels—IL-1β (**a**) and TNF-α (**b**). Data are presented as mean ± SD. *$p < 0.05$ vs. wt

because the animals present lack language repertoires. In this study, we evaluate three forms of memory—habituation, aversive, and object recognition. Habituation is a primitive form of non-associative learning, and it is classically defined as the waning of a response, elicited by repeated exposure to a novel stimulus not accompanied by any biologically relevant consequence [27]. The aversive memory is evaluated through step-down inhibitory avoidance task paradigm. This task represents one of the major determinants of survival behavior in all species [37], corresponding to many important examples of learning in humans. The novel object recognition task has emerged as a popular method for testing the non-spatial memory in mice. This task exploits the natural tendency of rodents to explore novel items [29]. We observed that, in this research, there were alterations in the habituation, aversive, and novel object recognition memories. These data reinforce those already described in the literature and support the involvement of more than one type of memory in the DMD pathophysiology process.

Studies with neuroimaging demonstrated a subtle, but significant, decrease in whole brain volume in the order of 5% for DMD patients compared to healthy controls [38]. In the mdx mice, the brain volume alteration is not found [39]. In recent study using mdx mice, the authors showed that dystrophin is expressed in oligodendrocytes and influences developmental myelination, which provides new insights into potential cellular contributors to brain dysfunction associated with DMD [40]. Another important molecule involved in cognitive process and found altered in the brain of mdx mice [41] and serum DMD patients [19] is the BDNF. Normal BDNF levels in the brain are essential to the maintenance of normal learning and memory function by a process referred to as synaptic consolidation.

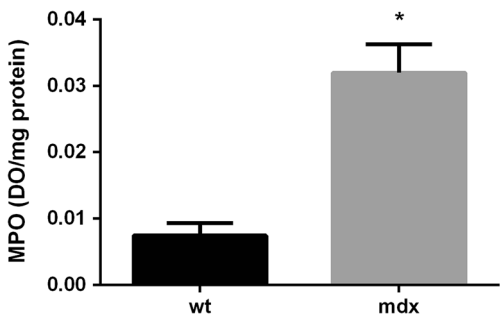

Fig. 8 MPO activity. Data are presented as mean ± SD. *$p < 0.05$ vs. wt

Alterations of BDNF levels are associated to cognitive impairment in several neurodegenerative disorders [42]. Recent evidences showed that alterations in the neuromodulatory immune molecules in the skeletal muscle and brain can be associated to cognitive impairment in the DMD [3]. In this study, we can observe a decrease of BDNF levels in mdx brain tissue.

Another important result observed in this study is that the mdx mice presented a depression- and anxiety-like behavior, evaluated through the increase of immobility time and increase of time spent in the close arms, respectively. In the DMD patients, comorbidity with neuropsychiatric disorders has been documented, including attention-deficit hyperactivity disorder, obsessive-compulsive disorder, autism, epilepsy, and anxiety [36, 43]. Anxiety is a complex phenomenon that cannot be described in a single test. However, there were few studies that showed anxiety-like symptoms in neither humans nor animal model. Depression and anxiety are moderately correlated to quality of life in adult patients with neuromuscular disease [44]. Depression appears to go underdiagnosed in patients with chronic somatic diseases [45]. In a recent study, it was demonstrated that 24% and 19% of DMD patients presented anxiety and depression disorders, respectively [46]. Vaillend and Chaussenot (2017) demonstrated that altered spatial localization of $GABA_A$ can be associated to an anxiety-related behavioral parameter characterized by an increase of exploration of the center zone of the open field in the mdx mice [7]. In this context, alterations of postsynaptic $GABA_A$ receptors are already described in mdx brain tissue [7].

In this context, our results demonstrated an increase of pro-inflammatory cytokines and MPO activity associated to an overexpression of synaptic proteins—synaptophysin and PSD-95—in the brain of mdx mice. In the skeletal muscle, the inflammation process is an important component in the pathophysiology of DMD and it is strongly associated with the severity of the disease. In mdx mice, it was observed that the use of TNF-α inhibitor presents a delay in muscle pathology, potentially supporting the use of TNF-α for slow disease progression [47]. Neuroinflammatory disease associated to a cognitive impairment is associated to increased levels of TNF-α and IL-1β [26]. However, the contribution of these pro-inflammatory markers in brain tissue during the physiopathology of DMD process is not clear [3].

Another inflammatory parameter evaluated was MPO activity. MPO is a heme protein produced by neutrophils and

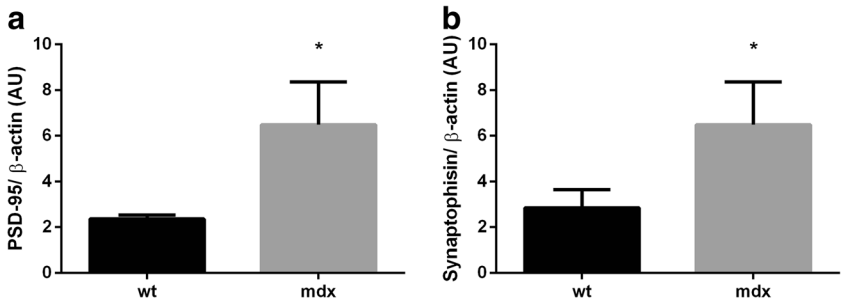

Fig. 9 PSD-95 (**a**) and synaptophysin expression (**b**). Data are presented as mean ± SD. *$p < 0.05$ vs. wt

monocytes, and its increase in the brain has been considered a marker for neurodegenerative diseases associated to an inflammatory and oxidative process [48]. Comim and colleagues [5] demonstrated the presence of oxidative stress in the mdx brain. Our study showed an increase of MPO activity in mdx brain tissue. This increase can be associated to increase of pro-inflammatory cytokines and the presence of oxidative stress. It is known that high levels of IL-1β and TNF-α affect cognitive impairment and synaptic process [26].

Cognitive damage is an important feature in DMD patients [3]. In the attempt to find ways to further elucidate the physiopathology of the cognitive damage, our study evaluated the expression of two proteins involved in the synaptic transmission process—PSD-5 and synaptophysin. We demonstrated an increase of PSD-5 and synaptophysin expression in brain tissue of mdx mice. Alder and colleagues [49] showed that overexpression of synaptophysin results in increased release of glutamate [49]. A recent study has shown that changes in calcium homeostasis and increased sensitization of NMDA receptors make neurons more sensitive to excitotoxicity [50]. The absence of dystrophin has been associated with a destabilization of cell membranes, favoring one of the first events involved in the pathophysiology of DMD, the increase of intracellular Ca^{2+} [51, 52]. This result may be associated with the hypothesis that, in mdx mice, there may be a process of excitotoxicity, since excessive activation of glutamate receptors may lead to an alteration of postsynaptic structures [53].

In conclusion, this study showed that adult mdx mice present habituation, aversive, and object recognition memory impairment associated with depression- and anxiety-like behavior. The data found in this study also showed a decrease in BDNF levels and an increase in levels of TNF-α and IL-1β and MPO activity in brain tissue. These alterations may be involved in the mechanism responsible for the increase in the expression of synaptophysin and PSD-95 and thus interfere in the synaptic connections, since these proteins play essential functions for the formation of learning and memory processes.

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Compliance with Ethical Standards

All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All protocols performed were approved by the ethics committee at UNISUL (protocol number 16.033.4.01.IV).

Conflict of Interest The authors declare that they have no conflict of interest.

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