











**ARTICLE**

## Excitation–Contraction Coupling

# Nerve-dependent distribution of subsynaptic type 1 inositol 1,4,5-trisphosphate receptor at the neuromuscular junction

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**Inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>Rs) are enriched at postsynaptic membrane compartments of the neuromuscular junction (NMJ), surrounding the subsynaptic nuclei and close to nicotinic acetylcholine receptors (nAChRs) of the motor endplate. At the endplate level, it has been proposed that nerve-dependent electrical activity might trigger IP<sub>3</sub>-associated, local Ca<sup>2+</sup> signals not only involved in excitation–transcription (ET) coupling but also crucial to the development and stabilization of the NMJ itself. The present study was undertaken to examine whether denervation affects the subsynaptic IP<sub>3</sub>R distribution in skeletal muscles and which are the underlying mechanisms. Fluorescence microscopy, carried out on in vivo denervated muscles (following sciactomy) and in vitro denervated skeletal muscle fibers from flexor digitorum brevis (FDB), indicates that denervation causes a reduction in the subsynaptic IP<sub>3</sub>R1-stained region, and such a decrease appears to be determined by the lack of muscle electrical activity, as judged by partial reversal upon field electrical stimulation of in vitro denervated skeletal muscle fibers.**

## Introduction

Motor innervation is required for the maturation and maintenance of diversity of both slow-twitch and fast-twitch fibers, thus regulating the contractile properties of skeletal muscle fibers, in part through the effect of electrical activity on the expression of distinct myosins. Motor innervation is also required for the attainment and maintenance of trophic homeostasis and muscle mass (Tintignac et al., 2015), with inactivity and denervation being paramount causes of skeletal muscle atrophy (Carlson, 2014).

Efficient neuromuscular transmission requires complex machineries both at pre- and postsynaptic compartments of the neuromuscular junction (NMJ). The motorneuron has developed molecular strategies to tune the adequate release of neurotransmitter acetylcholine (ACh); the skeletal muscle fiber has adopted strategies to maximize the binding of ACh to nicotinic

acetylcholine receptors (nAChRs) to trigger action potentials and to turn on the excitation–contraction (EC) coupling apparatus.

Expression, distribution, and stability of nAChR at the endplate are controlled by both the release of soluble factors from motor nerve endings and the electrical activity of muscle fibers (Duclert and Changeux, 1995; Missias et al., 1996; Sanes and Lichtman, 2001; Li et al., 2018; Shi et al., 2012). Skeletal muscle denervation causes nAChR cluster dispersal at the endplate zone, reduction in the half-life of synaptic nAChRs, and isoform switch from adult  $\epsilon$ -nAChRs to embryonic  $\gamma$ -nAChRs, the latter being diffusely distributed over the muscle plasma membrane (Midrio, 2006; Wu et al., 2014).

Among nerve-derived chemical messengers, a key role is played by the proteoglycan agrin (McMahan, 1990) via the activation of the Lrp4/MuSK receptor complex (Li et al., 2018).

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Neural agrin controls the growth and stabilization of the endplate nAChRs (Sanes and Lichtman, 2001) and the distribution and expression of a number of other components of the postsynaptic apparatus (Wallace, 1989; Meier et al., 1997; Brigue et al., 2000).

As to the regulating effect of the electrical activity, direct muscle stimulation alone is able to prevent the denervation effects at the endplate level. In denervated active/stimulated muscles, electrical activity prevents the denervation-induced decline of metabolic nAChR stability and the loss of endplate structure, and also suppresses the expression of the fetal  $\gamma$ -nAChRs (Brenner and Rudin, 1989). Moreover, direct muscle stimulation counteracts the reduction in the endplate nAChR density induced by blockade of the neuromuscular transmission (Akaaboune et al., 1999). The effects of the induced electrical activity are mediated by an increase in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ), through either ligand-gated, L-type channels or intracellular  $\text{Ca}^{2+}$  stores, and are, at least in part, dependent on nAChRs phosphorylation (Martinez-Pena y Valenzuela and Akaaboune, 2021).

The electrical activity of muscle fibers also exerts its role by changes in  $[\text{Ca}^{2+}]_i$ , definitely unrelated to RyR- $\text{Ca}^{2+}$  channels responsible for  $\text{Ca}^{2+}$  release from the SR during EC coupling (Ríos and Pizarro, 1991): evidence is accruing in favor of an inositol 1,4,5-trisphosphate receptor ( $\text{IP}_3\text{R}$ )-mediated  $\text{Ca}^{2+}$  signaling in the activity-dependent regulation of muscle gene expression (Araya et al., 2003), a process referred to as excitation-transcription (ET) coupling, through which skeletal muscle fibers decode the motoneuron stimulation pattern into a specific gene expression profile and phenotype (Casas et al., 2022).

The presence of all the intermediate steps involved in the metabolism of phosphoinositides, from synthesis to degradation of  $\text{IP}_3$  (Milani et al., 1988; Hidalgo and Jaimovich, 1989), the presence of  $\text{IP}_3\text{Rs}$ , as shown by *in situ* hybridization, immunohistochemistry, and  $[\text{H}^3]$ - $\text{IP}_3$  binding (Moschella et al., 1995), provides a role for  $\text{IP}_3$  in skeletal muscle function highly likely (Volpe et al., 1985; Vergara et al., 1985), although unrelated to proposals concerning the role of  $\text{IP}_3$  as a chemical messenger for EC coupling (Somlyo, 1985; Blaauw et al., 2012).

$\text{IP}_3\text{R}$  immunofluorescence analysis shows a staining pattern indicative of the localization of  $\text{IP}_3\text{Rs}$  at the Z-line in murine muscles different from that of type 1 RyRs. In addition, subcellular fractionation experiments show  $\text{IP}_3\text{R}$  to be enriched in longitudinal SR but not in terminal cisternae containing RyRs (Salanova et al., 2002; Powell et al., 2003).  $\text{IP}_3\text{Rs}$  are also enriched at postsynaptic components of the NMJ, surrounding the subsynaptic nuclei and close to nAChRs of the motor endplate (Powell et al., 2003). At the endplate level,  $\text{IP}_3\text{R1}$  is the prevalent isoform and is involved in synaptic gene expression, as determined by RNA interference-mediated  $\text{IP}_3\text{R1}$  gene silencing (Zhu et al., 2011).  $\text{IP}_3\text{R1s}$  could be placed either in walls of the synaptic folds, subsynaptic triads (Dauber et al., 2000), and/or junctions of unknown function between subsynaptic folds and rough SR (Dauber et al., 1999).

Nerve-driven electrical activity might trigger  $\text{IP}_3$ -associated, local  $\text{Ca}^{2+}$  signals in NMJ involved not only in gene regulation

but also crucial to the development and stabilization of the NMJ itself (Powell et al., 2003). In more detail, a functional interplay between nAChRs activity and  $\text{IP}_3\text{R1}$ -driven  $\text{Ca}^{2+}$  release has been proposed to be responsible for localized subsynaptic  $\text{Ca}^{2+}$  signaling (Zayas et al., 2007; Zhu et al., 2011). In pathophysiological conditions, abnormal activity of  $\text{IP}_3\text{Rs}$  could also be responsible for  $\text{Ca}^{2+}$  overload observed in the slow-channel myasthenic syndrome (Zayas et al., 2007) and for NMJ degeneration during excessive cholinergic activation (Zhu et al., 2011).

Although hypotheses have been formulated for the presence and role of subsynaptic  $\text{IP}_3\text{R}$ -driven  $\text{Ca}^{2+}$  signaling in innervated skeletal muscle fibers, it is not known whether and how innervation controls the subsynaptic  $\text{IP}_3\text{R1}$  membrane compartment. Based on the significant rearrangement of the endplate region following denervation and the supposed functional interaction between endplate nAChRs and  $\text{IP}_3\text{R1s}$ , the present study was undertaken to examine whether denervation affects the subsynaptic  $\text{IP}_3\text{R1}$  distribution and to unveil the mechanisms controlling the expression and localization of subsynaptic  $\text{IP}_3\text{R1s}$ . Fluorescence microscopy carried out on *in vivo* denervated muscles (sciactomy) and *in vitro* denervated adult skeletal muscle fibers (cultured flexor digitorum brevis [FDB] muscle fibers) indicates that denervation caused a reduction in the subsynaptic  $\text{IP}_3\text{R1}$ -stained region and that such a decrease appeared to be determined mainly by the lack of muscle electrical activity.

## Materials and methods

### Animals

Wistar rats and C57BL/6 mice were housed in certified animal facilities at the Universities of Padova and Trieste, respectively, in a 12-h light/12-h dark cycle, bred, and fed *ad libitum* within the guidelines and rules of the European legislation (2010/63/EU).

### In vivo denervation procedure

6-wk-old female Wistar rats (140–160 g of body weight) were anesthetized before surgery and were treated with antibiotics and pain reliever drugs after surgery, as specified in *Aut. Min.* 1089-2020. Denervation procedure, i.e., sciactomy, was performed on the right leg as previously described (Bortoloso et al., 2006) according to the recommendations provided by the European Convention for the Protection of Vertebrate Animals used for Experimental and Scientific Purposes (Council of Europe No. 123, Strasbourg, 1985) and authorized by the Animal Care Committee of the University of Padova and the Italian Health Ministry, in agreement with the European legislation (2010/63/EU), as per *Aut. Min.* 1089-2020. 9 d after sciactomy, rats were sacrificed and extensor digitorum longus (EDL) muscles were dissected from both left (contralateral, control) and right (denervated) legs.

### Isolation of adult skeletal muscle fibers

Single adult skeletal muscle fibers were obtained from the dissociation of FDB muscles of 6–8-wk-old C57BL/6 male mice (20–25 g of body weight). Mice were sacrificed by cervical

dislocation as approved by the local Animal Care Committee of the University of Trieste and the Italian Health Ministry, in agreement with the European legislation (2010/63/EU).

FDB muscle fibers were isolated from both hindlimb foot muscles of a single mouse for each independent preparation. Immediately after excision, FDB muscles were treated with type I collagenase 0.3% (w/v; Sigma-Aldrich) for 1 h in ice and 1 h at 37°C in Tyrode's solution containing (in mM) 137 NaCl, 2.7 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 0.35 Na<sub>2</sub>HPO<sub>4</sub>, 12 NaHCO<sub>3</sub>, 25.5 HEPES, and 5.5 D-glucose, pH 7.4 NaOH, plus 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS (Gibco). Single fibers (750–900 for each mouse) were isolated by mechanical dissociation with Pasteur pipettes with decreasing tip diameters and allowed to settle on Matrigel-coated (1 mg/ml; Corning) glass coverslips in 35-mm Petri dishes.

#### **In vitro denervation of adult skeletal muscle fibers**

Denervation effects at the endplate region were induced in vitro (Grohovaz et al., 1993) by culturing single adult FDB muscle fibers for up to 7 d in a medium composed of DMEM high glucose enriched (Sigma-Aldrich) supplemented with horse serum (5%; Gibco), L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 µg/ml) at 37°C in a 100% humid air atmosphere containing 5% CO<sub>2</sub>. The medium was renewed every 3 d. In these experimental conditions, progressive disarray of the endplate and the appearance of the fetal  $\gamma$ -nAChR isoform occurred after in vivo denervation (Henderson et al., 1987; Tarpey et al., 2018; Gupta et al., 2020). According to the experimental purposes, cultures were maintained in Tyrode's solution at 37°C in a 100% humid air atmosphere containing 5% CO<sub>2</sub> (control conditions) or underwent specific treatments (neural agrin exposure or field electrical stimulation; see below).

#### **Neural agrin treatment of adult, cultured skeletal muscle fibers**

The addition of 1 nM recombinant C-terminal fragment of rat agrin-z\* (550-AG; R&D Systems) to the culture medium started 24 h after dissociation. Each experimental point was obtained from skeletal muscle fibers isolated from the same animal and cultured in the absence (control) or the presence of neural agrin. Renewal of the medium was performed every 3 d and 1 nM neural agrin was added, where applicable, at each medium change.

#### **Field electrical stimulation of adult, cultured skeletal muscle fibers**

The effect of field electrical stimulation was analyzed by comparing adult skeletal muscle fibers isolated from the same animal, cultured at 37°C in a 100% humid air atmosphere containing 5% CO<sub>2</sub> in the absence (control) and the presence of electrical stimulation. Electrical stimulation was delivered to muscle fibers using a Grass S88 stimulator (Grass Instruments). The stimulator was connected to a device constituted by a 6-well plate with two connection cards associated with two parallel platinum-iridium electrodes (0.2 mm in diameter) in each well, placed 2 cm apart and positioned 1–2 mm over the cells. Field electrical stimulation was performed daily, for 5 h, and started

from 24 h after dissociation. Capacitors in series with the electrodes allowed the delivery of biphasic single pulses of 1 ms width at a frequency of 1 Hz. The medium was renewed every 3 d.

#### **nAChR and IP<sub>3</sub>R1 staining**

EDL muscles from contralateral and denervated hindlimbs of each rat were dissected and rapidly frozen in liquid nitrogen. Processing and staining were carried out on 30-µm longitudinal cryosections postfixed with 4% (w/v) paraformaldehyde in PBS for 45 min at room temperature, then blocked with 10% goat serum in PBS (v/v) supplemented with Triton-X100 0.3% (v/v), followed by incubation for 48 h at 4°C with polyclonal antibodies against IP<sub>3</sub>R1s (1:250 in PBS, PA3-901A; Thermo Fisher Scientific Life Sciences) or antibodies raised against a synthetic peptide corresponding to IP<sub>3</sub>R1 aa 1829–1848 developed by Volpe et al. (1993). Sections were extensively washed in PBS and stained with goat anti-rabbit IgG–Alexa Fluor 488 conjugated secondary antibodies (1:500 in PBS; A11034; ThermoFisher Scientific Life Sciences) together with 2 µg/ml snake toxin  $\alpha$ -bungarotoxin ( $\alpha$ -BuTX) Alexa Fluor 555 conjugate (B35451; ThermoFisher Scientific Life Sciences) overnight. After the secondary antibodies and the toxin were removed, slides were washed three times and mounted using Pro Long antifade with DAPI mounting medium (Thermo Fisher Scientific Life Sciences).

In single FDB muscle fibers, nAChRs and IP<sub>3</sub>R1s labeling was carried out from 24 h up to 7 d after dissociation. Fixation was carried out on coverslips with a solution of 4% (w/v) paraformaldehyde in PBS for 15 min at 4°C. Fibers were then permeabilized in PBS + Triton-X100 0.3% (v/v) for 15 min and incubated in a blocking solution containing PBS plus 2% BSA (Sigma-Aldrich) for 30 min. IP<sub>3</sub>R1s were stained with either rabbit polyclonal anti-IP<sub>3</sub>R1s antibodies PA3-901A (Thermo Fisher Scientific Life Sciences) or antibodies developed by Volpe et al. (1993). Rabbit polyclonal anti-IP<sub>3</sub>R1s antibodies were diluted 1:100 in blocking solution, and cell incubation was performed overnight at 4°C. Muscle fibers were incubated for 1 h at room temperature with the secondary goat anti-rabbit Alexa Fluor 568 conjugated antibody (1:500 in PBS; A11036; Thermo Fisher Scientific Life Sciences) and Hoechst solution (1:2,000 in PBS; Thermo Fisher Scientific Life Sciences). nAChRs were labeled by 2.5 µg/ml Alexa Fluor 488- $\alpha$ -BuTX in PBS supplemented with 0.1% BSA (v/v) for 1 h at room temperature. After staining, fibers were washed three times with PBS and finally rinsed with distilled water and mounted on a microscope glass.

#### **Analysis of nAChR and IP<sub>3</sub>R1 staining**

In single FDB muscle fibers, the distribution of nAChRs and IP<sub>3</sub>R1s was analyzed on images acquired by a Nikon C1 confocal microscope using a Plan-Apochromat 60 $\times$ /1.4 (NA) oil-immersion objective. Optical images were collected at either 0.30 or 0.35 µm z resolution by sequential line scanning. Volumetric analysis of the endplate nAChRs and corresponding subsynaptic IP<sub>3</sub>R1s staining was carried out by applying Fiji ImageJ software (ver 2.1.0/1.53c, National Institutes of Health). For each endplate, a stack of images was collected through the entire depth containing the  $\alpha$ -BuTX visible signal. The region of

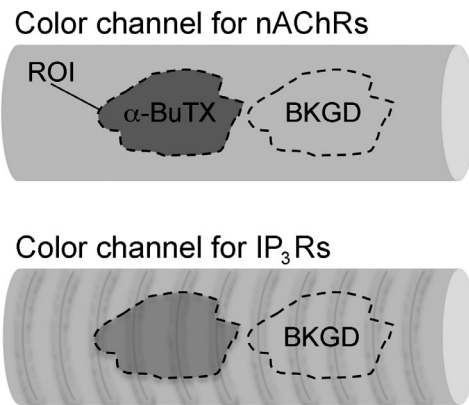


Figure 1. **Measurement of nAChR- and IP<sub>3</sub>R-stained volumes.** The sketch shows the selection of the ROI as an endplate profile identified by  $\alpha$ -BuTX labeling and an example of where the ROI was set to calculate the background value (BKGD) for each stack of the color channel.

interest (ROI) for the measurements was set by projecting through the z-axis planes the most intense pixels of the  $\alpha$ -BuTX signal. Endplate and subsynaptic IP<sub>3</sub>R1 volumes were calculated in the same ROI in the corresponding color channels (Fig. 1). To each stack of images, a threshold was applied to eliminate the fluorescent noise, and the background signal was subtracted. The background signal of each color channel was calculated in the ROI set as close as possible to the endplate avoiding inhomogeneous cell regions (BKGD; Fig. 1). The  $\alpha$ -BuTX- and IP<sub>3</sub>R-stained volumes were calculated as the sum of subtracted  $\alpha$ -BuTX- and IP<sub>3</sub>R1-stained images of each z-stack, taking into account the step size of acquisition.

In EDL cryosections, the analysis of nAChR and IP<sub>3</sub>R1 staining was performed using epifluorescence and confocal microscopy. Epifluorescence analysis was done under a Leica DMR microscope equipped with a Leica HCX PL Fluotar 40 $\times$ /0.75 (NA) dry objective and using the Leica Application Suite Advanced Fluorescence 4.0.0.11706 software (LASAF). Confocal analysis was carried out by a Leica SP5 confocal inverted microscope using a Leica HCX PL Fluotar L 40 $\times$ /0.60 (NA) dry objective. Confocal images were collected at 1  $\mu$ m z resolution by sequential line scanning. Due to the complexity of the tissue, the irregular IP<sub>3</sub>R1 signal made the background very variable and quantification of IP<sub>3</sub>R1 staining unreliable, thus the volumetric analysis (as described above for FDB muscle fibers) was limited to the  $\alpha$ -BuTX signal. As for IP<sub>3</sub>R1 quantification, we identified the IP<sub>3</sub>R1-free endplates, i.e., endplates in which the IP<sub>3</sub>R1 fluorescent signal in the ROI was equal to or below the minimum background signal value measured in the corresponding fiber.

#### Caged IP<sub>3</sub> photolysis and Ca<sup>2+</sup> measurements

FDB muscle fibers plated on 18-mm coverslips were incubated with 1  $\mu$ M Fluo-4/AM (Life Technologies), 0.02% pluronic F-127 (Sigma-Aldrich), 200  $\mu$ M sulfinpyrazone (Sigma-Aldrich), 1  $\mu$ M ci-IP<sub>3</sub>/PM (Tocris Bioscience), and 2  $\mu$ g/ml  $\alpha$ -BuTX Alexa Fluor 555 conjugate (B35451; Thermo Fisher Scientific Life Sciences) for 30 min at 37°C in mKRB medium (in mM: 140 NaCl, 2.8 KCl, 2 MgCl<sub>2</sub>, 10 HEPES, and 1 CaCl<sub>2</sub>, pH 7.4) supplemented with

10 mM glucose. After washing, coverslips were mounted in Ca<sup>2+</sup>-free, EGTA-containing (100  $\mu$ M) mKRB and visualized on an inverted microscope (Leica Microsystems DMI8 Thunder Imager 3D Cell Culture) by a 20 $\times$  ultraviolet-permeable objective (HC PL FLUOTAR 340; Leica Microsystems). Fluo-4 was excited with the white line of a CoolLED pE-340<sup>fura</sup> (CoolLED Limited), and the emitted fluorescence was collected with a Leica Dmi8 GFP-filter set. Where indicated, photolysis of ci-IP<sub>3</sub>/PM was obtained by exposing the sample (300 ms) to the output of the unfiltered 340 and 380 nm lines of a CoolLED pE-340<sup>fura</sup>. Fluo-4 images were acquired every 50 ms, with a 40-ms exposure time by a Hamamatsu Flash 4.0 V3 camera (Hamamatsu Photonics). After 100 s, 20  $\mu$ M cyclopiazonic acid (CPA; Sigma-Aldrich) was added to release Ca<sup>2+</sup> from the sarcoplasmic reticulum.

At the end of the experiments,  $\alpha$ -BuTX Alexa Fluor 555 was excited by the 555 nm line of LED8 (Leica Microsystems) to visualize the NMJ and to select the corresponding ROIs. Images were background subtracted and analyzed with LAS X Premium (Leica Microsystems), calculating the ratio  $F/F_0$  between the Fluo-4 fluorescence emission collected at each frame ( $F$ ) and that collected at the beginning of the experiment ( $F_0$ ) in the ROIs.

#### Statistical analysis

Results are expressed as mean  $\pm$  standard error (SEM). Data were analyzed with GraphPad Prism 4.00 (GraphPad Software) and Origin 2019b (OriginLab Corporation). Shapiro normality test was used to determine whether sample data were drawn from a Gaussian distributed population. For parametric data, statistical significance was determined using an unpaired  $t$  test; for data that do not belong to a normal distribution, statistical analysis was performed using Mann-Whitney test. Relationships between volumetric analysis of the endplate and corresponding subsynaptic IP<sub>3</sub>R1 stained volume were analyzed by Spearman's correlation test (for nonparametric data).  $P < 0.05$  was considered statistically significant.

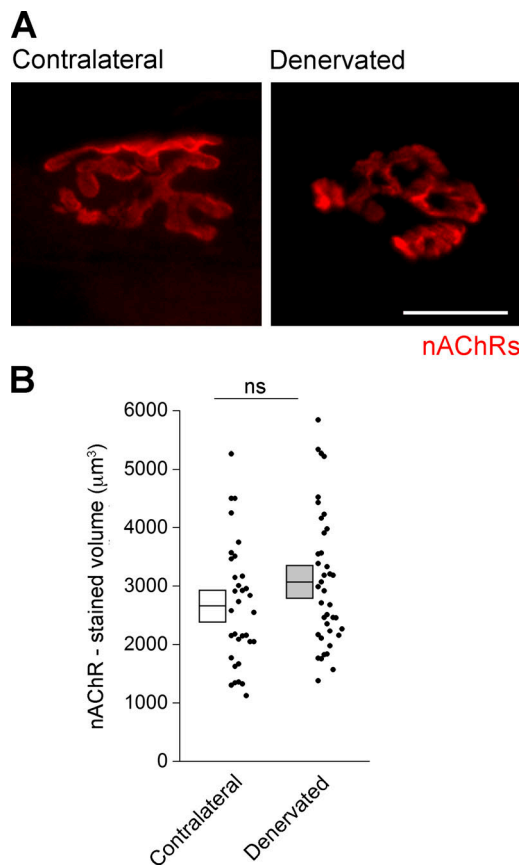
## Results

#### Effect of in vivo denervation on the subsynaptic IP<sub>3</sub>R1 distribution in EDL muscle

To investigate whether denervation affects the subsynaptic IP<sub>3</sub>R1 distribution, in vivo denervation was carried out in adult rats using the classical procedure of sciectomy. Sciectomy evokes a rapid ensuing and irreversible muscle atrophy, as judged by a marked decrease of CSA from denervation day 3 onward (Bortoloso et al., 2006; Qiu et al., 2021, and references therein).

In longitudinal muscle sections, identification of endplate was carried out by nAChR labeling with  $\alpha$ -BuTX, as shown in Fig. 1 A for both contralateral and denervated EDL; as judged by pretzel-like features (Fig. 2 A) and by measurements of mean nAChR-stained volumes, endplates appeared not to be changed at denervation day 9 (contralateral:  $2,664.32 \pm 181.42 \mu\text{m}^3$ ,  $n = 33$ ; denervated:  $3,074.21 \pm 183.55 \mu\text{m}^3$ ,  $n = 39$ ; Fig. 2 B), a finding in line with previous observations (Fumagalli et al., 1990; Gupta et al., 2020; Walter et al., 2021). Immune labeling of





**Figure 2. No change in endplate and  $\alpha$ -BuTX-stained volume was observed in 9-d denervated rat EDL longitudinal sections. (A)** Representative images for contralateral and denervated endplates. Scale bar, 20  $\mu$ m. **(B)** nAChR-stained volumes measured in confocal images from contralateral and denervated EDL are shown as a scatter plot (contralateral: 33 endplates; denervated: 39 endplates). White and grey boxes represent the mean volume  $\pm$  SEM. ns,  $P = 0.06$ , Mann-Whitney test: data are not significantly different.

contralateral EDL with anti-IP<sub>3</sub>R1 antibodies shows a double localization pattern, i.e., at the Z-line and in the subsynaptic area (Fig. 3 A), as previously reported by Salanova et al. (2002) and Powell et al. (2003). Endplates, nuclei, and IP<sub>3</sub>R1s were clearly detectable (Fig. 3 B). Confocal scanning of EDL samples shows that at variance with contralateral EDL ( $n = 33$  endplates), denervation caused a change in the subsynaptic IP<sub>3</sub>R1-immunolabeling. Tissue complexity and variable background signal discouraged any attempt at a reliable quantification, but in about 23% of the analyzed fibers ( $n = 39$  endplates) the subsynaptic IP<sub>3</sub>R1-immunolabeling was indistinguishable from the nearby regions or lower (representative image in Fig. 3 C; see Materials and methods for further details), and such endplates were deemed IP<sub>3</sub>R-free.

Data obtained in vivo, although preliminary and lacking quantification of the IP<sub>3</sub>R1-staining volume, are suggestive of a causal relationship between innervation and expression/localization of IP<sub>3</sub>R1s at the endplates. To substantiate this hypothesis and to explore which are the mechanisms controlling the expression and localization of subsynaptic IP<sub>3</sub>R1s, a well-established, in vitro denervation model was investigated, i.e., isolated FDB muscle fibers.

### Innervated and in vitro denervated FDB adult skeletal muscle fibers: nAChR- and IP<sub>3</sub>R1-stained volumes

The endplate nAChR- and the corresponding subsynaptic IP<sub>3</sub>R1-stained volumes were analyzed in single FDB adult skeletal muscle fibers by confocal fluorescence microscopy after 24 h and 7 d in culture as representative of innervated and in vitro denervated muscle cell elements. In fact, FDB skeletal muscle fiber in culture is a well-established protocol to reproduce in vitro the denervation effects at the endplate level: a decreased density of nAChRs and the appearance of the fetal  $\gamma$ -nAChRs isoform (Grohovaz et al., 1993).

24 h after plating, confocal scanning microscopy displayed nAChRs confined to the endplate region (Fig. 4, upper panel). Analysis of the fluorescent nAChR- and IP<sub>3</sub>R1-stainings revealed a mean nAChR-stained volume of  $1,100.03 \pm 128.51 \mu\text{m}^3$  and a mean IP<sub>3</sub>R1-stained volume of  $473.92 \pm 70.80 \mu\text{m}^3$  ( $n = 6$  mice, 44 fibers; Fig. 4 B). The correlation plot of matched endplate nAChR- and subsynaptic IP<sub>3</sub>R1-stained volumes indicated a direct proportionality: the larger the endplate, the larger the subsynaptic IP<sub>3</sub>R-stained volume (Fig. 4 C, upper graph). On average, the subsynaptic IP<sub>3</sub>R1-stained volume corresponded to  $\sim 40\%$  of the endplate region.

In in vitro denervated fibers, endplates displayed severe alterations, i.e., evident fragmentation (Fig. 4 A, lower panel). Mean fluorescent nAChR- and IP<sub>3</sub>R1-stainings were reduced respectively to  $425.16 \pm 50.61$  and  $132.77 \pm 25.27 \mu\text{m}^3$  ( $n = 6$  mice, 31 fibers; Fig. 4 B). The correlation between endplate nAChR- and corresponding subsynaptic IP<sub>3</sub>R1-stained volumes was maintained (Fig. 4 C, lower graph). However, the extent of IP<sub>3</sub>R1-stained volume reduction was larger than that for nAChRs, and the subsynaptic IP<sub>3</sub>R1-stained volume corresponded to  $\sim 25\%$  of the endplate region after 7 d of in vitro denervation.

### Subsynaptic IP<sub>3</sub>-mediated Ca<sup>2+</sup> release in innervated and in vitro denervated FDB skeletal muscle fibers

To investigate whether the reduction in the subsynaptic IP<sub>3</sub>R1-stained volume induced by in vitro denervation determined a change in the local IP<sub>3</sub>-mediated Ca<sup>2+</sup> signals, flash photolysis experiments of caged IP<sub>3</sub> were performed in Fluo-4 loaded fibers. Uncaging of IP<sub>3</sub> in in vitro denervated FDB adult skeletal muscle fibers caused a subsynaptic increase in the  $[\text{Ca}^{2+}]_i$ , significantly lower than in the innervated counterparts (Fig. 5 A). The mean peak values of the subsynaptic  $[\text{Ca}^{2+}]_i$  variations, measured as  $F/F_0$  (further details in Materials and methods), were  $1.07 \pm 0.01$  in innervated ( $n = 44$  endplates) and  $1.01 \pm 0.01$  in in vitro denervated fibers ( $n = 29$  endplates), corresponding to a decrease in the peak value (calculated as percentage versus basal  $[\text{Ca}^{2+}]_i$ ) of  $\sim 80\%$  in in vitro denervated versus innervated fibers; Fig. 5 A). In our experimental conditions, the IP<sub>3</sub> photolysis entails a 1-s interruption in the acquisition (i.e., 300 ms flash and the time required to restart postflash image acquisition), thus we cannot exclude concomitant differences in Ca<sup>2+</sup> release kinetics undetectable by our imaging unit.

Control experiments confirmed that UV flash pulse did not alter, per se, the levels of fluorescence (Fig. 5 A, inset).

In the analyzed Fluo-4 loaded fibers, application of 20  $\mu\text{M}$  CPA, an inhibitor of sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase

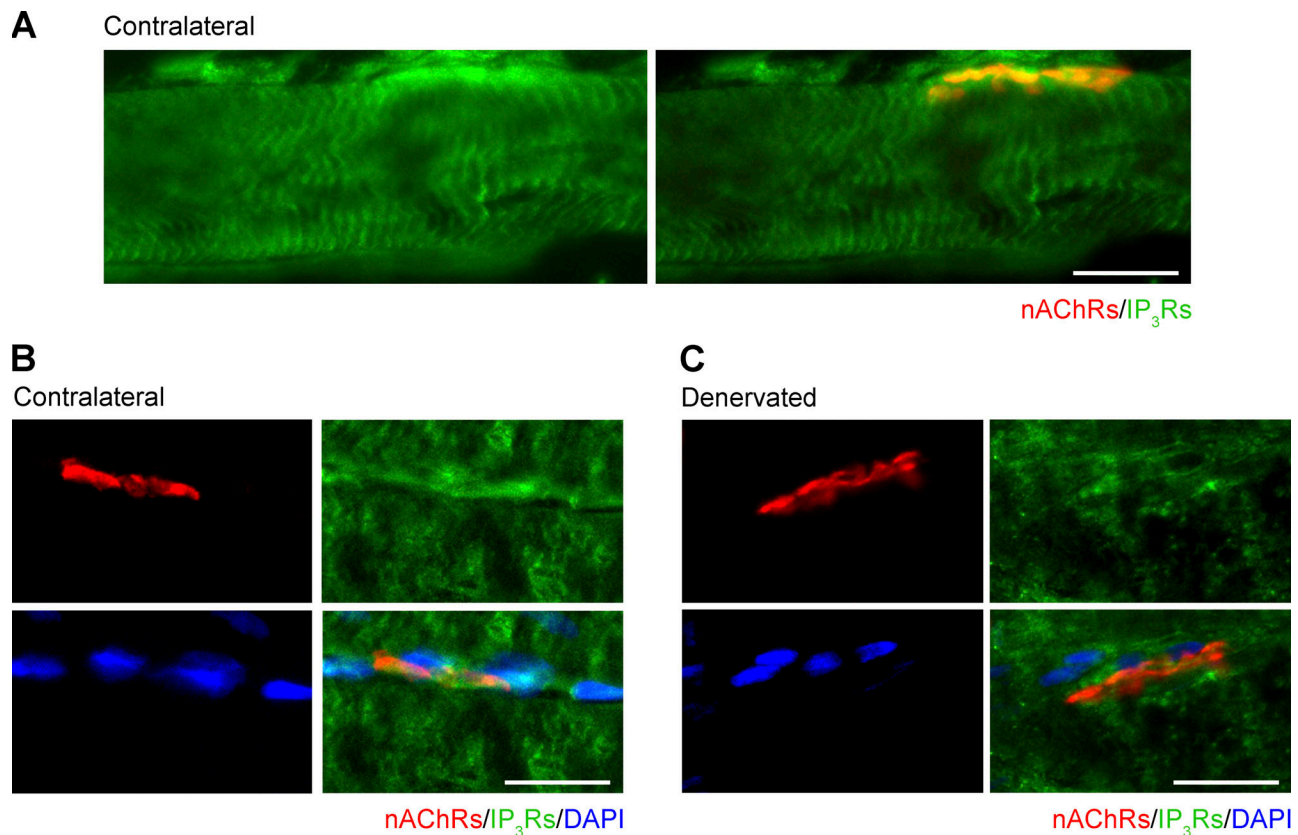


Figure 3. **Absence of subsynaptic IP<sub>3</sub>R1s at endplate level in 9-d denervated rat EDL longitudinal sections.** (A) In the left panel, contralateral EDL labeled with anti-IP<sub>3</sub>R1 antibodies; in the right panel, merged image after double labeling for nAChRs and anti-IP<sub>3</sub>R1s. Scale bar, 20  $\mu$ m. (B) Individual labeling of contralateral EDL with  $\alpha$ -BuTX, anti-IP<sub>3</sub>R1 antibodies, and DAPI. Scale bar, 20  $\mu$ m. (C) Individual labeling of 9-d denervated EDL with  $\alpha$ -BuTX, anti-IP<sub>3</sub>R antibodies, and DAPI. Scale bar, 20  $\mu$ m.

(Seidler et al., 1989), induced significantly different  $[Ca^{2+}]_i$  variations in innervated and in vitro denervated fibers (Fig. 5 B). The corresponding mean area values were  $101.00 \pm 12.25$  ( $n = 44$  endplates) and  $65.54 \pm 10.77$  ( $n = 29$  endplates), respectively, indicating a decrease of  $\sim 35\%$  of the releasable  $Ca^{2+}$  content in in vitro denervated versus innervated fibers (Fig. 5 B).

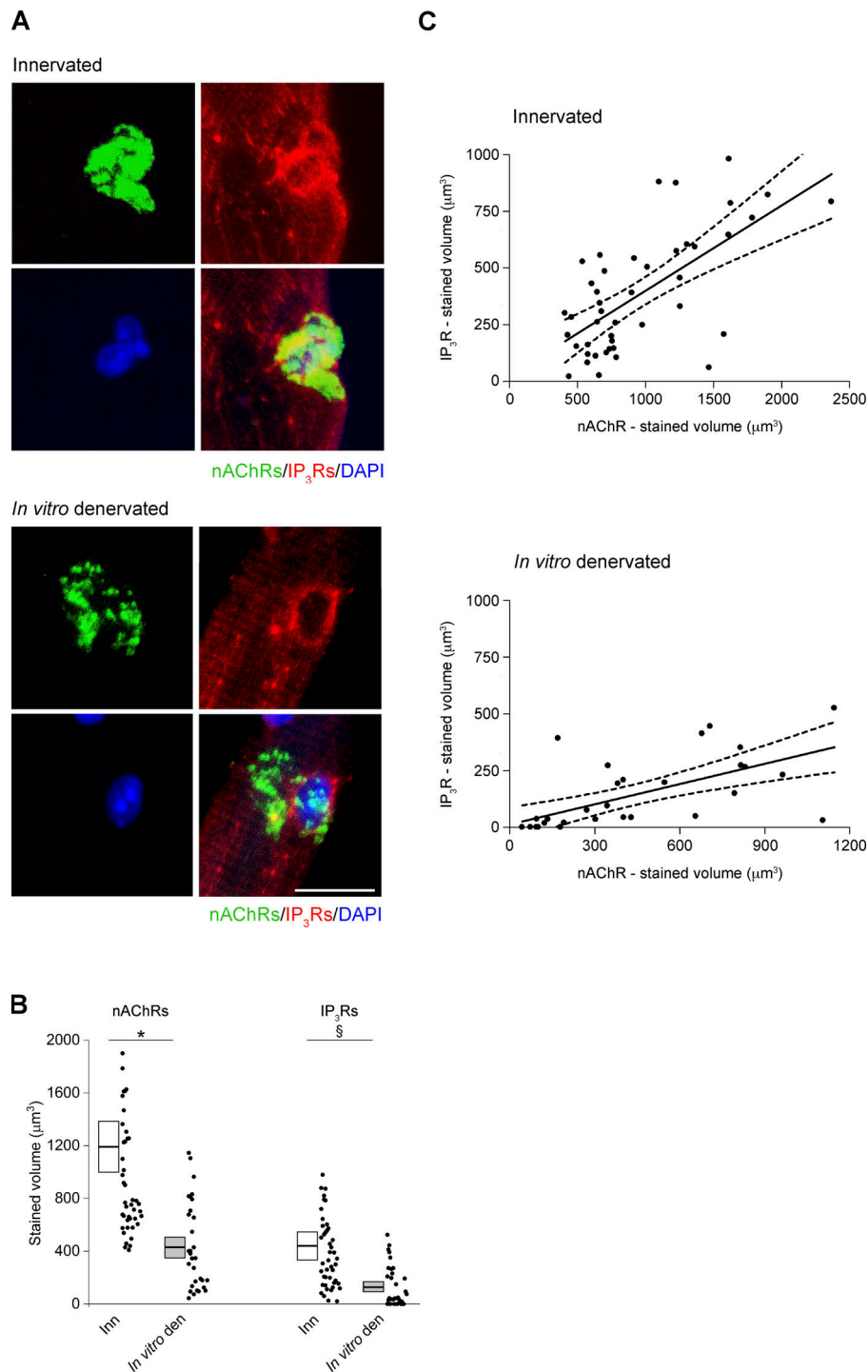
#### In vitro denervated FDB adult skeletal muscle fibers: Effect of neural agrin and field electrical stimulation on nAChR- and IP<sub>3</sub>R1-stained volumes

In innervated muscle, the maintenance of the endplate apparatus is controlled by neural agrin and electrical muscle activity (Lomo, 2003; Wu et al., 2010; Shi et al., 2012; Li et al., 2018). Some experiments were planned to investigate if neural agrin and/or muscle electrical activity control the subsynaptic distribution of IP<sub>3</sub>R1s. To pursue this aim, we have taken advantage of isolated cultured FDB skeletal muscle fibers, which offer the possibility to work in a controlled environment (i.e., in the absence of neurotrophic factors and electrical activity) and to check the specific contribution of neural agrin and electrical activity applied experimentally in the stabilization of the subsynaptic IP<sub>3</sub>R1s.

The effect of neural agrin was explored by comparing nAChR- and IP<sub>3</sub>R1-stained volumes in FDB adult skeletal muscle fibers cultured up to 7 d in control conditions and in the presence of 1 nM 90 kD recombinant C-terminal fragment of rat

neural agrin, which contains the binding site for Lrp4/MuSK receptor complex and induces nAChR clustering (Ferns et al., 1993). As expected, in the presence of neural agrin, the mean endplate nAChR-stained volume was larger ( $799.93 \pm 124.69 \mu m^3$ ; 5 mice, 42 fibers) than in control conditions ( $404.52 \pm 56.59 \mu m^3$ ; 5 mice, 28 fibers; Fig. 6). However, even if the treatment with the neurotrophic factor caused an increase in the mean IP<sub>3</sub>R-stained volume in in vitro denervated fibers ( $191.32 \pm 39.31 \mu m^3$ ; 5 mice, 42 fibers), it was statistically comparable ( $P = 0.18$ ) to the mean volume measured in the control counterparts ( $120.73 \pm 27.21 \mu m^3$ ; 5 mice, 28 fibers; Fig. 6).

Similar experiments were carried out to investigate the effect of the muscle electrical stimulation. Field electrical stimulation was delivered daily to the FDB adult skeletal muscle fibers in culture at a frequency of 1 Hz for 5 h. Endplate nAChRs and subsynaptic IP<sub>3</sub>R1s volumes were measured in electrical stimulated cultured FDB skeletal muscle fibers and compared to those measured in control conditions, i.e., without electrical stimulation. 4 d of electrical stimulation determined stabilization of the endplate nAChRs: in stimulated fibers, the mean nAChR-stained volume was  $441.59 \pm 38.81 \mu m^3$  (6 mice, 34 fibers) versus the mean volume of  $278.30 \pm 39.42 \mu m^3$  (6 mice, 39 fibers) in control cultures. Field electrical stimulation stabilized also the subsynaptic IP<sub>3</sub>R1s localization: the mean IP<sub>3</sub>R1-stained volume was  $138.20 \pm 21.67 \mu m^3$  in stimulated fibers (34 fibers, 6 mice) and



**Figure 4. nAChR- and IP<sub>3</sub>R1-stained volumes in FDB adult skeletal muscle fibers during in vitro denervation.** **(A)** Top: Endplate region in a FDB adult skeletal muscle fiber as a representative image of the innervated condition (24 h after plating). nAChR staining with Alexa Fluor 488- $\alpha$ -BuTX revealed that nAChRs were localized at the endplate level, and immunostaining for IP<sub>3</sub>R1s detected a subsynaptic localization of the receptor. Bottom: *In vitro* denervated FDB adult skeletal muscle fiber (7 d in culture) as an example of fragmented endplate and reduced subsynaptic IP<sub>3</sub>R1s staining. Nuclei were counterstained with DAPI. Images are shown as maximum intensity projection (for details, see Materials and methods). Scale bar, 20  $\mu\text{m}$ . **(B)** The nAChR- and corresponding IP<sub>3</sub>R1-stained volumes from each of the analyzed fibers are represented as a scatter plot ( $n = 44$  fibers). White and grey boxes represent the mean volume  $\pm$  SEM calculated under the assumption that the number of analyzed animals (six mice) is an independent variable. \*,  $P = 0.0006$ , unpaired  $t$  test; §,  $P < 0.0001$ , unpaired  $t$  test. **(C)** Top: Correlation plot of endplate nAChR- versus corresponding subsynaptic IP<sub>3</sub>R1-stained volumes from the innervated FDB adult skeletal muscle fibers shown in B.  $R = 0.57$ ;  $P < 0.0001$ , Spearman's rank correlation. Bottom: Correlation plot of endplate nAChR- versus corresponding subsynaptic IP<sub>3</sub>R-stained volumes from *in vitro* denervated FDB adult skeletal muscle fibers shown in B.  $R = 0.72$ ;  $P < 0.0001$ , Spearman's rank correlation.

$56.70 \pm 9.83 \mu\text{m}^3$  in control counterparts (6 mice, 39 fibers; Fig. 7 A). Although the electrical stimulation was effective on both volumes, the plot of matched endplate nAChR- versus subsynaptic IP<sub>3</sub>R1-stained volumes indicated that the two parameters were not correlated (Fig. 7 B).

## Discussion

Growing experimental evidence suggests that the motor neuron stimulation pattern encodes IP<sub>3</sub>-dependent  $\text{Ca}^{2+}$  signals

regulating muscle transcriptional activity and plasticity via the so-called ET-coupling mechanism. Direct electrical stimulation of skeletal muscle fibers reveals the existence of an IP<sub>3</sub>-dependent signaling cascade, which originates from activation of the voltage-gated  $\text{Ca}^{2+}$  channel (Cav1.1) and proceeds via ATP release through pannexin-1 channel and activation of purinergic receptors (Casas et al., 2022). In parallel, the localization of IP<sub>3</sub>R at the NMJ and the functional interplay between nAChRs activity and the subsynaptic IP<sub>3</sub>R-induced  $\text{Ca}^{2+}$  release indicate that the nerve-mediated electrical stimulation encodes also for localized

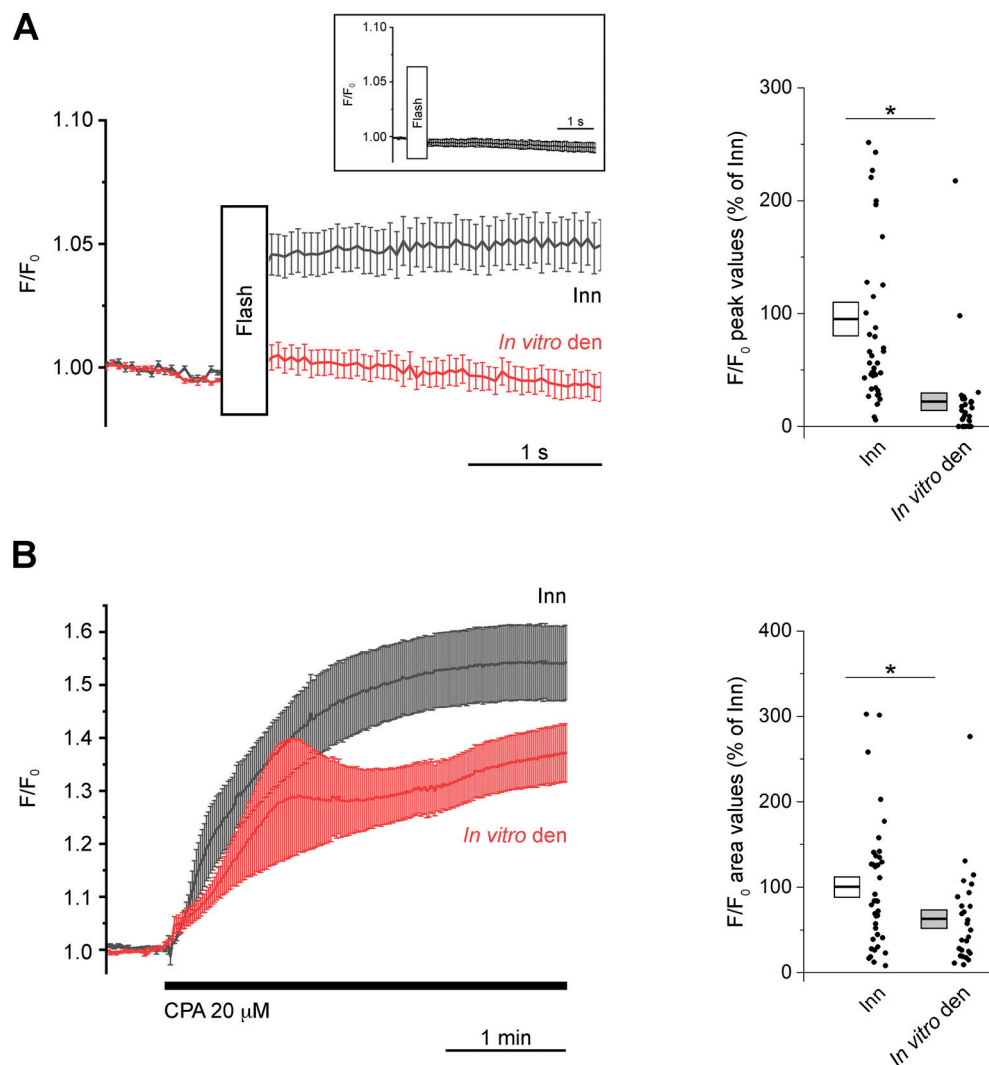


Figure 5. **Smaller subsynaptic IP<sub>3</sub>-releasable [Ca<sup>2+</sup>]<sub>i</sub> pool in in vitro denervated FDB adult skeletal muscle fibers.** (A) On the left, mean traces of subsynaptic [Ca<sup>2+</sup>]<sub>i</sub> increases measured in innervated (24 h after plating;  $n = 44$  endplates) and in vitro denervated FDB adult skeletal muscle fibers (7 d in culture;  $n = 29$  endplates). Trace interruption marks the time of flash photolysis of caged IP<sub>3</sub> (Flash). Inset: Mean trace of fluorescence measured after the UV flash in the absence of caged IP<sub>3</sub>. On the right, the scatter plot of the normalized peak values of the [Ca<sup>2+</sup>]<sub>i</sub> increases measured in in vitro denervated versus innervated fibers after flash photolysis. White and grey boxes represent the mean peak values  $\pm$  SEM. \*,  $P < 0.0001$ , Mann-Whitney test. (B) On the left, mean traces of subsynaptic [Ca<sup>2+</sup>]<sub>i</sub> increases after 20  $\mu$ M CPA addition upon basal [Ca<sup>2+</sup>]<sub>i</sub> recovery from photolysis. On the right, scatter plot of the normalized area values of the [Ca<sup>2+</sup>]<sub>i</sub> changes induced by CPA application (3.5 min) in in vitro denervated versus innervated fibers. White and grey boxes represent the mean area values  $\pm$  SEM. \*,  $P = 0.0037$ , Mann-Whitney test.

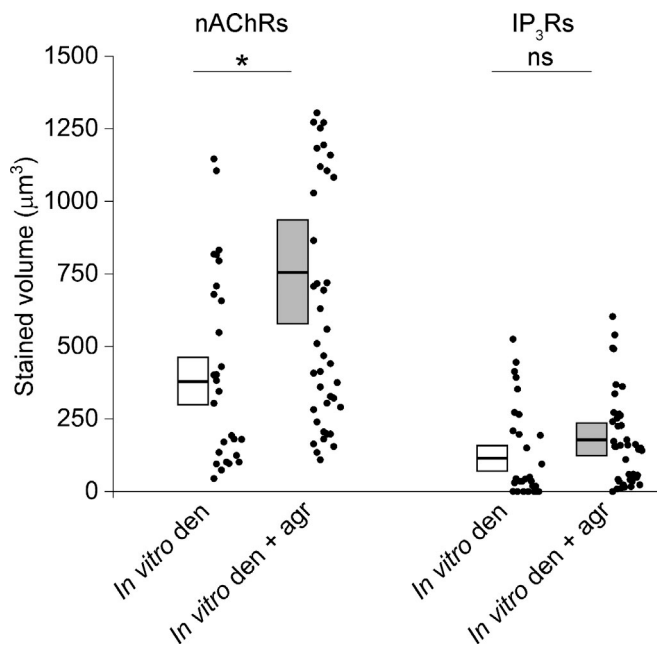
Ca<sup>2+</sup> signals (Zayas et al., 2007; Zhu et al., 2011), which could regulate a “synaptic” ET-coupling mechanism governing the stability of the NMJ apparatus (Zayas et al., 2007; Zhu et al., 2011). If IP<sub>3</sub>Rs are crucial in a synaptic ET-coupling, a reduction in their content/activity would impact directly on the efficient neuromuscular transmission and muscle plasticity.

As inferred from experiments carried out on in vivo denervated rat skeletal muscles obtained by sciactomy, it appears that innervation, i.e., an anatomical intact nerve-muscle junction, controls the distribution of the subsynaptic IP<sub>3</sub>Rs at the endplate. In denervated EDL muscle, although quantification of the IP<sub>3</sub>R1 staining volume is lacking, a significant number of IP<sub>3</sub>R1-free endplates was observed (~23%). In this respect, preliminary experiments carried out on human muscles after

60 d of bed rest, in which a marked disuse atrophy is provoked in the presence of an intact and active nerve-muscle junction (Blottner et al., 2006), indicate that subsynaptic IP<sub>3</sub>Rs are not changed (unpublished results). Thus, innervation appears to control distribution of IP<sub>3</sub>R1s at the NMJ, and its role seems to be prominent also in humans. However, since there are well-known differences in the mechanism of muscle atrophy models, in particular denervation versus disuse atrophy (MacDonald et al., 2014), a further experimental investigation is needed to unveil specific genetic programs and metabolic pathways involved.

The role of innervation on subsynaptic IP<sub>3</sub>R1 distribution was directly investigated in murine adult, isolated FDB muscle fibers. Quantification of denervation effects and investigation of the

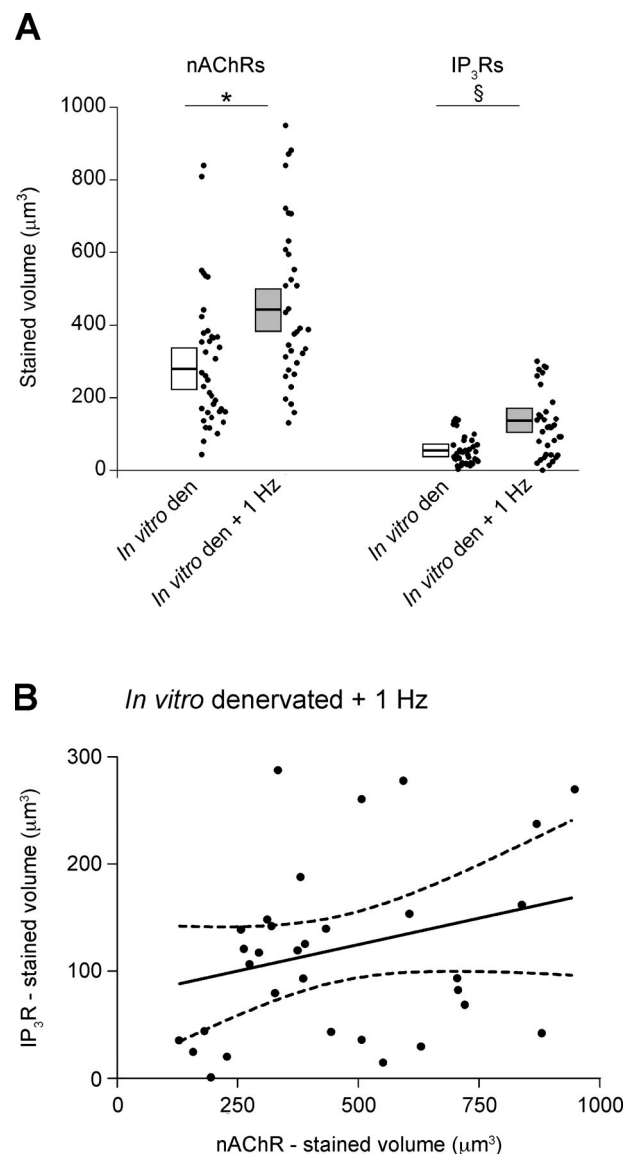




**Figure 6. Neural agrin stabilized endplate but did not prevent the decrease in the subsynaptic IP<sub>3</sub>R1s staining in FDB adult skeletal muscle fibers during in vitro denervation.** When FDB adult skeletal muscle fibers were maintained in culture in the presence of 1 nM rat neural agrin, the endplate nAChR-stained volume was significantly larger than that of in vitro denervated FDB adult skeletal muscle fibers in control conditions. However, the subsynaptic IP<sub>3</sub>R1-stained volume is similar to control. Data from each analyzed fiber are shown as a scatter plot (in vitro den: 28 fibers; in vitro den + agr: 42 fibers). White and grey boxes represent the mean volume  $\pm$  SEM calculated under the assumption that the number of analyzed animals (five mice) is an independent variable. \*,  $P = 0.02$ , unpaired  $t$  test. ns,  $P = 0.18$ , unpaired  $t$  test: data are not significantly different.

mechanisms governing the subsynaptic distribution of IP<sub>3</sub>R1s were pursued by comparing subsynaptic IP<sub>3</sub>R1 in freshly isolated (innervated) and in cultured FDB fibers (in vitro denervated) maintained in different conditions.

The volumetric analysis of nAChR and IP<sub>3</sub>R1 stainings in freshly dissociated FDB skeletal muscle fibers reveals a correlation between the endplate size and the subsynaptic IP<sub>3</sub>R1 volume, which supports the role of subsynaptic IP<sub>3</sub>-sensitive Ca<sup>2+</sup> stores as a key amplifier of Ca<sup>2+</sup> influx triggered by nAChRs activation (Zayas et al., 2007; Zhu et al., 2011). The same quantitative analysis performed on skeletal muscle fibers, upon in vitro denervation, revealed a decrease of the nAChRs-stained volume associated with a reduced IP<sub>3</sub>R1 volume. The apparent discrepancy between the substantially conserved volume of the endplate in vivo (nAChR-stained volumes: Den,  $\sim 2,600 \mu\text{m}^3$ ; Inn,  $\sim 3,074 \mu\text{m}^3$ ) and the fragmentation and reduced endplate volume in vitro (nAChR-stained volumes: in vitro Den,  $\sim 470 \mu\text{m}^3$ ; Inn,  $\sim 1,100 \mu\text{m}^3$ ) could be due to either the short denervation time in vivo (Fumagalli et al., 1990; Gupta et al., 2020; Walter et al., 2021) and/or the severe conditions of denervation in vitro, i.e., absence of the basal lamina (Glavinovic et al., 1987), absence of neurotrophic factors, and production of nerve-breakdown by-products, which might not occur in vivo (Cangiano, 1985).



**Figure 7. Field electrical stimulation stabilized endplate and the subsynaptic IP<sub>3</sub>R1s staining in FDB adult skeletal muscle fibers during in vitro denervation.** (A) After 4 d of electrical stimulation, both endplate nAChRs and subsynaptic IP<sub>3</sub>R1 volumes resulted in significantly larger volumes than those of in vitro denervated FDB adult skeletal muscle fibers in control conditions. Data from each analyzed fiber are shown as a scatter plot (in vitro den: 39 fibers; in vitro den + 1 Hz: 34 fibers). White and grey boxes represent the mean  $\pm$  SEM volumes calculated under the assumption that the number of analyzed animals (six mice) is an independent variable. \*,  $P = 0.01$ , unpaired  $t$  test; §,  $P = 0.006$ , unpaired  $t$  test. (B) The plot of endplate nAChR- versus corresponding subsynaptic IP<sub>3</sub>R1-stained volumes measured in electrically stimulated fibers, shown in A, indicated that the two parameters were not correlated in electrical stimulated FDB adult skeletal muscle fibers.  $R = 0.26$ ;  $P = 0.14$ , Spearman's rank correlation.

As far as in vitro denervated muscle fibers are concerned, apart from the reduced subsynaptic IP<sub>3</sub>R1 volume, the volumetric analysis shows a different correlation slope between IP<sub>3</sub>R1- and nAChR-stained volumes, i.e., a more severe reduction for that referable to IP<sub>3</sub>R1s. This in vitro finding agrees fairly well with the number of IP<sub>3</sub>R1-free NMJs detected after in vivo

denervation before any detectable alteration at the endplate level. To the best of our knowledge, our findings indicate for the first time that a nerve-dependent regulation of the IP<sub>3</sub>R1 distribution at the endplate level and that IP<sub>3</sub>R1-enriched membrane compartments are even more sensitive to the motor nerve absence than nAChRs membrane domains.

Flash photolysis experiments with caged IP<sub>3</sub> indicate that the reduction in the subsynaptic IP<sub>3</sub>R1 volume in *in vitro* denervated skeletal muscle fibers leads to local IP<sub>3</sub>-mediated Ca<sup>2+</sup> release reduced by ~80% compared to the innervated counterparts. Interestingly, the reduced subsynaptic IP<sub>3</sub>-mediated Ca<sup>2+</sup> release seems to exclude any compensation by other IP<sub>3</sub>R isoforms after denervation. Moreover, the slighter decrease of the [Ca<sup>2+</sup>]<sub>i</sub> transient areas observed after CPA addition (~35%) suggests that denervation might affect the mechanisms of IP<sub>3</sub>-dependent release more than the capacity of the subsynaptic Ca<sup>2+</sup> storage. In line with the intriguing hypothesis suggested by Powell et al. (2003), the precocious reduction of subsynaptic IP<sub>3</sub>R1s and the consequent, plausible Ca<sup>2+</sup> signaling alterations might bring about the inevitable NMJ destabilization. Whether or not denervation induces redistribution of IP<sub>3</sub>R1s, downregulates expression, and/or alters the metabolic stability of subsynaptic IP<sub>3</sub>R1s remains an open question. Denervation upregulates the lysosome, calpain, and ubiquitin-proteasome systems (Schiaffino et al., 2013), all major mechanisms of skeletal muscle catabolism; proteomic studies indicate that ~20% of total proteins were differentially expressed significantly within 2 wk after denervation compared with control muscles (Lang et al., 2017): it remains to be ascertained whether IP<sub>3</sub>R1 reduction is merely and only a quantitative, secondary feature of denervation-induced catabolism or whether the IP<sub>3</sub>R1 gene is specifically repressed upon denervation, i.e., it is one of the specific atrogenes downregulated during early events of muscle atrophy. Thus far, published data do not include IP<sub>3</sub>R among the growing list of atrogenes (cfr. Lang et al., 2017; Lecker et al., 2004; Raffaello et al., 2006; Sacke et al., 2007).

*In vitro* denervation of isolated FDB skeletal muscle fibers was implemented in searching for the causative role of either neural agrin and/or electrical activity in the subsynaptic distribution of IP<sub>3</sub>R1s.

Exogenous neural agrin added to the cell culture medium partially stabilized the endplate during *in vitro* denervation, whereas its effect on the denervation-induced reduction of the IP<sub>3</sub>R1-stained subsynaptic volume did not reach statistical significance. The statistical power of <0.8 revealed that our sample size was not numerous enough to rule out a less strong agrin-mediated mechanism controlling the subsynaptic localization of IP<sub>3</sub>R1s.

On the other hand, fiber activity induced by field electrical stimulation not only promoted the stability of the endplate during both *in vitro* denervation and *in vivo* denervation (Brenner and Rudin, 1989; Akaaboune et al., 1999) but also displayed an effect on the subsynaptic IP<sub>3</sub>R1s. In fact, electrical stimulation counteracted, although partially, the denervation-induced reduction of the IP<sub>3</sub>R1-stained volume, thus suggesting a prevalent activity-dependent control mechanism on subsynaptic IP<sub>3</sub>R1s.

In our experimental conditions, each electrical pulse delivered to the fibers at 1 Hz caused a [Ca<sup>2+</sup>]<sub>i</sub> transient associated with a single twitch (Bosutti et al., 2019). It is known that the stabilizing effect of electrical activity on nAChRs is mediated by an increase in [Ca<sup>2+</sup>]<sub>i</sub> (Martinez-Pena y Valenzuela and Akaaboune, 2021), whereas the mechanisms regulating the subsynaptic IP<sub>3</sub>R localization are still unknown. The larger subsynaptic IP<sub>3</sub>R1 volume, measured in electrically stimulated denervated FDB fibers, indicates the importance of fiber activity in the control of the subsynaptic IP<sub>3</sub>R1 membrane compartment as well. However, the lack of correlation between the IP<sub>3</sub>R1- and nAChR-stained volumes suggests that the direct electrical stimulation was efficacious but not sufficient to coordinate growth and interplay between the two receptor compartments. Whether the physiological electrical activity triggered by the nerve ending causing nAChR activation and localized synaptic Ca<sup>2+</sup> signals (Powell et al., 2003; Zayas et al., 2007; Zhu et al., 2011) is responsible for subsynaptic IP<sub>3</sub>R1s stabilization merits future investigations.

As judged by electron microscopy, upon denervation, secondary synaptic fold density falls (Brown et al., 1982), and primary grooves reveal a flattening or, more often, collapse (Labovitz et al., 1984). Since such morphological changes appear to be reversed by electrical stimulation (Brenner and Rudin, 1989), it is tempting to speculate that IP<sub>3</sub>R1-membrane compartments are somehow coupled to both secondary synaptic folds and primary grooves. As to the heterotypic organelle juxtaposition, i.e., the interaction between nAChR- and IP<sub>3</sub>R1-membrane compartments, identification of putative contact sites awaits further experimental investigation by way of specific sensor site probes (Cieri et al., 2018) in presence and absence of innervation. Moreover, considering the recent experimental evidence in favor of a possible role of Homer 2 scaffold protein in the endplate remodeling and plasticity (Lorenzon et al., 2021), Homer 2 could be one of the molecular players linking nAChRs to IP<sub>3</sub>R1s.

In conclusion, this study provides evidence that the subsynaptic IP<sub>3</sub>R1 distribution at the endplate level is controlled by innervation through a prevailing mechanism based on electrical activity. This opens a new scenario in which IP<sub>3</sub>R1 could be included in the growing list of atrogenes. Whether the IP<sub>3</sub>R1 downregulation causes an altered synaptic ET-coupling mechanism contributing to impaired neuromuscular transmission and muscle atrophy remains to be assessed.

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The authors declare no competing financial interests.

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Furlan, P. Caccin, R. Sacchetto, D. Sandonà, G. Trautmann, M. Salanova, A. Bosutti, G. Massaria, A. Bernareggi, and M. Sciancalepore carried out experiments and statistical analyses. P. Pizzo discussed experiments and revised the manuscript. All the authors revised and approved the manuscript.

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