

# A cellular memory mechanism aids overload hypertrophy in muscle long after an episodic exposure to anabolic steroids

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## Key points

- Training studio folklore suggests that previous strength training, with or without the use of anabolic steroids facilitates re-acquisition of muscle mass even after long intervening periods of inactivity. This ‘muscle memory’ has previously been attributed to motor learning, but our data suggest the existence of a cellular memory residing in the muscle fibres themselves.
- Muscle fibres have multiple nuclei, and the number of nuclei increases when muscle mass increases.
- When mice were briefly treated with steroids the muscle mass and number of nuclei increased. The drug was subsequently withdrawn for 3 months and the muscle mass returned to normal, but the excess cell nuclei persisted. When such muscles were subjected to overload they grew by 30% over 6 days while controls grew insignificantly.
- Our data suggest that previous strength training might be beneficial later in life, and that a brief exposure to anabolic steroids might have long lasting performance-enhancing effects.

**Abstract** Previous strength training with or without the use of anabolic steroids facilitates subsequent re-acquisition of muscle mass even after long intervening periods of inactivity. Based on *in vivo* and *ex vivo* microscopy we here propose a cellular memory mechanism residing in the muscle cells. Female mice were treated with testosterone propionate for 14 days, inducing a 66% increase in the number of myonuclei and a 77% increase in fibre cross-sectional area. Three weeks after removing the drug, fibre size was decreased to the same level as in sham treated animals, but the number of nuclei remained elevated for at least 3 months (>10% of the mouse lifespan). At this time, when the myonuclei-rich muscles were exposed to overload-exercise for 6 days, the fibre cross-sectional area increased by 31% while control muscles did not grow significantly. We suggest that the lasting, elevated number of myonuclei constitutes a cellular memory facilitating subsequent muscle overload hypertrophy. Our findings might have consequences for the exclusion time of doping offenders. Since the ability to generate new myonuclei is impaired in the elderly our data also invites speculation that it might be beneficial to perform strength training when young in order to benefit in senescence.

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**Abbreviations** CSA, cross-sectional area; EDL, extensor digitorum longus.

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## Introduction

Together with neurons, muscle fibres constitute one of the two major post-mitotic tissues of the body. They are by volume the largest cells in mammals (Bruusgaard *et al.* 2003), and represent one of very few syncytia in vertebrates. The permanent fibres can contain hundreds of nuclei (Bruusgaard *et al.* 2003), each surrounded by its own synthetic machinery synthesizing protein for a local domain in its immediate vicinity (Hall & Ralston, 1989; Pavlath *et al.* 1989). During overload hypertrophy the increase in fibre volume is preceded by the incorporation of new myonuclei from interstitial muscle stem cells (satellite cells; Schiaffino *et al.* 1976; Bruusgaard *et al.* 2010; Wang & Rudnicki, 2012). Recent evidence suggest that these 'extra' nuclei are permanent or at least long lasting even under subsequent conditions where muscle mass is lost (Wada *et al.* 2002; Aravamudan *et al.* 2006; Bruusgaard & Gundersen, 2008; Gundersen & Bruusgaard, 2008; Bruusgaard *et al.* 2010, 2012; van der Meer *et al.* 2011). Thus, while muscle hypertrophy is completely reversible when it comes to muscle mass, a previous hypertrophic condition seems to convey a lasting imprint on muscle fibres in the form of an elevated number of myonuclei.

We here report that episodic treatment with steroids induced larger fibres with more myonuclei. These extra myonuclei were not lost even months after the muscle size had returned to normal upon drug removal. When such muscles were subjected to overload exercise, however, they grew much faster than controls.

## Methods

### Animal experiments

Female NMRI mice weighing 25–30 g were used. The animal experiments were approved by the Norwegian Animal Research Authority and were conducted in accordance with the Norwegian Animal Welfare Act of 20th December 1974. The Norwegian Animal Research Authority provided governance to ensure that facilities and experiments were in accordance with the Act, National Regulations of January 15th, 1996, and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of March 18th, 1986.

Inhalation gas anaesthesia with 2% isoflurane in air was used for all non-terminal experiments. For terminal experiments, intraperitoneal injections of  $5 \mu\text{l} (\text{g body wt})^{-1}$  of Equithesin (Ullevål Sykehus, Norway;  $42.5 \text{ mg ml}^{-1}$  chloral hydrate and  $9.7 \text{ mg ml}^{-1}$  pentobarbitone) were used. All imaging and surgery was performed under deep anaesthesia. The depth of anaesthesia was checked regularly by pinching the

metatarsus region of the limb, and additional doses were given if necessary. Animals were killed by neck dislocation while deeply anaesthetized.

In order to overload the extensor digitorum longus muscle (EDL), approximately two-thirds of the distal end of the tibialis anterior muscle was excised. Overload of the soleus muscles was performed by excising similar parts of both gastrocnemius and plantaris.

Pellets containing testosterone propionate or sham pellets (A-211 Innovative Research of America, 2.5 mg, release time 21 days) were implanted subcutaneously by making a 5 mm long incision in the skin of the neck.

### Analysis of testosterone levels in blood

In six mice, blood samples were drawn before implantation of the pellet, then 1 and 14 days after implantation, and finally 7 days after removal of the pellet (see inset in Fig. 2A). The analyses were performed at the approved (NS-EN ISO/IEC 17025) Hormone Laboratory at Oslo University Hospital, using a radioimmunoassay (RIA) kit from Orion Diagnostica (Espoo, Finland) after ethyl ether extraction.

### In vivo myonuclear imaging

For *in vivo* labelling of myonuclei, single fibres in the EDL were injected with a solution containing a 5'-TRITC-labelled random 17-mer oligonucleotide with a phosphorothioated backbone (Yorkshire Biosciences Ltd, Heslington, United Kingdom) dissolved in an injection buffer (10 mM NaCl, 10 mM Tris, pH 7.5, 0.1 mM EDTA and 100 mM potassium gluconate). The oligonucleotides are taken up into the nuclei inside the injected fibres apparently by active transport, and serve solely as an intravital nuclear dye in our experiments. The methods have been described in detail previously (Utvik *et al.* 1999; Bruusgaard *et al.* 2003, 2010). In the present study surface fibres were removed to expose fibres in the interior of the muscles. This allowed us to inject fibres that are more representative of the muscle as a whole compared to just the surface fibres that we have injected previously. Thus, both surface and deeper fibres are included in the material. The oligonucleotides contained the randomly selected sequence TAGTCCTAAGTGGACGC, and a BLAST analysis confirmed that the sequence was not represented in the mouse genome either in the sense or antisense direction.

*In vivo* imaging was performed essentially as described previously (Balice-Gordon & Lichtman, 1994; Utvik *et al.* 1999; Bruusgaard *et al.* 2003, 2010). Fibre segments of 250–1000  $\mu\text{m}$  were analysed by acquiring images in different focal planes 5  $\mu\text{m}$  apart on an Olympus BX-50WI compound microscope with a  $\times 20$  0.3 NA long working

distance water immersion objective. All images were acquired with an Andor iXion+ camera, controlled by Andor SOLIS software. By importing the images to a Macintosh computer running Adobe Photoshop and NIH ImageJ software, a stack was generated and used to count all the nuclei in the segment. The counting of nuclei was performed by evaluating all of the images in each stack. Illustration images (Fig. 3C) are composites of several focal planes in order to show all the myonuclei in each fibre segment.

### Immunohistochemistry

Muscles were excised and embedded in OCT Tissue-Tek before being frozen slightly stretched in melting isopentane and stored at  $-80^{\circ}\text{C}$  followed by cryosectioning at  $10\ \mu\text{m}$ . The sections were subsequently blocked in 1% bovine serum albumin, and stained with anti-dystrophin monoclonal antibodies against Dystrophin (Abcam, clone MANDYS8 ab7163). Secondary antibody was goat anti-mouse TRITC conjugated IgG (Sigma T7782). Both antibodies were used at a dilution of 1:200. Nuclei were finally co-stained using Hoechst dye 33342 (Invitrogen;  $0.1\ \mu\text{g ml}^{-1}$  in PBS). As discussed previously (Gundersen & Bruusgaard, 2008; Bruusgaard *et al.* 2012), to ensure that only the nuclei inside muscle fibres were included in the analysis, myonuclei were defined as nuclei with their geometrical centre inside the inner rim of the dystrophin ring.

Histological analysis of myosin heavy chain fibre type was performed as described previously (Lunde *et al.* 2011) using the following panel of monoclonal antibodies: BA-D5 (I), SC-71 (IIa), 6H1 (IIX) and BF-F3 (IIb). All of these were grown in-house from hybridoma stocks obtained from ATCC/LGC Standards. In order to estimate fibre type composition, a grid was placed over the whole muscle section, and fibre type was determined for each fibre that was located at grid intersections.

### Statistics

Differences were tested by analysis of variation (ANOVA) and Bonferroni's correction for multiple comparisons. Values are given as means  $\pm$  SEM.

### Results

We have investigated the effects of anabolic steroids and overload exercise on muscle cells *in vivo*. Adult female mice had pellets releasing testosterone propionate implanted subcutaneously for 14 days. Some of the animals in both groups also had synergist muscles ablated in order to overload either the oxidative/slow soleus or the glycolytic/fast

extensor digitorum longus (EDL) muscle during the drug treatment.

In the soleus, steroid treatment alone increased the number of myonuclei by 66% compared to treatment with sham pellets whereas overload alone increased the number of myonuclei by 51% (Fig. 1A). When steroids and overload were combined, the increase was 92% (Fig. 1A). Qualitatively similar, but less dramatic effects were observed in the EDL (Fig. 1B). Steroid use has also been shown to increase the number of myonuclei in humans (Eriksson *et al.* 2005).

While steroids had no effects on muscle fibre type (Supplemental Fig. S1), changes in fibre size roughly mirrored those of the myonuclei (Fig. 1C and E). In the soleus, steroid treatment alone increased fibre cross-sectional area (CSA) by 77% compared to treatment with sham pellets, while overload alone only induced a 48% hypertrophy. When steroids and overload were combined, a hypertrophy of 118% was observed, and thus the effect of the two treatments was roughly additive. As for the myonuclei, qualitatively similar, but less dramatic effects were observed in the EDL (Fig. 1D). These findings in mice were similar to the effects of resistance exercise and testosterone treatment in humans (Bhasin *et al.* 1996).

We next asked if steroids could have long lasting effects on the number of myonuclei, and if the extra nuclei could aid in building muscle mass after the drug was withdrawn and the size had reverted to normal. The variation in the blood testosterone concentration during the pellet implantation and after its removal is shown in Fig. 2A (inset), and we determined that the testosterone levels were below the detection limit ( $<0.05\ \text{nm}$ ) less than a week after pellet removal. When soleus muscles were investigated 3 weeks after pellet removal, the number of myonuclei was not significantly reduced and remained on average 42% higher than in the sham group (Fig. 2A). The CSAs were, however, completely reversed and the fibre size of steroid and sham treated muscles was indistinguishable (Fig. 2B and C). When overload was introduced at this time and for a subsequent period of 14 days, the steroid group displayed a hypertrophy of 44%, while in the sham treated mice the cells grew by only 17% (Fig. 2B and C).

Even though the EDL responded less strongly to steroids than the soleus (Fig. 1), it was important to investigate EDL further. The soleus is an oxidative postural muscle, and is probably less relevant for strength performances. Moreover, EDL is more suitable for *in vivo* imaging, allowing myonuclei to be observed directly in single fibres *in situ* in the living animal. This was desirable since conventional *ex vivo* histological techniques can introduce artefacts confounding the interpretations in this type of experiments (Gundersen & Bruusgaard, 2008; Bruusgaard *et al.* 2012). The *in vivo* single fibre observations in EDL supported the *ex vivo* analysis of the soleus. Thus, 2 weeks of steroid treatment lead to an increase in the

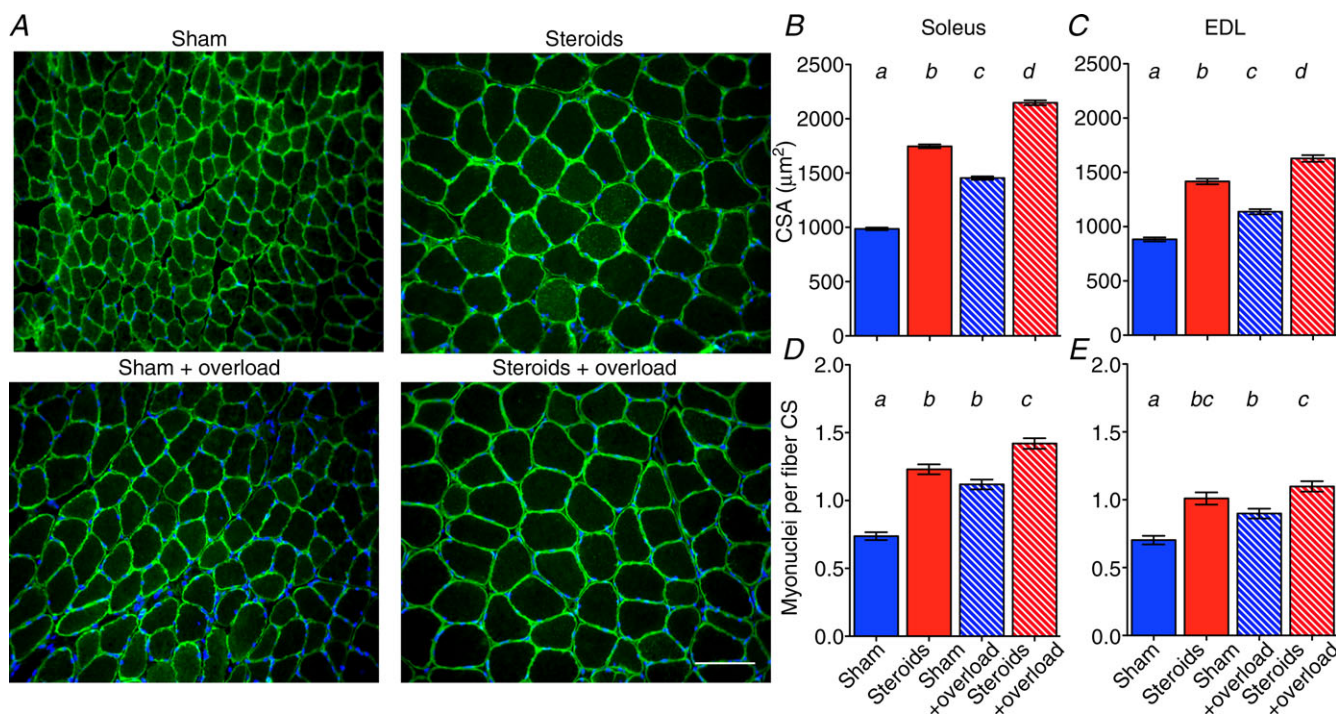
number of myonuclei from 46 to 56 nuclei per millimetre fibre length at sarcomere lengths of  $3\ \mu\text{m}$ , i.e. up 20% (Fig. 3A and C). Fibre diameters were increased by 18%, and assuming cylindrical fibres this corresponds to an increase in CSA of 39% (Fig. 3B and C). Three weeks after pellet removal fibre diameters in the steroid and sham groups were indistinguishable (Fig. 3B and C) while the number of nuclei had not changed significantly in any of the groups (Fig. 3A and C). When overload was introduced at this time, the increases in fibre diameters indicated that during 14 days CSA grew by 42% in the steroid group and by 21% in the sham group (Fig. 3B and C).

In order to demonstrate a longer term memory, we also investigated the effects of the episodic steroid treatment 3 months after pellet removal. NMRI mice in captivity live for about 2 years (Lhrke *et al.* 1984), so 3 months constitutes  $\approx 12\%$  of their lifespan, and would correspond to approximately a decade in humans. At this time, while there was no difference in CSA or fibre type (Supplemental Fig. S2), the number of nuclei was 28% higher in the steroid group than in the sham group (Fig. 4A). When overload was introduced, CSA increased by 31% in the steroid group during the first 6 days, while in the sham group it increased by only 6% (non significant; Fig. 4B

and C). The two groups afterwards grew in parallel, but the CSA was still 20% higher in the steroid group after 14 days of overload (Fig. 4B).

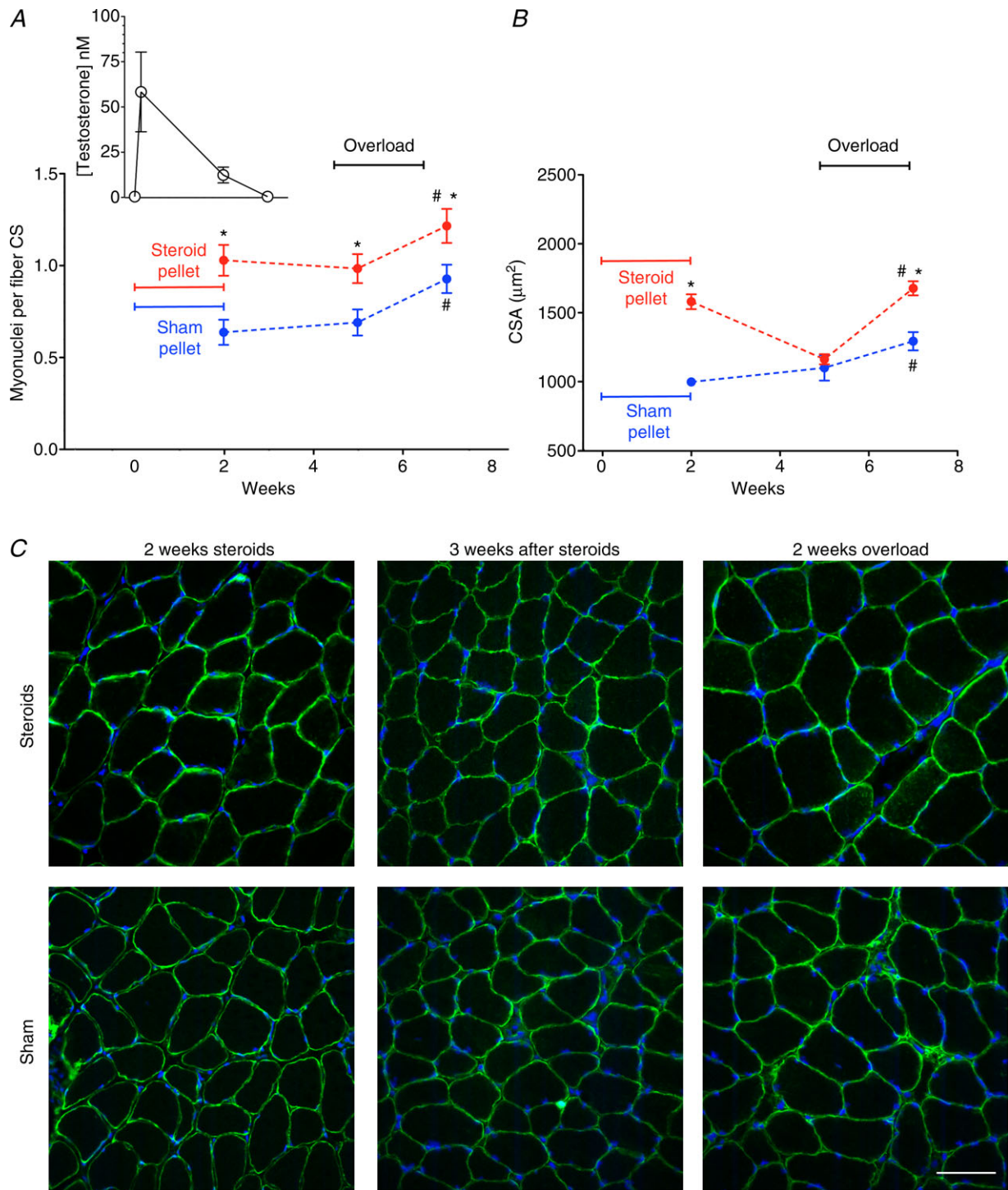
## Discussion

We here provide evidence for a new epigenetic mechanism in the form of an elevated number of nuclei within the muscle syncytium aiding restoration of muscle mass in muscles that have previously been large. Such mechanisms might have been evolutionary important since it would allow, for example, individuals performing seasonal strength-demanding tasks to regain strength quickly without having to maintain a large permanent muscle mass. In modern society it has been commonly observed, that previous strength exercise seems to make it easier to regain muscle mass later in life even after long intervening periods of inactivity and mass loss, and this phenomenon has been dubbed 'muscle memory' (Staron *et al.* 1991; Taaffe & Marcus, 1997). There has previously been no known mechanism for a cellular memory in muscle, and long lasting effects of previous training was solely attributed to motor learning in the CNS (Rutherford & Jones, 1986).



**Figure 1.** Effect of anabolic steroids and functional overload on cross-sectional area (CSA) and the number of myonuclei per fibre found per fibre on cross-sections (CS)

A, representative micrographs of cryosections stained with Hoechst dye 33342 (blue) to label DNA, and antibodies against dystrophin (green) from soleus muscles after 2 weeks of sham or steroid treatment and with or without simultaneous functional overload. Scale bar is  $100\ \mu\text{m}$ . CSA, and number of myonuclei per fibre cross-section (CS) from soleus (B and D), and EDL (C and E). Bars show means  $\pm$  SEM. Letters a–d above columns indicate statistical significant differences ( $P < 0.01$ ); columns designated with the same letter were not statistically different. Each column represents 317–896 fibres from 6 muscles.

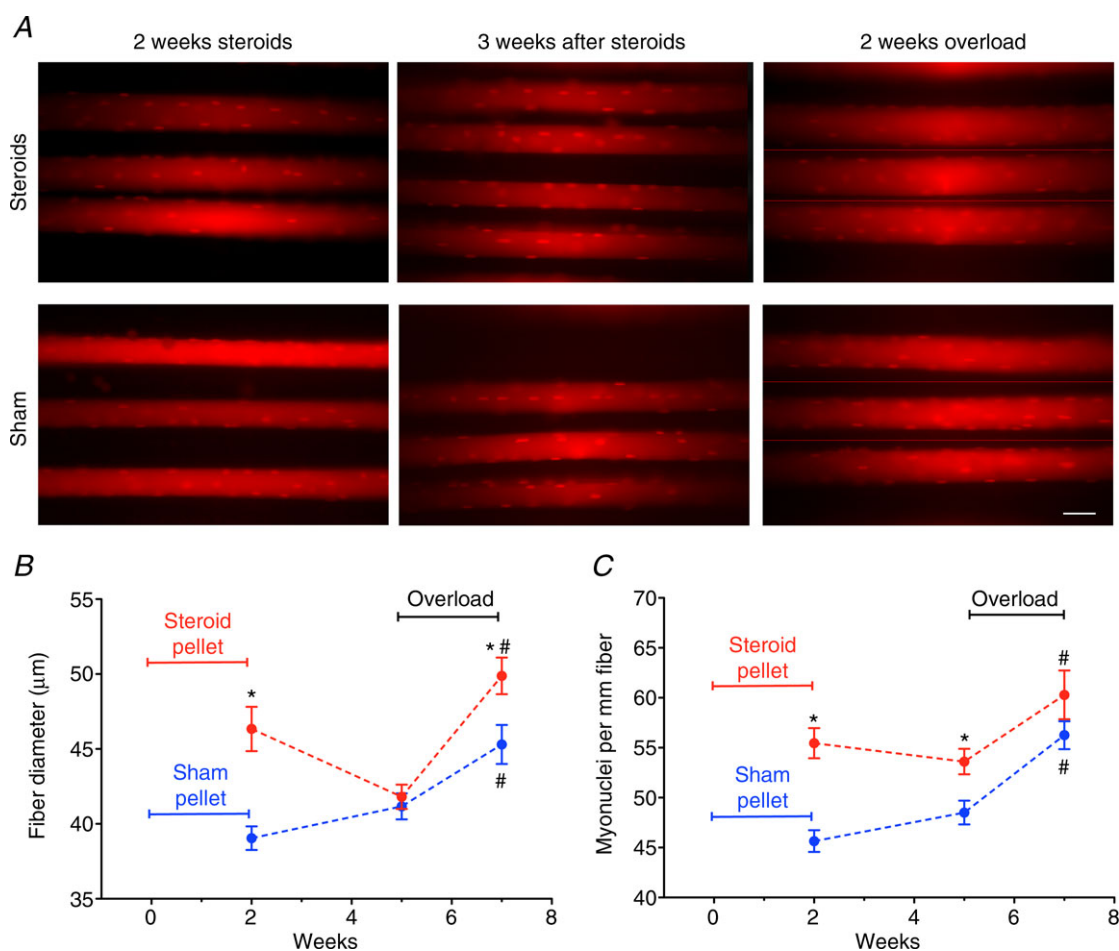


**Figure 2. The effect of prior administration of anabolic steroids on the number of nuclei myonuclei per fibre found on cross-sections (CS; A) and fibre cross-sectional area (CSA; B)**  
 Inset in A shows blood testosterone concentration on same time axis as in main figure. \*Statistically significantly different from sham ( $P < 0.05$ ); #statistically significantly different from overload ( $P < 0.05$ ). Each data point represents 300 fibres from 6 muscles. C, representative micrographs of cryosections stained with Hoechst dye 33342 (blue) to label DNA, and antibodies against dystrophin (green) after treatment with anabolic steroid or sham pellets for 2 weeks, 3 weeks after pellet removal, and followed by functional overload for 2 weeks. Scale bar is 50  $\mu\text{m}$ .

### Mechanisms for muscle hypertrophy and memory

The ability of muscle cells to maintain large cytoplasmic volumes spanning vast intracellular distances without a well developed intracellular transport system seems to be related to having a high number of nuclei optimally distributed throughout the cell (Bruusgaard *et al.* 2003; Metzger *et al.* 2012; Qaisar *et al.* 2012). A dislocation of nuclei is commonly observed in many myopathies, and, for example, in Emery–Dreifuss syndrome the muscular dystrophy is frequently linked to mutations in nuclear envelope genes (Puckelwartz & McNally, 2011). During overload, recruitment of new nuclei from satellite cells precedes the increase in cytoplasmic volume (Bruusgaard *et al.* 2010), but previous literature concluded that myonuclei were lost during subsequent atrophy, suggesting a

complete reversibility in cytoarchitecture. These findings were, however, based mostly on conventional histology that might not have provided a clear separation between myonuclei and the other nuclei in the tissue (e.g. satellite cells). Recently (and in this paper), *in vivo* and *ex vivo* imaging of single fibres has, however, revealed that no myonuclei are lost and that the ‘extra’ nuclei remain in the cells during atrophy (Wada *et al.* 2002; Aravamudan *et al.* 2006; Bruusgaard & Gundersen, 2008; Gundersen & Bruusgaard, 2008; Bruusgaard *et al.* 2010, 2012; Jackson *et al.* 2012a). This represents a cytoarchitectural *hysteresis* that allowed us to hypothesize that the elevated number of nuclei could be a mechanism for a muscle memory related to muscle mass. The present data indicate the existence of such a cellular memory that is beneficial and residing in the muscle cells themselves.



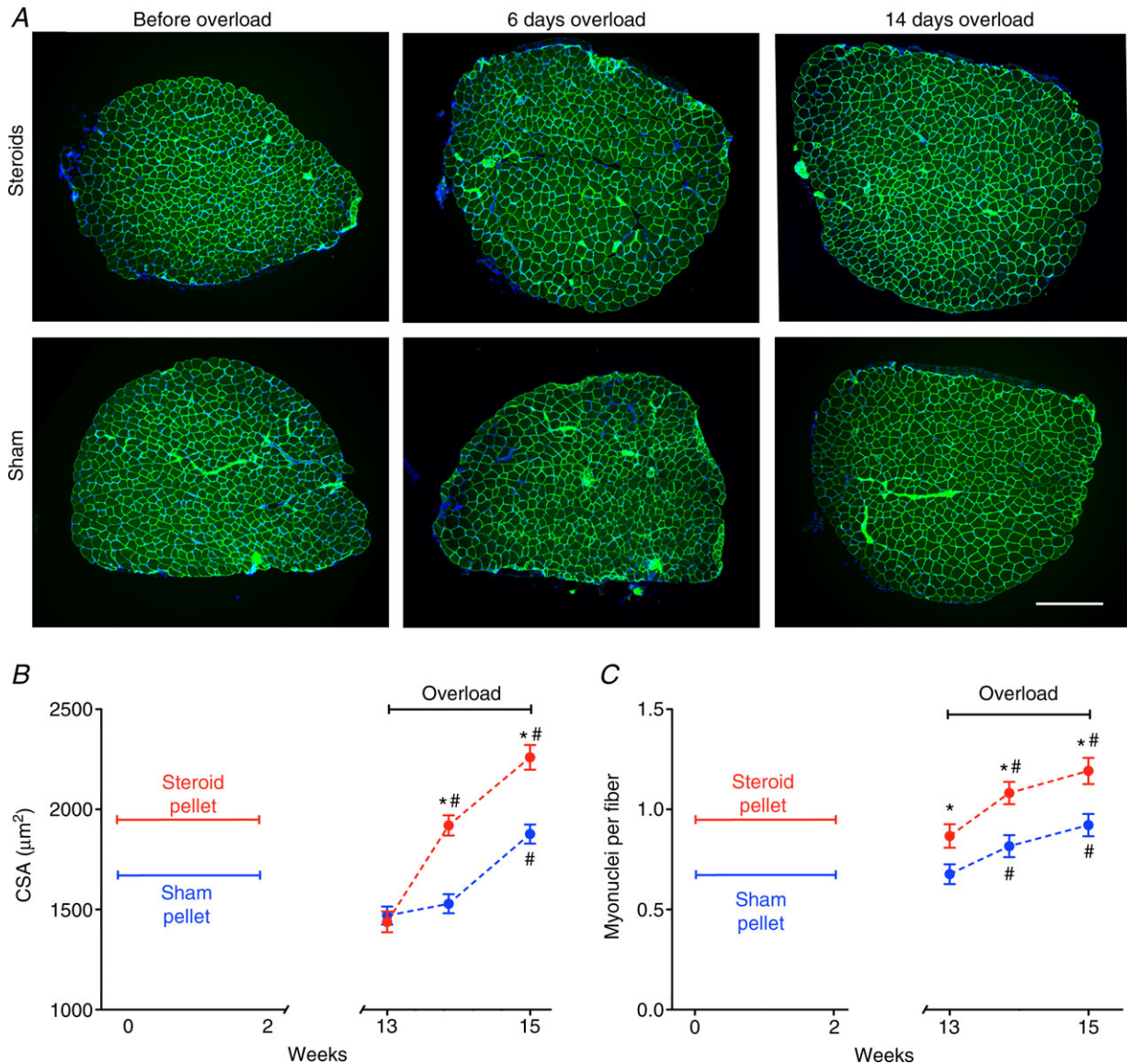
**Figure 3.** *In vivo* imaging demonstrated that prior administration of anabolic steroids increased the number of myonuclei per mm fibre (A and B) and enhanced a subsequent hypertrophy response to overload in EDL muscles (A and C)

\*Statistically significantly different from sham ( $P < 0.001$ ); #statistically significantly different from overload ( $P < 0.001$ ). Each data point represents 33–50 fibres from 6–8 muscles. C, representative *in vivo* images of muscles each showing three oligo-injected muscle fibres labelling myonuclei and the outline of single fibres immediately after treatment with anabolic steroid or sham pellets for 2 weeks, 3 weeks after pellet removal, and followed by functional overload for 2 weeks. In A hairlines indicate boundaries when different focal planes are depicted. Scale bar is 50 µm.

In normal animals the addition of new nuclei seems to accompany hypertrophic growth under a variety of conditions (Enesco & Puddy, 1964; Moss, 1968; Cheek *et al.* 1971; Schiaffino *et al.* 1976; Seiden, 1976; Cabric & James, 1983; Cabric *et al.* 1987; Giddings & Gonyea, 1992; Winchester & Gonyea, 1992; Allen *et al.* 1995; McCall *et al.* 1998; Kadi *et al.* 1999; Roy *et al.* 1999; Bruusgaard *et al.* 2010; Wang & Rudnicki, 2012). It has been speculated that addition of nuclei is related mainly to conditions likely to induce muscle damage, such as after elimination of synergistic muscles, or after heavy

exercise with eccentric contractions (Schiaffino *et al.* 2013), implying that it is connected more to muscle repair than hypertrophic growth of pre-existing fibres. Addition of myonuclei, however, seems also to accompany hypertrophy related to treatment with hormones such as testosterone (the present data and Kadi, 2008) or IGF-1 (Barton-Davis *et al.* 1999), which is less likely to produce damage.

In overload hypertrophy studies, it has been shown both by <sup>3</sup>H-thymidine labelling (Aloisi *et al.* 1973) and *in vivo* imaging (Bruusgaard *et al.* 2010), that new



**Figure 4. Long-term effects of a short period of anabolic steroid administration on myonuclei (A) and fibre growth in response to overload in EDL muscles (B and C)**

\*Statistically significantly different from sham ( $P < 0.001$ ); #statistically significantly different from overload ( $P < 0.001$ ). Each data point represents 669–856 fibres from 6 muscles. C, representative micrographs of cryosections stained with Hoechst dye 33342 (blue) to label DNA, and antibodies against dystrophin (green) immediately after treatment with anabolic steroids or sham pellets for 2 weeks and muscles that 3 months later were functionally overloaded for 6 and 14 days. Scale bar is 300  $\mu\text{m}$ .

myonuclei are incorporated before any apparent change in CSA, suggesting a causal role for myonuclei in contributing to hypertrophy. We are, however, not suggesting that the number of myonuclei is the only determinant of muscle fibre size; for example, we show here that in spite of a larger number of myonuclei induced by steroid treatment the muscle fibres shrink, and are no larger than control fibres with fewer nuclei when the steroids are withdrawn. Moreover, there is no addition of nuclei during regrowth back to normal fibre size after hindlimb suspension (Bruusgaard *et al.* 2012; Jackson *et al.* 2012b), leading us to suggest that the number of nuclei might represent the largest size the fibre has had in its history rather than its current size (Bruusgaard *et al.* 2012). Moreover, with similar *in vivo* imaging methods to those used in the present paper we have previously found that the number of nuclei in fibres of the same size is higher in slow than in fast fibres (Bruusgaard *et al.* 2003). This might be related to the higher turnover and higher demands on protein synthesis in slow muscles (Goldberg, 1967; Kelly *et al.* 1984). Generally there seems to be a stronger correlation between number of nuclei and fibre size under conditions of relatively high protein turnover (Bruusgaard *et al.* 2006). We suggest that fibre size is determined by: (i) proteolysis, (ii) protein synthesis per nucleus and (iii) number of nuclei.

Several gene-manipulated mice have displayed hypertrophy without a corresponding increase in the number of nuclei, such as mice overexpressing the proteins Akt (Blaauw *et al.* 2009), JunB (Raffaello *et al.* 2010), or Ski (Bruusgaard *et al.* 2005), or mice harbouring loss-of-function mutations in the myostatin gene (Amthor *et al.* 2009). The Ski mice have a reduced content of contractile material (Bruusgaard *et al.* 2005) and the myostatin mice display reduced specific force (Amthor *et al.* 2007; Mendias *et al.* 2011), abnormalities that could be related to the low number of nuclei. In contrast, the Akt mice seemed to have normal specific force after 3 weeks (Blaauw *et al.* 2009). Similarly, specific force appeared normal after 2 weeks of overload hypertrophy in mice where 90% of satellite cells were ablated using an inducible Pax7–diphtheria toxin A transgene (McCarthy *et al.* 2011).

For the latter models more research is required to determine if hypertrophy without the normal addition of myonuclei is sustainable over time, is more costly, or leads to suboptimal functional properties (other than short term specific force). Alternatively, hypertrophy without the addition of nuclei might represent mechanistic redundancy, which is quite common in biological systems. Although we cannot extrapolate to other experimental models or exclude the possibility that testosterone has other long lasting effects on muscle than to increase the number of nuclei, we suggest that muscles with more nuclei seem better adapted to a rapid regrowth, and thus are bearers of a muscle memory.

## Muscle memory in sport and public health

Hypertrophy induced by overload is greatly attenuated in older animals (Carson *et al.* 1995; Alway *et al.* 2002), and in humans more than 50% of the individuals currently fill the clinical criteria for frailty at ages >80 years (Matthews *et al.* 2011), creating major health problems in the ageing western population (Dutta & Hadley, 1995; Hughes & Schiaffino, 1999). Hard resistance training in the elderly has, however, proven to have benefits even after years of detraining, in particular if the individuals maintain a regime of moderate-intensity training (Smith *et al.* 2003). The ability to generate new myonuclei is, however, impaired with ageing (Schultz & Lipton, 1982), possibly due to reduced notch signalling (Conboy *et al.* 2003; Conboy & Rando, 2005). Our data suggests the existence of a long-term muscle memory related to myonuclei. Investigations are needed to establish whether a similar nuclei-related muscle memory related to strength exercise in humans exists; and if it does, public health advice for strength training in younger individuals should be considered, since it might aid in maintaining muscle mass more easily in senescence.

Our data demonstrate that in least in mice, an episode of testosterone use may recruit a long lasting pool of excess myonuclei, and a persistent increased ability to regain muscle mass by resistance exercise in the absence of further steroid exposure. Thus, the benefits of even episodic drug abuse might be long lasting, if not permanent, in athletes. Our data suggest that the World Anti-Doping Code calling for only 2 years of ineligibility after a conviction for steroid use (WADA, 2009) should be reconsidered.

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## Additional information

### Competing interests

None.

### Author contributions

Conception and design of experiments: I.M.E., J.C.B. and K.G. Collection and analysis of data I.M.E., J.C.B. and E.E. Drafting the article K.G. and revising it critically for important intellectual content: all authors.

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