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ULTRASTRUCTURAL STUDY OF MUSCLE FIBERS AND NEUROMUSCULAR JUNCTIONS IN THE OPOSSUM THYROARYTENOID MUSCLE

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ABSTRACT

Some muscle groups of mammals, such as the laryngeal muscle, present tonic muscle fibers among fast twitch muscle fibers. The latter are supplied by single en plaque neuromuscular junctions (NMJs) and the former by multiple NMJs. The aim of the present study was to characterize the muscle fiber types and their NMJs in the thyroarytenoid (TA) muscle of the opossum. Five adult opossums (*Didelphis albiventris*) were anesthetized and perfused with Karnovsky solution. The TA muscles of the right side were processed for TEM. The contralateral TA muscles were submitted to connective tissue digestion with HCl before scanning electron microscopy processing. Based on myofibril morphology, the number and arrangement of mitochondria, sarcoplasmic reticulum and T tubule profiles and Z-line width, three fast twitch muscle fiber types were identified. Tonic fibers characterized by small and compact myofibrils were also found. Although tonic muscle fibers were present, only single NMJs were observed. In these NMJs the axon terminals occupy the synaptic clefts, which have variable depths. The sarcolemmal folds were not homogeneously arranged along the NMJ cleft. The Schwann cell bodies and their cytoplasmic projections were covering the axon terminals. Scanning electron microscopy analysis revealed empty synaptic clefts with irregular distribution of junctional folds. At some NMJs, the axon terminals were not removed and were present, filling up the synaptic cleft. The presence of only the en plaque NMJ type is discussed in view of the functions performed by the opossum TA muscle. Moreover, we demonstrate the similarity in NMJ distribution between the opossum TA muscle and those of rats and humans, with the opossum thus representing another useful experimental animal model for studies regarding intrinsic laryngeal muscles.

Key words: Neuromuscular junctions, opossum, thyroarytenoid muscle, ultrastructure

INTRODUCTION

The South American opossum belongs to the Theria subclass of the class Mammalia and presents incomplete intrauterine development due to the presence of a placental lake [16]. When facing a stress situation, this animal opens its mouth and emits a characteristic sound. In most mammals the sound comes from the larynx that is positioned in the oral cavity. Coutinho *et al.* [3] reported that in the opossum the larynx is positioned in the nasal cavity. This organ projects through an opening of the palatopharyngeal muscle. The intranasal larynx allows the animal to eat and breathe at the same time. This functional characteristic permits the young to survive until the end of their development inside the marsupial pouch, where the fetuses present a fusion of their oral epithelium with the mother's nipple epithelium [9].

Studies conducted on opossum extraocular muscles have revealed the presence of slow and fast twitch fibers [22]. Using transmission electron microscopy (TEM) and scanning electron microscopy (SEM), Matheus and Soares [19,21] demonstrated the presence of multiple and single en plaque neuromuscular junctions (NMJs) in the same muscles. In the retractor bulbi muscle, which only contains fast twitch fibers, only en plaque NMJs were found [20].

The larynx muscles develop from the branchial arcs, are supplied by cranial nerves and present slow and fast twitch fibers [7,10,29,30].

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Many mammalian species present "fast-contracting" and "fatigue-resistant" muscles. These properties are related to the muscle fiber type and are determined by oxidative enzyme activity. The myosin heavy-chain (MHC) determines the maximum contraction speed of a muscle fiber. Recent immunohistochemistry studies have located this muscle fiber type in the extraocular and thyroarytenoid muscles of mammals [33]. Wu *et al*. [36], analyzing muscles of the dog larynx, demonstrated that each laryngeal muscle contains hybrid fibers (polymorphic) that can co-express multiple forms of MHC. According to Rhee *et al*. [28], the thyroarytenoid muscle expresses MHC, found in the extraocular muscle, as also observed for the isoforms found in the legs and arms. Yamagata *et al*. [37], using SEM, identified two types of NMJs in the rat thyroarytenoid muscle based on subneural apparatus morphology.

Dysphonia is an important idiopathic laryngeal motor control disorder that affects voice control during speech [4]. The primary treatment for the most common type of this disorder involves direct injection of botulinum toxin into the NMJ region [1,31,32,35]. The rat has been used as an experimental model in spasmodic dysphonia studies [12 -15].

Literature data regarding the morphological characteristics of the opossum laryngeal muscles are available. The opossum is a marsupial that has been used as an important animal model in biomedical and ecological studies. The location of the NMJs within the opossum thyroarytenoid muscle described in this study might be useful as another animal model for these procedures. Therefore, the aim of the present study was a) to describe the morphology of the thyroarytenoid muscle, b) to determine the location of its NMJs, and c) to characterize the ultrastructure of its muscle fibers.

MATERIAL AND METHODS

Five adult opossums (*Didelphis albiventris*) of both sexes weighing 400 to 800 g were used (IBAMA - permission number 033/2001). After anesthetic intraperitoneal injection of sodium pentobarbital (40 mg/kg), the animals were perfused through the left ventricle with Karnovsky fixative (2.0% glutaraldehyde and 4.0% paraformaldehyde, 1:1, in 0.1 M sodium phosphate buffer, pH 7.4). After perfusion, the tongue-pharynx set was removed and the thyroarytenoid muscles were dissected.

The right-side muscles were routinely processed for TEM. The contralateral muscles were first submitted to the nonspecific esterase technique for location of the NMJs [17], followed by digestion with HCl [5] to remove the intramuscular connective tissue. This method consists of washing the muscle in PBS at room temperature (3 changes of 15 minutes each). The muscles were then digested with 8 N HCl at 60°C for about 20 minutes. Treatment with collagenase, as proposed in the original method, was omitted. After digestion, the specimens were washed several times in PBS and submitted to routine processing for SEM.

RESULTS

Muscle fiber ultrastructure

Ultrastructural analysis of the muscle fibers revealed three fast twitch fiber types (Fig. 1). Type 1 fibers were characterized by well-defined myofibrils, especially at the A-band level. Intermyofibrillar mitochondria were present isolated or forming columns, sometimes giving a discontinuous appearance to the myofibrils. These mitochondria presented variable shapes ranging from round to elongated (Fig. 1a). The Z-line was broad and straight. In some preparations the M-band with some filaments was seen (Fig. 1a'). Type 2 fibers were closely similar to type 1 fibers, but exhibited numerous lipid droplets associated with the intermyofibrillar mitochondria (Fig. 1b and 1b'). The third muscle fiber type had a homogeneous appearance and their myofibrils were packed and well organized. This type 3 fiber did not present lipid droplets and mitochondria were rare (Fig. 1c). The Z-line was continuous, straight and thinner compared to the other fiber types. The H-band and M-line were not clearly visible (Fig. 1c'). Another type of muscle fiber was identified and was characterized as "tonic" slow twitch fiber (Fig. 2). Its myofibrils were small and compact (Fig. 2a), many triads and T tubules were present, and the Z-line was broad (Fig. 2a').

Neuromuscular junctions

The nonspecific esterase technique permitted the observation of the distribution of the NMJ along the thyroarytenoid muscle. In contrast to what is observed in most muscles, the NMJs were distributed at random along the thyroarytenoid muscle. This morphology resembled the "en plaque" type junctions, with most of them being elliptical and their long axis running parallel to the muscle fiber major axis. The synaptic cleft was branched and presented cross-striations related to the junctional

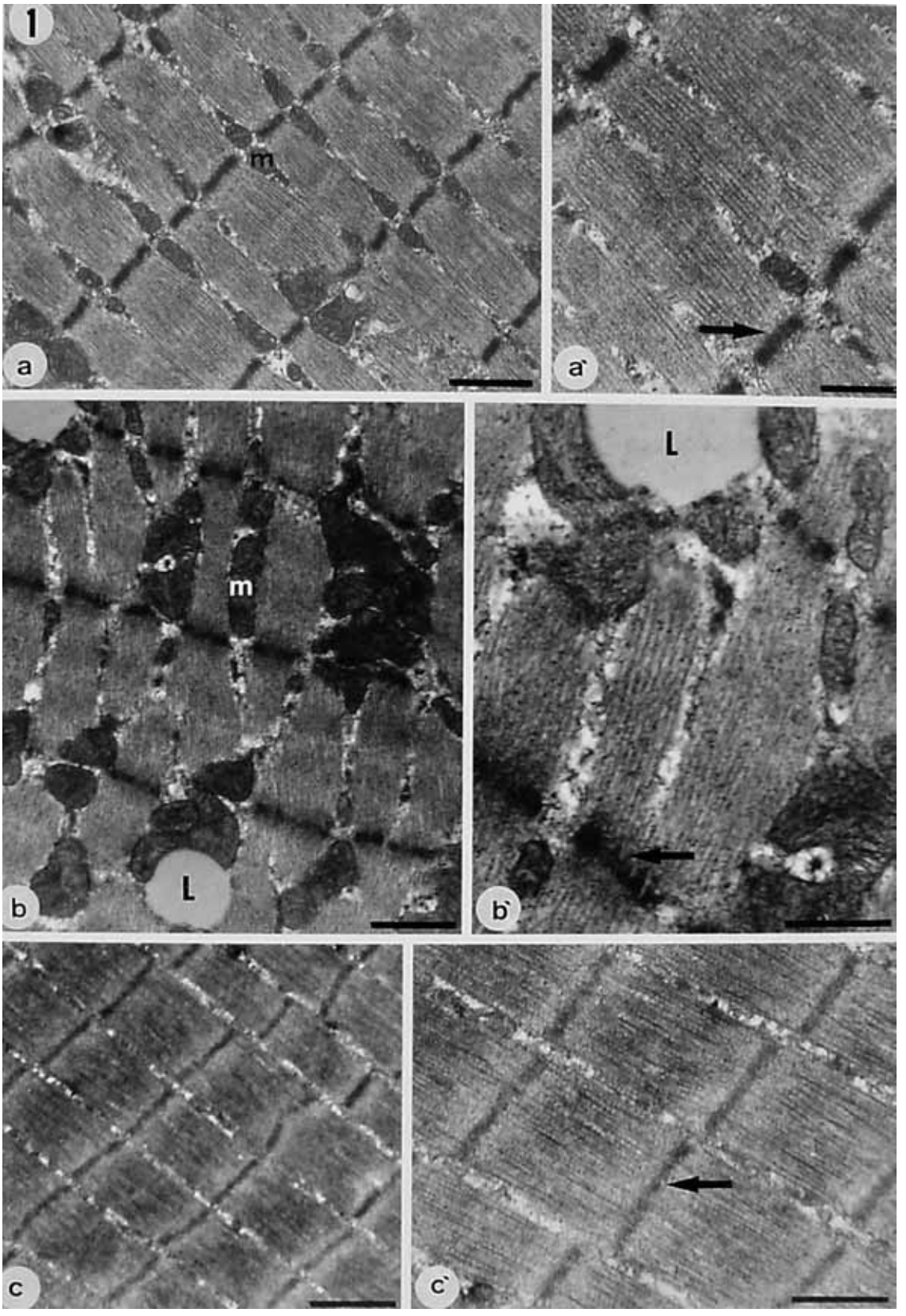

Figure 1. Electron photomicrographs of type 1 (a,a'), type 2 (b,b') and type 3 (c,c') fibers present in the thyroarytenoid muscle. Mitochondria (**M**), lipids (**L**), Z-line (**arrow**). Bars a,b,c = 1 μm; a',b',c'= 0.5 μm.

folds. Some NMJs presented a less branched synaptic cleft and small terminal projections. Other less numerous NMJs were elliptical and very compact, a fact impairing detailed observation. Intermediate forms such as open, irregular and compact NMJs were rare and characterized the polymorphism of the NMJ (Fig. 3).

SEM analysis revealed only "en plaque" type NMJs. In these places where only partial digestion of the connective tissue occurred, two or three terminal Schwann cells can be seen over the synapses. In these muscle fibers it is possible to observe the nerve ending fitting the peripheral depressions in the synaptic cleft (Fig. 4c). In the case of the muscle fibers where the connective tissue and the nerve terminal were totally removed, the characteristics of the synaptic cleft can be described in detail. In some NMJs the clefts were deep and branched and exhibited a central elevation of the sarcolemma (Fig. 4d and 4e). Other muscle fibers showed shallow and discontinuous synaptic clefts, and depressions where the terminal axons had made focal contacts (Fig. 4a, 4b, 4d). The junctional folds were clearly visible in both NMJs (Fig. 4f). The different synaptic clefts demonstrated in this study represent the NMJs associated with different "fast twitch" muscle fibers observed in the thyroarytenoid muscle. The "en grappe" multiple NMJs associated with tonic slow twitch muscle fibers were not observed in this study.

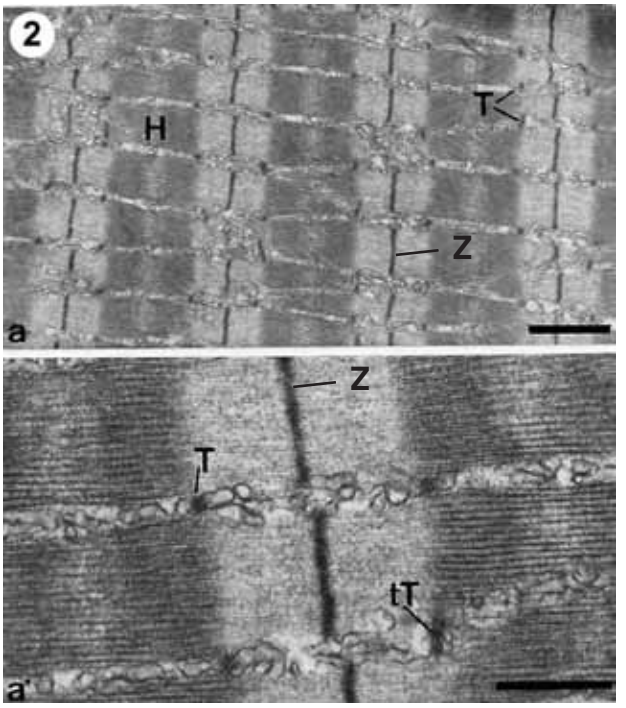

Figure 2. Electron photomicrographs of tonic fibers (a,a') present in the thyroarytenoid muscle. H-band (**H**), T tubules (**tT**), triads (**T**), Z-line (**Z**). Bar = 1 μm.

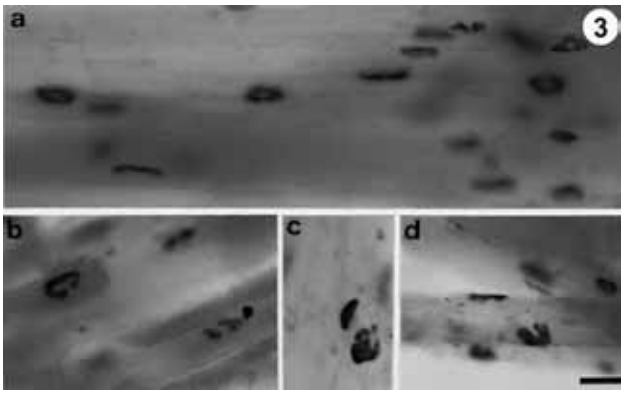

Figure 3. Neuromuscular junction of the thyroarytenoid muscle. Whole-mount. acetylcholinesterase technique. Bar = 10 μm.

DISCUSSION

The NMJ is a chemical synapse which is structurally and functionally adapted to transmit electrical signals from the nerve terminal to a circumscribed postsynaptic region of the muscle fiber. The position and number of NMJs in the muscle fiber, the distribution of nerve terminals at the junctions and the complexity of the postsynaptic region may vary according to phylum and species, between different muscles of a given species, and between different fibers of the same muscle [6].

The method used by Lehrer and Ornstein [17] and in the present study to label the NMJs, although described for TEM, has been largely used for light microscopy. This method labels the NMJ site in a rapid way and is of easy execution. In addition, since the muscles are previously immersed in the fixative used for TEM, after the reaction the NMJ places become sharply visible in the muscles and can be easily removed for processing. During observation of the thyroarytenoid muscle submitted to the nonspecific esterase technique, multiple NMJs associated with tonic fibers were not identified, as also reported previously for the extraocular muscle of this same animal [21].

In the opossum, the single "en plaque" NMJs are distributed randomly in the muscle. The authors agree that this distribution pattern of the NMJs may be related to the geometry of the thyroarytenoid muscle, whose fibers present different points of origin and

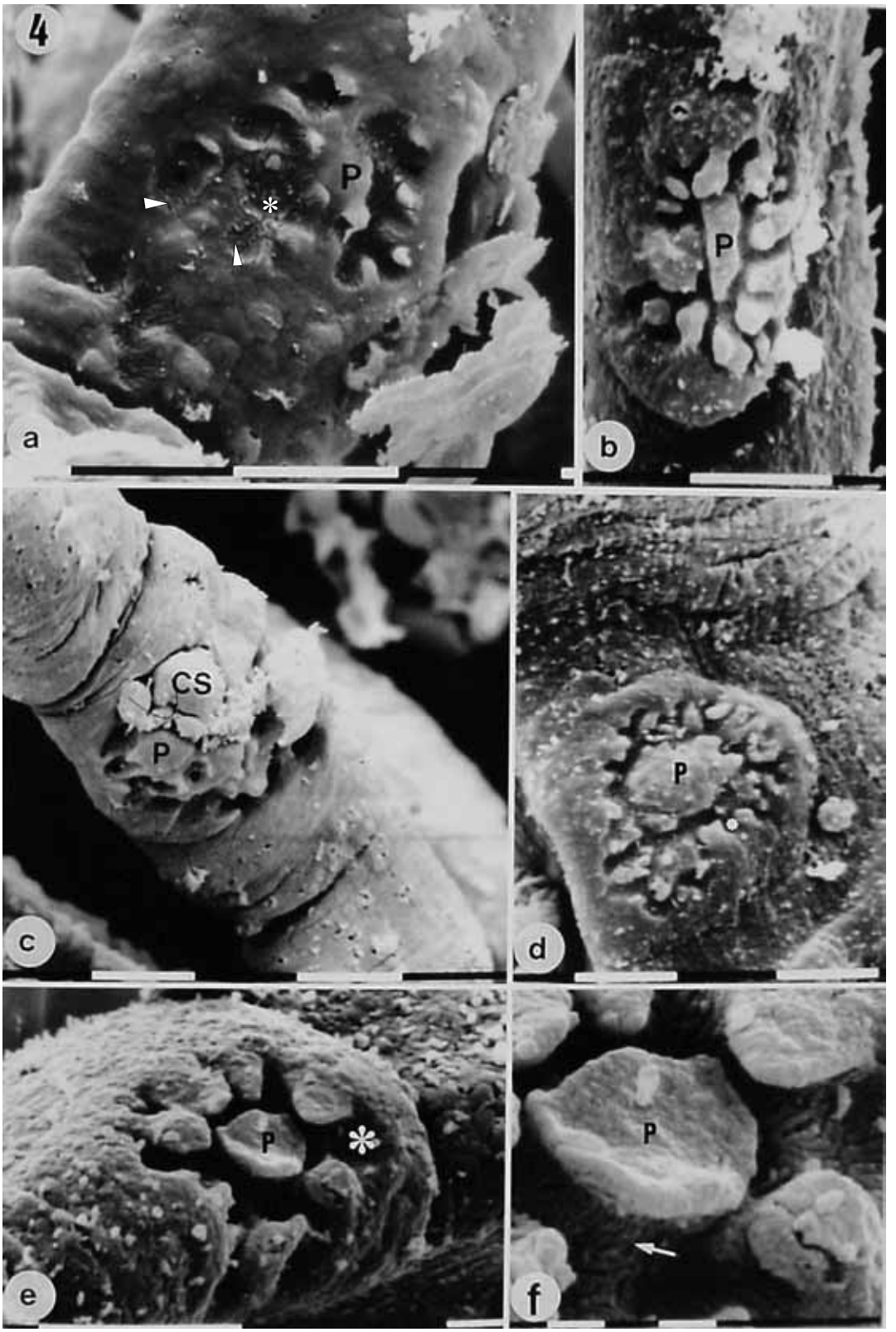

Figure 4. Scanning electron microscopy images of the neuromuscular junction after HCl digestion. (**a, b, d, e, f**) Total digestion of connective tissue with exposed postsynaptic elements. Note the presence of an unoccupied synaptic groove (*) and the secondary clefts (**arrowheads**). Sarcoplasmic protuberance (**P**). In panel (**c**), partial digestion of connective tissue. Note the Schwann cell body (**CS**). Bars a,b,c,d,e = 10 μm; f =1 μm.

insertion. Gacek [8] found this NMJ distribution in the human thyroarytenoid muscle, whereas Sheppert *et al*. [32], using a three-dimensional reconstruction model, verified that 74% of the NMJs were located in the middle third and less than 7% in the anterior third.

In an ultrastructural study, Yamagata *et al*. [37] identified two types of NMJs in the thyroarytenoid muscle of rats and also demonstrated morphological differences in the subneural apparatus of the NMJs of the posterior cricoarytenoid muscle. The authors suggested that these NMJs have different functional and evolutive characteristics when compared to other striated skeletal muscles. The observed NMJs matched with the rat extraocular NMJs and the authors inferred that both muscles have a functional and sharp regulation.

The MHC determines the maximum contraction speed of a muscle fiber. Immunohistochemical studies have located this muscle fiber type in extraocular and thyroarytenoid muscles of mammals. The distribution pattern of MHC is related to the specific function of the laryngeal muscle. The high density of MHC in the rat thyroarytenoid muscle may be explained by the role of this muscle in airflow protection and in the glottis closing reflex [33].

On the basis of the literature [11,21,25,37,38] and considering the embryonic origin of the thyroarytenoid muscle from the branchial arc [24], we expected to find multiple NMJs in this muscle, but only single "en plaque" NMJs were observed. NMJs thus present peculiar characteristics depending on the muscle fiber type with which they are associated [26,27,34]. "En plaque" NMJs are widely distributed among the fast-contracting twitch fibers [6]. Although the opossum emits a characteristic sound when facing a stress situation, the larynx of this animal does not act as a phonation organ. The larynx and its muscles are important for swallowing, ventilation, coughing, sneezing and Valsalva's maneuver [23]. The intrinsic muscles control the size and shape of the laryngeal inlet and the tension of the vocal folds. The fast and constant action of these muscles protects the lower airways from foreign bodies and during swallowing. It is also responsible for the precise timing and coordination of contractile activity and a well-developed sustained work capacity [23]. Another exclusive biological characteristic is that the young opossum in the marsupium attaches to the mother's nipples and its larynx permits it to breathe while sucking [3,9].

In general, the intrinsic laryngeal muscle shows fast-twitch kinetics and velocity of shortening. These functional characteristics might be explained by the prevalence of the "en plaque" NMJ in the thyroarytenoid muscle of the opossum.

Some authors [2,23] have raised the hypothesis that the thyroarytenoid muscle may become weaker, slower and fatigable with age and that the dynamic remodeling of NMJ structure would be a cause of age-related muscular atrophy. This may cause reduced voice strength and swallowing function. Other authors have studied laryngeal muscle atrophy induced by nerve injury [18], to better understand the spasmodic dysphonia syndrome [1,32,35]. The primary treatment for the most common type of this disorder involves direct injection of botulinum toxin into the thyroarytenoid muscle [12], a procedure for which it is essential to know the location of the NMJ.

Thus we conclude that the similarity of the random distribution of the NMJ of the opossum thyroarytenoid muscle to that observed in rats and humans [8,15] may represent an anatomical reference and may be used as another animal experimental model for these studies.

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