Molecular and Cellular Neuroscience 80 (2017) 1–17

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

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

NGF-dependent axon growth and regeneration are altered in sympathetic neurons of dystrophic *mdx* mice

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ARTICLE INFO

Article history:
Received 9 May 2016
Revised 9 December 2016
Accepted 29 January 2017
Available online 2 February 2017

Keywords:
Axon growth
Axon regeneration
Duchenne muscular dystrophy
Dystrophin-dystroglycan complex
mdx mice
NGF signaling
TrkA receptor

ABSTRACT

Duchenne muscular dystrophy (DMD) is a lethal disease, determined by lack of dystrophin (Dp427), a muscular cytoskeletal protein also expressed by selected neuronal populations. Consequently, besides muscular wasting, both human patients and DMD animal models suffer several neural disorders. In previous studies on the superior cervical ganglion (SCG) of wild type and dystrophic *mdx* mice (Lombardi et al. 2008), we hypothesized that Dp427 could play some role in NGF-dependent axonal growth, both during development and adulthood. To address this issue, we first analyzed axon regeneration potentials of SCG neurons of both genotypes after axotomy *in vivo*. While noradrenergic innervation of *mdx* mouse submandibular gland, main source of nerve growth factor (NGF), recovered similarly to wild type, iris innervation (muscular target) never did. We, therefore, evaluated whether dystrophic SCG neurons were poorly responsive to NGF, especially at low concentration. Following *in vitro* axotomy in the presence of either 10 or 50 ng/ml NGF, the number of regenerated axons in *mdx* mouse neuron cultures was indeed reduced, compared to wild type, at the lower concentration. Neurite growth parameters (*i.e.* number, length), growth cone dynamics and NGF/TrkA receptor signaling in differentiating neurons (not injured) were also significantly reduced when cultured with 10 ng/ml NGF, but also with higher NGF concentrations. In conclusion, we propose a role for Dp427 in NGF-dependent cytoskeletal dynamics associated to growth cone advancement, possibly through indirect stabilization of TrkA receptors. Considering NGF activity in nervous system development/remodeling, this aspect could concur in some of the described DMD-associated neural dysfunctions.

1. Introduction

Duchenne muscular dystrophy (DMD) is a common X -linked recessive disease, in which patients exhibit progressive and irreversible muscle degeneration, together with significant neurological abnormalities (Blake and Kroger, 2000; Mehler, 2000; Waite et al., 2009; Pilgram et al., 2010). The disease is caused by mutations in the DMD gene (Koenig et al., 1987), which encodes dystrophin, a protein of the cortical cytoskeleton of 427 kDa (Dp427) expressed in skeletal, cardiac and smooth muscles, in several brain areas (Mehler, 2000; Waite et al., 2009) and in autonomic neurons (De Stefano et al., 1997). Dp427 is part of a large trans-membrane glycoproteic complex, the dystrophin-associated glycoprotein complex (DGC), characterized by structural and signaling properties (Blake et al., 2002; Davies and Nowak, 2006). In contrast to the situation in muscles, where Dp427 expression reaches a plateau already in fetal life (Chelly et al., 1990), dystrophin expression in the brain appears to be developmentally regulated, possibly modulating aspects of neurogenesis, neuronal migration and differentiation, neuronal size and dendritic arborization (Jagadha and Becker, 1988; Mehler, 2000). Indeed, first major neuronal alterations in DMD patients and *mdx* mice, an animal model of the disease, occur during development, leading after birth to various degrees of cognitive and behavioral abnormalities (Pilgram et al., 2010; Anderson et al., 2002; Vaillend et al., 2004; Cyrulnik and Hintoon, 2008).

The peripheral nervous system (PNS) is also affected, as autonomic dysfunctions have been reported in DMD patients (Yotsukura et al., 1995). We previously described several structural and functional alterations in the superior cervical ganglion (SCG) of *mdx* mice, a sympathetic ganglion, which innervates different muscular (*i.e.* heart, iris) and non-muscular (*i.e.* salivary glands) targets and expresses Dp427 in wild type animals (De Stefano et al., 1997). In particular, between postnatal day 5 (P5) and 10 (P10), coinciding with the natural occurring cell death, the number of *mdx* mouse neurons projecting to muscular targets significantly decreased compared to wild type. However, well before the beginning of neuronal death (*i.e.* P0), noradrenergic innervation of muscular targets was also reduced, and all ganglionic neurons, regardless of the type of target they innervate, showed less axon

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http://dx.doi.org/10.1016/j.mcn.2017.01.006
1044-7431/

defasciculation and terminal branching (De Stefano et al., 2005; Lombardi et al., 2008). For these same post-natal dates, we also reported significant alterations in the protein levels of NGF receptors (TrkA and p75NTR), which suggested an imbalance in the NGF signaling cascade (Lombardi et al., 2008), and a differently modulated expression of genes encoding proteins involved in neuron survival and differentiation (Licursi et al., 2012).

Purpose of this study was to investigate the role of Dp427 in NGF-dependent axonal growth and regeneration, by using *in vivo* and *in vitro* experimental paradigms. Our results highlight a novel role for the Dp427-dystroglycan complex in axon growth of both maturing and regenerating neurons. This could be achieved by indirect stabilization of membrane-bound TrkA receptors and consequent mediation of the NGF-dependent cytoskeletal dynamics associated to growth cone advancement. These data could provide the incentive for new research aimed at developing therapeutic strategies to reduce neural dysfunctions and autonomic failures in DMD patients.

2. Materials and methods

2.1. Animals

Wild type and genetically dystrophic *mdx* C57BL/10 mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were used. All studies were carried out in accordance with the guidelines promulgated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Guide, revised 1996), in the European Convention for the protection of Vertebrate Animals used for Experimental and Other Scientific Purposes of the Council of Europe (no. 123, June 15th, 2006), and in accordance with The Code of Ethics of the EU Directive 2010/63/EU. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques. The experimental procedures and protocols were approved by the Ethical Committee for Animal Research of the Italian Ministry of Public Health.

2.2. In vivo axotomy

Postganglionic nerve crush was performed as previously described (Zaccaria et al., 1998). Briefly, male adult mice were anesthetized by an intra-peritoneal injection of chloral hydrate (400 mg/kg body mass), the right SCG was exposed, and both the internal and external carotid nerves were crushed. The wound was sutured and the mice were left to recover from anesthesia. Mice that had been operated (at least 4 mice for each time point) were killed 1, 24 or 60 days (d) after surgery, for the biochemical experiments, and 1, 6, 12 and 24 d after surgery for the immunohistochemical studies.

2.3. Cell cultures

All reagents used for SCG neuron cultures were from Sigma-Aldrich (Milan, Italy), unless otherwise indicated. Cultures were prepared according to established protocols (Di Angelantonio et al., 2010). SCGs were dissected from P0 to P2 mouse pups of either sex, collected in a pre-warmed L15 medium containing antibiotics (100 unit/ml penicillin and 0.1 mg/ml streptomycin) and cleaned of the surrounding tissues. Ganglia were treated, for 30 min at 37 °C, with 2 mg/ml collagenase in sterile phosphate buffered saline (PBS) and, after collagenase removal, incubated, for 30 min at 37 °C, with 0.5% trypsin in Ca^{2+}-free and Mg^{2+}-free Hanks' balanced salt solution (HBSS). SCG were transferred into warm L15 medium containing 10% fetal bovine serum (FBS) and antibiotics; cells were then mechanically dissociated using a sterile pipette, and their density was determined in a counting chamber. Depending on the type of experiments we performed, cells were plated on either 12 mm coverslips, 25 mm coverslips or 35 mm Petri dishes, at different densities (as indicated in the specific paragraphs), and maintained, at 37 °C and humidified atmosphere with 5% CO_2, in DMEM/F12 medium containing antibiotics, 10% FBS and different NGF concentrations (1, 10, 50 or 100 ng/ml) (Peprotech EC Ltd., London, UK). Both coverslips and plates had been previously coated with 10 μg/ml poly-L-ornithine (3–4 h at 37 °C) and 20 μg/ml laminin (overnight at 37 °C).

2.4. Western immunoblot

The primary antibodies used for Western immunoblots are listed in Table 1.

2.4.1. Preparation of tissue extracts and cell lysates

Six to seven-week old operated wild type and *mdx* mice and matching non-operated controls were anesthetized with isoflurane (Merial, Milan, Italy) and killed by decapitation. Irises and submandibular glands (SbGl) were quickly removed on ice, individually frozen and stored at −80 °C until use. To obtain tissue extracts, each iris was placed in 15 μl of ice-cold RIPA buffer (50 mM Tris/HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% SDS, 1% Triton X-100, 1× inhibitor cocktail, 1 mM PMSF, 0.2 mM Na_3VO_4 and 1 mM NaF) and sonicated for 30 s at a frequency of 30 kHz with a UP100H Ultrasonic Processor (Dr. Hielsher GmbH, Teltow, Germany). In contrast, each SbGl was placed in RIPA buffer (20 μl RIPA/mg fresh tissue mass), triturated with an Ultraturrax and then sonicated.

Cell lysates were obtained from SCG cultures (300,000–500,000/35 mm Petri dishes) by adding 50 μl of the same RIPA buffer used for tissue homogenization directly into the dish, and were collected after 10 min incubation at room temperature (RT). Tissue homogenates and cell lysates were centrifuged (14,000 rpm for 10 min at 4 °C) and a measured aliquot of the supernatant was used to determine protein concentration by using the Micro BCA kit (Pierce, Rockford, IL, USA). Loading buffer (4×: 200 mM Tris/HCl pH 6.8, 4% SDS, 30% glycerol, 4% β-mercaptoethanol, 4% blue bromophenol) was added to the remaining

Table 1
Primary antibodies used in Western immunoblot and immunofluorescence experiments.

Antibody	Dilution	Company
Western immunoblot		
Rabbit anti-tyrosine hydroxylase	1:1000	Millipore (Billerica, MA, USA)
Rabbit anti-actin	1:2000	Sigma-Aldrich (Milan, Italy)
Mouse anti-dystrophin rod domain (Dys1)	1:15	Novocastra Laboratories (Newcastle upon Tyne, United Kingdom)
Mouse anti-dystrophin C-terminal domain (Dys2)	1:10	Novocastra Laboratories
Rabbit anti-TrkA,	3 μg/ml	Abcam (Cambridge, UK)
Mouse anti-phospho-TrkA	1:500	Santa Cruz Biotechnology (Dallas, TX, USA)
Rabbit anti-phospho-TrkA	1:500	Sigma-Aldrich
Rabbit anti-Akt	1:1000	Cell Signaling (Beverly, MA, USA)
Rabbit anti-phospho-Akt	1:1000	Cell Signaling
Rabbit anti-PI3K p85/p55	1:1000	Cell Signaling
Rabbit anti-phospho-PI3K p85/p55	1:1000	Cell Signaling
Rabbit anti-p44/p42 MAPK (ERK1/2)	1:1000	Cell Signaling
Rabbit antiphospho-p44/p42 MAPK (ERK1/2)	1:1000	Cell Signaling
Rabbit anti-S6	1:1000	Cell Signaling
Rabbit anti-phospho S6	1:1000	Cell Signaling
Rabbit anti-eEF1A1 + 2 + L3	1 μg/ml	Abcam
Immunofluorescence		
Mouse anti-neuron specific βIII-tubulin (Tuj1)	1:3000	Covance (Emeryville, CA, USA)
Rabbit anti-caspase 3 cleaved	1:300	Cell Signaling
Mouse anti-β-dystroglycan	1:10	Monosan (Uden, The Netherlands)
Mouse anti-β-dystrobrevin diluted 1:500;	1:500	Transduction Laboratories (Franklin Lakes, NJ, USA)
Rabbit anti-TrkA	1:750	AbCam