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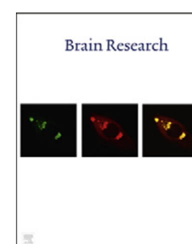

Research Report

Quantitative changes of nicotinic receptors in the hippocampus of dystrophin-deficient mice

Paulo César Ghedini[a,1], *Maria Christina W. Avellar*[b], *Thereza Christina M. De Lima*[c], *Maria Teresa R. Lima-Landman*[a], *Antônio José Lapa*[a,d], *Caden Souccar*[a,*]

[a]*Department of Pharmacology, Section of Natural Products, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil*
[b]*Department of Pharmacology, Section of Experimental Endocrinology, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil*
[c]*Laboratory of Neuropharmacology, Department of Pharmacology, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil*
[d]*Laboratory of Pharmacology and Toxicology, Amazon Biotechnology Center, Manaus, AM, Brazil*

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A B S T R A C T

Lack of dystrophin in Duchenne muscle dystrophy (DMD) and in the mutant *mdx* mouse results in progressive muscle degeneration, structural changes at the neuromuscular junction, and destabilization of the nicotinic acetylcholine receptors (nAChRs). One-third of DMD patients also present non-progressive cognitive impairments. Considering the role of the cholinergic system in cognitive functions, the number of nAChR binding sites and the mRNA levels of α4, β2, and α7 subunits were determined in brain regions normally enriched in dystrophin (cortex, hippocampus and cerebellum) of *mdx* mice using specific ligands and reverse-transcription polymerase chain reaction assays, respectively. Membrane preparations of these brain regions were obtained from male control and *mdx* mice at 4 and 12 months of age. The number of $[^3H]$-cytisine (α4β2) and $[^{125}I]$-α-bungarotoxin ($[^{125}I]$-αBGT, α7) binding sites in the cortex and cerebellum was not altered with age or among age-matched control and *mdx* mice. A significant reduction in $[^3H]$-cytisine (48%) and $[^{125}I]$-αBGT (37%) binding sites was detected in the hippocampus of *mdx* mice at 12 months of age. When compared with the age-matched control groups, the *mdx* mice did not have significantly altered $[^3H]$-cytisine binding in the hippocampus, but $[^{125}I]$-αBGT binding in the same brain region was 52% higher at 4 months and 20% lower at 12 months. mRNA transcripts for the nAChR α4, β2, and α7 subunits were not significantly altered in the same brain regions of all animal groups. These results suggest a potential alteration of

Abbreviations: αBGT, α-Bungarotoxin; nAChR, Nicotinic acetylcholine receptor; CA1, Cornus ammonis 1 of hippocampus; cDNA, Complementary DNA; CNS, Central nervous system; DGC, Dystrophin glycoprotein complex; DMD, Duchenne muscle dystrophy; dNTPS, Deoxynucleotide triphosphates; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; $GABA_AR$, γ-Aminobutyric acid subtype A receptor; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; mRNA, Messenger ribonucleic acid; NMDA, N-methyl-D-aspartate; PMSF, Phenylmethyl sulphonyl fluoride; RNAse, Ribonuclease; RT-PCR, Real-time polymerase chain reaction; Tris–HCl, Tris(hydroxymethyl)aminomethane hydrochloride

**Corresponding author.* Fax: +55 11 55764499.
E-mail address: csouccar@unifesp.br (C. Souccar).
[1] Present address: Institute of Biological Sciences, Department of Physiological Sciences, Federal University of Goiás, Campus Samambaia, Goiânia-GO, Brazil.

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the nicotinic cholinergic function in the hippocampus of dystrophin-deficient mice, which might contribute to the impairments in cognitive functions, such as learning and memory, that have been reported in the dystrophic murine model and DMD patients.

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1. Introduction

Duchenne muscle dystrophy (DMD) is an X-linked muscle disease that manifests as a progressive and irreversible muscle degeneration with a high incidence in boys (Emery, 2002). The myopathy is caused by mutations in the dystrophin gene resulting in a lack of protein expression (Hoffman et al., 1987). One-third of DMD patients also present non-progressive cognitive impairment, as well as behavioral and neuropsychiatric disorders of variable intensities (Cyrulnik et al., 2008; Hendriksen and Vles, 2008; Mehler, 2000).

Dystrophin is a 427-kDa protein expressed in striated muscles and the central nervous system (CNS). It is localized at the cytoplasmic face of the plasma membrane linking the intracellular cytoskeleton with the extracellular matrix (Ervasti, 2007; Pilgram et al., 2010). Three full-length dystrophin (Dp427) isoforms, which are transcribed from distinct promoters of the *DMD* gene, are expressed in muscles (Dp427M), throughout the brain (Dp427B), and in cerebellar Purkinje cells (Dp427P). Four other shorter isoforms (Dp260, Dp140, Dp116, and Dp71), regulated by internal promoters, are expressed in the CNS and other tissues (Blake and Kroger, 2000; Perronnet and Vaillend, 2010). The mutant *mdx* mouse is a well-studied model of DMD that lacks the expression of full-length dystrophin in both the muscles and brain (Sicinski et al., 1989). At the cell membrane, dystrophin is associated with a complex of glycoproteins (DGC) comprised of dystroglycans, syntrophins, dystrobrevins, sarcoglycans, and sarcospan (Blake et al., 2002; Ervasti, 2007). In striated muscles, the lack of dystrophin disrupts the macromolecular complex and damages the plasma membrane, resulting in muscle degeneration and necrosis (Petrof, 2002). At the neuromuscular synapse, dystrophin and some components of the DGC are necessary for the maturation of post-junctional folds and the regulation of nicotinic acetylcholine receptors (nAChRs) (Ghedini et al., 2008; Grady et al., 2000; Huh and Fuhrer, 2002). The protein complex also plays a role in Ca^{2+} homeostasis and cell signaling pathways involved in the maintenance of cell membrane integrity (Batchelor and Winder, 2006).

In normal brains, dystrophin is abundant in the cerebral cortex, hippocampus, cerebellum, and amygdala, where it is located at postsynaptic densities of the neuronal synapses (Lidov, 1996; Sakamoto et al., 2008; Sekiguchi et al., 2009). In the hippocampus, cerebellum (Knuesel et al., 1999), and amygdala (Sekiguchi et al., 2009) of the *mdx* mouse, the lack of dystrophin and DGC has been shown to cause a reduction in the size and number of $GABA_A$ receptor ($GABA_AR$) clusters containing the α1 and α2 subunits. A decrease in kainate-type glutamate receptor density has also been described in different brain regions of dystrophin-deficient mice (Yoshihara et al., 2003). These observations suggest that dystrophin and the DGC play a role in the stability of receptors and synaptic function in the brain, indicating that cognitive impairment in the murine model and DMD patients might be related to dysfunction in synaptic transmission (Perronnet and Vaillend, 2010; Pilgram et al., 2010). Moreover, compared with control animals, *mdx* mice have been shown to exhibit a decreased response to nicotine in the passive avoidance test, suggesting a possible downregulation of nAChRs in the CNS (Coccurello et al., 2002). Decreased mRNA expression of the α3 nAChR subunit has also been observed in the cortex and hippocampus of *mdx* mice (Wallis et al., 2004), suggesting possible dysfunctions in nicotinic cholinergic transmission in the brain.

nAChRs are widely distributed in the brain, where they play a role in cognitive functions, such as attention, memory and learning. They are also involved in pathological conditions, such as Alzheimer's disease, Parkinson's disease, schizophrenia, anxiety, depression, and epilepsy (Newhouse et al., 2004; Sacco et al., 2004). These receptors are ligand-gated cationic channels formed by the pentameric combination of different α (α2–α10) and β (β2–β4) subunits expressed in the nervous system (Dani and Bertrand, 2007). In the brain, the most frequent nAChR subtypes are α4β2 (heteropentamers), which bind nicotine with high affinity (Flores et al., 1992), and α7 (homopentamers), which bind nicotine with low affinity and α-bungarotoxin with high affinity (Séguéla et al., 1993).

In view of the importance of the cholinergic system in cognitive functions (Gold, 2003), which are impaired in some DMD patients, the aim of this work was to quantify the binding sites for α4β2 and α7 nAChRs subtypes and to measure their mRNA levels in both whole brain tissue and in brain regions normally enriched in dystrophin (i.e., the cortex, hippocampus and cerebellum) of *mdx* mice. Considering that the progression of muscle disease in *mdx* mice is very slow compared to that in DMD patients, and that behavioral alterations and biochemical abnormalities in the brain are more evident in older (>6 months) *mdx* mice (Rae et al., 2002), the analysis was conducted at two stages of muscle disease: in young adults (4 months old), after maximal muscle degeneration has occurred (DiMario et al., 1991), and in old (12 months old) *mdx* mice, when the murine model exhibits some features of the muscle disease (Pastoret and Sebille, 1995).

2. Results

2.1. Binding sites for [^{3}H]-cytisine

The specific binding of [^{3}H]-cytisine to whole brain membranes from control and *mdx* mice was saturable and represented 80–85% of total ligand binding. The number of [^{3}H]-cytisine binding sites (B_{max}) in these membrane preparations decreased from 4 to 12 months of age by 20% in the control, and by 35% in *mdx* mice. However, there were no significant changes in the affinity of the ligand binding (K_d) in

Table 1 – Saturation binding of [^{3}H]-cytisine and [^{125}I]-α-bungarotoxin to whole brain membranes of 4- and 12-month-old control and *mdx* mice.

Ligand	4-months		12-months	
	Control	*mdx*	Control	*mdx*
[^{3}H]-cytisine				
B_{max} (fmol/mg protein)	97.4±2.1	101.4±2.4	78.2±5.2*	65.8±3.3*
K_d (nM)	1.16±0.08	1.27±0.09	1.52±0.27	1.33±0.18
[^{125}I]-α-bungarotoxin				
B_{max} (fmol/mg protein)	46.9±3.9	49.5±3.9	51.5±5.4	77.4±10.3
K_d (nM)	0.90±0.17	1.04±0.25	1.00±0.18	1.13±0.12

Data are means±SEM of three assays each in triplicate.
* Different from the 4-month-old group in a same animal strain ($p<0.05$).

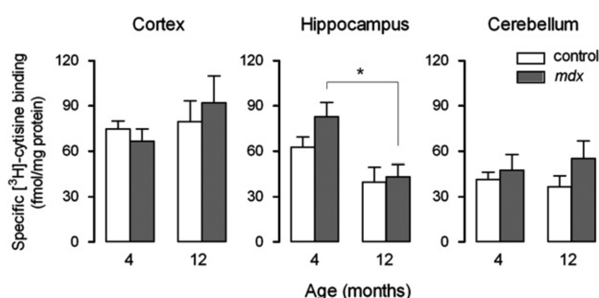

Fig. 1 – Specific binding of [^{3}H]-cytisine in the cortex, hippocampus, and cerebellum of 4- and 12- month-old control and *mdx* mice. The data are given as the means±SEM of three experiments repeated in triplicate. $^*p<0.05$ compared with the 4-month-old *mdx* group (ANOVA and Tukey–Kramer's *post-hoc* test).

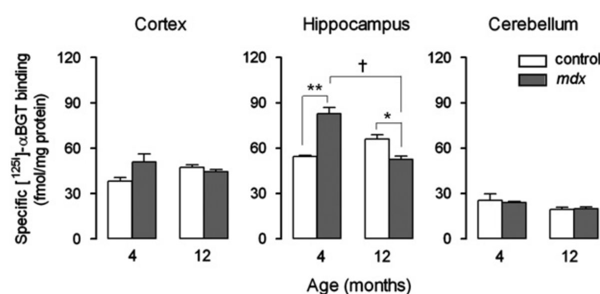

Fig. 2 – Specific binding of [^{125}I]-α-bungarotoxin ([^{125}I]-αBGT) in the cortex, hippocampus and cerebellum of 4- and 12-month-old control and *mdx* mice. The data are given as the means±SEM of three experiments repeated in triplicate. $^*p<0.05$, $^{**}p<0.001$ compared to the age-matched control group. $^†p<0.05$ compared with the 4-month-old *mdx* group (ANOVA and Tukey–Kramer's post-hoc test).

either animal group (Table 1). When compared with age-matched control groups, the B_{max} and K_d values of [^{3}H]-cytisine binding in the *mdx* brain membranes did not differ at either age (Table 1).

In the cortex and cerebellum, there was no significant age-related difference in the number of [^{3}H]-cytisine binding sites in either animal strain or between age-matched control and *mdx* mice (Fig. 1). In the hippocampus, there was no significant age-related difference in the number of [^{3}H]-cytisine binding sites in the control group; however, it decreased by 48% with age in *mdx* mice from 4 (82.9±9.4 fmol/mg protein) to 12 (43.2±8.2 fmol/mg protein) months (Fig. 1). When compared with age-matched control groups, the number of [^{3}H]-cytisine binding sites in the *mdx* hippocampus did not differ at either age (Fig. 1). These results indicated that the number of [^{3}H]-cytisine binding sites decreased with age in the hippocampal region of *mdx* mice, and in whole brain preparations of control and *mdx* mice.

2.2. *Binding sites for [^{125}I]-α-bungarotoxin ([^{125}I]-αBGT)*

The specific binding of [^{125}I]-αBGT to whole brain membranes from control and *mdx* mice was saturable and represented 90–95% of total ligand binding. The B_{max} and K_d values of [^{125}I]-αBGT binding were not significantly altered with age in either animal strain, and they did not differ between the age-matched control and *mdx* mice at either age (Table 1).

In the cortex and cerebellum, the number of [^{125}I]-αBGT binding sites did not change from 4 to 12 months of age or between the age-matched control and *mdx* mice (Fig. 2). In the hippocampus, there were no age-related differences in [^{125}I]-αBGT binding in the control group; however, it was decreased by 37% with age in *mdx* mice from 4 (82.9±4.0 fmol/mg protein) to 12 (52.6±2.4 fmol/mg protein) months (Fig. 2). When compared with age-matched control groups, *mdx* mice showed 52% higher [^{125}I]-BGT binding in the hippocampus at 4 months and 20% lower binding at 12 months (Fig. 2). These results indicated that [^{125}I]-αBGT binding decreased with age in the hippocampus of *mdx* mice and that [^{125}I]-αBGT binding is altered in the hippocampus of *mdx* mice when compared with age-matched controls.

2.3. *Relative quantification of α7, α4, and β2 nAChR subunit mRNAs.*

Fig. 3 shows the relative mRNA levels of α4, β2, and α7 nAChR subunits that were determined in the cortex, hippocampus, and cerebellum of 4- and 12-month-old control and *mdx* mice. In all three brain regions, no significant difference was detected in the level of transcripts for α4, β2,

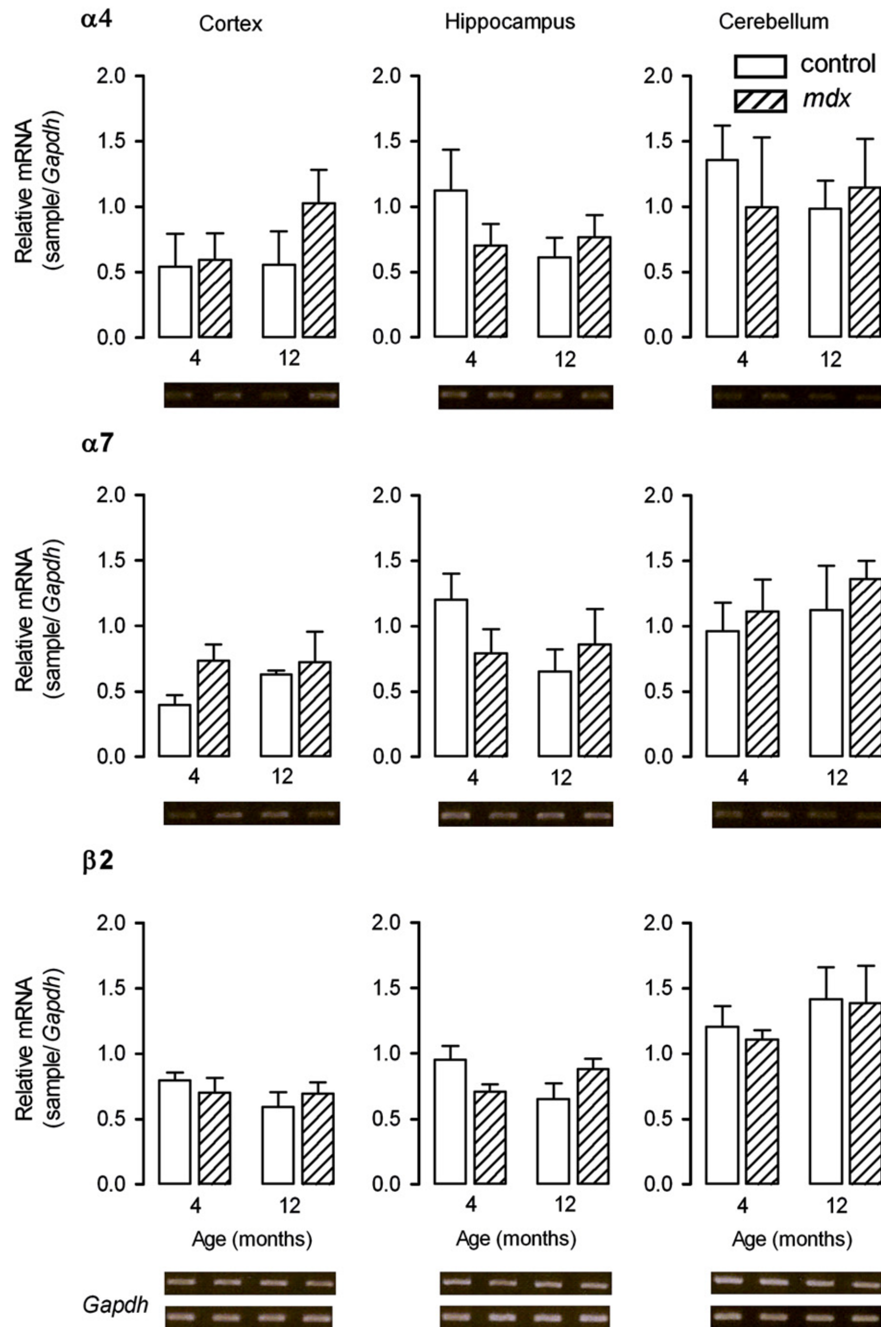

Fig. 3 – Representative semi-quantitative analysis of the relative abundance of the transcripts for the α4, α7, and β2 nAChR subunits in the cortex, hippocampus and cerebellum from 4- and 12-month-old *mdx* compared with age-matched controls. The data are expressed as the ratio of the densitometric measurement for each specific gene compared with the corresponding internal standard (GAPDH) and presented as the means ± SEM for three independent experiments.

and α7 subunits with age or between age-matched control and *mdx* mice.

3. Discussion

To investigate whether central cholinergic dysfunction is associated with a lack of dystrophin, the number of [^{3}H]-cytisine and [^{125}I]-αBGT binding sites and mRNAs levels of α4, β2, and α7 nAChR subunits were determined in membrane preparations from young (4 months old) and old (12 months old) control and *mdx* mice. The brain regions selected were those that are normally enriched in dystrophin, i.e., the cortex, hippocampus, and cerebellum. The results show that dystrophin-deficient mice exhibited significant differences in the number of [^{3}H]-cytisine (predominantly α4β2 nAChRs) and [^{125}I]-αBGT (α7 nAChRs) binding sites in the hippocampus; this may be indicative of alterations in nicotinic cholinergic neurotransmission. Changes in the number and function of nAChRs in various human brain regions have

been implicated in several neurological and cognitive disorders, such as Alzheimer's disease, Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder and autism (Gotti et al., 2006; Guan et al., 2002; Sacco et al., 2004). Similar cognitive impairments, as well as behavioral and neuropsychiatric disorders have also been described in boys with DMD (Hendriksen and Vles, 2008; Mehler, 2000). As the data suggest these impairments might involve alterations in nicotinic cholinergic function associated with a lack of dystrophin in the brain.

The significant decrease in [^{3}H]-cytisine binding sites detected in the hippocampal region of old *mdx* mice was consistent with those observed in whole brain membranes of age-matched control and *mdx* groups, thus reflecting the potential age-related reduction in the α4β2 nAChRs, as previously reported (Gahring et al., 2005). However, the decrease in this receptor subtype was approximately two fold greater in whole brain membrane preparations of old *mdx* mice than in those from control, indicating that the absence of dystrophin facilitates the age-related degenerative effects. This observation was confirmed by the age-related reduction in [^{125}I]-αBGT binding sites obtained in the hippocampal membranes of *mdx* mice, but not in the membranes from control mice. These results are consistent with a disruption in membrane stability being associated with age and lack of dystrophin, as well as to an increase in choline-containing compounds (glycerocholine and phosphocholine), as previously reported in the hippocampus and cerebellum of old (>6 months) *mdx* mice (Rae et al., 2002).

Several studies have shown the importance of dystrophin and DGC in the anchoring and stabilization of receptors and ionic channels (Connors et al., 2004; Pilgram et al., 2010). The lack of dystrophin and DGC disrupts the cell membrane structure, resulting in a reduction in the number and size of $GABA_AR$ clusters in the hippocampus and cerebellum, where the protein is colocalized with these receptors (Graciotti et al., 2008; Knuesel et al., 1999). Decrease in the α3β2/β4 subtypes of nAChR in the superior cervical ganglion (Del Signore et al., 2002; Di Angelantonio et al., 2011), fragmentation of the neuromuscular junction, and upregulation of the embryonic-type nAChRs have also been reported in dystrophin-deficient mice (Ghedini et al., 2008; Grady et al., 2000; Huh and Fuhrer, 2002). Thus, it seems conceivable that the structural disorganization of the cell membrane, associated with a lack of dystrophin, might also be involved in the altered number of [^{3}H]-cytisine and [^{125}I]-αBGT binding sites observed in the hippocampus of *mdx* mice. With regard to the number of α4β2 and α7 nAChR binding sites in *mdx* mice, the hippocampus appears more responsive to the lack of dystrophin than the cortex and cerebellum do, as evidenced by the fact that these regions normally express high levels of dystrophin (Lidov, 1996); however, they did not present marked changes in [^{3}H]-cytisine and [^{125}I]-αBGT binding.

A decrease in the mRNA expression of the α3 nAChR subunit has been reported in the cortex and hippocampus of young (<4 months) *mdx* mice (Wallis et al., 2004). In contrast, the alterations in [^{3}H]-cytisine and [^{125}I]-αBGT binding in the hippocampus of *mdx* mice observed in our study were unaccompanied by significant changes in the expression of α4, β2, and α7 transcripts, indicating that the effects are related to post-transcriptional mechanisms. Similar observations have been reported for the cerebellum of *mdx* mice, in which a decrease in the number of $GABA_AR$ clusters (Knuesel et al., 1999) was unrelated to changes in the expression of the $GABA_AR$ α1-subunit, thereby indicating an alteration in the receptor clustering rather than changes in the receptor protein expression (Kueh et al., 2008). Our results are unrelated to structural changes in the hippocampus of *mdx* mice because there were no significant alterations detected in the gross anatomy and no apparent signs of neuronal loss or cellular pathology found in the same brain region (Miranda et al., 2009). This is in contrast to findings described for the cortex (Carretta et al., 2001; Sbriccoli et al., 1995) and spinal trigeminal nucleus of the murine model (Pinto et al., 2008).

The hippocampus is a brain structure involved in attention, memory, and learning mechanisms in which functional α7, α4β2, and α3β4 nAChR subtypes have been identified (Alkondon and Albuquerque, 2004; Graham et al., 2003). These receptors are expressed at presynaptic and postsynaptic sites in GABAergic (inhibitory) and glutamatergic (excitatory) neurons, and are involved in regulating neurotransmitter release, mediating fast nicotinic synaptic transmission, and modulating synaptic plasticity (Dani and Bertrand, 2007). α7 nAChRs predominate in the hippocampus, and their activation by endogenous ACh release or nicotinic agonists has been shown to facilitate long-term synaptic potentiation, a form of synaptic plasticity underlying learning and memory (Drever et al., 2011; McKay et al., 2007). α7 nAChRs are also densely expressed on GABAergic interneurons in the hippocampus, and their activation in vitro inhibits or disinhibits $GABA_A$ synaptic activity on CA1 pyramidal neurons, thus enhancing or suppressing synaptic potentiation (Alkondon and Albuquerque, 2001; Ji et al., 2001). Our data show that the [^{125}I]-αBGT binding sites in the hippocampus were upregulated in young and reduced in old *mdx* mice with respect to their age-matched controls. The mechanisms underlying the observed quantitative changes in the expression of α7 nAChRs in the hippocampus of *mdx* mice are still unclear. However, considering the influence of α7 nAChRs on GABAergic synaptic activity in the hippocampus (Albuquerque et al., 2009), it is possible that the observed alterations in this receptor subtype might reflect an adjustment of the excitatory–inhibitory circuits in the hippocampal region, related to the decreased number of $GABA_A$ receptors and the lack of dystrophin (Kueh et al., 2008). Consistent with this hypothesis, we have recently shown that [^{3}H]-ACh release evoked by nAChR activation in hippocampal synaptosomes was also significantly altered in preparations from both young and old *mdx* mice compared with those from their age-matched controls (Souccar et al., 2011). These results indicate a potential nicotinic cholinergic dysfunction in the hippocampus of dystrophin-deficient mice; this dysfunction may in turn affect cognitive functions such as learning and memory, both of which involving α4β2 and α7 nicotinic receptors (Levin et al., 2006). Indeed, deficits in attention and memory, among other cognitive and behavioral abnormalities, have been reported in boys with DMD (Cyrulnik et al., 2008), whereas impaired consolidation of certain forms of long-term memory with unaltered acquisition in learning tasks has been described in *mdx* mice

(Vaillend et al., 2004). Vaillend and coworkers have also reported an abnormal facilitation of N-methyl-D-aspartate (NMDA) receptor-mediated synaptic transmission in the hippocampal CA1 region of *mdx* mice, which has been attributed, in part, to neuronal disinhibition associated with a reduced number of $GABA_AR$ clusters and lack of dystrophin (Vaillend and Billard, 2002; Vaillend et al., 2004). Nevertheless, the functional role of the observed quantitative changes in α7 and α4β2 nAChRs in the disrupted NMDA receptor-mediated synaptic plasticity in the hippocampus of *mdx* mice is still unknown, and should be further evaluated.

4. Conclusion

The absence of dystrophin alters the number of α7- and β2-containing nAChRs in the hippocampal region of *mdx* mice, thus supporting a role for dystrophin and DGC in stabilizing these receptors in central synapses. The observed quantitative changes of both nAChR subtypes in the hippocampus of *mdx* mice indicate that putative dysfunction of nicotinic cholinergic synapses in the absence of dystrophin might contribute to the impairment of cognitive functions, such as learning and memory, that have been reported in dystrophic mice and DMD patients.

5. Experimental procedures

5.1. *Animals*

Normal (control) and mutant *mdx* 4- and 12-month-old male mice from an C57Bl/10 strain that was bred in the Animal Facility at the Instituto Nacional de Farmacologia e Biologia Molecular, Escola Paulista de Medicina/Universidade Federal de São Paulo were used. All animals were housed under a controlled 12 h/12 h light/dark cycle and at 22 ± 2 °C with free access to food and water. The animals were sacrificed using cervical dislocation, and the brain was rapidly removed. The cerebral cortex, hippocampus, and cerebellum were dissected over a cold plate and frozen in liquid nitrogen. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the USA National Institutes of Health (Bethesda, Maryland), and the local Animal Investigation Ethical Committee approved all procedures (Protocol # 1400/03).

5.2. *Membrane preparation*

The membranes from the whole brain, cerebral cortex, hippocampus, and cerebellum of control and *mdx* mice were prepared according to Davies et al. (1999). The brain regions of 10 to 12 pooled animals were homogenized in 10 volumes of ice-cold phosphate buffer (50 mM NaH_2PO_4; 50 mM Na_2HPO_4; 1 mM EDTA; 0.1 mM PMSF; 0.01%(w/v) sodium azide, pH 7.4) containing 0.32 M sucrose (Sigma Chemical Co., St Louis, MO). The homogenate was centrifuged at 1,700 *g* for 10 min at 4 °C, the pellet was re-suspended in 20 volumes of sucrose buffer and re-centrifuged. The supernatant fractions were combined, and the pellet was washed twice by centrifugation (15,000 *g*, 4 °C, 30 min). The final pellet was re-suspended in buffer, and the protein content was determined using the Bradford (1976) method.

5.3. *Ligand binding studies*

Binding to α4β2 and α7 nAChR subtypes in the whole brain, cortex, hippocampus, and cerebellum of control and *mdx* mice was determined using the ligands [^{3}H]-cytisine and [^{125}I]-α bungarotoxin ([^{125}I]-αBGT), respectively

5.3.1. *[^{3}H]-cytisine binding assays*

The analysis of the equilibrium binding of [^{3}H]-cytisine to whole brain membranes was performed as previously described (Pabreza et al., 1991). The membrane samples (0.1 mg) were incubated with [^{3}H]-cytisine (specific activity 25 Ci/mmol, Perkin Elmer Inc., Waltham, MA) at concentrations ranging from 0.1 nM to 5.0 nM for 75 min at 4 °C in a buffer solution (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EDTA, and 0.1 mM PMSF, pH 7.4) (Sigma) to a final volume of 0.5 ml. The number of binding sites for [^{3}H]-cytisine in the cerebral cortex, hippocampus, and cerebellum were measured using membrane samples (0.1 mg) incubated with a saturating concentration of the ligand (10 nM, final concentration) and 50 mM Tris–HCl buffer for 75 min at 4 °C. Non-specific binding was determined in the presence of 10 μM nicotine (Sigma). The specific binding was determined as the total binding minus the nonspecific binding. The incubations were terminated using vacuum filtration through A/E glass fiber filters pre-soaked in 0.05%(v/v) polyethyleneimine. The filters were washed twice with ice-cold buffer, and the radioactivity was counted in a scintillation beta counter (Packard Instruments Co., Meriden, Connecticut).

5.3.2. *[^{125}I]-αBGT binding assays*

Saturation binding experiments for [^{125}I]-αBGT were performed using whole brain membranes (0.1 mg protein) incubated in triplicate with 0.1 nM to 10 nM [^{125}I]-αBGT (specific activity 200 μCi/mmol, Amersham Biosciences, U.K.) in 0.5 ml phosphate buffer (final volume), for 120 min at 25 °C. The [^{125}I]-αBGT binding sites for the cortex, hippocampus, and cerebellum were determined by incubating the membrane samples (0.1 mg) from each structure with 10 nM [^{125}I]-αBGT (final concentration) in phosphate buffer for 30 min at 25 °C. The non-specific binding was determined using samples containing 1 μM unlabeled toxin. The incubations were terminated with the addition of 1 ml ice-cold phosphate-buffered saline containing 0.5% BSA followed by centrifugation (15,000 *g*, 20 min, 4 °C). This step was repeated, and samples were washed overnight in the buffer solution at 4 °C. Subsequently, the samples were centrifuged and the radioactivity in the pellet was determined using a gamma counter (Packard Instruments Co., Meriden, Connecticut).

5.4. *Reverse-transcription polymerase chain reaction (RT-PCR)*

Total RNA was extracted from the cortex, hippocampus and cerebellum using Trizol® reagent (Invitrogen, San Diego, CA)

Table 2 – Primer sequences used for the amplification of each transcript coding for nAChR subunits (α4, α7, and β2) and internal control GAPDH, corresponding base sites, and the sizes of PCR products (Δ). Each set of primers spans at least one intron to ensure that the PCR products were from cDNA and not genomic DNA.

Gene	Primer	Oligonucleotide sequence (5′–3′)	Nucleotide position	Δ (bp)
α4	Forward	cagcttccagtgtcagacca	1668–1687	395
	Reverse	tggaagatgtgggtgactga	2043–2062	
α7	Forward	cattccacaccaacgtcttg	414–433	357
	Reverse	tgagcacacaaggaatgagc	751–770	
β2	Forward	gtaccgctggtgggaaagta	1059–1078	472
	Reverse	tccaatcctccctcacactc	1511–1530	
Gapdh	Forward	ggaagctggtcatcaacggg	258–277	357
	Reverse	ggcagtgatgccatggactg	595–614	

GenBank accession numbers: NM_015730 (α4); NM_007390 (α7); NM_009602 (β2); and X02231 (*Gapdh*).

according to the manufacturer's instructions. The RNA (1 μg) was reverse-transcribed using 3 U of ThermoScript reverse transcriptase (Invitrogen) and oligo d(T)$_{20}$ primers. The reaction was performed in a solution containing 50 mM Tris acetate (pH 8.4), 75 mM KOAc, 8 mM Mg $(AO_C)_2$, 5 mM DTT, 1 mM dNTPs and 40 U of RNAse inhibitor at 55 °C for 1 h. The amplification was performed in a thermal cycler (Applied Biosystems, Foster City, CA) with 1 μl of the cDNA in 20 μl of buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 U of Taq DNA polymerase (Invitrogen) and 0.4 μM of each sense- and antisense-specific primer to amplify the specific nucleotide sequences present in the nAChR subunit (α4, α7, and β2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; used as internal control) gene transcripts (Table 2). The PCR cycling conditions for the amplification of each transcript in the linear range and analysis of final DNA products on an agarose gel containing ethidium bromide (0.5 μg/ml) were conducted as previously described (Ghedini et al. 2008). The gel photographs were scanned and the results were quantified using Scion Image Analysis (Scion Corporation, Frederick, Maryland) for the densitometric analysis. The data were normalized against that of the GAPDH mRNA in each sample.

5.5. *Statistical analysis*

The data were presented as the means ± SEM values. The saturation binding data for both ligands were analyzed using nonlinear regression. The differences between the control and *mdx* groups were determined using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer's *post-hoc* test. Student's *t* test (two-tailed not paired) was used to compare two groups. The data were analyzed using Graphpad software (GraphPad Prism version 5.00, San Diego, CA). A probability value of 0.05 or less was accepted as significant.

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