Experimental Gerontology 106 (2018) 159–164

Contents lists available at ScienceDirect

Experimental Gerontology

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

An evaluation of common markers of muscle denervation in denervated young-adult and old rat gastrocnemius muscle

Paul Hendrickse[a,b], Magdalena Galinska[a], Emma Hodson-Tole[a], Hans Degens[a,b,*]

[a] School of Healthcare Science, Manchester Metropolitan University, UK
[b] Institute of Sport Science and Innovation, Lithuanian Sports University, Kaunas, Lithuania

ARTICLE INFO

Keywords:
Atrophy
Sarcopenia
Biomarkers
Neuromuscular junction

ABSTRACT

A large part of age-related muscle wasting is due to incomplete reinnervation of fibres that have become denervated following motoneuron loss. Neural cell adhesion molecule (NCAM) and sodium channel NaV1.5 are considered markers for denervation, but the time course of changes in their expression following denervation has never been systematically evaluated in young-adult and old muscle. To assess the time course of denervation-induced changes in their expression, the left gastrocnemius muscle in 15 young-adult (5-month) and 10 old (25-month) male Wistar rats was denervated for 1, 2 or 4 weeks, while the right muscle served as an internal control. Sections were stained for α-bungarotoxin, to visualise the neuromuscular junctions, combined with NCAM, polysialylated NCAM (PSA-NCAM) or NaV1.5.

In young-adult animals, denervation induced a transient decrease in junctional and cytoplasmic NCAM expression, while in the old NCAM expression was increased after 2 weeks. Cytoplasmic PSA-NCAM was increased in both young-adult and old fibres after 2 weeks denervation with a further increase after 4 weeks in the young only. The junctional PSA-NCAM was transiently increased or decreased in the young and old muscles, respectively. NaV1.5 expression decreased after 1 and 2 weeks of denervation in NaV1.5 in young muscle fibres before returning to control levels, whereas old muscle fibres displayed a transient increase after 1 week followed by a decrease and a return to control levels after 2 and 4 weeks respectively.

In conclusion, NCAM and NaV1.5 are not unequivocally elevated with denervation and consequently are not adequate markers of fibre denervation.

1. Introduction

During the 20th century, a 30-year increase in life expectancy was gained in many parts of the western world, including the United Kingdom (Christensen et al., 2009), leading to an increased burden on healthcare. One of the causes of the increased demand on healthcare is the dramatic loss of muscle mass and strength, known as sarcopenia (Vandervoort and McComas, 1986; Deschenes, 2004; Larsson et al., 1979; Kallman et al., 1990; MacIntosh et al., 2006; Nilwik et al., 2013; Overend et al., 1992; Lexell et al., 1988) that can be as much as 55% between the age of 30 and 80 years (Vandervoort and McComas, 1986). While a significant part of the muscle wasting is attributable to fibre atrophy as a consequence of disuse (Degens and Alway, 2006), there is also a dramatic loss of muscle fibres (Lexell et al., 1988). The latter is considered a consequence of the age-related denervation-reinnervation following motoneuron loss (Faulkner et al., 2007), where denervated fibres are non-functional and contribute to the lower specific tension in old than young-adult rats (Urbanchek et al., 2001). Neural Cell Adhesion Molecule (NCAM) was used by Urbanchek et al. (Urbanchek et al., 2001) to determine the proportion of denervated fibres in young and old rat muscle. Gillon and Sheard (2015) showed that old muscles had a diminished capacity to increase the expression of extra-junctional NCAM during 2 weeks of denervation and suggested this was an indication of an impaired regenerative drive in older individuals (Gillon and Sheard, 2015). In human studies, the increased expression of the sodium channel NaV1.5 has been suggested to reflect the increased number of denervated fibres in old muscle (Rowan et al., 2012). However, so far, no studies have systematically investigated whether these markers are indeed adequate indicators of fibre denervation and whether the expression in a denervated fibre may change after denervation. Therefore, the aim of the present study was to assess the expression of the markers – NCAM, PSA-NCAM and NaV1.5 – in young-adult and old rat muscles that had been denervated for 1, 2 or 4 weeks.

* Corresponding author at: Manchester Metropolitan University, Manchester M1 5GD, United Kingdom.
E-mail addresses: paul.w.hendrickse@stu.mmu.ac.uk (P. Hendrickse), h.degens@mmu.ac.uk (H. Degens).

https://doi.org/10.1016/j.exger.2018.03.007
Received 4 December 2017; Received in revised form 6 February 2018; Accepted 7 March 2018
Available online 08 March 2018
0531-5565/

2. Methods

2.1. Animals

Male Wistar rats were kept two to a cage with *ad libitum* access to food and water in an environment of 22 °C on a 12 h light/12 h dark cycle. The rats were randomly allocated to groups in which their left gastrocnemius and soleus muscles were denervated for 1, 2 or 4 weeks, while the right leg served as an internal control as described previously (van der Meer et al., 2011; Degens et al., 2008). In the old group, there were 3, 3 and 4 animals for the 1-, 2- and 4-week-denervated time point, respectively. In the young group, there were 5 animals for each time point. To denervate the muscles, the tibial nerve was exposed proximal to the head of the gastrocnemius and the medial and lateral branches of the tibial nerve innervating the soleus and gastrocnemius muscles were cut and sewn into the biceps femoris muscle to prevent reinnervation. The surgery was performed under aseptic conditions while the rats were anaesthetised with isoflurane. After surgery, rats were given a subcutaneous injection of Rimadyl (0.5 mg kg^{-1}) as an analgesic. To prevent bias related to age differences within groups, denervation occurred so that each of the young-adult rats were 5 months old and the old rats were 25 months old when sacrificed, regardless of whether they were subjected to 1, 2 or 4 weeks of denervation. After sacrifice, the gastrocnemius muscles were quickly removed, blotted dry and weighed. The muscles were then stretched to slightly above slack length, pinned on cork, frozen in liquid nitrogen and stored at −80 °C until analysis. The local research ethics committee of the Radboud University Nijmegen Medical Centre approved the study.

2.2. Immunofluorescence

Muscle cross-sections (10 µm) were cut in a cryostat at −20 °C, mounted onto slides and stored at −80 °C until use. The slides were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and incubated at room temperature for 10 min in PBS Tween-20 (PBS-T) to permeabilise the sections. Then the sections were incubated for 30 min with blocking solution, containing 4% goat serum (Vector Laboratories, California, USA) in PBS to prevent non-specific staining. Sections were incubated at room temperature in a dark sealed container with conjugated NCAM antibody (1:50, CD56 rabbit polyclonal antibody conjugated with fluorescein isothiocyanate [FITC] Bioss, Massachusetts, USA) for 1 h, or 2 h with a primary antibody against PSA-NCAM (rabbit polyclonal at 1:100 dilution in blocking solution, Millipore, Massachusetts, USA, no. AB5032), or NaV1.5 for 1 h (rabbit polyclonal; 1:150 dilution in blocking solution, Abcam, Cambridge, UK, ab56240). The sections stained for PSA-NCAM or NaV1.5 were then incubated in the dark with AlexaFluor 488 conjugated secondary goat anti-rabbit antibody (1:200, ThermoFisher, Massachusetts, USA). All sections were co-stained with α-bungarotoxin (α-BTX) (1:200, T0195, Sigma-Aldrich, Missouri, USA) to identify the neuromuscular junction (NMJ). Sections stained for NaV1.5 were co-stained with wheat germ agglutinin (1:400, W32466, Thermofisher). The slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield mounting medium with DAPI, Vector Laboratories, California, USA).

2.3. Quantitative analysis

Images of the stained sections were taken using a fluorescent microscope (Zeiss Axio Imager Z1, Zeiss, Germany). The illumination and exposure times were kept constant for the whole experiment for each separate antibody: exposure times were 1400, 4000, 750 and 11,800 ms for α-BTX, NCAM, PSA-NCAM and NaV1.5, respectively. Images were taken at 20× for each section, and for each gastrocnemius muscle the optical density (for cytoplasmic NCAM and PSA-NCAM) and cross-sectional area were measured for 25 muscle fibres with ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.net/Downloads). Preliminary studies had shown that increasing the number of fibres per region of interest beyond 20 did not result in further decreases in the standard deviation for any of the parameters of interest. To determine the levels of junctional NCAM and PSA-NCAM, each NMJ (9–349 per cross-section) of each section for each antibody was photographed at 40× magnification.

Image size was calibrated on ImageJ using a slide micrometre. The NMJs for each section were outlined from 40× images and their optical density (for junctional NCAM and PSA-NCAM) was measured relative to a negative control.

2.4. Statistical analysis

A two-way ANOVA was used with age and duration of denervation as between-subject factors. If a significant duration effect was identified, Bonferroni-corrected *post-hoc* tests were performed to locate the differences (IBM SPSS, version 23, New York, USA). If a significant interaction was found, an ANOVA was performed on the young-adult and old muscles separately to assess the time course in the young-adult and old muscles. The normality of the data was tested using the Shapiro–Wilk test. Effects were considered significant at $p < 0.05$. Data are presented as the mean ± SEM.

3. Results

3.1. Muscle mass fibre size

Data on muscle mass have been presented before (van der Meer et al., 2011), but are given again here for completeness. Both muscle mass (Fig. 1A) and fibre size (Fig. 1B) decreased in both old and young rats after 1 week of denervation ($p < 0.01$) and had decreased further after 2 weeks ($p < 0.01$), with no further significant decrease between

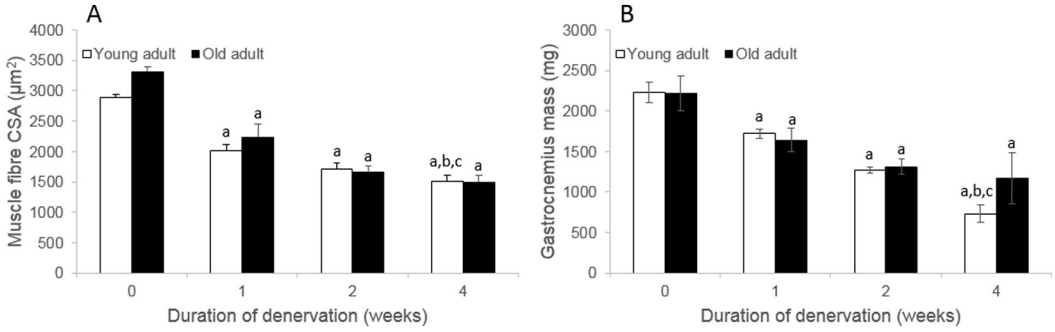

Fig. 1. Effect of four weeks of denervation on A) muscle fibre cross-sectional area (CSA) and B) gastrocnemius muscle mass.
a: different to week 0; b: different to week 1; c: different to week 2, all at $p < 0.01$.