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RESEARCH ARTICLE

The ultrastructural anatomy of the nuclear envelope in the masseter muscle indicates its role in the metabolism of the intracellular Ca^{++}

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ABSTRACT

Specific ultrastructural anatomy of masticatory muscles is commonly referred to a general pattern assigned to striated muscles. Junctional feet consisting of calcium channels of the sarcoplasmic reticulum (*i.e.* the ryanodine receptors, RyRs) physically connected to the calcium channels of the t-tubules build triads within striated muscles. Functional RyRs were demonstrated in the nuclear envelopes of pancreas and of a skeletal muscle derived cell line, but not in muscle *in situ*. It was hypothesized that ryanodine receptors (RyRs) could also exist in the nuclear envelope in the masseter muscle, thus aiming at studying this by transmission electron microscopy. There were identified paired and consistent subsarcolemmal clusters of mitochondria, appearing as outpockets of the muscle fibers, usually flanking an endomysial microvessel. It was observed on grazing longitudinal cuts that the I-band-limited mitochondria were not strictly located in a single intermyofibrillar space but continued transversally over the I-band to the next intermyofibrillar space. It appeared that the I-band-limited transverse mitochondria participate with the column-forming mitochondria in building a rather incomplete mitochondrial reticulum of the masseter muscle. Subsarcolemmal nuclei presented nuclear envelope-associated RyRs. Moreover, t-tubules were contacting the nuclear envelope and they were seemingly filled from the perinuclear space. This could suggest that nucleoplasmic calcium could contribute to balance the cytosolic concentration via pre-built anatomical routes: (i) indirectly, via the RyRs of the nuclear envelope and (ii) directly via the communication of t-tubules and sarcoplasmic reticulum through the perinuclear space.

1. Introduction

The specific ultrastructural anatomy of masticatory muscles is commonly referred to a general pattern assigned to striated muscles. The basic ultrastructural anatomy of the masseter muscle was previously evaluated (Abe et al., 2000; Bani et al., 1999; Bani and Bergamini, 2002; Seibel et al., 1978), but here we present evidence of a very peculiar aspect of the intimate relation between t-tubules and nucleus. Although the masseter's fiber composition has certain peculiarities (Table 1) and is species-specific (Bani et al., 1999; Tuxen and Kirkeby, 1990), the t-tubules ultrastructure is not fiber-dependant (Greising et al., 2012).

The excitation-contraction coupling (signal transmission from the sarcolemma to the actin/myosin apparatus) is mediated by Ca^{++} (Al-Qusairi and Laporte, 2011). In striated muscles there is a bidirectional communication between mitochondria and the sarcoplasmic reticulum (SR) (Boncompagni et al., 2009; Rossi et al., 2009), the two organelles being linked by small bridges or tethers (Boncompagni et al., 2009). Such tethers involve mitochondria close to the I-band and the junctional SR cisterna facing the Z-disc of the sarcomere (Boncompagni et al., 2009). The opposite face of that junctional (terminal) SR cisterna is building a triad (CRU, calcium-release unit). A triad is a complex bond between two SR cisternae and a transverse tubule (or t-tubule), united by junctional feet (Ferguson et al., 1984). To ensure a uniform spread of excitation to all the Ca^{++} release sites, the plasma membrane internalizes to form the t-system - a dense network that is responsible for excitation–contraction coupling (Jayasinghe and Launikonis, 2013) and present exclusively in striated muscle (Al-Qusairi and Laporte,

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Table 1
Types of fibers of masseter muscle.

Fibers types	Ultrastructural pattern Bani et al. (1999)	Histochemical characterization Tuxen and Kirkeby (1990)
Type I – red/slow fibers	Numerous large perinuclear and subsarcolemmal clusters of mitochondria long intermyofibrillar rows of mitochondria thick Z-discs moderate sarcoplasmic reticulum	Weak ATPase activity
Type II – white/fast	Scarce small mitochondria, usually paired at the Z-discs level thin Z-discs consistent sarcoplasmic reticulum	Strong ATPase activity
Type IM - intermediate	Intermediate features	Moderate ATPase activity

2011). The junctional feet consist of the SR Ca^{++} channels named ryanodine receptor (RyR) which are physically connected to the voltage-sensitive Ca^{++} channels of the t-tubule, named dihydropyridine receptors (DHPR) (Cadot and Gomes, 2016).

Different models of the ultrastructural players involved in the intracellular Ca^{++} handling were recently presented (Marcucci et al., 2018).

The RyR is a tetramer (Fill and Coronado, 1988) with a tetragonal symmetry resembling four-leaf clovers (quatrefoils) and consists of four opposed and eventually distorted spherical subunits surrounding a central area (Ferguson et al., 1984; Fill and Coronado, 1988). Due to this peculiar morphology, a RyR could be accurately identified under transmission electron microscopy (TEM). The SR domains not associated with membranes but bearing RyRs are named corbular (extended junctional or non-junctional) SR (Dolber and Sommer, 1984; Franzini-Armstrong et al., 1999; Kim et al., 2017). Functional RyRs not only exist in endoplasmic reticulum but were also found expressed in the nuclear envelope of pancreatic beta-cells (Zheng et al., 2012) and of a skeletal muscle-derived cell line (Marius et al., 2006). We hypothesized that RyRs could also exist *in situ* in the nuclear envelope of striated muscles. Thus we aimed at studying using TEM the ultrastructure of the masseter muscle.

2. Materials and methods

We decided to use rabbit as an experimental model for several reasons, listed below: first of all, the consistency of animal status is superior to other lab animals, and since muscle ultrastructure specimens may vary a lot, any variable out of the equation is a significant strength. Secondly, the access to the masseter muscle was prefered from a surgical point of view to other mammals.

We included in this study three male Oryctolagus cuniculus rabbits weighing 2.5–3 kg, aged between six to twelve months. Experimental animals were housed in individual cages in 12/12-h light/dark cycle and fed *ad libitum*. All rabbits were acclimatized for one week before the experiments started. After eight days, the animals were sacrificed with lethal intravenous injections of phenobarbitone. All procedures were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. The masseter muscles were dissected out and further processed for TEM study. Small tissue fragments about 1–2 mm^3 were prefixed in fresh, ice-cold 4% glutaraldehyde in sodium cacodylate buffer (pH 7.4) for four hours at 4 °C. After fixation, the tissues were washed six times in 0.05 M sodium cacodylate buffer (pH 7.4) at 4 °C, postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate at room temperature for 2.5 h, stained *en bloc* with 0.5% aqueous uranyl acetate overnight at 4 °C and washed with 0.05 M sodium cacodylate buffer. After dehydration in graded series of ethanol and infiltration with propylene oxide, specimens were embedded in Glycid ether (Epon 812-equivalent) and were finally polymerized at 60 °C for 48 h. Semithin sections were stained with 1% toluidine blue for light microscopy. Ultrathin sections (80–100 nm) were cut using a diamond knife and collected on 200 mesh copper grids, and double counterstained with uranyl acetate and subsequently lead citrate. The grids were examined with a Philips electron microscope EM 208S operated at an acceleration voltage of 80 kV. We used an image acquisition system previously described (Rusu et al., 2017).

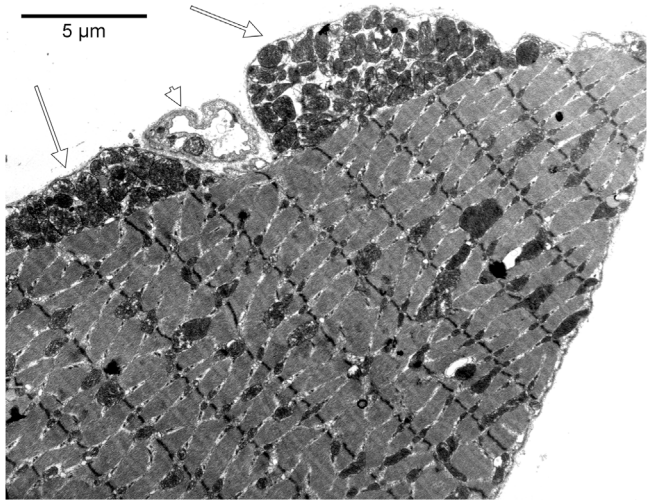

Fig. 1. Rabbit masseter muscle. Transmission electron microscopy. Subsarcolemmal clusters of mitochondria (arrows) are separated by an endomysial microvessel (arrowhead).

3. Results

The general sarcomeric structure of the masseter muscle myofibrils was clearly identified on grids. Sarcomere constituents, such as the Z-discs, A-bands and I-bands were unequivocally recognized.

The first peculiar ultrastructural feature of the muscle fibers were the paired and consistent subsarcolemmal clusters of mitochondria which were found as outpockets of the muscle fibers, usually flanking an endomysial microvessel (Fig. 1). Other than these deposits, any evidence of subsarcolemmal mitochondria was scarce or absent. With this peculiar disposition, the respective microvessels were related to both deposits of mitochondria but also to the myofibrils between those deposits. These subsarcolemmal clusters of mitochondria were indicative for RFs.

Endothelial cells of those microvessels showed abundant vesicles and scarce vacuoles (Fig. 2). Most of the time, but not exclusively (Fig. 3), these vessels were devoid of pericytes, thus assessed as capillaries. Sarcolemma lining these “grooves” for endomysial capillaries inserted transverse tubules (Fig.2). Subsarcolemmal caveolae as well as large vesicles were found (Figs. 2 and 4). Nevertheless, some of these large vesicles appeared to have an intramitochondrial location (Fig. 2). Occasionally, filamentous mitochondria were found projecting from the subsarcolemmal clusters within the intermyofibrillar spaces (Fig. 3). The subsarcolemmal mitochondria were densely packed, with their outer membranes in contact; among these mitochondria we identified microtubules, ribosomes (free and grouped), SR (cisterns, as well as corbular - Fig. 4).

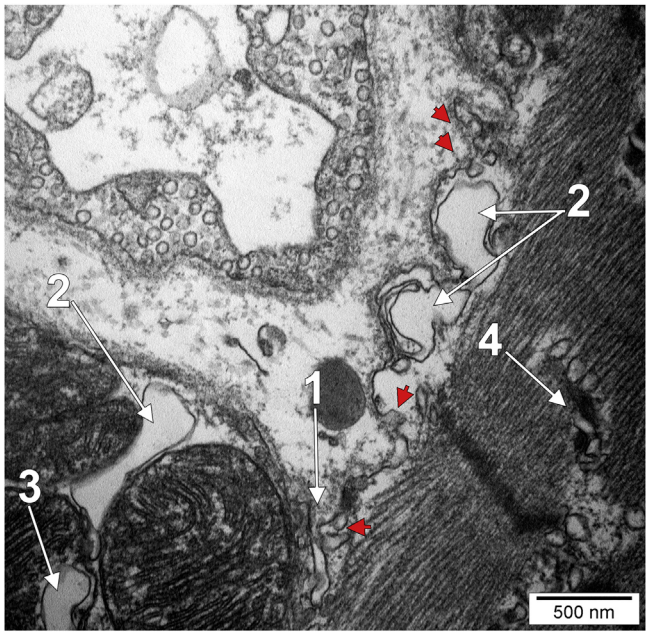

Fig. 2. Rabbit masseter muscle. Transmission electron microscopy. (1) transverse tubule; (2) large subsarcolemmal vesicles; (3) large intramitochondrial vesicle; (4) intermyofibrillar triad. The arrowheads indicate caveolae.

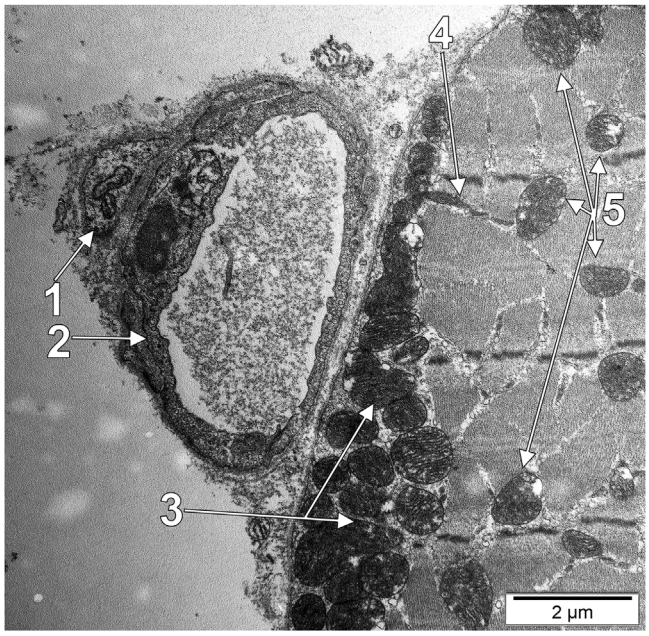

Fig. 3. Rabbit masseter muscle. Transmission electron microscopy. An endomysial microvessel is built up by pericytes (1) and endothelial cells (2). A cluster of subsarcolemmal mitochondria (3) is linked by a mitochondrial filament (4) to intermyofibrillar mitochondria (5).

Occasionally, within the subsarcolemmal collections of mitochondria were embedded nuclei (Fig. 5). However, subsarcolemmal nuclei were not exclusively related topographically with such collections of mitochondria (Fig. 6). On grazing (Fig. 5) and transverse (Fig. 6) cuts of nuclei there were observed nuclear envelope-associated RyRs with their characteristic four-leaf clovers appearance – four subunits around a central area (Fig. 5). Such RyRs had distinctive intranuclear extensions (Fig. 6). T-tubules were found directly contacting the nuclear envelope (Fig. 7). Moreover, such t-tubules were seemingly filled from the perinuclear space (Fig. 8).

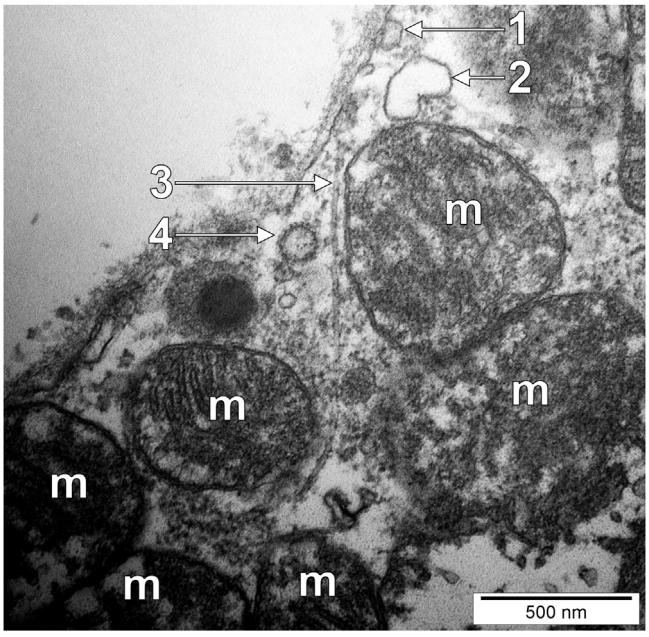

Fig. 4. Rabbit masseter muscle. Transmission electron microscopy. A large subsarcolemmal caveola is indicated (1). Between subsarcolemmal mitochondria (m) there are: sarcoplasmic reticulum (2), a microtubule (3), and corbular sarcoplasmic reticulum (4).

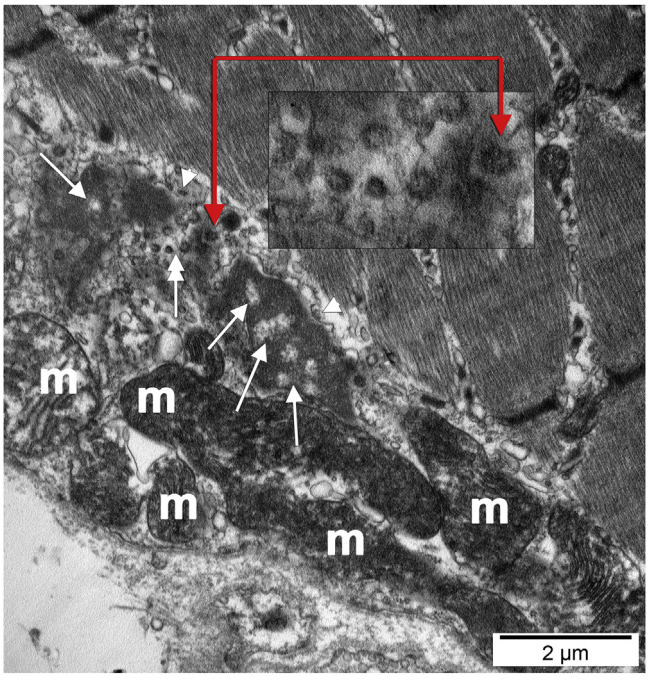

Fig. 5. Rabbit masseter muscle. Transmission electron microscopy. Grazing section of a subsarcolemmal nucleus. Arrows indicate nuclear pores. Ryanodine receptors of the nuclear envelope are cut transversally (arrowheads) and tangentially (double-headed arrow). The latter are depicted at higher magnification (inset, the connector indicating corresponding areas). m: mitochondria.

Except the subsarcolemmal mitochondria, we observed the intermyofibrillar mitochondria (Figs. 3,9–12).

On longitudinal cuts there were usually I-band-limited paired mitochondria, located on both sides of the Z-discs (Fig. 1). This was however not exclusive, as longitudinally disposed large mitochondria (column-forming mitochondria) were paralleling the sarcomeres between successive I-band-limited mitochondria

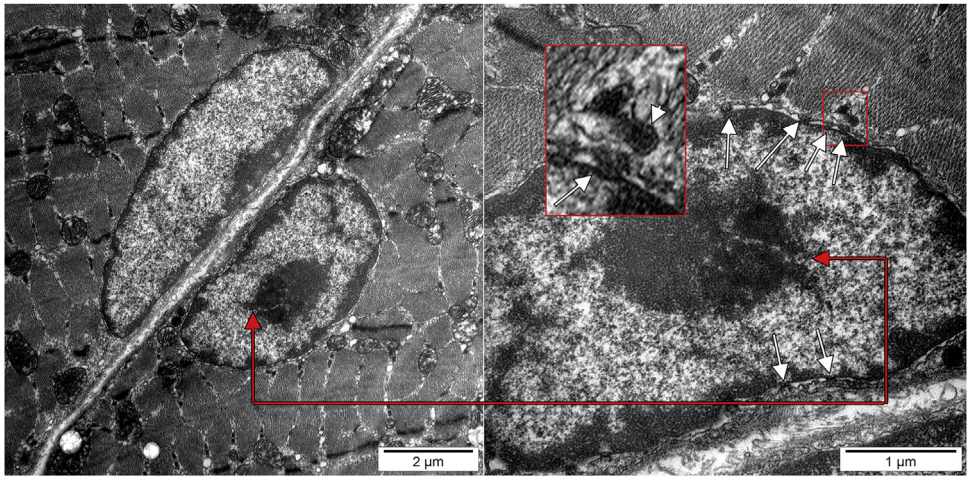

Fig. 6. Rabbit masseter muscle. Transmission electron microscopy. Facing nuclei of two adjacent muscle fibers. Corresponding areas are indicated by the red connector. The nuclear envelope has ryanodine receptors (white arrows) which extend into the nucleus. These receptors are morphologically similar to those in adjacent junctional feet (inset, digital magnification, arrowhead). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

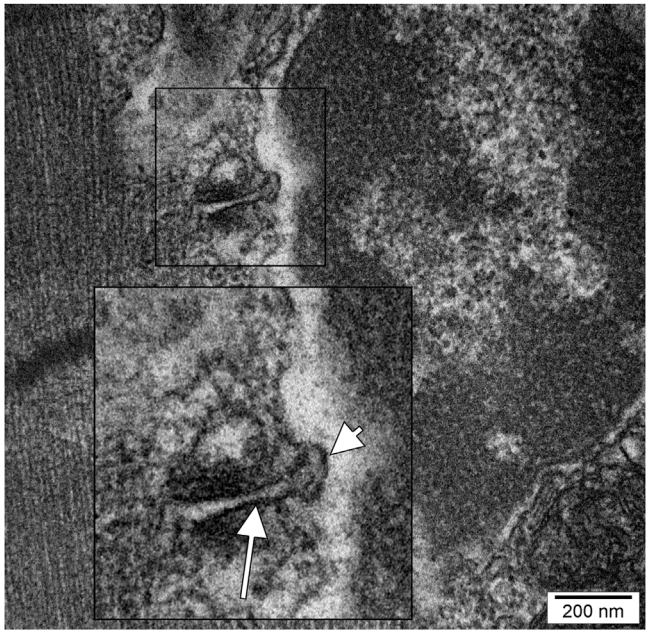

Fig. 7. Rabbit masseter muscle. Transmission electron microscopy. Perinuclear triad (inset, digitally magnified) demonstrating the contact between the t-tubule (arrow) with the nuclear envelope (arrowhead).

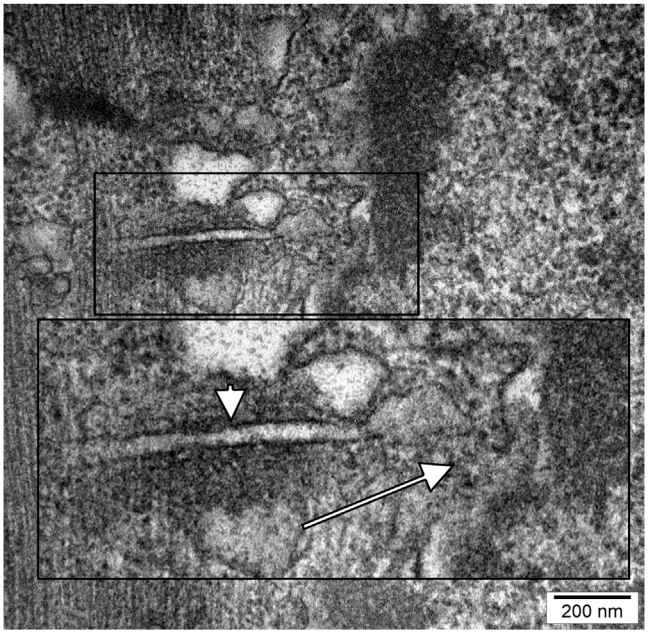

Fig. 8. Rabbit masseter muscle. Transmission electron microscopy. A t-tubule (arrowhead) reaches the nuclear envelope and is seemingly filled from the perinuclear space (arrow).

(Figs. 1,9,10). These patterns were suggestive for an intermediate type of fibers (IM).

Regular-sized mitochondria and megamitochondria were building incomplete longitudinal columns (Fig. 10). Noteworthy, we observed on grazing longitudinal cuts that the I-band-limited mitochondria were not strictly located in a single intermyofibrillar space but continued transversally over the I-band to the next one (Fig. 12). It appeared that the I-band-limited transverse mitochondria participate with the column-forming mitochondria in building a rather incomplete mitochondrial reticulum of the masseter muscle. Filamentous mitochondria or mitochondrial filaments link this reticulum to the subsarcolemmal deposits of mitochondria. Intermitochondrial connections were found: (a) tight tetralaminate contacts resulted from the apposed mitochondrial membranes; (b) fused mitochondria (Fig. 9) and (c) outer mitochondrial membranes separated by a narrow space filled with granular osmiophilic material.

The I-band-limited mitochondria were usually lined by triads located at the level of A/I junctions of sarcomeres (Figs. 9–12). We found symmetrical and asymmetrical triads built-up by paired thick junctional feet and, respectively, by thick and thin feet (Fig. 11). Moreover, we found that the thick junctional feet are elongated along the corresponding t-tubule and transversally across the A/I junction of that sarcomere — such transversally disposed triads neighboured the I-band-limited transverse mitochondria (Fig. 12). Tethers were found linking mitochondria with the junctional SR of triads as well as with cisterns of tubular SR (Fig. 12).

4. Discussion

4.1. Muscle fiber types of the masseter muscle in rabbit

In this study we noticed peculiar pockets of large subsarcolemmal mitochondrial clusters which were not united by

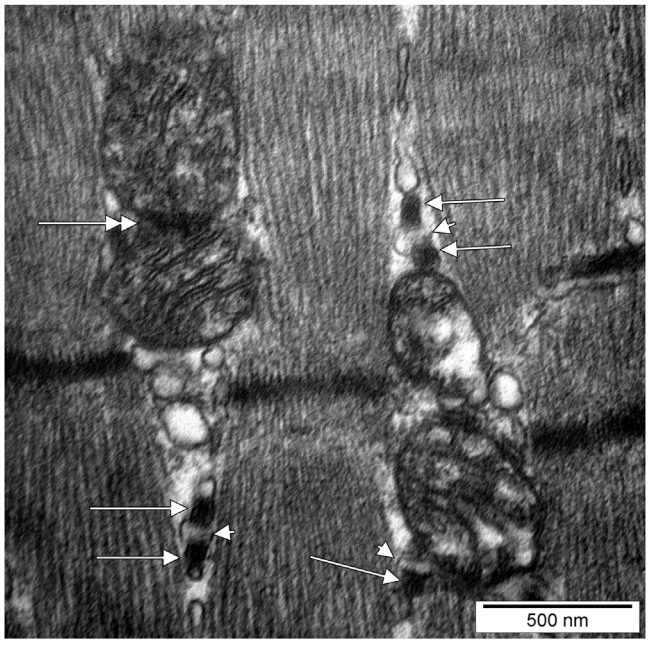

Fig. 9. Rabbit masseter muscle. Transmission electron microscopy. Thick junctional feet (arrows) are linked to transverse tubules (arrowheads) within triads. Two successive intermyofibrillar mitochondria appear to be fused (double-headed arrow).

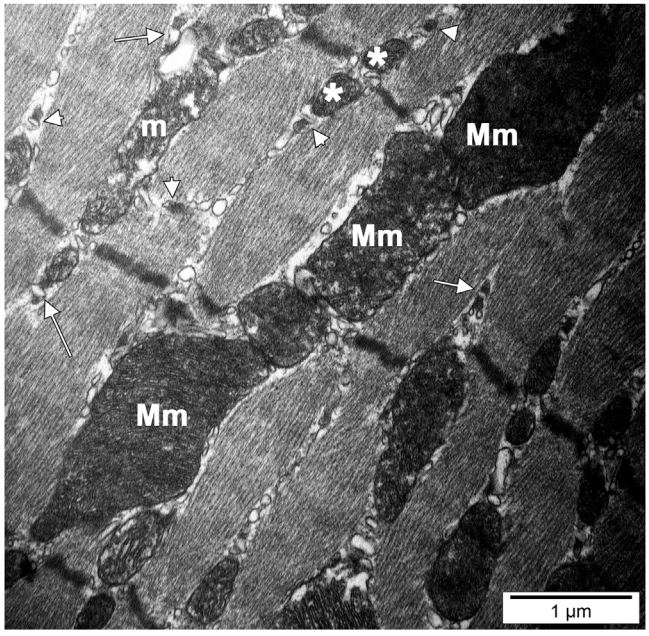

Fig. 10. Rabbit masseter muscle. Transmission electron microscopy. Within a longitudinally cut muscle fiber there are several longitudinal cords formed by regular-sized mitochondria (m), or by megamitochondria (Mm), or just pairs of mitochondria (*) on both sides of Z-discs. At the level of A–I junctions of myofibrils, there are dyads (arrowheads) and triads (arrows).

subsarcolemmal mitochondrial strands. As found by Ogata and Yamasaki (1985), the most consistent deposits of subsarcolemmal mitochondria are in the red fibers. Subsarcolemmal mitochondria are only sporadically found in intermediate fibers while they are absent in white fibers (Ogata and Yamasaki, 1985). Accordingly, we considered the pairs of sarcolemmal pockets of mitochondria as indicative for red fibers (RFs). Rabbit's masseter muscle was documented (Tuxen and Kirkeby, 1990) as with exclusive content of white fibers (WFs) or with an equal content of RFs and

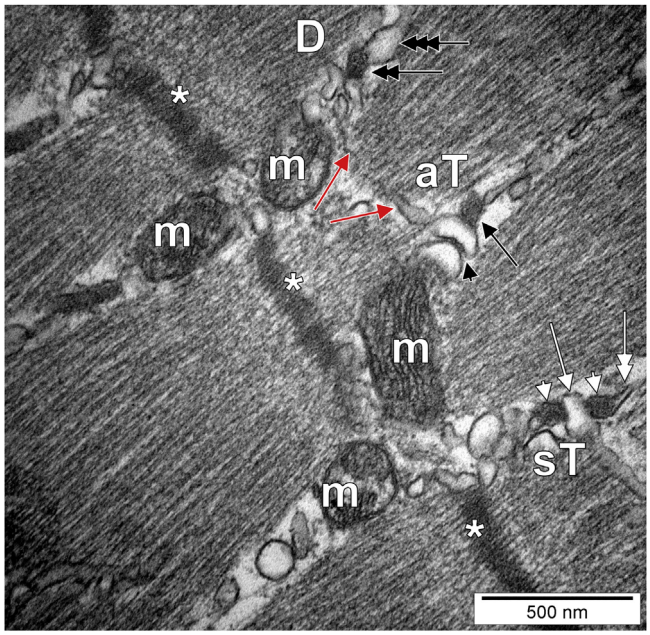

Fig. 11. Rabbit masseter muscle. Transmission electron microscopy. Within intermyofibrillar spaces mitochondria (m) are located on both sides of the respective Z-disc (*). A transverse tubule (diverging red arrows) connects a dyad (D) and an asymmetrical triad (aT). Within the dyad a thick junctional foot (double-headed black arrow) links the transverse tubule to a dilated cistern (triple-headed arrow) of sarcoplasmic reticulum. Within the asymmetrical triad a thick junctional foot (black arrow) links the transverse tubule to a narrow tubule of sarcoplasmic reticulum and a thin junctional foot (black arrowhead) links the t-tubule to a dilated cistern of sarcoplasmic reticulum. There is also indicated a symmetrical triad (sT) in which the t-tubule (white arrow) is united by thick junctional feet (white arrowheads) to narrow tubular cisterns of sarcoplasmic reticulum (double-headed white arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

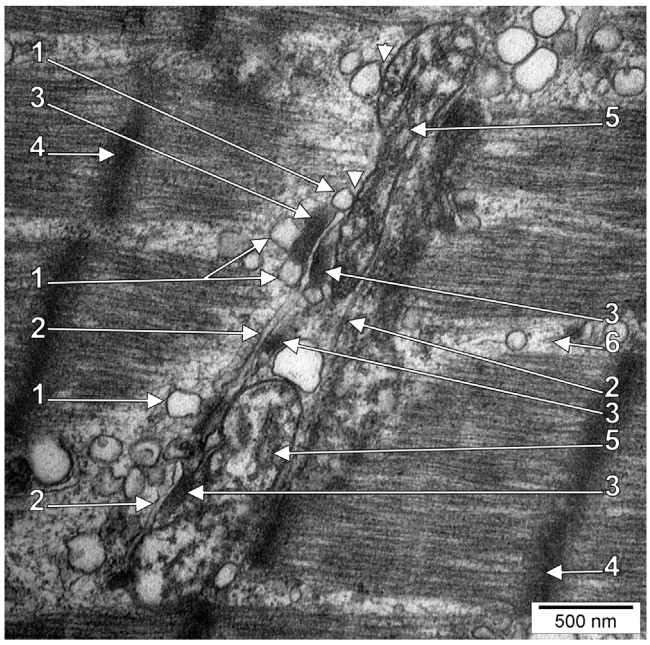

Fig. 12. Rabbit masseter muscle. Transmission electron microscopy. Longitudinal grazing cut of myofibrils. 1.junctional sarcoplasmic reticulum cisternae; 2.transverse tubules; 3.transversal junctional feet of triads; 4.Z-discs; 5.transversal mitochondria; 6.intermyofibrillar space. The arrowheads indicate tethers/bridges between the junctional sarcoplasmic reticulum and mitochondria.

Table 2
Masseter muscle fibers types in different species/models (Tuxen and Kirkeby, 1990).

Rat mouse	Type II only
Rabbit	Either type II only or types I and II
Cat, Dog	Predominant type II
Cow	Predominant type I
Pig, human	Types I, II and IM

WFs (Table 2). These subsarcolemmal pockets should have a functional impact, as they bring mitochondria very close to endomysial microvessels, thus favouring a prompt metabolic input through a thin barrier built-up by the endothelial and sarcolemmal basal laminae and the thin ECM layer between them. According to the ultrastructural pattern of intermyofibrillar mitochondria we found, the rabbit's masseter muscle seems also consisting of intermediate fibers, such as in humans (Table 2), thus being an adequate model for study. However, an analysis of fiber types using the ATPase approach or the use of myosin heavy chain isoform analyses through SDS-PAGE electrophoresis could better evaluate the fiber types. This does not impede on our study as "*there is no evidence that there are fiber type differences in excitation-contraction coupling at the level of the t-tubule and triad junction*" (Greising et al., 2012).

4.2. Triads in the masseter muscle of rabbit

Franzini-Armstrong and Nunzi (1983) described three morphological types of junctional feet, as follows: (a) Type 1 or parallel lines: feet which are elongated in a plane parallel to the adjacent membranes of the SR and t-tubule; (b) Type 2: feet forming single dense columns between the apposed membranes they touch and (c) Type 3 or pillars: feet with distinctly less dense centres which give them of two lines perpendicular on the facing membranes they unite (Franzini-Armstrong and Nunzi, 1983).

Type 1 of junctional feet is variable in regards of the extent of the attachment of feet to the facing membranes (Franzini-Armstrong and Nunzi, 1983). We found symmetrical (sTs) and asymmetrical (aTs) triads, built-up on longitudinal cuts by two thick junctional feet or by a thin and a thick one. The sTs, as well as dyads, consisted of thick Type 1 junctional feet - elongated feet paralleling the t-tubules and the junctional SR. The thin feets in the aTs were Type 2, according to the same classification.

4.3. Topography of mitochondria in the masseter muscle of rabbit

Disposition of mitochondria is important due to their known cooperation with intracellular calcium deposits (the sarcoplasmic reticulum) in muscular cells, hence the difference in their distribution pattern compared to the endothelial cells. We showed under TEM that within the masseter muscle mitochondria equally form intermyofibrillar columns and transverse bars lying on the I-bands, therefore a mitochondrial reticulum (that can neither be considered complete, nor incomplete). Transversally disposed mitochondria were also previously found in different muscles and species (Ogata and Yamasaki, 1985, 1997). Therefore, it seems that studies or textbooks exclusively indicating solely longitudinal columns of mitochondria in striated muscles should be reconsidered. On the other hand, these few proofs of mitochondrial cables within skeletal muscles support the concept regarding mitochondria as electrically united systems (Bakeeva et al., 1978; Skulachev, 2001), able of generating traveling depolarization and Ca^{++} signals (Ichas et al., 1997).

The functional significance of different types of mitochondria underlies the response pattern in the calcium release in the muscular cell. The lower the distance between ATP-producing mitochondria and Ca^{++}-containing sarcoplasmic reticulum, the faster the contraction generation in which Ca^{++} plays a pivotal role.

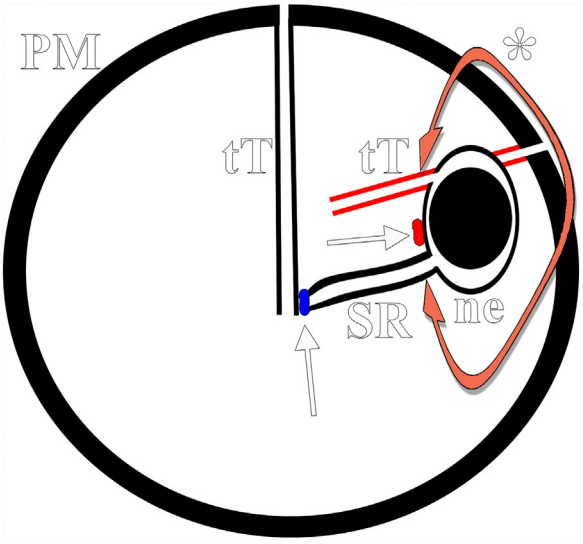

Fig. 13. Diagram of the t-tubules (tT) and sarcoplasmic reticulum (SR) connection via the perinuclear space (*) beneath the nuclear envelope (ne). Ryanodine receptors (arrows) are present equally on the junctional SR (blue) and the nuclear envelope (red). the tT maintain an extracellular-intracellular interface. PM: plasmalemma. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hence, the Ca^{++} intracellular traffic and everything is related to it is of utmost importance.

4.4. Ryanodine receptors of the masseter muscle

We found in TEM bona fide RyRs located on the junctional SR as well as on the nuclear envelope. This is reasonable, as the SR results as an extension of the nuclear envelope, which ensures the communication of the perinuclear space with the SR cisterns. Moreover, we found t-tubules contacting the nuclear envelope and even communicating with the perinuclear space. In these regards, the t-system and SR cisterns communicate with each other via the perinuclear space (Fig. 13).

The nucleoplasmic reticulum functions as a Ca^{++} release channel and is continuous with the sarcoplasmic reticulum. The t-system surrounds the nucleus at a high density determined by the abutting myofibrils that run parallel to the long axis of the nuclei; the t-tubules and the SR are simply brought into close proximity to the nuclear envelope by this arrangement (Jayasinghe and Launikonis, 2013). The t-tubules of rat and toad fibers fully or partially encase the nucleus while the t-tubules in frog skeletal muscle form dyadic junctions with the nuclear envelope (Jayasinghe and Launikonis, 2013). Our findings support that also in rabbit the excitation-contraction coupling is active in this microdomain. It was suggested that Ca^{++} released from SR during excitation-contraction coupling influences Ca^{++} levels inside the nuclear envelope and nucleoplasm (Jayasinghe and Launikonis, 2013). However, also the reverse, nuclear-to-cytosolic pathway of Ca^{++} could be speculated about reasonably.

Although it is known that intranuclear Ca^{++} release occurs through the inositol triphosphate pathway, it was tested whether a similar release can occur via RyRs (Marius et al., 2006). We found gathered evidence, which supports our findings, that intranuclear extensions of the nuclear envelope have functional RyRs regulating Ca^{++} signals in discrete nuclear regions and Ca^{++} release from RyRs occurs in both the nucleus and cytoplasm of a skeletal muscle derived cell line (Marius et al., 2006). RyRs were also found in the nuclear envelope of non-muscle cells — the primary pancreatic β-cells (Zheng et al., 2012). We hereby bring in situ evidence

demonstrating the presence of RyRs on the nuclear envelope of striated muscle cells.

4.5. Limitations of the study

The masseter muscle is complex in anatomy and function thus analyzing small tissue fragments in TEM could appear simplistic. However, studies on the masseter muscles do (Bredman et al., 1992), or do not indicate exactly from which parts the samples were taken (Tuxen and Kirkeby, 1990). An ultrastructural documentation of muscle fiber type thus appears necessary. The ultrastructural discrimination of fiber types seems mandatory in the rabbit masseter because there is a spatiotemporal pattern of fiber types (Bredman et al., 1992) which could not be discriminated when samples are dissected out. This, however, does not impede on anatomical analyses of the t-system.

5. Conclusion

The close connection between mitochondria and endomysial microvessels ease the metabolic exchanges. This might prove very important for all masticatory muscles, as it could explain the high resistance to short-time continuous effort and high-load/increase force paradigm of these muscles. The peculiar distribution of mitochondria that we presented, represents a novel point of view, backed by electron microscopic evidence and which fits the electrophysiological features of the analyzed muscles. Further electron microscopic analysis of other masticatory muscles would reveal any common ultrastructural patterns and putative specificity that might exist at this level.

The nuclear envelope is seemingly equipped to support a contribution of the nucleoplasmic calcium to balance the cytosolic concentration via pre-built anatomical routes: (i) indirectly, via the RyRs of the nuclear envelope and (ii) directly via the communication of t-tubules and sarcoplasmic reticulum through the perinuclear space.

Contribution of authors

All the authors have equally contributed to this paper.

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Ethics

All procedures were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and were tacitly approved by the affiliated institutions.

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None.

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