

# Sensorimotor restriction during development impairs rat skeletal muscle maturation

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## Research Article

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## Abstract

Early postnatal development is a critical period during which mechanical and neural inputs shape the acquisition of the mature skeletal muscle phenotype. This study investigated whether early sensorimotor restriction uniformly impairs muscle development or induces distinct responses across functionally different muscles. Rat pups underwent sensorimotor restriction from birth to postnatal day 28 (P28). The soleus, plantaris, extensor digitorum longus, and tibialis anterior muscles were collected at P15 and P28. Muscle growth and morphology, fibre-type composition, centronucleation, PAX7 expression and neuromuscular junction maturation were assessed using histology, immunofluorescence and protein analyses. Sensorimotor restriction induced muscle-specific responses according to their functional characteristics. The postural soleus, displayed sustained growth impairment and a slow-to-fast fibre-type transition at P28. Conversely, the extensor digitorum longus and tibialis anterior showed transient hypertrophy at P15, consistent with the passive stretch associated with the restriction model, followed by a fast-to-intermediate fibre type transition. The plantaris remained structurally resilient. Centronucleated fibres were more frequent in the soleus, extensor digitorum longus and tibialis anterior of restricted animals, suggesting altered myofibre maturation. At P28, PAX7 protein abundance increased in the extensor digitorum longus and tended to increase in the soleus. Neuromuscular junction maturation was disrupted, with increased polyinnervation at P15 and persistent structural alterations at P28, consistent with delayed synaptic refinement. In conclusion, the divergent responses across muscles suggest that functional identity and mechanical environment influence developmental adaptations to reduced sensorimotor experience. Thus, early postnatal sensorimotor input emerges as a key factor in shaping muscle-specific developmental trajectories.

**Keywords:** Postnatal development, immobilization, muscle phenotype, centronucleated fibres, muscle stem cells, neuromuscular junction.

## INTRODUCTION

Skeletal muscle is a highly plastic tissue whose structural and functional properties are shaped by contractile activity and mechanical loading. In adults, increased activity promotes muscle growth, enhances force production (Hughes et al., 2018; Lim et al., 2022), mitochondrial density and function (Granata et al., 2018), capillarization (Prior et al., 2004), and calcium handling (Tupling et al., 2003), and maintains neuromuscular junction integrity. Whilst this plasticity is advantageous for adaptation, it also renders muscle vulnerable to inactivity (Campbell et al., 2013; Canu et al., 2019; Egan and Zierath, 2013). Consequently, reduced physical activity, often exacerbated by ageing, triggers a maladaptive shift leading to progressive muscle atrophy and impaired performance, potentially evolving in sarcopenia (Cruz-Jentoft et al., 2019; Naruse et al., 2023; Wilkinson et al., 2018). While the detrimental effects of disuse are extensively documented in adults, the consequences of early life hypoactivity remain poorly understood.

Early postnatal period represents a critical window of heightened plasticity (Walton et al., 1992), including for the muscles, which undergo significant growth and maturation at this stage. Indeed, this period is essential for maturation of myofibres and is characterized by muscle adaptation to the increased functional demand, as the newborn begins to produce movements. In rodents, muscle growth and specialization are highly responsive to mechanical loading and neural activity during the first weeks of life. A hallmark of this maturation is synaptic pruning, during which muscle fibres evolve from poly- to mono-innervation guided by activity-dependent mechanisms (Sanes and Lichtman, 2001; van Ooyen and Willshaw, 1999). Concurrently, muscle fibres progressively acquire their metabolic and contractile identity to support muscle function, highlighting the importance of neuromuscular interactions in shaping mature muscle properties. First, an increase in muscle mass is achieved through the rise in the number and size (length and cross-sectional area) of myofibres (Bachman et al., 2018; Gokhin et al., 2008; White et al., 2010; Xu et al., 2022). Second, myofibres undergo profound structural reorganization: nuclei migrate from a central to a peripheral position and aggregate beneath the neuromuscular junction (Cadot et al., 2015), while fibre morphology evolves from a rounded to an angular profile (Xu et al., 2022). Third, muscle fibre phenotype transition takes place (Punkt et al., 2004). For instance, the postural soleus (SOL) muscle undergoes a fast-to-slow transition, shifting from 50% of fast-twitch fibres expressing type II isoform of myosin heavy chain (MyHC II) at postnatal day 8 (P8), to approximately 80% of type I fibres (MyHC I) in adulthood. Conversely, fast-twitch muscles such as extensor digitorum longus (EDL) refine their phenotype by shifting from MyHC IIa to MyHC IIb isoform (Punkt et al., 2004).

Disrupting sensorimotor experience during this period may therefore have consequences that differ fundamentally from disuse in mature muscle: rather than modifying an already

established phenotype, early hypoactivity may interfere with the acquisition of that phenotype. This issue is clinically relevant, as reduced mobility, abnormal posture, and altered sensorimotor interactions occur in several paediatric motor disorders and may contribute to persistent musculoskeletal abnormalities (Stackhouse et al., 2005). Yet, despite the importance of the topic, there remains a paucity of studies on the consequences of hypoactivity and sensorimotor impairment on the growth and maturation of skeletal muscles. A sensorimotor restriction (SMR) model consisting in immobilizing in extended position the hindquarter of rodent pups from birth (P1) until weaning (P28), for 16 hours a day, was developed to reproduce the main clinical features of developmental coordination disorders (Delcour et al., 2018). Research using the SMR model were mainly focused on neurological outcomes and revealed significant developmental delay at different levels of the sensorimotor system. At muscular level, SMR was associated with a significant atrophy of the gastrocnemius muscle at P28 and a sustained expression of neonatal MyHC isoforms on SOL muscle (Canu et al., 2022; Delcour et al., 2018). These findings suggest a muscle adaptive impact of early SMR. However, whether early sensorimotor restriction produces a generalized developmental impairment or affects individual muscles differently according to their functional and mechanical characteristics remains unknown.

Our hypothesis is that a sensorimotor restriction during the first four postnatal weeks would alter the postnatal growth (mass, cross-sectional area), structure (fibre shape), regenerative capacity (satellite cells quantification) and maturation (precise nuclear position, MyHC isoforms) of hindlimb muscles as well as NMJ morphology. To provide a comprehensive understanding of these effects, we selected four hindlimb muscles representing a broad spectrum of biomechanical roles, developmental trajectories and mature phenotypes. Specifically, we analysed extensors (the slow-postural SOL and the fast-locomotor plantaris, PLT) and antagonist flexors (the fast-phasic EDL and tibialis anterior, TA). Investigating this phenotypically diverse set is crucial to determine whether early disuse induces a generalized impairment of muscle development, or if cellular and structural alterations are muscle-specific, depending strictly on their anatomical compartment and inherent functional destiny.

## **MATERIALS & METHODS**

### **Animals**

All procedures described below were performed on female and male OFA-Sprague-Dawley rats purchased from Charles River Laboratories (L'Arbresle, France). Rats were housed in standard plastic cages in controlled conditions of humidity, temperature and light (20 – 24°C, 40 – 70% humidity, 12:12 hour light-dark cycle) with *ad libitum* access to water and food. All experiments were conducted in accordance with European and French legislation on animal

experimentation and were approved by the Regional Committee on the Ethics of Animal Experiments of the Haut-de-France region (CEEA 75, reference number: APAFIS#51113 - 2024051608522519). After mating, pregnant females were housed in individual cages. At birth, litters were standardized to 10 pups (5 males and 5 females when possible) and assigned to control (CTRL) or SMR group. A total of 48 rats was used: 8 rats from 2 different litters per group (CTRL or SMR) and age (P15 and P28); 16 other rats were used for NMJ analysis (n=2 per group and age).

### **Sensorimotor restriction model**

SMR pups underwent immobilization from P1 to P28 for 16 hours per day (from 5 p.m to 9 a.m, i.e., mainly during the dark phase) and were free to move for 8 hours. When pup body weight was under 10 grams, hind limbs were tied up together with medical tape. When pups exceeded 10 grams, a size-appropriate cast was added to the medical tape, keeping hind limbs in an extended position. The separation with the mother for immobilization and cast removal did not exceed 15 minutes twice a day. The casts did not prevent the pups from urinating, sucking, or other physiological needs. CTRL pups were also separated from their mother and handled daily in equal proportion.

### **Tissue preparation**

P15 or P28 animals were euthanized in the morning between 8 and 11 a.m. After deep isoflurane anaesthesia, animals received a lethal I.P. injection of sodium pentobarbital (Dolethal). After total exsanguination by intracardial infusion of NaCl, the SOL, EDL, TA and PLT muscles were harvested and weighed. For histological analysis, muscles were frozen extended in isopentane cooled with liquid nitrogen and stored at -80°C. For protein analysis, muscles were flash-frozen directly in liquid nitrogen.

### **Haematoxylin and eosin staining (H&E)**

H&E staining was performed on 10 µm thick cryosections. Tissue sections were first incubated in Mayer's hemalum solution (Sigma-Aldrich) for 6 minutes followed by a water bath for 10 minutes to remove excess stain. The sections were then immersed in an eosin Y solution (Sigma-Aldrich) acidified with 0,5% glacial acetic acid for 6 min and then rinsed with water for 1 minute. Then a progressive dehydration was performed through rising ethanol concentration (80%, 90%, 95% and 100%) baths. To finish, two successive baths of toluene were performed. Finally, the slides were mounted using mounting medium (EUKITT®, Chem-Lab NV).

## **Immunofluorescent labelling of muscle fibres**

**Immunofluorescent staining.** For myosin heavy chain (MyHC) staining, 10 µm thick muscle cryosections made perpendicularly of the belly of the muscles (Leica CM3050 S) were first incubated at room temperature with BSA 4% (Bovine Serum Albumin, Sigma Aldrich) diluted into PBS. Then, they were incubated overnight at room temperature with a cocktail containing four primary antibodies diluted in BSA 3%: anti-laminin (1/200; ab11575; Abcam), anti-MyHC I (1/100; BA-D5; DSHB), anti-MyHC IIa (1/50; SC-71; DSHB) and anti-MyHC IIb (1/50; BF-F3; DSHB). Slides were then rinsed three times with PBS. Afterwards, a cocktail of secondary antibodies containing Alexa Fluor 405 (1/500; 115-477-185; Jackson ImmunoResearch), Alexa Fluor 488 (1/500; A11008; Invitrogen), Alexa Fluor 555 (1/500; 1021-32; Clinisciences) and Alexa Fluor 647 (1/500; 1090-31; Clinisciences) and diluted in PBS, was applied for 2 hours at room temperature protected from light, followed by three rinses with PBS. Finally, the samples were mounted with ProLong Gold Antifade Mountant (P36934, Invitrogen) and coverslipped.

For PAX7/DAPI staining, 10 µm thick muscle sections made perpendicularly of the belly of the muscles were first fixed with 4% PFA at room temperature for 10 minutes and then rinsed with PBS three times. Sections were then permeabilized with a 0.22 µm filtrated solution of 0.1% Triton X-100 and 0.1M glycine diluted in PBS 1X for 10 minutes and rinsed two times with PBS. Subsequently, the sections were blocked with a 0.22 µm filtrated solution of 3% BSA and 5% of goat serum diluted in PBS for 1 hour at room temperature. Slices were then incubated overnight at 4°C with anti-laminin (1/200; ab11575; Abcam) and anti-PAX7 (1/100; sc-81647; Santa Cruz Biotechnology) primary antibodies diluted in blocking solution. The following day, slices were rinsed three times with PBS and then incubated with a cocktail of secondary antibodies containing Alexa Fluor 488 (1/500; A11008; Invitrogen) and Alexa Fluor 647 (1/500; 406618; Thermofisher Scientific) diluted in blocking solution for 1 hour at room temperature. Finally, slices were rinsed another three times with PBS and then mounted using a specific mounting medium containing DAPI (ab104139; Abcam) applied directly to the sections for 5 minutes before coverslipping.

**Image acquisition and analysis.** Images were acquired using an inverted fluorescent microscope (Leica DMI8, Leica Microsystems, Wetzlar, Germany) equipped with a Leica DFC450 C digital CCD camera (2560 x 1920 pixels, 5 megapixels) and an automated motorized stage for mosaic imaging. Images were acquired using Leica Application Suite X Software (LAS X, version 3.7.6.25997) with a HC PL FIUOTAR L 20x/0.40 objective. Exposure times ranged from 250 and 350 ms depending on the fluorophore/channel imaged.

For MyHC and CSA quantification, fully digitized images composed of four channels, each corresponding to a protein, were used. The channel containing laminin staining was preprocessed using cell segmentation algorithm (<https://www.cellpose.org>) to generate a “mask” that isolated each fibre present in the muscle (Stringer et al., 2021).

At the same time, the three other channels, with MyHC I, IIa and IIb staining, were split and binarized using manual thresholding in Fiji/ImageJ to ensure that each image matched the initial staining. The “LabelsToROIs” plug-in was then used on ImageJ/Fiji to extract the CSA, perimeter (to calculate shape index) and grey intensity (ranging from 0 to 255 pixels for MyHC determination) for each identified fibre (Waisman et al., 2021). Shape index was computed as:  $(Perimeter^2)/(4\pi \times CSA)$ .

For MyHC typing, the fibre was considered as positive for a MyHC type when its grey intensity was higher than 50 pixels. If two channels exhibited grey intensity higher than the threshold (50 px), the fibre was classified as hybrid. Based on this criterion, fibres were identified as type I, IIa, IIb, or hybrids (I/IIa or IIa/IIb). “Unstained” refers to fibres that were unlabelled.

The determination of number and position of nuclei in fibres was performed on fully digitized images composed of three channels: one for DAPI (nuclei), one for laminin and one for PAX7. Images were analysed using ImageJ/Fiji software with MuscleJ2 plug-in (Danckaert et al., 2023; Mayeuf-Louchart et al., 2018). This plug-in allows the automatic assignment of each nucleus to its respective fibre and determines its position as central or peripheral and the quantification of satellite cells. The percentage of centronucleated fibres (CNF) was calculated as:  $(\text{Number of CNF}) / (\text{Total number of fibres}) \times 100$ . The number of muscle stem cells (MuSCs) was manually counted on crops of  $1000 \times 1000$  px and was calculated as:  $(\text{Total number of MuSCs}) / (\text{Total number of fibres})$ .

### **Immunofluorescent staining and analysis of neuromuscular junction**

The NMJ was analysed on SOL and EDL muscles from CTRL and SMR groups (n=4 per muscle and per group from 2 different litters). After dissection, whole SOL and EDL were fixed on a small support with pins and stored in PFA for at least 24 hours. Muscles were then washed 30 min in 1X PBS, and dissected under dissection microscope in at least 5 thin bundles per muscle. Bundles were washed 3 x 20 min in PBS. They were subsequently incubated in a neutralization solution (0.1 M Glycine diluted in PBS 1X) for 1 hour, washed in 0,5% triton X-100 in PBS for 3 x 10 min; blocked 2 hours at RT with 0,5% Triton X-100, 5% goat serum and 4% BSA in 1X PBS. Finally, bundles were incubated with primary antibody cocktail against neurofilament (1/500; 2H3; DSHB) and synaptophysin (1/500; 9020s; Cell Signaling) diluted in blocking solution for at least 48 hours at 4°C on a rotating mixer. After that, SOL and EDL bundles were washed 3 x 20 min in 0,5% triton X-100 in PBS and incubated in a cocktail of Alexa Fluor 647 Goat anti-Mouse (1/1000; A-21240; ThermoFisher Scientific) and  $\alpha$ -Bungarotoxin conjugated with Alexa Fluor 594 (1/500; B13423; Invitrogen) diluted in blocking solution. Finally, bundles were washed 4 x 20 minutes in 1X PBS and mounted between SuperFrost microscope slides (VWR) and coverslipped with ProLong Gold Antifade Mountant (P36934, Invitrogen) by applying homogeneous pressure to flatten the bundles. When sealed,

slides were stored in the dark at 4°C until imaging with confocal microscopy (LSM 980 AiryScan Zeiss) at 25X magnification (Objectif LCI Plan-Neofluar 25x/0,8 Imm Korr DIC M27) using a camera GaAsP Detectors (number of pixels based of Nyquist-Shannon criteria) using Zen Blue software (v 3.7).

Images of 20 *en-face* NMJs in each muscle of each animals were analysed for all the morphological variable of interest using a home-made automated adapted version of the NMJ-morph workflow (Jones et al., 2016) available upon request.

### **Western blot analysis**

**Protein Extraction.** Frozen muscle samples were pulverized into powder, weighed, and homogenized in ice-cold RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails (Roche). The buffer-to-tissue ratio was 10 µL/mg. Lysates were sonicated and incubated for 1 hour at 4°C with gentle agitation to ensure solubilization. Following centrifugation (13,000 rpm, 10 min, 4°C), supernatants were collected and stored at -20°C. Protein concentrations were determined using the Bradford assay (Bio-Rad).

**Western Blotting.** Protein samples were diluted in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.02% bromophenol blue) and denatured at 95°C for 10 min. Proteins were separated on pre-cast Criterion TGX Stain-Free polyacrylamide gels (Any kD, Bio-Rad) using a running buffer (25 mM Tris base, 190 mM Glycine, 0.1% SDS) at a constant voltage of 280 V. Total protein load was visualized using a ChemiDoc MP imager (Bio-Rad) for subsequent Stain-Free normalization. Proteins were then transferred onto a 0.2 µm nitrocellulose membrane (Trans-Blot Turbo RTA Midi Kit, Bio-Rad) using the Trans-Blot Turbo Transfer System (25 V, 10 min). Transfer efficiency was confirmed by UV imaging.

**Immunodetection.** Membranes were blocked for 1 hour at room temperature in TBS-Tween (15 mM Tris-HCl pH 7.6, 140 mM NaCl, 0.05% Tween-20) containing 5% non-fat dry milk. Following blocking, membranes were incubated overnight at 4°C with a mouse monoclonal anti-PAX7 antibody (1:1000, sc-81648, Santa Cruz Biotechnology). After three washes in TBS-Tween (10 min each), membranes were probed for 2 hours at room temperature with an HRP-conjugated anti-mouse IgG secondary antibody (1:1000, #7076S, Cell Signaling Technology). After final washing, proteins were detected using Clarity Western ECL Substrate (Bio-Rad). Signals were acquired with the ChemiDoc MP imager and quantified using Image Lab software 6.1 (Bio-Rad), normalizing target signal intensities to the total protein load.

## Statistical analysis

Statistical analyses were performed using PRISM software (10.3.0; GraphPad) or RStudio (version 2025.05.1+513). Males and females were first compared and, as no sex-related differences were detected, data were pooled. Normality was assessed using the Shapiro-Wilk test and outliers were identified using the ROUT method (Q=1%). Muscle weight normalized to body weight and the proportion of centronucleated fibre were analysed using two-way ANOVA followed by Tukey's post-hoc test when appropriate in GraphPad Prism. PAX7 protein levels were compared using unpaired t-test. CSA and Shape index were analysed in RStudio using linear mixed-effects models (LMM). For each muscle (SOL, EDL, TA and PLT) at P15 and P28, Group (CTRL vs. SMR), Fibre type, and their interaction were included as fixed effects, with Animal as a random intercept. Fixed effects were tested using type III ANOVA with Satterthwaite approximation, and post-hoc contrasts (SMR vs. CTRL within each fibre type) were performed on estimated marginal means with Holm correction. Fibre type composition was analysed using generalized LMM, with Group as a fixed effect and Animal as a random intercept. For each fibre type, counts were modelled relative to the remaining typed fibres within the same sample, and global differences in fibre type distribution between groups were additionally assessed using chi-squared tests. Neuromuscular junction morphometric parameters were analysed in RStudio using LMM for continuous variables, with Group, Age, and their interaction as fixed effects and Animal as a random intercept. Type III ANOVA was used to test fixed effects, and pairwise post-hoc contrasts were performed on estimated marginal means with Holm correction. Polyinnervation, considered as binary outcome, was analysed separately using binomial generalized LMM with the same fixed- and random-effects structure. For SOL, an additional model restricted to P15 was fitted because no polyinnervated junctions were observed at P28.

## Results

### Impact of SMR on muscle weight and morphological features

To evaluate the global impact of SMR on muscle development, we first assessed relative muscle weight (i.e., muscle weight / body weight), histological organization, and fibre cross-sectional area (CSA) in the four muscles at both P15 and P28.

Muscle weight analysis revealed that SMR primarily affected the slow twitch (SOL) and mixed (PLT) muscles of the posterior compartment of the hind limb (Fig. 1A,B). In the SOL, a significant reduction in weight was observed in SMR animals at both P15 (-38%  $p < 0.0001$ ) and P28 (-32%,  $p < 0.0001$ ). A significant *Group* effect was also found for the PLT ( $p = 0.0117$ ), although the weight reduction was more moderate compared to the SOL (-12% and -12% at P15 and P28 respectively). In contrast, the weight of the fast-twitch EDL and TA muscles

remained unaffected by SMR at both developmental stages (*Group* effect: 0.1381 and 0.1491 for EDL and TA respectively; Fig. 1C,D).

Histological analysis via haematoxylin and eosin staining reveals that CTRL muscles displayed well-organized fibres with characteristic polygonal shape, while SMR SOL muscles showed smaller, irregularly shaped fibres and increased interstitial spaces, particularly at P15 (Fig. S1). To quantify these morphological changes, a shape index was calculated (Table 1). In the SOL at P15, SMR rats exhibited a lower shape index in comparison to the CTRL rats (-4.6%,  $p=0.005$ ). This finding suggests that SMR rats had not yet acquired the typical mature polygonal geometry. Interestingly while SMR did not affect the TA weight, its shape index showed a slight but significant increase at P15 (+2.1%,  $p<0.001$ ), suggesting subtle changes of the architectural maturation even in muscles with preserved mass.

| Muscle | Age | CTRL               | SMR                | %Δ    | p value           |
|--------|-----|--------------------|--------------------|-------|-------------------|
| SOL    | P15 | 1.45 (1.35 – 1.59) | 1.38 (1.30 – 1.50) | -4.6% | <b>0.005</b>      |
|        | P28 | 1.43 (1.34 – 1.55) | 1.41 (1.33 – 1.52) | -1.6% | 0.089             |
| PLT    | P15 | 1.43 (1.33 – 1.57) | 1.40 (1.32 – 1.53) | -1.8% | 0.350             |
|        | P28 | 1.42 (1.34 – 1.54) | 1.42 (1.34 – 1.52) | -0.2% | 0.261             |
| EDL    | P15 | 1.42 (1.33 – 1.54) | 1.42 (1.33 – 1.56) | +0.4% | 0.220             |
|        | P28 | 1.42 (1.34 – 1.53) | 1.43 (1.34 – 1.55) | +0.8% | 0.395             |
| TA     | P15 | 1.39 (1.32 – 1.50) | 1.42 (1.33 – 1.56) | +2.1% | <b>&lt; 0.001</b> |
|        | P28 | 1.43 (1.34 – 1.54) | 1.44 (1.35 – 1.57) | +0.7% | 0.162             |

**Table 1: Shape index value of the SOL, PLT, EDL and TA in CTRL and SMR animals at P15 and P28.** Values are as median (Q1-Q3). Shape index reflects fibre circularity with a value of 1 representing a perfect circle. Values were compared using linear mixed-effects models. For each muscle (SOL, EDL, TA and PLT) at P15 and P28, Group (CTRL vs. SMR), fibre type, and their interaction were included as fixed effects, with animal as a random intercept. Fixed effects were tested using type III ANOVA with Satterthwaite approximation, and post-hoc contrasts (SMR vs. CTRL within each fibre type) were performed on estimated marginal means with Holm correction.

These changes in global muscle geometry and weight were closely reflected at the cellular level by shifts in individual fibre surface (Fig. 2A,B). In the SOL, the significant loss of muscle mass was associated with a marked reduction in the cross-sectional area (CSA) median at both P15 (-26%,  $p=0.008$ ) and P28 (-35%,  $p=0.002$ ) (Fig. 2C,D). Detailed analysis of CSA according to fibre type (Table S1) revealed that the atrophic process impacted primarily type I (P15: -32%,  $p=0.01$ ; P28: -51%,  $p<0.001$ ) and type I/IIa hybrid fibres (P15: -21%,  $p=0.019$ ; P28: -46%,  $p=0.004$ ). At P28, type IIa and unstained fibres were also affected, (-31%,  $p=0.005$  and -21%,  $p=0.032$  respectively). Conversely, the EDL and TA exhibited a transient hypertrophic response at P15 (Fig. 2C). Median CSA increased significantly by 36% in the EDL ( $p=0.003$ ) and 25% in the TA ( $p<0.001$ ). In the EDL, this early expansion was driven by significant increases in the size of type I, I/IIa, IIa and unstained fibres. In the TA, significant hypertrophy was observed for all fibre type. However, this enlargement was temporary, since in both muscles, fibre size returned to levels comparable to CTRL by P28 (EDL:  $p=0.449$ ; TA:  $p=0.530$ ; Fig. 2D). Finally, in line with more moderate weight variation observed, the median fibre CSA of the PLT remained remarkably similar in both groups, at both P15 ( $p=0.639$ ; Fig. 2C) and P28 ( $p=0.399$ ; Fig. 2D).

### Fibre type composition

Given that changes in fibre size are often associated with shifts in phenotype during postnatal development and in response to altered mechanical loading, we next investigated whether SMR also impacted fibre type composition. Thus, the distribution of MyHC isoforms was evaluated using multicolour immunofluorescence across all four muscles at both P15 and P28 ( $\chi^2<0.0001$  for all muscles; Fig. 3).

In the SOL, SMR induced a progressive remodelling of the fibre profile. At P15, the proportion of type I fibres was lower in SMR rats (-21 %,  $p=0.0183$ ) while that of unstained fibres was higher (+70%,  $p=0.0067$ ) (Fig. 3B). At P28, SMR animals exhibited a massive reduction in the proportion of slow-twitch type I fibres, compared to CTRL animals (-24%,  $p<0.0001$ ). This loss was counterbalanced by a significant increase in the faster type IIa (+28%  $p=0.0048$ ) and hybrid I/IIa fibres (+88%,  $p=0.0468$ ) (Fig. 3C). This observation confirms the classical slow-to-fast shift typically associated with mechanical unloading (Pette and Staron, 2000).

The fast twitch muscles (PLT, EDL and TA) also underwent a significant remodelling, although the patterns differed from the SOL, showing a shift toward intermediate-fast profile rather than slow fibres. The TA showed a significant reduction in the fastest type IIb fibres as early as P15 (-43%,  $p=0.0456$ ), a phenomenon that became much more pronounced at P28 (-55%,  $p<0.0001$ ). This was mirrored by a robust increase in type IIa (+169%,  $p<0.001$ ) and hybrid IIa/IIb fibres (+313%,  $p=0.0115$ ), suggesting a shift from “very fast” to “intermediate-fast” contractile phenotype.

Similar to the TA, the EDL was characterized at P28 by a sharp decrease in type IIb fibres (-44%,  $p < 0.0001$ ) (Fig. 3C). This loss was accompanied by a significant expansion of the type IIa pool (+45%,  $p = 0.0193$ ) and hybrid populations, including IIa/IIb (+66%,  $p = 0.0262$ ) and I/IIa (+258%,  $p = 0.0144$ ) (Fig. 3C).

Finally, the PLT followed a unique trajectory. At P15, unlike the EDL and TA, it showed an increased proportion of type IIb fibres (+29%,  $p = 0.0152$ ) alongside a decrease in IIa (-26%,  $p = 0.0055$ ) and I (-56%,  $p = 0.0084$ ) fibres (Fig. 3B) indicating a different adaptive response to SMR compared to its fast-twitch counterparts.

### **Muscle maturity and regenerative status**

Such extensive remodelling of fibre type composition suggests a disruption of normal postnatal maturation, led us to assess markers and actors of muscle maturation. The positioning of nuclei within the fibres serves as an indicator of muscle maturity and stability. While peripheral nuclei are characteristic of mature fibres, centronucleated fibres (CNF) typically reflect ongoing regeneration or delayed maturation during development.

SMR induced a significant and persistent increase in the proportion of CNF in all muscles except the PLT, suggesting a disruption in normal muscle maturation (Fig. 4). At P15, CTRL animals displayed a baseline CNF incidence of approximately 16%. This proportion was significantly exacerbated by SMR in the SOL ( $p = 0.0033$ ), the EDL ( $p = 0.0152$ ) and the TA ( $p = 0.0146$ ). A similar phenotypic pattern was maintained at P28; although the baseline for the CTRL group decreased to 7.5% the SMR group exhibited a sustained and significant elevation in the SOL ( $p = 0.0041$ ), the EDL ( $p = 0.0152$ ) and the TA ( $p = 0.0146$ ). Notably the PLT was the only muscle that was not affected, with SMR failing to significantly modify the CNF ratio (Group effect:  $p = 0.3686$ ).

To further explore the impact of SMR on muscle homeostasis and determine whether the change in CNF percentage was accompanied by a change in the myogenic pool, we quantified PAX7+ muscle stem cells (MuSCs). Preliminary histological count ( $N = 2$ ) suggested a trend toward an increased MuSCs per fibre ratio at P28 in the SOL, EDL and TA of SMR animals (Fig. 5A-B). To validate these observations with greater statistical power, we performed western blot analysis of PAX7 protein expression levels in the SOL and the EDL. At P15, no significant differences in PAX7 expression were detected in either muscle (Fig. 5C-D). However, at P28, SMR led to a significant increase in PAX7 levels in the EDL (+61%,  $p = 0.0344$ ). In the SOL, a 79% increase was also observed but it did not reach significance level ( $p = 0.0767$ ) (Fig. 5E-F). Together, these findings suggest altered myogenic regulation but do not establish changes in satellite-cell number, activation, or cell-cycle progression.

### Neuromuscular junction morphology

Muscle maturation is tightly coordinated with NMJ development. Thus, we next investigated whether NMJ structure was affected by SMR. To address this, a complete analysis of NMJs was conducted at P15 and P28 in the SOL and EDL of 16 rats. The structure of the presynaptic element was evaluated by neurofilament and synaptophysin staining, while postsynaptic component was visualized with  $\alpha$ -bungarotoxin staining. Complete data are presented in Table 2.

In CTRL rats, a polyneuronal innervation of a few synapses was observed for both muscles at P15 while monoinnervation was achieved at P28. SMR delayed synapse pruning. Indeed, the percentage of polyinnervated synapses was higher in SMR rats at P15 in the SOL (+656%,  $p=0.005$ ) and in the EDL (+262%,  $p<0.001$ ) (Fig. 6A). At P28, a few NMJ were still polyinnervated in the EDL although this was not significant. SMR was also associated with a reduced axon diameter at P15 and P28 in the SOL (respectively -22%,  $p=0.005$ ; -23%,  $p<0.001$ ), and at P28 (-19%,  $p<0.001$ ) in the EDL. These data suggest an impaired presynaptic development and a delay in synapse elimination.

On the postsynaptic side, SMR induced marked alterations in NMJ maturation. In the SOL, NMJs were predominantly altered at P15. SMR led to a reduction in AChR area (-26%,  $p=0.037$ ), endplate perimeter (-16%,  $p=0.046$ ) and endplate area (-30%,  $p=0.024$ ), reflecting an overall smaller and less developed postsynaptic apparatus (Fig. 6B). Compactness was also slightly increased (+6%,  $p=0.049$ ) suggesting a more condensed and less complex receptor organization. At P28, the alterations observed at P15 were no longer significant, indicating partial structural recovery. However, the number of AChR clusters was markedly increased (+86%,  $p<0.001$ ) (Fig. 6C,D), suggesting ongoing postsynaptic remodelling or fragmentation at later stages.

In the EDL, no difference was observed at P15 with respect to CTRL rats. At P28, most NMJ morphological parameters in the EDL remained comparable between groups, except the number of AChR clusters that increased significantly (+95%,  $p=0.006$ ), suggesting persistent postsynaptic remodelling despite the apparent normalization of global NMJ size.

Finally, the overlap between pre- and postsynaptic elements remained unchanged in both muscles at both time points, suggesting that SMR primarily affects structural maturation rather than synaptic apposition *per se*. Overall, these data reveal a delay in NMJ maturation affecting both pre- and postsynaptic compartments, with a more pronounced impact in the SOL.

| Variable                               |     | CTRL           | SMR            | %Δ             | P Value          |
|----------------------------------------|-----|----------------|----------------|----------------|------------------|
| <b>SOLEUS</b>                          |     |                |                |                |                  |
| <b>Presynaptic</b>                     |     |                |                |                |                  |
| Polyinnervation (%)                    | P15 | 2.50 ± 1.44    | 18.89 ± 1.11   | <b>+655.6%</b> | <b>0.005</b>     |
|                                        | P28 | 0.00 ± 0.00    | 0.00 ± 0.00    |                | <b>NA</b>        |
| Nerve terminal area (μm <sup>2</sup> ) | P15 | 101.76 ± 7.17  | 77.78 ± 10.90  | -23.6%         | 0.162            |
|                                        | P28 | 159.28 ± 17.47 | 139.01 ± 4.14  | -12.7%         | 0.206            |
| Axon diameter (μm)                     | P15 | 1.70 ± 0.03    | 1.32 ± 0.12    | <b>-22.2%</b>  | <b>0.005</b>     |
|                                        | P28 | 2.36 ± 0.09    | 1.82 ± 0.07    | <b>-23.1%</b>  | <b>&lt;0.001</b> |
| <b>Postsynaptic</b>                    |     |                |                |                |                  |
| AChR area (μm <sup>2</sup> )           | P15 | 135.89 ± 7.00  | 101.04 ± 9.21  | <b>-25.6%</b>  | <b>0.037</b>     |
|                                        | P28 | 189.07 ± 16.51 | 173.38 ± 5.32  | -8.3%          | 0.306            |
| Endplate diameter (μm)                 | P15 | 20.78 ± 0.73   | 18.50 ± 1.82   | -10.9%         | 0.195            |
|                                        | P28 | 24.62 ± 1.32   | 23.87 ± 0.71   | -3.0%          | 0.654            |
| Endplate perimeter (μm)                | P15 | 55.36 ± 1.53   | 46.71 ± 2.82   | <b>-15.6%</b>  | <b>0.046</b>     |
|                                        | P28 | 72.03 ± 4.36   | 66.88 ± 1.02   | -7.1%          | 0.198            |
| Endplate area (μm <sup>2</sup> )       | P15 | 177.52 ± 9.94  | 124.36 ± 12.90 | <b>-29.9%</b>  | <b>0.024</b>     |
|                                        | P28 | 266.33 ± 22.33 | 230.91 ± 8.81  | -13.3%         | 0.099            |
| Compactness                            | P15 | 76.92 ± 1.05   | 81.60 ± 1.19   | <b>+6.1%</b>   | <b>0.049</b>     |
|                                        | P28 | 72.05 ± 2.22   | 75.81 ± 1.01   | +5.2%          | 0.082            |
| Number of AChR Clusters                | P15 | 1.00 ± 0.00    | 1.00 ± 0.00    | 0.0%           | 1.000            |
|                                        | P28 | 1.22 ± 0.09    | 2.27 ± 0.22    | <b>+85.9%</b>  | <b>&lt;0.001</b> |
| <b>Merge</b>                           |     |                |                |                |                  |
| Overlap (%)                            | P15 | 68.27 ± 1.09   | 71.12 ± 2.31   | +4.2%          | 0.184            |
|                                        | P28 | 75.45 ± 1.55   | 73.98 ± 0.94   | -1.9%          | 0.490            |
| <b>EDL</b>                             |     |                |                |                |                  |
| <b>Presynaptic</b>                     |     |                |                |                |                  |
| Polyinnervation (%)                    | P15 | 8.64 ± 3.63    | 31.25 ± 6.25   | <b>+261.8%</b> | <b>&lt;0.001</b> |
|                                        | P28 | 0.00 ± 0.00    | 3.75 ± 2.39    |                | 0.977            |
| Nerve terminal area (μm <sup>2</sup> ) | P15 | 99.84 ± 8.43   | 131.24 ± 16.62 | +31.5%         | 0.097            |
|                                        | P28 | 196.12 ± 3.70  | 211.47 ± 18.32 | +7.8%          | 0.476            |
| Axon diameter (μm)                     | P15 | 1.54 ± 0.04    | 1.47 ± 0.06    | -4.3%          | 0.366            |
|                                        | P28 | 2.39 ± 0.06    | 1.93 ± 0.01    | <b>-19.2%</b>  | <b>&lt;0.001</b> |
| <b>Postsynaptic</b>                    |     |                |                |                |                  |
| AChR area (μm <sup>2</sup> )           | P15 | 129.03 ± 11.95 | 157.73 ± 16.69 | +22.2%         | 0.179            |
|                                        | P28 | 221.96 ± 8.99  | 241.95 ± 20.58 | +9.0%          | 0.412            |
| Endplate diameter (μm)                 | P15 | 20.44 ± 1.39   | 21.66 ± 2.06   | +6.0%          | 0.557            |
|                                        | P28 | 25.21 ± 0.67   | 28.74 ± 1.42   | +14.0%         | 0.123            |
| Endplate perimeter (μm)                | P15 | 51.10 ± 2.27   | 57.58 ± 3.03   | +12.7%         | 0.141            |
|                                        | P28 | 71.76 ± 1.63   | 80.78 ± 4.76   | +12.6%         | 0.060            |
| Endplate area (μm <sup>2</sup> )       | P15 | 153.93 ± 13.48 | 196.41 ± 22.91 | +27.6%         | 0.241            |
|                                        | P28 | 303.81 ± 16.84 | 350.91 ± 42.29 | +15.5%         | 0.247            |
| Compactness                            | P15 | 83.86 ± 0.78   | 81.24 ± 0.68   | -3.1%          | 0.154            |
|                                        | P28 | 74.49 ± 1.51   | 71.18 ± 2.18   | -4.4%          | 0.141            |
| Number of AChR Clusters                | P15 | 1.00 ± 0.00    | 1.00 ± 0.00    | 0.0%           | 1.000            |
|                                        | P28 | 1.00 ± 0.00    | 1.95 ± 0.44    | <b>+95.0%</b>  | <b>0.006</b>     |
| <b>Merge</b>                           |     |                |                |                |                  |
| Overlap (%)                            | P15 | 72.90 ± 2.83   | 72.93 ± 2.56   | +0.0%          | 0.998            |
|                                        | P28 | 77.92 ± 1.78   | 76.64 ± 1.30   | -1.6%          | 0.673            |

**Table 2: Morphological variable of the neuromuscular junction of the SOL and the EDL in CTRL and SMR animals at P15 and P28.** Values are presented as mean ± SEM. Values were compared using LMM with Group, Age and their interaction as fixed effects and Animal as a random intercept. Type III ANOVA was used to test fixed effects, and pairwise post-hoc contrasts were performed on estimated marginal means with Holm correction. Polyinnervation,

considered as binary outcome, was analysed separately using binomial generalized LMM with the same fixed- and random-effects structure. For SOL, an additional model restricted to P15 was fitted because no polyinnervated junctions were observed at P28.

## Discussion

The present study investigated how early sensorimotor restriction (SMR) affects skeletal muscle growth and maturation during a critical period of postnatal development. Few studies have examined the consequences of immobilization in newborn rats, but available data remain fragmented across separate studies focusing on individual muscles or a limited number of muscles. Here, by combining morphological, phenotypic, cellular and neuromuscular analyses across muscles with distinct functional properties, we show that SMR does not induce a uniform developmental response. Rather, its effects strongly depend on the muscle considered.

By analysing a critical window of rat development (from birth to P28), we found that the slow-twitch postural muscle (SOL) was particularly vulnerable, displaying sustained growth impairment together with marked remodelling of its contractile phenotype. In contrast, the fast-twitch muscles (EDL, TA) maintained their relative muscle mass and exhibited a transient increase in fibre size at P15, associated with a shift from very fast towards more intermediate MyHC phenotypes. The PLT showed comparatively preserved fibre size and nuclear organization despite moderate changes in muscle mass and fibre-type composition. Importantly, SMR also alters key markers of muscle maturation, as evidenced by an increased proportion of centronucleated fibres and altered PAX7-associated myogenic response. In addition, neuromuscular junction structure is disrupted at both the presynaptic (persistent polyinnervation) and postsynaptic (altered endplate morphology) levels, in a muscle-specific manner.

Together, the consequences of SMR appear to reflect an interaction between reduced activity, the mechanical state imposed on each muscle and its intrinsic functional characteristics, rather than a generalized delay in muscle development. Overall, our findings suggest that during the critical developmental period, mechanical loading and contractile activity act not only as trophic factors, but as essential instructive signals required for the proper execution of the postnatal myogenic program.

### ***SMR induces muscle-dependent adaptations in postnatal growth***

Our results partially mirror classical findings in adult unloading models, where adaptation is governed by the muscle's initial function during immobilization. In the posterior compartment of the hind limb, the SOL exhibited the most pronounced and persistent growth impairment, evidenced by a marked reduction in weight and median CSA at both P15 and P28. The PLT,

although located in the same posterior compartment, was substantially less affected at the fibre level. Conversely, the fast-twitch EDL and TA muscles displayed a high degree of mass preservation, as their global weights remained unaffected by SMR. As a slow-twitch postural muscle, it is widely accepted that the SOL is the most responsive to mechanical unloading and the EDL or TA the least responsive, while the PLT muscle shows an intermediate response (Jaspers and Tischler, 1984).

Nevertheless, contractile phenotype alone is unlikely to explain the present findings. Muscle adaptation to immobilization also strongly depends on muscle length. Indeed, in adults, immobilization in a chronically shortened position accentuates muscle loss, whereas chronic stretch can preserve or even promote muscle growth (Booth, 1982; Fitts et al., 2001; Goldspink, 1977; Thomason and Booth, 1990; Williams and Goldspink, 1978). In the present SMR model, the SOL and PLT are maintained in a relatively shortened position, whereas the EDL and TA are placed under passive stretch. This difference in mechanical state likely contributes to the divergent responses observed between anterior and posterior muscles.

In the SOL, the combination of reduced contractile activity and chronic shortening may therefore contribute to the marked atrophic response, triggered by molecular pathways. Indeed, the atrophic response is typically driven by the activation of the ubiquitin-proteasome system, notably through the up-regulation of muscle-specific E3 ubiquitin ligases such as Atrogin-1 (MAFbx) and MuRF-1 (Bodine et al., 2001; Gomes et al., 2001). The induction of these atrogenes is often controlled by FoxO transcription factors when anabolic signalling is suppressed (Sandri et al., 2004).

Conversely, the EDL and TA displayed a striking but transient increase in fibre CSA at P15 despite preservation of overall muscle weight. Such early fibre enlargement is consistent with an effect of passive stretch imposed by the SMR position. Chronic mechanical tension has long been recognized as a potent inducer of sarcomerogenesis and radial hypertrophy (Goldspink, 1977). More recently, mechanical stretch has been shown to activate anabolic signalling pathways, including mTOR-dependent mechanisms (Wackerhage et al., 2019). However, because the present experimental design does not dissociate reduced sensorimotor activity from the imposed muscle position, passive stretch cannot be identified as the sole cause of this transient hypertrophic response.

The contrasting behaviour of the PLT is particularly informative in this respect. Although it is shortened together with the SOL, its fibre CSA remained largely preserved at both developmental stages, despite a moderate reduction in relative muscle weight. Thus, muscle position alone is insufficient to predict the response to SMR. Instead, the present data suggest that postnatal muscle growth reflects the combined influence of mechanical state, functional identity and baseline recruitment pattern.

### ***SMR induces MyHC transitions and disrupts postnatal contractile maturation***

The innovative aspect of our findings is that SMR alters the normal postnatal evolution of MyHC expression during the postnatal window of heightened plasticity, a period during which contractile activity is essential for the proper muscle specialization (Walton et al., 1992). In adult models of hindlimb unloading or microgravity, the SOL and PLT typically undergo a significant slow-to-fast fibre type transition, where the lack of weight-bearing and tonic neural discharge prevents the maintenance of the slow-twitch identity (Baldwin et al., 2013; Pette and Vrbova, 1999). In these unloading paradigms, slow oxidative fibres are consistently reported as the primary targets of disuse-induced atrophy, whereas fast-twitch glycolytic fibres (EDL, TA) are generally less affected (Fitts et al., 2001; Thomason and Booth, 1990).

However, our results demonstrate that in the postnatal context, the response is fundamentally different from a simple adult-like reversion, because the mature contractile phenotype has not yet been established. Rather than modifying a stable adult profile, SMR interferes with a highly dynamic ontogenetic program. During normal postnatal development, rat skeletal muscle undergoes a tightly regulated MyHC transition (MyHC II to MyHC I in postural muscles) with the stabilization of adult isoform distribution occurring around weaning (Ohira et al., 2001). Consequently, instead of the slow-to-fast (SOL, PLT) or fast-to-slow (EDL, TA) transition that can be observed in adults, our study reveals first that SMR reduced the proportion of type I fibres and increased type IIa and hybrid I/IIa fibres in the SOL, indicating impaired establishment of the mature slow phenotype. Thus, reduced sensorimotor experience appears to alter the course of SOL maturation, rather than simply reversing it. Second, the EDL and TA shifted from a very fast towards a more intermediate-fast phenotype, with fewer type IIb fibres and increased type IIa and hybrid populations. Because these muscles are chronically stretched in the SMR position, this response may partly reflect mechanically driven remodelling, as altered loading can influence MyHC expression independently of neural firing patterns (Schiaffino and Reggiani, 2011). Interestingly, our data suggest a dissociation between muscle growth and phenotypic maturation, since the transient increase in fibre CSA observed in the EDL and TA was accompanied by marked changes in MyHC profile and persistent centronucleation. Such a dissociation between muscle mass and MyHC content has already been described. For instance, in the hindlimb suspension model, increased neural input through electromyostimulation prevents the slow-to-fast transformation of rat SOL without preventing the loss of muscle mass or force (Leterme and Falempin, 1994) whereas activation of afferent input by means of tendinous vibration prevents the atrophy but not the MyHC transition (Falempin and Fodili In-Albon, 1999). Thus, muscle size and phenotype are influenced by interacting neural, mechanical and metabolic signals (Blaauw et al., 2013), and the present design cannot determine their respective contribution during development. Third, the PLT followed yet another pattern, with an early increase in type IIb fibres and reductions in

type I and IIa fibres, indicating a transition towards a faster phenotype. Together, these contrasting responses show that SMR does not produce a uniform alteration of MyHC maturation but redirects contractile specialization in a muscle-specific manner.

Interpretation of the unstained fibre population also requires caution. Such population is supposed to contain embryonic and neonatal forms of MyHC. Indeed, these MyHC progressively decline during the first postnatal weeks but may still be present in neonatal skeletal muscle, with significant amounts of embryonic MyHC being detected in 7- and even 10-day neonatal muscle (Schiaffino et al., 1988). Previous studies have also reported the persistence of small amounts of neonatal isoforms after immobilization of hind limbs in the postnatal period (Canu et al., 2022; Picquet et al., 1998). However, the fibres not identified by the antibodies used in the present study could contain developmental MyHC isoforms, but also MyHC type IIx, or a combination of these isoforms. Consequently, the unstained population cannot by itself be considered evidence that fibres remain trapped in a neonatal state. Specific immunodetection of developmental and MyHC IIx isoforms will be required to distinguish between persistent developmental expression and remodelling towards alternative adult isoforms.

Overall, our findings suggest that early SMR modifies the trajectory of postnatal contractile specialization rather than inducing a simple, generalized delay in MyHC maturation.

### ***Cellular markers reveal altered maturation and remodelling***

The modification of developmental trajectory is confirmed by the identification of centronucleated fibres (CNF) and muscle stem cell dynamics as key indicators of impaired maturation. During normal postnatal development, myonuclei migrate from a central position to the fibre periphery, a process essential for structural stability and functional specialization (Cadot et al., 2015). In our study, CTRL animals showed a marked decline in CNF from P15 to P28, marking the completion of this developmental process. Conversely, SMR animals maintained significantly elevated CNF levels at P28 in the SOL, EDL and TA, demonstrating a failure in nuclear dispersion. In this developmental context, the persistence of central nuclei, particularly in the TA and EDL may reflect delayed maturation or activation of the satellite pool to facilitate the stretch induced hypertrophy and/or fibre damage.

This interpretation is supported by the PAX7 analysis. PAX7 is essential for satellite cell maintenance and postnatal muscle growth (Relaix and Zammit, 2012; Seale et al., 2000). At P28, SMR significantly increased PAX7 protein expression in the EDL, while a similar increase in the SOL did not reach statistical significance. Preliminary histological observations also suggested a higher number of PAX7-positive cells relative to fibre number in several SMR muscles. However, because these histological counts were exploratory and PAX7 protein

abundance does not directly measure satellite-cell proliferation, these findings should not be interpreted as evidence of satellite-cell activation or failure to return to quiescence.

During normal maturation, satellite cells progressively exit the cell cycle and return to quiescence as fibres stabilize (Lepper et al., 2009; White et al., 2010).

Thus, the combined centronucleation and PAX7 findings support the presence of persistent cellular remodelling following SMR but do not establish a single mechanism. In the SOL, these changes are compatible with delayed structural maturation, whereas in the stretched EDL and TA, they may reflect a combination of developmental perturbation and adaptive growth responses.

### ***Neuromuscular junction alterations as a driver of delayed maturation***

Although one study has been conducted recently to evaluate the consequences of decreased activity on the neuromuscular junction (NMJ) in adults (Deschenes et al., 2025), whether early SMR might affect NMJ morphology remains to be elucidated. The NMJ findings provide particularly strong evidence that early SMR interferes with neuromuscular maturation. Indeed, postnatal maturation of skeletal muscle is tightly coordinated with the progressive refinement of neuromuscular connectivity, including synapse elimination, axonal growth and postsynaptic specialization. We observed a higher number of polyinnervated fibres in SMR rats at P15 in both SOL and EDL muscles, indicating a delay in synapse elimination, a hallmark of immature neuromuscular circuits (Sanes and Lichtman, 2001). This observation is particularly relevant, as the transition from poly- to monoinnervated fibres is a critical step in the functional maturation of motor units during early life. The persistence of polyinnervation suggests that SMR disrupts activity-dependent synaptic pruning, a process known to rely on coordinated neural activity and muscle contraction (Buffelli et al., 2003; Witzemann et al., 2013).

In addition to these early defects, we identified alterations in presynaptic structure at P28, notably a reduction in axon diameter in both muscles. This finding suggests that SMR not only delays early synaptic refinement but may also impair axonal maturation and stability. Given that axonal calibre is closely linked to conduction velocity and neurotransmission efficiency (Waxman, 1980), such changes could contribute to impaired neuromuscular transmission and altered muscle function. Postsynaptic effects also differed between muscles. In the SOL, the reduction in AChR area and endplate size observed at P15 further supports the presence of an immature NMJ phenotype (Shi et al., 2012; Slater, 2017). Although most of these alterations were partially restored by P28, the increased number of AChR clusters observed at this stage in both muscles suggests ongoing remodelling or fragmentation, reflecting a persistent instability of the postsynaptic apparatus. Importantly, pre- and postsynaptic overlap was preserved in both muscles. This suggests that SMR does not produce a major loss of synaptic

apposition but rather modifies the temporal and structural maturation of the neuromuscular junction.

These NMJ alterations may contribute to the muscle phenotypes observed in the present study, since neuromuscular activity is a major regulator of muscle fibre growth, contractile identity and satellite cell dynamics (Tintignac et al., 2015). Impaired NMJ development may lead to reduced or altered activation patterns, ultimately preventing the proper execution of the postnatal myogenic program. Together, these data suggest that SMR disrupt the coordinated development of skeletal muscle and neuromuscular junction.

### ***Conclusion and clinical perspectives***

To conclude, the present study provides evidence that early sensorimotor restriction from birth to weaning profoundly modifies the normal coordination of skeletal muscle and neuromuscular development. Importantly, the response is not uniform across muscles. These findings indicate that the consequences of early hypoactivity cannot be understood solely in terms of disuse-induced atrophy. During postnatal development, sensorimotor restriction modifies the mechanical and neural signals that normally guide muscle-specific growth, contractile specialization and synaptic refinement. The resulting phenotype therefore reflects the interaction between developmental stage, functional identity and the mechanical environment of each muscle.

Given the negative implications of low muscle mass, strength and physical function on children health and adverse outcomes in adulthood (Inoue et al., 2024), these observations are highly relevant to clinical conditions inducing paediatric sarcopenia such as cerebral palsy, spinal muscular atrophy or prolonged immobilization during childhood, where altered mechanical loading, impaired neural activity and lack of mobility contribute to abnormal muscle architecture, long-term motor deficits and/or abnormal expression of MyHC isoforms (Edman et al., 2026; Hedberg-Oldfors et al., 2024; Lieber and Ward, 2011).

It is now well documented that early-life stimuli may affect skeletal muscle function in adult life. Muscles have an epigenetic memory, defined as “the capacity of skeletal muscle to respond differently to environmental stimuli in an adaptive or maladaptive manner if the stimuli have been previously encountered” (Seaborne et al., 2018; Sharples et al., 2016). Accordingly, future research must determine whether the observed maturation delay leaves permanent epigenetic marks on muscle promoters, potentially inducing irreversible developmental divergence and limiting the muscle’s plastic response to rehabilitation later in life. Whether a stimulus such as SMR during early life may have adverse functional consequences in adulthood remains to be determined.

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**Ethics approval statement:** All experiments were conducted in accordance with European and French legislation on animal experimentation and were approved by the Regional Committee on the Ethics of Animal Experiments of the Haut-de-France region (CEEA 75, reference number: APAFIS#51113 -2024051608522519).

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## Figure legends:

### **Fig. 1 Evolution of relative weight of hind limb muscles between P15 and P28 development stages**

Data represent the ratio of muscle weight (mg) to body weight (g) for the Soleus (SOL), the Plantaris (PLT), the Extensor Digitorum Longus (EDL) and the Tibialis Anterior (TA). Values are mean  $\pm$  SEM and were compared using two-way ANOVA and Tukey post-hoc analysis. Main effects of Group (CTRL vs SMR) and Age (P15 vs P28) are represented beneath the corresponding graph.

### **Fig. 2 Distribution of muscle fibre cross-sectional area in SOL, PLT, EDL and TA at P15 and P28**

(A-B) Representative immunofluorescence staining of laminin in muscle cryosections of CTRL and SMR soleus (A) and EDL (B) at P15 and P28. Scale bars = 50  $\mu$ m. (C-D) Density plot and boxplot representation of fibre cross-sectional area of SOL, PLT, EDL and TA in CTRL and SMR groups at P15 (C) and P28 (D). Data are shown as mean  $\pm$  SEM in the half violin. CSA bins = 100  $\mu$ m<sup>2</sup>. Boxplots represent Q1, Median, Q3 and 5-95 percentiles.  $\beta$  indicates the estimated difference in CSA between SMR and CTRL groups, expressed in  $\mu$ m<sup>2</sup>  $\pm$  SE. Values were compared using LMM with Group, Fibre type and their interaction as fixed effects and animal as a random intercept to account for measurements from the same animal. Fixed effects were assessed using type III ANOVA with Satterthwaite approximation.

### **Fig.3 Change in MyHC typing in SOL, PLT, EDL and TA in response to SMR**

(A) Representative immunofluorescence staining of Laminin (green), MyHC I (magenta), MyHC IIa (blue) and MyHC IIb (red) for CTRL and SMR SOL, PLT, EDL, TA at P15 and P28. Scale bar: 100 $\mu$ m. (B-C) Fibre type distribution (in %) in all four muscles at P15 (B) and P28 (C). For each muscle and age, chi-squared test assessed overall differences in fibre-type distribution between CTRL and SMR groups. Fibre-type-specific comparisons were performed using GLMM, with group as a fixed effect and animal as a random intercept. Each fibre type was compared against the remaining typed fibres within the same sample.

### **Fig. 4 Proportion of centronucleated fibres as an indicator of muscle stability and maturation**

(A) SOL, (B) PLT, (C) EDL and (D) TA percentage of CNF in CTRL and SMR animals at P15 and P28. Values are mean  $\pm$  SEM and were compared using two-way ANOVA and Tukey post-hoc analysis. Main effects of Group (CTRL vs SMR) and Age (P15 vs P28) are represented beneath each corresponding graph.

### **Fig. 5 Quantification of the myogenic pool and PAX7 protein levels**

(A) Representative immunofluorescence images of muscle cross-sections stained for DAPI (Blue), Laminin (green) and PAX7 (magenta). White arrowheads indicate PAX7+ muscle stem cells (MuSCs). Scale bar = 50  $\mu$ m. (B) Ratio of MuSCs per muscle fibre in SOL, PLT, EDL and TA, at P15 and P28 (N=2 per group). (C-F) Western blot analysis and quantification of PAX7 protein levels in the SOL and EDL at P15 (C-D) and P28 (E-F). Representative blot for PAX7 (50 kDa) and the corresponding stain-free total protein gel (used for normalization) are shown for each condition. Statistical comparisons between CTRL and SMR were performed using parametric t-test. Values are mean  $\pm$  SEM expressed in arbitrary units (A.U.).

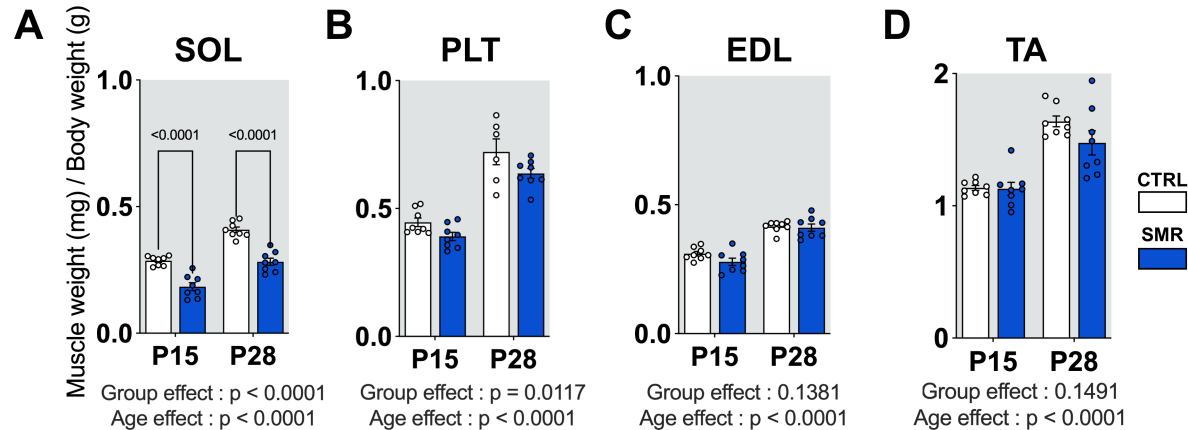
### **Fig. 6 Representative neuromuscular junction (NMJ) immunofluorescence**

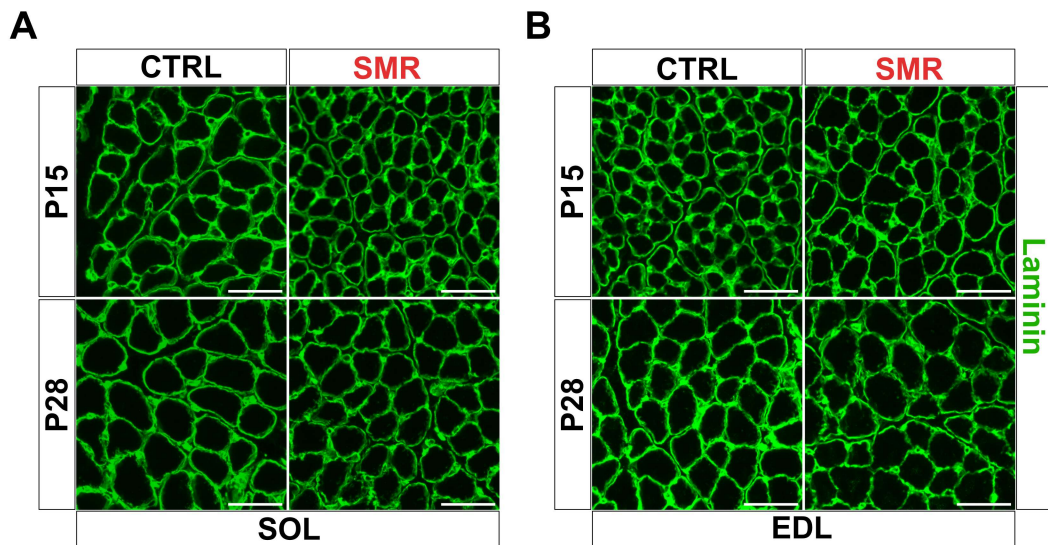
Representative confocal images of SOL NMJs stained with neurofilament/synaptophysin (green) and  $\alpha$ -bungarotoxin (red). (A) Example of polyinnervation observed in SMR rats at P15. (B) Representative NMJs from CTRL and SMR SOL muscles at P15. (C-D) Representative NMJs from CTRL (C) and SMR (D) SOL muscles at P28, showing increased fragmentation in the SMR rats. Scale bars = 10 $\mu$ m.

### Supplementary material

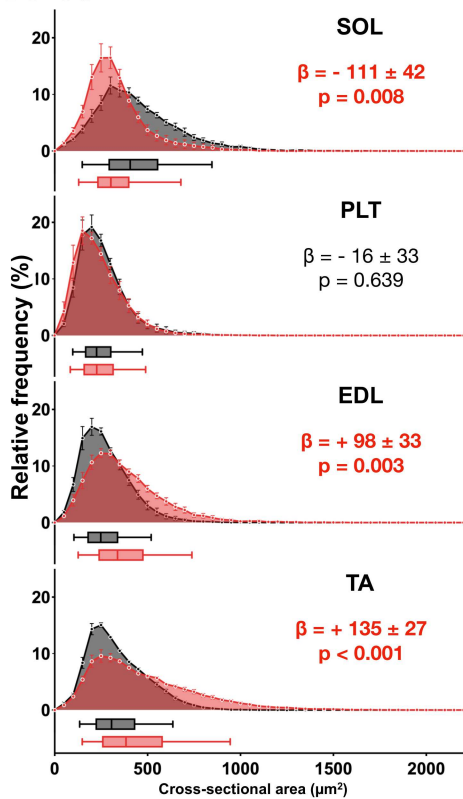
**Fig. S1: Representative cross-sections of skeletal muscle stained with haematoxylin and eosin (H&E).** Soleus, extensor digitorum longus, tibialis anterior and plantaris muscle sections of CTRL and SMR groups were stained with haematoxylin (to highlight nuclei) and eosin (to highlight cytoplasm) at both P15 and P28. Scale bar = 50  $\mu\text{m}$ .

**Table S1: Cross-sectional area value of the Soleus, the plantaris, the Extensor digitorum longus and the tibialis anterior in CTRL and SMR animals at P15 and P28.** Values are as median (Q1-Q3).

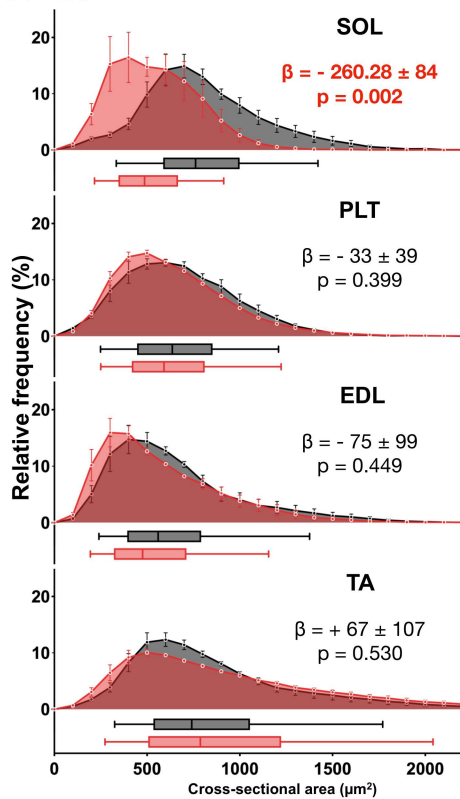




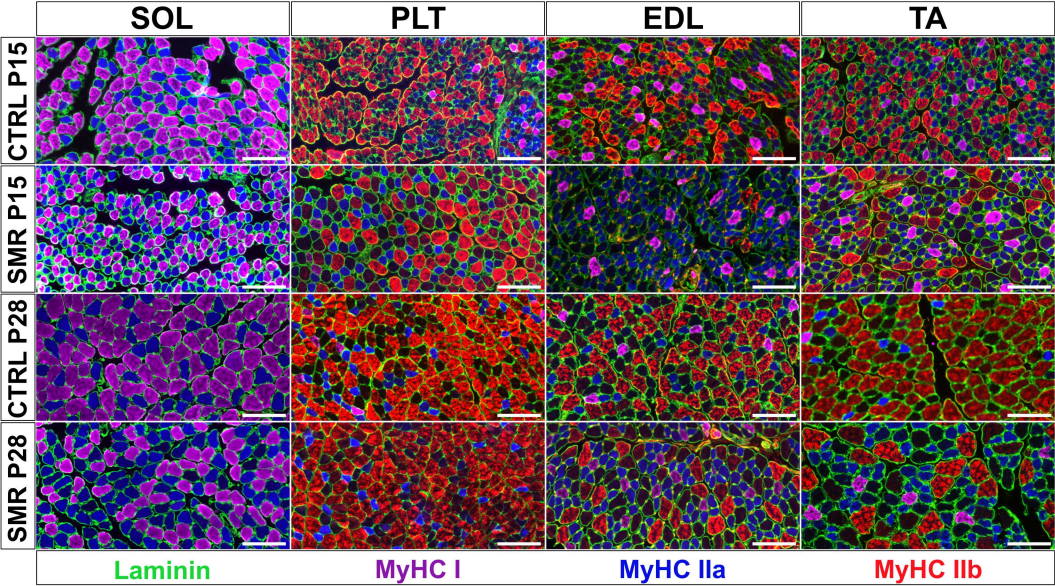
**C: P15**



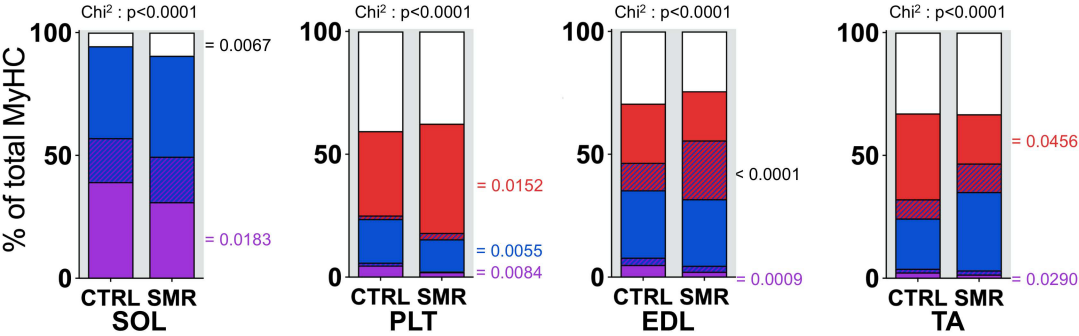
**D: P28**



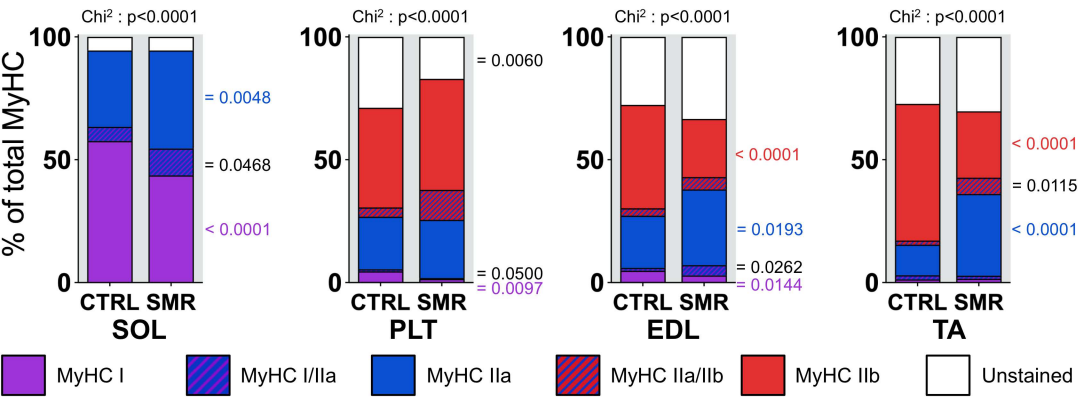
A

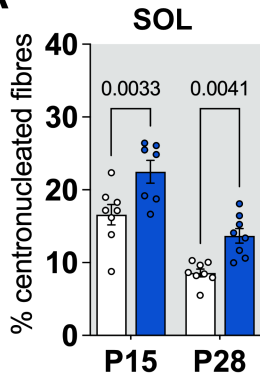


B: P15

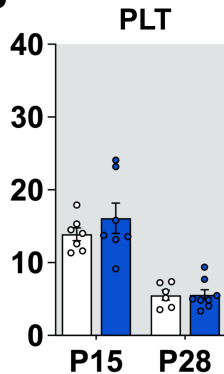


C: P28

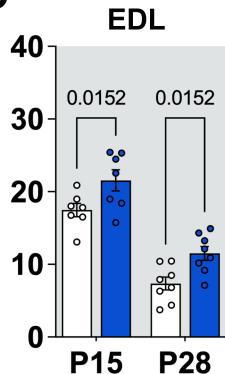


**A**

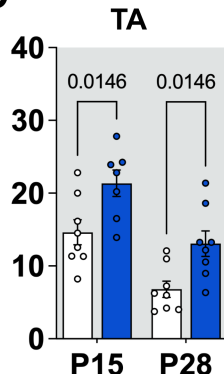
Group effect:  $p < 0.0001$   
Age effect:  $p < 0.0001$

**B**

Group effect:  $p = 0.3686$   
Age effect:  $p < 0.0001$

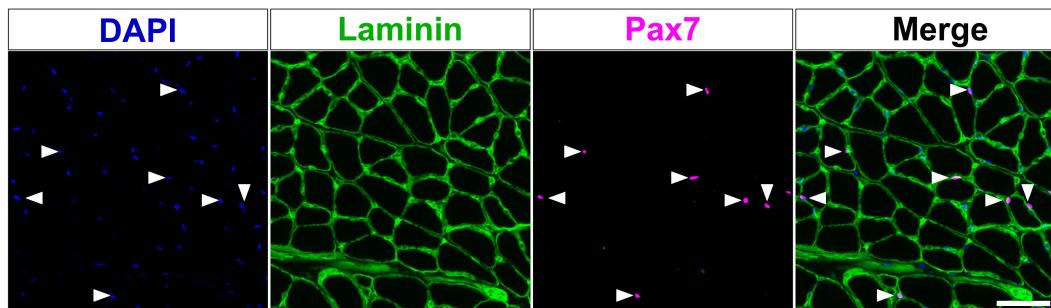
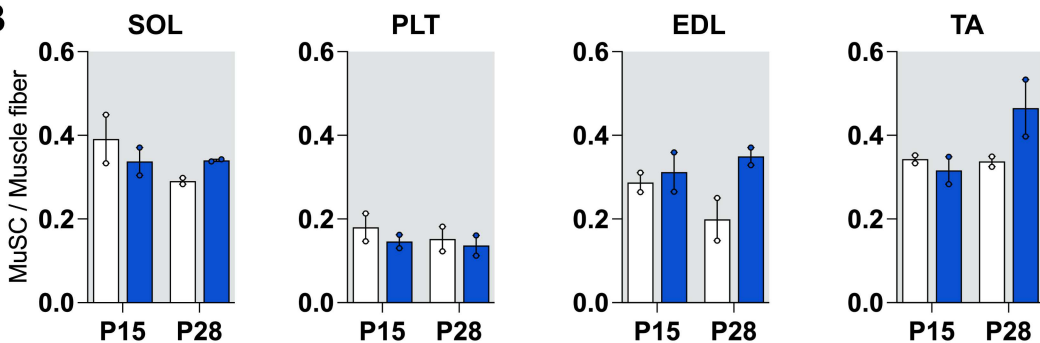
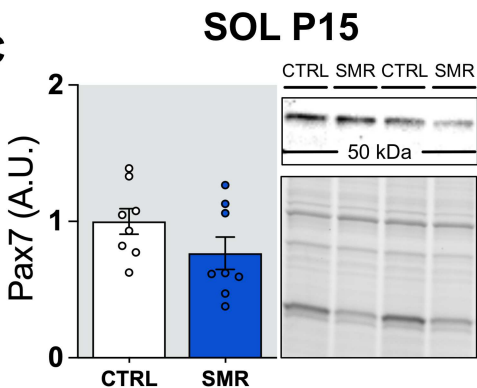
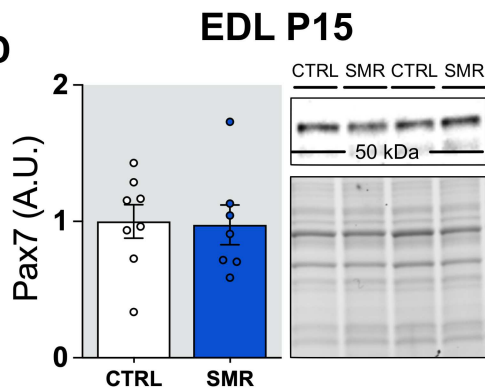
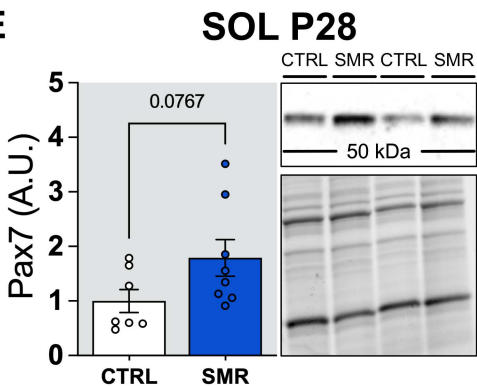
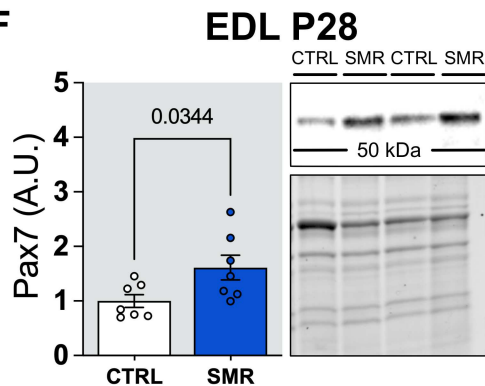
**C**

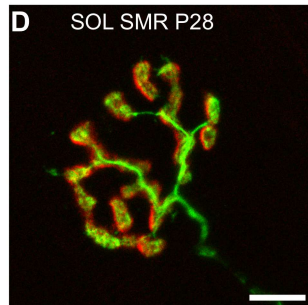
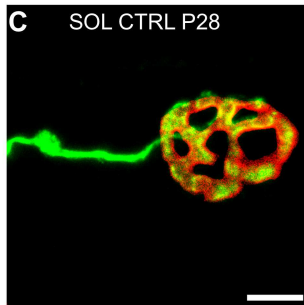
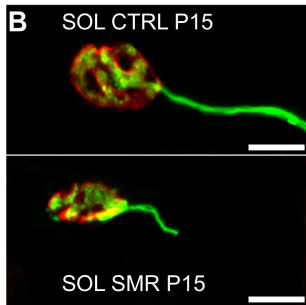
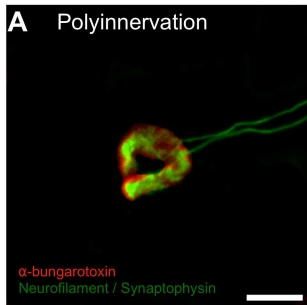
Group effect:  $p = 0.0006$   
Age effect:  $p < 0.0001$

**D**

Group effect:  $p = 0.0004$   
Age effect:  $p < 0.0001$

CTRL  
SMR

**A****B****C****D****E****F**



## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SuppTableS1.pdf](#)
- [SuppFigS1.tiff](#)