Distribution of Neuromuscular Junctions in Laryngeal and Syringeal Muscles in Vertebrates

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ABSTRACT

Vertebrates are capable of producing a variable sound spectrum. In mammals, lissamphibia, and reptiles, the larynx is the vocal organ responsible for sound production, whereas in birds it is produced by the syrinx, an avian organ located at the base of trachea. The distribution of neuromuscular junctions responsible for the fine control of laryngeal muscle (LM) and syringeal muscle (SM), although studied with some detail in human LM, remains mostly unknown in other vertebrates. In the present study, we analyzed the distribution of motor end plates (MEPs) in LM/SM of different vertebrate classes using the histochemical detection of acetylcholinesterase: the thyroarytenoid and cricoarytenoid LM of mammal (human, rat, and rabbit) and cricoarytenoid LM of nonmammalian (frog and avian) species and the tracheobronchial SM of rooster and pigeon. In humans and frogs/avians, MEPs were distributed diffusely along, respectively, the thyroarytenoid-cricoarytenoid and the cricoarytenoid LM fibers, whereas in rats and rabbits, MEPs were concentrated in a transverse band located in the middle of thyroarytenoid and cricoarytenoid muscle fibers. In roosters and pigeons, MEPs were distributed diffusely along SM fibers. The highly diffuse MEP distribution along human thyroarytenoid and cricoarytenoid fibers indicates that these muscles can markedly change their degree of contraction, which may contribute for the large range of different sounds produced by human vocal folds. The same rationale was applied to discuss the possible functional significance of the morphological distribution of MEPs along the LM/SM of the other vertebrates analyzed. Anat Rec Part A 288A:543–551, © 2006 Wiley-Liss, Inc.

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It is well known that vocalization varies significantly among vertebrates (Kardong, 2002). Although most aspects of vocal production are essentially similar between the vocal tracts of humans and other animals, a few key differences underlie vocal specificity along vertebrates: the importance of resonance capacity of the higher portion of the vocal tract, the position of the larynx in the throat, the capacity of vocal imitation, and the sophistication of nervous motor control over vocal articulates (Fitch, 2000; Fitch and Hauser, 2002). Even between mammals, two gross morphological differences are particularly prominent in nonhuman mammals and do not exist in humans: air sacs and vocal membranes. The former are present in bats and primates, whereas the latter are present in several primates, including apes (Mergell et al., 1999). Moreover, the organ responsible for sound production is not the same along vertebrates with the larynx and vocal folds being responsible for sound production in mammals, rep-

tiles, and lissamphibians (Kardong, 2002), whereas in birds, this role is played by a special subtracheal structure, the syrinx. Finally, the thyroid cartilage is present in the larynx of mammals but is absent in the other verte-

brate classes. This fact implies that the role of the thyroarytenoid laryngeal muscle in phonation and as anatomical glottal sphincter in mammals is played by the cricoarytenoid muscle in lissamphibia (phonation and anatomical sphincter) and avians (just as glottal sphincter) (George and Berger, 1966; Storer et al., 1979; Kardong, 2002).

In anuran lissamphibians, vocal communication is crucial in their social behavior and thus they can have a complex vocalization pattern, mainly in males (Storer et al., 1979; Boyd et al., 1999; Kelley, 2004). The elaborated song production in songbirds is thought to parallel human speech in several aspects, namely, dependence on learning (Marler, 1970), gradual motor development (Marler and Peters, 1982; Podos et al., 1995), lateralized brain specialization areas for production and perception (Nottebohm, 1971; Vicario, 1993; Wild, 1993), and importance of vocal tract movements in many aspects of song production (Hoese et al., 2000). In contrast, very little is known about reptile vocalization (Young et al., 1995; Hartdegen et al., 2001; Sacchi et al., 2004), and little or nothing is known about vocal production in most nonpasserine birds and most mammalian orders (Fitch and Hauser, 2002).

The understanding of animal vocal production and its motor innervation is still largely unknown (Fitch, 2000). However, taking into account that the contraction of muscle fibers is mediated by motor units and their neuromuscular junctions, it is possible that the degree of vocal variability depends, at least in part, on the number and distribution of motor end plates (MEPs) along laryngeal muscle (LM) and syringeal muscle (SM). However, very few studies have focused on the fine anatomy of motor units in vocal muscles. In what concerns mammals other than humans, only two other studies analyzed the distribution of the motor innervation of the rat larynx (Pais-Clemente and Lima-Rodrigues, 1996; Inagi et al., 1998). To the best of our knowledge, no studies have been performed on the anatomy of laryngeal/syringeal fine muscle motor control in other mammals and other vertebrate taxa, including lissamphibia and birds.

In humans, the lateral cricoarytenoid and thyroarytenoid muscles are very important in sound production since they are essential in closing the glottis (by rotating the arytenoid cartilages medially) and in pitch control (Greene, 1989; Williams et al., 1999). The increasing clinical importance of botulinum toxin therapy to block thyroarytenoid and cricoarytenoid MEP in laryngeal distonia (Blitzer et al., 1986; Castellanos et al., 1994; Bielamowicz et al., 2002; Tisch et al., 2003; Maronian et al., 2004) requires a deeper knowledge of human laryngeal motor innervation in order to better understand the nature of this disease. However, the pattern of motor innervation of the thyroarytenoid and cricoarytenoid muscles is still a matter of discussion. MEPs distributed diffusely along LM with no recognized band or any cluster arrangement (Rosen et al., 1983; Périé et al., 1997), covering two-thirds of the vocal folds (Rossi and Cortesina, 1965a, 1965b), or with a clear higher density in LM middle third (Pais-Clemente and Lima-Rodrigues, 1996; Sheppert et al., 2003) have been described.

Taking into account the relevant role of thyroarytenoid muscles in mammals (phonation and glottal sphincter), the cricoarytenoid muscles in mammals (phonation), lissamphibia (phonation and glottal sphincter), and avians (glottal sphincter) and of the syringeal muscles in avians

(phonation), we evaluate the pattern of fine motor innervation of these muscles in vertebrates. The present study analyzes the general distribution and morphology of MEPs in the thyroarytenoid and/or cricoarytenoid LM of three mammalian (human, rat, and rabbit), two avian (rooster and pigeon), and one lissamphibian (frog) species and in the tracheobronchial SM of the rooster and pigeon.

MATERIALS AND METHODS

Six laryngeal thyroarytenoid muscles from male adult rat (Wistar strain, obtained from Charles Rivers, Barcelona, Spain), rabbit (*Oryctolagus cuniculus*), and frog (*Rana perezi*) larynxes, six cricoarytenoid muscles from the rat, rabbit, frog, rooster (*Gallus gallus*), and male pigeon (*Columba livia*) larynxes, and six syringeal (bronchotracheal) muscles from the rooster and pigeon syrinxes were obtained after anesthetizing the animals with ether. Human vocal folds were obtained from six autopsy specimens. LM and SM were removed and immediately immersed in buffered 10% formalin, at pH 7.4, for 24 hr at room temperature. In order to obtain serial longitudinal sections of the muscle fibers, LM and SM were oriented appropriately and cut into 50 μm sections in a cryostat.

For identification of MEPs, we performed a histochemical detection of acetylcholinesterase activity by adapting the method described by Koelle and Friedenwald (1949). Briefly, sections were incubated in Koelle's medium for 2 hr with final staining in a 5% ammonium sulfide solution for 15 min. Sections were then placed in polylisine slides and mounted in entellan. The maintenance of proper pH of the reaction mixture and the addition of a selective pseudocholinesterase inhibitor (Iso-OMPA), combined with control sections where the reaction was performed without substrate (acetyltiocholine iodide), allowed the identification of a specific staining for acetylcholinesterase activity. All LM and SM serial sections were then analyzed in a light microscope Axioskop 2 plus (Carl Zeiss, Germany) and appropriate images of MEP distribution in the different species studied were taken using an Axiocam HRC camera and AxioVision 3.1 software (Carl Zeiss).

RESULTS

In humans, both the thyroarytenoid (Fig. 1B–D) and cricoarytenoid laryngeal muscles presented a diffuse pattern of MEP distribution along their muscle fibers, with the middle zone (Fig. 1C) showing a higher density of MEPs, followed by the posterior (Fig. 1D) and the anterior (Fig. 1B) parts of the muscles. By contrast, in the rabbit (Fig. 1A) and the rat (Fig. 2A and B), both the thyroarytenoid (Figs. 1A and 2A) and cricoarytenoid (Fig. 2B) muscles presented their MEPs concentrated in a transverse band located in the middle of the muscle fibers. As in humans, the frog (Fig. 2C), the rooster (Fig. 3B), and the pigeon (Fig. 3D) showed MEPs diffusely distributed along the cricoarytenoid muscles. In what concerns the syrinx of the rooster (Fig. 4A) and pigeon (Fig. 4C), the distribution of MEPs along tracheobronchial syringeal muscles showed, in both cases (Fig. 4B and D, respectively), a scattered pattern along their entire extension.

In what concerns the morphology of laryngeal MEPs, they were round in the rat (Fig. 5A), rabbit, and human (Fig. 5D), whereas in the rooster (Fig. 5B), pigeon, and frog (Fig. 5C), they were elongated, reaching frequently a long fusiform profile. Syringeal MEPs were elongated in
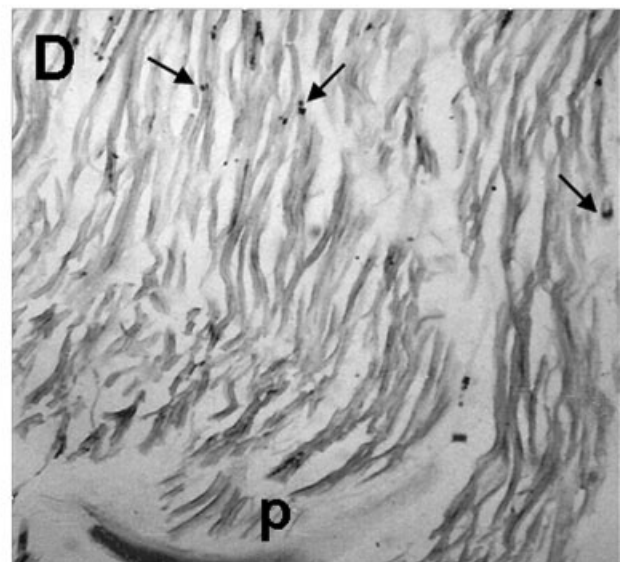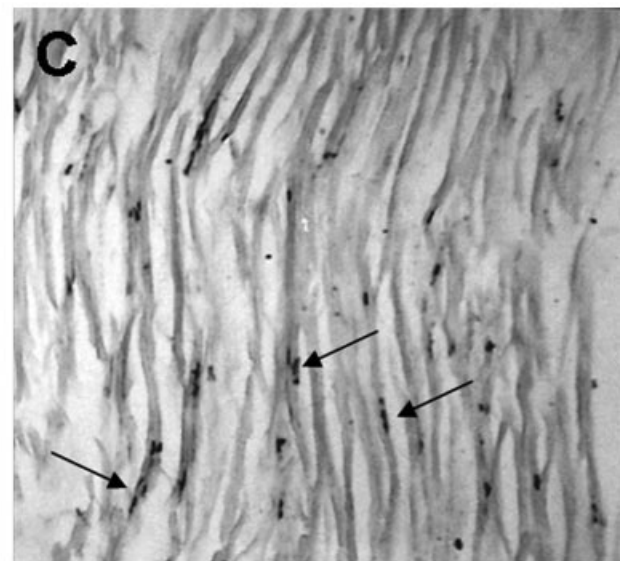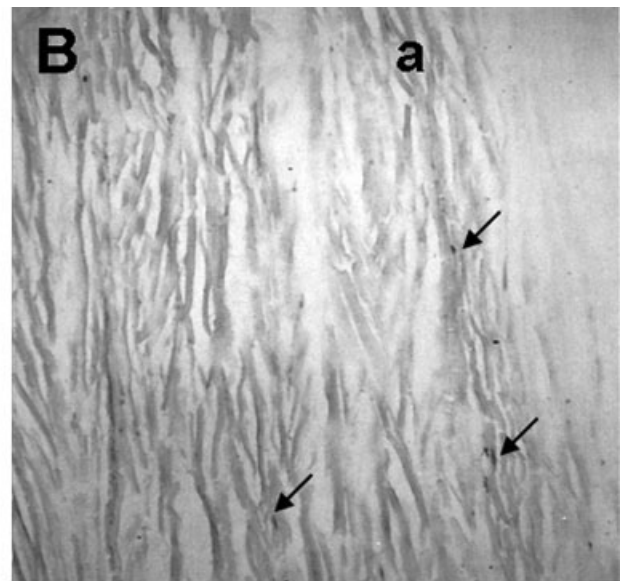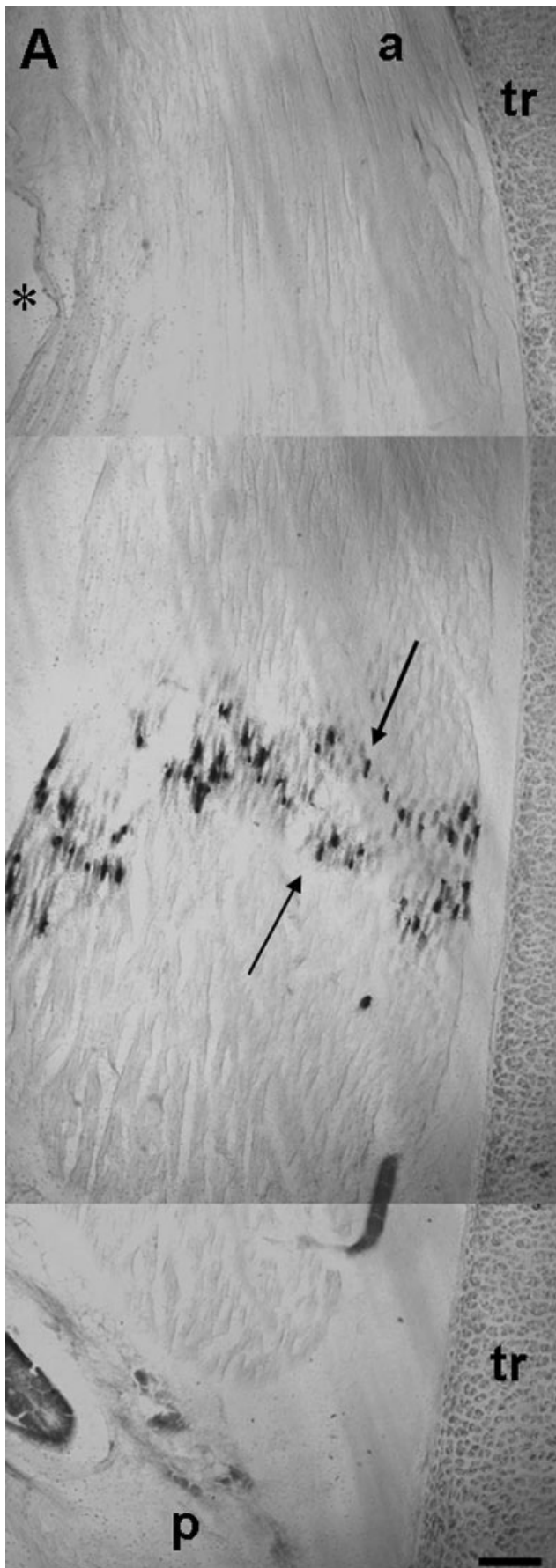
Fig. 1. Distribution pattern of motor end plates in the thyroarytenoid muscles of rabbit (**A**) and human (**B–D**). Note in the rabbit the concentration of MEPs in a transverse middle band (large arrows) of the thyroarytnoid muscle, whereas in human they are diffusely distributed along different areas of this glottal muscle, namely, the anterior (B), middle (C), and posterior (D) portion, although with a higher density in the middle portion. In the human larynx, small arrows indicate a few MEPs in the anterior and posterior areas, whereas in the medial zone, large arrows indicate multimotor end plates. Asterisk, laryngeal tract; tr, thyroid cartilage; p, posterior; a, anterior. Scale bar = 75 μm (A); 200 μm (B–D).
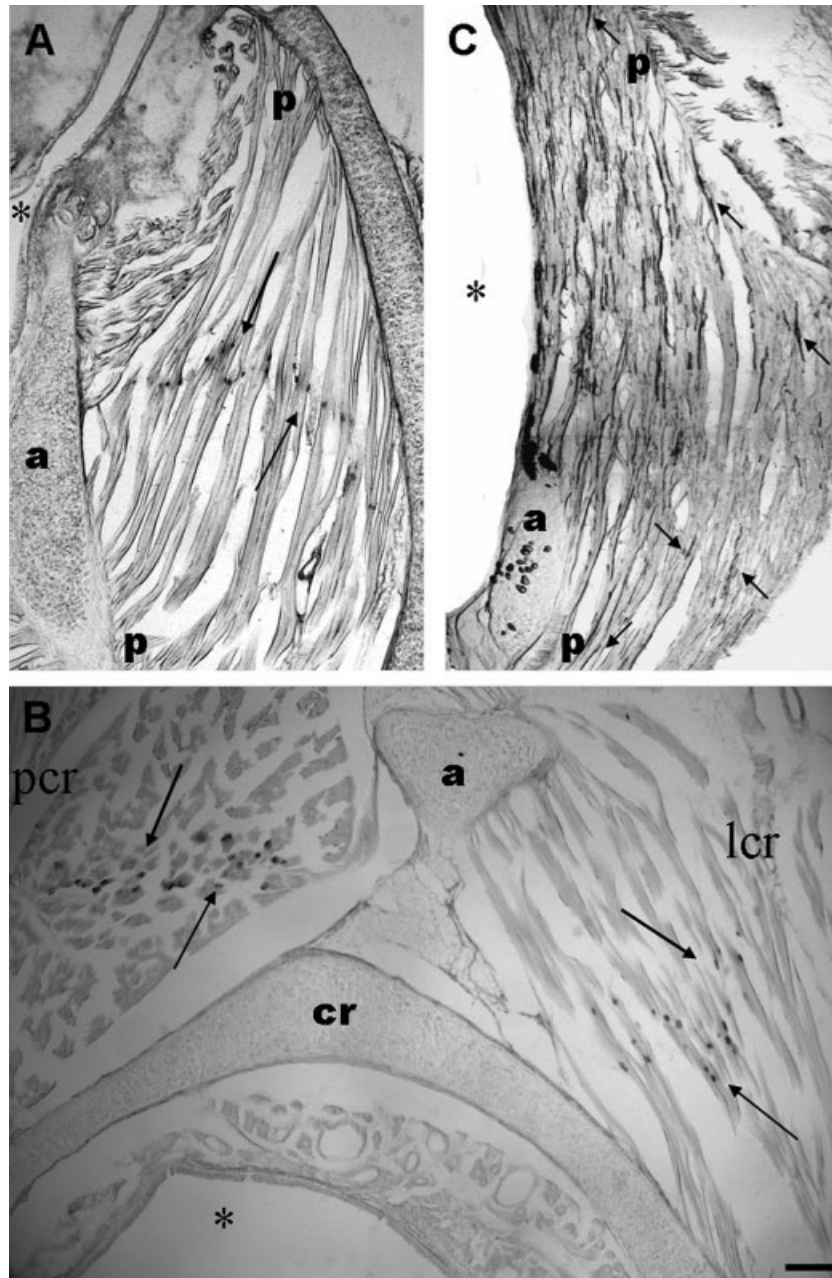
Fig. 2. Distribution pattern of motor end plates in the thyroarytenoid (**A**) and lateral/posterior cricoarytenoid (**B**) muscles of the rat and the cricoarytenoid muscles of the frog (**C**). In both laryngeal muscles analyzed in the rat, MEPs are concentrated in a transverse middle band (large arrows), whereas in the frog cricoarytenoid muscle, they are scattered along the muscle fibers (small arrows). Asterisk, laryngeal tract; a, arytenoid cartilage; cr, cricoid cartilage; lcr, lateral cricoarytenoid muscle; pcr, posterior cricoarytenoid muscle; p, muscle insertion points. Scale bar = 100 μm.

both the rooster (Fig. 5E) and pigeon (Fig. 5F). In humans, MEPs were aggregated in groups in the same fiber, forming multimotor end plates (Fig. 5D). In the frog vocal muscles (Fig. 4B) and in SM (Fig. 3B and D) and LM (Fig. 2B and D) of the rooster and pigeon, the fibers seem also to have several MEPs along their extension.

DISCUSSION

The data obtained in the present study indicate that the fine motor innervation of the LM and SM analyzed, which have important functions in vocalization, varies within different mammals and vertebrate taxa. Interestingly, the distribution pattern of neuromuscular junctions along the extension of LM in anuran lisamphibia and SM in birds is more similar to that present in human vocal folds than to the other mammals studied (rat and rabbit).

The sound source in mammals is the larynx (Fitch and Hauser, 2002), with the thyroarytenoid and cricoarytenoid muscles being relevant muscles supporting phonation in humans (Greene, 1989; Williams et al., 1999) and other
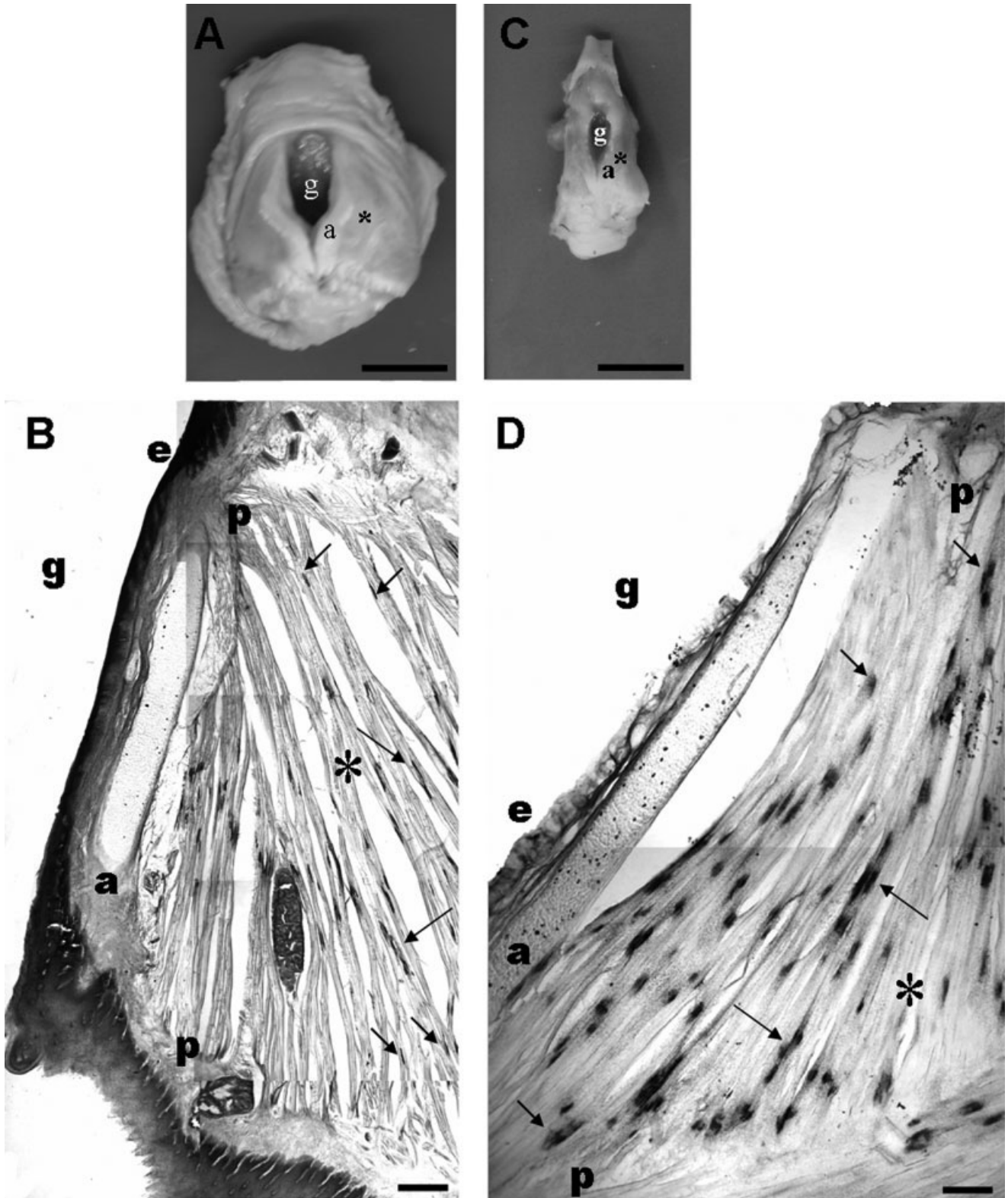
Fig. 3. Distribution pattern of motor end plates in the cricoarytenoid muscles of the rooster (**A** and **B**) and pigeon (**C** and **D**). In the external macroscopic morphology of the rooster (A) and pigeon (C) larynxes, it is possible to identify the crycoarytenoid cartilage (a), the crycoarytenoid muscle (asterisk), and the glottal aperture (g), which are shown at the microscopic level in B (rooster) and D (pigeon). In both avians, MEPs (arrows) are diffusely distributed (B, D) along the muscles, which may present several MEPs innervating the same muscle fiber (large arrows). e, laryngeal epithelium; p, muscle insertion points. Scale bar = 1 cm (A and C); 300 μm (B); 100 μm (D).
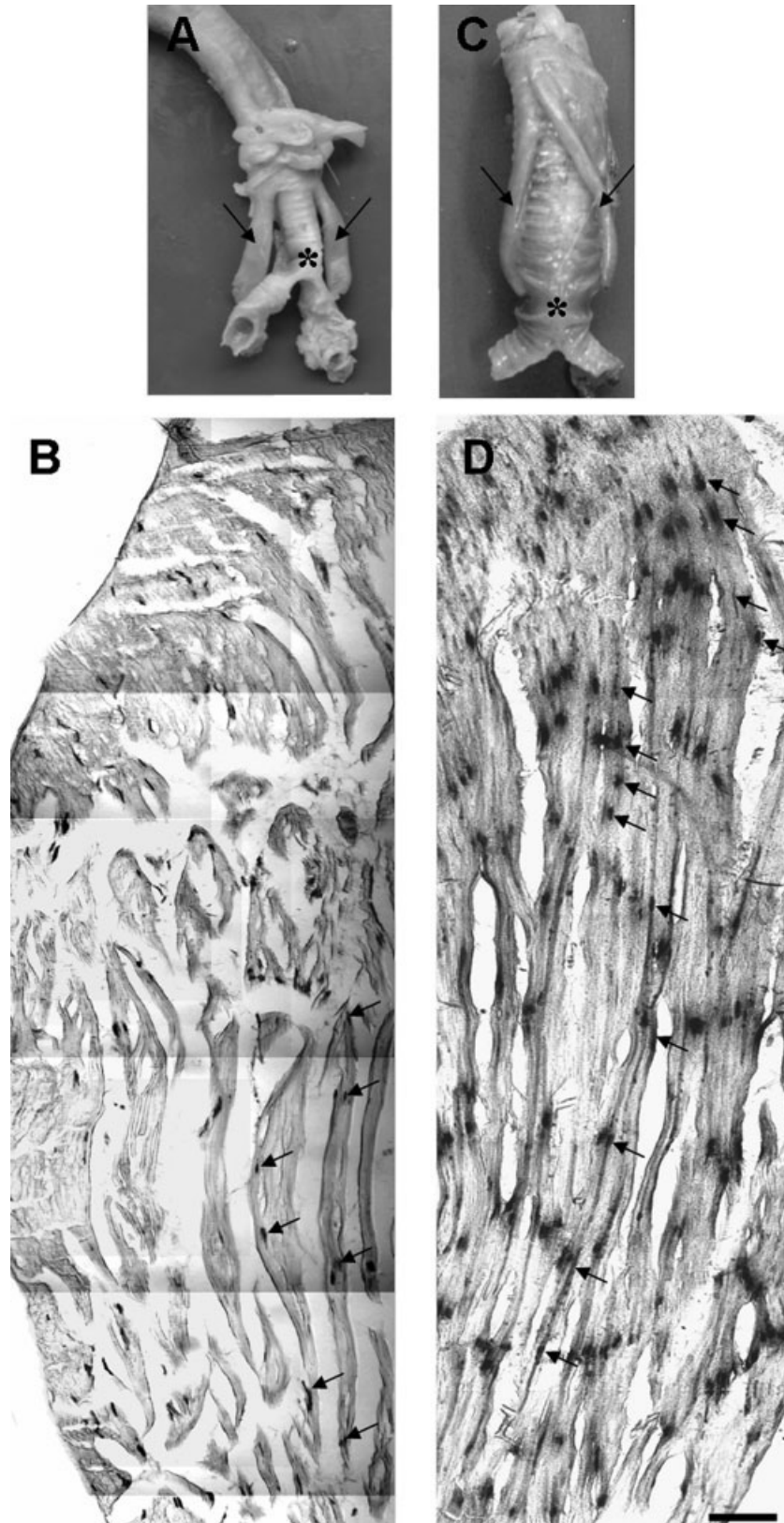
Fig. 4. Distribution pattern of motor end plates in the syringeal muscles of the rooster (**A** and **B**) and pigeon (**C** and **D**). Note in the external morphology of the rooster (A) and pigeon (C) the tracheobronchial syrinxes (asterisks) and syringeal muscles (arrows). In both avians, MEPs are diffusely distributed (B and D) along the extension of the syringeal muscles, with arrows indicating different series of neuromuscular junctions located apparently along the same fibers. Scale bar = 1.2 cm (A); 220 μm (B); 0.4 cm (C); 150 μm (D).

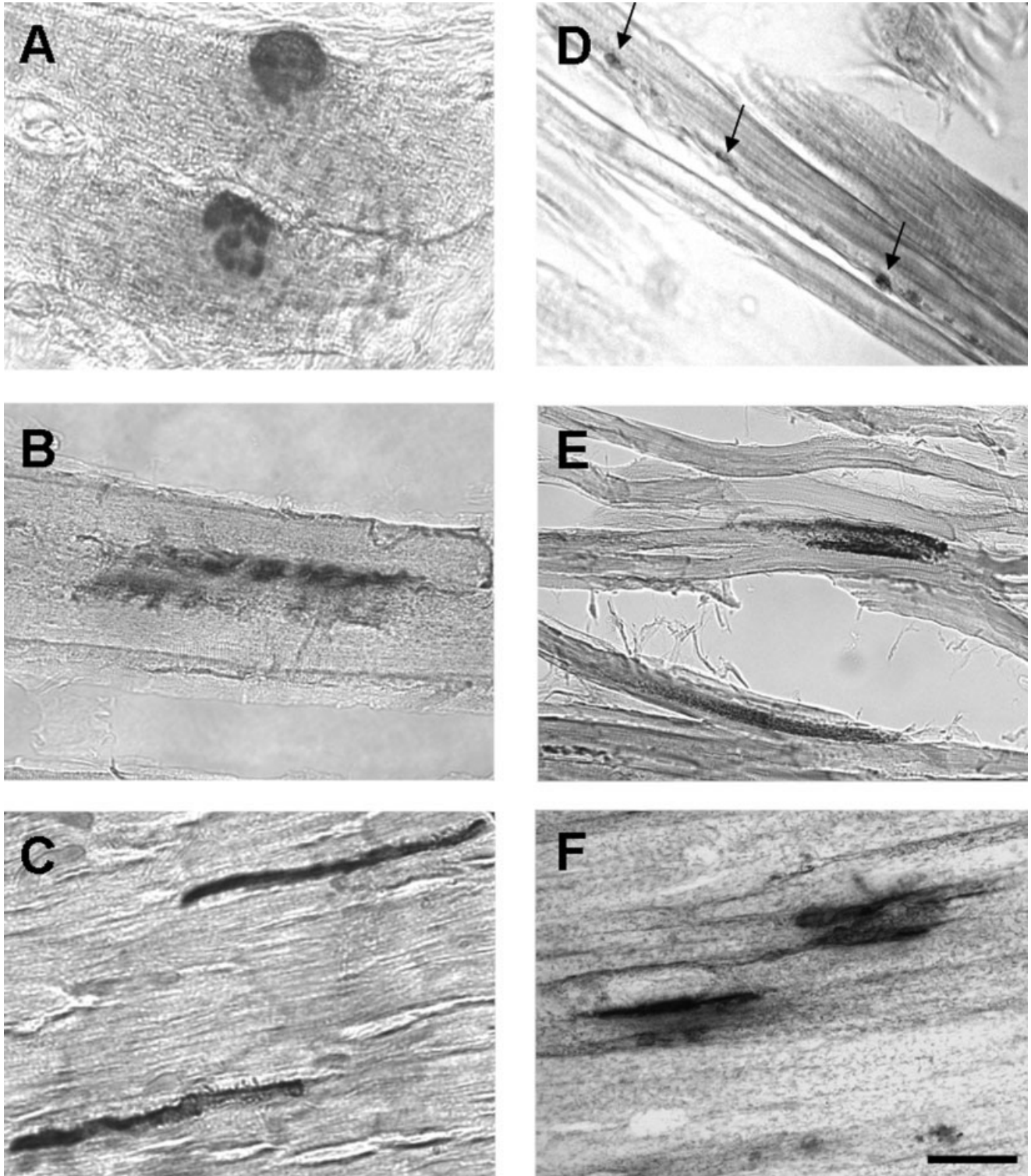Fig. 5. Morphology of MEPs in the laryngeal muscles of mammalian [rat (**A**) and human (**D**)] and nonmammalian [rooster (**B**) and frog (**C**)] vertebrate classes and in syringial muscles of the rooster (**E**) and pigeon (**F**). A similar round configuration of MEPs in laryngeal muscles of mammals (A and D) is in contrast with an elongated/fusiform morphology of MEPs in birds (B) and lissamphibia (C). An elongated morphology is also a characteristic of MEPs in avian syringial muscles (E and F). Note in the human vocal muscles several MEPs in a row are present in the same muscle fiber (multimotor end plates; arrows). Scale bar = 10 μm (A); 80 μm (B); 10 μm (C); 100 μm (D).

mammals. The differences between the human and rat vocal folds in what concerns MEP distribution confirmed our and other previous studies in humans (Pais-Clemente and Lima-Rodrigues, 1996; Sheppert et al., 2003) and rat (Pais-Clemente and Lima-Rodrigues, 1996; Inagi et al., 1998). Human vocal folds showed a clear scattered distribution of MEPs along LM, with a higher density in the middle third of the muscle, whereas in the rat (and rabbit), MEPs were concentrated along a narrow band at the midbelly of the vocal muscles. Although several factors are important for vocalization (see Introduction), the different MEP distribution between human and rat/rabbit vocal folds suggests a different motor innervation of laryngeal intrinsic muscles in humans when compared to the rat and rabbit. Vocal fold muscles in humans present an elaborated motor control since different muscle fibers are innervated at different rostrocaudal positions, thus allowing a complex pattern of possibilities for muscle contraction. By contrast, the fact that all muscle fibers in the rat and rabbit vocal folds are innervated approximately at the same rostrocaudal location suggests a more restricted mode of contraction of the entire LM muscles.

It is well known that some anuran lissamphibia and avian species can produce a complex pattern of different sounds, respectively, from the larynx and the syrinx (Storer et al., 1979). Applying the same rationale used for mammals, the diffuse distribution of MEPs (as in human vocal muscles) in lissamphibia cricoarytenoid muscles and avian SM may contribute to their vocal versatility. In what concerns the avian larynx, it does not appear to have the capacity for producing sound (McClelland, 1989), but may be used instead to modify sound originated from the syrinx (Harris et al., 1968; White, 1968). The diffuse distribution of MEPs in the rooster glottal muscles suggests some elaboration on the functions played by the avian cricoarytenoid muscles.

Given the present results, it is possible that the diffuse distribution of MEPs in the thyroarytenoid and cricoarytenoid in humans, in the latter muscle in anuran lissamphibia, and in the SM in birds may contribute to the fact that humans can talk and produce voice, as some lissamphibia and birds can produce very complex sounds. This difference in the fine motor innervation of vertebrate vocal muscles does not seem to be correlated with the dimension of muscle extension. In fact, animals with large larynxes can have a scattered (human, rooster) or centered (rabbit) MEP distribution in the cricoarytenoid muscles, whereas larynxes from smaller species can also have a scattered (frog, pigeon) or centered (rat) MEP distribution in the same muscles. Thus, in what concerns LM/SM motor innervation, humans are members of a group including birds and lissamphibia and excluding other mammals. Interestingly, several nonprimate species are able to mimic human speech to a remarkable degree, as highly trained parrots can have a large vocabulary of sounds that they use with a communication objective (Pepperberg, 1991). This indicates that in terms of speech by vocal imitation, humans are also members of an apparently uncharacteristic group that includes birds (and aquatic mammals such as dolphins) but excludes nonhuman primates (Fitch, 2000). However, physiological studies are needed to elucidate on a possible correlation between MEP distribution in vocal muscles and vocalization.

The morphological analysis of MEPs in the LM/SM of animal species studied also revealed clear differences be-tween species: in mammalians (rat, rabbit, and human), the motor end plates had round configuration, whereas in birds (rooster, pigeon) and lissamphibia (frog), MEPs were elongated and, frequently, fusiform. This suggests that, contrary to the distribution of MEPs, the morphological pattern of these structures is similar between species of the same vertebrate taxa. Studies are needed in order to elucidate the physiological significance of the change in laryngeal MEP morphology along different vertebrate classes.

The higher concentration of MEPs in the middle third of the human vocal folds implicates a stronger tension of contraction in that particular area. This may contribute to the higher incidence of vocal cord nodes (kissing nodes) in the correspondent region of the vocal fold epithelium (Pontes et al., 2002). This observation suggests that the intramuscular injection of botulinum toxin in the middle third of the thyroarytenoid and cricoarytenoid LM may be of clinical importance not only for the treatment of spasmodic disphonia (Blitzer et al., 1986; Castellanos et al., 1994; Bielamowicz et al., 2002; Tisch et al., 2003; Maronian et al., 2004), but also for recovering from kissing nodes. However, other phonetic parameters such as aerodynamics, subglottal pressure, and amount/mode of phonation are important etiological factors that should also be taken into account for the treatment of vocal cord nodes (Gunter, 2004).

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