Stretching prior to resistance training promotes adaptations on the postsynaptic region in different myofiber types

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ABSTRACT

The morphology of the neuromuscular junction adapts according to changes in its pattern of use, especially at the postsynaptic region according to the myofibrillar type and physical exercise. This investigation revealed the morphological adaptations of the postsynaptic region after static stretching, resistance training, and their association in adult male Wistar rats. We processed the soleus and plantaris muscles for histochemical (muscle fibers) and postsynaptic region imaging techniques. We observed muscle hypertrophy in both groups submitted to resistance training, even though the cross-section area is larger when there is no previous static stretching. The soleus postsynaptic region revealed higher compactness and fragmentation index in the combined exercise. The resistance training promoted higher adaptations in the postsynaptic area of plantaris; moreover, the previous static stretching decreased this area. In conclusion, the neuromuscular system's components responded according to the myofiber type even though it is the same physical exercise. Besides, static stretching (isolated or combined) plays a crucial role in neuromuscular adaptations.

Key words: Neuromuscular junction; motor endplate; muscle hypertrophy; static stretching; resistance training.

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Introduction

Exercise training promotes increased neuromuscular activity and demonstrates the plasticity of the neuromuscular junction (NMJ), resulting in improved endurance and resistance training in different ages.[1,2] Moreover, the NMJ presents potential in postsynaptic component adaptations. The increase in acetylcholine receptors (AChR) and endplate compactness, ensuring efficient communication between the pre and postsynaptic region and prolonged muscle fatigue.[3-5]

Resistance training (RT) can promote chronic muscular adaptations in humans, and it is advocated as a fundamental exercise modality to improve muscle size and function development.[6,7] These adaptations are predominantly due to increasing the muscle cross-section area (CSA) through the differentiation and proliferation of satellite cells by the release of inflammatory cytokines and growth factors.[8] The manipulation of their variables (series, repetitions, frequency, overload, and rest interval) is directly proportional to the adaptive biological responses progress.[9] Static stretching has been widely practiced among individuals of all physical activity levels, commonly prescribed during the warm-up of strength training.[10,11] Furthermore, static stretching does not require much time or effort, has a low risk of injury in young adults, and has shown beneficial postural stability results.[12,13] Evidence suggests that stretching performed before resistance training can directly and negatively influence strength production, number of repetitions, total volume, and muscle hypertrophy.[14-16]

It is essential to understand the possible effects in the postsynaptic region of the NMJ caused by static stretching and resistance training, especially in your combination. Furthermore, the composition of a muscle fiber type can be determined according to intrinsic and extrinsic responses such as genetics, hormones, aging, and type of exercise.[17,18] The soleus muscle is located deep into the gastrocnemius muscle, and it is responsible for plantar flexion acting in jumping, walking, knee flexion, and like a peripheral heart in the return of venous blood.[19,20] The plantaris is the vestigial remains of a muscle that originally continues the plantar aponeurosis as seen in some quadrupedal primates to aid in the prehension like the palmaris longus muscle.[21] The plantaris muscle is also fixed in the superficial posterior compartment of the lower leg as an accessory soleus muscle.[22] Although these muscles seem to work simultaneously, they present a different composition of fiber types. Soleus presents high content of Type I muscle fibers; besides, plantaris presents Type IIx (or IIb) muscle fiber predominance.[23] Therefore, we investigated the postsynaptic region plasticity of soleus and plantaris muscle in 8 weeks of static stretching, resistance training, and their protocol combination in adult male Wistar rats.

Materials and Methods

Animals

Thirty-two 60-day-old male Wistar rats were divided into 4 groups (n=8): No-Training (NT); Resistance Training (RT); Stretching Training (ST); and Stretching-Resistance Training (SRT). During the experimental period, the animals were kept in cages (33 x 40 x 16 cm) (n=4), under the conditions of temperature monitoring (23± 2°C) and 12 h light/dark period, with food and water *ad libitum*. The Ethics Committee on the Use of Animals (CEUA) of the Biosciences Institute of the São Paulo State University (UNESP) approved this study (nº 09/2019).

Stretching training protocol

The ST and SRT groups performed an 8-week (24 sessions) stretching protocol (3 times/week), which consisted of using the non-quantified manual force applied to the plantar portion of the right posterior limb with the movement of dorsiflexion up to the range of motion limit of the talocrural joint. With the manual assistance of a specific investigator, the Wistar rats performed 10 movements of 30 s of static stretching, followed by a 30-s interval resting in the neutral joint position.[24] The same investigator executed all sessions. SRT group performed the stretching and immediately followed by resistance training in a vertical ladder.

Resistance training protocol

The Wistar rats of RT and SRT groups performed an 8-week (24 sessions) resistance training protocol (3 times/week) in a vertical ladder (110 x 18 cm, 2 cm grid, 80° incline). The sessions consisted of 4 to 9 progressive load climbs. They were allowed to rest for 120 s at the top of the ladder after each climb.[25] The animals performed the first four climbs with 50%, 75%, 90%, and 100% of their body mass additional loads fixed to the tail's proximal region. In the subsequent climbs, 30 g of the extra progressive load was added until the 9th climb or exhaustion occurred.[26] The Wistar rats performed the training protocols at the same time of the day across the experimental period.

Histochemistry and morphometric analysis

The soleus and plantaris muscle belly samples (n=5 in each) of experimental groups were dissected and cryofixed, then transverse sections were made (10 µm) (Cryostat HM 505 E, MICROM™, CA, USA) on microscope slides (n=75). The histochemical reaction was used to differentiate the fiber types (type I and II in soleus / type I, IIa, and IIx in plantaris muscle).[27]

Twenty-five slides were incubated for 30 min at 37°C in a solution containing adenosine triphosphate (ATP - 10 mg) dissolved in three drops of distilled water, glycine/$NaCl_2$/$CaCl_2$ buffer (10 mL), and 46.5 mg of dithiothreitol for 30 min until reaching pH 9.4. For pH 4.3 and 4.6, the slides (n = 25 in each pH) were pre-incubated in 0.1 M sodium acetate buffer solution and 10 mM ethylenediaminetetraacetic acid (EDTA) solution for 10 min at 4°C. After, the slides were incubated in a solution containing ATP (10 mg), glycine/$NaCl_2$/$CaCl_2$ buffer (10 mL), and 46.5 mg of dithiothreitol for 30 min. The final stage for all slides was incubation in 2% cobalt chloride for 7 min, dehydration in a series of alcohol concentrations (70%, 90%, 95% and 100%), then the slides were finished in xylol.[28]

The cross-section area (CSA) of type I and II of soleus myofibers and the CSA of type I, IIa, and IIx of plantaris myofibers (n = 100/fiber type/group) were observed using a 20x objective lens with 10X ocular magnification. The images were obtained by a Zeiss™ Axioskop (Jena, Germany) light microscope.[29] After, the morphometry was performed in each pH by the ImageJ™ software (National Institutes of Health, Bethesda, MD, USA). Subsequently, we performed the normality test of the data and analyzed it using Kruskal-Wallis with Dunn's *post-hoc* test ($p<0.05$).

Postsynaptic imaging - Tissue preparation

The samples of soleus and plantaris muscle (n=3 in each) of experimental groups were dissected and cryofixed in liquid nitrogen (-196°C), then longitudinal sections (100 µm thickness) were made (Cryostat HM 505 E, MICROM™, CA, USA) to visualize the postsynaptic component of each NMJ. The sections were collected in silanized slides, pre-treated with 0.1% Triton-X solution, and washed (3×5 min) in phosphate-buffered saline (PBS). The sections were incubated overnight at 4°C in a solution containing α-bungarotoxin conjugated with rhodamine (BTX; Molecular

Probes, Eugene, OR, USA; T-1175), diluted 1:600 in PBS containing 1% bovine serum albumin (BSA); washed in PBS before being coated with Prolong (Molecular Probes; P10144), coverslips applied and stored at −20°C until analysis.[30]

Postsynaptic region morphometric analysis

The images (n=20) for morphometric analysis were captured using a 100x objective lens with 10x ocular magnification by an Olympus BX61™ Fully Motorized Fluorescence Microscope (Shinjuku, Japan); equipped with a Fluorescence UIS2 optical system by Texas Red filter (Texas red excitation 596 nm/emission 620 nm), obtained by a monochromatic camera Orca-Flash 2.8 (Hamamatsu, Japan) with the Software CellSens v.11 (Olympus™). With the ImageJ™ Software (National Institutes of Health, Bethesda, Maryland, USA), it was measured the endplate area (μm²): the entire region composed of stained and unstained receptors; AChR area (μm²): the area composed of AChR; endplate perimeter (μm): the whole length covering the endplate composed of stained and unstained receptor; AChR perimeter (μm): the size covering around the AChR region; endplate diameter (μm): the maximum diameter formed by stained and unstained receptors; and the AChR clusters number (un) in the motor endplate (Figure 1).

The endplate compactness (%) was measured for an accurate descriptor of receptors number within a given area,[31] in other studies, we can find this index as a "dispersion":[32]

$$\text{Compactness} = (\text{AChR area})/(\text{endplate area})\times 100$$

The endplate fragmentation index was measured, whereby an index of 0 means a solid plaque-like endplate, and an index close to 1 means a highly fragmented endplate:[31]

$$\text{Fragmentation Index} = 1-(1/(\text{number of AChR clusters})$$

We performed the Shapiro-Wilk's normality test (α=0.05) to verify the data's normal distribution, which presents a non-normal distribution; therefore, we analyzed it using Kruskal-Wallis with Dunn's *post-hoc* test ($p<0.05$).

Results

We reveal the alterations in the muscle fiber and postsynaptic region from soleus and plantaris of NT, ST, RT, and SRT groups.

Cross-section area (CSA)

In the histochemical analysis, the type I and II CSA from the soleus muscle and the type I, IIa, and IIx cross-section areas from plantaris of NT, ST, RT, and SRT groups were obtained (Table 1).

Soleus: the CSA of types I ($p<0.05$) and II fibers ($p<0.0001$) of the ST group were smaller compared to NT. The CSA of Type I fibers ($p<0.0001$) of RT was larger compared to NT. Additionally, the CSA of Type II fibers ($p<0.01$) of RT was smaller than that found in NT. The CSA of types I ($p<0.05$) and II ($p<0.001$) fibers of SRT were larger than the NT group. The CSA of types I and II ($p<0.0001$) fibers of SRT were larger than ST. Besides, the CSA of type I ($p<0.0001$) fibers of SRT were smaller, and the Type II ($p<0.0001$) fibers were larger than that found in the RT group.

Plantaris: the CSA of types I, IIa ($p<0.0001$), and IIx ($p<0.001$) fibers of ST were both larger compared to NT. The CSA of types I ($p<0.0001$), IIa ($p<0.0001$), and IIx ($p<0.0001$) fibers of RT were both larger compared to NT. Furthermore, the CSA of Type I ($p<0.0001$) and IIa ($p<0.0001$) fibers of SRT were larger, and the type IIx fibers were smaller than those found in NT. In the SRT Group, the CSA of types I ($p<0.0001$) and IIa ($p<0.01$) fibers were larger, and type IIx ($p<0.0001$) fibers were smaller compared to ST. Additionally, the CSA of types IIa ($p<0.001$) and IIx (p 0.0001) fibers of SRT were both smaller, and the type I ($p<0.0001$) fibers were larger than that found in RT.

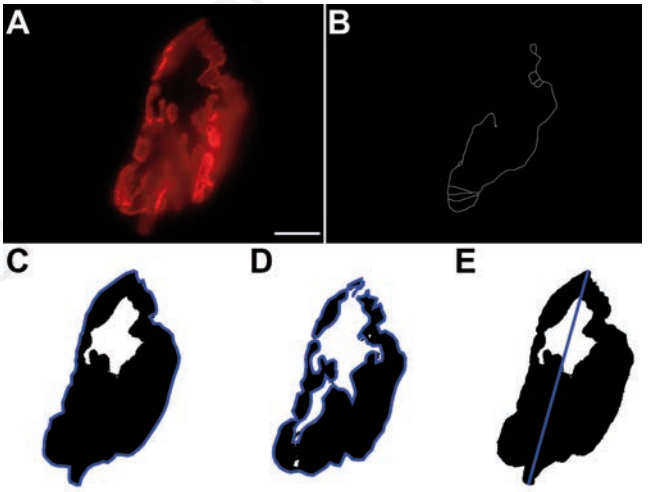

Figure 1. Postsynaptic region morphometry. (A) Original image of the postsynaptic region; scale bars: 10 μm. B) The example of AChR clusters measured. C) Binary image and the endplate perimeter and area measured. D) Binary image and the AChR perimeter and area measured. E) Binary image and the endplate diameter measured.

Table 1. Mean ± standard deviation of cross-section area (CSA - μm²) of myofibers type I and II in the soleus muscle and I, IIa, IIx in the plantaris muscle of the No-Training (NT), Stretching Training (ST), Resistance Training (RT),and Stretching-Resistance Training (SRT) groups.

Muscle	CSA	Groups			
		NT	ST	RT	SRT
Soleus	Type I	3584.0±773.3	3504.5±887.1	4326.5±903.1†,‡	3847.3±793.6
	Type II	3297.9±747.2	2759.2±588.4†	2892.4±354.4§	3819.6±523.9‡
Plantaris	Type I	1132.1±187.2	1247.3±356.6	1769.9±466.1‡	1801.2±339.5‡
	Type IIa	1207.3±219.8	1594.0±331.1‡	1918.9±419.1‡	1791.7±334.6‡,¶
	Type IIx	2763.7±832.0	3266.4±843.1†	3718.5±910.2‡	2741.2±524.1†,‡

Soleus: type I: †NT≠RT ($p<0.001$), ‡ST≠RT ($p<0.0001$), and ‡SRT≠RT ($p<0.0001$); type II: †NT≠ST ($p<0.001$), §NT≠RT ($p<0.05$), ‡NT≠SRT ($p<0.0001$), and ‡RT≠SRT ($p<0.0001$). Plantaris: type I: ‡NT≠RT ($p<0.0001$), ‡NT≠SRT ($p<0.0001$), and ‡ST≠SRT ($p<0.0001$). Type IIa: ‡NT≠ST ($p<0.0001$), ‡NT≠RT ($p<0.0001$), ‡NT≠SRT ($p<0.0001$), and ¶ST≠SRT ($p<0.01$); type IIx: ‡NT≠RT ($p<0.0001$), †NT≠ST ($p<0.001$), ‡RT≠SRT ($p<0.0001$), and †ST≠SRT ($p<0.001$).

Postsynaptic region

The area and perimeter of the endplate and AChR of soleus present smaller values in the ST group than NT (Figure 2). Moreover, in the ST group, the diameter, compactness, and number of AChR clusters of the endplate were smaller than that found in the NT group. The area and perimeter of the endplate and AChR of soleus were both smaller in the RT group compared to NT. Furthermore, in the RT group, the diameter, and compactness of the endplate were smaller; and the number of AChR clusters was higher than that found in the NT. The area and perimeter of the endplate and AChR of soleus were both smaller in the SRT group compared to NT. Besides, the endplate diameter and the number of AChR clusters of SRT were both smaller compared to NT. In SRT, the compactness endplate showed larger than NT. The area and perimeter of the endplate of SRT of soleus were smaller compared to ST. However, the AChR area and perimeter, and endplate compactness were larger in SRT than that found in the ST group. Furthermore, the endplate diameter and the number of AChR clusters of SRT showed minor values compared to ST.

The area of endplate and AChR of SRT was smaller than RT. The endplate perimeter and diameter of SRT were both smaller than that found in the RT. Besides, the AChR perimeter, the endplate compactness, and the number of AChR clusters of SRT were larger than RT. There was no statistical difference in the results of the postsynaptic region of the soleus (Figure 2). The AChR area and perimeter, and the area and perimeter of the plantaris muscle endplate were larger ($p<0.01$) in the ST group than in the NT. The endplate diameter, compactness, and the number of AChR clusters of ST were larger than those found in the NT. The AChR area and perimeter, and the area and perimeter of the endplate were larger ($p<0.05$) in RT than in the NT. Furthermore, the endplate diameter, compactness, and the number of AChR clusters ($p<0.01$) of RT were both larger than NT. The endplate area of SRT of plantaris was smaller compared to NT. Additionally, the endplate perimeter and the AChR perimeter and area of SRT were larger than the NT; the endplate compactness ($p<0.01$) was even larger. The endplate diameter and the number of AChR clusters of SRT were smaller compared to NT. The area ($p<0.01$) and perimeter ($p<0.05$) of the endplate, and the AChR area and perimeter ($p<0.01$) of plantaris were smaller in SRT than that found in the ST. Besides, the AChR area, the endplate compactness ($p<0.05$), and the number of AChR clusters ($p<0.01$) of SRT were larger than ST. The endplate diam-

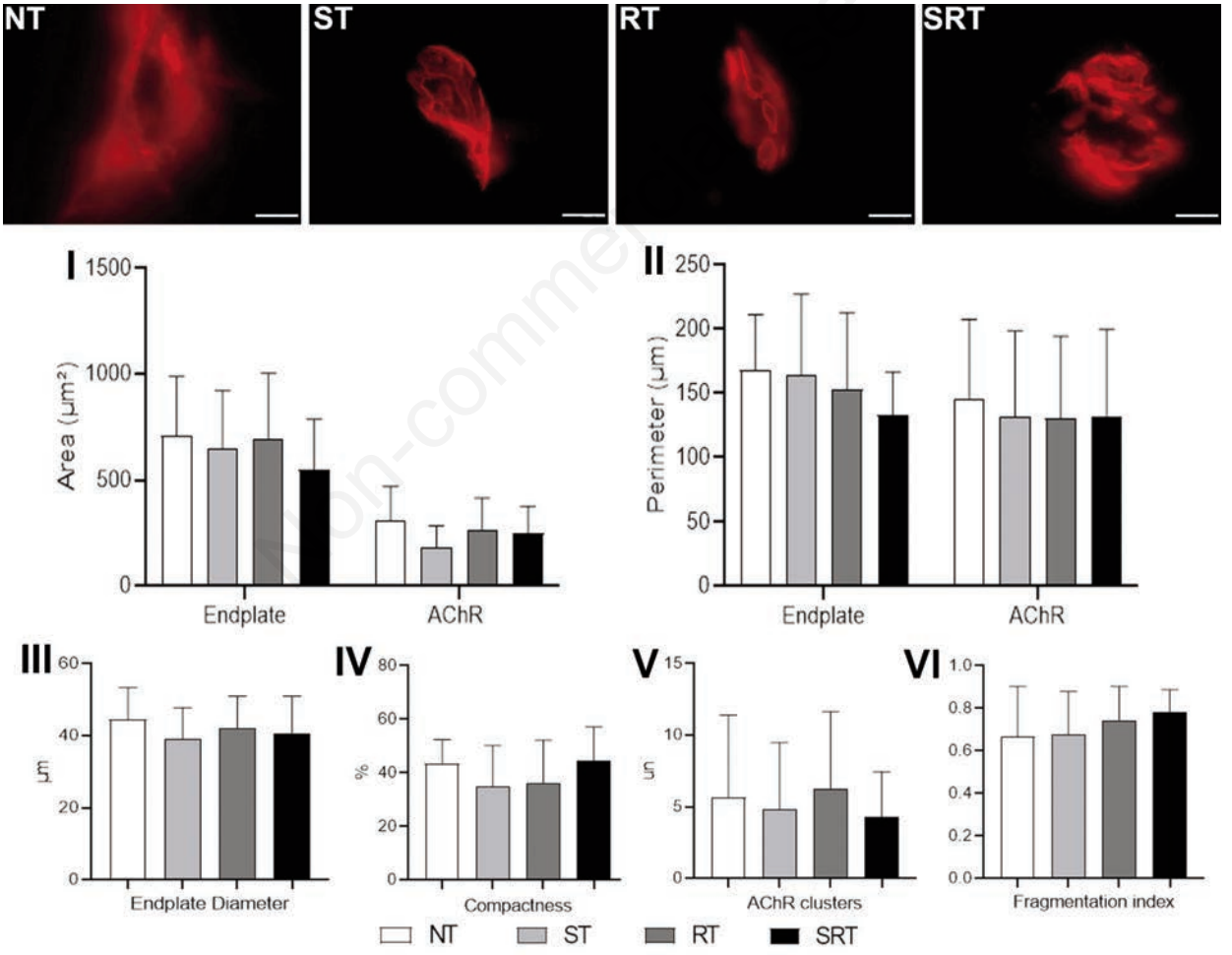

Figure 2. Postsynaptic AChR region of the soleus muscle of No-Training (NT), Stretching Training (ST), Resistance Training (RT), and Stretching-Resistance Training (SRT) groups; scale bars: 10 µm. I) Mean ± standard deviation values of the endplate and AChR areas (µm²). II) Mean ± standard deviation values of the endplate and AChR perimeters (µm). III) Mean ± standard deviation values of endplate diameter (µm). IV) Mean ± standard deviation values of endplate compactness (%). V) Mean ± standard deviation values of the number of AChR clusters (un). VI) Mean ± standard deviation values of the fragmentation index.

eter was smaller in SRT than in ST. In SRT, the AChR ($p<0.01$) area and perimeter, and the area and perimeter of the endplate ($p<0.0001$) were smaller compared to RT. Moreover, the endplate diameter of SRT was smaller than that found in RT ($p<0.05$). The endplate compactness and the number of AChR clusters in SRT were both larger than RT (Figure 3).

Discussion

These results suggest an increase in NMJ activity, specifically in the postsynaptic region after resistance training.[33] Our recent finding revealed the effects of static stretching, resistance training, and their association with the myofibers CSA and postsynaptic region development. These findings are distinct between the muscles regarding the morphofunctional characteristics.

Regular resistance training is the main intervention to promote muscular adaptations, such as increased muscle mass, CSA, myofibrillar protein synthesis, and sarcomeres length.[7,26,27,34] In the RT group, we observed hypertrophy of muscle fibers through the higher CSA of type I of soleus and type IIx myofibers of plantaris muscle. These adaptations in the muscle fibers architecture are directly associated with muscle strength production, where fibers with higher CSA values can develop higher muscle strength production due to the new sarcomeres' formation.[35,36] Also, Allen *et al.*[37] suggested that muscle hypertrophy is correlated with the addition of new myonuclei through satellite cells' activity. A recent study by Rocha *et al.*[29] showed soleus muscle hypertrophy in both fiber types after 24 sessions of resistance training in the vertical ladder which corroborates our results.

Regarding the static stretching response in the CSA of the muscle fibers, Peviani *et al.*[38] demonstrated no difference in the soleus of experimental groups after 10 and 15 days of static stretching. In humans, Simpson *et al.*[39] observed an increase in the lower limb's muscular thickness after 6 weeks of overloaded stretch training. These adaptations seem to occur according to stretching protocol intensity.

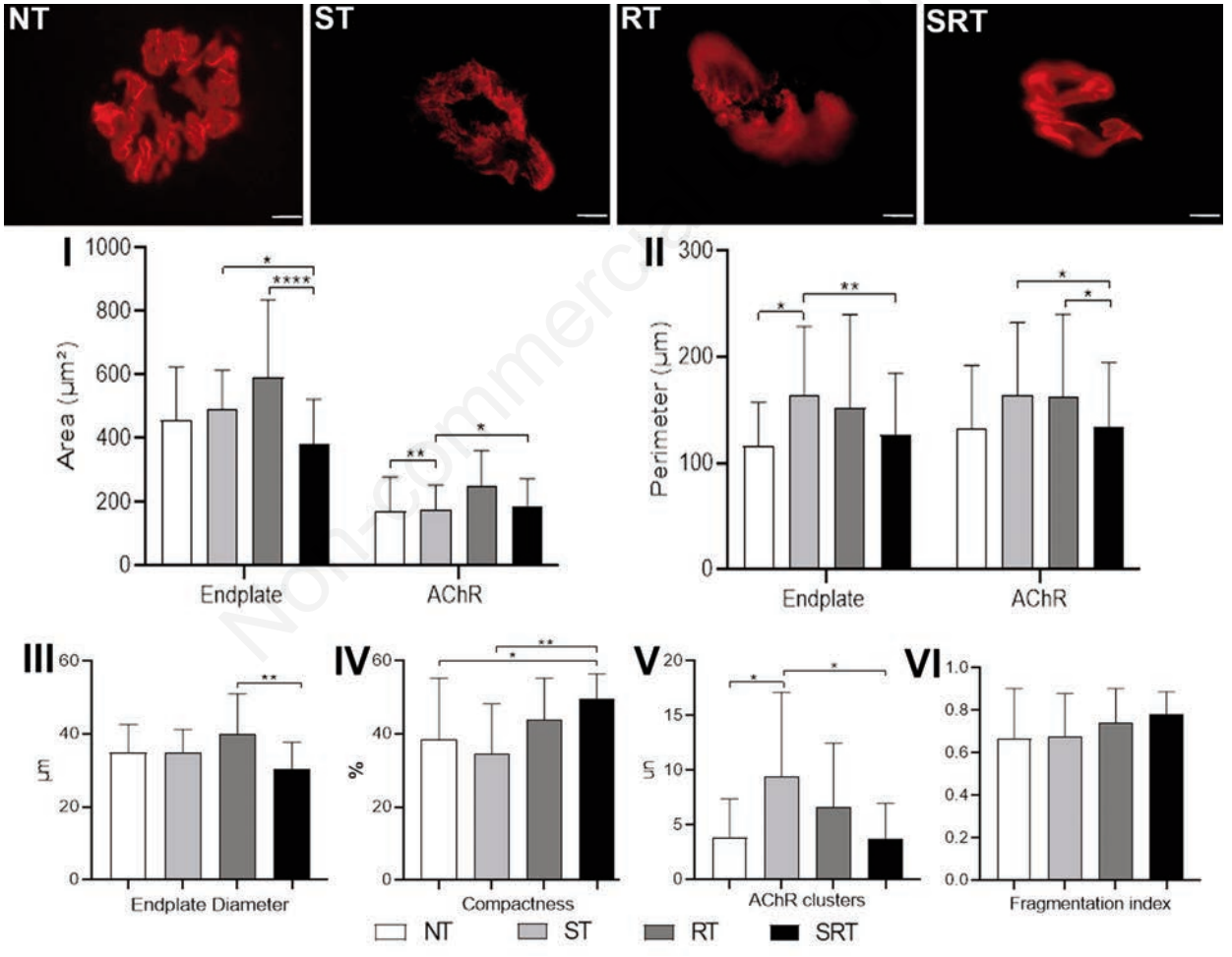

Figure 3. Postsynaptic AChR region of the plantaris muscle of No-Training (NT), Stretching Training (ST), Resistance Training (RT), and Stretching-Resistance Training (SRT) groups; scale bars: 10 µm. I) Mean ± standard deviation values of the endplate and AChR areas (µm²); *ST ≠ SRT ($p<0.05$); ****RT ≠ SRT ($p<0.0001$); **NT ≠ ST ($p<0.01$); *ST ≠ SRT ($p<0.05$). II) Mean ± standard deviation values of the endplate and AChR perimeters (µm); *NT ≠ ST ($p<0.05$); **ST ≠ SRT ($p<0.01$); *ST ≠S RT ($p<0.05$); *RT ≠ SRT ($p<0.05$). III) Mean ± standard deviation values of endplate diameter (µm); **RT ≠ SRT ($p<0.01$). IV) Mean ± standard deviation values of endplate compactness (%); *NT ≠ SRT ($p<0.05$); *ST ≠S RT ($p<0.05$). V) Mean ± standard deviation values of the number of AChR clusters (un); *NT ≠ ST ($p<0.05$); *ST ≠ SRT ($p<0.05$). VI) Mean ± standard deviation values of the fragmentation index; **NT ≠ ST ($p<0.01$); **ST ≠ SRT ($p<0.01$).

The stretching with overload does have a hypertrophic muscle effect, and changes in muscle size and architecture did not happen in the low-intensity stretch.[40] Barbosa *et al*.[41] indicated that there was no increase in CSA of myofibers in the gastrocnemius muscle after 2 months of static stretching. Our results revealed a smaller CSA of myofiber compared to the NT group in soleus, differently in plantaris that presented muscle hypertrophy. This fact suggests that this static stretching protocol is enough and beneficial to adapt only in plantaris muscle fibers.

Similar to resistance training, manipulating variables in static stretching training seems to play a key role in neuromuscular adaptations. In soleus type I fibers, when static stretching is combined with resistance training, we can observe a non-significant increase in CSA. In contrast, the highest CSA value was considered in type II fibers after previous static stretching to resistance training. This indicates the hypertrophic response induced by stretching before resistance exercise seems to affect fast fibers, presumably due to the stretching of these fibers caused by static stretching and the tension generated through the load additional in resistance training.

The current literature suggests that low-intensity muscle stretching does not seem to respond significantly to muscle hypertrophy; however, high-intensity stretching can cause possible adaptations that are still limited.[42] The practical implication of not performing the previous stretching is a shorter duration of the training sessions, improving adherence to exercise.[43,44] In this study, changes in the postsynaptic region regarding static stretching and resistance training provided new data of plasticity in the peripheral nervous systems. We observed a reduction in the AChR and endplate perimeters, AChR and endplate areas, and consequently, the soleus postsynaptic region's compactness. Indeed, we observed an enlargement of the plantaris endplate and AChR area in ST and RT Group, interrupted when the stretching was performed before the resistance training (SRT Group). Morphological remodeling of the AChR area may be associated with desensitization during intense and repeated neural stimulation.[33] This area's increase has a functional consequence of decreasing peripheral muscle fatigue during high-intensity muscle contractions.[33] However, this area's reduction does not imply that the motor neuron's innervation in the muscle fibers is restricted considering that the postsynaptic potential usually has an amplitude that significantly exceeds the necessary to obtain a postsynaptic action potential.[45]

Regarding resistance training on a vertical ladder, Deschenes *et al*.[31] presented data on young and elderly male Fischer 344 rats, where there were adaptations only in the postsynaptic components of the NMJ, as is evident in our RT data. Resistance training after static stretching (SRT group) resulted in higher endplate compactness than other experimental groups. The term compactness has been used as a descriptor of quantity receptors in the postsynaptic area;[32] in other studies, we can find this index as a "dispersion".[46] Deschenes *et al*.[47] documented that compactness (or dispersion) is related to the intensity of exercise in which the resistance training increased soleus postsynaptic compactness, and in low intensity running, reduced endplate dispersion. The resistance training after stretching requires high neuromuscular activity, whereas the alterations in the distribution of synaptic receptors within the endplate region are induced by exercise type.[41] We can verify that the training intensity can influence the receptors' alterations in the postsynaptic region.

The endplate diameter showed changes similar to the endplate area, which are minor values in stretching groups. In Boehm *et al*.[48] work, each animal species has a different endplate diameter value. Indeed, Estrada-Bonilla *et al*.[49] observed the NMJ morphometric characteristics of the upper limbs of healthy and diabetes mellitus-induced rats. These data differ from the lower limbs found in this study. There were also minor values in the diameter in conditions of diabetes compared to the control.[49] However, the climbing training with and without an additional load in alternate sessions increases the endplate diameter and even the number of the biceps brachii muscle postsynaptic cleft.[26]

Regarding the fragmentation index, Prakash and Sieck[50] found the effects of aging on NMJs morphological adaptations such as altered fragmentation. Although fragmentation may result from redistribution of the pre-synaptic components over a greater synaptic area, these alterations can also represent an increase in denervation and reinnervation, which may compromise motor innervation and contribute to muscle weakness.[35] As a result, these exercise-induced alterations need to be considered in training protocols or rehabilitation exercises to improve neuromuscular function.[46]

In conclusion, both groups submitted to resistance training showed a larger cross-section area of soleus myofibers, mainly without previous static stretching. The muscle-specific adaptation in the postsynaptic region occurs according to the type of exercise. However, the previous static stretching changes this remodeling by smaller AChR and endplate areas. Furthermore, static stretching contributes to the CSA muscle fiber development and can be proposed in functional and recovery exercise protocols. Despite the resistance training without static stretching is most beneficial for AChR remodeling and muscle hypertrophy, therefore the most recommended.

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