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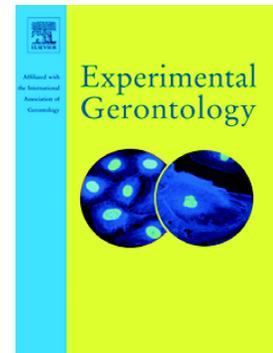
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AN EVALUATION OF COMMON MARKERS OF MUSCLE DENERVATION IN DENERVATED YOUNG-ADULT AND OLD RAT GASTROCNEMIUS MUSCLE

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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.

Keywords: Atrophy, sarcopenia, biomarkers, neuromuscular junction

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 [1], 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 [2-9] that can be as much as 55% between the age of 30 and 80 years [2]. While a significant part of the muscle wasting is attributable to fibre atrophy as a consequence of disuse [10], there is also a dramatic loss of muscle fibres [9]. The latter is considered a consequence of the age-related denervation-reinnervation following motoneuron loss [11], where denervated fibres are non-functional and contribute to the lower specific tension in old than young-adult rats [12]. Neural Cell Adhesion Molecule (NCAM) was used by Urbanchek *et al.* [12] to determine the proportion of denervated fibres in young and old rat muscle. Gillon & 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 [13]. 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 [14]. 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.

Methods

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 hour light/12 hour 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 [15, 16]. 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 whilst 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.

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 hour, or 2 hours 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 hour (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).

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 11800 ms for α-BTX, NCAM, PSA-NCAM and NaV1.5, respectively. Images were taken at 20x 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 40x magnification.

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

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 were tested using the Shapiro-Wilk test. Effects were considered significant at $p < 0.05$. Data are presented as the mean ± SEM.

Results

Muscle mass fibre size

Data on muscle mass have been presented before [15], 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 2 and 4 weeks.

NCAM

There were significant effects of denervation and age ($p < 0.01$), and an age*denervation interaction on NCAM at both cytoplasmic and junctional locations. In young-adult rats, both cytoplasmic (Fig. 2B) and junctional (Fig. 2C) NCAM levels were transiently lower than control after 2 weeks of denervation ($p < 0.01$). In denervated old rat muscle, the expression of NCAM in the cytoplasm (Fig. 2B) was increased after 4 weeks ($p < 0.05$), while at the NMJ levels (Fig. 2C) increased briefly after 1 week ($p < 0.05$) followed by a return to control levels at 2 weeks and an increase again after 4 weeks of denervation ($p < 0.01$).

PSA-NCAM

For cytoplasmic PSA-NCAM there were effects of denervation and age ($p < 0.01$) and a significant age*denervation interaction ($p < 0.05$; Fig. 3B). In young-adult rats, cytoplasmic PSA-NCAM increased after 1 week ($p < 0.01$) followed by a further increase after 4 weeks ($p < 0.01$) of denervation (Fig. 3B). The junctional PSA-NCAM was transiently reduced after 1 and 2 weeks of denervation, followed by return to control levels after 4 weeks (Fig. 3C). In old rats, 1 week of denervation induced an increase in both cytoplasmic (Fig. 3B) and junctional (Fig. 3C) PSA-NCAM above control levels ($p < 0.01$) which persisted in the cytoplasm ($p < 0.01$), but returned to normal levels in the NMJ to non-significant levels after 4 weeks of denervation.

NaV1.5

There was a significant age*denervation interaction on NaV1.5 sodium channel expression. In young-adult rat muscle NaV1.5 expression increased transiently ($p <$ 0.05), reduced after 2 weeks below control ($p < 0.05$) and had returned to normal levels after 4 weeks (Fig. 4B). In muscles from old rats, the intensity of NaV1.5 staining was transiently lower than in controls after 1 and 2 weeks of denervation ($p < 0.05$) after which staining intensity returned to levels not significantly different to controls.

Discussion

The effect of denervation upon the time course of NCAM, PSA-NCAM and NaV1.5 expression in skeletal muscle fibres differed between young and old rats. In contrast to our expectations, the levels of NCAM and NaV1.5 did not increase with increased duration of denervation, but became lower than control levels in young rats, while NCAM and NaV1.5 expression in muscle fibres from older rats were only transiently elevated. Thus, our findings suggest that NCAM and NaV1.5 are not suitable markers for denervation.

Time course of NCAM expression

It has been proposed that the denervation-reinnervation process occurs in skeletal muscle throughout life and that NCAM expression in muscle fibres is stimulated by neuronal activity, which is lost with denervation [17]. In line with this, it has been reported that NCAM expression is reduced in denervated muscle and disappears after reinnervation [18-20]. This is not unequivocal, however, as the multiple methods used to study the effects of denervation on NCAM expression in muscle fibres, such as nerve crush [13, 21], removal of a section of nerve [22], cutting the nerve without preventing reinnervation [18] and using the action potential blocker tetrodotoxin (TTX) [18] have produced conflicting data in respect to NCAM production. While cutting or crushing the nerve, or using TTX induced increases in

NCAM post-denervation in adult rats [18], others using a nerve crush model in mice found no change in cytoplasmic or junctional NCAM levels [13]. Part of the discrepancies between studies may be related to the duration of denervation, where after long-term (25 months) denervation through the removal of a section of the nerve and implantation of the nerve stumps into muscular tissue a reduction, rather than an increase, in NCAM-positive muscle fibres was found [22]. Indeed, the time course of NCAM expression following denervation remains unknown and we found that the junctional and cytoplasmic expression of NCAM is not consistently elevated during 4 weeks after denervation. This indicates that NCAM is not a suitable marker for either short term (4 weeks; this study) or long-term (25 months, [22]) muscle fibre denervation.

In contrast to NCAM, the cytoplasmic, but not the junctional, PSA-NCAM was elevated at all time points after denervation in both young and old rats, something also seen by others [13] and may thus be used as a marker of denervation.

Time course of NaV1.5 sodium channel expression

The NaV1.5 sodium channel has been used in multiple studies as a marker for denervation as it was thought not to be detectable in healthy adult skeletal muscle whilst being present during development and after denervation [13, 14]. It has also been used to identify denervated fibres in old and young muscles subjected to partial denervation [13]. Similar to NCAM, the time course of NaV1.5 expression following denervation has not been studied and the fluctuations in levels of the sodium channel found in the present study after denervation indicate that NaV1.5 is also an inappropriate marker of denervation.

Potential contribution of NCAM to reinnervation

Old muscle fibres have a reduced capacity for complete and effective reinnervation, leading to an accumulation of denervated fibres that contributes to the age-related muscle wasting and weakness [9, 14, 23]. There are many known causes for this, including attenuated regenerative axon sprouting and destabilisation of the NMJ [24], which has, at least partly, been attributed to a diminished NCAM production in old

denervated fibres [13]. We observed, however, increased rather than decreased junctional and cytoplasmic NCAM levels in denervated muscles from old and transiently decreased levels in muscles young rats (Fig.2B, 2C). A similar pattern was observed in junctional PSA-NCAM (Fig. 3C). Although part of the discrepancy between the previous [13] and our study may be due to the species, muscle type and method of denervation, the transient reduction in NCAM in young rats lead us to suggest that the impaired reinnervation of older muscle fibres is not due to a lack of NCAM or PSA-NCAM expression.

Conclusions

The findings of this study demonstrate that NCAM and NaV1.5 are not suitable markers of denervation, but cytoplasmic PSA-NCAM may be used a marker. As even PSA-NCAM is not an all-or-nothing marker further study is required to identify reliable markers for denervation.

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FIGURE LEGENDS

Figure 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 $p < 0.01$.

Figure 2: A: Immunofluorescent stains of cytoplasmic and junctional neural cell adhesion molecule (NCAM) with bungarotoxin (BTX) stain for the neuromuscular junctions (NMJs) B: Effect of four weeks of denervation and age on levels of NCAM at cytoplasmic and junctional locations. a: different to week 0; b: different to week 1; c: different to week 2, at all at $p < 0.05$.

Figure 3: A: Immunofluorescent stains of cytoplasmic and junctional polysyialated neural cell adhesion molecule (PSA-NCAM) with bungarotoxin (BTX) stain for neuromuscular junctions (NMJs) B: Effect of four weeks of denervation and age on levels of PSA-NCAM at cytoplasmic and junctional locations. a: different to week 0; b: different to week 1; c: different to week 2 all at $p < 0.05$.

Figure 4: A: Immunofluorescent stains of cardiac sodium channel (NaV1.5) B: Effect of four weeks of denervation and age on levels of NaV1.5. Image shows bungarotoxin (BTX) stains to highlight the neuromuscular junction and NCAM stains at the same region of interest for young and old animals.

FIGURES

FIGURE 1 Hendrickse et al
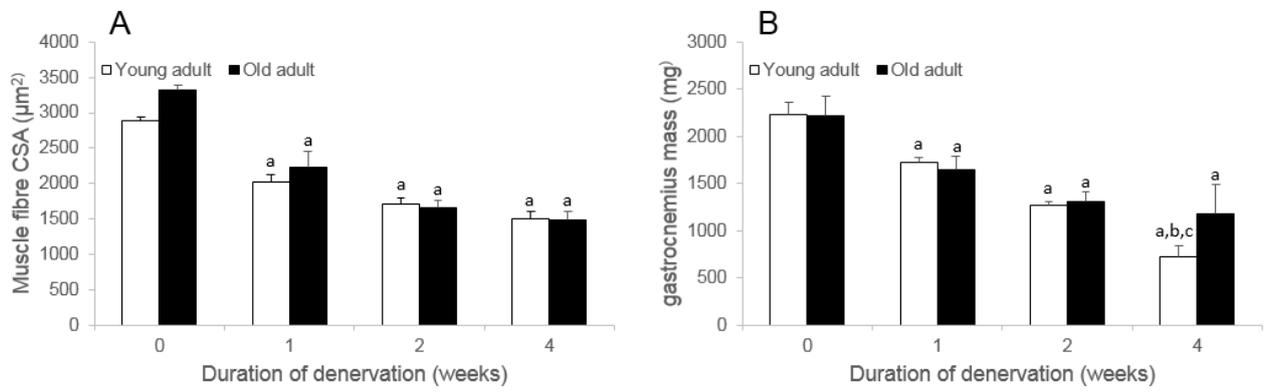
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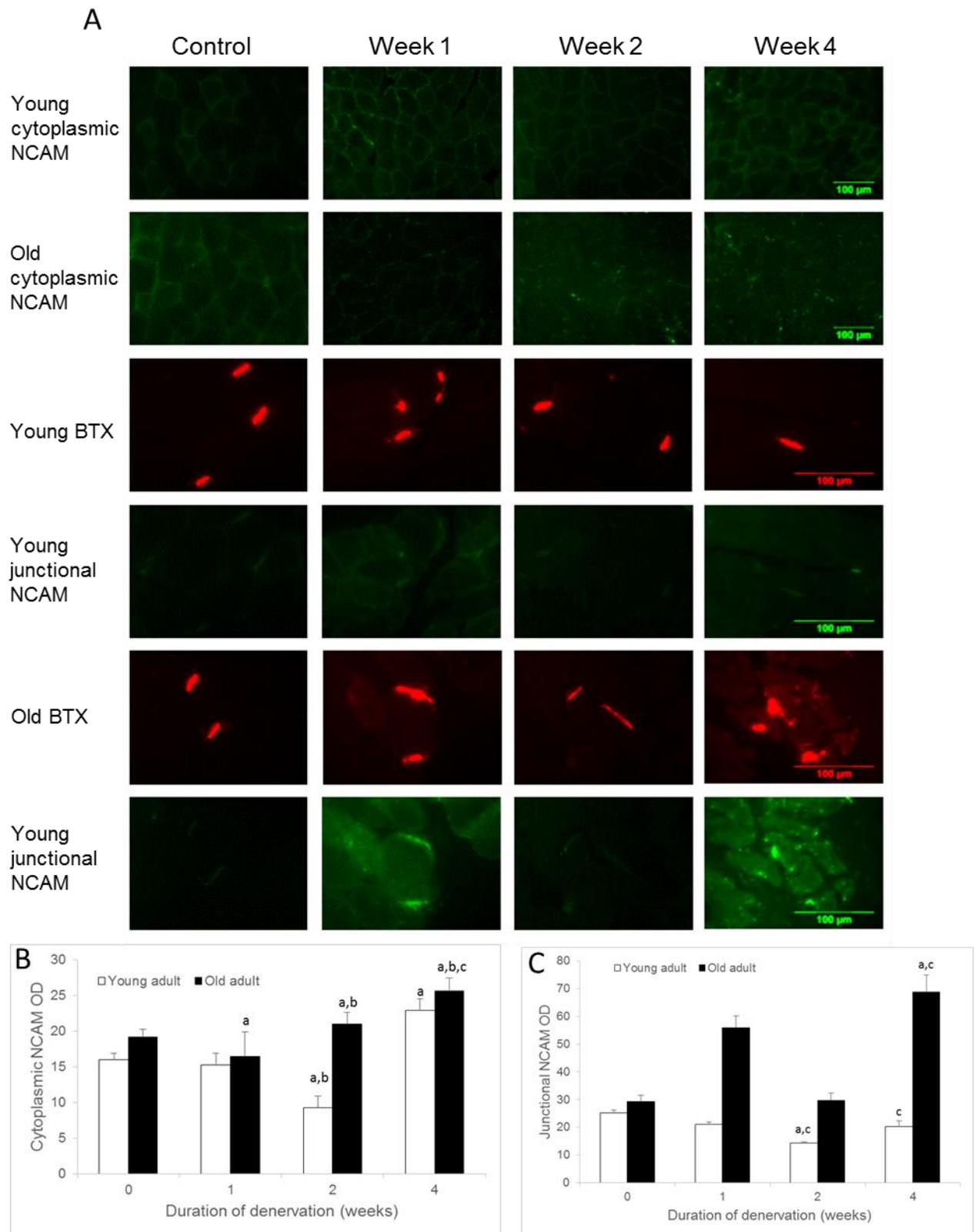
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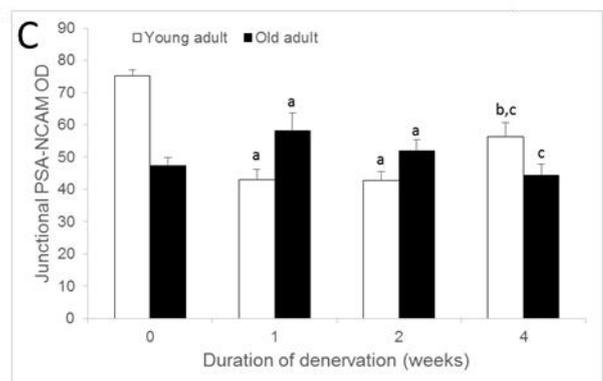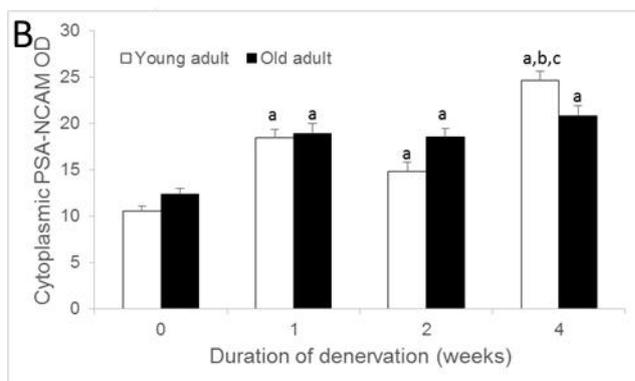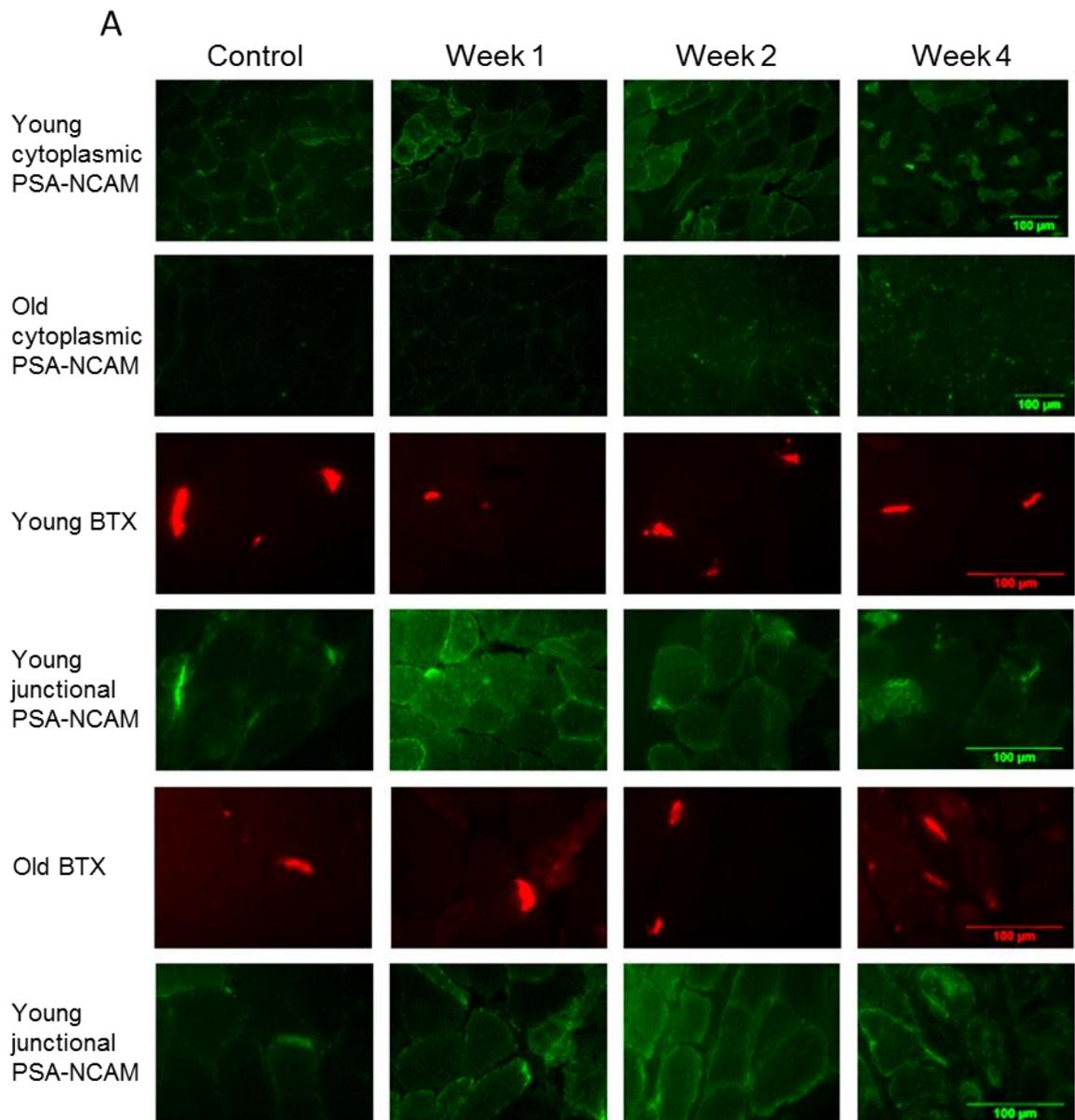
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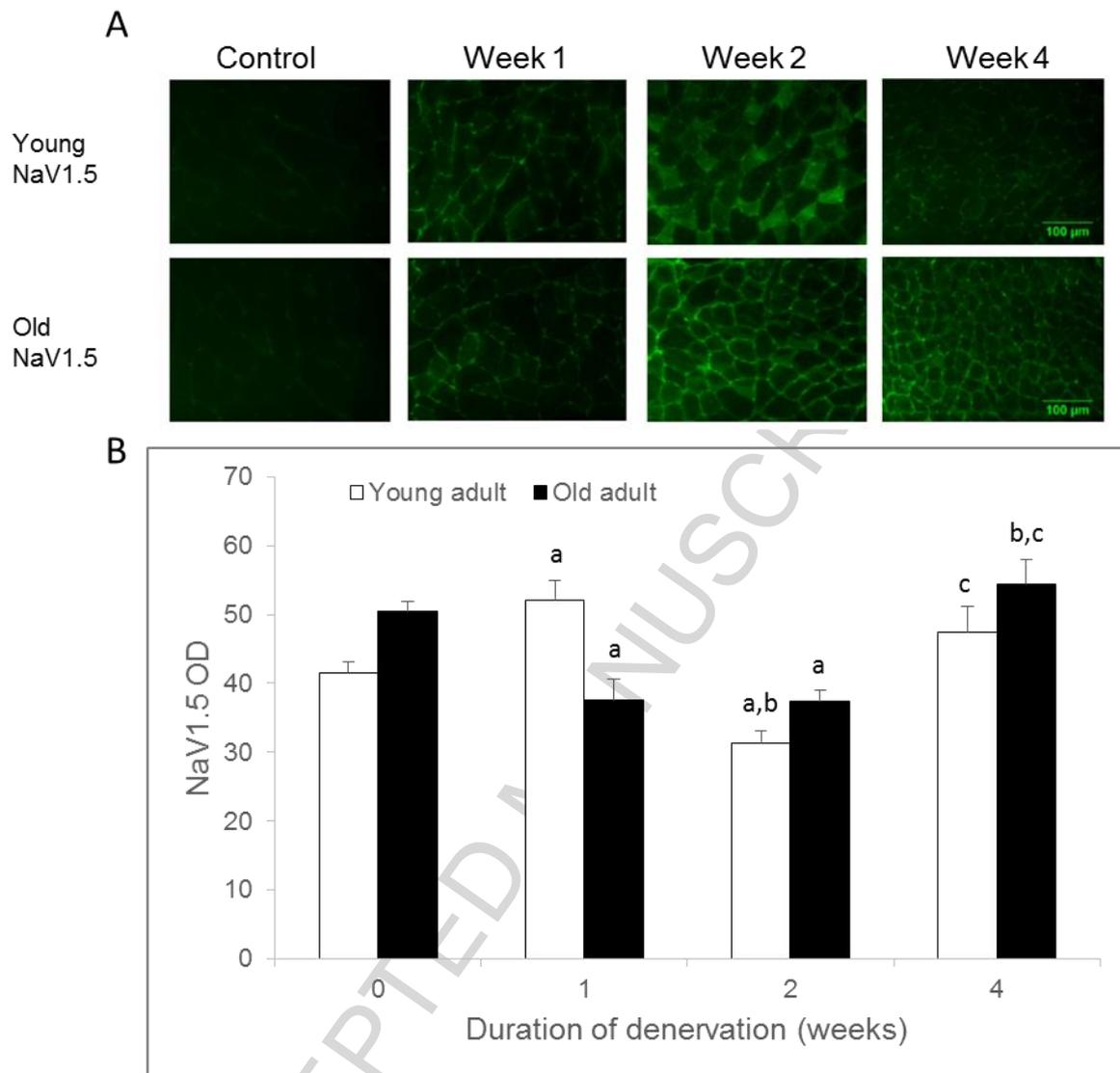
Highlights:

- Changes in NCAM and NaV1.5 expression in fibres after muscle denervation was assessed

- Gastrocnemius muscles of 5- and 25-month-old rats were denervated

- The expression of NCAM and NaV1.5 were determined 1, 2 and 4 weeks after denervation

- NCAM and NaV1.5 are not unequivocally elevated with denervation

- NCAM and NaV1.5 are not adequate markers of fibre denervation