

Neuroanatomy of the equine dorsal cricoarytenoid muscle: Surgical implications

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Summary

Reason for performing study: Studies are required to define more accurately and completely the neuroanatomy of the equine dorsal cricoarytenoid muscle as a prerequisite for developing a neuroprosthesis for recurrent laryngeal neuropathy.

Objective: To describe the anatomy, innervation, fibre types and function of the equine dorsal cricoarytenoid muscle.

Methods: Thirty-one larynges were collected at necropsy from horses with no history of upper airway disease and 25 subjected to gross dissection. Thereafter, the following preparations were made on a subset of larynges: histochemical staining (n = 5), Sihler's and acetylcholinesterase staining for motor endplates (n = 2). An additional 6 larynges were collected and used for a muscle stimulation study.

Results: Two neuromuscular compartments (NMC), each innervated by a primary nerve branch of the recurrent laryngeal nerve, were identified in all larynges. Stimulation of the lateral NMC produced more lateral displacement of the arytenoid cartilage than the medial NMC (P<0.05). The medial NMC tended to rotate the arytenoid cartilage dorsally. Motor endplates were identified at the junction of the middle and caudal thirds of each NMC. If fibre type grouping was present it was always present in both NMCs.

Conclusions: The equine dorsal cricoarytenoid muscle has 2 distinct muscle NMCs with discrete innervation and lines of action. The lateral NMC appears to have a larger role in increasing cross-sectional area of the *rima glottidis*.

Potential relevance: This information should assist in planning surgical reinnervation procedures and development of a neuroprosthesis for recurrent laryngeal neuropathy.

Introduction

Recurrent laryngeal neuropathy (RLN) is a major cause of poor performance in racehorses and affects 1.6–8% of Thoroughbreds (Goulden and Anderson 1982; Lane *et al.* 1987; Dixon *et al.* 2001; Brown *et al.* 2005). The caudal (recurrent) laryngeal nerve

innervates the dorsal cricoarytenoid (DCA) muscle and produces abduction of the vocal process of the arytenoid cartilage (Quinlan *et al.* 1982; Dyce *et al.* 2002; König and Liebich 2004). RLN results in progressive atrophy of the DCA muscle and associated loss of arytenoid cartilage abduction (Cole 1946; Duncan *et al.* 1974; Cahill and Goulden 1986). At exercise, this produces *rima glottidis* narrowing and increased inspiratory impedance and noise (Derksen *et al.* 1986; Brown *et al.* 2005).

The first prosthetic laryngoplasty was described by Marks *et al.* (1970). This technique is the current standard for treating equine RLN (Kidd and Slone 2002; Dixon *et al.* 2003a; Brown *et al.* 2004) and has a 48–66% success rate in racehorses (Strand *et al.* 2000; Davenport *et al.* 2001; Dixon *et al.* 2001; Kidd and Slone 2002), although a much higher success rate (73–91%) is reported in horses performing at submaximal exercise (Dixon *et al.* 2003b). Restoration of physiological function through reinnervation and nerve-muscle pedicle transplant techniques has produced positive results (Ducharme *et al.* 1989a,b; Fulton *et al.* 1991, 1992). These reinnervation techniques have similar success rates, lower morbidity, but longer post operative recovery than prosthetic laryngoplasty. In canine and ovine studies, functional electrical stimulation of the DCA muscle has produced vocal fold abduction and overcome airway obstruction (Broniatowski *et al.* 1985; Kim *et al.* 1987; Sanders 1991; Zrunek *et al.* 1991). In addition, functional neuromuscular stimulation has been shown to increase muscle contractility in denervated canine DCA muscle (Zealear *et al.* 2000). Stimulation also protected the muscle from atrophy by preventing muscle weight loss and *type 2* fibre deterioration and rescued muscle fibres from undergoing fibrosis (Zealear *et al.* 2000). A neuroprosthesis offers the possibility of rapid restoration of function and this requires a more thorough neuroanatomical knowledge. Current descriptions of the equine DCA muscle refer only to one muscle neuromuscular compartment (NMC) (Nickel *et al.* 1979; Dyce *et al.* 2002; König and Liebich 2004). The human literature suggests that the human DCA muscle (referred to as the posterior cricoarytenoid muscle) consists of 2 functional compartments (Sanders *et al.* 1994; Brandon *et al.* 2003). Three compartments have been described in the dog (Sanders *et al.* 1993). Little is known about the internal neuromuscular characteristics, such as the presence of discrete

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muscle NMCs, nerve supply patterns and banding of motor endplates of the equine DCA muscle. This is partially because conventional anatomical methods cannot trace individual nerve branches in a given muscle. Recently, Sihler's stain has been used to investigate the innervation of skeletal muscles, especially in the upper respiratory tract (Wu and Sanders 1992; Mu and Sanders 2000, 2001; Ren and Mu 2005; Zur *et al.* 2004). Sihler's stain is a whole mount technique that stains all nerve tissues while clearing soft tissue, allowing the distribution and branching of any nerves within the tissue to be precisely identified.

The aims of this study were to develop a more precise biomechanical understanding of the equine DCA muscle; determine the location of the motor endplates, the exact pathway of the abductor branch of the recurrent laryngeal nerve and describe the intramuscular morphological characteristics of the equine DCA muscle.

Materials and methods

Thirty-one larynges were collected at necropsy less than 60 min after euthanasia from horses with no history of upper airway disease (mean age 8 years; 13 geldings, 12 mares; 15 Thoroughbred, 3 Standardbred, 6 Quarter Horses, 1 Warmblood; mean bodyweight 442 kg, range 380–450 kg). The first 25 larynges were subject to gross dissection. The following preparations were then made on subsets of larynges: histochemical staining ($n = 5$); Sihler's staining to characterise innervation ($n = 3$); acetylcholinesterase staining to identify neuromuscular endplates ($n = 2$); and an electrophysiological study ($n = 6$).

Gross dissection

Isolated larynges were obtained from fresh cadavers. Each DCA muscle was dissected and photographed with a digital camera¹ from a dorsal and dorsolateral aspect bilaterally to catalogue the presence of individual NMCs. The DCA appearance, number of NMCs, and mode of attachment to the underlying laryngeal cartilages were described. The fibre angle of each NMC midpoint relative to the sagittal ridge of the cricoid cartilage was measured using a goniometer. In 5 larynges, the DCA muscle was elevated laterally from the cricoid cartilage to seek further NMCs at a deeper level.

Dorsal cricoarytenoid muscle specimens from 2 animals were isolated and fixed near their resting length in 10% formalin. Muscle fibres were identified with a dissection microscope (Wild M5) and gross fibre bundle length was measured. The whole muscles were placed in a 10% nitric acid solution for 7–10 days. The preparation was observed daily until there was connective tissue breakdown. Fibre bundles were placed in 100% glycerin for storage and to facilitate manipulation.

Sihler's stain

Sihler's stain was used to map the i.m. nerve supply of 4 equine DCA muscles (Mu *et al.* 2000, 2001). Briefly, specimens were fixed in 10% formalin for 4–8 weeks; macerated in 3% KOH solution for 3 weeks with several changes; decalcified in Sihler's Solution I (acetic acid, glycerin, chloral hydrate) for 2 weeks with several changes; stained with Sihler's Solution II (contains Ehrlich's haematoxylin) for 4 weeks; destained in Sihler's

Solution I for 3–4 h; nerve darkening with 0.05% lithium carbonate solution for 1 h; cleared in 50% glycerin for 2 or 3 days; and finally preserved with 100% glycerin. At the completion of staining, the stained specimens were removed from the cartilage through careful dissection and the muscle transilluminated by a xenon light source (Model 610)² and photographed.

Acetylcholinesterase stains

Two whole DCA muscles were stained using acetylcholinesterase (AChE) as described previously (Ypey 1978; Meyers and Hermanson 1994). Briefly, the freshly dissected whole muscle was placed into acetate-buffered (pH 5.0–6.0) acetylthiocholine iodide, CuSO₄ and copper glycine for 48–72 h at 37°C, washed with distilled water, then dipped briefly (approximately 30 s) into dilute ammonium sulphide solution. This technique produced white staining of motor endplates and light brown staining of muscle fibres. The stained specimens were photographed.

Histochemical study

The DCA muscle was sharply dissected bilaterally from 7 larynges and weighed. The midsection of each NMC removed, mounted on a cork block with 5% gum tragacanth and quick frozen in isopentane cooled to approximately -140°C by liquid nitrogen, then sectioned at 10 μ m using a freezing microtome³. The muscle samples were mounted so that cross-sectional slices of the muscle fibres were presented. Sections from each NMC were stained with myosin-ATPase. Myosin-ATPase reactions followed preincubation in barbital acetate buffer at pH 4.3, 4.4, or 4.5 for 5 min, with subsequent incubation in ATP-barbital acetate buffer at pH 9.4 for 30 min at 37°C (Hermanson and Cobb 1992). This stain renders *type I* (slow twitch) muscle fibres black and *type II* (fast twitch) muscle fibres unstained (Brooke and Kaiser 1970).

Low power fields were selected randomly from each section and fibres identified as *type I* or *type II* until at least 1000 fibres were counted. The presence of intermediate stained fibres, suggestive of *type IIa* or *IIb* myosin isoforms, was not quantified. The presence or absence of fibre type grouping, an indication of reinnervation, was assessed subjectively (Cahill and Goulden 1986; Fulton *et al.* 1992).

Electrophysiological study

In 6 specimens, the oesophagus and *cricopharyngeus* were removed, and spherical fluorescent markers⁴ attached to the abaxial and ventral aspect of the corniculate process of each arytenoid cartilage. Lateral movement was assessed in a transverse plane (x), dorsal movement in a frontal plane (y) and caudal movement in a sagittal plane (z). Planes were defined relative to each fluorescent sphere. Two high-speed cameras⁵ (200 frames/s) were arranged orthogonally to record movement of the fluorescent spheres in each plane. A ruler was used to provide scale. Two electrodes were placed 1.5 cm apart in a rostrocaudal direction. A rostral electrode was placed in the medial NMC and a second electrode, 1.5 cm caudally in the lateral NMC. Any connective tissue lying over the sagittal ridge of the cricoid cartilage was transected to avoid inadvertent stimulation of the contralateral DCA muscle. The DCA muscle was stimulated at 50 Hz and 0.3 V for 0.3 s. In order to isolate the affect of contraction of each NMC, the left and right DCA muscles were

tested in each of 2 conditions. In the first condition, a series of 3 pulses 1 s apart were applied to both NMCs. In the second condition, the insertion of one NMC onto the muscular process of the arytenoid cartilage was transected randomly, so only the remaining NMC (lateral or medial) could exert a force. The transection was continued until the cricoarytenoid joint was entered to ensure none of that NMC's fibres remained. A series of 3 pulses was again applied. The other NMC was transected on the contralateral side. The time from euthanasia to completion of both testing conditions was less than 30 min for each larynx.

The video images from each camera were digitised using a video analysis software program⁶ and imported into a digital imaging program⁷ with a scale used to determine a coordinate at abduction (x, y, z) during stimulation relative to the resting position of each fluorescent marker (0,0,0). Figure 1 shows representative still images of the resting and abducted positions of the arytenoid cartilages viewed with orthogonal cameras.

Data analysis

The gross appearance of each muscle, fibre alignment, nerve branching and banding patterns of motor endplates within the DCA muscle were reported through sketches, photographs and written descriptions. Fibre type distribution, expressed as a percentage of total fibre numbers, for the lateral and medial NMCs was compared using a mixed effect model with specimen as a random factor. The presence or absence of fibre type grouping, defined as loss of normal mosaic pattern with clustering of several fibres of the same type, in each NMC was compared using Fisher's exact test. For the electrophysiological study, the data for

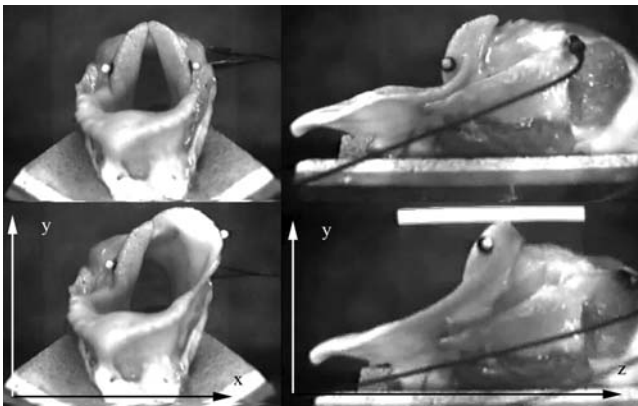


Fig 1: Representative still images of the resting and abducted positions of the arytenoid cartilages viewed with high speed orthogonal cameras.

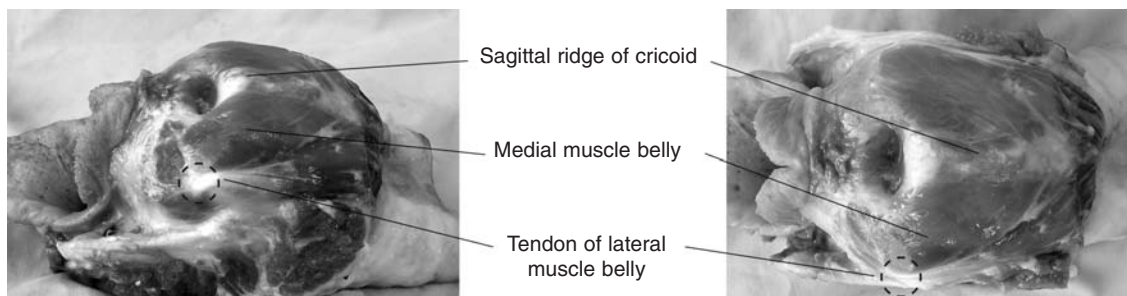


Fig 2: Dorsolateral and dorsal views of equine larynx. Rostral is to the left. Lateral NMC with rostral tendon and medial NMC with fibres running more acutely to sagittal ridge of cricoid. Dotted circle indicates muscular process of arytenoid cartilage.

each individual NMC were transposed to compare displacements between contralateral NMCs. The mean maximally abducted position produced by each NMC was compared in each plane using a mixed linear model with NMC as a fixed factor and larynx as a random factor. The maximally abducted position was correlated to the identity of the pulse (1–3) to assess the possible effect of fatigue. All statistical analyses were performed using S-plus⁸. Significance was set at $P < 0.05$.

Results

Morphological features

Each DCA muscle had 2 distinct NMCs ($n = 25$) (Fig 2). The lateral NMC was multi-penate caudally, with fibres running at an oblique angle (mean \pm s.d. $16 \pm 1.5^\circ$) to the sagittal ridge. These fibres converged at a superficial rostral tendon and inserted on the dorsolateral aspect of the muscular process of the arytenoid cartilage. The medial NMC inserted on the dorsomedial aspect of the muscular process of the arytenoid cartilage, and no tendon was present. The fibres of the medial NMC ran at an acute angle ($34 \pm 2^\circ$) relative to the sagittal ridge of the cricoid cartilage. This difference was significant ($P < 0.001$).

No discrete NMCs were identified deep to the medial and lateral NMCs. Over approximately 1.5 cm^2 of the rostral and medial portion of the cricoid lamina, muscle fibre attachment was absent. Thirty bundles were isolated containing 1–10 fibres near the mid-NMC of each muscle. The mean \pm s.d. length of the unattached muscle fibres in the 2 animals studied was $29.6 \pm 3.4 \text{ mm}$. The sample size was too small for further analysis.

Electrophysiological study

Stimulation of both NMCs together produced a lateral displacement (adjusted mean \pm s.e.) of $9.27 \pm 1.67 \text{ mm}$ and a dorsal (y) displacement of $15.11 \pm 1.58 \text{ mm}$. The lateral (x) displacement (adjusted mean \pm s.e.) produced by the lateral and medial NMCs was $10.23 \pm 1.70 \text{ mm}$ and $8.61 \pm 1.68 \text{ mm}$, respectively. This difference was significant ($P < 0.05$). The dorsal (y) displacement (adjusted mean \pm s.e.) produced by the lateral and medial NMCs was $14.7 \pm 1.61 \text{ mm}$ and $14.0 \pm 1.59 \text{ mm}$, respectively. This difference was not significant ($P = 0.17$). The medial NMC produced significantly less dorsal (y) displacement than both NMCs together ($P < 0.05$). There was no significant evidence of fatigue over the sequence of pulses ($P = 0.17$). Failure of one camera to record data prevented acquisition of complete data for displacement in the sagittal (z) plane.

In addition, the medial NMC appeared to have a tendency to rotate the arytenoid cartilage in a dorsocaudal direction. The degree of rotation could not be quantified by the single point reference system employed in this study.

Motor endplate banding pattern

The whole mount specimens stained for AChE showed that each DCA muscle NMC had a single motor endplate (MEP) band. On the dorsal surface, this was approximately 3 mm wide and located at the junction of the caudal and middle thirds of each muscle NMC (Fig 3). There was some staggering at the junction between the 2 NMCs. On the ventral surface against the dorsal fossa of the cricoid cartilage, the MEP band was approximately 2 mm wide and ran parallel to each branch of the recurrent laryngeal nerve.

Sihler's stain

In 4 larynges studied, Sihler's stain demonstrated 2 discrete primary branches of the recurrent laryngeal nerve innervating the medial and lateral NMCs in both the left and right DCA muscles

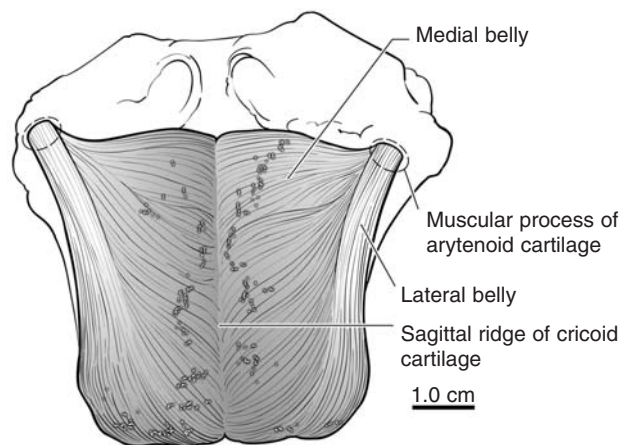


Fig 3: Motor endplate banding pattern in medial and lateral NMCs viewed from dorsal aspect.



Fig 4: Sihler's staining of right DCA muscle viewed from dorsal showing: Two primary nerve branches to the DCA muscle (white arrows); branch to the transverse arytenoid muscle (a); transected branch to the lateral cricoarytenoid muscle (b); muscle attachment to the sagittal ridge of the cricoid cartilage (c); and main recurrent laryngeal nerve (d).

(Fig 4). The recurrent laryngeal nerve ran on the dorsal aspect of the cricoid lamina before turning ventrally under the DCA muscle and dividing to enter each muscle NMC on its ventral surface at its longitudinal midpoint with the most rostral branch innervating the medial NMC of the DCA. The adductor branch of the recurrent laryngeal nerve continued and innervated the transverse arytenoid, lateral cricoarytenoid, *vocalis* and *ventricularis* muscles. Sihler's stain was effective in staining nerve tissue and rendering the surrounding tissue translucent. Some dissection and compression of the muscle sections was required to optimise characterisation of the nerve distribution.

Histochemical study

Mean weight of the left and right DCA muscles was 10.5 g (range: 5.4–15.9 g and 5.4–15.6 g, respectively). A distinct *epimyseum* was identified histologically in all sections. The medial NMC contained (adjusted mean \pm s.e.) 35.2 \pm 4.2% type 1 fibres and 64.8 \pm 4.3% type 2 fibres. The lateral NMC contained 38.9% \pm 4.2% type 1 fibres and 61.1 \pm 4.3% type 2 fibres. There was no significant difference in fibre type distributions between left and right sides or lateral and medial NMCs.

Fibre type grouping was absent in all of the right DCA muscles and present in 5 out of 7 of the left DCA muscles (Fig 5). This difference was significant ($P < 0.02$). If fibre type grouping was present, it was present in both NMCs of each DCA muscle.

Discussion

This study characterised the i.m. morphological characteristics of the equine DCA muscle, the exact pathway of the abductor branch of the recurrent laryngeal nerve, and the location of motor endplates and i.m. fibre type distribution.

The human literature (Sanders *et al.* 1994; Brandon *et al.* 2003) also describes the DCA muscle in terms of 2 NMCs as in the present study. In order to classify a muscle subunit as a NMC, it should be innervated by a discrete primary nerve branch and be separated from adjacent subunits by connective tissue (English and Letbetter 1982; English and Weeks 1987). In the present study it was noted that each muscle NMC is supplied by a primary nerve branch of the recurrent laryngeal nerve, and a connective tissue division between the lateral and medial compartments was found. The term neuromuscular compartment was used to describe the i.m. division of this muscle.

The electrophysiological technique described in this study was able to produce maximal abduction of the arytenoid cartilages as

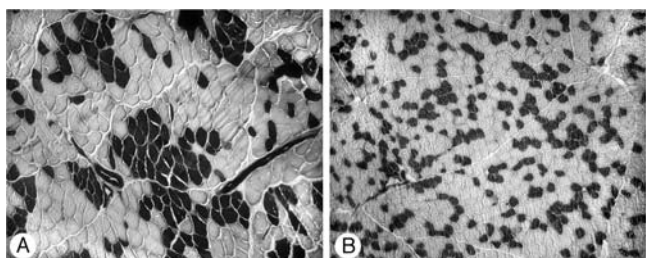


Fig 5: Myosin ATPase staining with acidic preincubation (type I fibres stain black and type II fibres stain light grey) demonstrating: (A) The presence of fibre type grouping characteristic of reinnervation in the lateral NMC of the left DCA muscle and (B) Normal mosaic fibre distribution in the lateral NMC of the right DCA muscle from the same horse.

described by Hackett *et al.* (1991). The lateral NMC appears to be most important in terms of lateral displacement and elevation of the arytenoid cartilage. The medial NMC tended to produce dorsal rotation of the arytenoid cartilage, although this could not be quantified. These results suggest that 2 NMCs perform similar but distinctive functions. This information is important, as it may suggest that the implantation of neuromuscular pedicle grafts should be targeted towards the lateral rather than the medial NMC. It is possible that concurrent inadvertent stimulation of the adductor muscles could damp the degree of abduction observed during stimulation, however, contraction of the adductor muscles was not observed in this study.

Although these horses were not subjected to endoscopic examination prior to euthanasia, there was no gross evidence of DCA muscle atrophy or a difference in left and right DCA muscle weights in any specimen. It is possible that a number of the horses could have had low *grade 2* (Hackett *et al.* 1991) RLN, however, *grade 3* and *4* RLN is usually associated with DCA muscle atrophy, which was not identified in these specimens.

The fibre type distribution within the equine DCA muscle was not uniform. The lateral NMC contained relatively more *type I* and less *type II* fibres than the medial NMC of the same muscle ($P < 0.05$). There was a wide variation in fibre type distribution across larynges. The prevalence of fibre type grouping was significantly higher in the left DCA muscles than in the right ($P < 0.02$). Fibre type grouping occurs due to collateral sprouting, when denervated muscle fibres stimulate healthy adjacent nerve fibres to send out fine sprouts to reinnervate them (Dubowitz 1967; Karpati and Engel 1968; Cahill and Goulden 1986). As a result, denervated fibres in any given region assume the same metabolic and myosin type as adjacent fibres. Our findings are consistent with previous studies that suggest the denervation-reinnervation cycle occurs for some time before gross morphological changes can be identified (Cahill and Goulden 1986; Duncan and Baker 1987; Harrison *et al.* 1992).

The neuromuscular specialisation seen in the equine DCA muscle may have a number of clinical implications. First, it appears that, from the perspective of increased cross sectional area of the *rima glottidis*, the prosthetic laryngoplasty suture should mimic the action of the lateral NMC. An alternate or additional suture placement in a medial site, such as the sagittal ridge, could possibly result in more rotation and warrants further investigation. Although we focused on the degree of abduction and size of the *rima glottidis*, the different modes of arytenoid displacement and possible associations with lower morbidity, for example tracheal aspiration, should also be considered. Second, laryngeal reinnervation techniques should target reinnervation of both NMCs if the native abductor function is desired. Third, the documentation of the motor endplates is important because reinnervation occurs most readily at sites of the original motor endplates and it has been suggested that placement of a reinnervating neuromuscular pedicle near a large population of motor endplates could improve outcome (Goding 2005). This description of the motor endplates should provide information to the surgeon allowing more precise placement of neuromuscular pedicle graft for motor reinnervation of the equine DCA. Fourth, photographic identification of the recurrent laryngeal nerve branch distribution and branch topography *in situ* should be useful for planning nerve cuff placement for direct nerve stimulation. In addition, the dorsal cricoid *lamina* area without DCA muscle attachment could serve as a potential space reservoir for an implant or deposition of an agent to stimulate DCA muscle activity.

An electrical device has been placed in the subperichondrial layer of the posterior (dorsal) cricoid *lamina* in human laryngeal pacing trials (Goding and Bierbaum 1999). Furthermore, development of more physiological (i.e. dynamic) surgical treatments for equine RLN may require stimulation of the equine DCA neuromuscular compartments as 2 discrete entities.

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- ⁴Motion Analysis, Santa Rosa, California, USA.
- ⁵NEC Corporation, Santa Clara, California, USA.
- ⁶WOMBLE Multimedia, Cupertino, California, USA.
- ⁷Adobe Systems Incorporated, San Jose, California, USA.
- ⁸Insightful Corporation, Seattle, Washington, USA.

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