Revisiting the peculiar regional distribution of muscle fiber types in rat Sternomastoid Muscle

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Abstract

The sternomastoid (SM) muscle in rodents is known to have a peculiar distribution of fiber types with a steep gradient from surface to deep region. We here further characterize this peculiar regional distribution by quantitative histochemical morphometrys. In Hematoxylin-Eosin (H-E) stained transverse cryosections harvested in the medial portion of the muscle we counted around 10.000 myofibers with a mean diameter of 51.3 ± 12.6 (µm). Cryisections of the SM stained by SDH reaction clearly show two distinct regions, toward the deep surface of the muscle a 40% area that contains packed SDH-positive myofibers, while the remaining area of the SM toward the external surface presents a more checker-board appearance. On the other hand, in the deep region of SM type 1 (slow contracting) muscle fibers, caracterized by positive acidic ATPase pH 4.35 reaction, are only the 24.5% of the fibers in the deep area of SM muscles, being restricted to the deepest region. The 75.5% of the myofibers in the deep region are of the fast contracting types (either 48.4% 2A, SDH –positive fibers or 27.1% 2B, SDH-negative fibers, respectively). As expected the 2B muscle fibers, acidic ATPase pH 4.3-negative and SDH-negative, present the largest size, while Type 1 fibers, acidic ATPase pH 4.3-positive and SDH-positive, present the smallest size in rat SM muscle. Based on present and previous observations, comparison of change in absolute number and/or percentage of the fiber types in any experimental model of muscle atrophy/hypertrophy/plasticity/pathology /recovery in the rat SM, and possibly of all mammals, will ask for morphometry of the whole muscle cross-sections, muscle sampling by bioptic approches will provide only comparable data on the size of the different types of muscle fibers.

Key Words: rat, Sternomastoid muscle, muscle fiber types, SDH, myofibrillar ATPases, regional distribution

Eur J Transl Myol 28 (1): xxx-xxx, 2018

The sternomastoid muscle (SM) in rodents is known to have a peculiar distribution of fiber types with a steep gradient from surface to deep region.[1] This behavior is also present in leg muscles (e.g., tibialis anterior muscle), but not at extent of the SM muscle.[2-4] We are characterizing in normal animals this peculiar regional distribution by quantitative histochemical analyses of transverse sections in the third medial portion of the muscle. We will present results suggesting that comparison of change in absolute number and/or percentage of the fiber types in the rat SM will ask for morphometry of the whole muscle cross-section, while muscle bioptic samplings will provide only consistent results on size of muscle fibers.
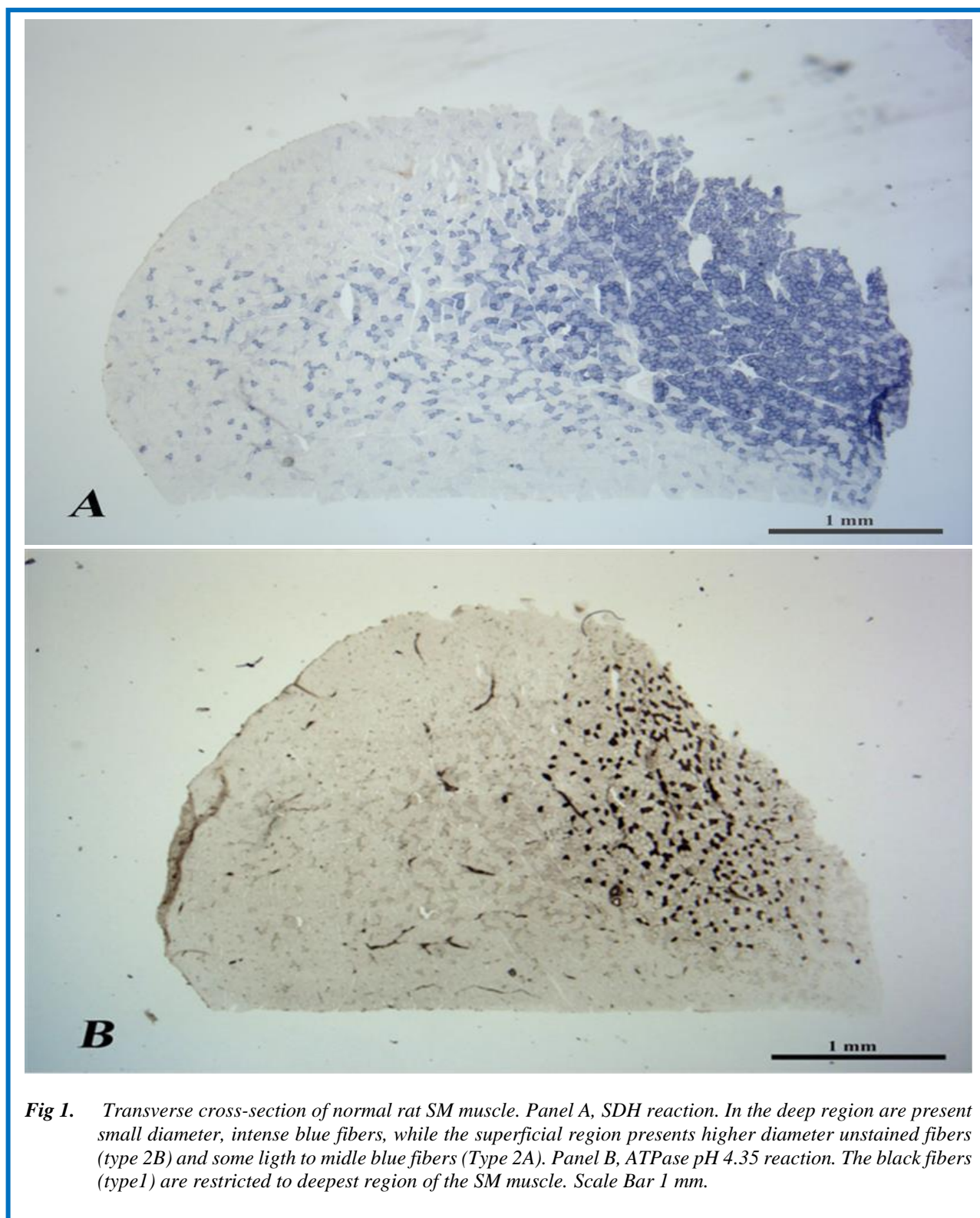
Fig 1. *Transverse cross-section of normal rat SM muscle. Panel A, SDH reaction. In the deep region are present small diameter, intense blue fibers, while the superficial region presents higher diameter unstained fibers (type 2B) and some ligth to midle blue fibers (Type 2A). Panel B, ATPase pH 4.35 reaction. The black fibers (type1) are restricted to deepest region of the SM muscle. Scale Bar 1 mm.*

Materials and Methods

Animals and harvesting of muscles

Adult females Wistar rats, 300 g of weight were used. These animals come from the vivarium of the Department of Neurosciences – University of Padova.

The animals were kept in cages with light/dark cycle of 12h, room temperature (24±2), food and water *ad libitum*. All procedures in this study were according to the international ethical principles of animal experimentation and approved by the Ethics on Animal Experimentation Committee (EAEC) of University of

***Table 1.** Mean myofiber diameter (μm) in the SM rat muscle stained by H-E. Values are mean±SD*

All muscle fibers	SM dx	SM sx
	51.6±26.7	50.9±12.1
Mean of two SM	51.3±12.7	
Total fibers in Deep and Superficial region	9981	

Padova. Animals were killed by deep anaesthesia and muscles were excised, weighted, frozen in liquid nitrogen (-196°C) and kept at -80°C until use.

Histology and histochemistry of the sternomastoid muscle

Size, and absolute or percent contents of different muscle fiber types were determined in transverse cryosections of the SM muscle harvested in the medial third of the muscle. Serial 10μm cross-sections were cut on a cryostat microtome at -25°C, mounted on slides and stained in small glass jars. From stained sections digital images were collected at magnification of x 2.5 and x10 using a transmission light microscope mounting a HDMI camera. Quantitative evaluations were performed on the collected pictures using Scion Image for Windows version Beta 4.0.2 (2000 Scion Corporation). Muscle fiber sizes and regional distribution of the muscle fiber type were determined using the Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA).

Absolute number of muscle fibers in rat SM muscle

To determine the absolute number of muscle fibers in the rat SM muscle, cryosections were stained with standard Hematoxylin-Eosin (H-E) procedure.[5]

Fiber typing of the muscle fibers was performed by the following histochemical methods

Succinate Dehydrogenase (SDH) reaction

SDH reaction was performed to distinguish between oxidative, "less" oxidative and non-oxidative muscle fibers. Muscle sections were incubated in SDH incubation solution for 60 min a 37°C. Sections were rinsed in distilled H2O, 3x1 min and removed unbound NBT from sections with 3x1 min exchanges of acetone solutions 30, 60, 90 % in distilled H2O, in increasing then decreasing concentrations. Sections were rinsed in distilled H2O, 3x1 min and dehydrated in graded alcohol solutions (ethanol 70, 90, 100%) cleared in xylene and mounted in permanent medium: Canada balsam. SDH incubating solution: NBT (nitro blue tetrazolium): 10mg; SDH stock solution: 10ml. Adjust to pH 7.2 to 7.6 with 0.1 N HCl or NaOH. SDH stock solution: 0.2M sodium succinate solution 100ml, 0.2M Phosphate buffer, pH 7.4 100ml, make freshly at least every two weeks.

Histochemistry by Myofibrillar actomyosin ATPase

Two different procedures were used for staining myofibrillar actomyosin ATPase using essentially the same methods described by Brooke and Kaiser,[6,7] and by Guth and Samaha.[8-10] For determination of the alkali preincubated myofibrillar ATPase activity the following procedures were employed. cryosections were air dried for 10 min a room temerature and fixed for 3 min in 5% (w/v) formaldehyde in 200mM sodium cacodylate, 68mM CaCl2 2H2O and 340 mM sucrose (adjusted with HCl to pH 7.6 before adding the formaldehyde).After washing in wash solution 100mM Tris, 18mM CaCl2 (pH 7.8), an alkaline pre-incubation was performed for 15 min a room temperature in a solution containing 200mM 2-amino-3-methyl-1 propanol (adjusted with HCl to pH 10.4). After two successive 1 min incubation in the wash solution, sections were incubated for 45 min a 37°C in ATP solution (2,7mM ATP, 90mM CaCl2, 100mM sodium barbital adjusted with NaOH to pH 9.4). For the acid preincubation myofibrillar ATPase activity (10, 11), the following procedures were utilized. Sections were air dried for 10 min at room temperature and incubated in a solution consisting of 100mM sodium acetate (adjusted with HCl to pH 4.35) for 10 min a room temperature. After washing 2x1 min in 18 mM CaCl2 and 100mM Tris HCl pH 7.8, the sections were incubated a 37°C for 45 min in ATP solution together to serial sections with alkali preincubation. After washing 2x30 sec in distilled H2O, sections were incubated for 5 min in 2% (w/v) CoCl2, washed 2x30 sec in distilled H2O, sections were incubated 5 min in 2% (v/v) (NH4)2S, washed in distilled H2O 2x30 sec, and dehydrated in graded alcohol solution (ethanol 70-90-100%) cleared in xilene, and mounted with Canada balsam medium.

Results

Morphometry: number and size of rat SM muscle fibers in H-E stained cryosections.

In the rat SM muscle cryosections harvested in the medial portion of the muscle we counted around 10.000

myofibers. The mean diameter in these muscle sections stained by H-E was 51.3±12.7 µm (Table 1).
Histochemistry of sternomastoid rat muscle
Fiber typing by SDH reaction
In Fig.1A is presented a transverse section from the medial region of a rat SM muscle stained by SDH reaction. The cryosection clearly shows two distinct regions, a right part of the section that cover around 40% area that contains SDH positive myofibers (right part of Fig.1A) and a the left part of Fig.1A; the latter encopasses the other 60% area of the SM with a more checker-board appearance. The rigth part of the cryosection, peculiarly rich in SDH positive muscle fibers, corresponds to the deep part of the SM, while the left part of the figure correspond to the superficial region of the SM. Table 2 shows that SDH-positive myofibers are the 72.9% of the total myofibers of SM deep region. In the superficial region (left part of the cryosection) based on SDH staining (Fig.1A), the mitochondrial rich, 2A muscle fibers are 31.1%. Since very few or none of type 1 muscle fibers are present there (see ATPase histochemistry reaction). The 68.9% 2B negative fibers are that of the myofibers present in this region of the SM muscle. Figure 2 shows six rappresentative fields at highter magnification taken from periferal, intermediate, and deep portions of cryosections stained either by SDH (A, B, C) or acidic ATPase (D, E, F).
Histochemistry by Myofibrillar actomyosin ATPase
In the deep region (right part of Fig.1B), the type 1 (slow contracting) muscle fibers, identified by positive acidic ATPase pH 4.35 (Fig.1B), are only the 24.5% of the fiber of this part of SM muscle (Table2). On the other hand, the slow type muscle fibers, i.e., those positive at the

acidic ATPase pH 4.35 are restricted in the deepest region of SM muscle. Thus, 75.5% of the myofibers in the deep region are of the fast contracting types (either 48.4% 2A and 27.1% are 2B, respectively).
In the superficial region, left part of the cryosection, based on acidic ATPase pH 4.35, the type 1 positive fibers are absent, the type 2A and muscle fibers characterized by an intermediate intensity decrease to 20.3%. Therefore the vast majority of the muscle fibers present in the superficial region of the SM muscle are of the 2B type (68.9% as SDH- negative fibers or 79.7% when counted in the acidic ATPase pH 4.35).
Fiber size of the three different fiber types of the SM muscle
As expected, Table 1, shows that the acidic ATPase pH 4.35 negative and SDH-negative 2B type myofibers, present the largest size among the three types of muscle fiber of rat SM muscle. In the superficial region based on acid ATPase pH 4.35, the 2B fiber size result 47.6 µm and the 2A fiber 35.3 µm. In the deep region the fiber size of 1 fibers (slow contracting) result to be the smallest: 29.2 µm; while the 2A and 2B fiber size are 36.8 µm on average in size. Based on mitochondrial enzyme activities, SDH reaction, in the superficial region the size of 2B fiber recognizable as the larger ones are 53.3 µm and in the deep region 41.1µm, while the 1 and 2A fiber size in the superficial region and in the deep region is respectively 35.8µm and 33.8µm.

Discussion

The peculiarity of the SM rat muscle is the presence of two distinct regions, where the fiber type composition is characterized in both zone by 2B fibers that are easily

Table 2. *Mean myofiber diameter (µm) in deep and superficial region of rat SM stained by SDH or ATPase, pH 4.35 reactions. Values are mean±SD.*

SDH reaction

	Deep region	Superficial region
Type fibers 1 + 2A	33.8±7.5	35.8±9.2
Type fibers 2B	41.1±9.2	53.3±10.8
Total number fibers	5211	3975

ATPase pH 4.35

	Deep region		Superficial region	
Type fibers 1	29.2±6.5	Type fibers 2A	35.3±8.5
Type fibers 2A + 2B	36.8±9.2	Type fibers 2B	47.6±11.9
Total number fibers	5802		4179

Table 3 . *Number and percentage of SM fiber types in deep or superficial regions by SDH or ATPase pH 4.3*

SDH reaction

	Deep region	Superficial region
Type fibers 1+2A		
Number	3800	1235
Percentage	72.9%	31.1%
Type fibers 2B		
Number	1411	2740
Percentage	27.1%	68.9%
Total number fibers	5211	3975
Total fibers Deep plus Superficial region	9186	

ATPase pH 4.35

	Deep region		Superficial region
Type fibers 1		Type fibers 2A	
Number	1422		849
Percentage	24.5%		20.3%
Type fibers 2A+ 2B		Type fibers 2B	
Number	4380		3330
Percentage	75.5%		79.7%
Total number fibers	5802		4179
Total fibers Deep and Superficial region		9981	

recognizable from their major size, while the 1 and 2A are the smallest. The SM muscle presents a very heterogeneous distribution of muscle fiber types with a large predominance of the type 2B in the superficial region while moving to deep regions there is a progressive enrichment of 2A fibers. Only in the deepest region of the SM muscle there are type 1 muscle fibers (Fig 1B and Table 3).

It remains to be determined if the content of fiber types varies along the muscle length and if applying new imaging processing as the Machine-learning algorithms (Deep Neural Networks) that have been proved to be very powerful methods for automatic image segmentation, especially in the field of histological analyses,[11] some of the probleams we are facing with non homogeneous muscles would be overcommed. Anyhow, as demonstrated here and by previously,[1] in the rat SM muscle comparative analyses for experimental models of muscle plasticity, in normal and diseased muscles,[12-26] will require morphometry of the whole cross-sections. In conclusion, in case of bioptic fragments of SM muscle, in small, but conceivably also large, mammals, only the size of the different fiber types could be evaluated to avoid systematic errors related to diferent regional sampling.

List of acronyms

ATPase - myofibrillar Actomyosin ATPase histochemistry
EAEC - Ethics Committee on Animal Experimentation
H-E – Hematoxylin-Eosin reaction
SDH - succinate dehydrogenase reaction
SM - sternomastoid muscle

Author's contributions

Authors equally contributed to the manuscript.

Acknowledgments and Funding

Conflict of Interest

The authors declare no conflicts of interests.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this

typescript is consistent with those guidelines.

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