Neurochemistry International 55 (2009) 802–805

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Oxidative variables and antioxidant enzymes activities in the *mdx* mouse brain

Clarissa M. Comim [a], Omar J. Cassol-Jr [a], Leandra C. Constantino [a], Larissa S. Constantino [b], Fabrícia Petronilho [b], Lisiane Tuon [a], Mariz Vainzof [c], Felipe Dal-Pizzol [b], João Quevedo [a,*]

[a] *Laboratório de Neurociências and Instituto Nacional de Ciência e Tecnologia Translacional em Medicina, Programa de Pós-Graduação em Ciências da Saúde, Unidade Acadêmica de Ciências da Saúde, Universidade do Extremo Sul Catarinense, 88806-000 Criciúma, SC, Brazil*

[b] *Laboratório de Fisiopatologia Experimental and Instituto Nacional de Ciência e Tecnologia Translacional em Medicina, Programa de Pós-Graduação em Ciências da Saúde, Unidade Acadêmica de Ciências da Saúde, Universidade do Extremo Sul Catarinense, 88806-000 Criciúma, SC, Brazil*

[c] *Human Genome Research Center, Biosciences Institute, University of São Paulo, São Paulo, Brazil*

ARTICLE INFO

Article history:
Received 28 May 2009
Received in revised form 11 July 2009
Accepted 4 August 2009
Available online 12 August 2009

Keywords:
mdx mouse
Brain
Oxidative variables

ABSTRACT

Dystrophin is a protein found at the plasmatic membrane in muscle and postsynaptic membrane of some neurons, where it plays an important role on synaptic transmission and plasticity. Its absence is associated with Duchenne's muscular dystrophy (DMD), in which cognitive impairment is found. Oxidative stress appears to be involved in the physiopathology of DMD and its cognitive dysfunction. In this regard, the present study investigated oxidative parameters (lipid and protein peroxidation) and antioxidant enzymes activities (superoxide dismutase and catalase) in prefrontal cortex, cerebellum, hippocampus, striatum and cortex tissues from male dystrophic *mdx* and normal C57BL10 mice. We observed (1) reduced lipid peroxidation in striatum and protein peroxidation in cerebellum and prefrontal cortex; (2) increased superoxide dismutase activity in cerebellum, prefrontal cortex, hippocampus and striatum; and (3) reduced catalase activity in striatum. It seems by our results, that the superoxide dismutase antioxidant mechanism is playing a protective role against lipid and protein peroxidation in *mdx* mouse brain.

1. Introduction

Duchenne's muscular dystrophy (DMD) is a lethal X-linked myopathy characterized by the near absence of dystrophin protein (Ragusa et al., 1990). This protein is located at the inner-side of cell membrane in muscles and brain cells, in association with a membrane-bound cytoskeletal protein complex known as the dystrophin-associated protein (Blake and Kröger, 2000). Brain dystrophin is enriched in the postsynaptic densities of pyramidal neurons specialized regions of the subsynaptic cytoskeletal network, which are critical for synaptic transmission and plasticity (Lidov et al., 1990).

The lack of dystrophin in brain structures such as in hippocampus and neocortex has been involved with impaired cognitive functions in these regions (Anderson et al., 2002). However, the nature, magnitude and biological support of the cognitive deficits involving dystrophin deficiency still remain unclear, although they have been partly addressed by studies in the dystrophin-deficient *mdx* mouse, a genetic model of DMD (Anderson et al., 2002; Vaillend et al., 2002). The brain dystrophin is abundant in the hippocampus and absent in other subcortical areas (Lidov et al., 1990). Selective behavioral deficits involving hippocampal function were predicted to occur in the *mdx* mouse mutant. Pioneer studies showed that dystrophin deficiency in *mdx* mouse is associated with impaired memory retention at long delays in certain learning and spatial alternation tasks (Muntoni et al., 1991; Vaillend et al., 1995, 2002). At cellular level, the absence of dystrophin in *mdx* mouse causes altered calcium homeostasis and hippocampal neuronal function (Mehler et al., 1992), primarily by altering hippocampal long-term potentiation, a form of plasticity widely believed to be critical for memory formation (Vaillend et al., 2002).

Additionally, the brain is particularly vulnerable to reactive oxygen species (ROS) production, since it metabolizes 20% of total body oxygen and has 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. ROS can cause cell damage by enzyme inactivation, lipid peroxidation, and DNA modification (for review see: Floyd, 1999).

Oxidative stress is well known to contribute to neuronal degeneration in the central nervous system (CNS), in the process of aging as well as in neurodegenerative diseases such as

* Corresponding author. Fax: +55 48 34312759.
E-mail address: quevedo@unesc.net (J. Quevedo).

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doi:10.1016/j.neuint.2009.08.003

amyotrophic lateral sclerosis (Rosen et al., 1993), Alzheimer's dementia (Durany et al., 1999), Parkinson's disease (Abraham et al., 2005), and major depression (Lucca et al., 2009a,b).

On top of that, *mdx* mouse model appears to have impaired bioenergetic function and abnormal calcium homeostasis, also reported in DMD (Rae et al., 2002). Based on the findings above mentioned, we hypothesize that oxidative stress may play an essential role in the physiopathology of *mdx* mouse cognitive impairment. Thus, the main purpose of the present study was to investigate oxidative stress parameters and enzyme activities in prefrontal cortex, cerebellum, hippocampus, striatum and cortex of dystrophin-deficient *mdx* mouse, compared to normal mouse controls.

2. Materials and methods

We used male dystrophic (*mdx*) and normal C57BL10 mice (3 months: wild-type $n = 5$, *mdx* $n = 5$), ceded by Human Genome Research Center, Biosciences Institute, University of São Paulo. They were housed five per cage with food and water available *ad libitum* and were maintained on a 12 h light/dark cycle (lights on at 7:00 am). The mice were killed by decapitation and prefrontal cortex, cerebellum, hippocampus, striatum, and cortex were immediately dissected, isolated, and stored at −80 °C for analyses of oxidative stress parameters and enzyme activities.

All experimental procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. All procedures were approved by the Animal Care and Experimentation Committee of UNESC, Brazil.

2.1. Biochemical measurement

In order to assess oxidative damage, the formation of thiobarbituric acid reactive species (TBARS) was measured during an acid-heating reaction, as previously described (Esterbauer and Cheeseman, 1990). The samples were mixed with 1 mL of trichloroacetic acid (TCA) 10% and 1 mL of thiobarbituric acid 0.67% and were then heated in a boiling water bath for 15 min. TBARS was determined by the absorbance at 535 nm. Oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH), as previously described (Levine et al., 1994). Proteins were precipitated by the addition of 20% trichloroacetic acid and were redissolved in DNPH; the absorbance was read at 370 nm. To determine CAT activity, the brain tissue was sonicated in 50 mmoL/L phosphate buffer (pH 7.0), and the resulting suspension was centrifuged at 3000 × *g* for 10 min. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm (Aebi, 1984). SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described (Bannister and Calabrese, 1987). All biochemical measures were normalized to the protein content, with bovine albumin as standard (Lowry et al., 1951).

2.2. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) 17.0 was utilized for statistical analyses. All data are expressed as mean ± S.D. of *n* animals, and have been statistically analyzed with the Student's *t*-test for unpaired data. *P* values less than 0.05 were considered statistically significant.

3. Results

We demonstrate in Fig. 1A and B, that oxidative damage, assessed by TBARS and the protein carbonyl assays, occurred in several brain regions of *mdx* mouse. The TBARS levels were decreased in the striatum ($p < 0.05$; Fig. 1A) compared with wild-type. And carbonyl levels were decreased only in the cerebellum and prefrontal ($p < 0.05$; Fig. 1B) compared with wild-type.

To determine enzymatic antioxidant status in the brain of *mdx* mouse, the activity of the main central nervous system antioxidant enzymes (CAT and SOD) was determined. Except for the cortex, we

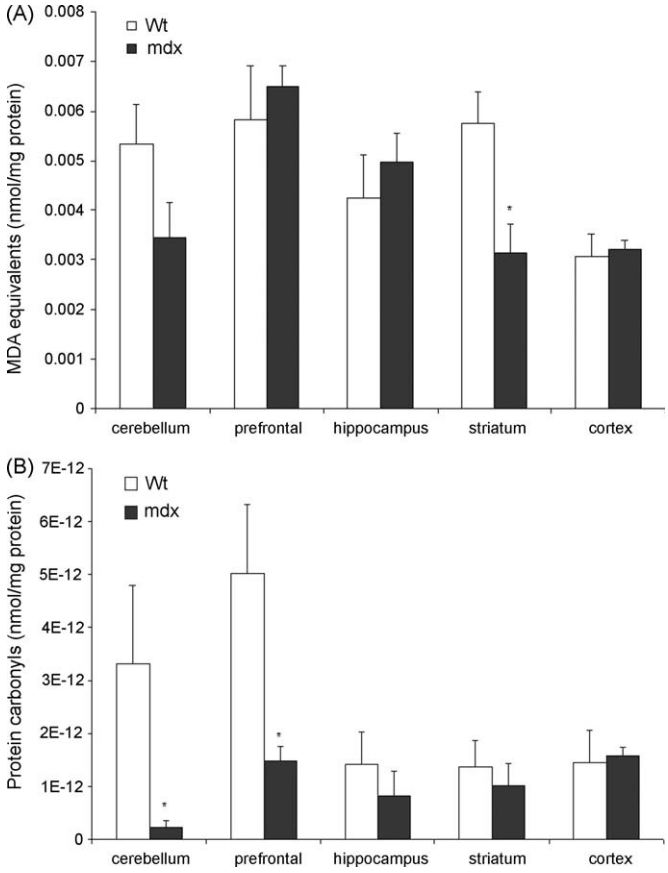

Fig. 1. Oxidative variables in the brain *mdx* mouse. Thiobarbituric acid reactive species (TBARS) – MDA equivalents (A) and carbonyl protein (B). Bars represent means ± S.D. of 5 rats. $^{*}p < 0.05$ vs. wild-type according to *Student t test*.

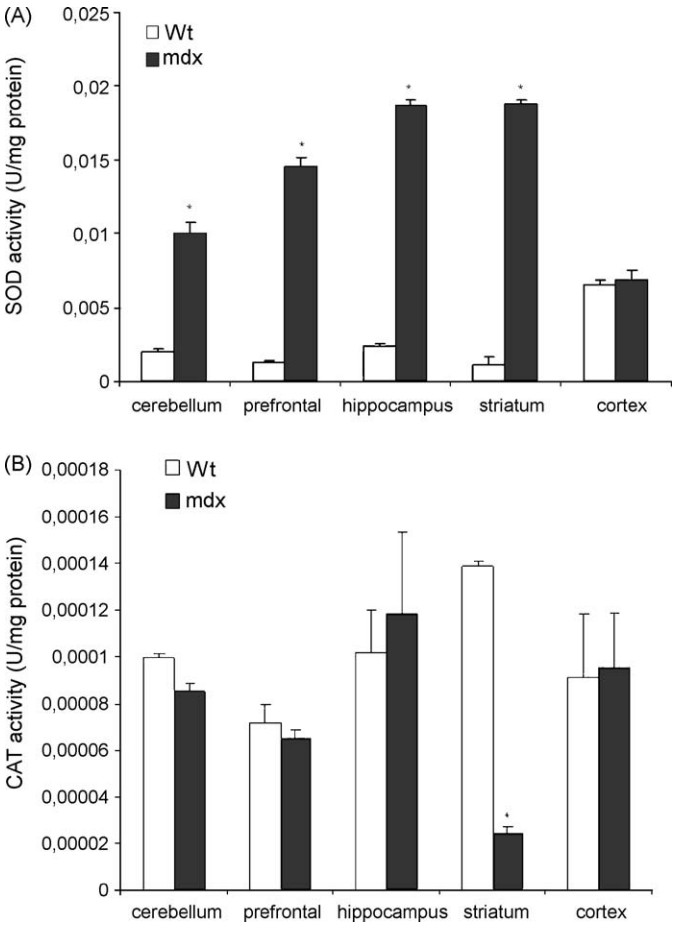

Fig. 2. Oxidative variables in the brain *mdx* mouse. Catalase (CAT) (A) and superoxide dismutase (SOD) (B) activities. Bars represent means ± S.D. of 5 rats. $^{*}p < 0.05$ vs. wild-type according to *Student t test*.

demonstrated an increase in SOD activity in all brain regions ($p < 0.05$; Fig. 2A). In contrast, the striatum had a decrease in CAT activity ($p < 0.05$; Fig. 2B), compared with the wild-type.

4. Discussion

The lack of dystrophin can affect the brain in several ways. First, dystrophin is expressed in the developing brain (Sogos et al., 1992), and mutations that affect the dystrophin complex can affect neuronal migration and differentiation (Mehler et al., 1992). Second, the lack of dystrophin affects neuronal excitability, since this protein is found in the postsynaptic apparatus and serves to anchor receptors, including the $GABA_A$ receptor (Haenggi and Fritschy, 2006). The absence of dystrophin also affects long-term synaptic plasticity (Culligan and Ohlendieck, 2002). Third, the loss of dystrophin may lead to neuronal death (Jagadha and Becker, 1988). Fourth, there are decreased brain-derived neurotrophic factor (BDNF) levels, a regulator of neuronal survival, fast synaptic transmission, and activity-dependent synaptic plasticity, in *mdx* mouse brain (Comim et al., 2009). Such brain abnormalities might help explain the cognitive impairment presented in DMD patients.

The present work demonstrated that *mdx* mice showed: (1) reduced lipid peroxidation (striatum) and protein peroxidation (cerebellum and prefrontal cortex); (2) increased superoxide dismutase activity (cerebellum, prefrontal cortex, hippocampus, and striatum); and (3) reduced catalase activity (striatum). Moreover, the enhanced susceptibility of dystrophin-deficient neurons to hypoxia-induced loss of synaptic transmission, suggests that Ca^{2+}-dependent pathways of neurotoxicity might have a role in the CNS pathology seen in DMD (Mehler et al., 1992). In fact, DMD and *mdx* mouse model appear to impair intracellular calcium homeostasis in CNS and disrupt multiple protein–protein interactions that normally promote information transfer and signal integration from the extracellular environment to the nucleus within regulated microdomains (Rae et al., 2002). The bioenergetic abnormalities reported in DMD brain have also been documented in the *mdx* mouse, with an increased inorganic phosphate to phosphocreatine ratio, increased intracellular brain pH, alteration in metabolism of glucose, and abnormally clustered $GABA_A$ receptors (Rae et al., 2002). Furthermore, cultured cerebellar granule-cell neurons from *mdx* mice have 24% more free Ca^{2+} compared with those from normal mice (Hopf and Steinhardt, 1992). These results could indicate that similar abnormalities in Ca^{2+} homeostasis in dystrophin-deficient muscle are also reflected in neurons (Niebrój-Dobosz and Hausmanowa-Petrusewicz, 2005). In Dystrophin muscle, oxidative stress is a potential pathogenetic factor which may determine the severity of the pathology. The action of free radicals should be limited by several defense mechanisms (Niebrój-Dobosz and Hausmanowa-Petrusewicz, 2005). Free radicals are known to be responsible for chemical and molecular damage of DNA, nucleotides, proteins, lipids, carbohydrates, and cell membrane structure (Slater, 1984).

Taken together, our data suggest an up-regulation of antioxidant enzyme activity and lipid peroxidation characterized in *mdx* muscle tissue (Vajda et al., 2004). A previous study in *mdx* mice found a net increase in total SOD activity in the diaphragm, gastrocnemius, and soleus muscles (Vajda et al., 2004). In some psychiatric diseases, there has been found the up-regulation of antioxidant enzyme activity (Lucca et al., 2009a,b; Frey et al., 2006). These data corroborate to our results in *mdx* brain tissue (cerebellum, prefrontal cortex, hippocampus, and striatum). These findings can be interpreted as the result of elevated superoxide radical production in mitochondria, the locus of the induced superoxide radical scavenging enzyme, SOD. On the other hand, we found CAT activity to be decreased in striatum and normal in the all other brain regions. CAT in known to clean an excess of peroxide, diminishing the oxidative effects of hydrogen peroxide. Thus, an imbalance between SOD and CAT activity could lead to oxidative stress (Slater, 1984).

The brain is particularly sensitive to oxidative damage due to its relative high content of peroxidizable fatty acids and limited antioxidant capacity (Floyd, 1999). Several enzymes generate free radicals, including xanthine, urate, coproporphyrinogen III, glucose, lysyl, monoamine, D-amino acid oxidases, and superoxide dismutase (Halliwell and Gutteridge, 1997). Because reactive oxygen species 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 animal muscular dystrophies in which the expression and localization of dystrophin is unaffected (Lidov et al., 1990; Blake and Kröger, 2000), increases in antioxidant enzyme activities and measures of lipid peroxidation are consistent with enhanced oxidative stress (Blake and Kröger, 2000).

In conclusion, this study showed the first evidence of oxidative variables and antioxidants enzymes in the *mdx* mouse brain tissue. It seems that the increase in superoxide dismutase activity may be playing an important protective role against the oxidative stress effects, paradoxically to the fact that in situations in which superoxide dismutase levels are increased without a concomitant catalase increase. As seen in our results, the intermediate product (hydrogen peroxide) may accumulate and generate hydroxyl radicals that may lead to lipid and protein oxidation. These data might be of use in conducting further studies to help in elucidating the possible physiopathology mechanism of oxidative stress and antioxidant defenses in *mdx* mouse brain.

Acknowledgements

This research was supported by grants from CNPq, FAPESC, Instituto Cérebro e Mente and UNESC (FD-Pand JQ). FD-P and JQ are CNPq Research Fellows. FP is a holder of a CAPES studentship, CMC is a holder of a CNPq studentship, and LSC is a holder of a FAPESC/CAPES studentship.

References

Abraham, S., Soundararajan, C.C., Vivekanandhan, S., Behari, M., 2005. Erythrocyte antioxidant enzymes in Parkinson's disease. Indian J. Med. Res. 121, 111–115.
Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Anderson, J.L., Head, S.I., Rae, C., Morley, J.W., 2002. Brain function in Duchenne muscular dystrophy. Brain 125, 4–13.
Bannister, J.V., Calabrese, L., 1987. Assays for superoxide dismutase. Methods Biochem. Anal. 32, 279–312.
Blake, D.J., Kröger, S., 2000. The neurobiology of duchenne muscular dystrophy: learning lessons from muscle? Trends Neurosci. 23, 92–99.
Comim, C.M., Tuon, L., Stertz, L., Vainzof, M., Kapczinski, F., Quevedo, J., 2009. Striatum brain-derived neurotrophic factor levels are decreased in dystrophin-deficient mice. Neurosci. Lett. 459, 66–68.
Culligan, K., Ohlendieck, K., 2002. Diversity of the brain dystrophin–glycoprotein complex. J. Biomed. Biotechnol. 2, 31–36.
Durany, N., Münch, G., Michel, T., Riederer, P., 1999. Investigations on oxidative stress and therapeutical implications in dementia. Eur. Arch. Psychiatry Clin. Neurosci. 249, 68–73.
Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. 186, 407–421.
Floyd, R.A., 1999. Antioxidants, oxidative stress, and degenerative neurological disorders. Proc. Soc. Exp. Biol. Med. 222, 236–245.
Frey, B.N., Valvassori, S.S., Réus, G.Z., Martins, M.R., Petronilho, F.C., Bardini, K., Dal-Pizzol, F., Kapczinski, F., Quevedo, J., 2006. Changes in antioxidant defense enzymes after d-amphetamine exposure: implications as an animal model of mania. Neurochem. Res. 31, 699–703.
Haenggi, T., Fritschy, J.M., 2006. Role of dystrophin and utrophin for assembly and function of the dystrophin glycoprotein complex in non-muscle tissue. Cell. Mol. Life Sci. 63, 1614–1631.
Halliwell, B., Gutteridge, J.M., 1997. Lipid peroxidation in brain homogenates: the role of iron and hydroxyl radicals. J. Neurochem. 69, 1330–1331.

Hopf, F.W., Steinhardt, R.A., 1992. Regulation of intracellular free calcium in normal and dystrophic mouse cerebellar neurons. Brain Res. 578, 49–54.
Jagadha, V., Becker, L.E., 1988. Brain morphology in Duchenne muscular dystrophy: a Golgi study. Pediatr. Neurol. 4, 87–92.
Levine, R.L., Williams, J.A., Stadtman, E.R., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. 233, 346–357.
Lidov, H.G., Byers, T.J., Watkins, S.C., Kunkel, L.M., 1990. Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons. Nature 348, 725–728.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Lucca, G., Comim, C.M., Valvassori, S.S., Réus, G.Z., Vuolo, F., Petronilho, F., Gavioli, E.C., Dal-Pizzol, F., Quevedo, J., 2009a. Increased oxidative stress in submitochondrial particles into the brain of rats submitted to the chronic mild stress paradigm. J. Psychiatr. Res. 43, 864–869.
Lucca, G., Comim, C.M., Valvassori, S.S., Réus, G.Z., Vuolo, F., Petronilho, F., Dal-Pizzol, F., Gavioli, E.C., Quevedo, J., 2009b. Effects of chronic mild stress on the oxidative parameters in the rat brain. Neurochem. Int. 54, 358–362.
Mehler, M.F., Haas, K.Z., Kessler, J.A., Stanton, P.K., 1992. Enhanced sensitivity of hippocampal pyramidal neurons from mdx mouse to hypoxia-induced loss of synaptic transmission. Proc. Natl. Acad. Sci. USA 89, 2461–2465.
Muntoni, F., Mateddu, A., Serra, G., 1991. Passive avoidance behaviour deficit in the mdx mouse. Neuromuscul. Disord. 1, 121–123.
Niebrój-Dobosz, I., Hausmanowa-Petrusewicz, I., 2005. The involvement of oxidative stress in determining the severity and progress of pathological processes in dystrophin-deficient muscles. Acta Biochim. Pol. 52, 449–452.
Rae, C., Griffin, J.L., Blair, D.H., Bothwel, l, J.H., Bubb, W.A., Maitland, A., Head, S., 2002. Brain biochemical abnormalities associated with lack of dystrophin; studies in the mdx mouse. Neuromuscul. Disord. 12, 121–129.
Ragusa, R.J., Chow, C.K., Porter, J.D., 1990. Oxidative stress as a potential pathogenic mechanism in an animal model of Duchenne muscular dystrophy. Neuromuscul. Disord. 7, 379–386.
Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S.M., Berger, R., Tanzi, R.E., Halperin, J.J., Herzfeldt, B., Bergh, R.V., Hung, W.-Y., Bird, T., Deng, G., Mulder, D.W., Smyth, C., Laing, N.G., Soriano, E., Pericak–Vance, M.A., Haines, J., Rouleau, G.A., Gusella, J.S.H., Horvitz, R., Brown Jr., R.H., 1993. Mutations in Cu/Zn-superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62.
Slater, T.F., 1984. Free-radical mechanisms in tissue injury. Biochem. J. 222, 1–15.
Sogos, V., Curto, M., Reali, C., Gremo, F., 1992. Developmentally regulated expression and localization of dystrophin and utrophin in the human fetal brain. Mech. Ageing Dev. 123, 455–462.
Vaillend, C., Rampon, C., Davis, S., Laroche, S., 2002. Gene control of synaptic plasticity and memory formation: implications for diseases and therapeutic strategies. Curr. Mol. Med. 2, 613–628.
Vaillend, C., Rendon, A., Misslin, R., Ungerer, A., 1995. Influence of dystrophin-gene mutation on mdx mouse behaviour. I. Retention deficits at long delays in spontaneous alternation and bar-pressing tasks. Behav. Genet. 25, 569–579.
Vajda, Z., Pedersen, M., Doczi, T., Sulyok, E., Nielsen, S., 2004. Studies of mdx mice. Neuroscience 129, 993–998.