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Morphological changes in the trigemino-rubral pathway in dystrophic (*mdx*) mice

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Abstract

The lack of dystrophin that causes Duchenne muscle disease affects not only the muscles but also the central nervous system. Dystrophin-deficient *mdx* mice present changes in several brain fiber systems. We compared the projections from the trigeminal sensory nuclear complex to the red nucleus in control and *mdx* mice using retrograde tracers. Injection of 200 nL 2% fluorogold into the red nucleus caused labeling in the mesencephalic trigeminal nucleus, the principal sensory nucleus and the oral, interpolar, and caudal subnuclei of the spinal trigeminal nucleus in both control and *mdx* mice. Injection of latex microbeads labeled with rhodamine and fluorescein gave results similar to those seen with fluorogold. The number of labeled neurons in the trigeminal sensory nuclear complex was significantly reduced in *mdx* mice. In the oral subnucleus of the spinal trigeminal nucleus this reduction was 50%. These results indicate that the trigemino-rubral pathway is reduced in dystrophin-deficient mice.

Keywords: Dystrophin; Duchenne muscle disease; *mdx* mouse; Red nucleus; Trigeminal sensory nuclear complex; Fluorogold

Duchenne muscular dystrophy (DMD) is an X-linked myopathy caused by lack of dystrophin. Dystrophin is a 427 kDa protein on the cytosolic face of the sarcolemma that appears to stabilize the membrane during repeated cycles of muscular contraction [3]. Several animal models have been used to study this disease [3], among them the *mdx* mouse [26], which carries a spontaneous mutation that results in dystrophin deficiency.

Dystrophin is also present in neuromuscular synapses and in the central nervous system (CNS) [21,28], where it is believed to affect cellular motility, the shape of the soma, intracellular transport, and development [1,18]. In the CNS, dystrophin is found in aggregates on the post-synaptic membrane and in short segments along the soma and dendrites, but not on axons [14,17,21]. Dystrophin density is especially high in the cerebral cortex, cerebellum, and the CA1 and CA3 regions of the hippocampus [13,17], but it is also detected in regions of the brain stem, including the inferior olive complex, the reticular formation, and the trigeminal complex [5,17–19].

In humans, the lack of dystrophin causes moderate non-progressive cognitive and memory impairment [17]. Dystrophin-deficient mice rapidly forget newly learned information [22,29,30] and present changes in cerebellar neurotransmission [1,2]. Dystrophin deficiency in mice also causes structural changes in the brain, including a reduction of the number of neurons of the cortico-spinal pathway, changes in the shape of these neurons [25], alterations in the blood–brain barrier [23,24,31], and reduced cerebral kainic-acid receptor density [33].

It was recently reported that connections from the red nucleus to the spinal cord are compromised in the dystrophin-deficient *mdx* mouse [4]. The red nucleus is a structure involved in the control of distal muscles and orofacial movement that receives sensory input from the trigeminal sensory nuclear complex (TSNC) [11,20,27].

To investigate the projections from the TSNC to the red nucleus in *mdx* mice, we injected tracers (fluorogold and latex microbeads coated with fluorescein or rhodamine) into the red nucleus and examined the retrograde transport of the tracers to the sensory nuclei of the trigeminal complex.

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All experimental procedures conformed to the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the Ethics in Research Committee of the Federal University of São Paulo (protocol 0362/04). We used male C57BL10 mice, 20–30 g, 3 months old, 14 control, and 9 mutant dystrophic mice (*mdx*). Mice were housed individually in plastic cages on a floor of wood chips, in a room with controlled temperature (22–25 °C) and light-dark cycle (lights on from 6 to 18 h). They had free access to water and chow.

Mice were anesthetized by i.p. injection of 1 mg ketamine and 0.2 mg xylazine. They were placed in a stereotaxic instrument, and small holes were drilled in the skull above the left and right red nucleus. Injections were made with a 0.5 μL Hamilton syringe, connected with polyethylene tubing to a stainless steel injection needle with an outer diameter of 0.2 mm. The injection coordinates were 3.35 mm rostral, 0.55 mm lateral, and 4.3 mm ventral of the bregma [10]. In one side, 200 nL 2% fluorogold dissolved in sterile saline was injected. In the contralateral side, 400 nL of a suspension of latex microspheres (20–200 nm diameter) impregnated with a tracer was injected. In half of the cases the label was rhodamine, in the rest, fluorescein (red and green retrobeads, respectively, Lumafluor Inc., Naples).

Five to seven days after injection of the tracers, the mice were anesthetized again and perfused through the heart with 100 mL 0.9% NaCl, followed by 200 mL 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed, incubated for 24 h in the fixative, and incubated in phosphate buffer with 30% sucrose for 48 h. The brain stems were cut on a cryostat in 35 μm coronal slices and mounted on microscope slides with Jung Tissue freezing medium (Leica, Nussloch, Germany).

The spread of the tracers was analyzed on an Olympus BX 60 epifluorescence microscope with appropriate filters (fluorogold: excitation 340–360 nm, absorbance 420 nm; rhodamine: excitation 510–560 nm, absorbance 590 nm; fluorescein: excitation 450–490 nm, absorbance 520 nm). Because the spacing between the slides in the atlas of Franklin and Paxinos is large for our aims, we prepared from a different mouse a set of serial 10 μm sections stained with Klüver-Barreira to locate the fluorescent label with more precision.

The following nuclei in the trigeminal sensory nuclear complex were distinguished: the mesencephalic trigeminal nucleus (Me5), the principal sensory nucleus (Pr5), and the oral, interpolar, and caudal subnuclei of the spinal trigeminal nucleus (Sp5O, Sp5I, and Sp5C). The spinal trigeminal nucleus was subdivided as described previously [15,16]. Briefly, the rostral and caudal limits of the oral subnucleus are the same as those of the nucleus of the facial nerve. Laterally and rostrally the oral subnucleus borders to the principal sensory nucleus. The interpolar subnucleus extends from the oral subnucleus to the substancia gelatinosa at the level of the obex. The caudal subnucleus extends from the interpolar subnucleus to the spinal medulla, where it fuses with the dorsal horn.

The number of fluorogold-labeled cell bodies in the oral subnucleus of the spinal trigeminal nucleus was determined as the total of labeled cells in each section that contained this subnucleus (about eight sections per mouse). Similar methods have been used by other authors to measure retinal ganglion cell loss [6] and the loss of neurons that project from the spinal cord to the brainstem [34].

Table 1
Sites of injection of fluorogold and nuclei labeled by retrograde transport in control and dystrophin-deficient mice (*mdx*)

Case	Size (mm)	Location		Me5	Pr5	Sp5		
		Type	Level			O	I	C
Wild-type mice								
8	1.4	p + m	3.08	*	*	*	*	*
5	1.1	p + m	3.40	*	*	*	*	*
19	1.1	p + m	3.40	*	*	*		
2	1.1	p + m	3.40	**	**	**	**	**
10	1.2	p + m	3.40	**	**	**	**	**
16	1.7	p + m	3.40	**	**	**	**	**
20	1.3	p + m	3.40	**	**	**	**	**
22	1.2	p + m	3.40	**	**	**	**	**
9	1.1	p + m	3.40	**	**	**	**	**
23	1.7	m	3.52	**	**	**	**	**
24	1.8	m	3.52	**	**	**	**	**
25	1.3	m	3.64	**	**	**	**	**
21	1.1	m	3.80	**	**	**	**	**
4	1.1	m	4.04	**	**	**	**	**
mdx mice								
16	1.2	p	3.28	*	*	*		
2	1.3	p + m	3.40	*	*	*		
17	1.3	p + m	3.40	*	*	*		
1	1.4	m	3.52	**	**	**	**	**
11	0.9	m	3.64	**	**	**	**	**
15	1.4	m	3.64	**	**	**	**	**
7	1.3	m	3.80	**	**	**	**	**
12	1.3	m	3.80	**	**	**	**	**
13	1.1	m	3.80	**	**	**	**	**

Column 2 shows the anterior–posterior extension of the injection site. Column 3 shows injection type: p = parvocellular, m = magnocellular part of the red nucleus. Column 4 shows the rostro-caudal coordinates of the injection center, relative to the atlas of Franklin and Paxinos [10]. Italicized cases were used for quantification of labeled neurons in the Sp5O. * and ** indicate low and high percentage of labeled cells, respectively.

Projection patterns from the trigeminal sensory nuclear complex to the red nucleus appeared similar in control and *mdx*. In both groups, fluorogold injections that labeled the magnocellular part of the red nucleus caused bilateral staining in the TSNC, including the mesencephalic trigeminal nucleus, the principal sensory nucleus, and the oral, interpolar, and caudal subnuclei of the spinal trigeminal nucleus (Table 1). Contralateral staining was more intense than ipsilateral staining. The trigeminal motor nucleus was not labeled. Examples of injection sites and of label transported retrogradely are shown in Fig. 1.

Injections in the parvocellular (rostral) part of the red nucleus caused bilateral retrograde transport to the same trigeminal nuclei, but the intensity was much lower than with magnocellular injections. With injections in the intermediate part of the red nucleus, which contains both magnocellular and parvocellular neurons, the amount of transported tracer was higher when the injections labeled more magnocellular regions of the red nucleus.

Injection sites were smaller with fluorescent beads (mean ± S.E.: 713 ± 31 μm, $n = 16$ versus 1249 ± 46 μm, $n = 23$). The pattern of transport of microbeads and fluorogold was similar.