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EFFECT OF DYSTROPHIN DEFICIENCY ON SELECTED INTRINSIC LARYNGEAL MUSCLES OF THE mdx MOUSE

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Health Sciences
at the University of Kentucky

By
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and Dr. Anne L. Harrison, Associate Professor of Physical Therapy

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The intrinsic laryngeal muscles are recognized as a highly specialized allotype of skeletal muscle. To date, much of the research examining the properties of this muscle group has been conducted on 2 primary muscles: the thyroarytenoid and posterior cricoarytenoid. Consequently, it is unknown whether the remaining intrinsic laryngeal muscles evidence this highly refined phenotype or if they retain a phenotype more similar to prototypical skeletal muscle.

The purpose of this study was to further define the biologic properties of the interarytenoid (IA) and cricothyroid (CT) muscles of the larynx using the dystrophin deficient *mdx* mouse model. Previous work in this model has demonstrated sparing of select craniofacial muscles in the disease. Interestingly, a vast body of literature also supports the uniqueness of these spared muscles in a number of other areas including: fiber types, motor unit size, proprioceptive mechanisms, myosin isoform expression, remodeling behaviors, and sarcomeric structure. It follows, then, that muscle response to dystrophin deficiency serves as a sensitive marker of a muscle's level of biological specialization and its similarity to or departure from classic limb muscle.

Larynges and gastrocnemius muscles from 8 *mdx* and 8 C57BL control mice were examined histologically for typical markers of dystrophinopathy. Immunocytochemical testing examined the distribution of dystrophin and its homolog, utrophin, in control and *mdx* muscles.

Results demonstrated that despite the absence of dystrophin, the laryngeal muscles did not show the classic markers of disease. The *mdx* superior cricoarytenoid muscle (SCA; mouse counterpart of human IA) demonstrated no evidence of damage, inflammation, necrosis, or regeneration. The *mdx* CT evidenced subtle markers of regeneration (eg, slight increase in centrally nucleated fibers) but no evidence of degeneration. The authors concluded that the SCA was spared from the effects of dystrophin deficiency, while the CT was strongly protected. The results demonstrate that the SCA and CT muscles of the larynx possess a specialized nature that separates them from prototypical limb muscle.

Information from the study offers insight into the unique biology of the laryngeal muscles and holds implications for the translational study of voice and voice disorders.

KEYWORDS: Dystrophin Deficiency, Larynx, Skeletal Muscle, Interarytenoid, Cricothyroid

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DISSERTATION

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CHAPTER 1 – INTRODUCTION

The craniofacial muscles are a highly specialized and diverse set of skeletal muscles intricately involved in the processes of respiration, deglutition, sensation, and communication. From the very beginning of their development, these muscles make fundamental departures from prototypical limb skeletal muscle anatomy and physiology.[1, 2] These specializations permit the craniofacial muscles to meet the high-level functional demands required in the above activities. Much of the literature surrounding the craniofacial muscle phenotype has focused on the extraocular muscles; however, research has now identified special features in other craniofacial muscle groups, including the laryngeal muscles.[3-10]

The intrinsic laryngeal muscles have primary responsibilities in respiration, swallowing, airway protection, and phonation. The group is comprised of thirteen muscles (6 paired, 1 unpaired) which work in concert to adduct, abduct, tense, and relax the vocal folds.[11, 12] In recent years, a growing body of evidence has emerged supporting the distinctive nature of these muscles.[3, 4, 6, 7, 13-25] Key areas in which the laryngeal muscles diverge from prototypical limb muscle include: innervation,[13, 14, 19, 20] contractile proteins,[17, 18, 21-23, 26, 27] regenerative capacity,[3, 6] sensitivity to disease and insult,[4, 7, 25, 28-30] and response to aging.[24] Their level of specialization has led some to propose these muscles as a separate allotype of skeletal muscle.[2]

However, a closer examination of the literature identifies that much of the research supporting the laryngeal musculature's unique and highly refined biology has emerged from the study of 2, select laryngeal muscles: the adductory thyroarytenoid (TA) and the abductory posterior cricoarytenoid (PCA). While the intensive study of these 2 muscles has yielded an abundance of information related to specializations within the larynx, it has, perhaps, failed to appreciate the biology of the broader group of intrinsic muscles. Two muscles, in particular, which have received little attention in the literature are the interarytenoid (IA) and cricothyroid (CT).

The IA muscle is positioned in the posterior aspect of the larynx and is the primary adductor of the cartilaginous vocal folds.[11, 12] As such, the IA is a vital contributor to laryngeal closure during swallowing, coughing, throat clearing, and

voicing.[31] Available literature indicates that this muscle diverges from its laryngeal counterparts structurally as well as functionally. Contractile protein profiles of the IA are strikingly similar to those of classic limb muscle.[32] Specifically, the IA shows a mosaic of the basic skeletal muscle myosin isoforms and the complete absence of the atypical, specialized isoforms observed in other laryngeal muscles, a finding which suggests that the IA's contraction times are more indicative of limb, than laryngeal, muscle.[32] Further, the IA is the only laryngeal muscle displaying muscle spindles, and consequently, the only laryngeal muscle relying upon the classic mechanisms of proprioception used by limb skeletal muscle.[32] Similarly, the CT muscle, a primary regulator of vocal fold tension,[12] shows marked departures from its sister laryngeal muscles. Embryologic origins of the CT are traced to the fourth branchial arch, whereas all other laryngeal muscles are traced to sixth arch.[33] As cell lineage is an important contributor to muscle phenotype, some have suggested that the CT's unique developmental history separates it structurally and functionally from laryngeal muscle.[34] The CT also stands apart in terms of its contractile proteins. As with the IA, the CT displays a mixture of basic fast and slow myosins and an absence of specialized isoforms, a profile similar to that of fast limb muscle.[17, 22, 35]

While the body of evidence pertaining to the IA and CT is limited relative to that of the TA and PCA, the above findings point to the possibility of phenotypic diversity among the intrinsic laryngeal muscles. The lack of a comprehensive examination of IA and CT biology has, to this point, made confirmation of diversity impossible.

The purpose of this study was to further define the biological characteristics of the IA and CT muscles. While a number of methods are available to examine features of these muscles of interest, one model in particular serves as a sensitive indicator of a muscle's level of specialization and its similarity to or departure from prototypical limb muscle. The *mdx* mouse model of dystrophin deficiency is the genetic equivalent of human Duchenne muscular dystrophy (DMD).[36] The disease is a result of a spontaneous mutation of the Xp21 gene, which results in the absence of the cytoskeletal protein dystrophin.[37, 38] In the absence of this pivotal support protein, the muscle's cell membrane is subject to the mechanical forces of muscle contraction.[38] Sarcolemmal tearing often results, permitting the entry of extracellular calcium into the muscle cell. High levels of

intracellular calcium trigger the activity of protein destroying enzymes and the subsequent destruction of the muscle fiber. Over time, the disease results in widespread necrosis and fibrosis throughout the muscle.[38, 39]

Diseases of skeletal muscle, such as DMD, are expected to trigger their predictable pathological cascades across the entire class of skeletal muscle. When muscles paradoxically escape the cascade, questions are raised regarding their similarity to and/or departure from the prototypical skeletal muscle. Duchenne muscular dystrophy was once believed to affect all skeletal muscles; however, it has been realized that a few select muscles are spared, chief among these are the extraocular muscles and the thyroarytenoid, posterior cricoarytenoid, and lateral cricoarytenoid muscles of the larynx.[4, 7, 25, 40] For reasons yet to be elucidated, these muscles retain normal structure and function in the absence of dystrophin. Interestingly, a vast body of literature also supports the uniqueness of these spared muscles in the areas of: fiber types, motor unit size, proprioceptive mechanisms, myosin isoform expression, remodeling behaviors, and sarcomeric structure.[3, 4, 6, 7, 13, 15, 17-25, 41-45] It follows, then, that muscle sensitivity to DMD serves as a sensitive marker of a muscle's level of biological specialization and its similarity to or departure from classic limb muscle. Recent studies suggesting that constitutive, biological differences separate DMD-affected and DMD-spared muscles appear to support this assertion.[42] Examination of the IA and CT muscles with the *mdx* model can, therefore, provide insight into the biological properties of these muscles and offer researchers a better understanding of their similarity to or divergence from the prototypical limb muscle phenotype.

Hence, the study examined the effects of dystrophin deficiency on the transverse IA and CT muscles of the larynx. The posterior cricoarytenoid (PCA) muscle of the larynx served as the spared muscle control, while the gastrocnemius served as the affected muscle control. For the initial phase of the study, serial 10-μm-thick cryosections of the above muscles were obtained. Histological sections were air-dried and stained with hematoxylin and eosin. Sections were later examined under light microscopy for evidence of muscle fiber degeneration (ie, inflammation, necrosis, and fibrosis) and attempted regeneration (ie, pleomorphic fibers, central nucleation). In the second phase of the study, dystrophic and normal mice were injected with Evans blue dye, a vital dye

used to assess the integrity of cell membranes. Approximately 18 hours after injection, the mice were killed, and tissues were collected as described above. Serial 10-µm-thick cryosections of the aforementioned muscles were fixed, washed, and mounted. The presence of Evans blue-positive fibers, indicating cell membrane disruption, was evaluated with fluorescence microscopy. Finally, a polyclonal antibody against dystrophin and monoclonal and polyclonal antibodies against utrophin were used to confirm the presence and/or absence of the vital proteins in *mdx* and control muscles.

Results of the study will provide a more thorough understanding of the IA and CT muscles and a greater appreciation of the potential differences that exist among the intrinsic laryngeal muscles. Further, the in-depth investigation of these muscles will hold important implications for the study of medical conditions affecting the larynx and for the clinical management of voice and swallowing disorders.

This chapter has offered an introduction to the intrinsic laryngeal muscles and has presented the rationale for this study. The following chapter reviews pertinent literature related to this study. Topics reviewed in the next chapter include: laryngeal muscle structure and function, laryngeal muscle specialization, evidence for laryngeal muscle heterogeneity, and history and use of the *mdx* mouse.

CHAPTER 2 – REVIEW OF THE LITERATURE

Introduction

Pertinent literature supporting this study is presented in 5 sections. In the first section, an overview of the craniofacial musculature is presented, including the musculature's developmental origin, specialization, and deviation from prototypical limb skeletal muscle. In the second section, the intrinsic laryngeal musculature is examined in detail. The discussion reviews the basic structure and function of the intrinsic laryngeal muscles, the role of the laryngeal muscles in voice production, specialized features of the intrinsic laryngeal musculature, points of laryngeal muscle deviation from limb muscle, and evidence of diversity among the intrinsic laryngeal muscles. The third section reviews 2 muscles emerging as distinct among the laryngeal muscles: the interarytenoid (IA) and the cricothyroid (CT). In this section, details of each muscle's anatomy, morphogenesis, function, innervation, sensory mechanisms, and contractile properties are offered. Areas in which the muscles deviate from their sister laryngeal muscles are highlighted, and the functional implications of the deviations are noted. The importance of detailed study of the IA and CT is discussed. The fourth section presents the *mdx* model of dystrophin deficiency as the suggested model for study of the IA and CT. The section includes a description of the model, a discussion of its previous use, and a review of its use with the laryngeal muscles. The section concludes with comments on the implications of the study's findings beyond this model of dystrophin deficiency. In the last section, the purpose of the study is presented, and hypotheses are shared. Implications of the study's findings for the understanding of voice and voice disorders are discussed.

Craniofacial Musculature

Muscles of the craniofacial region represent a diverse group of skeletal muscles responsible for the processes of respiration, deglutition, speech production, vision, hearing, and the display of emotion. These muscles evidence a remarkable degree of specialization which permits their successful engagement in life-supporting functions. Because the functional demands placed upon craniofacial muscles differ from those imposed upon other skeletal muscles, the craniofacial muscles show marked anatomical and physiological deviations from prototypical limb skeletal muscle. The uniqueness of

the craniofacial muscle phenotype has led to their being described as "paradoxical" members of the skeletal muscle group[1] and, more recently, to the search for and description of a separate craniofacial muscle allotype.[46, 47]

The anatomical and physiological differences that exist between craniofacial and limb skeletal muscle are vast. Architectural differences related to muscle insertion patterns, muscle fiber size, and sarcomeric structure have been identified.[2, 44, 45, 48-50] Additionally, differences in contractile protein expression, mitochondrial content, motor innervation, and proprioceptive mechanisms have emerged. These later specializations produce functional differences in contraction times, tension generation, endurance, and precision of movement.[2, 12, 14, 15, 19-23, 27, 43, 51-54] The exact mechanism of the distinctive phenotype has yet to be elucidated; however, it has been suggested that the diversity is established during morphogenesis and later regulated by muscle-group specific patterns of gene expression.[1, 43, 46, 47] The consequences of the diversity between craniofacial and limb muscle are significant. The specialized phenotype permits the craniofacial muscle group to engage in extremely rapid and prolonged contraction, perform highly refined patterns of movement, escape the pathological cascade of some neuromuscular diseases, recover amid mechanical and neurological insult, and resist the influence of aging.[2, 4, 5, 7, 9, 10, 12, 25, 41, 43, 46, 55]

Interestingly, the above specializations are observed in some, but not all, muscles of the craniofacial region. The lack of universal specialization is not surprising given that the craniofacial muscles have been described as the most diverse set of muscles in the human body.[1] However, one subset of the craniofacial muscles emerging as highly specialized is the intrinsic laryngeal muscle group. Muscles of this group are intricately involved in the life-sustaining functions of respiration, airway protection, swallowing, and vocalization.

Laryngeal Musculature

The larynx is a sophisticated sphincteric structure with primary roles in the modulation of airflow during respiration, protection of the airway during swallowing, and production of voice in oral communication.[56, 57] Structurally, the larynx is a jointed, cartilaginous tube covered by a mucosal layer. Housed within and protected by the cartilaginous framework are the paired vocal folds, which project toward one another in

the transverse plane.[12] Movements of the cartilaginous larynx and membranous vocal folds are controlled by the action of the intrinsic laryngeal muscles, under the guidance of an exquisite mechanism of neuromuscular control.

Overview of the Intrinsic Laryngeal Muscles

Muscles which have their origin and insertion on laryngeal cartilages are termed intrinsic laryngeal muscles.[12] There are 13 muscles within this group[δ]: 6 paired and 1 unpaired.[12] Contraction of these muscles modifies the relationship between the laryngeal cartilages, and thereby, alters the position, length, and tension of the vocal folds. Typical classification schemes place the muscles into 4 groups on the basis of function: vocal fold adductors, abductors, tensors, and relaxors.[12] Three muscles play an adductory role: the lateral cricoarytenoid (LCA), the interarytenoid (IA), and the thyroarytenoid (TA). The paired LCA muscles course from the lateral aspect of the cricoid cartilage to the muscular process of the ipsilateral arytenoid cartilage. Contraction of the LCA rotates the vocal process of the arytenoid medially, and thereby, adducts the membranous vocal fold. The IA muscles are located in the posterior aspect of the larynx and are often discussed as 2 separate muscles. The paired oblique IAs course diagonally from the base of one arytenoid to the apex of the opposing arytenoid. Their contraction yields medial movement of the arytenoid apices and corniculate cartilages and the closure of the superior aspect of the posterior glottis. The unpaired transverse IA muscle runs horizontally from the lamina of one arytenoid to the lamina of the contralateral arytenoid. Transverse IA contraction pulls the arytenoid bodies to midline, adducting the posterior, cartilaginous region of the glottis.[11, 12] The final adductory muscle, the thyroarytenoid (TA), makes up the bulk of the true vocal fold complex. The TA is often discussed as 2 separate muscles: the medially positioned vocalis and the more laterally positioned thyromuscularis.[11, 12] The muscle attaches anteriorly to the angle of the thyroid cartilage just below the thyroid notch and posteriorly to the vocal process and fovea oblonga of the arytenoid cartilage.[11, 12, 58] Contraction of the TA moves the thyroid and arytenoid cartilages into closer proximity, thereby, shortening and relaxing the folds.[φ] A single

[δ] The number of muscles within the larynx varies in the literature, pending the author's perspective of the thyroarytenoid muscle as a single muscle or as two separate muscles: the vocalis and the thyromuscularis.

[φ] Isometric contraction of the vocalis results in a tensing of the medial aspect of the vocal fold.

paired muscle is responsible for vocal fold abduction.[11, 12] The posterior cricoarytenoid (PCA) muscles course from the posterior aspect of the cricoid cartilage to the muscular process of the ipsilateral arytenoid, creating a broad, fan-shaped distribution of muscle fibers. Contraction of the PCA causes lateral rotation of the vocal process and an associated movement of the folds away from midline. The final intrinsic muscle, the paired cricothyroid (CT), acts as the primary vocal fold tensor. The muscle originates along the anterolateral aspect of the cricoid cartilage and inserts into the thyroid cartilage as 2 separate units: the pars recta which courses vertically to insert along the inner aspect of the thyroid cartilage's lower margin; and the pars oblique which courses superiorly and posteriorly to insert into the inferior horn of the thyroid. Contraction of the CT alters the perspective between the thyroid and cricoid cartilages, thereby, elongating and tensing the vocal fold.[11, 12] Together these muscles offer the larynx a remarkable degree of versatility and sophistication of movement. Their level of functional refinement is perhaps best appreciated during voice production.

Intrinsic Laryngeal Musculature in Voice Production

Voice is the result of a highly refined interplay between the respiratory, phonatory, and resonance systems. During voice production the paired vocal folds are brought together at midline via the action of the adductory muscles. Subglottic air pressure from the exhaled air stream builds below the adducted and closed folds, eventually blowing open the membranous portion of the folds and permitting the release of an air pulse. Rapid changes in transglottal air pressure brought about by the glottal opening and the elasticity of the displaced tissue quickly return the membranous folds to midline. Subglottic pressure again builds below the folds, and the cycle is repeated.[59] Pressures from the supraglottic region help to maintain vocal fold oscillation.[11, 60] Upward movement of the air through the glottis causes the compression and rarefaction of air molecules above the glottis and associated changes in supraglottic air pressure. These supraglottal pressure modifications facilitate a "top-down" loading effect on the vocal folds which perpetuates their oscillatory motions.[60] Beyond this basic mechanism of vibration, vocal fold tensors and relaxors fine tune the length and tension of the folds, permitting a wide array of vocal manipulations[12] and enabling the voice to meet the demands of the emotional, acoustic, and physical environments. The above processes

require that the laryngeal muscles maintain an internal balance with one another as well as an external balance with the forces of the respiratory and supraglottic resonance systems. The highly refined phenotype of the intrinsic laryngeal muscles permits them to successfully meet these complex physiologic demands.

Intrinsic Laryngeal Muscle Deviation from Limb Skeletal Muscle

As with certain other skeletal muscles of the head and neck, the laryngeal muscles stand apart from prototypical limb skeletal muscle along several fronts, including their morphogenesis, innervation patterns, fiber size and architecture, myosin isoform expression, metabolic profile, regenerative capacity, response to disease, and pattern of aging.[3, 4, 6, 7, 13-25] It is believed that the unique phenotype of laryngeal muscles has evolved to permit their successful participation in vital systemic functions, including modulation of upper airway airflow during respiration, provision of airway protection in swallowing, and fixation of thoracic cavity pressures for heavy lifting and defecation. It is further recognized that in a number of species, the specialization of laryngeal muscles has permitted the development of sophisticated systems of vocal communication. The sections that follow highlight the primary parameters along which the laryngeal muscles depart from prototypical limb muscle and discuss the relevance of these departures to laryngeal function.

Morphogenesis

Background. Laryngeal muscles possess a unique developmental history – standing apart from other craniofacial muscles as well as from muscles of the trunk and limbs.[1, 57, 61, 62] Skeletal muscle throughout the body is formed from the paraxial mesoderm (PAM), regions of mesenchymal tissue extending on either side of the neural tube from the primitive streak rostrally to the tip of the notochord caudally.[1, 61] The most rostral aspects of the PAM (pre-otic vesicle) which give rise to many of the craniofacial muscles do not segment during development, whereas caudal aspects (post-otic vesicle) which give rise to a portion of the craniofacial muscles and all trunk and limb muscles segment into 30+ distinct folds of mesenchymal tissue termed somites.[57, 61, 63] The origin of skeletal muscle as either somitic or non-somitic is of note in considering the characteristics of muscle, as distinct patterns of gene expression have been observed on either side of the otic vesicle boundary. A discussion of craniofacial and limb/trunk

muscle development is offered below and is contrasted with the development of the intrinsic laryngeal musculature.

General Craniofacial Muscle Development. Many of the craniofacial muscles emerge from the unsegmented PAM, and are, therefore, classified as non-somitic.[1, 57, 61] Specifically, extraocular muscles (EOM) innervated by the oculomotor nerve emerge from the axial prechordal mesoderm, a sparse assembly of mesenchymal cells located just beneath the rostral neural plate.[57, 64] Remaining EOM arise from a more organized collection of tissue in the pre-otic region of the PAM. While not fully segmented, certain of these pre-otic cells coalesce into 7 pseudo-segmentations, referred to as "somitomeres."[57, 65] The 2 most rostral somitomeres yield the EOM.[62] Neural crest cells found throughout the region migrate with the muscle precursors during development and yield the connective and neural tissues associated with the EOM.[57]

Also emerging from the pre-otic unsegmented PAM are the branchial muscles.[1] These include muscles associated with the jaw, hyoid bone, and branchial skeletal structures (eg, masseter, temporalis, digastricus, buccinator, stylopharyngeus). As with the EOM, branchial muscle primordia are established in the somitomeres; however, unlike the EOM, these muscles continue their development within the branchial arch environment (arches 1-3).[1, 57, 66] Neural crest cell on the surface of the branchial arch produce connective and neural tissues associated with the musculature.

Tongue and Laryngeal Muscle Development. Just caudal to the otic vesicle, the somitic pairs develop into the skeletal muscles of the tongue, larynx, trunk, and extremities.[57] Tongue muscles as well as the intrinsic laryngeal muscles arise from lateral borders of the most rostral somite pairs, termed the occipital somites.[1, 62, 67, 68] During the development of these muscles, mesodermal cells of occipital somites migrate to form a bilateral hypoglossal cord. A portion of the muscle precursor cells within the cord migrate rostrally, joining with neural crest populations of the first three branchial arches to form the tongue musculature. Meanwhile, additional muscle precursor cells, move away form the cord and toward the laryngotracheal space. These cells become integrated into branchial arches 4 and 6 and later develop into the intrinsic laryngeal muscles.[1, 62, 67, 68] As with the extraocular and branchial muscles noted above, neural crest cells in the region produce the connective and neural tissues associated with the glossolaryngeal

musculature.[57] This pattern of development involving progression from somitic to branchial arch phases is unique within the craniofacial muscle family. Indeed, the somitic origin of laryngeal muscle places it in line, embryologically, with muscles of the trunk and extremities; however, its involvement in the branchial arch system parallels that of the majority of craniofacial muscles. Consequently, the glossolaryngeal muscles often described as hybrids among the set of craniofacial muscles.[1, 64]

Limb Muscle Development. Finally, the more caudal somite pairs of the PAM are grouped into cervical, thoracic, lumbar, sacral, and coccygeal regions and are precursors to muscles of the extremities.[57] Progenitor cells from the dorsal aspect of the somites migrate to the region of interest. Once there the cells proliferate and differentiate into skeletal muscle.[69]

Summary. The intrinsic laryngeal muscles possess a complex and unique developmental history. The occipital somites, from which these muscles emerge, sit on the border of the segmented-unsegmented boundary and progress through the branchial arch system, producing hybrid or mixed muscles with characteristics of both head and body skeletal muscle.[1, 64]

Motor Innervation

The laryngeal muscles have long been recognized for their rich neural support from branches of the vagus nerve.[13, 19, 20, 51, 70] The TA, LCA, PCA, and IA muscles receive primary innervation the recurrent laryngeal nerve (RLN),[12, 70, 71] and, in a small percentage of cases, supplemental innervation from the internal and/or external branches of the superior laryngeal nerve (SLN).[70] The CT stands alone in receiving its primary innervation via the external branch of the SLN. For all intrinsic muscles except the transverse IA, innervation from the above branches is unilateral. The unpaired nature of the transverse IA offers it the advantage of bilateral neural input from the RLN.[70]

Laryngeal muscles are highly innervated, characterized by motor units with only a small number of fibers per individual motor neuron.[13, 19, 20, 51] While an exact ratio of fibers to a single motor neuron has not been established, it has been suggested that the ratio is far smaller than that reported in limb muscle (100-2000 fibers per motor neuron)[72] and perhaps comparable to that observed in the extraocular musculature (13-20 fibers per motor neuron).[2] Most laryngeal muscle fibers possess a single neuromuscular junction

(NMJ); however, fibers with multiple NMJs have been reported in all laryngeal muscles innervated by the RLN (TA, IA, LCA, PCA).[19] In these fibers, NMJs are generally scattered in a grape-like pattern along the middle 2/3 of the long axis of muscles.[13, 19, 20] This end-plate distribution is strikingly different from the single-NMJ, mid-muscle end plate zone typical of limb skeletal muscle.[72] Interestingly, patterns of innervation for the laryngeal muscles are comparable to those identified within the highly specialized extraocular muscles.[2, 19, 43] The exquisite nature of laryngeal muscle innervation speaks to the muscle group's high level of specialization and its importance in the finely-tuned activities of respiration, swallowing, and voicing.

Sensory Mechanisms

Sensory information from the larynx is conveyed via 2 branches of the vagus nerve: the SLN and the RLN. Somatic sensory information from mechanoreceptors, chemoreceptors, taste buds, and free nerve endings within the glottal and supraglottic mucosa are transmitted via the internal branch of the SLN; similar information from subglottic regions is transmitted via the RLN.[71] Unfortunately, a thorough description of the larynx's *proprioceptive* sense has proven more elusive. The presence of proprioceptive organs, such as the muscle spindle, within the laryngeal muscles has been a matter of much debate.[14, 19, 73-79] Muscle spindles are small organs housed within skeletal muscle which respond to muscle stretch. During muscle contraction, spindles activate sensory neurons, which prompt alpha motor neurons of the associated muscle to respond to the stretch. Spindles are classically recognized by the presence of intrafusal fibers resting within a connective tissue capsule and surrounded by a neural network.[80] Early studies using histological staining methods consistently identified the organs in the intrinsic laryngeal muscles of humans.[75-79] However, recent studies using more refined methods of examination have brought these earlier findings into question. More recent investigations on the topic are presented below.

Histologic and Immunocytochemical Studies of Laryngeal Proprioception.

Studies of the TA. Brandon et al[14] used immunohistochemical methods to consider spindle presence in human TA muscles. Larynges used in the study were excised during total laryngectomy procedures. The group employed antibodies against 2 specialized myosins isoforms (tonic myosin, neonatal myosin) commonly found in the intrafusal

fibers of spindles. Their study found no evidence of the specified myosins in the TA, leading to the conclusion that this primary muscle of the larynx was devoid of classic muscle spindles. Kersing and Jennekens[81] used histologic staining to examine the TA muscles of 23 cadaveric and surgically excised larynges. Staining failed to identify spindles in the vocalis region of the TA in infant, middle-aged, and old-aged larynges, findings which supported the earlier work of Brandon et al. Interestingly, one recent study by Sanders disputes the above findings. The group stained 50-µm human TA muscle sections with hematoxylin and eosin. Spindles were defined based upon the following features: (1) round or oval structure, (2) a 2-layer external capsule, (3) a wide subcapsular space, (4) intrafusal muscle fibers in the inner capsule, and (5) nerve fibers surrounding the intrafusal fibers. The group identified spindles along the entire anterior to posterior aspect of the vocal fold, with the greatest concentration of spindles being in the fold's superior medial compartment. **Studies of the Other Intrinsic Muscles.** Perie et al[19] examined the motor and sensory innervation of 4 intrinsic laryngeal muscles in humans: TA, PCA, LCA, and CT. The group incubated 60-µm muscle sections in 5-bromoindoxyl acetate, an indicator of cholinesterase activity. Nerve endings positive for cholinesterase activity were identified within connective tissue capsules, a finding suggestive of muscle spindles. However, the absence of intrafusal fibers near the capsules led the authors to propose that the structures represented another form of sensory receptor and not true muscle spindles. The authors concluded that the intrinsic laryngeal muscles did not rely on muscle spindles for proprioception. Finally, Tellis et al,[32] used histochemical and immunohistochemical methods such as those used by Brandon et al to look for spindles in the human IA. Tonic and neonatal myosins typical of the spindle's intrafusal fibers were identified throughout the muscle. Spindles were complex in design and morphologically distinct from classic limb spindles in terms of their connective tissue capsules, elongated extrafusal fibers, and peculiar change in orientation at the muscle's insertion into the arytenoid cartilage. The authors concluded that spindles were present in the IA and that the unique morphology of these spindles made them remarkably sensitive to dynamic movement.

Clinical Studies of Laryngeal Proprioception. Two recent studies have used servomotor-induced mechanical displacement of laryngeal cartilages to examine the

presence of laryngeal muscle stretch reflexes and have, thereby, indirectly considered the presence of muscle spindles. In the first of these studies, Andreatta et al[82] used hooked wire electrodes to monitor laryngeal adduction responses from the feline TA during posterior displacement of the arytenoid cartilages. The experiment considered the TA's response under 2 conditions: vocal fold mucosa intact and vocal fold mucosa removed. With mucosa intact, recordings from the TA showed consistent adductor responses; however, with mucosa removed, recordings of the TA's adductor responses were markedly reduced in number and intensity. The authors concluded that mucosal mechanoreceptors, rather than classic muscle spindles, mediated reflexive activity of the TA. In a follow-up study, Loucks et al[16] used hooked wire electrodes to record activity in the human TA, CT, and sternothyroid muscles during servomotor displacement of the thyroid cartilage. No TA or CT activity was identified simultaneous to the mechanical displacement, suggesting the absence of a stretch reflex in these laryngeal muscles. Interestingly, activity was identified in the extrinsic sternothyroid muscle during displacement. The authors proposed that the TA and CT were lacking in muscle spindles and that afferent feedback for voice control was mediated via other forms of sensory receptors within the larynx.

Summary of Laryngeal Proprioception. Thus, debate continues as to the mechanism of proprioceptive feedback used by the laryngeal muscles. Those maintaining the presence of spindles highlight the importance of the organs in managing the refined biomechanics of voice production.[73] However, those refuting their presence propose that, as a non weight-bearing organ, the larynx does not require afferent feedback to guide muscle response to stretch and external load[2, 14] and that the proprioceptive sense for laryngeal movement is likely mediated outside of the muscle layer through the mucosal mechanoreceptors located in the posterior aspect of the larynx.[16, 82] While the final word on laryngeal proprioception has not emerged, it appears clear that laryngeal sensory mechanisms do not parallel the well-defined mechanisms observed in limb muscle.

Fiber Size

The laryngeal muscles have been identified as having smaller diameter fibers than prototypical skeletal muscle.[19, 50, 83, 84] Sadeh et al[50] examined the size of human CT, PCA, LCA, and vocalis muscles and reported an average diameter of 40-50µm. Rodeno

et al[84] found similar results in their examination of the TA and PCA muscles. In their study, TA diameter ranged from 38μm to 39μm, while PCA fibers ranged from 43μm to 47μm, pending fiber type. Finally, Perie et al[19] reported slightly smaller fibers than the above authors, with mean diameters of 20-35μm for the TA, LCA, PCA, and CT. These fiber size estimations suggest that laryngeal muscles are small relative to limb muscle (35-75 μm)[72] but comparable to that of extraocular muscle (20-50 μm).[43]

The fiber size of skeletal muscles is critical, as it holds implications for the muscle's mechanical properties. Studies conducted on small-diameter craniofacial muscles confirm that these muscles exert significantly lower levels of mechanical force than other skeletal muscles.[52-54] These lower levels of force generation remain even when correction is made for fiber cross sectional area. Such differences in force generation are of note, as they have been suggested as a potential mechanism whereby laryngeal and other craniofacial muscles may be spared from select neuromuscular diseases.[7, 25, 85, 86]

Contractile Properties

Skeletal muscle is often classified according to 2 key contractile properties: speed of contraction and sustainability of contraction. These functional properties are determined by the muscle fiber's myosin heavy chain (MyHC) composition and its method of energy production: two domains in which laryngeal muscles have proven to be distinctive.

Myosin Isoforms. The MyHC is the primary determinant of the speed of muscle contraction.[87] Three basic isoforms of myosin are commonly expressed in human skeletal muscle: MyHC-I yields slow contractions, whereas MyHC IIA and IIX produce rapid contractions.[88] Additional isoforms (eg, MyHC-neonatal, MyHC-embryonic, and MyHC-extraocular) are present in developing fibers and in the fibers of some specialized craniofacial muscles.[89] Studies in non-human animal models reveal additional skeletal muscle isoforms[89]; however, of these, only the fast isoform, IIB is pertinent to the discussion that follows. Contractile speeds of the primary isoforms discussed above adhere to the following sequence, from slowest to fastest: I, IIA, IIX, IIB, and extraocular.[89]

Laryngeal Myosin Expression. Laryngeal myosin expression varies across species in both the diversity and distribution of isoforms.[90, 91] **Animal Models**. In animal models,

laryngeal muscles express Type I, IIA, IIX, and IIB myosins.[35, 92-94] Specialized myosin isoforms (MyHC-extraocular, MyHC-IIL) capable of extremely rapid contraction have also been identified in laryngeal muscles of rats and rabbits.[17, 18, 22] Of the laryngeal muscles, the TA has been the most widely studied in terms of its MyHC composition. Studies in rats and non-human primates suggest that the TA is nearly homogenous in its MyHC expression, being composed almost entirely of fast, type II myosins and the specialized MyHC-extraocular (MyHC-eo).[83, 90, 92, 93] Authors have related the nearly universal display of fast isoforms in these animals to the muscle's role as a glottal adductor. Interestingly, the canine model deviates from the above showing a heterogeneous display of myosins in the medial TA, an arrangement which could be beneficial in fine tuning the muscle's action to meet specific force needs.[95] Studies considering muscle MyHC composition across various intrinsic laryngeal muscles have been rare. However, 2 studies conducted in rats suggest that the LCA and PCA muscles demonstrate profiles similar to that described above in the TA: a strong presence of MyHC-IIB and the scattered presence of MyHC-eo.[22, 93] The above studies point to an exquisite mechanism for contractile speed in the intrinsic laryngeal muscles of some animals. Profiles such as those noted above are found in few other muscle groups, most notably the extraocular muscles.[89] **Human Models.** Human laryngeal muscles evidence 3 basic myosin isoforms (MyHC I, IIA, IIX). Unlike certain of the animal models described above, human laryngeal muscles display a combination of fast and slow isoforms within a single muscle, a feature which permits the muscle to recruit the specific combination of fibers most in keeping with the desired form of contraction. The exact combination of isoforms varies across laryngeal muscles and appears reflective of the muscle's functional role and its requirement for contractile speed (ie, muscles responsible for glottic closure and airway protection demonstrate faster MyHC isoforms profiles than do abductors[23, 84, 96]). Work comparing human laryngeal and limb muscle suggest that some laryngeal fibers are capable of contractile speeds which far exceed those of limb muscle.[21] Despite this apparent contractile advantage of laryngeal muscle over limb muscle, studies comparing laryngeal to extraocular muscles, indicate that myosin expression in the human larynx is not as refined as that of these other specialized craniofacial muscles.[96]

It should be noted that one study by Han et al[97] reported the presence of an extremely rare, "slow tonic" MyHC in the human TA. This rare isoform produces a long-lasting muscle contraction, rather than the classically observed twitch response to repeated stimulation,[89] a feature believed to be important in functions requiring prolonged contraction. Prior to Han et al's study, human expression of the isoform was believed to be limited to the extraocular musculature.[89, 97] Expression of the isoform in human vocal folds reflects the TA's remarkable potential for prolonged contraction, such as that used in voicing.[97]

The above findings reveal differences in laryngeal muscle MyHC composition between human and animal models (eg, presence of MyHC-eo and MyHC-IIB, uniformity of myosin expression within single muscles). More importantly, however, they demonstrate the refined and rapid nature of laryngeal muscles contraction relative to limb muscle contraction.

Metabolism. The muscle's method of energy production is the primary determinant of its ability to sustain contraction and resist fatigue.[98] Energy for the muscle fiber can be produced via 2 pathways. In the oxidative pathway, oxygen and nutrients are delivered to the cell by the vascular system. These substances are taken up by the mitochondria where they are processed to produce ATP for muscle contraction. The efficient nature of this pathway (netting 36 ATP molecules per exchange) results in the muscle being able to sustain contraction for long periods of time. Other fibers rely upon glycolytic pathways of energy production. Glycolytic processes are faster than oxidative pathways and can transpire in the absence of oxygen; however, they yield only 2 ATP molecules per exchange. Thus, while capable of generating energy for rapid and forceful contractions, fibers using glycolytic pathways are generally less resistant to fatigue than oxidative fibers.[98]

Studies considering metabolic profile of human laryngeal muscles have been conducted primarily on the TA and PCA muscles.[50, 81, 84, 99] These studies suggest that both oxidative and glycolytic fibers are present within individual muscles. Differences in study methodology and methods of reporting make exact distributions of oxidative and glycolytic fibers difficult to ascertain; however, some basic conclusions have been drawn. Slowly contracting, oxidative fibers (fatigue resistant) have been identified at the TA's

medial edge while more rapidly contracting, glycolytic fibers (fatigable) have been found in the muscle's lateral aspect.[90, 99] It has been suggested that this distribution is functionally advantageous, permitting the medial TA to engage in the sustained contractions required for speech production while ensuring the lateral TA's ability to rapidly close the airway.[90, 99] Interestingly, comparisons of the PCA and TA suggest that the PCA demonstrates a more oxidative profile than the TA.[84] Some have proposed that the increased level of oxidative (fatigue resistant) metabolism in the PCA is reflective of the muscle's continuous activity in modulating the glottal opening during respiration.[84]

Finally, recent work conducted in animal models has suggested that nonpathological TA, PCA, and CT muscles demonstrate usually high densities of mitochondria when compared to limb skeletal muscle.[83, 100] Similar findings have been observed in the extraocular muscles and have been related to the high oxidative activity, sophisticated vascularity, and requirement for fatigue resistance in these muscles.[2] Hinrichsen and Dulhunty[100] have proposed a similar explanation for the elevated mitochondrial counts in laryngeal muscles. They suggest that continuous muscle activity during respiration dictates a need for fatigue resistance that far exceeds that required by typical limb skeletal muscle activities.

Thus, the laryngeal muscles appear refined for prolonged contraction. Their ability to rapidly contract and resist fatigue sets them apart from other skeletal muscles and reflects the unique physiological demands placed upon them.

Regenerative Capacity and Ability to Recover Post Insult

It has been well-established that prototypical limb skeletal muscle has the capacity to regenerate in the face of injury via the action of satellite cells.[101] After myofiber injury, satellite cells progress from a quiescent state to an active state. Once active, the cells move to the site of injury, fuse with one another, and differentiate into the new myofiber.[101] However, recent work in the extraocular and laryngeal muscles of rabbits suggests that myofiber remodeling is ongoing in these fibers in the absence of apparent fiber injury.[3, 6, 41] Seminal work in this area by McLoon et al[41] found evidence of continual myonuclear removal and addition in uninjured single fibers of rabbit extraocular muscles. Remodeling proceeded at a rate of one myonuclear addition per 1,000 myofibers in cross section every 12 hours. Follow-up work by Goding et al[3]

identified similar patterns of uninjured fiber remodeling in rabbit TA and PCA muscles. The group estimated that myonuclear addition in the laryngeal muscles occurred at a rate of 2 myonuclei per 1,000 myofibers in cross section per 24 hours. These findings suggested that muscle precursor cells, generally quiescent in limb skeletal muscle, are strangely active and ever cycling in specialized craniofacial muscles. Authors of the above studies propose that the enhanced remodeling observed in these muscle may play a role in their recovery after insult and their resistance to age-related change.[3, 41]

Along this same vein, the laryngeal muscles have long been recognized for their ability to survive and reinnervate following neurological insult.[28, 102, 103] Following denervation of a vocal fold, reinnervation ensues in a portion of cases, even after extended periods of time.[103] This striking ability to reinnervate has not been fully explained; however, it has been suggested that regenerating axons from the damaged nerve or supplemental innervation from the superior laryngeal nerve may play a role.[102, 103] More recently, work by Shinners et al[6] has related the survivability of laryngeal muscles after neurological insult to the distinctive remodeling capacity discussed above. The group identified heightened levels of fiber remodeling immediately following RLN nerve section which were maintained for 24 weeks post injury. The authors concluded that it was the remarkable regenerative capacities of the muscles which facilitated their ability to survive and regenerate following neurological insult. Regardless of the precise mechanism at play, spontaneous reinnervation of the laryngeal musculature does not often restore normal vocal fold abduction and adduction. It does, however, appear to offer sufficient nerve input to prevent or impede severe muscle atrophy in a number of cases.[11, 28, 102-104] Such patterns of reinnervation and muscle maintenance post insult are not observed in limb skeletal muscle, where reinnervation is less common and denervation atrophy can be marked.[105-107]

Sensitivity to Disease

Most neuromuscular diseases exert their pathological cascade universally across skeletal muscles. However, as previously noted, some craniofacial muscles respond paradoxically to neuromuscular diseases which target classic skeletal muscle. Preferential sparing of the extraocular muscles in Duchenne muscular dystrophy (DMD) and amyotrophic lateral sclerosis (ALS) and preferential involvement the muscles in

myasthenia gravis and mitochondrial myopathy have been well-established.[2, 40, 43, 47, 85] Similarly, early involvement of the laryngeal musculature in myasthenia gravis, bulbar-manifesting ALS, and mitochondrial myopathy has been reported in the clinical literature[29, 30, 108-110]; sparing of the muscles in dystrophin deficiency has just recently been realized.[4, 7, 25] Reasons for the laryngeal muscles' paradoxical response to the above disease processes has not been fully explained. The early laryngeal manifestation of some disease processes appears to be a function of the preferential involvement of the cranial nerve nuclei responsible for laryngeal function.[111] However, the mechanism of the group's preferential sparing in dystrophin deficiency has not yet been determined. Current theories suggest that constitutive features of the laryngeal muscles (eg, exquisite remodeling capabilities, fiber types, refined calcium sequestration mechanisms, and/or lower levels of mechanical force generation during contraction)[85, 86, 112, 113] may play a role.

Patterns of Aging

An abundance of literature supports the remodeling of limb skeletal muscle in later life. Most notable among the age-related morphological changes are: a reduction in overall muscle mass,[114, 115] a loss of Type I and Type II muscle fibers,[116, 117] preferential atrophy of Type II fibers,[117-119] fiber type grouping,[114, 120] and infiltration of connective tissue.[116] Functional consequences of the above changes include reduced speed,[121] force,[121] strength,[122-125] and endurance[126] of muscle contraction. Examination of laryngeal muscle aging has been less comprehensive and limited primarily to the TA and PCA muscles. Yet, available literature suggests that laryngeal muscles do remodel as part of the aging process; however, often in ways that diverge from the above limb muscle patterns. While the typical reduction in overall muscle mass is evidenced in the human TA muscle,[99, 127-129] specific fiber changes underlying the atrophy are unclear. Some authors point to a loss of Type I and II fibers,[128] while others suggest a loss of Type I fibers[99, 130] or Type II[81] fibers only. Further, there appears to be a maintenance of Type II fiber size in human laryngeal muscles,[84, 130] a finding contrary to that of limb muscle. Patterns of connective tissue infiltration in the laryngeal muscle have not been clearly defined; some authors report infiltration of the non-contractile tissue,[81, 84, 99, 129] while others report no change in its distribution.[130] Finally, methodological concerns have

prevented the study of age-related functional changes in human intrinsic laryngeal muscles; however, studies in rodent models suggest a reduction in contractile speed, force, and endurance with age,[83] patterns similar to those observed in limb muscle.

Summary of Laryngeal Muscle Specializations

The preceding discussion highlighted numerous points of laryngeal muscle departure from limb skeletal muscle. Areas of laryngeal muscle specialization are broad and include both structural and functional features. The gradual emergence of the above literature base has raised awareness of and appreciation for the laryngeal muscle phenotype. Research is currently ongoing to further define distinctive aspects of these muscles.

Diversity within the Intrinsic Laryngeal Musculature

The above discussion demonstrates that, as a group, laryngeal muscles depart from classic limb muscle in a number of respects. However, much of the literature demonstrating the exceptional phenotype of laryngeal muscles has been based upon the study of 2 key muscles: the TA, a primary adductor, and the PCA, a primary abductor. As a result, relatively little is known about other intrinsic laryngeal muscles. Moreover, due to the diversity of individual craniofacial muscles,[1] what is known of the TA and PCA can not be easily generalized to their sister laryngeal muscles. Consequently, the intrinsic laryngeal muscles must be considered individually for their biological properties and their similarity to or departure from limb skeletal muscle.

Two intrinsic laryngeal muscles which have received minimal attention in the literature but which make significant contributions to laryngeal function are the IA and CT. Previous work suggests that these muscles may be phenotypically distinct from their sister laryngeal muscles. Among the intrinsic muscles, the IA stands apart for its unpaired nature, bilateral RLN innervation, well-defined mechanisms of proprioception, and prototypical MyHC profile.[32, 131, 132] These differences have led some to suggest that the IA may, in fact, be more closely aligned with typical limb skeletal muscle than with its laryngeal counterparts.[32] Similarly, consideration of the CT suggests that this muscle deviates from other laryngeal muscles in its embryonic development, primary innervation source, contractile properties, and response to neuromuscular disease,[4, 17, 22, 33-35, 94, 133-135] facts which have led some to discuss the CT as a hybrid or transitional form of skeletal

muscle which shares properties of laryngeal, pharyngeal, and limb skeletal muscle.[34, 135, 136] The discussion that follows reviews the literature pertaining to the IA and CT. Each muscle is first considered for its basic structure and function. This overview is followed by a review of studies examining the muscle's biological properties. Summary statements pertaining to the muscle's distinctive features conclude each section.

The Interarytenoid

The IA muscles, located in the posterior aspect of the larynx, are composed of paired oblique fibers, which course from the muscle process of one arytenoid to the apex of the opposite arytenoid, and unpaired transverse fibers, which run from the posterior-lateral margin of one arytenoid to the same point on the opposite arytenoid.[12] The IAs function as vocal fold adductors, bringing together the posterior, cartilaginous region of the folds and allowing for full glottic closure.[137-139 θ]

Morphogenesis

The human IA originates in occipital somites 1 and 2 and then progresses through development in the branchial arch system.[1, 57] At approximately the 4th week of gestation, mesodermic tissue in the ventral aspect of the foregut divides into 5 bilateral projections termed the branchial arches (arches 1, 2, 3, 4, 6).[57] Each branchial arch is comprised of a mesenchymal core which is surrounded by neural crest cells and angiogenic mesenchyme. The core of the branches produces myoblast cells which will eventually develop into skeletal muscle. The IA forms from the mesoderm of the sixth arch. Also forming from the sixth arch are the TA, LCA, and PCA muscles and the recurrent laryngeal nerve, the source of innervation for the muscles.[33, 57]

Function

The function of the IA has been considered with electromyographic (EMG), in vitro, and in vivo investigations. Studies point to the muscle's participation in phonatory as well as non-phonatory tasks. The results of these studies are detailed below.

EMG Studies. Electromyographic studies are helpful in delineating the contribution of individual muscles to various functional tasks. Two EMG examinations of

θ Investigations of the IA have not differentiated between the transverse and oblique muscles. Therefore, in the discussion that follows, the transverse and oblique IAs are considered as a single muscle.

the IA were identified in the literature. **Phonation.** Gay et al[140] used hooked wire electrodes to examine laryngeal muscle activity during variations of pitch, loudness, and vocal onset in 5 normal speaking adults. Comments related to IA activity were limited; however, the authors did report increased IA activity in some, but not all, subjects at high loudness levels. Interarytenoid activity was not related to control of pitch or vocal onset. In the second study, Hillel[31] used monopolar fine-wire electrodes to track the behavior of the IA during sustained phonation, prolonged phonation, pitch elevation, and repeated vowel production. During sustained phonation and repeated production of vowels, TA and LCA muscles showed high levels of activity at onset of phonation which reduced in intensity after onset. The IA was slower to reach peak activity but remained active throughout the phonatory task. The author concluded that the TA and LCA were critical in positioning the folds for phonation, while isometric contraction of the IA was important in maintaining adduction throughout phonation. During prolonged phonation tasks aimed at examining the laryngeal response to reduced respiratory support at the end of phonation, IA activity increased at the end of voicing in all subjects. These results suggested the IA's importance in securing and/or tightening glottal closure to enhance glottal efficiency. Interestingly, in some subjects, heightened TA and/or LCA activity was also observed during prolongation tasks, indicating that in a segment of the population, additional muscles are recruited to valve airflow in the face of reduced respiratory support. Finally, IA activity was unchanged during pitch elevation tasks, indicating no role for the muscle in this pitch control. Hillel concluded that the IA was primarily responsible for maintaining vocal fold adduction during sustained voice production.[31] **Swallowing and Other Sphincteric Functions.** Only the aforementioned study by Hillel has examined IA participation in non-phonatory tasks. During swallowing, the IA showed an increase in activity approximately 20msec after the initiation of the swallow, supporting the muscle's role as an important laryngeal adductor during swallowing. In addition, the muscle showed heightened activity during laryngeal closure for coughing, throat clearing, and production of the Valsalva maneuver.[31] **Respiration.** Hillel's work found that the IA was not active during exhalatory tasks.[31] The muscle was active during inhalation, but only when subjects were engaging in vigorous, active breathing.

In Vivo Studies. Three in vivo studies of IA function have been completed in the canine larynx. In the first of theses studies, Nasri et al[138] stimulated IA and TA activity in tracheotomized and anesthetized dogs. Videostroboscopic images of laryngeal activity were recorded during muscle stimulation. Stimulation of the IA alone resulted in adduction of the vocal processes of the arytenoids, the presence of a mid-vocal fold gap, and the inability to initiate phonation. Similarly, stimulation of the TA alone yielded a bulging of the membranous fold, the presence of a sizeable posterior gap, and the inability to initiate phonation. Combined stimulation of the IA and TA yielded full adduction and closure along the entire vocal fold length and the ability to phonate. The authors concluded that the IA was necessary for closure of the posterior glottis and for the production of voice. In a later study, Nasri et al[139] examined all 3 primary adductors (IA, TA, and LCA) in a group of tracheotomized dogs. Adductory force at the vocal processes of the arytenoids was recorded during stimulation of the adductory muscles in isolation. Force measurements showed the LCA to be the strongest of the adductors, followed by the TA, and finally the IA. The authors suggested that the IA was primarily involved in adduction of the vocal processes, acting as an accessory muscle to the TA and LCA. Finally, Choi et al[137] used the canine model to examine function of the laryngeal adductors. The group electrically stimulated RLN branches leading to adductory muscles to determine the individual and combined contributions of these muscles to vocal fold adduction. They identified the TA as the primary adductor of the anterior, membranous folds; the LCA as the primary adductor of the region between the vocal processes; and the IA as the primary adductor of the posterior commissure between the arytenoid bodies. Choi and colleagues further demonstrated a correlation between IA activity and increases in subglottic pressure, intensity, and fundamental frequency. The authors concluded that the IA was critical in adducting the most posterior aspect of the glottis and in the subsequent building-up of subglottic pressure for vocal fold vibration. Interarytenoid influence on intensity and fundamental frequency was felt to be indirect and mediated via the muscle's influence on subglottic pressure.

In Vitro Studies. Hirano and Kakita reviewed the work of colleagues (Hirano, 1975; Koike et al,1975; Morio, 1976) performed on excised canine larynges.[141] The researchers electrically stimulated individual laryngeal muscles and visually recorded still

images of alterations in vocal fold length, position, thickness, appearance, and stiffness at the time of stimulation. Interarytenoid stimulation resulted in adduction of the cartilaginous vocal fold. The muscle's influence on the membranous, vibrating aspect of the vocal fold was minimal, consisting of a slight reduction in vocal fold length, a slight thickening of the fold's edge, and a mild reduction in the fold's stiffness. The authors concluded that the primary contribution of the IA was adduction of the posterior, cartilaginous aspect of the vocal folds.

Summary of Function Studies. The above studies point to an important role of the IA in adducting the cartilaginous glottis for voice production, swallowing, and other sphincteric functions. Further, during phonation, the IA appears to acts in concert with its fellow adductors, the TA and LCA, to posture the folds for the onset of phonation, while the IA retains the posture for prolongation of the tone.

Innervation

The IA is privileged to have the most complex neural supply of all the laryngeal muscles.[70] The transverse IA is the only unpaired muscle within the larynx, and consequently, the only laryngeal muscle receiving bilateral innervation from the recurrent laryngeal nerve.[70, 131, 132] Additionally, in most cases the IA receives a degree of supplemental innervation from the internal branch of the SLN.[70, 132] Specifically, this supplemental innervation is offered when the lower branch of the SLN joins the RLN to create a nerve plexus in the superior aspect of the IA.[70, 132] This unique innervation pattern offers the transverse IA the benefit of continued neural input in the presence of unilateral recurrent laryngeal nerve injury,[132] and a potentially heightened resistance to physical injury.[32] Interestingly, the IA also demonstrates a more rich supply of multi-innervated fibers (9% to 21% of fibers show multi-innervation) than other laryngeal muscles.[19]

Sensory Mechanisms

The IA also diverges from the intrinsic laryngeal muscles in its mechanism of proprioceptive feedback.[32, 77, 78] Katto et al[77] presented one of the earliest studies examining muscle spindles within the IA. The histological study was performed using a single human larynx removed during laryngectomy. Muscles block-stained with saturated uranyl acetate were viewed initially under light microscopy to determine the presence or

absence of spindles. When spindles were identified, ultrathin sections were cut, stained with uranyl acetate and lead citrate, and viewed under electron microscopy. The researchers identified spindles in the IA which were approximately 1/3 the size of those typically observed in limb muscle. Further, spindles of the IA possessed primary morphological differences from classic limb muscle spindles (ie, thinner connective tissue capsule, narrowed periaxial space, atypical intrafusal fiber appearance, atypical patterns of twisting and swelling at nerve endings), leading authors to conclude that spindles of the IA were refined to respond to both muscle stretch and pressure. A second study by Okamura and Katto was performed on human larynges removed during total laryngectomy.[78] Initially, muscle sections from the IA, TA, PCA, LCA, and CT were stained with Lee's methylene blue-basic solution and examined under light microscopy. Once spindles were localized, ultrathin sections were stained with toluidine blue in preparation for electron microscopy. Spindles were identified in all laryngeal muscles except the LCA. Within the IA, spindle presence was abundant, and most spindles were localized to the muscle's central region. Unusual spindle morphology was again identified in the IA, and the authors concluded that spindles the IA were refined to convey both stretch and pressure information. Most recently, Tellis et al[32] used histological and immunohistochemical (anti-neonatal polyclonal and anti-tonic polyclonal antibodies) methods to examine human IA muscles obtained from total laryngectomy cases. An average of 7 spindles was identified per IA, with most being localized to the mid-belly of the muscle. Spindles were complex in design and morphologically distinct from classic limb muscle spindles in terms of their connective tissue capsules, their elongated extrafusal fibers, and their peculiar orientation at the muscle's insertion into the arytenoid cartilage. The peculiarity of the spindles led Tellis et al to suggest that spindles of the IA were unusually sensitive to dynamic muscle activity[32, 77, 78].

The above studies, which include both histological and immunocytochemical methods, point to the presence of muscle spindles in the IA. However, the spindles appear to be morphologically distinct from those classically observed in limb muscle. The authors of the above studies propose that spindles within the IA may be refined to offer the muscle maximal sensitivity to pressure, stretch, and complex movement.

Contractile Properties

While myosin isoforms in laryngeal muscle has been an area of intense interest, the IA muscle has frequently been omitted from studies on this topic. However, 2 studies of myosin expression in the IA were identified in the literature. **Human Studies.** In the first study, Shiotani, Westra, and Flint used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots to determine the MyHC distribution within the IA, TA, LCA, PCA, CT, and vocalis muscles. The IA was comprised of 21.5% MyHC I, 57.9% MyHC IIA, and 20.6% MyHC IIB.[φ] The IA's MyHC composition was most similar to that of the LCA, a partner vocal fold adductor. Tellis et al[32] examined the IA muscles of 5 human larynges excised during laryngectomy. Interarytenoid fiber types were determined by a combination of histochemical and immunocytochemical assays. Histochemical stains included myofibrillar ATPase (marker of fast vs. slow myosin isoforms), glyceraldehyde 3-phosphate dehydrogenase (marker of glycolysis), and succinate dehydrogenase (marker of oxidative phosphorylation). Immunocytochemical assays used monoclonal antibodies against basic and specialized myosin isoforms. The IA contained 35% Type I fibers, 45% Type IIA fibers, and 15% Type IIX fibers. Less than 5% of fibers co-expressed more than one MyHC, and no atypical isoforms were identified. The authors concluded that fiber types within the IA were similar to those of limb muscle and dissimilar to those reported in other laryngeal muscles. While studies of the IA's contractile properties have been limited, current work shows an IA profile similar to that of limb muscle: presence of Type I, IIA, and IIX fibers, the absence of coexpressing fibers, and the absence of atypical myosin isoforms.[32]

Summary of the Interarytenoid Literature: Strengths, Limitations, and Future Directions

The above review demonstrates a relative paucity of studies examining the biological features of the IA muscle. Available studies have focused primarily on the muscle's gross structure, function, and innervation. The literature supports the IA as the primary adductor of the posterior larynx, contributing to the maintenance of laryngeal closure during voicing, swallowing, coughing, and throat clearing. Further, the above studies offer early evidence supporting the IA's divergence from laryngeal muscle along

[φ] MyHC IIB is now known not to exist in humans. The MyHC IIB referred to in this study is likely correctly identified as MyHC IIX.

several fronts. First, the IA's innervation has been described as the most sophisticated among the laryngeal muscles. The muscle's unique bilateral support from the RLN, supplemental innervation from the SLN, and high percentage of multi-innervated fibers offer it a neurological advantage over its sister laryngeal muscles. Further, the muscle's sensory mechanisms differ from that observed in most laryngeal muscles. At present, the IA is the only intrinsic laryngeal muscle where the presence of spindles is not under debate. Interestingly, IA spindles appear refined beyond those observed in limb muscle, a point which has led some to propose the dual mediation of stretch and pressure by these organs. Finally, IA fibers show the presence of MyHC I, IIX, and IIA, the absence of atypical myosin isoforms, and the absence of co-expressing fibers: a profile analogous to that of limb muscle, but divergent from that of laryngeal muscle.

Tellis et al[32] were the first to comprehensively examine the IA and the first to comment on the muscle's unexpected similarity to limb muscle. In their discussion, the authors highlighted the importance of further defining the phenotype of the IA in order to refine the clinical management of voice and swallowing disorders. Unfortunately, since that study, no additional examinations of the IA have been reported in the literature, and no further discussion of the IA's divergent phenotype has ensued.

The Cricothyroid

The CT is a two-bellied muscle coursing between the anterior aspects of the cricoid and thyroid cartilages. The muscle arises from the anterolateral arch of the cricoid cartilage, inserting into the thyroid cartilage as 2 separate units: the pars recta which courses vertically to insert along the inner aspect of the thyroid cartilage's lower margin, and the pars oblique which courses superiorly and posteriorly to insert into the inferior horn of the thyroid.[12] The more vertically positioned pars recta elevates the cricoid ring, whereas the diagonally positioned pars oblique retracts the entire cricoid cartilage relative to the thyroid. The combined action of the 2 bellies increases the distance between the angle of the thyroid and the vocal processes of the arytenoids, elongating and tensing the vocal folds. Because of its ability to manage vocal fold length and tension, the muscle plays a primary role in pitch control and a supportive role in vocal fold adduction.[12, 34, 142]

Morphogenesis

As with the IA, cricothyroid muscle development is traced to the occipital somites. However, unlike its sister laryngeal muscles that emerge from the sixth branchial arch, the CT forms from the mesoderm of the fourth arch. Also forming from the fourth arch are pharyngeal constrictors, select muscles of the tongue and palate, and the SLN, the primary innervation source for the CT.[33] This origin sets the CT apart from the remaining intrinsic laryngeal musculature and has caused some to propose that the muscle is embryologically more closely aligned with the pharyngeal musculature than the laryngeal musculature.[34]

Function

Function of the CT has been examined using EMG, in vivo, and in vitro methods. Studies suggest CT activity during a myriad of phonatory and non-phonatory tasks.

EMG Studies. Electromyographic studies have permitted investigators to examine the activity of the intrinsic laryngeal musculature of humans during the performance of vegetative and phonatory activities. **Phonation:** An early study by Yanagihara and von Leden[143] examined CT activity as related to airflow, subglottic pressure, pitch, and intensity. Three males with normal voice control participated in the study. Cricothyroid activity was recorded during production of /a/ at varying pitch and loudness levels via a concentric needle electrode positioned in the left CT. The authors found heightened CT activity during production of high-frequency and high-intensity tones. The authors concluded that the CT regulated pitch and loudness by manipulating vocal fold tension and vocal fold resistance to the air stream. Gay and colleagues[140] used hooked wire electrodes to examine the activity of the intrinsic laryngeal muscles during modifications in pitch, loudness, and vocal onset. The CT was identified as a key muscle of pitch elevation, acting in conjunction with the vocalis to control pitch in the chest and falsetto registers. Contrary to the findings of Yanagihara and von Leden, the CT did not make primary contributions to the control of intensity in this study. The CT also offered no contribution to the control of vocal onset. Hillel[31] conducted perhaps the most comprehensive EMG examination of the intrinsic laryngeal musculature to date. Monopolar hooked wire electrodes were positioned in the laryngeal muscles of 12 normal speaking adults. The CT was most specifically active during pitch elevation. The

muscle's participation in sustained phonation (modal register) varied across individuals. When the CT was active during sustained phonation, it remained engaged throughout phonation, showing phonatory behavior similar to that of the IA. The author concluded that the CT's primary contribution to phonation was in the regulation of pitch. Finally, only one study has compared the activity of the pars recta and pars oblique segments of the CT during phonatory tasks. Hong et al[144] placed hooked wire electrodes in the pars recta and pars oblique bellies of the CT in 8 adults status-post ipsilateral thyroid lobectomy. The investigators found simultaneous activity of the bellies at the onset of sustained phonation; however, the two bellies offered distinctive contributions related to vocal fold lengthening. The pars oblique was more active than its sister belly during the initial posturing and fine tuning of vocal fold length at the onset of speech, whereas the pars recta showed greater activity than the oblique during notable modifications of pitch. The authors suggested that the combined activity of the bellies guided the establishment, and later adjustment, of vocal fold length during voicing. **Vegetative Tasks:** Only one EMG study has examined CT activity in the activities on respiration and swallowing.[31] Hillel used monopolar hooked wire electrodes to track CT activity in 12 normal adults. During swallowing, the CT acted in synchrony with primary vocal fold adductors: the TA and LCA. These findings pointed to a potential role of the CT in glottic closure during the swallow. Cricothyroid function during respiration was variable across individuals. The muscle showed heightened activity during inspiration in all subjects tested; however, activity during exhalation in only a portion (44%) of subjects.

In Vivo Modeling. In vivo studies of CT function have also contributed to the understanding of the muscle's role in phonatory as well as non-phonatory tasks. **Phonation.** In an early study, Hirose et al[145] electrically stimulated the TA, PCA, and CT muscles of tracheotomized cats. Single impulse stimulation of the SLN and RLN resulted in the development of vocal fold tension. Contraction of the CT in isolation yielded rapid tension development, whereas, contraction of the TA in isolation yielded slower and more prolonged tension development. The group concluded that the CT worked with the TA to manage fold tension, with the CT acting as the primary *external* controller of gross tension and the TA acting as an intrinsic regulator of fine tension. Several years later, Tanaka and Tanabe subglottically insufflated canine larynges to determine glottal

adjustments used in intensity control.[74] Intrinsic muscle (CT, LCA, TA, and PCA) movements were simulated using mechanical retraction; sound pressure level, subglottic pressure, mean airflow rate, aerodynamic power, and glottal resistance were recorded during the simulated motion. The LCA and TA muscles were identified as the primary contributors to intensity. Cricothyroid activity contributed only slightly to intensity, adding, on average, only 0.2 dB to the tone. The authors concluded that the CT's role in intensity control was minimal and mediated via its influence on vocal fold adduction. Hong and colleagues[142] compared function of the pars recta and pars oblique in a canine model. The group tracked changes in fundamental frequency of vibration (Fo), intensity, subglottic, pressure, vocal fold length, and cricothyroid distance during stimulation of the pars recta and oblique. Stimulation of the pars recta yielded a greater increase in the frequency of vocal fold vibration than stimulation of the pars oblique; however, simultaneous stimulation of both branches brought about the most significant changes in Fo. The elevation in pitch was mediated via a two-part action of the CT upon the cricoid and thyroid cartilages. The pars recta displaced the thyroid on the cricoid along the vertical axis, while the pars oblique displaced the cartilages along their horizontal plane. The authors concluded that changes in cricothyroid joint position during pitch elevation were complex and multi-dimensional, the result of a coordinated effort of the pars recta and pars oblique to readjust the relationship of the cricoid and thyroid cartilages for vocal fold elongation. **Respiration.** Amis et al[146]examined the pharyngeal responses to CT activity during respiration. Muscle activity was induced via supramaximal electrical stimulation of the external branch of the SLN. Supraglottal and upper airway resistances were calculated; pharyngeal movement was tracked via computerized axial tomography. Cricothyroid contraction resulted in pyriform sinus dilation and an associated a reduction in supraglottic and upper airway resistance. The authors concluded that the CT served as a pharyngeal dilator during respiration. The same group later examined the CT's influence on laryngopharyngeal geometry and airway resistance using the methods discussed above.[147] Cricothyroid stimulation yielded: (1) lateral movement of the thyroid cartilage alae and subsequent dilation of the pyriform sinuses, (2) glottal lengthening, and (3) slight vocal fold movement toward midline. Subsequent reductions in supraglottic and upper airway resistance to airflow were observed. From the 2 studies, the authors

concluded that the CT played a role in widening the pharyngeal outlet and, consequently, reducing upper airway resistance to airflow during respiration.

In Vitro Modeling. Hirano and Kakita reviewed the in vitro work of Hirano (1975), Koike et al (1975), and Morio (1976).[141] The series of studies involved the electrical stimulation of individual laryngeal muscles in excised canine larynges. Changes in vocal fold position, length, thickness, appearance, and stiffness were recorded via still photographs taken superior and medial to the vocal folds. Stimulation of the CT resulted in: lowering of the vocal folds in the larynx, elongation and thinning of the vocal folds, sharpening of the vocal fold edge, and stiffening of the vocal fold's 3 primary layers (ie, body, transition, and cover). The authors concluded the CT exerted notable influence over vocal fold vibration by influencing vocal fold geometry and modifying the mechanical properties of the vocal fold layers during voicing. Hirano et al[148] examined TA, LCA, CT, and PCA roles in glottic shaping in 10 male and 10 female cadaveric larynges. Ventricular folds and epiglotti were removed to enhance viewing of the glottal area, and larynges were positioned in a support frame. Nylon threads attached along various points of the laryngeal cartilages were manipulated to simulate muscle activity. Cricothyroid activity was simulated by a ventrocaudal pull on the superior aspect of the thyroid cartilage. The primary contribution of CT activity was elongation of the membranous vocal folds; however, a supportive role in vocal fold adduction and abduction was recognized. Interestingly, CT influence over vocal fold length was more pronounced in females. The authors proposed that the gender difference was due to increased cricothyroid joint mobility in females or an increased extensibility of the female vocal fold tissue.

Summary of CT Function Studies. The above studies point to CT participation in phonation, swallowing, and respiration. It is, however, the CT's contribution to voice production that has been of greatest interest to researchers. In phonation, the CT acts as an external regulator of vocal fold length and tension and as a vital contributor to vocal pitch. The muscle exerts additional influence over voicing by altering the mechanical properties vibrating vocal folds. The muscle's action during vegetative activities has been less extensively examined; however, the evidence points to a role in inhalation, perhaps as a supraglottal and pharyngeal dilator.

Innervation

The CT is innervated by the external branch of the SLN.[12] Hence, it is the only intrinsic laryngeal muscle receiving primary innervation from a source other than the RLN. As the external branch nears the larynx, it courses under the sternothyroid muscle before dividing to supply muscle fibers of the pars recta, pars oblique, and inferior constrictor.[71] According to DeVito, Malmgren, and Gacek,[149] motor endplates of the human CT are randomly and widely distributed throughout the medial 2/3 of the muscle (anterior to posterior) and limited at the muscle's extreme ends. The above pattern diverges from the narrow, mid-muscle band of motor end plates classically observed in limb muscle. The authors speculated that the CT's unique innervation pattern was secondary to the geographic complexity of the muscle's fibers and/or the possible presence of multiple neuromuscular junctions per muscle fiber.

Sensory Mechanisms

Histological Studies. Attempts to identify proprioceptive organs within the CT have been limited. Keene et al[79] used hematoxylin and Bierbrich scarlet stain and the Romanes silver method to study the distribution of spindles across human intrinsic laryngeal muscles. While the group reported finding spindles in all laryngeal muscles, they indicated that spindles were particularly abundant in the CT and PCA. Raman and Devanandan used a modification of DeCastro's silver technique to examine spindle presence in the intrinsic and extrinsic laryngeal muscles of bonnet monkeys.[150] In contrast to Keene et al's findings, the group found the intrinsic laryngeal muscles (CT, TA, PCA) to be devoid of spindles, while suprahyoid and infrahyoid extrinsic muscle controls evidenced the structure. Hence, the presence of spindles in the CT has yet to be determined. More recent histologic studies of laryngeal proprioception have not included the CT. Therefore, more sophisticated methods of muscle spindle identification have not been applied to this muscle group.

Clinical Studies. In an attempt to more clearly define laryngeal proprioception and particularly the mechanisms employed to track changes in vocal fold length, Loucks et al[16] used hooked wire electrodes to record activity in the CT, TA, and sternothyroid muscles during servomotor displacement of the thyroid cartilage. Electromyographic activity in the CT and TA did not change during moments of mechanical displacement,

suggesting the absence of a stretch reflex within these intrinsic muscles. Interestingly, the investigators found opposing results in the extrinsic sternothyroid muscle. The authors concluded that the CT and TA were lacking in muscle spindles and that afferent feedback for voice control was mediated via other sensory receptors within the larynx.

Thus, histological studies of the CT have been limited and have failed to offer a clear picture as to the presence or absence of spindles in the muscle. Recent clinical studies support the findings of Raman and Devanandan and suggest that proprioception for the CT is mediated without the muscle spindle.

Contractile Properties

Myosin Isoform Profile. **Animal Models.** Jung and colleagues[94] used reverse transcription polymerase chain reaction (RT-PCR) to determine the precise RNA transcript levels of laryngeal MyHC isoforms. Specifically, the authors considered transcription levels for MyHC I, IIA, IIB, IIX, IIL, embryonic, and neo-natal in the rat larynx. The CT contained primarily fast isoforms: 72.1% MyHC IIX, 25.2% MyHC IIA, 2.2% MyHC I, 0.4% MyHC IIB, and 0.04% embryonic and neonatal. The MyHC IIL (a fast myosin considered by some to be MyHC-eo) was not identified. Despite the abundance of fast isoforms, the CT was found to have among the slowest myosin profile of the laryngeal muscles examined. Rhee, Lucas, and Hoh[35] compared myosin expression between the CT and TA muscles of rats. Monoclonal antibodies against MyHC-I, IIA, IIX, IIB, and extraocular were employed. The CT evidenced all forms of limb skeletal muscle fiber types in the following distributions: 61.2% MyHC IIX, 19% MyHC I, 12.5% MyHC IIA, and 4.9% MyHC IIB. The TA demonstrated a faster profile consisting primarily of fibers expressing or co-expressing MyHC IIB, extraocular, and IIX. The authors concluded that the myosin heavy chain profile of the CT was unlike that of the TA, but analogous to that of classic limb muscle. Lucas et al[17] used monoclonal antibodies against MyHC-eo to consider the isoform's expression in rabbit CT and TA muscles. While the specialized isoform was identified in the TA, it was not found in the CT. The authors concluded that the CT possessed a rate of contraction more indicative of fast limb muscle than laryngeal muscle. Later work by Shiotani and Flint[22] in the rat model supported the above results. Results of SDS-PAGE and Western blotting confirmed the presence of MyHC I, IIB, IIX, and IIA in the CT. MyHC-eo was identified

in some intrinsic laryngeal muscles (TA, PCA, LCA) but not within the CT. The group concluded that the CT stood apart from laryngeal muscles on 2 fronts: (1) it possessed the slowest myosin profile of the intrinsic laryngeal muscles examined in their study, and (2) it was the only laryngeal muscle devoid of MyHC-eo. The authors suggested that the CT's departure from laryngeal muscle was secondary to its differing embryological origin and/or its differing source of primary innervation. **Human Studies.** Two groups have examined MyHC distribution across the primary intrinsic laryngeal muscles in humans. Shiotani et al[23] used SDS-PAGE and Western blots to examine myosin isoforms in 6 cadaveric larynges. They defined CT myosin composition as 61.1% MyHC IIA, 34.6% MyHC I, and 4.3% MyHC IIB[ϕ], results which placed the CT alongside the PCA as the slowest of the laryngeal muscles. Finally, findings of Li et al[96] supported the above study. The group used SDS-PAGE and Western blotting to examine myosin isoform expression in 5 cadaveric larynges. All laryngeal muscles except the IA were considered in the study. The authors found only MyHC IIA (60-65%) and I (30-35%) in the CT. The fastest of the basic human isoforms, MyHC IIX, was absent in the muscle. When comparing results across laryngeal muscles, the authors concluded that the CT demonstrated a slower profile than the group of laryngeal adductors.

Hence, studies in animal and human larynges point to the CT's myosin heavy chain profile as slow relative to that of its sister laryngeal muscles but comparable to that of fast limb muscle. It has been suggested that this deviation from other laryngeal muscles may be a result of the muscle's differing embryonic development and/or its differing mode of innervation.[22]

Fiber Size and Arrangement

Four studies considering the fiber size of the CT relative to other laryngeal and limb skeletal muscles were identified in the literature. A study by Sadeh et al[50] used 2 human larynges obtained from laryngectomy to compare the fiber diameter of 4 intrinsic laryngeal muscles (CT, PCA, LCA, vocalis) to referenced fiber sizes of limb muscle. In both larynges, mean CT fiber diameter was 40μm, notably less than their referenced limb muscle diameter (60-70μm). Interestingly, in one specimen CT diameter was similar to

[ϕ] MyHC IIB is now known not to exist in humans. The MyHC IIB referred to in this study is likely correctly identified as MyHC IIX.

that of the PCA and vocalis muscles; however, in the second specimen, CT fibers were notably smaller than those of the vocalis (60µm). The study suggested that CT fiber sizes are generally similar to those of fellow laryngeal muscles but smaller than those of classic limb muscle. A second study used the canine model to compare fiber diameter across 4 intrinsic laryngeal muscles: the CT, cricoarytenoid lateralis, cricoarytenoid dorsalis, and TA muscles.[151] The largest fibers (38.19µm to 43.25µm) were found in the CT, whereas the smallest fibers (29.38µm to 32.05µm) were found in the TA. The study suggested that a degree of variability in fiber size exists across laryngeal muscles and that CT fibers appear to be largest in the canine larynx. Two additional studies have considered CT fiber diameters in the rat. Mean CT fiber diameters were similar to comparison laryngeal muscles (TA and PCA) and ranged from 15-30µm.[135, 152] These studies suggest that the CT is comprised of small diameter fibers typical of the intrinsic laryngeal musculature.

Two studies have examined the arrangement of muscle fibers within the CT. In the first of these studies, Hyodo et al[135] considered the myotendinous junctions of the CT and PCA muscles. Junctions in the PCA were conical with multiple longitudinal clefts, a simple, primitive architecture relative to classic limb junctions. The CT, however, demonstrated 2 forms of myotendinous junctions, one simple form as described above in the PCA and one more complex junction likened to that of limb muscle. The authors concluded that the CT was a transitional form of muscle, falling into a category between the more primitive laryngeal muscle phenotype and the more evolved limb muscle phenotype. A second study examined the network among individual fibers within the CT.[152] Most fibers ran parallel to one another along the long aspect of the muscle; however, some fibers branched and interdigitated with nearby fibers. The result of the branching was a complex network of myomyous junctions not observed in classic limb muscle, but previously reported in cardiac, extraocular, and other laryngeal (ie, TA) muscles. The reason for this unique architecture is unknown; however, the authors suggest that it may offer the muscle a more refined and efficient pattern of contraction.[152]

Sensitivity to Disease

Laryngeal muscles have been recognized for their early involvement in some diseases and their preferential sparing in others.[4, 7, 25, 29, 30, 108-110] However, one recent study by Marques et al[4] has suggested that laryngeal response to disease may vary across

muscle. The authors examined the effects of dystrophin deficiency on the medial TA, lateral TA, LCA, PCA, and CT muscles in 4 month (adult) and 18 month (aged) dystrophin deficient *mdx* and C57Bl/10 (control) mice. No evidence of myofiber degeneration or regeneration was observed in the medial TA, lateral TA, LCA, and PCA muscles. Interestingly, mild markers of disease (eg, central nucleation) were evidenced in the CT muscle of *mdx* mice. While percentages of central nuclei in the *mdx* CT (adult M = 9.3, SD 4.0; aged M = 18.0, SD = 1.5) did not approach those of the stereotypically affected tibialis anterior (adult M = 50.0, SD 1.0; aged M = 96.0, SD = 2.0), they were significantly higher ($p < .05$) than those observed in other *mdx* laryngeal muscles (range 1.0 to 2.5) and in control CT muscles (adult M = 4.8, SD 1.1; aged M = 5.3, SD 1.1). The authors proposed that mild disease effects in the CT in the face of otherwise widespread laryngeal muscle sparing may have been secondary to the CT's biochemical and/or structural differences from other intrinsic laryngeal muscles.

Summary of CT Literature: Strengths, Limitations, and Future Directions

Literature pertaining to the CT has focused primarily on the muscle's function, contractile properties, and innervation. Available literature highlights a primary role for the CT in voice production and a supportive role for the muscle in respiration and swallowing. During phonation, the muscle acts as a primary external regulator of vocal fold length and tension, and consequently, a controller of vocal pitch.

While functional roles of the CT have been well-defined, its biological properties have yet to be thoroughly described. Morphologically, the CT appears similar to other laryngeal muscles in its fiber size and general architecture. However, its morphogenesis, innervation, myosin heavy chain profile, contractile patterns, and sensitivity to disease set it apart from laryngeal muscle and place it more in line with pharyngeal and/or limb skeletal muscle. This highly unique phenotype of the CT has led some to classify it as a hybrid or transitional form of muscle. Unfortunately, comprehensive studies of the CT capable of thoroughly describing the muscle's phenotype relative to other laryngeal muscles have not been completed. The CT plays a unique role within the larynx, acting as the sole modulator of static vocal fold tension. Its activity is required for the preservation of the vocal fold's medial aspect and for proper glottal valving. Hence, an appreciation of this muscle's biological properties and its response to disease and aging is critical.

Summary of the IA and CT Muscles

The above review suggests the presence of heterogeneity among the intrinsic laryngeal musculature. Specifically, the literature intimates that the IA and CT muscles diverge from their sister laryngeal muscles and demonstrate a phenotype more similar to that of limb skeletal muscle. However, comprehensive investigations of IA and CT biology have not been performed to confirm this diversity. While a number of methods are available for further examining the biology of these muscles, one model, in particular – the *mdx* mouse model of dystrophin deficiency – has served as an indicator of a muscle's level of specialization and its similarity to or departure from classic limb skeletal muscle. A description of the model, a review of its use, and a discussion of its application to the IA and CT muscles follow.

The Model

Duchenne muscular dystrophy is a genetic, lethal disease that results from the lack of the cytoskeletal protein, dystrophin.[39] The disease was once believed to affect all skeletal muscles; however, recent work has identified the paradoxical sparing of some muscles, most notably the extraocular muscle group and the TA, PCA, and LCA muscles of the larynx.[4, 7, 25, 40] The absence of the pathological cascade in these muscles highlights their uniqueness among skeletal muscle. In addition to their sparing in this disease, the extraocular and laryngeal muscles are recognized for their departures from limb muscle in the areas of: fiber diameter, fiber types, motor unit size, proprioceptive mechanisms, myosin isoform expression, remodeling behaviors, and sarcomeric structure.[2, 41, 44, 47-49, 52] Hence, response to dystrophin deficiency may serve as a sensitive marker of a muscle's level of biological specialization and its similarity to or departure from classic limb muscle. Examination of the IA and CT muscles with this model will offer greater insight into their biological characteristics and level of specialization.

The Rodent Larynx

The rodent larynx has been useful in the study of laryngeal biochemistry, vascularity, and neuromuscular function as well as in the examination of laryngeal response to irradiation, aging, and disease.[22, 83, 153-158] Most laryngeal studies involving rodents have employed the rat model. As a result, the rat larynx has become the most

well-defined in the rodent family[159-161] and, therefore, becomes basis for emerging study of the mouse larynx.

The Rat Larynx: Gross Anatomy and Myology

Skeletal aspects of the rat larynx include the hyoid bone, epiglottis, thyroid cartilage, cricoid cartilage, and paired arytenoid cartilages.[159-161] An additional, wing-shaped alar cartilage has been identified in the anterior larynx near the base of the epiglottis.[160, 161] The broad-faced thyroid cartilage encases other laryngeal cartilages laterally and ventrally and serves as the attachment for key intrinsic muscles. The ring-shaped cricoid rests between the first tracheal ring and the thyroid cartilage and articulates dorsally with the caudal horn of the thyroid. Rostral and caudal aspects of the cricoid cartilage serve as attachment points for intrinsic laryngeal muscles. The paired, V-shaped arytenoid cartilages articulate with the cricoid lamina. The arytenoids demonstrate three distinct processes: the muscular process which articulates with a rostral ridge of the cricoid cartilage; the vocal process which projects ventrally toward the thyroid lamina; and the corniculate process which projects anteriorly toward its counterpart on the opposing side. The muscular and vocal processes serve as key attachments for intrinsic muscles.[159-161]

The rat TA, CT, LCA, and PCA muscles are positioned as within the human larynx.[160, 161] However, the rat larynx demonstrates two additional muscles not found in humans.[160, 161] The first of these muscles courses from the alar cartilage anteriorly to the lateromedial aspect of the muscular process and cricoid cartilage posteriorly. The muscle has been termed both the alar cricoarytenoid muscle and the cricovocal muscle. Speculation as to its function as not been offered. The second muscle, termed the superior cricoarytenoid (SCA) and the rostral cricoarytenoid, courses posteriorly and medially from the lateral face of the arytenoid to the cricoid lamina's midline tubercle.[160, 161] Authors suggest that the SCA muscle may function to draw the arytenoids toward one another at midline, in a fashion similar to that of the IA in humans. Interestingly, only one source has described the presence of a transverse arytenoid muscle in the rat.[159] In 1976, Hebel and Stromberg[159] identified fibers coursing between the paired arytenoid cartilages in a manner similar to that of the transverse IA of humans. More recent works,

such as those noted above by Inagi et al[160] and Kobler et al,[161] have not identified this muscle.

The Mouse Larynx

Investigations using the mouse larynx have been infrequent in the literature.[4, 7, 25, 162-164] A number of these studies have considered only superficial aspects of the larynx (eg, epithelium, taste bud function) and have not examined the skeletal and myologic aspects of the mouse larynx. Consequently, gross and fine aspects of mouse laryngeal anatomy remain largely unexplained.

Early descriptions of the mouse laryngeal framework indicate a hyoid bone, epiglottis, thyroid cartilage, cricoid cartilage, and paired arytenoid cartilages.[165, 166] No defining works on mouse laryngeal myology could be identified in the literature. Two studies examining the effects of dystrophin deficiency on mouse laryngeal muscles did offer early information regarding the presence of certain muscles in the mouse model. Thomas and colleagues[25] identified TA and PCA muscles in the mouse, while Marques et al[4] identified the aforementioned muscles as well as the LCA and CT. Details of the muscles' anatomy and positioning within the larynx were not described by either author.

Thus, the study of the mouse larynx remains in its infancy. In particular, laryngeal myology remains to be investigated and the presence, location, and properties of the intrinsic muscles described.

Rodent Larynx: Summary

While aspects of rat laryngeal structure continue to emerge, the generalization of this knowledge to the mouse larynx may be inappropriate. Anatomical differences in the presence and location of neck muscles have been confirmed across various rodent species.[167] As a result, independent investigations of mouse laryngeal anatomy are needed to further define gross and fine aspects of the organ.

History and Features of the mdx *Mouse*

Dystrophin is a large (~400kd) cytoskeletal protein coded at gene locus Xp21.[36, 38] The protein is recognized as the pivotal member of the elaborate dystrophin-glycoprotein complex (DGC),[39] which mechanically links the muscle fiber's contractile filaments to the extracellular matrix,[38, 168] as shown in Figure 2.1. Dystrophin is comprised of 4 domains: (1) the N-terminus domain, which interacts with cytoskeletal actin filaments;

(2) the central-rod domain, which also interacts with cytoskeletal actin; (3) the cysteine-rich domain, which binds to the membrane-spanning protein, β-dystroglycan; and (4) the C-terminus domain which interacts with 2 additional cytoskeletal protein families, the dystrobrevins and syntrophins.[169] The linkage of the components in this way permits stabilization and support of the fragile cell membrane during muscle contraction.[37, 38, 168, 170] In addition to this support role, dystrophin has been implicated as playing a role in transmembrane signaling and in the regulation of intracellular calcium.[37, 169]

Pathophysiology of DMD

In DMD, a spontaneous mutation of the Xp21 gene results in the absence of dystrophin,[37, 38] and the subsequent disruption of the DGC's integrity.[38, 169] Without the DGC's structural support, the sarcolemma becomes vulnerable to the excessive mechanical forces applied by muscle contraction; focal sarcolemmal tearing often results.[38] The loss of sarcolemmal integrity permits the influx of extracellular calcium into the muscle fiber and the subsequent activation of protein-destroying enzymes. Gradually, fiber necrosis results.[39] Attempts at myofiber regeneration ensue, as evidenced by the presence of pleomorphic and centrally nucleated fibers. Over time, however, continued cycles of fiber degeneration and failed attempts at regeneration result in widespread fibrosis and fatty cell infiltration throughout the muscle.[171]

In recent years, it has been argued that the mechanical theory described above is not sufficient to explain the pathological cascade associated with dystrophin deficiency.[37, 171] As a result, an additional theories related to calcium regulation have been proposed.[171] Some have identified the presence of dystrophin-controlled mechanosensitive calcium channels in the membranes of skeletal muscle.[37, 172-174] In the absence of dystrophin, the channels remain open for prolonged periods and permit the entry of excessive amounts of calcium into the muscle cell. Consequently, intracellular calcium levels increase, and the enzyme-triggered fiber damage described above ensues.[37] Others have suggested a calpain-triggered increase in calcium leak channel activity in dystrophin-deficient muscles.[175, 176] Under this theory, extracellular calcium enters the muscle cell via leak channels, eventually triggering the action of proteases and the degeneration of the muscle fiber.

It is clear that high levels of intracellular calcium play a role in the pathophysiology of DMD. At present, however, researchers are unclear as to whether the high levels of intracellular calcium observed in dystrophin deficient muscles are a mechanism of the pathology, as suggested by calcium regulation theories, or a consequence of the pathology, as suggested by mechanical theories.

The mdx *Strain*

The *mdx* mouse strain is considered the standard animal model for the study of human DMD,[36, 177] and muscle sections taken from human DMD and *mdx* specimens confirm the genetic equivalency of the 2 models.[36] The *mdx* strain, first identified by Bulfield et al[178] in 1984, was the result of a spontaneous mutation of the Xp21 gene location in the C57BL/10ScSn mouse. The mutation yields the impaired expression of full-length dystrophin in skeletal muscle.[36] Histological markers of the disease (eg, fiber degeneration and regeneration, inflammation, fiber necrosis, centrally positioned nuclei) are present in the both *mdx* mouse and humans models; however, the mouse displays a milder clinical phenotype and a near normal lifespan.[177] Since discovery of the *mdx* model of dystrophin deficiency, it has been successfully used in a number of investigations of the pathophysiology and treatment of DMD.[42, 112, 177, 179-183]

The lifespan for the wild-type mouse is estimated at 24 months, but sexual maturity and the adult mouse form are identified by the 8th week.[184] Histologic markers of the dystrophin deficiency (eg, fiber degeneration and regeneration) are evidenced in *mdx* mice by the 3rd to 4th week.[177] By the 8th week, markers (eg, inflammation, protein destruction, and muscle regeneration) are clearly observed.[181] As a result, the 8-week mouse has been used frequently in the study of dystrophin deficiency.[7, 25, 42, 181]

Assays Used in the Study of Dystrophin Deficiency

A variety of histological and immunocytochemical assays are used to establish a muscle's response to dystrophin deficiency. Histological assays examine the overall morphology of the muscle fiber and the integrity of its membrane, whereas immunocytochemical methods confirm the presence and/or absence of key proteins of the DGC.

Histologic Assays

Histological Staining. Muscle fibers affected by dystrophin deficiency evidence fiber degeneration and regeneration. Degeneration is recognized by the presence of inflammation, necrosis, fibrosis, and fatty infiltration, while regeneration is identified by the presence of centrally located nuclei and pleomorphic fibers.[39, 105, 185] Basic histologic stains, such as hematoxylin and eosin,[186] are used to examine these aspects of basic muscle fiber structure.

Hematoxylin and eosin staining is a commonly used stain for overall tissue morphology which clearly reveals the general structure of the tissue sample, including the presence and location of nuclei, fibrous and fatty tissue, inflammatory cells, and fibrosis.[186] Hematoxylin stains cell nuclei blue/black, whereas eosin stains the cytoplasm and other cellular components in shades of pink, orange, and red. Classic hematoxylin and eosin protocols for frozen sections involve: tissue fixation, hematoxylin staining, eosin counter staining, dehydration in an ethanol series, tissue clearing, and mounting with an appropriate mounting medium.[186, 187] Tissues stained with hematoxylin and eosin are then viewed under light microscopy for evaluation of markers of interest.

Hematoxylin and eosin staining has been a commonly used assay in the study of dystrophin deficiency.[4, 7, 25, 86, 178] In the early stages of the disease process, affected muscles demonstrate central nucleation, inflammation, and necrosis. In the later stages, widespread fibrosis and fatty tissue infiltration are also observed. Spared muscle groups retain normal morphology across all ages tested, showing peripherally positioned nuclei and the absence of inflammation, fibrosis, necrosis, and fatty tissue collection.

Vital Dyes. Live cells have the capacity to manage the uptake and distribution of injected dyes, whereas, damaged cells do not.[188] Vital dyes can, therefore, be used to assess the health and integrity of individual cells. Vital dye protocols call for the injection of dye into living animals, with the subsequent sacrifice of the animal approximately 18 hours post injection. Muscle sections are prepared and evaluated under florescence microscopy to determine the dye's retention in the extracellular space or its incorporation into the intracellular space.

The vital dye Evans blue has been used previously in the *mdx* mouse to determine the integrity of the cell membrane.[189, 190] Muscle responses to the dye are binary.

Unaffected fibers in control and spared muscles retain the dye in the extracellular space and show an absence of florescent fibers under fluorescence microscopy. However, affected muscles with a loss of sarcolemmal integrity are unable to restrict dye entry into the muscle fiber and consequently fluoresce brightly under microscopy.

Immunocytochemical Assays

A variety of proteins of interest to researchers are not visible under basic light microscopy. Immunocytochemistry (ICC) uses antigen-antibody interactions to reveal cell and/or tissue components of interest.[191, 192] Immunofluorescence is one form of ICC which uses fluorescent labels to localize the target molecules. In studies of dystrophin deficiency, these methods have been used to confirm the presence of the dystrophin in control muscle and its absence in *mdx* muscle, to identify the presence, absence, and/or re-localization of associated members of the DGC and to examine the inflammatory response to dystrophin deficiency.[4, 7, 25]

Background. Antigens are proteins, carbohydrates, and lipid molecules that possess highly individualized binding sites, termed epitopes. Antibodies are serum proteins (immunoglobulin class) produced the humoral immune system, capable of identifying and linking with a specific antigen at its binding site. By locating the site of antigen-antibody binding, researchers can verify the presence of the antigen of interest.[191, 192] In immunofluorescence, the site of antigen-antibody binding is recognized in one of 2 ways: direct methods which conjugate a florescent label to the primary antibody and indirect methods which apply a florescent secondary antibody against the primary antibody. Florescence microscopy excites the fluorescent probe to reveal areas of antigen-antibody binding, and thereby, the presence and distribution of the antigen.[191, 192] Methods related to the production and use of antibodies in ICC are reviewed below.

Monoclonal Antibodies. Monoclonal antibodies used in ICC are homogeneous antibodies produced by a single B-cell line and capable of linking to a single epitope on the antigen of interest.[193] For production, antigens are injected into a live animal prompting an immune response. Spleen cells are removed from the immunized animal and combined in vitro with an immortal myeloma cell line. A hybrid cell, sharing properties of the antibody and the immortality of the cell line, is produced. Hybrid cells are screened to identify those producing the specific antibody of interest; selected hybrids

are cloned for repeated production of the desired antibody.[192] Characteristics of the monoclonal antibody are specified based upon the class of immunoglobulin (Ig) to which it belongs (IgA, IgD, IgE, IgG, or IgM) and the host animal in which the antibody was produced. As a result of the production process, monoclonal antibodies offer the advantages of consistency and homogeneity.[193] Monoclonal antibodies against key components of the DGC have been used in previous studies of dystrophin deficiency to identify the presence or absence of dystrophin, to determine the integrity of the DGC, and to consider modifications of protein expression in response to the loss of dystrophin.[4, 7, 25, 112, 194-196]

Polyclonal Antibodies. Polyclonal antibodies are a complex mixture of serum proteins (immunoglobulins) produced by the immune system against a specific antigen.[193] Polyclonal antibodies are produced by multiple B cell lines, and the resultant serum is a composite antibody capable of recognizing a variety of epitopes. For production, a host animal is presented with the antigen of interest, and an immune response ensues. Serum from the animal is harvested and purified to yield the polyclonal antibody. The generation of polyclonal antibodies from multiple B cell clones offers them a high degree of specificity, as they are able to link with multiple epitopes on the target antigen.[193] Polyclonal antibodies for dystrophin have not been previously used in examining the effects of dystrophin deficiency on the laryngeal muscles. However, successful use of a rabbit polyclonal antibody against dystrophin in skeletal muscle (ab15277, Abcam, Cambridge, MA) has been reported recently in the literature.[197]

Species Selection with Primary Antibodies. The species from which primary antibodies (monoclonal and polyclonal) are produced is an important consideration in immunological investigations. Primary antibodies generated from a species which is closely related, phylogenetically, to the species of study can yield an altered reaction to the antibody.[193] As a result, selection of a phylogenetically diverse antibody-generating species is preferred.[193]

Secondary Antibodies. In indirect ICC, a secondary antibody produced against the primary antibody is used to permit visualization and localization of the antigen-antibody binding site.[191, 192] In immunofluorescent studies, the secondary antibody linking with the primary antibody is a fluorochrome, a molecule capable of absorbing radiation and

moving into an excited state. Excitation of the fluorochrome by a given wavelength of light causes repositioning of the fluorochrome's electrons and the subsequent emission of a visible wavelength of light, generally longer than the exciting wavelength. Emitted light from the fluorochrome can be observed under fluorescent microscopy to localize the secondary antibody, and hence, the site of primary antibody-antigen binding. Critical characteristics of secondary antibodies that must be considered prior to use include: (1) the host animal in which the secondary antibody was produced, (2) the specific species against which the primary antibody was raised, and (3) the absorbance wavelength of the fluorochrome.[191, 192]

Fluorescence Microscopy. Tissue specimens prepared using the above methods are examined under fluorescence microscopy. In this process, a beam containing all wavelengths of light is produced and passed through an initial, heat-absorbing filter. The light is then directed through additional filters which permit only the desired (exciting) wavelength of light to pass. Upon projecting through the tissue sample, the fluorochrome is excited as described above, and the microscope's objective collects both the exciting and the emitted (fluorescent) wavelengths. The exciting wavelength is then filtered out, and the fluorescent wavelength is projected on to the viewer's eye.[198]

Immunocytochemistry in the Study of Dystrophin Deficiency. As noted above, ICC has previously been used to examine the presence and/or absence of dystrophin and its related DGC proteins and to monitor the inflammatory response in dystrophin deficiency.[4, 25, 112, 194-196] In the first of these uses, ICC reveals an absence of dystrophin and other vital proteins of the DGC in dystrophin deficient muscles and the normal distribution of these proteins in control muscles. When used to track inflammation, ICC shows markers of the inflammatory response (eg, myeloperoxidase presence in activated neutrophils[199]) in affected *mdx* muscles and the absence of such markers in spared *mdx* muscles.

Summary

The combination of histological assays and ICC has provided researchers a broad view of a muscle's response to dystrophin deficiency. Gross changes in tissue integrity and morphology as well as subcellullar changes in protein distribution are appreciated, helping to separate spared and affected muscle groups.

Previous Model Use in the Laryngeal Muscles

Two studies have used the *mdx* mouse and the above assays to consider the effects dystrophin deficiency on laryngeal muscles. In the first study, Thomas et al[7, 25] used histological methods and ICC to determine the effects of dystrophin deficiency on the TA and PCA muscles of 8-week old *mdx* mice. An examination of general morphology after hematoxylin and eosin staining revealed no evidence of muscle fiber degeneration or regeneration in the laryngeal and extraocular muscles. In contrast, notable disease markers (eg, fiber necrosis, fibrosis, and central nucleation) were widespread in the *mdx* gastrocnemius/soleus and diaphragm. Additionally, Evans blue dye staining showed an absence of dye-positive fibers in the *mdx* laryngeal and extraocular muscles and the presence of dye-positive fibers in the *mdx* gastrocnemius/soleus and diaphragm. The authors concluded that the TA and PCA muscles of the larynx were spared from the effects of dystrophin deficiency in the *mdx* mouse.

Marques et al[4] extended the above study by examining the influence of dystrophin deficiency on the medial TA, lateral TA, PCA, LCA, and CT muscles in adult (4 months) and aged (18 months) *mdx* mice. Using the same histologic assays employed by Thomas et al, the authors found no evidence of disease the medial TA, lateral TA, PCA, and LCA muscles of adult or old mice, while widespread fiber degeneration and regeneration was found in the *mdx* tibialis anterior muscle (stereotypically affected limb muscle). Interestingly, mild markers of disease (eg, central nucleation, Evans blue positive fibers) were observed in the CT in both age groups of mice examined; however, these effects were more pronounced in the aged group. While the disease effects on the CT did not approach the magnitude of those in the tibialis anterior, the authors proposed that the CT showed mild evidence of disease and a response to disease which differed from that of its laryngeal counterparts.

Potential Mechanisms of Laryngeal Muscle Sparing

The mechanism of extraocular, and now, laryngeal muscle sparing has not yet been elucidated. However, prominent theories suggest that either constitutive properties and/or adaptive mechanisms mediate the sparing. Primary theories are reviewed below.

Muscle Fiber Types and Utrophin Upregulation. The DGC is comprised of a series of cytoplasmic, transmembrane, and extracellular proteins that link the muscle's

cytoskeleton (ie, F-actin components) to the extracellular matrix and, thereby, provide support to the sarcolemma during muscle contraction (Figure 2.1).[169] Dystrophin is a pivotal protein within the complex, linking to actin at its N-terminus domain and to β-dystroglycan at its cysteine-rich and C-terminal domains. Dystrophin's subsarcolemmal linkage to β-dystroglycan secures the sarcolemma's glycoprotein complex. Consequently, the absence of dystrophin disrupts the entire DGC, and the link between cytoskeletal and extracellular structures is lost. It has been proposed, however, that muscles spared in DMD compensate for the absence of dystrophin through the overexpression of other structural proteins.[42, 180, 195] One protein that has received much attention as a potential substitute is the dystrophin homolog utrophin. The 395 kDa protein has a structural sequence which is strikingly similar to that of dystrophin.[200] As with dystrophin, utrophin possesses an actin-binding N-terminus domain, a central region of spectrin-like repeats, and a DGC-binding C-terminus domain.[200] This structural similarity to dystrophin along most of the protein's length makes it capable of mimicking dystrophin's role as a link between cytoskeletal F-actin filaments and transmembrane components.[195, 200, 201] In contrast to dystrophin, utrophin within skeletal muscle is classically found near the neuromuscular and myotendinous junctions of mature muscle fibers and near the sarcolemma of developing or regenerating fibers.[202, 203] While utrophin is typically expressed in normal muscle throughout the body, some researchers report differences in utrophin expression and functioning across various muscle fiber types.[42, 201] The implications of such differences for muscle physiology have not been fully elucidated and are a current area of study.

Studies demonstrating an upregulation and sarcolemmal localization of utrophin in cases of dystrophin deficiency, [42, 180, 195, 204] a negative correlation between utrophin levels and DMD disease severity,[205] an increase in muscle degeneration corresponding with the developmental decline of utrophin in early life,[206] prevention of DMD pathological cascade with the application of utrophin,[183] and the loss of disease protection in double knock-outs lacking both dystrophin and utrophin[182] point to the homolog's potential role in mediating the effects of the disease process. However, studies specifically examining utrophin expression in spared muscles (eg, extraocular and laryngeal) have failed to clearly show utrophin upregulation as the sole mechanism of

sparing. Thomas et al[25] found no evidence of sarcolemmal localization of utrophin in spared PCA or TA muscles of *mdx* mice and concluded that factors other than utrophin replacement were at play in the sparing. Work by Porter et al[42] in the EOM supported the above conclusions. Porter and his colleagues found that certain fiber types within *mdx* EOM had the unique ability to retain utrophin levels into their maturity and consequently the ability to retain the linkage between the muscle's cytoskeleton and the sarcolemma. Interestingly, however, EOM fiber types *which failed to demonstrate* the utrophin retention ability were also spared from the effects of the disease, leaving the authors to conclude that factors other than utrophin retention / presence were responsible for EOM sparing.

Thus, work continues to determine the role of utrophin in cases of dystrophin deficiency. The above studies point to a role for the dystrophin homolog in managing the disease cascade in some affected muscles; however, to date, they fail to support utrophin as the primary means of muscle sparing.

Reduced Mechanical Strain. A second potential mechanism of sparing relates to the muscle fiber size and functional mechanics of spared muscle groups. The extraocular muscles are composed of notably small muscle fibers,[48, 49] which possess greater surface to volume ratios than larger fibers. Consequently, smaller fibers are able to distribute the strain of contraction over a larger region of the sarcolemma. Some propose that the reduction in focal strain is advantageous to the weakened sarcolemma of DMD, preserving the integrity of the membrane in the absence of dystrophin.[86] Further, the small fiber size extraocular muscles exert mechanical forces well below those of other skeletal muscles.[52-54] These lower levels of force generation remain even when correction is made for fiber cross sectional area. Some have theorized that the reduced level of force generated by these smaller fibers places less stress and strain on the sarcolemma during contraction, and thereby, helps to preserve myofiber architecture in the absence of the supportive DGC.[85, 86] However, some reject such an explanation of sparing. Porter et al[42] argue that the mechanical workload of extraocular muscles is significant and of sufficient magnitude to impose damage upon DGC-deficient sarcolemmas. Others deny mechanical explanations based upon the severe manifestation of the disease in the diaphragm,[207] a muscle with relatively low mechanical stresses.[208-210] Finally, some suggest that purely

mechanical explanations for sparing are becoming less likely, as the field appreciates a more complex, signaling and homeostatic function of the DGC.[42]

Sarcoplasmic Reticulum Development and Mechanisms of Calcium Homeostasis. Some have considered patterns of calcium regulation as a potential mechanism of sparing. The loss of sarcolemmal integrity and the subsequent entry of extracellular calcium into the muscle fiber are the primary mechanisms leading of myofiber necrosis in DMD.[39] One structure within the muscle cell, the sarcoplasmic reticulum, is capable of sequestering intracellular Ca^{2+} and storing it within an isolated compartment. The development and sophistication of the sarcoplasmic reticulum varies across muscle groups, with rapidly contracting muscles exhibiting more refined Ca^{2+} uptake systems than those with slower profiles.[211] Interestingly, the spared extraocular muscles are rapidly contracting muscles that have been recognized for their well-developed sarcoplasmic reticulum and their exquisite ability to uptake free calcium.[43, 113] The spared laryngeal muscles demonstrate contraction rates similar to those observed in extraocular muscles[6] and can, therefore, be assumed to have similar mechanisms of Ca^{2+} handling.

Interestingly, dystrophin-deficient extraocular muscles do not present with the classically observed increased levels of Ca^{2+} or with increased levels of the Ca^{2+} sequestering enzyme: Ca^{2+} ATPase.[42, 112] To determine the degree to which normal calcium levels were explained by this unique mechanism of calcium handling or by the retention of sarcolemmal integrity, Khurana et al[112] pharmacologically induced sarcolemmal injury in extraocular muscle fibers. Despite the disruption of integrity, the fibers maintained Ca^{2+} homeostasis. The authors concluded that the maintenance of proper levels of Ca^{2+} in the presence of a disrupted sarcolemma supported a superior mechanism of calcium handling rather than an intact sarcolemma as the means of sparing. However, later work by Porter et al[42] contradicted the above study. Porter and colleagues found normal levels of Ca^{2+} and Ca^{2+} ATPase, as well as intact sarcolemmas in *mdx* extraocular muscles. The group concluded that calcium handling was not, therefore, a plausible explanation of sparing. The role of calcium handling in extraocular muscle sparing remains unresolved.

Regenerative Capacity. Finally, some authors have offered that preferentially spared muscle groups possess greater regenerative capacities than affected muscles.[41, 47]

Under this model, all muscle groups experience the damaging effects of the disease process; however, spared muscles avert the full pathological cascade by engaging superior regenerative processes. Recent work by Shinners et al[6] has shown that the laryngeal muscles, like extraocular muscles, do indeed possess remarkable regenerative abilities, a fact which may be interpreted as supporting this theory of sparing. However, studies of the spared laryngeal and extraocular muscles fail to demonstrate any markers of regeneration (eg, central nucleation, pleomorphic fibers) in dystrophin-deficient fibers.[4, 7, 25] This finding suggests that regeneration is not ongoing within spared fibers,[4, 7, 25, 112] leaving the door open for alternative explanations of sparing.

Summary. Thus, questions remain as to the mechanism of muscle sparing in DMD. Current work suggests that constitutive properties of the muscles, not compensatory strategies, mediate the sparing.[42] Constitutive differences known to exist between spared and affected muscles that have been proposed as playing a role in sparing include: muscle fiber type distribution, muscle biochemistry, endoplasmic reticulum development and calcium sequestration ability, cell signaling patterns, and remodeling capabilities.[41, 42, 212] Research into these areas will certainly continue as investigators work to uncover the specific muscle properties responsible for protection against disease.

Translational Extensions from Use of the mdx *Model*

As use of the *mdx* model can aid in separating highly specialized muscle groups from prototypical groups, the study of muscle response to dystrophin deficiency has implications that reach beyond the consideration of the disease process. Study of the IA and CT using this model will permit the investigation of potential phenotypic diversity among laryngeal muscles and offer a more thorough understanding of the biological properties of the IA and CT muscles of the larynx. Findings from the study have the potential to make notable contributions to the clinical management of voice disorders.

Voice disorders plague an estimated 6% of the general population of the United States.[213] Studies conducted in treatment-seeking populations suggest that a significant portion of these disorders contain an element of laryngeal muscle dysfunction.[214, 215] As discussed earlier, voice production is the result of a balanced and highly refined interplay between the intrinsic laryngeal muscles, the respiratory tract, and the supraglottal resonators.[216] Perturbation of the system brought about by laryngeal pathology,

neuromuscular impairment, and/or learned patterns of muscle misuse has the potential to disturb this balance and trigger compensatory responses which further complicate the pathological sequela. Over the years, behavioral voice therapies have been applied to reduce aberrant patterns of laryngeal muscle behavior and restore normal patterns of intrinsic muscle control over the system.[217-223] Many of these physiologic treatments have emerged from the skeletal muscle literature and have been founded upon 3 basic tenants: (1) programs of exercise can improve skeletal muscle mass, strength, and endurance; (2) laryngeal muscle is characteristic of the larger class of skeletal muscle; (3) laryngeal muscles are, as a group, are homogenous.

A vast body of research conducted on limb skeletal muscle supports of the initial assertion.[123, 224-231] Resistance exercise has been consistently related to improvements in limb muscle mass and strength,[123, 224, 225, 228, 230] while endurance training has been associated with the shifting of limb muscle metabolic profiles toward a more oxidative, fatigue-resistant form.[227, 229, 231] However, research supporting the final 2 principles has proven more elusive. In fact, a growing body of evidence supports the fact that certain members of the intrinsic laryngeal muscle group deviate significantly from the classic profile of skeletal muscle.[3, 4, 6, 7, 13-15, 18-23, 25, 27, 51, 96] Differences in neural support, contractile protein composition, methods of energy production, and regenerative capacity raise questions as to the appropriateness of generalizing limb skeletal muscle literature to this highly specialized muscle group. Finally, the above research argues against the presence of a single laryngeal muscle phenotype.[4, 17, 32, 34, 35] Within the larynx, there appears to be a duality of muscle phenotypes: one form characteristic of the larger class of prototypical limb skeletal muscles and one form highly specialized and divergent from classic muscle. As a result, the universal application of exercise principles across these 2 broad phenotypes may be inappropriate.

The realization of laryngeal muscle deviation from limb muscle and the recognition of heterogeneity among the laryngeal muscles may make the blanket application of limb muscle rehabilitation principles to the laryngeal muscles inappropriate. Determining the phenotype of individual laryngeal muscles will be an important step in identifying specific muscular targets for therapy.

Purpose Statement

The purpose of this study was to use the *mdx* mouse model of dystrophin deficiency to further demonstrate the biological characteristics of the IA and CT muscles. These specific muscles were selected for study because of their previously demonstrated deviations from other laryngeal muscles and their functional importance in voice production. Information from the study will be helpful in further defining the biological properties of the IA and CT and in determining their similarity to or divergence from limb skeletal muscle. Additionally, the study's findings will be helpful in considering the presence of phenotypic diversity within the intrinsic laryngeal muscle group. In time, an improved understanding of individual laryngeal muscle biology will be important in further defining the biomechanics of voice production, the potential laryngeal response to neuromuscular disease and/or impairment, and expected laryngeal muscle response to medical and/or behavioral treatments.

Sparing of the muscles would point to their similarity to laryngeal muscles and the preservation of their important contribution to respiration, swallowing, and voicing in DMD. However, indicators of pathology in the dystrophin-deficient IA and CT muscles would highlight their similarity to limb skeletal muscle and suggest heterogeneity among the intrinsic laryngeal muscles. Mild evidence of pathology within the IA and CT, such as that found in earlier studies of the masseter and the CT,[4, 9] would support the categorization of these muscles as hybrid forms of skeletal muscle, evidencing features of both limb and specialized muscles.

Finally, as mouse laryngeal anatomy has not been clearly defined, a secondary purpose of this study was to define the myology of the mouse larynx and to identify anatomical and functional counterparts of the human IA and CT muscles for study.

Hypotheses

The study will test the primary hypothesis that the mouse counterparts of the IA and CT muscles are affected by dystrophin deficiency. Specific null and alternative hypotheses under investigation in the study are detailed below.

Null Hypothesis 1. Qualitative, histological assessment of the *mdx* IA, or its counterpart, will show no evidence of pathology (ie, fibrosis, necrosis, pleomorphism, Evans blue dye infiltration).

Alternative Hypothesis 1. Qualitative, histological assessment of the *mdx* IA, or its counterpart, will show evidence of pathology (fibrosis, necrosis, pleomorphism, Evans blue dye infiltration).

Null Hypothesis 2. Qualitative, histological assessment of the *mdx* CT will show no evidence of pathology (eg, fibrosis, necrosis, pleomorphism, Evans blue dye infiltration).

Alternative Hypothesis 2. Qualitative, histological assessment of the *mdx* CT will show evidence of pathology (eg, fibrosis, necrosis, pleomorphism, Evans blue dye infiltration).

Null Hypothesis 3. There will not be a significantly greater percentage of centrally positioned nuclei in *mdx* IA, or its counterpart, than in the IA control.

Alternative Hypothesis 3. There will be a significantly greater percentage of centrally positioned nuclei in the *mdx* IA, or its counterpart, than in the IA control.

Null Hypothesis 4. There will not be a significantly greater percentage of centrally positioned nuclei in *mdx* CT than in the CT control.

Alternative Hypothesis 4. There will be a significantly greater percentage of centrally positioned nuclei in the *mdx* CT than in the CT control.

The above hypotheses will be tested using the *mdx* model reviewed in this chapter. In the following chapter, the study's methodology will be considered in detail. Animals and experimental assays selected for study are presented, and methods of data analysis are discussed.

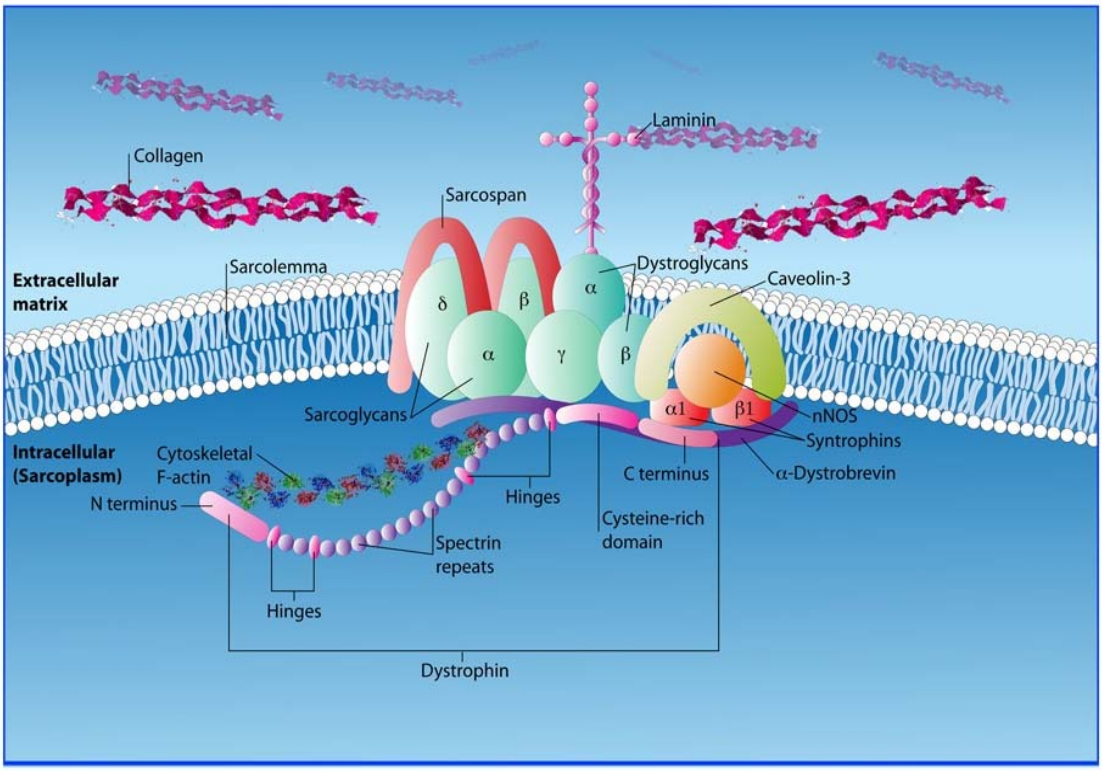

Figure 2.1. Model of the dystrophin-glycoprotein complex. In DMD, the absence of dystrophin results in disruption of the entire complex and subsequent disruption of sarcolemmal integrity.

CHAPTER 3: METHODOLOGY

In Chapter 2, the literature supporting this study and the rationale behind this study were discussed. The purpose of this study was to determine the effects of dystrophin deficiency on the IA and CT muscles of the larynx. The study was designed to offer additional insight into the physiology of the IA and CT muscles and their similarity or dissimilarity to laryngeal muscle through use of the *mdx* mouse model of dystrophin deficiency. In the section below, the methodology used in this study is reviewed.

Animals

Use of experimental animals was approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Six C57BL/6J mice were obtained from Jackson laboratories for a preliminary investigation of mouse laryngeal anatomy. For the primary investigation, an additional 16 mice (8 C57BL/10SnJ; 8 C57BL/10Sc-Sn-Dmd *mdx*/J) were obtained from Jackson Laboratories

Preliminary Investigation of Mouse Laryngeal Anatomy

Eight to twelve-week old male C57BL/6J (n = 6) were obtained from Jackson Laboratories. Mice were euthanized by CO_2 asphyxia. Whole larynges were quickly dissected. For consideration of gross anatomy, three larynges were dissected and viewed from the anterior, posterior, lateral, and mid-sagittal aspects under a Nikon SMZ1500 stereotactic microscope (Nikon, Inc., Melville, NY). Larynges were then transferred to a Nikon E600 microscope (Nikon Inc., Melville, NY). Brightfield illumination was used along with supplemental external lighting. Select images were captured with a Spot RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and a PowerMAC G4 computer (Apple Computer, Inc., Cupertino, CA) equipped with Spot RT software version 4.0 (Diagnostic Instruments, Inc., Sterling Heights, MI). All views were analyzed by a muscle physiologist, a speech physiologist, and two speech-language pathologists with expertise in laryngeal anatomy. Consensus was reached prior to the identification and labeling of laryngeal structures.

Three larynges were quickly embedded in an optimal cutting temperature medium, and frozen in 2-methylbutane cooled to its freezing temperature with liquid nitrogen. Serial 10-µm thick frontal, sagittal, and transverse sections of whole larynges were collected. Frozen sections were later stained with hematoxylin and eosin[186] and

examined under a Nikon E600 microscope (Nikon Inc., Melville, NY). Serial images within each plane were captured with a Spot RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and a PowerMac G4 computer (Apple Computer Inc., Cupertino, CA) equipped with Spot RT software, version 4.0 (Diagnostic Instruments, Inc.).

Stored serial images from each of the planes of study were examined by a muscle physiologist, a speech physiologist, and 2 speech-language pathologists with expertise in laryngeal anatomy to determine the presence and location of laryngeal cartilages and the intrinsic laryngeal muscles. Information obtained from these views was combined with knowledge gained during the examination of whole larynges to formulate a description of mouse laryngeal muscle structure and function. At each point, consensus was reached among group members before inclusion of the feature in the final model. Results from the preliminary investigation were used to select muscles for study in the primary investigation.

Primary Investigation

Eight-week old male C57Bl/10SnJ (n = 8) and C57BL/10ScSn-Dmd *mdx*/J mice (n = 8) were obtained from Jackson Laboratories. The *mdx* mouse strain is the result of a spontaneous X-linked mutation of the C57BL/10ScSn strain[178] resulting in impaired expression of the full-length dystrophin in skeletal muscle.[36] The *mdx* mouse strain is considered to be the standard animal model for the study of human Duchenne muscular dystrophy (DMD)[177] and was, therefore, the strain of choice for this investigation. The wild type C57Bl/10SnJ was used as control.[φ]

Eight-week old (adult) mice were selected for study, following a model established in other published studies of the *mdx* mouse.[181, 182] The mouse strain evidences muscle damage at 3-4 weeks of age and significant inflammation and muscle regeneration are evidenced by 8 weeks.[181, 182] The only muscle known to worsen with aging beyond the 8-week point is the diaphragm.[207] As a result, 8-week mice permitted assessment of disease markers.

[φ] The C57BL/10SnJ and C57BL/10ScSn are minor substrains of C57BL. Both strains are considered appropriate controls for the *mdx* mutation.

Four skeletal muscles were considered. The IA and CT muscles were the experimental muscles used in the study. The PCA was examined because of its documented status as a spared muscle. The gastrocnemius displays the classic pathological cascade of DMD, and its inclusion permitted the comparison of the laryngeal muscles to a stereotypically affected muscle group.

Mice were euthanized by CO_2 asphyxia. Whole larynges and the gastrocnemius muscle were quickly dissected, embedded in an optimal cutting temperature medium, and frozen in 2-methylbutane cooled to its freezing temperature with liquid nitrogen.

Histology and Immunocytochemistry

Four *mdx* and four control mice were used for histological and immunocytochemical investigations. Serial 10-µm thick sagittal and transverse sections of whole larynges were collected to permit cross-sectional examination of the IA (or murine counterpart), CT, and PCA muscles. (Sagittal cuts provided the IA and CT in cross section, whereas transverse cuts offered the PCA muscle in cross section.) Finally, 10-µm thick cross sections of whole gastrocnemius muscles were collected. For each histological or immunocytochemical assay, slides from the above muscle groups were processed concurrently to allow for the comparison across muscles.

Morphology

For overall morphology and central nuclei counts (a marker of fiber regeneration[105, 185]), 2 slides (3-5 sections per slide) were selected from each muscle (CT, IA counterpart, PCA, gastrocnemius) from each mouse (4 control and 4 *mdx*) for a total of 64 slides. Slides were stained with hematoxylin and eosin.[186] After staining, slides were dehydrated in an ethanol series, cleared with xylene, mounted in Permount and viewed with a Nikon E600 microscope (Nikon Inc., Melville, NY). Images were captured with a Spot RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and a PowerMac G4 computer (Apple Computer Inc., Cupertino, CA) equipped with Spot RT software, version 4.0 (Diagnostic Instruments, Inc.). To determine the percentage of nuclei in the central position, the total number of muscle fibers and the number of fibers with centrally positioned nuclei were counted in control and *mdx* CT (full muscle, all fields), transverse IA (full muscle, all fields), PCA (full muscle, all fields), and gastrocnemius muscles (3-5 random fields per muscle). The quantitative

analysis was performed by 2 trained personnel blinded to the experimental conditions. Training of personnel was provided by the primary investigator (LBT) previously trained in nuclei counting in the Department of Physiology. Twenty-percent of fields were randomly selected for examination by both raters to determine inter-rater reliability.[232-235]

Sarcolemmal Integrity

Live cells are capable of controlling the uptake and distribution of injected dyes, whereas damaged cells are not.[188] Consequently, vital dyes can be injected into a living animal and its distribution can be assessed to determine the integrity of cell membranes. In this study, the vital dye Evans blue (Sigma Chemical Corp.) was used to examine sarcolemmal integrity in control and *mdx* mice.[189, 190] The clear and binary results associated with Evans blue (emission of a bright red signal when activated by green light in fluorescence microscopy[189]) indicated that a sample size of 4 animals per group was appropriate for testing of this parameter. Dye was injected into 4 control and 4 *mdx* mice (50mg/kg body weight, i.p.). Animals were sacrificed approximately 18 hours after injection.[236] Whole larynges and gastrocnemius muscles were quickly dissected, embedded in an optimal cutting temperature medium, and frozen in 2-methylbutane cooled to its freezing temperature with liquid nitrogen. Unfixed muscle sections (cut as per the above description) were mounted with SlowFade Gold Antifade reagent (Invitrogen, Carlsbad, CA) and examined by fluorescence microscopy to determine the extent of intracellular dye incorporation.[236] Infiltration of dye into the muscle cell indicated a loss of sarcolemmal integrity, and maintenance of the dye in the extracellular space confirmed the presence of an intact cell membrane. Photographs were taken under standardized exposure settings within muscle groups to permit comparison of dye infiltration across control and *mdx* animals.

Immunocytochemistry

Immunocytochemical assays were used to: (1) document the presence and general distribution of dystrophin in control and *mdx* mice, and (2) consider the potential compensatory sarcolemmal re-localization of utrophin in spared muscles.

Dystrophin was examined using a rabbit polyclonal antibody against dystrophin (ab15277) purchased from Abcam Incorporated (Cambridge, MA). Ten-µm thick frozen sections of control and *mdx* muscles were rehydrated in PBS and incubated with the

primary antibody (diluted 1:100 in PBS/Tris/goat serum) in a humid chamber at 4°C overnight; negative controls were rehydrated and incubated with non-immune serum (PBS/Tris/goat serum) under the same conditions. After washing in PBS, immunoreactivity was visualized by incubation for 1 hour at room temperature with AlexaFluor 555 goat anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA) diluted 1:100 in PBS. After washing, sections were fixed in 2% paraformaldehyde in PBS, rinsed with PBS, and mounted with SlowFade Gold antifade reagent (Invitrogen, Carlsbad, CA). Sections were visualized under fluorescence microscopy, and qualitatively considered for protein presence and distribution. Negative controls (no application of the primary antibody) were used to document the extent of non-specific binding of the secondary antibody. Photographs were taken under standardized exposure settings within muscle groups to permit comparison across *mdx* and control animals. A second run conducted as described above was used to confirm the results of the initial experiment.

The dystrophin homolog, utrophin, was considered using both monoclonal and polyclonal antibodies. A mouse monoclonal antibody against utrophin's N-terminus domain (DRP3/20C5) was purchased from Abcam Laboratories (Cambridge, MA). Ten-µm thick frozen sections of control and *mdx* muscles were fixed in 2% paraformaldehyde in PBS and rinsed with PBS/Tris. To control the reaction of the mouse tissue to the mouse monoclonal antibody, sections were blocked with Histomouse BEAT blocking solutions (Invitrogen, Carlsbad, CA). After blocking, sections were rinsed with PBS/Tris and incubated with the primary antibody (1:10 in PBS/Tris/goat serum) and an α-bungarotoxin-Alexa Fluor 488 conjugate (Invitrogen, Carlsbad, CA) double label (1:500 in PBS/Tris/Goat) in a humid chamber at 4°C overnight. Use of the double label permitted identification of neuromuscular junction sites[192] and the localization of utrophin relative to those sites. Negative controls were incubated with non-immune serum in place of the primary antibody. After washing with PBS, immunoreactivity was visualized by incubation for 1 hour with Texas Red secondary antibody (Invitrogen, Carlsbad, CA) diluted 1:200 in PBS/Tris/goat serum. Sections were rinsed in PBS/Tris, mounted with SlowFade Gold antifade reagent (Invitrogen, Carlsbad, CA), visualized by fluorescence microscopy, and qualitatively considered for protein presence and

distribution. Negative controls (no application of the primary antibody) were used to document the extent of non-specific binding of the secondary antibody. A second run conducted as described above was used to confirm the results of the initial experiment.

A goat polyclonal antibody (sc-7459; Santa Cruz Biotechnology, Santa Cruz, CA) against utrophin's C-terminus domain was also used. Ten-µm thick frozen sections of control and *mdx* muscles were rehydrated in PBS and blocked in 4% goat serum for 1 hour. Sections were then incubated with the primary antibody (1:50 in PBS/Tris/goat serum) and an α-bungarotoxin-Alexa Fluor 488 conjugate (Invitrogen, Carlsbad, CA), Eugene, OR) double label (1:500 in PBS/Tris/Goat) in a humid chamber at 4°C overnight. Use of the double label permitted identification of neuromuscular junction sites[192] and the localization of utrophin relative to those sites. Negative controls were rehydrated and incubated with non-immune serum under the same conditions. After washing in PBS, sections were incubated in AlexaFluor-350 donkey anti-goat IgG secondary antibody (Invitrogen, Carlsbad, CA) for 1-hour at room temperature. After washing in PBS, sections were fixed in 2% paraformaldehyde in PBS and mounted with SlowFade Gold antifade reagent (Invitrogen, Carlsbad, CA). Sections were visualized by fluorescence microscopy, and qualitatively considered for protein presence and distribution. Negative controls (no application of the primary antibody) were used to document the extent of non-specific binding of the secondary antibody. A second run conducted as described above was used to confirm the results of the initial experiment.

Data Analysis

Overall Morphology

Each muscle was considered under light microscopy for the presence or absence of salient markers of the disease process, including: inflammation, fibrosis, necrosis, fatty infiltration, and pleomorphism. Examination of the features was qualitative, and findings were supported visually with photographs.

Central Nucleation

Two raters blind to the purpose of the study viewed photographs of each muscle for the purpose of counting central nuclei. The rater counted the total number of fibers in the image (IA, CT, PCA - full muscle, all fields; gastrocnemius – 3 to 5 random fields per muscle) and the number of fibers with centrally positioned nuclei. Mean percentages of

central nuclei were compared across control and *mdx* samples within each muscle group using the Wilcoxon Rank-Sum Test, the non-parametric equivalent of the independent *t*-test .[237] The non-parametric option was selected due to heterogeneity of sample size and heterogeneity of variance across muscle groups. The Wilcoxon Rank-Sum test is based upon ranked data. As such, nuclei counts for control and *mdx* samples within each muscle group (gastrocnemius, CT, IA counterpart, and PCA) were listed, ranked, and then re-allocated to their original *mdx* or control classification.[237, 238] When control and *mdx* groups were equal in size, the test statistic (Ws) was equal to the smaller summed rank. When control and *mdx* groups were not equal in size, the test statistic (Ws) was equal to the sum of ranks in the group containing fewer samples. Interpretation of the test statistic was based upon its conversion to the *z*-score.[238] For the conversion, sample sizes were used to determine the mean (Ws-bar) and standard error (SEw_s) of the test statistic. Results of these calculations were then entered into the following equation to calculate *z*.[238]

$$z = \frac{Ws - Ws\text{-bar}}{SEw_s}$$

Alpha levels for all one-tailed non-parametric tests were set at 0.05, indicating significance if the observed *z*-scores exceed 1.645. All calculations were performed using SPSS 15.0 (SPSS Inc., Chicago, IL).

Inter-rater reliability for central nuclei counts was determined using the intra-class correlation coefficient, two-way mixed model.[239] The coefficient determines the proportion of variance in an observation that is attributable to between-rater variability in scores.[240] The correlation is appropriate for use with continuous data and is preferred to Pearson's *r* when sample size is small ($n < 15$). Variations of the intra-class correlation coefficient are available, and the optimal version of the statistic is chosen based upon the conditions of the raters and the observations in the study. The two-way mixed model (single measure, absolute agreement) was selected for this study, as the coefficient was comparing a fixed set of raters on a random set of observations.[239] Interpretation of the coefficient is based upon a maximum value of 1.0, indicating perfect agreement among

raters.[240] For the purpose of this study, 20% of the photographs used in determining central nucleation were randomly selected for examination by both raters; the coefficient was then calculated based upon the raters' central nuclei measures on the selected set of tokens. The criterion of 20% of the total token sample for reliability calculation was selected based upon reliability methods recently published in the speech-language pathology literature.[232-235] All calculations were performed using SPSS 15.0 (SPSS Inc., Chicago, IL).

Sarcolemmal Integrity

Muscles were examined under fluorescence microscopy to determine the location of Evans blue dye relative to the cell membrane. A visual assessment of each muscle identified the presence or absence of fluorescent dye within the fiber. Muscles evidencing dye penetration into individual fibers were classified as having a disrupted sarcolemmas (dystrophic), whereas muscles retaining dye in the extracellular space were classified as intact (unaffected). The results of dye testing were expected to be binary, with dystrophic muscles evidencing Evans blue positive fibers and unaffected muscles showing no positive fibers. Consequently, qualitative methods involving the visual examination of muscle cross sections were considered appropriate for analysis. Results were supported visually with photographs.

Immunocytochemistry

Muscles were examined under florescence microscopy to qualitatively evaluate the presence and distribution of dystrophin and utrophin in control and *mdx* muscles. Negative controls (no application of primary antibody) were used to mark the extent of nonspecific binding. Fluorescence of the secondary antibody beyond that observed in the negative controls indicated the presence of the target protein in the sample.

This chapter has provided an overview of the study's methodology. In the following chapter, results of the study are detailed.

CHAPTER 4: RESULTS

In the previous chapter, the methods used in this investigation were presented. In this chapter, results of the histological and immunocytochemical assays are reviewed.

Preliminary Investigation

Three mice larynges were examined under an Olympus SZX9 stereomicroscope (Olympus, Center Valley, PA) to define the gross anatomy of the mouse larynx. All larynges were viewed by a muscle physiologist, speech physiologist, and 2 speech-language pathologists with expertise in laryngeal anatomy. During viewing, portions of the larynx were systematically removed by the muscle physiologist to permit viewing from superficial to deep aspects of the larynx. Select portions of the larynx were further examined at 40X under a Nikon E600 microscope (Nikon Inc., Melville, NY) using supplemental illumination positioned above the stage. Images were photographed with a Spot RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and a PowerMac G4 computer (Apple Computer Inc., Cupertino, CA) equipped with Spot RT software, version 4.0 (Diagnostic Instruments, Inc.). Further, 10-µm thick serial cryosections of 3 additional mouse larynges were obtained in the sagittal, horizontal, and frontal planes. Serial sections within each plane were stained with hematoxylin and eosin[186] and later viewed under a Nikon E600 microscope (Nikon Inc., Melville, NY). Images were captured as described above and considered by the aforementioned group of investigators to gain an appreciation of mouse laryngeal anatomy.

Framework

The larynx was bordered caudally by the signet-shaped cricoid cartilage. Ventral and rostral to and jointed with the cricoid cartilage was the shield-shaped thyroid cartilage. Paired cornu projected in the rostral and caudal directions along the dorsal aspect of the cartilage. The relationship between the thyroid and cricoid cartilages was, therefore, similar to that observed in the human larynx. Further, bilateral arytenoid cartilages articulated with the cricoid lamina dorsally. Arytenoid cartilages were characterized by a vocal process which projected caudally toward the thyroid angle and a muscle process which projected laterally. Patterns for these processes were similar to that observed in the human larynx. Arytenoid apices demonstrated a notable arch toward the dorsal aspect of the larynx. The epiglottis originated along the interior of the thyroid

cartilage and projected rostrally toward the hyoid bone as observed in the human larynx. A U-shaped cartilage, previously described by shape in the mouse[162] and as the alar cartilage in the rat,[160] was observed ventrally, just rostral to the vocal folds and caudal to the epiglottis. Corniculate and cuneiform cartilages present in the human larynx were unable to be appreciated in the mouse from the views obtained (Figures 4.1 - 4.6).

Musculature

Posterior cricoarytenoid, LCA, and TA muscles were positioned as in the human larynx and were presumed to function as their counterparts in humans (Figures 4.4, 4.5, and 4.7). Cricothyroid muscles were notable along the lateral aspect of the laryngeal complex. More dorsal fibers of the CT coursed perpendicular to the long edge of the vocal fold, while more caudal fibers coursed at a slightly oblique angle with respect to the folds. In contrast to the CT's more vertical orientation in the human larynx, the murine CT coursed more horizontally between the thyroid and cricoid cartilages (Figures 4.4 and 4.8). This orientation of the murine CT suggested that the muscle works to move the thyroid upon the cricoid in a shearing, rather than rocking, motion. Finally, the IA muscle was not identified in the mouse larynx. A thin muscle was observed coursing from the medial aspect of the dorsal face of the arytenoid cartilages to a midline prominence on the rostral aspect of the cricoid lamina. The angle of fibers ranged from a near horizontal course in those fibers originating from the lower aspect of the arytenoid cartilage to an oblique course in fibers originating from the superior aspect of the arytenoid (Figures 4.9-4.11). The position and direction of fibers suggested that the muscle played a role in drawing the arytenoid bodies to midline and tilting of the arytenoid apices toward midline. It was proposed that this dual action would result in complete adduction of the cartilaginous vocal fold and would serve the role of the transverse and oblique IA fibers observed in humans. The novel muscle appeared similar to the superior cricoarytenoid (SCA) muscle described in the rat and was, therefore, labeled the SCA muscle by the investigators.[Ψ]

[Ψ] This murine counterpart of the IA is hereafter referred to in this document as the superior cricoarytenoid or SCA muscle.

Additional Observations

Aryepiglottic folds were positioned as in the human larynx and marked the entry into the laryngeal vestibule (Figure 4.5). A small, recessed area was noted at the midline of the ventral wall of the larynx. The pouch, located just rostral to the vocal fold and caudal to the epiglottal base, was bordered by the alar cartilage described above (Figure 4.6). These findings confirmed a previous notation of the sac by Nakano and Muto.[162] The ventricular fold and ventricle were absent in the mouse (Figure 4.3).

Primary Investigation

Histology

Overall Morphology

Muscles from control and *mdx* mice were examined histologically after staining with hematoxylin and eosin. Control gastrocnemius muscles showed large, regular fibers with peripheral nuclei. By contrast, dystrophin-deficient gastrocnemius muscles were pleomorphic and demonstrated notable areas of inflammation, necrosis, and fibrosis. Control laryngeal muscles (PCA, CT, SCA) showed small, regular fibers with peripherally-positioned nuclei. Laryngeal muscles of *mdx* mice also showed small, regular fibers with peripheral nuclei: *mdx* PCA, CT, and SCA muscles did not evidence the pathological changes of inflammation, fibrosis, and fatty infiltration obvious in the *mdx* gastrocnemius (Figures 4.12 and 4.13).

Central Nucleation

Random samples from control and *mdx* mice were examined for nuclei position (single sample of each muscle from each mouse, 3-5 random fields for gastrocnemius, full muscle all fields for PCA, CT, and SCA). In mature muscle fibers, nuclei are located in the periphery of the fiber.[185] However, in regenerating muscle fibers, such as those observed in limb and respiratory skeletal muscles affected by DMD, nuclei are positioned centrally.[105, 185] Wilcoxon Rank-Sum tests were conducted for control-*mdx* comparisons of central nucleation percentages within each muscle group. Results indicated that the *mdx* gastrocnemius ($n = 16$) had significantly higher percentages of central nucleated fibers than control gastrocnemius muscles ($n = 16$), $W = 136.00$, $z = -4.826$, $p < .000$, suggesting the presence of ongoing fiber regeneration in *mdx* gastrocnemius muscles. *mdx* PCA, CT, and SCA muscles did not show significantly higher percentages of central

nucleation than their control muscle counterparts. The results indicated no evidence of increased muscle regeneration in dystrophin-deficient laryngeal muscles (Tables 4.1 - 4.2 and Figure 4.14).

It should be noted that in some cases the central nuclei percentages (Table 4.1) are higher than those previously reported for control and *mdx* skeletal muscle. Elevated percentages were felt to be a factor of: (1) use of a more liberal definition of central nucleation in this study and (2) difficulty obtaining strict cross-sectional cuts of the laryngeal muscles.

Inter-Rater Reliability. Twenty percent of histologic images used in the determination of central nucleation were randomly selected and examined by both raters. Inter-rater reliability was assessed using the intraclass correlation coefficient (two-way, mixed model; single measure). Results demonstrated a high degree of agreement between raters (Intraclass Correlation Coefficient = .978).

Sarcolemmal Integrity

The vital dye Evans blue was used to assess sarcolemmal integrity. In dystrophin-deficient gastrocnemius muscles, numerous clusters of Evans blue-positive fibers were found throughout the muscle, indicating a lack of sarcolemmal integrity. In addition, *mdx* strap muscles of the neck (dissected and embedded en bloc with the larynx) showed multiple clusters of dye-positive fibers. However, the PCA, SCA, and CT muscles of control and *mdx* mice and the gastrocnemius and strap muscles of control mice did not evidence Evans blue positive fibers. In these cases, the dye did not penetrate the sarcolemmal boundary and remained, instead, in the extracellular space (Figure 4.15). These results verify the sarcolemma's loss of integrity in *mdx* leg and strap muscle and its retention in *mdx* laryngeal muscles.

It should be noted that non-distinct areas of dye collection were observed in anterior aspects of the CT. These areas were examined by a muscle physiologist (FH) with 18 years of experience and were determined to be an artifact. These collections of dye were light in comparison to dye-positive fibers observed in affected gastrocnemius and strap muscles. Further, the areas of dye collection were non-distinct and were not confined to the interior of muscle fibers.

Immunocytochemistry

Dystrophin

Dystrophin was identified along the perimeter of the sarcolemma in gastrocnemius and laryngeal muscles of control mice but was absent in the corresponding muscles of *mdx* mice. Sparse evidence of non-specific binding of the secondary antibody to *mdx* tissue was identified in some sections; however, clear peripheral demonstration of the protein was not present as in control muscles. These results verify the presence of the protein in both leg and laryngeal muscles of control mice and its absence in both muscle groups in *mdx* models (Figure 4.16).

Utrophin

With use of the goat polyclonal antibody against utrophin, labeling was identified along the perimeter of the cell membrane in control gastrocnemius and laryngeal muscles. Areas of labeling did not correspond with the location of neuromuscular junctions. Limited evidence of non-specific binding of the secondary antibody to *mdx* gastrocnemius and laryngeal tissue was identified in some sections; however, clear localization of the protein to NMJ sites or the sarcolemmal boundary was not observed in *mdx* muscle (Figure 4.17).

The above results showing utrophin localization along the cell membrane of control tissue and a lack of utrophin labeling in *mdx* tissue suggested potential cross-staining with dystrophin, a concern previously reported in the literature.[202] Consequently, monoclonal antibodies against utrophin were used to achieve a more specific protein response.

A mouse monoclonal antibody against utrophin revealed utrophin localization at the NMJs of control gastrocnemius muscles. In *mdx* gastrocnemius muscles, utrophin was delocalized from NMJs and identified along the perimeter of some fibers. *mdx* gastrocnemius fibers showing sarcolemmal utrophin were present in clusters across the muscle. Utrophin was not identified at NMJ sites of control or *mdx* laryngeal muscles. Sparse non-specific binding of the secondary antibody was observed along the perimeter of some control and *mdx* laryngeal fibers, a finding likely secondary to the staining of extraneous proteins or the staining of dystrophin (in control muscle), a protein with a high degree of homology to utrophin.

To consider the potential cross-staining of dystrophin with the monoclonal antibody, the investigator used a Basic Local Alignment Search Tool (BLAST) to compare the utrophin amino acid sequence targeted by the monoclonal antibody with other mouse proteins. Utrophin antibody (DRP3/20C5; Abcam, Cambridge, MA) targets the first 261 amino acids of human utrophin. Hence, this portion of the human protein was considered using the SwissProt database. Results showed a 79% positive match between the amino acid sequence targeted by the monoclonal antibody against utrophin and murine dystrophin, indicating a high potential for dystrophin cross-staining with the monoclonal antibody used in the study.

Results in Relation to Hypotheses

This study tested the main hypothesis that the *mdx* SCA and CT muscles would be affected by dystrophin deficiency. Results as they pertain to specific hypotheses are noted below.

Hypothesis 1

Null. Qualitative, histological assessment of the *mdx* IA, or its counterpart, will show no evidence of pathology (ie, fibrosis, necrosis, pleomorphism, Evans blue dye infiltration).

Alternative. Qualitative, histological assessment of the *mdx* IA, or its counterpart, will show evidence of pathology (fibrosis, necrosis, pleomorphism, Evans blue dye infiltration).

Results. Results of histological tests showed no evidence of fibrosis, necrosis, pleomorphism, or Evans blue dye infiltration in the SCA muscle of the *mdx* mouse. The null hypothesis was retained.

Hypothesis 2

Null. Qualitative, histological assessment of the *mdx* CT will show no evidence of pathology (eg, fibrosis, necrosis, pleomorphism, Evans blue dye infiltration).

Alternative. Qualitative, histological assessment of the *mdx* CT will show evidence of pathology (eg, fibrosis, necrosis, pleomorphism, Evans blue dye infiltration).

Results. Results of histological assays demonstrated no evidence of fibrosis, necrosis, pleomorphism, or Evans blue dye infiltration in the CT muscle of the *mdx* mouse. The null hypothesis was retained.

Hypothesis 3

Null. There will not be a significantly greater percentage of centrally positioned nuclei in *mdx* IA, or its counterpart, than in the IA control.

Alternative. There will be a significantly greater percentage of centrally positioned nuclei in the *mdx* IA, or its counterpart, than in the IA control.

Results. Results showed that the percentage of centrally positioned nuclei in the *mdx* SCA was not significantly greater than the SCA control. The null hypothesis was retained.

Hypothesis 4

Null. There will not be a significantly greater percentage of centrally positioned nuclei in *mdx* CT than in the CT control.

Alternative. There will be a significantly greater percentage of centrally positioned nuclei in the *mdx* CT than in the CT control.

Results. Results showed that the percentage of centrally positioned nuclei in the *mdx* CT was not significantly greater than the CT control. A two-fold, but non-significant increase in central nucleation was observed in this muscle. The null hypothesis was retained.

Thus, the results of the study demonstrated the SCA and CT muscles of the mouse larynx are spared from myonecrosis dystrophin deficiency. Interestingly, the *mdx* CT showed subtle histologic changes (ie, increased central nucleation), indicative of increased regeneration.

Table 4.1. Percentage Centrally Nucleated Fibers by Muscle

Muscle	N (# sections rated)	Mean Percent Central Nucleated Fibers	SD
Gastrocnemius			
Control	16	3.93	3.08
mdx	16	65.93	7.99
PCA			
Control	3	7.13	3.83
mdx	4	4.88	2.14
SCA			
Control	4	5.33	3.01
mdx	4	1.60	1.62
CT			
Control	4	5.83	4.30
mdx	4	11.63	2.76

Table 4.2. Wilcoxon Rank Sum Statistics for Control-*mdx* Muscle Comparisons

Muscle Group	**n**	**Mean Ranks**	**Observed *z***	**Significance**
Gastrocnemius			-4.826	<.000
Control	16	8.50		
mdx	16	24.50		
PCA			-.707	.313
Control	3	4.67		
mdx	4	3.50		
SCA			-1.89	.045
Control	4	6.13		
mdx	4	2.88		
CT			-1.73	.058
Control	4	3.0		
mdx	4	6.0		

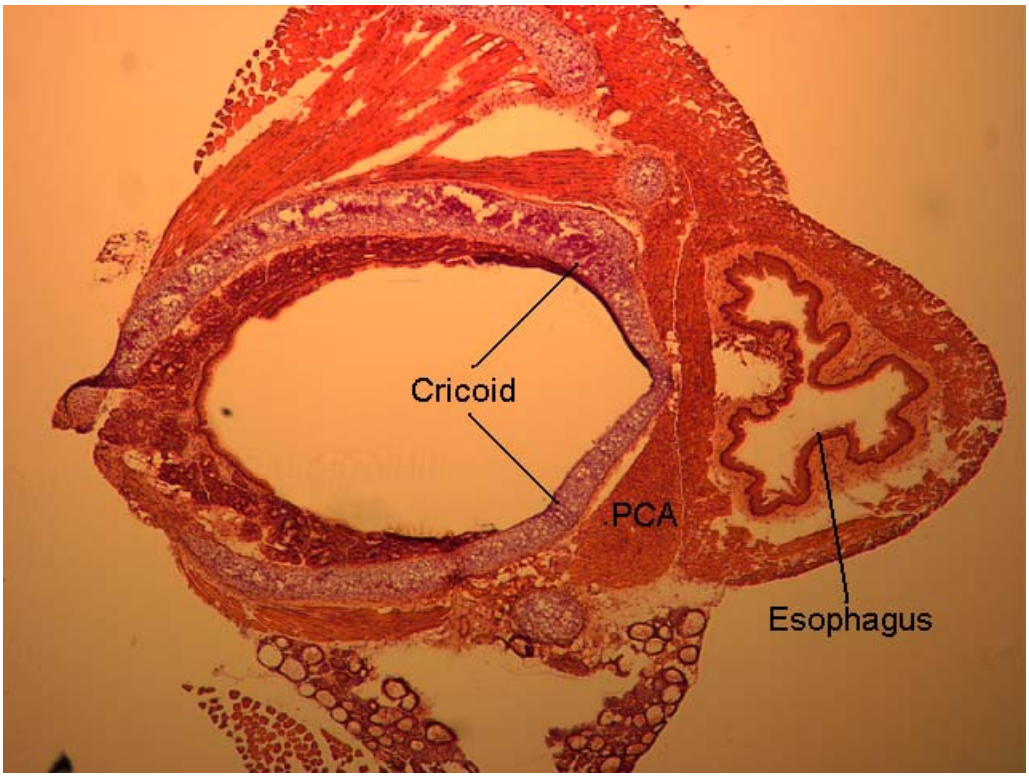

Figure 4.1. Hematoxylin and Eosin Staining of Mouse Larynx Viewed from Superior Aspect (40X). Image shows the ring-shaped cricoid cartilage (stained purple) bordered posteriorly by the esophagus.

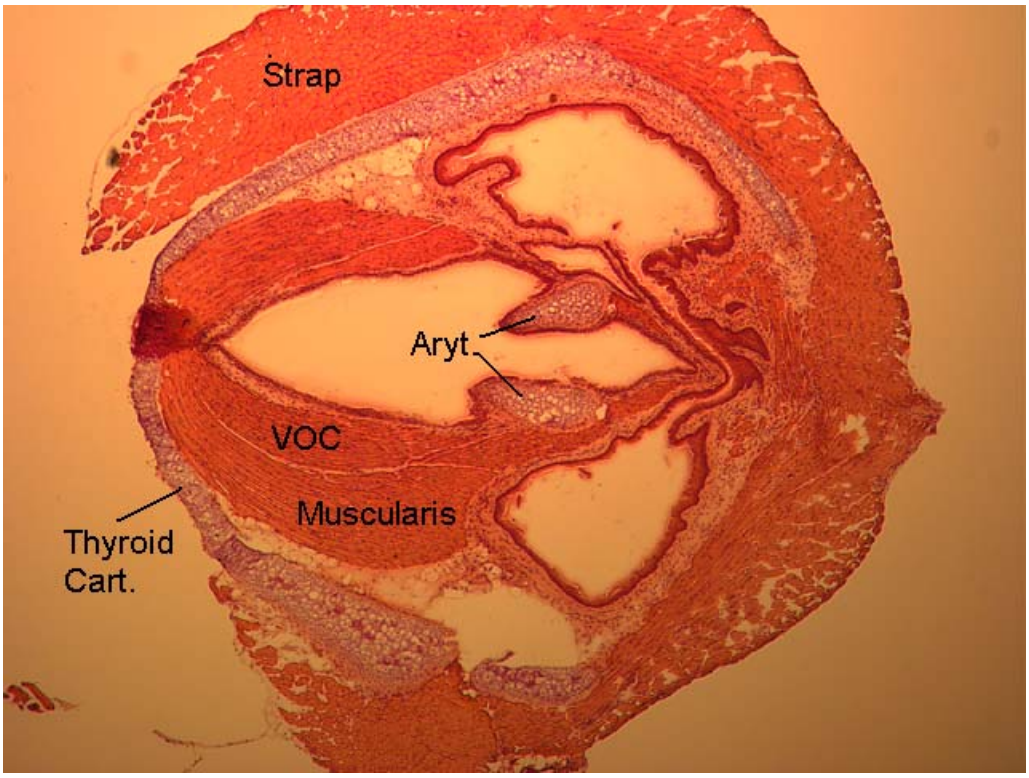

Figure 4.2. Hematoxylin and Eosin Staining of Mouse Larynx Viewed from Superior Aspect (40X). Image shows thyroid cartilage bordering the anterior larynx and bilateral arytenoid cartilages positioned posteriorly. Vocalis (VOC) and muscularis portions of the thyroarytenoid are visualized coursing from the thyroid to arytenoid cartilages.

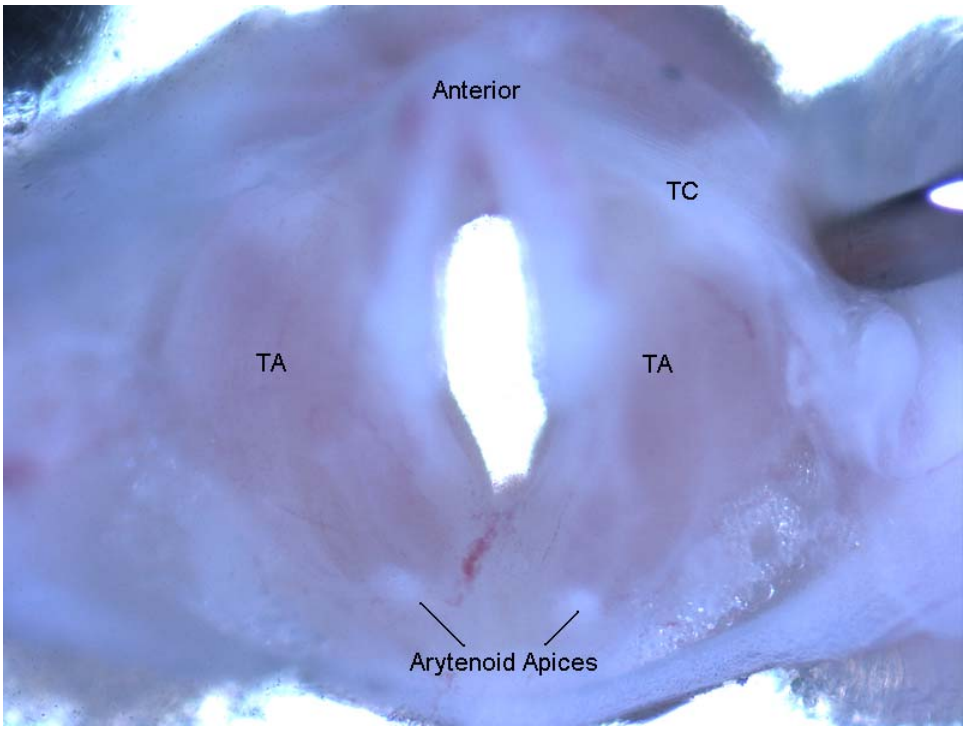

Figure 4.3. Superior View of C57BL Mouse Larynx (40X). Image shows thyroid cartilage and arytenoid cartilages positioned as in the human larynx. Bilateral TA muscles are observed coursing between the thyroid cartilage (TC) and arytenoid cartilages. Note: Ventricular folds and ventricle absent in the mouse model.

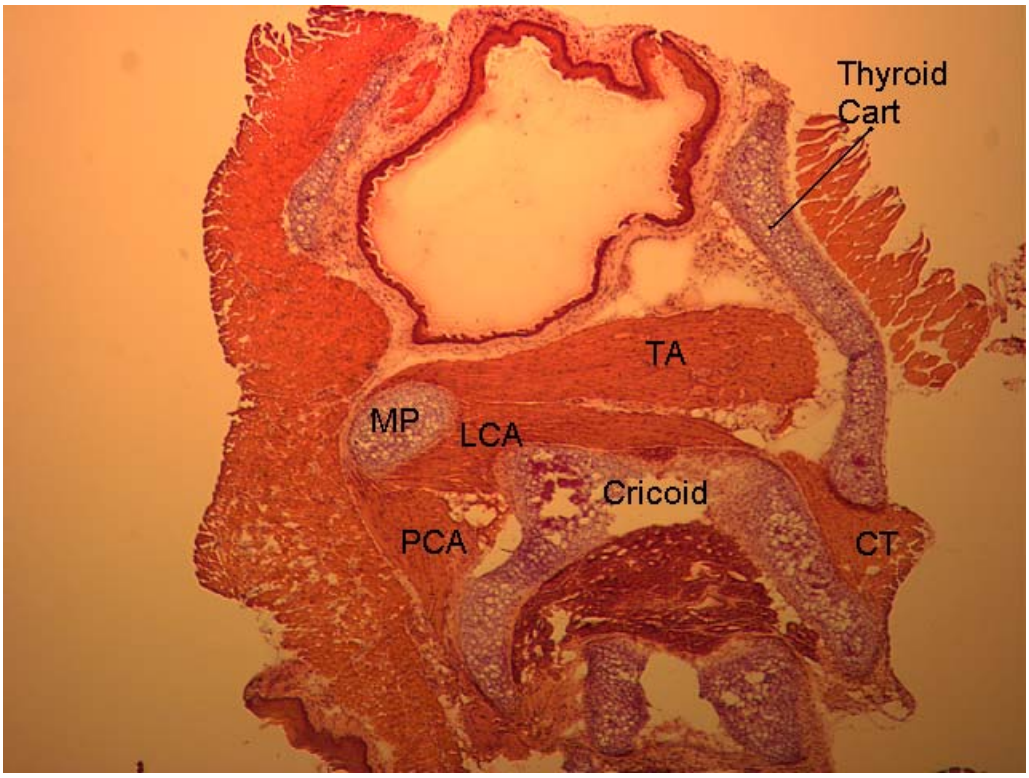

Figure 4.4. Hematoxylin and Eosin Staining of Mouse Larynx Viewed along a Sagittal Cut (40X). Image illustrates positioning of thyroid, cricoid, and arytenoid cartilages as in the human larynx. Attachment of the TA, LCA, and PCA muscles to the muscle process (MP) of the arytenoid is appreciated. Extreme anterior aspect of CT muscle is observed coursing between cricoid and thyroid cartilages.

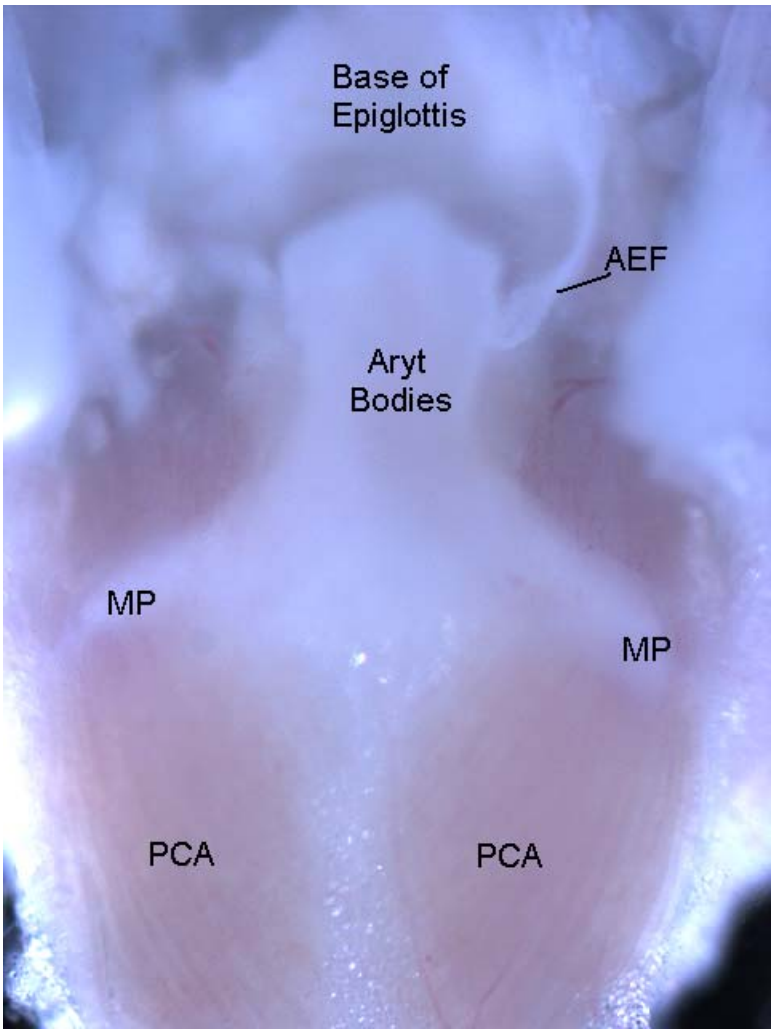

Figure 4.5. Posterior View of Laryngeal Inlet (40X). Image illustrates bilateral arytenoid bodies in adducted position (center of image) and bilateral muscle processes (MP) projecting laterally. Base of the epiglottis is observed at the entry to the laryngeal inlet, and inlet is bordered by the aryepiglottic folds (AEF). Bilateral PCA muscles are observed coursing from the cricoid lamina to the muscle processes of the arytenoid cartilages.

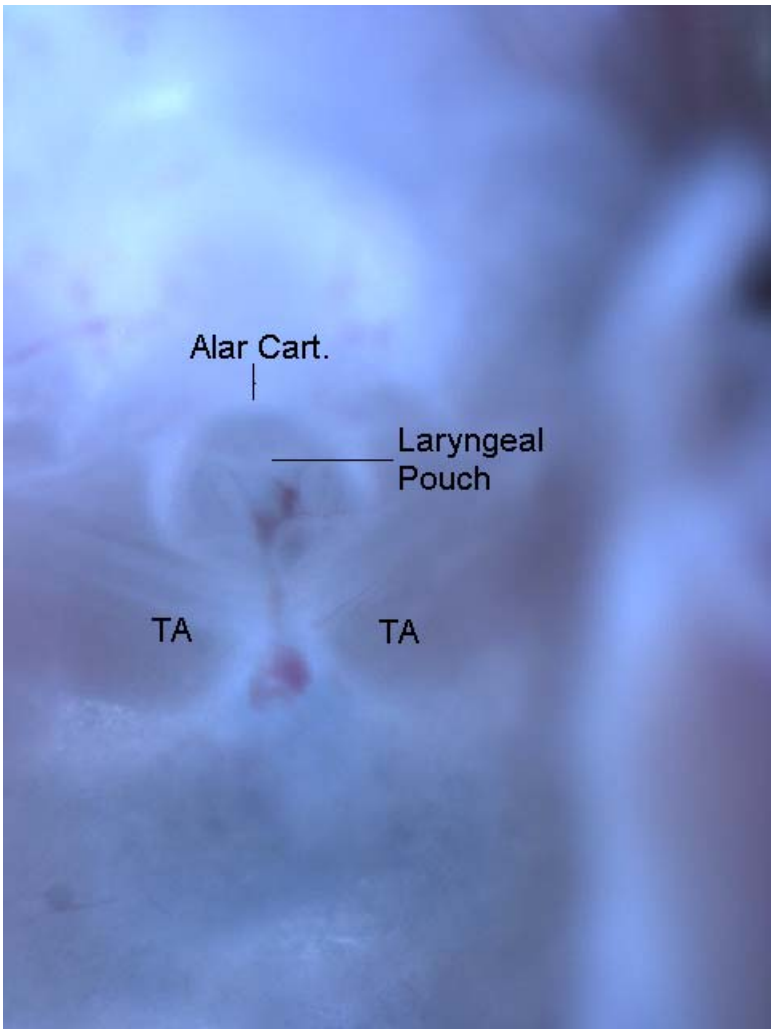

Figure 4.6. Mid-sagittal Image of C57BL Mouse Larynx (40X). Bilateral TA muscles are observed coursing posteriorly on either side of the images' focal point. The U-shaped alar cartilage and laryngeal pouch are positioned at midline, just superior to the TA muscles.

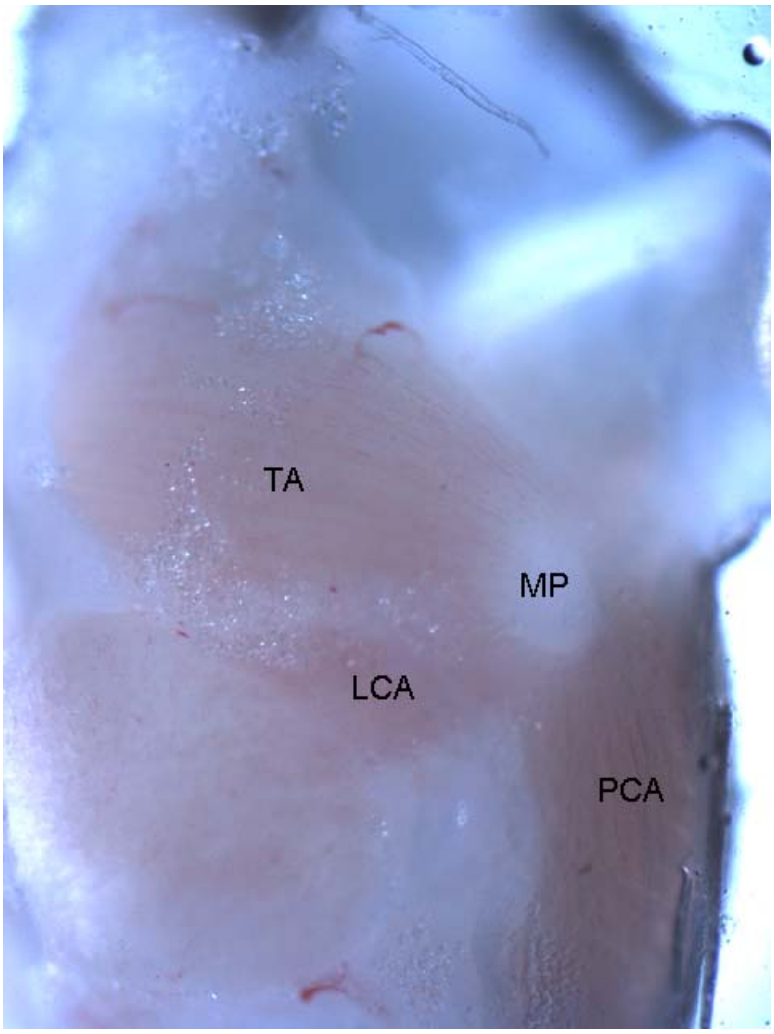

Figure 4.7. Sagittal View of C57BL Mouse Larynx (40X). Photograph taken after removal of the lateral portion of the thyroid lamina. View shows TA muscle coursing anteriorly from the muscle process (MP) of the arytenoid cartilage toward the thyroid cartilage. Fibers of the LCA muscle (cut-away) are observed at their attachment to the muscle process of the arytenoid. PCA muscles are viewed coursing inferiorly from their attachment to the muscle process.

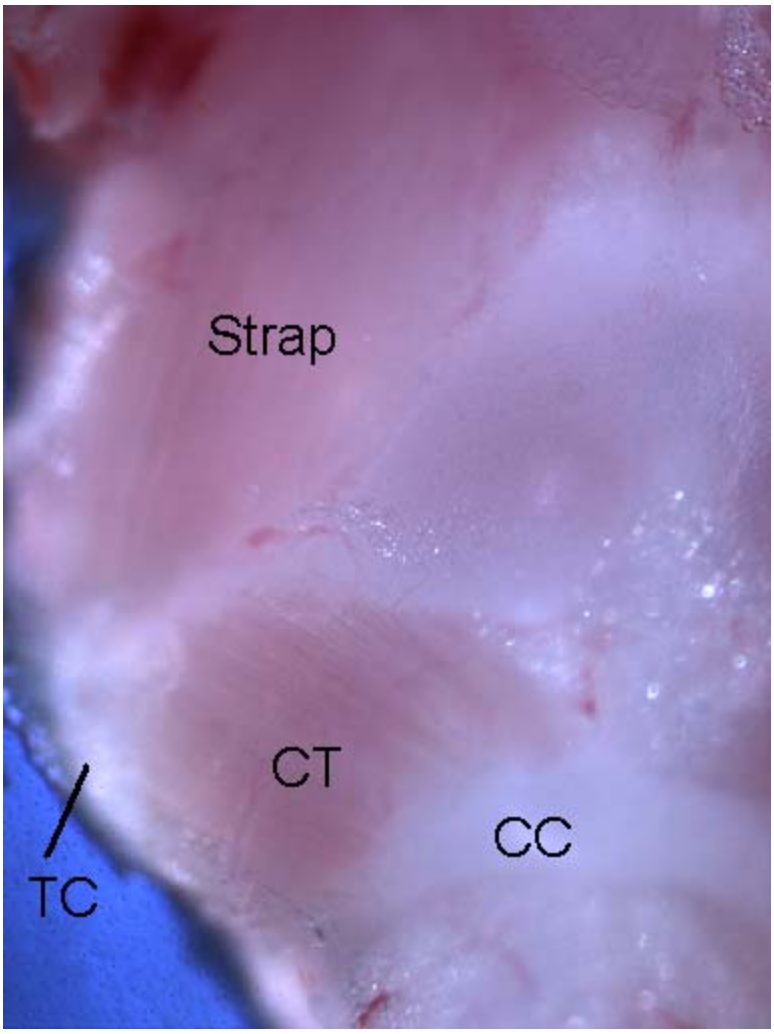

Figure 4.8. Anterior Larynx Viewed from Sagittal Cut (40X). Thyroid (TC) and cricoid cartilages (CC) are viewed at their anterior articulation. CT muscle observed coursing between the thyroid and cricoid cartilages. A strap muscle of the neck (thyrohyoid) is noted superior to the CT.

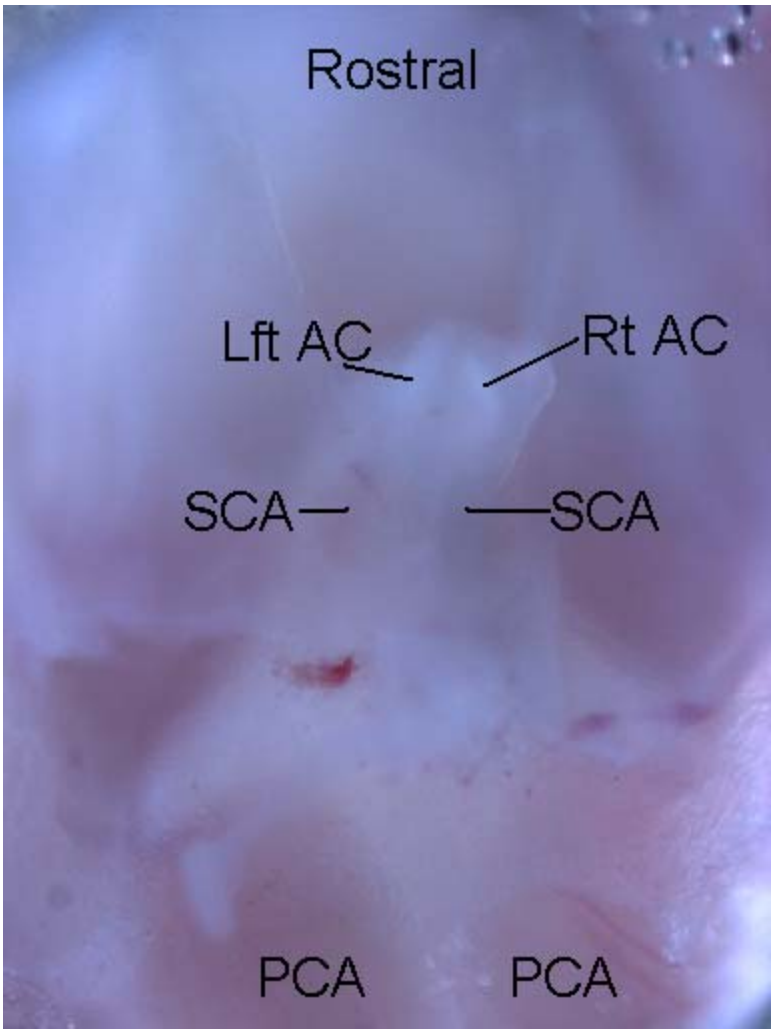

Figure 4.9. Posterior View of Larynx (40X) Showing Bilateral Arytenoid Cartilages (adducted). Portion of bilateral PCA muscles are observed at bottom of image. Bilateral SCA muscles are observed at their origin along the posterior aspect of the arytenoid cartilages (AC).

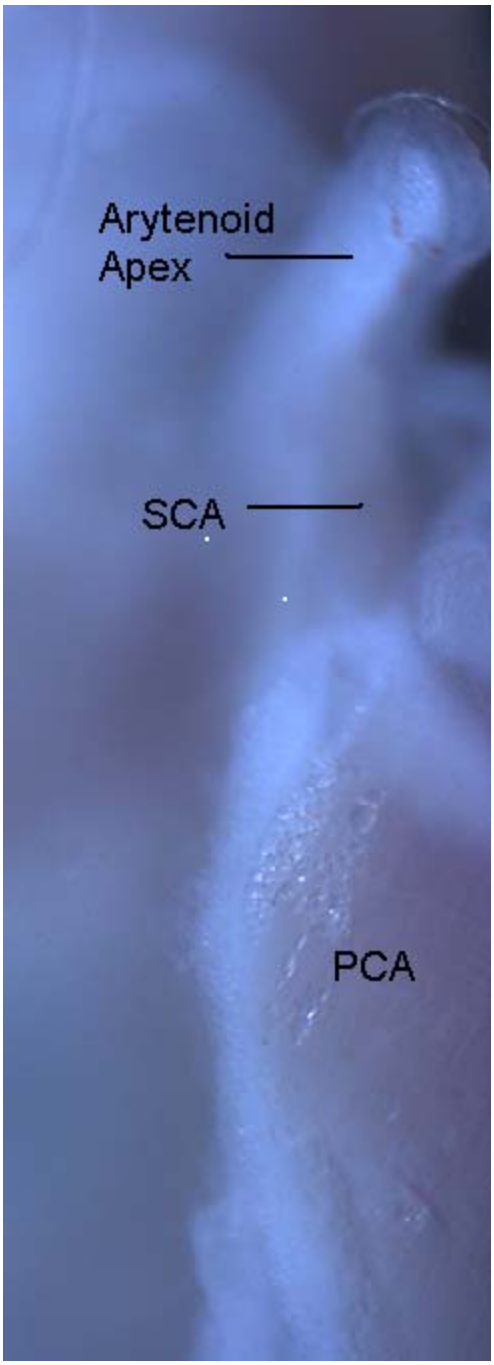

Figure 4.10. Mid-sagittal Image of SCA. Note SCA muscle positioned along the posterior aspect of the arytenoid cartilage (40X). PCA muscle noted at bottom of image.

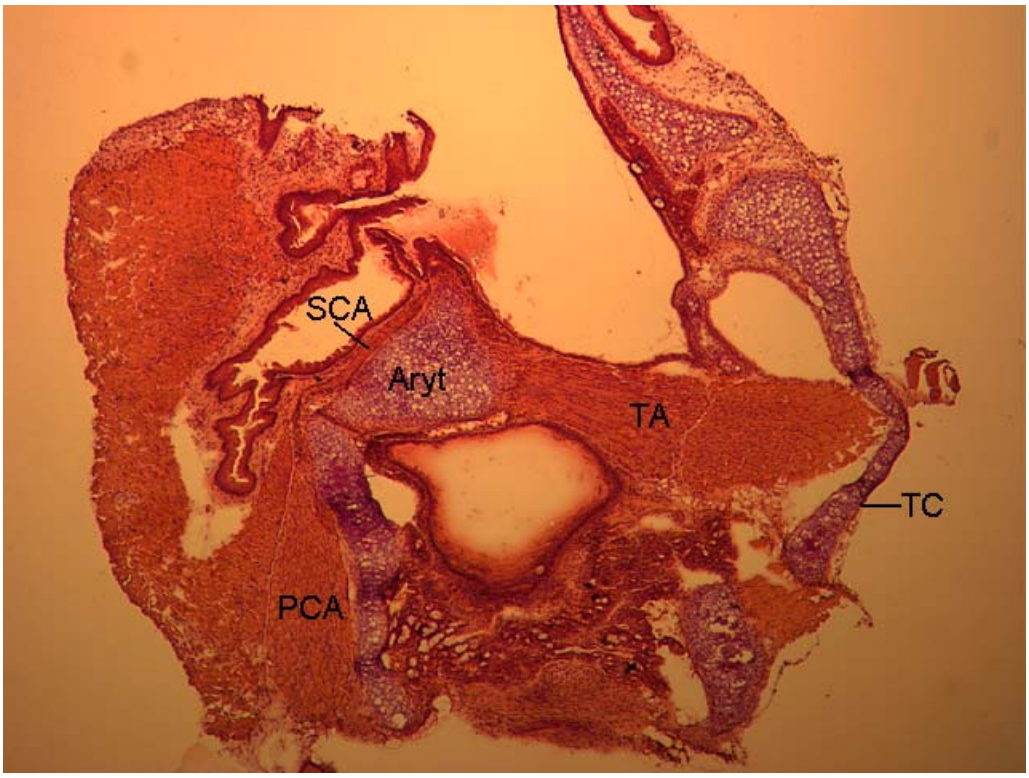

Figure 4.11. Hematoxylin and Eosin Staining of Mouse Larynx Viewed from Sagittal Cut (40X). Image illustrates SCA positioned at the posterior aspect of the arytenoid cartilage (Aryt).

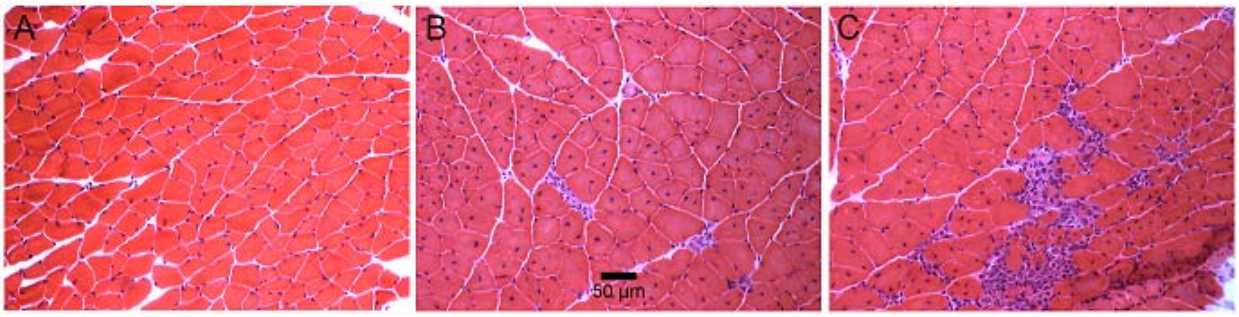

Figure 4.12. Hematoxylin and Eosin Staining of Gastrocnemius Muscles (20X). A. Control gastrocnemius shows normal gastrocnemius morphology with rectangular muscle fibers and peripheral nuclei. B.C. *mdx* gastrocnemius evidences fibrosis, pleomorphic fibers, and central nuclei.

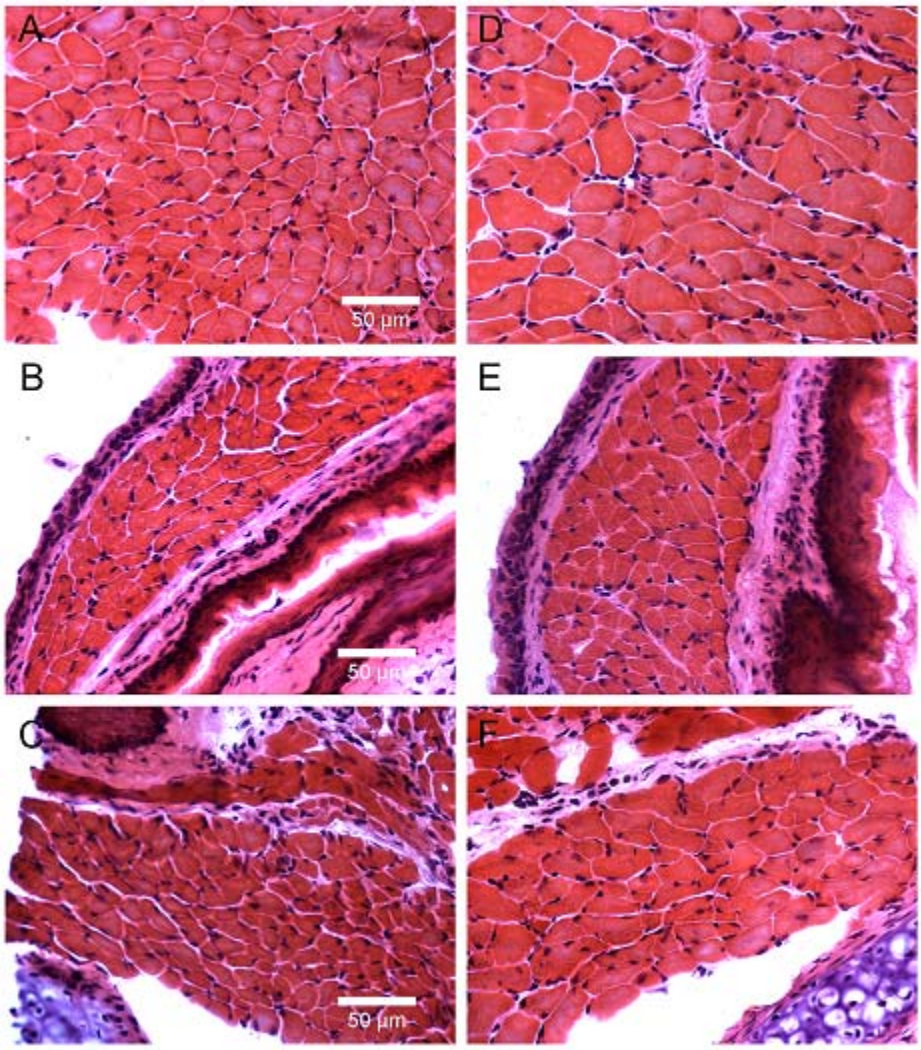

Figure 4.13. Hematoxylin and Eosin Staining of Control (A, B, C) and *mdx* (D, E, F) Laryngeal Muscles (40X). Control CT (A), SCA (B), and PCA (C) and *mdx* CT (D), SCA (E), and PCA (F) fibers demonstrate peripheral nuclei and consistent fiber size and shape.

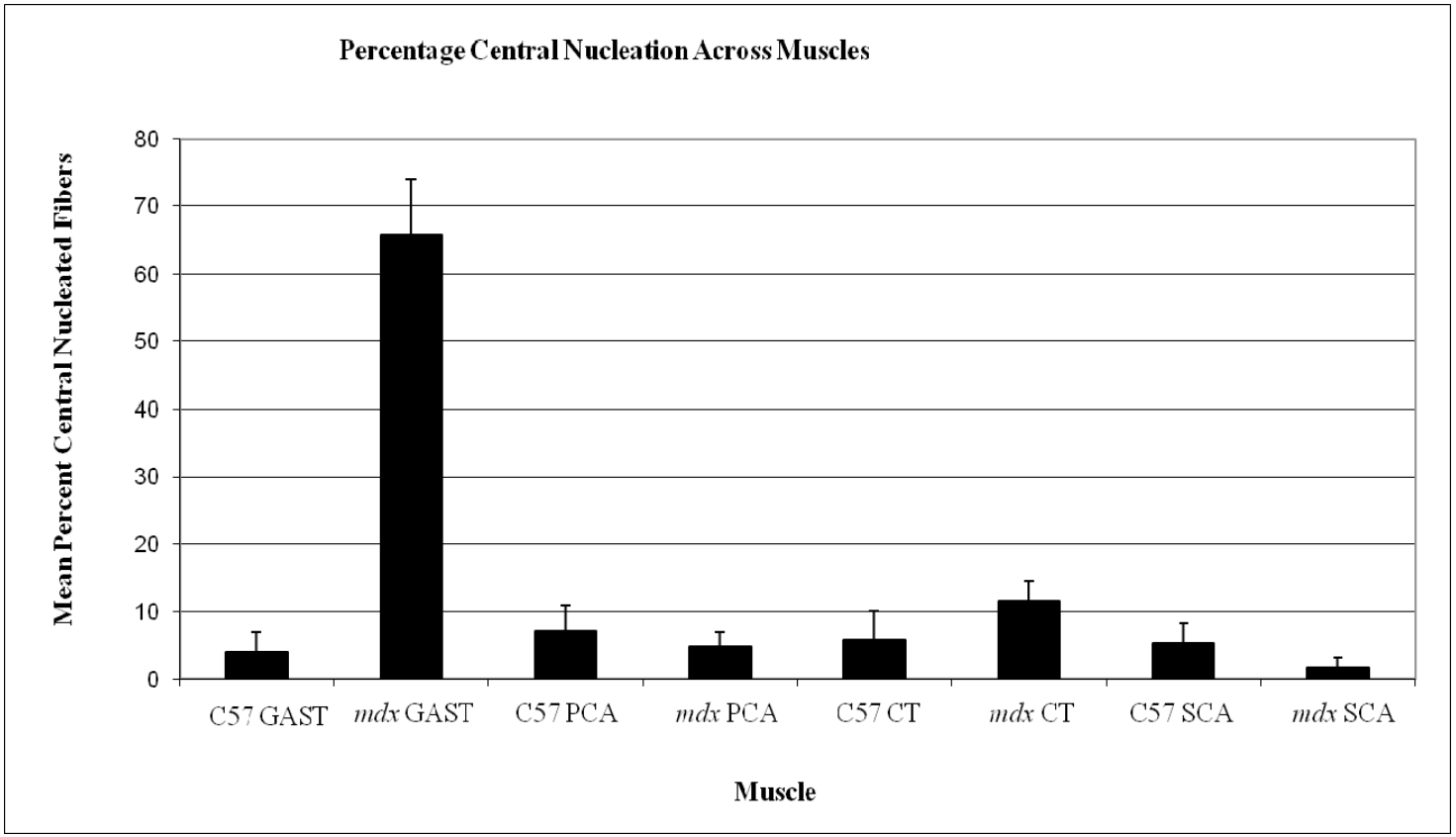

Figure 4.14. Central Nuclei Counts for Control and *mdx* Muscles of the Hindlimb and Larynx. Mean percentages of central nuclei (a marker of fiber regeneration) were elevated in the *mdx* gastrocnemius, while they remained unchanged in *mdx* laryngeal muscles.

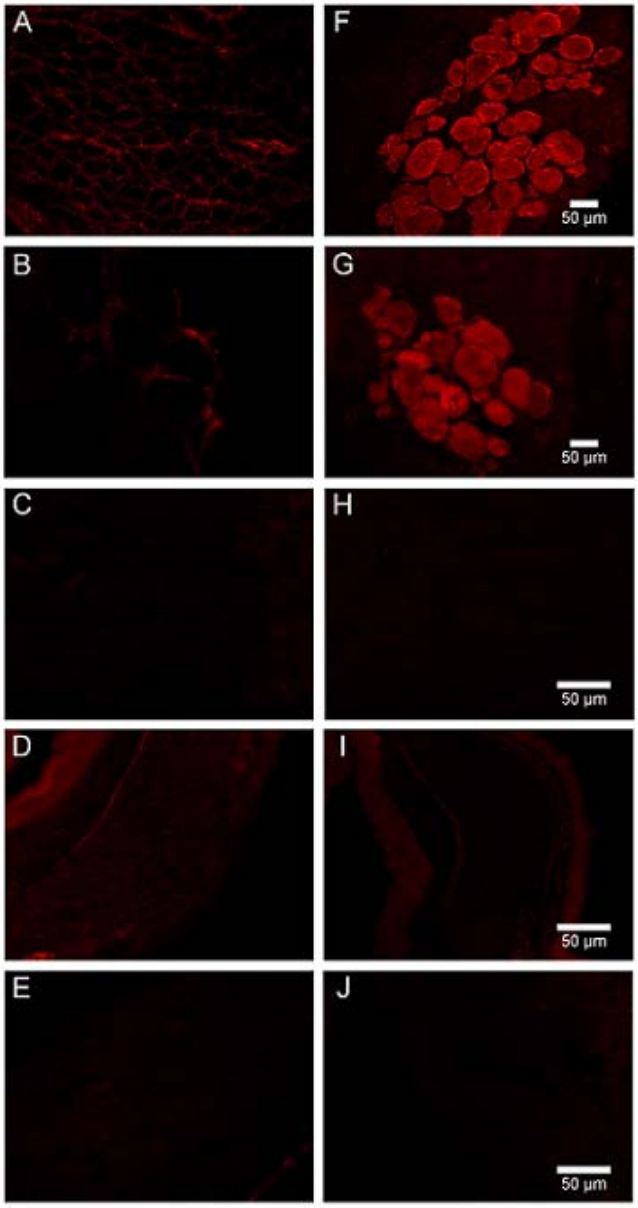

Figure 4.15. Results of Evans Blue Dye Testing. Pictures demonstrate the effects of Evans Blue dye injection on control and *mdx* gastrocnemius (A, F), neck strap muscle (B, G), cricothyroid (C, H), superior cricoarytenoid (D, I), and posterior cricoarytenoid (E, J). Frames F and G illustrate that dye penetrated the sarcolemma and rested within the intracellular space of affected muscles (*mdx* gastrocnemius, *mdx* strap). Dye did not penetrate the sarcolemma of control muscles (frames A – E) or *mdx* laryngeal muscles (frames H, I, J). The results demonstrate the maintenance of sarcolemmal integrity in *mdx* laryngeal muscles. Gastrocnemius photos taken at 20X, laryngeal muscles at 40X.

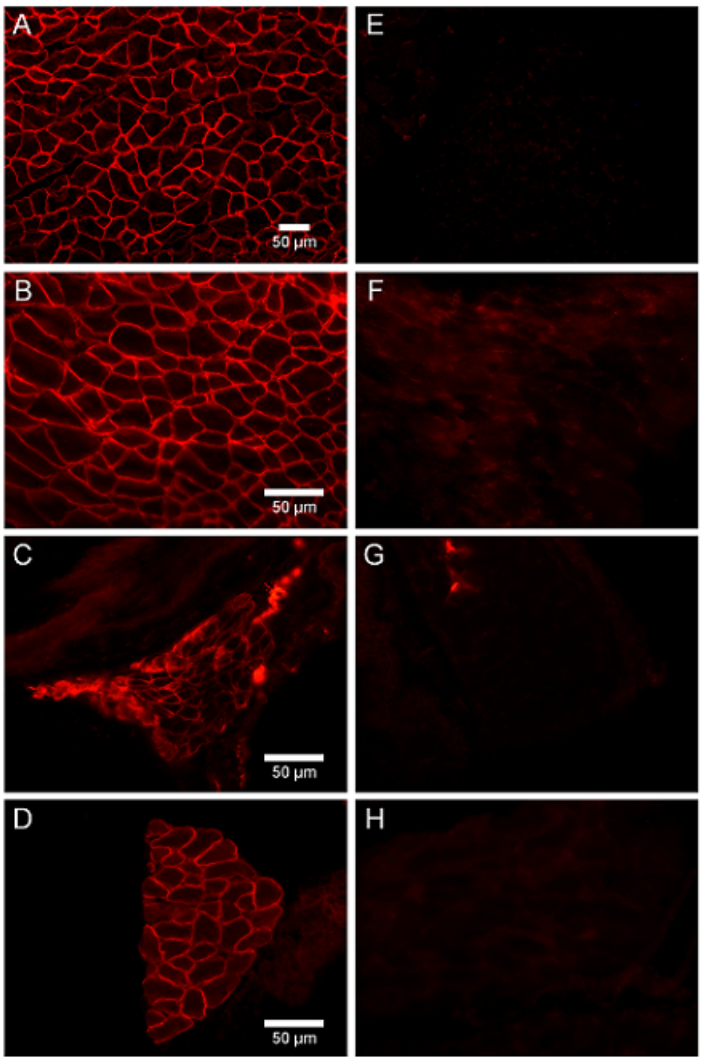

Figure 4.16. Dystrophin Distribution. Immunocytochemisty of gastrocnemius (A, E), cricothyroid (B, F), superior cricoarytenoid (C, G), and posterior cricoarytenoid (D, H) muscles. Control muscles (left) show the presence of dystrophin in the sarcolemmal boundaries of muscle fibers. *mdx* muscles fail to show dystrophin. Gastrocnemius photos taken at 20X, laryngeal muscles at 40X.

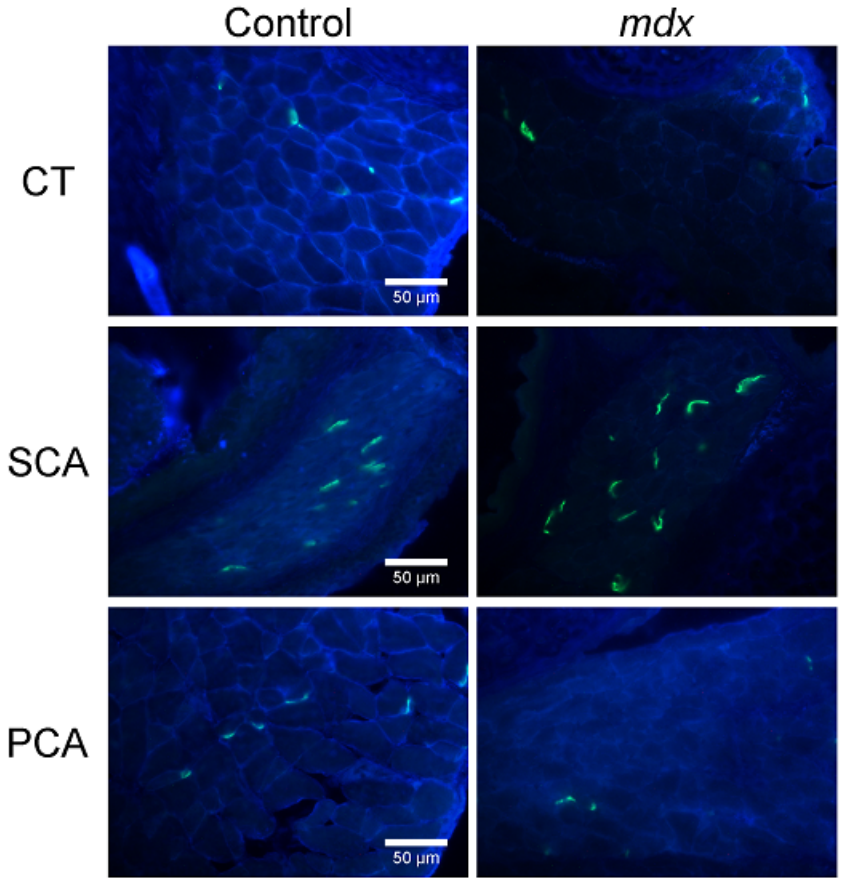

Figure 4.17. Utrophin (Polyclonal) Distribution. Immunocytochemistry of laryngeal muscles (40X) using a polyclonal antibody against utrophin (blue labeling). Neuromuscular junctions are labeled as green. In control laryngeal muscles, utrophin labeling is noted along the sarcolemmal boundaries of fibers. Utrophin labeling did not correspond with the neuromuscular junction sites in control muscle. In *mdx* laryngeal muscle, sarcolemmal utrophin labeling was notably reduced, and labeling did not correspond with neuromuscular junction sites. Findings suggest the cross-labeling of dystrophin by the polyclonal utrophin antibody. Gastrocnemius photos taken at 20X, laryngeal muscles at 40X.

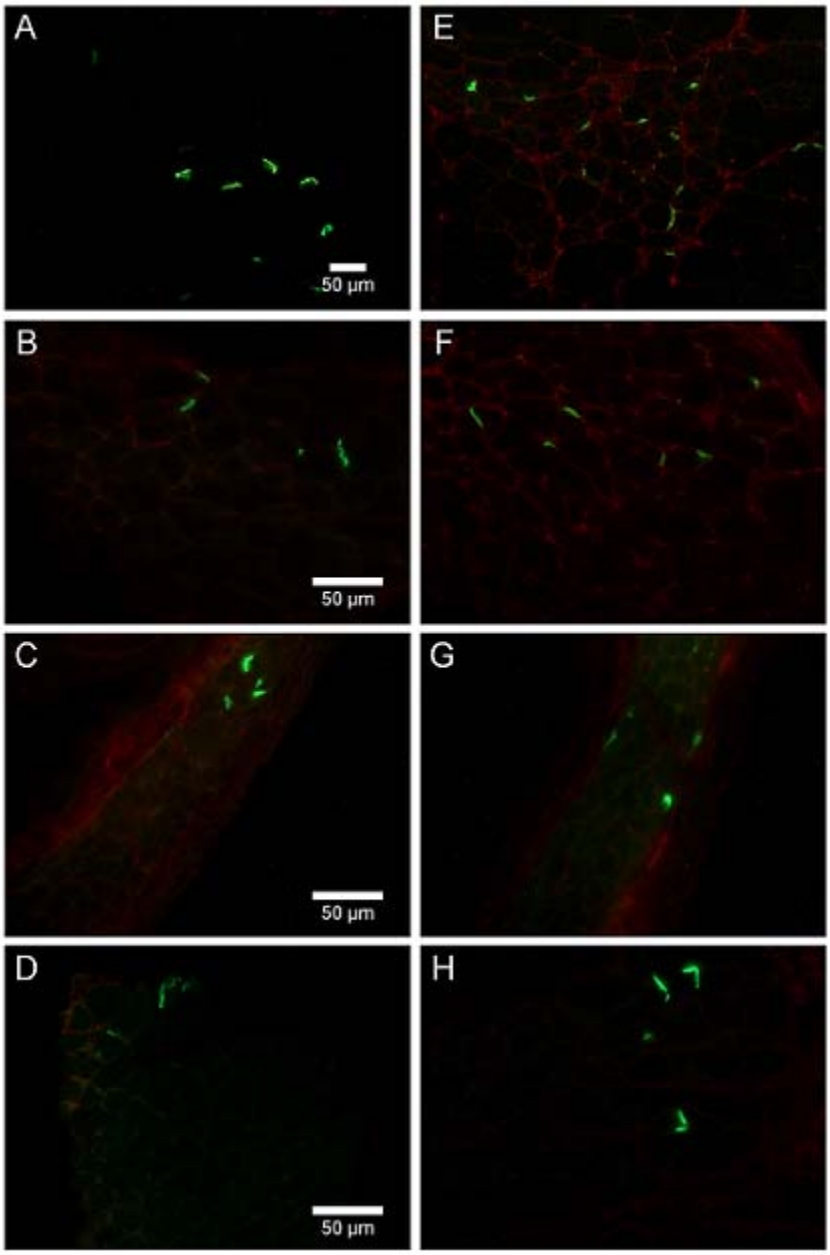

Figure 4.18. Utrophin (Monoclonal) Distribution. Immunocytochemistry of control (left) and *mdx* (right) muscles using a monoclonal antibody against utrophin (red labeling). Neuromuscular junctions are labeled in green. Control gastrocnemius muscles (A) show utrophin localized to NMJ sites, while *mdx* gastrocnemius muscles (E) show sarcolemmal localization of utrophin. In control CT (B), SCA (C), and PCA (D) muscles, utrophin is not present at NMJ sites. Finally, *mdx* CT (F), SCA (G), and PCA (H) muscles do not demonstrate sarcolemmal utrophin patterns as observed in the *mdx* gastrocnemius. Gastrocnemius photos taken at 20X, laryngeal muscles at 40X.

CHAPTER 5: DISCUSSION

Results indicate that the SCA muscles of the larynx are spared from the pathological consequences of dystrophin deficiency in the *mdx* mouse. These results parallel those of earlier studies showing sparing of the TA, PCA, and LCA muscles.[4, 7, 25] Further, the findings suggest that the SCA muscle possesses a refined mechanism of sarcolemmal management comparable to that of the well-studied TA and PCA muscles.

Findings also suggest significant disease protection in the CT muscle. The *mdx* CT evidenced subtle morphologic changes, characterized by percentages of central nuclei that were twice that of control muscles. While the increased presence of centrally nucleated fibers in the *mdx* CT was not significant per non-parametric statistical treatment, the increase did approach significance ($p = .058$) and similar increases were not observed in the CT's sister laryngeal muscles. The increased central nucleation was noted in the absence of fibrosis, necrosis, inflammation, and sarcolemmal disruption. This pattern of increased central nucleation in the absence of myofiber degeneration is a pattern previously described in mildly affected craniofacial muscles,[9, 85] suggesting a minimal disease effect in this muscle.

Findings of the current study add to the literature knowledge of the SCA, its response to disease, and its level of biological similarity to other laryngeal muscles. Results for the CT support a 2007 study[4] showing subtle changes within the *mdx* CT and raise questions as to areas of biological or mechanical difference between the CT and other laryngeal muscles.

In the sections that follow, findings related to the SCA and CT are discussed in relation to previous reports of their biological properties. Thoughts as to the muscles' properties, level of specialization, and contribution to laryngeal function are offered.

The SCA

The investigator hypothesized that the IA, or its counterpart in the mouse larynx, would be affected by dystrophin deficiency. The hypothesis was based upon previous work showing the IA to be similar to limb musculature in its contractile protein profile and proprioceptive mechanisms.[32, 77, 131] The investigator theorized that the IA's similarity to limb muscle in these areas would translate into a similar response to dystrophin deficiency. The findings of the study, however, disproved this hypothesis.

The finding of SCA sparing is novel to the current study; however, it parallels the results of previous studies showing sparing of the intrinsic laryngeal muscles emerging from the sixth branchial arch (TA, PCA, LCA).[4, 7, 25] Thomas et al[7, 25] were the first to examine the effects of dystrophin deficiency on the intrinsic laryngeal muscles, identifying the sparing of the TA and PCA muscles. Marques and colleagues[4] confirmed the sparing of the TA (medial and lateral aspects) and PCA muscles and added to the list of spared muscles the LCA.

Implications Regarding the Nature of the SCA

The results of this study showing specialized sarcolemmal management of the SCA, considered in the light of previous studies of the posterior adductor,[32, 77] suggest that the SCA is a "blended" muscle, possessing some features of prototypical skeletal muscle and other specialized features capable of protecting it from the pathological cascade of dystrophin deficiency. The mixed nature of the IA/SCA may be explained by one or both of the following points: (1) the differentiation between classic limb skeletal muscle and specialized laryngeal muscle is not explicit and some muscles may be "blended," sharing properties of both classic and specialized muscle, and/or (2) features of the IA/SCA (and other laryngeal muscles) differ across species and IA similarity to limb muscle identified in humans may not be generalized to rodent musculature. Each of these possibilities is discussed in more detail below.

Ambiguity of "Classic" vs. "Specialized" Muscle

In recent years, certain of the craniofacial muscles (eg, extraocular, masseter, intrinsic laryngeal) have been presented as highly specialized forms of skeletal muscle that deviate from classic (ie, limb) muscle in many key respects.[1, 2, 5, 24, 241] In fact, some have proposed that these muscles may represent a different allotype of skeletal muscle.[2] Chief among the areas of craniofacial deviation and specialization are: myosin isoforms, metabolic properties, innervation patterns, regenerative capacity, sarcomere structure, and response to disease.[2, 3, 5, 7-10, 13-20, 22-25, 41, 44, 85, 241] Select craniofacial muscles, such as the TA, PCA, and extraocular muscles, benefit from all of the aforementioned features.[2, 3, 7, 17, 19, 22-25, 41, 44] However, the findings of this and other studies suggest that other craniofacial muscles possess some, but not all, of the features. For instance, the SCA demonstrates a response to disease which is characteristic of specialized muscle but

myosin isoforms and innervation patterns representative of classic muscle.[32] Conversely, the masseter demonstrates mild effects of dystrophin deficiency[9] but myosin isoforms characteristic of highly specialized muscle.[8] These findings show that the separation between classic skeletal muscle and specialized muscle is not explicit and that certain of the craniofacial muscles may be best considered as "blended" muscles whose degree of specialization is driven by the level and type of functional demand placed upon them.

The characterization of the SCA as a blended muscle suggests that the muscle's level of specialization does not meet that of the TA and PCA muscles. While the SCA possesses a mechanism of sarcolemmal management similar to that of other laryngeal muscles, it maintains contractile speeds and proprioceptive features comparable to that of limb muscle.[32, 77] This unique nature may be reflective of the muscle's important role in positioning and holding the arytenoid cartilages at midline for voicing, an action requiring strength and endurance, rather than speed and refined motion.

Finally, recognition of the blended nature of many craniofacial muscles may be of great importance in the study of muscle sparing in dystrophin deficiency. The study of blended (classic + specialized) muscles, such as the SCA, may help researchers rule out prototypical features (ie, myosin isoforms, innervation) as contributory to the sparing and may, thereby, aid them in concentrating their efforts on other specialized muscle features.

Generalization across Species

Studies of the SCA/IA and its properties are rare. The current study highlighting the specialized nature of the murine SCA in response to disease is the only study to describe specialized features in this primary glottal adductor. Interestingly, all other studies demonstrating the muscle's similarity to prototypical limb muscle have been conducted in humans.[32, 77, 78] Thus, the possibility exists that the adductory muscle may retain different properties across species. It may follow then that the IA/SCA's level of specialization in humans and other animal models may differ secondary to the functional demands placed upon the larynx across models.

The CT

Marques and colleagues[4] were the first to examine the CT's response to dystrophin deficiency. The group used methods similar to those applied in the current study to examine the effects of dystrophin deficiency on the lateral TA, medial TA, PCA,

LCA, and CT muscles. While sparing was identified in the lateral TA, medial TA, PCA, and LCA muscles, the CT evidenced mild markers of fiber damage (ie, Evans blue positive fibers) and regeneration (ie, increased percentage of central nuclei). Within the CT, significant differences in the percentage of centrally nucleated fibers between control and *mdx* muscles were found at both 4 and 18 months. Markers of degeneration (eg, sarcolemmal disruption, inflammation) were more pronounced at 18 months. The authors concluded that the CT showed a mild and delayed response to dystrophin deficiency. Findings from the current study supported the work of Marques et al; however, slight differences in study findings should be discussed.

Results of the current study showed central nucleation shifts in the *mdx* CT similar to those observed in the Marques et al study (ie, *mdx* CT showing 2 to 3- fold increase over control CT); however, it did not identify markers of degeneration in the muscle. Slight differences in the findings of the two studies may be explained by methods used by investigators. First, Marques et al[4] examined adult (4-month) and old (18-month) mice, while the current study examined 8-week mice. As markers of degeneration appear delayed in the CT, the current study's use of the 8-week model may have limited its appreciation of degenerative changes which transpire later in the lifespan. Further, differences in the percentage of centrally nucleated fibers between control and *mdx* muscles were significant in the work of Marques and colleagues but non-significant (p = .058) in the current study. It is likely that the smaller sample size in this study and the requirement for non-parametric statistics made its statistical treatments less sensitive to group differences in central nucleation.

Interestingly, mild and delayed disease effects, such as those identified in the CT have also been noted in other craniofacial muscles, most notably the masseter and 2 accessory extraocular muscles, the levator palpebrae superioris and the retractor bulbi.[9, 85] In each of these mildly affected muscles, the primary marker of pathology was an increased occurrence of centrally nucleated fibers, indicating increased rates of fiber regeneration. Typical markers of degeneration (ie, inflammation, necrosis, fibrosis) associated with the disease were not observed or were observed only in late stages of the disease. The finding of increased regeneration in the absence of widespread degeneration has not been fully explained; however, it may be proposed that mildly affected muscle

groups possess regenerative abilities that outpace apoptotic or necrotic degeneration and, thereby, preserve the overall morphology of the muscle.

Further, if findings of slight, delayed disease effects within the CT are proven correct, the nature of the CT becomes a matter of some interest in the study of dystrophin deficiency. As with the EOM group, the group of intrinsic laryngeal muscles would possess both spared and mildly involved muscles and would offer an excellent mechanism for the study of muscle response to disease. Indeed, the systematic comparison of divergent muscles within a single muscle group could offer significant insight into factors associated with preservation and factors associated with pathology.

Implications Regarding the Nature of the CT

Mild involvement of the CT led Marques et al[4] to discuss the CT as a blended, or mixed, muscle which shared the properties of laryngeal and prototypical limb skeletal muscle. Such representations of the CT have been recorded previously in the literature. Hyodo et al[135] found variable forms of myotendinous junctions in the CT, some characteristic of laryngeal muscle and others characteristic of limb muscle. The authors concluded that the CT was a transitional muscle, sharing properties of both limb and laryngeal muscle. In addition, a number of studies examining the myosin heavy chain composition and contractile speeds of the CT have placed it more in line with fast limb muscle than with laryngeal muscle.[17, 22, 35] Authors identifying the CT's divergence from its sister muscles have suggested an embryological foundation for the differences.[22] The CT emerges from the fourth branchial arch, whereas all other intrinsic laryngeal muscles develop from the sixth arch.[33] Such a history places the CT more in line, developmentally, with pharyngeal muscles than with other laryngeal muscles.[34] The implications of this divergent embryology are unknown; however, it raises the possibility that the CT may differ from other laryngeal muscles in its developmental programming and patterns of gene expression and that the search for mechanisms of muscle protection should begin at the genetic level.

Finally, some have suggested that muscle response to dystrophin deficiency is a factor of a muscle's mechanical requirements.[85, 86] If the CT's differential response is considered from a purely mechanical viewpoint, several important questions are raised. First, does the finding of slight involvement of the CT suggest that the muscle possesses a

different, and perhaps less refined, sarcolemmal management system than its sister laryngeal muscles? Results of ICC assays show similar patterns of dystrophin staining in the CT and other laryngeal muscles and suggest that at least this component of the DGC is shared between the CT and its sister muscles. However, other components of the DGC (eg, β-dystroglycan, sarcoglycans, etc.) were not examined in this study, and the possibility of differences in those proteins across laryngeal muscles remains to be examined.

The CT's differential response also raises questions as to its mechanical requirements and properties. For instance, does the CT's role as the primary lengthener and external tensor of the vocal folds result in mechanical requirements or stressors that exceed those of other laryngeal muscles? Additionally, is the CT's design more or less able to handle load than other laryngeal muscles. Functional studies of the CT are limited and are primarily centered on its speed of contraction. However, a 2007 study does suggest that the CT and TA muscles differ from other laryngeal muscles in their mechanical properties.[242] Hunter and Titze have identified lower levels of stiffness in the CT and TA relative to the primary group of adductors/abductors (LCA, IA, PCA), a finding which may hold implications for the CT's load-bearing capacity. Consideration of this mechanical difference in light of the current study's findings suggests that mechanical properties of the CT may play a role in its differential response to dystrophin deficiency and that additional studies of the muscle's mechanics are indicated.

Finally, any discussion of CT mechanics and its possible relationship to disease effects should be considered in light of the current debate on muscle mechanics and response to dystrophin deficiency. It has been proposed for some time that variable disease effects across muscle groups arise due to differences in the mechanical stress placed upon the muscles during contraction.[85, 86] The small fiber size and reduced load of the EOM[52-54] as well as the realization of milder disease effects in distal muscle groups (eg, toe)[180] appear to support this mechanical explanation of sparing. However, the severe manifestation of the disease in the diaphragm, a muscle with low mechanical stress,[208-210] indicates that a purely mechanical explanation is unlikely. Thus, the debate continues as to the role of muscle mechanics in the pathophysiology of the disease. Until a definitive

answer emerges on mechanical explanations for sparing, it will be important to consider the mechanics of differentially affected muscles, including those of the CT.

Mechanisms of Sparing and Protection

The finding of selected intrinsic laryngeal muscle sparing and protection in dystrophin deficiency raises the question of the mechanism of sparing. While a number of theories of sparing have been proposed in the literature (see Chapter 2 for a description of key theories), one theory of sparing – utrophin upregulation – was investigated as part of this study.

Utrophin Upregulation

Utrophin is the structural homolog to dystrophin.[200, 202] The protein's structural similarity to dystrophin along key domains makes it capable of acting as dystrophin to link cytoskeletal actin to the extracellular matrix.[200, 243] This property has led some to suggest that utrophin may act as a substitute for dystrophin in DMD and that it may be responsible for the preferential sparing of some muscle groups.[180, 183, 195] Findings from the current study, however, failed to demonstrate re-localization of utrophin to the sarcolemma in spared *mdx* laryngeal muscles. Consequently, the study did not support utrophin upregulation as the primary mechanism of laryngeal muscle sparing. It should be noted that *mdx* CT muscles showed a clearer perimeter staining for utrophin than *mdx* PCA and SCA muscles (Figure 4.18), suggesting the possibility of sarcolemmal utrophin in this muscle. However, the perimeter staining was not identified in all positive sections and was not appreciably different from the pattern observed in some CT negative controls. Consequently, the author proposes that perimeter staining in the CT likely was the product of non-specific binding of the secondary antibody and suggests that alternative methods (ie, Western blotting) be used in the future to further evaluate utrophin upregulation in the *mdx* CT.

While this and other studies have failed to support utrophin upregulation as the sole explanation for sparing, a review of the DMD literature indicates that the dystrophin homolog is involved in some manner in the disease process. In fact, studies suggest that the protein's expression may be modified in both affected and spared muscle groups.

Utrophin Upregulation in Affected Muscles

Studies of dystrophin deficiency show marked increases in utrophin expression and sarcolemmal localization of the protein in affected muscle groups ,[182, 195, 205, 244] adaptations believed to occur post-transcriptionally.[244] Immunocytochemical assays of dystrophin deficient muscles show staining of utrophin at the perimeter of muscle fibers in a pattern indicative of dystrophin. Curiously, this pattern of localization appears restricted to select clusters of fibers within affected muscles. According to Porter and colleagues,[182] the modified distribution of the protein in these muscles does not prove biologically significant, as it fails to halt the pathological cascade. Interestingly, other studies showing an inverse relationship between naturally occurring utrophin levels and disease severity[180, 183, 195]suggest that utrophin's role may be biologically important, limiting the impact of disease.

Thus, the exact role of utrophin upregulation in affected muscle is unclear. A review of the studies suggests that the protein attempts to replace dystrophin at the sarcolemma but that the compensatory response is, in large part, inadequate and unable to halt progression of the disease.[182] Work continues to better define the role of utrophin in affected muscle groups, with hopes of someday applying the protein as a therapeutic agent in the disease.[180, 183, 195, 243, 245-247]

Utrophin Expression in Spared and Protected Muscle Groups

The current study and a previous study of the laryngeal muscles[25] fail to demonstrate widespread sarcolemmal localization of utrophin in dystrophin deficient laryngeal muscle. Consequently, the studies argue against utrophin upregulation as the sole mechanism of laryngeal muscle sparing. However, work conducted on other protected muscle groups, most notably the extraocular muscles, suggests that the protein does play a role in the sparing of some fibers. Two studies, in particular, highlight the complex nature of utrophin involvement in extraocular muscle sparing.

In contrast to the laryngeal findings noted above, sarcolemmal localization of utrophin has been identified in intact (ie, spared) extraocular muscle fibers of *mdx* mice.[42] Specifically, upregulation was noted in 3 of the 6 specialized EOM fiber types: orbital singly innervated, global multiply innervated, and orbital multiply innervated. The preservation of a normal phenotype in fibers exhibiting sarcolemmal utrophin pointed to

a potential rescuing role for utrophin in these fibers. Interestingly, other intact extraocular fibers, belonging to the remaining classes of EOM fibers, did not evidence sarcolemmal utrophin, a finding which suggests an alternative mechanism of sparing in these fibers. These results point to a fiber-type specific role for utrophin in the EOM, whereby some spared fiber types engage utrophin as a dystrophin substitute and other fibers engage an alternative mechanism.

In another elegant study, Porter et al[182] examined EOM characteristics in three mouse models: the dystrophin deficient *mdx* mouse, a single utrophin knock-out mouse, and a double utrophin/dystrophin knock-out mouse. The EOM of dystrophin-deficient mice and utrophin-deficient mice showed no evidence of pathology. However, the EOM of utrophin/dystrophin deficient double-knock out mice evidenced severe markers of disease. Interestingly, select muscle fibers within affected EOM remained spared in the double knock-out. The preserved fibers belonged to extraocular fiber types previously described as: global multiply innervated, orbital singly innervated, and orbital multiply innervated.[43] Results again suggested that the role of utrophin in muscle sparing is complex and perhaps specific to fiber type.

Thus, the role of utrophin in muscle sparing remains largely unresolved. Work conducted in the EOM suggests that the protein is a factor in sparing, but that its role is mediated by fiber type. Further, these studies demonstrate that additional, yet to be defined, mechanisms of sparing are at play in some muscle fiber types. Unraveling the mystery of utrophin's role in dystrophin deficiency remains a priority for many.[42, 179, 180, 182, 183, 245, 246] Unfortunately, scientists are just beginning to appreciate the complexity of this protein and the implications of its complexity for the study of dystrophin deficiency.[201, 204, 244, 248]

Utrophin Complexity and Implications for Study

Utrophin is ubiquitously expressed throughout the body.[204, 249] In skeletal muscle, the protein is recognized in the vasculature, peripheral nerves, and neuromuscular junctions of mature fibers and at the sarcolemma of developing or regenerating fibers.[202] Specific patterns of the protein's expression in muscle are, however, highly complex, varying across body tissues,[204] across muscle fiber types,[201, 250] and across the lifespan.[202]

Such intricate expression patterns prove challenging to investigators searching for altered patterns of the protein's expression in disease processes.

In addition to the varied expression patterns noted above, recent work reveals that both full-length and short forms of the protein exist. Two full-length forms, identified as utrophin-A and utrophin-B, have been described.[251] In skeletal muscle, utrophin-A is localized to the NMJ and peripheral nerves, whereas utrophin-B is identified within capillaries of the endomysium.[244] While the two utrophin forms may be co-expressed in some tissues, they are differentially regulated.[251] These independent patterns of regulation are clearly evidenced in dystrophin deficient models, where utrophin-A shows notable upregulation in response to disease and utrophin-B expression remains unchanged. Such findings show the complex nature of utrophin expression in disease and highlight the importance of developing and using utrophin-A and -B-specific antibodies in the study of dystrophin deficiency.

Future Studies of Utrophin in Laryngeal Muscles

The exact role of utrophin in dystrophin deficiency remains to be elucidated. While the current study did not show re-localization of utrophin in ICC assays performed on spared muscles, follow-up investigations of utrophin expression in the laryngeal muscles are warranted for several reasons: (1) to define normal patterns and levels of utrophin expression in this muscle group, (2) to consider variability in utrophin expression across muscle fiber types, and (3) to ensure the specific tracking of utrophin-A and B patterns in the laryngeal muscles.

Allotype-Based Perspectives on Sparing

Muscle response to dystrophin deficiency has been shown to vary widely across muscle groups – from severe progressive involvement in the limb and diaphragm, to mild involvement in some craniofacial muscles (eg, masseter), to complete sparing in a number of extraocular and intrinsic laryngeal muscles. Such patterns of response correspond strongly with 3 allotypes of skeletal muscle – limb/diaphragm, masticatory, and extraocular – previously discussed by Porter et al.[182] In fact, Porter has proposed that allotype-specific features may be central to determining a muscle's response to disease, a position that has piqued interest in the unique features of spared muscle groups.

Multiple specializations (eg, regenerative capacity, myosin isoforms, innervation patterns) of the extraocular phenotype have been presented.[2, 43] It has been suggested that certain of these refinements evolved to permit the EOM to meet demands for constant activity, rapid contraction, and exquisite motor control. Curiously, because of their role in respiration and airway protection, the laryngeal muscles share many of these same functional demands and many of these same biologic properties.[3, 14, 15, 17-19, 22, 24, 35]

Attempts to link muscle sparing to constitutive, allotype-specific features have proven fruitful in some areas. For instance, it is now recognized that utrophin expression in skeletal muscle is related to fiber type and oxidative capacity,[42, 182, 201] two areas of extraocular and laryngeal muscle specialization. Such knowledge has helped researchers focus their study of utrophin in spared and affected muscles. Additional allotype-specific features have been offered as potential links to sparing (eg, continuous myofiber remodeling, calcium management systems, developmental programming).[5, 41, 112] As of yet, however, none have been definitively tied with muscle protection in DMD. Thus, the search continues to identify the specific allotypic features responsible for sparing and protection. The recent realization of laryngeal muscle sparing may facilitate this research, as areas of extraocular and laryngeal phenotypic similarity will make clear targets for study.

Embryologic Links to Sparing

It has been suggested that muscle sparing in DMD may be attributed to the differing patterns of gene expression found in muscles emerging from the highly specialized, non-somitic head mesoderm.[5, 9] Curiously, however, the intrinsic laryngeal musculature is somitic in nature and yet spared in the disease process. This realization points to the fact that genes governing the sparing are not isolated to non-somitic tissues and widens the search for genes contributing to the sparing. Future investigations of genetic links to muscle sparing should include regions where gene expression of the somitic laryngeal muscles overlaps those of the non-somitic extraocular muscles.

Summary Remarks on Sparing

To date, the majority of studies examining mechanisms of muscle sparing have been conducted on the EOM. Such studies propose that effects of disease are averted in these muscles either via properties that prevent the disease's progression (ie, constitutive

properties) or via properties that permit exquisite adaptation to the disease (ie, adaptive properties). Recent studies on EOM point away from sparing as an adaptive response to deficiency and toward sparing as a consequence of biological specialization.[42] If constitutive properties are foundational to a muscle's response to dystrophin deficiency, the realization of laryngeal muscle sparing may facilitate the search for a mechanism of sparing, as it permits the identification of shared areas of extraocular and laryngeal divergence from classic limb muscle.

Implications

Laryngeal Muscle Diversity and Its Implications for Laryngeal Function

Findings from this study suggest that the SCA muscles of the mouse are spared from the effects of dystrophin deficiency, while the CT muscles remain strongly protected. Previous studies showing sparing of the highly-specialized TA and PCA muscles[4, 7, 25] of the larynx suggest that the SCA possesses a sarcolemmal management system which is comparable to that of its sister muscles and unlike that of prototypical limb muscle. Conclusions regarding the nature of the CT's support system are less clear; however, findings would suggest that the significant level of protection offered the muscle speaks to specialized support system in this muscle as well.

At the onset of the study, the author raised the question of diversity within the laryngeal musculature. The results of this and other studies suggest that subtle variations do exist within the group of intrinsic muscles, with some muscles showing a more refined phenotype than others. In regard to the IA/SCA, previous studies have shown that fiber types and proprioceptive patterns in the IA/SCA are less specialized (ie, more typical) than those of the TA and PCA and have, consequently, described the muscle as prototypical skeletal muscle.[32] However, the SCA's response to dystrophin deficiency suggests that the muscle is more refined than classic skeletal muscle and that it should be considered more fully for its biological specialization.

Similarly, the CT has shown fiber types and muscle organization patterns that are less specialized (ie, more typical) than the TA and PCA and more like classic skeletal muscle. The results of this study, however, show that while the CT's response to dystrophin deficiency may be slightly less sophisticated than that of its sister laryngeal muscles, it is markedly more advanced than that of classic limb/respiratory muscle. Such

findings suggest that the biologic mechanisms of the CT should be further examined relative to limb and laryngeal muscle.

DCG Organization of Laryngeal Muscles

The study identified the presence of dystrophin along the sarcolemma of both control gastrocnemius and control intrinsic laryngeal muscles. These results indicate similar patterns of dystrophin expression in leg and laryngeal muscles of 8-week old C57BL mice and suggest that the laryngeal muscles may possess a mechanism of sarcolemmal management similar to that found in leg muscles.

However, results of the current study bring into question patterns of utrophin expression in laryngeal muscles. Utrophin was not present at the NMJ sites of control laryngeal muscle fibers, as would be expected in mature skeletal muscle and as was clearly demonstrated in control gastrocnemius muscles. Sparse non-specific staining was identified around the sarcolemmal boundary of some control laryngeal muscle fibers; however, this staining was felt to be a consequence of the non-specific binding of the secondary antibody to extraneous proteins or to dystrophin, a protein with a high degree of homology to utrophin. Thus, the current study did not identify expected patterns of utrophin localization in laryngeal muscles.

In an attempt to interpret the unexpected results of utrophin staining in healthy laryngeal muscle fibers, the investigator was limited by the fact that utrophin distribution had not been previously described in these muscles. It is, therefore, unknown whether the laryngeal muscles exhibit a pattern of utrophin distribution similar to or divergent from other skeletal muscle. The identification of the protein at the NMJ in control gastrocnemius muscles in the current study suggests that procedural issues with the antibody were not a factor in the unexpected laryngeal muscle staining and that utrophin expression within the larynx demands further and more in-depth study. Immunocytochemistry methods used in the study are qualitative in nature and not sufficient to quantify potential low-levels of the protein. Consequently, more advanced, quantitative methods, such as Western blotting, should be applied to the study of utrophin in laryngeal muscles. Indeed, the need for further examination of utrophin is supported by the results of recent Western blotting in this laboratory failing to demonstrate utrophin in the PCA and TA muscles of rats (F.H. Andrade, personal communication, February 1,

2008). Finally, if continued study of utrophin in laryngeal muscle supports a unique expression pattern, it will be important to consider avenues for studying additional DGC components (eg, β-dystroglycan, sarcoglycans) and their expression in the laryngeal musculature.

Murine Model in Laryngeal Study

Over the years, animal models have played a significant role in the study of laryngeal structure and function. Rodent (ie, rat) models have helped to elucidate the myosin heavy chain composition and metabolic features of the laryngeal muscles.[15, 22, 35, 83, 156] Feline and primate models have increased the field's understanding of the neural aspects of laryngeal function.[82, 150, 252-255] Finally, canine models have permitted the study of laryngeal biomechanics and the testing of promising laryngeal surgeries.[133, 242, 256-262] However, it has not been until recently that the mouse model has been considered for its potential contributions to laryngeal study.[4, 25] Recent studies examining laryngeal muscle response to dystrophin deficiency and the current study's anatomical description of the murine larynx have opened the door for a broader use of the murine model in the study of laryngeal response to disease.

Because of its amenability to genetic engineering, the mouse has long been recognized as the premiere model for the study of human disease.[263] At present, mouse models of hundreds of diseases and conditions are available.[263] These models offer researchers a tool for further examining disease processes and a mechanism for testing potential therapeutic interventions. The introduction of the mouse model for laryngeal study will permit researchers an improved understanding of this unique organ and its response to a number of diseases.

The opportunities for study in this area are extensive. A number of disease processes (eg, myasthenia gravis, Parkinson's disease) produce highly characteristic voice and swallowing symptoms. At present, the precise neuromuscular mechanism(s) by which some diseases bring about early and distinctive voice and swallowing changes is unclear. Transgenic study using the mouse model will allow the field to better appreciate the laryngeal response to these diseases. In addition, a number of conditions have yet to be considered for their effect on laryngeal function. As rates of chronic disease continue

to rise, it will be important that the field understands potential changes in laryngeal structure and function with disease.

Clinical Implications

Voice and Swallowing Concerns Associated with DMD

Discussion of speech and voice changes in DMD has been limited; however, a 1961 study by Mullendore and Stoudt reported the presence of a "dystrophic voice" in children with DMD. The exact nature of the voice concerns was not discussed. The results of this study suggest that voice abnormalities such as those reported in the above study are likely secondary to a compromised respiratory support system rather than to laryngeal dysfunction. With the completion of this study, all intrinsic laryngeal muscles have been examined for their response to dystrophin deficiency, and all have shown protection against the disease. In contrast, the respiratory musculature required to support voice production is severely affected by DMD. Consequently, it can be assumed that vocal difficulties associated with DMD rest outside the laryngeal complex and most likely correspond with deterioration of the respiratory musculature.

Similarly, oropharyngeal and esophageal dysphagia has been reported in individuals with DMD.[264-266] However, the results of this study would point to an etiology of swallowing impairment external to the larynx. A review of research in the field supports this assertion. Typical swallowing concerns in DMD involve oral, pharyngeal, and esophageal concerns and include: increased oral phase time, difficulty chewing, difficulty in bolus propulsion, reduced pharyngeal contraction, increased pharyngeal residue, heartburn, vomiting, and esophageal constriction.[264-266] These concerns, often presenting in the mid-teens, are commonly attributed to reductions in tongue and pharyngeal constrictor muscle strength and alterations in the musculature of the esophagus.[265, 266] Interestingly, reports of choking and aspiration-related pneumonias are uncommon in this population.[266] These findings again support the fact that swallowing difficulties associated with DMD are not linked to deficits in airway protection at the level of the larynx but to structural and functional changes in other aspects of the digestive tract.

Laryngeal Muscle Physiology and Vocal Rehabilitation

In recent years, management of voice disorders has shifted from a symptomatic to a physiologic approach.[267-269] Certain of the physiologic methods work to restore normal patterns of muscle use via laryngeal exercise.[218, 267] Such methods have been patterned after approaches used in physical therapy for the treatment of conditions involving the limb musculature and assume a similar physiology and training response across muscle groups.[268] The current study adds to the growing base of research demonstrating marked differences between limb and laryngeal muscle and raises questions as to the laryngeal musculature's potential for responding to traditional exercise programs. The continued development of this new generation of voice therapy will rely upon a more thorough understanding of normal laryngeal muscle physiology, its degree of plasticity, and its patterns of remodeling post exercise, areas heretofore unexamined.

In skeletal muscle, muscle remodeling relies upon the activity of satellite cells, generally quiescent mononucleated cells which become active when fiber remodeling is required. Differences in the activity levels of these cells have been demonstrated across muscle groups,[3, 5, 41, 55] and these differences are believed to underlie variability in remodeling capacity across muscles.[55] The laryngeal muscles have recently been identified as one muscle group demonstrating non-typical regenerative and remodeling behavior.[3] As a consequence, remodeling patterns typically observed and expected in limb skeletal muscle following exercise can not be easily generalized to laryngeal muscle. Consequently, as concepts of laryngeal muscle exercise and physiologic therapies emerge, the study of laryngeal muscle remodeling and its capacity for plastic change with exercise will be essential. This line of research has the potential to offer biological support to current treatment methods or guide the field toward alternative approaches to vocal rehabilitation.

Mechanisms of Vocal Aging

Age-related changes in voice are well documented.[270-277] It is believed that changes in vocal pitch, loudness, and quality seen in the elderly correspond with age-related alterations within the vocal fold and across the laryngeal mechanism at large.[81, 128, 130, 278-290] At present, however, molecular mechanisms underlying laryngeal remodeling with age are not clearly defined. A review of the aging literature suggests that

much of the research has focused on age-related modifications of the vocal fold mucosa.[278-280, 287, 289] While recent years have brought an interest in vocal fold muscle (ie, TA muscle) aging,[24, 81, 83, 130, 282, 284] minimal attention has been given to other intrinsic laryngeal muscles. Consequently, the examination of intrinsic laryngeal muscle remodeling with age emerges as a priority area of research.

Certainly, aging of various biological systems is best understood in the context of normal anatomy and physiology. The current study offers an improved understanding of the normal biology of 2 rarely studied intrinsic laryngeal muscles. As information from this and other studies like it emerges, the foundation will be laid for the study of age-related change in the laryngeal musculature and its possible contribution to vocal aging.

Limitations

Methods used in this study were consistent with currently accepted protocols for examining sparing in dystrophin deficiency; however, some procedural limitations were observed.

Muscle Sections

Laryngeal muscle sections were collected in a manner that permitted all 3 muscles of interest (ie, PCA, SCA, CT) to be obtained from a single larynx. Whole larynges were mounted in OCT for cryosectioning. Larynges were initially approached from the lateral aspect. Ten-µm thick cryosections of the CT (sagittal plane) were obtained until the muscle's most medial aspect was observed. Sectioning continued along this plane until most proximal SCA was fully sectioned and the distal SCA was observed. The larynx was then re-mounted with OCT and approached from the subglottic aspect. Ten-µm thick transverse sections were taken through the extent of the PCA. The above approach allowed the acquisition of all muscles from a single organ and reduced by half the number of animals required for the study.

The above method permitted clear cross sections of the PCA. However, because of the angled nature of the SCA, early sections of the muscle obtained near the muscle's attachment to the posterior arytenoid were not in strict cross section; later sections of the SCA obtained near the muscle's insertion with the cricoid cartilage were in well-defined cross section. Finally, fibers of the CT changed angle from the muscle's posterior to anterior aspect, resulting a portion of fibers being cut at an angle and a portion being cut

in strict cross section. Certain of the histologic tests used in this study require (eg, counting of central nuclei) that fibers be viewed in cross section. The angled nature of the SCA and CT within the larynx limited the number of strict cross-sections which could be obtained. As a result, judgment of central nucleation may have been limited by the muscle view presented. High rates of interrater reliability do, however, suggest that the views examined were sufficient to permit accurate judgment of nuclei position.

Finally, the cutting of larynges as described in the above paragraphs resulted in only a small section of the PCA remaining for sectioning after acquisition of the SCA. As PCA response to dystrophin deficiency had been documented in two previous studies, including one in this laboratory, the decision was made to ensure adequate sampling of the CT and SCA and accept smaller samples/sections from the PCA. It was concluded, however, that samples of PCA were adequate to permit examination of the muscle's overall morphology, percentage of centrally positioned nuclei, sarcolemmal integrity, and protein presence and distribution.

Utrophin Antibodies

Utrophin upregulation was examined in this study as a potential mechanism of laryngeal muscle sparing. Utrophin is a large, cytoskeletal protein with notable similarity to dystrophin.[200] The protein presents along the perimeter of immature or regenerating muscles fibers and at the NMJ of mature fibers.[202]

During the completion of this study, the investigator used both polyclonal and monoclonal antibodies against utrophin. Unfortunately, within the monoclonal category, only mouse monoclonal antibodies were available. The use of mouse antibodies on mouse tissue was considered problematic, as using primary antibodies generated from a species which is closely related, phylogenetically, to the species of study often yields an altered reaction to the antibody.[193] To deal with these concerns, the investigator used a special blocking solution (BEAT Blocking Solution; Invitrogen, Carlsbad, CA) prior to the application of the monoclonal antibody. Despite the incorporation of the blocking step, some images contained higher than preferred levels of non-specific binding. To better deal with this concern, primary and secondary antibodies were prepared in a 10% PBS/Tris/goat serum base. The additional goat serum reduced non-specific binding and offered a more clear appreciation of the protein's distribution.

Concerns also arose with the goat polyclonal antibody against utrophin. By the nature of their formation, polyclonal antibodies are less specific than their monoclonal counterparts. Consequently, staining with polyclonal antibodies often yields high levels of non-specific binding and significant background staining. In the current study, the polyclonal antibody against utrophin showed strong staining along the perimeter of the muscle fiber in control tissue, in a pattern similar to that observed with dystrophin. Perimeter staining was markedly reduced in *mdx* muscle. Such patterns were present in both leg and laryngeal muscle. These findings pointed to the antibody's cross-labeling of dystrophin and its lack of acceptable levels of specificity for the target protein.

Hence the study suggests that currently available antibodies against utrophin are not adequate to permit highly specific staining of the protein in murine models. As utrophin has been identified as a replacement for dystrophin in some muscles affected by dystrophin deficiency, the identification of a high quality antibody against utrophin produced in an animal other than the mouse will be an important step in the continued study of the protein's role in neuromuscular disease processes.

Use of the Animal Model

The *mdx* mouse is recognized as the standard animal model for the investigation of dystrophin deficiency; however, differences do exist in the between mouse and human models. Whereas the genetic mutation and pathophysiology of the disease is comparable in the two models, mice demonstrate a milder phenotype, fewer functional deficits, and a near normal lifespan.[177] Consequently, some may argue that the gold standard for the investigation of the effects of DMD on specific muscle groups would be the human model.

Unfortunately, at present, the delicate nature of the larynx prohibits study of the disease's effects in the human organ. The intrinsic laryngeal muscles are small, delicate muscles positioned within and around a cartilaginous framework. Biopsy of the muscles is challenging for several reasons. First, because of their location within the laryngeal complex, the muscles are difficult to access for biopsy. Further, certain of the muscles (eg, bilateral TA muscles) are part of the layered vocal fold structure and, thereby, one aspect of the complex waveform achieved during vocal fold vibration. Penetration of the

delicate superficial layers of the vocal fold during biopsy may result in vocal fold scarring and subsequent limitations in the vibratory waveform required for voice.

Thus, until scientific advances permit the biopsy of human laryngeal muscles, the mouse model of dystrophin deficiency remains the model of choice for the study of this muscle group. Readers of the research should be aware of limitations in the use of the model, including differences in disease severity across models, differences in laryngeal anatomy across models, and potential differences in muscle specialization across models brought about by differing functional requirements for the system.

Concluding Remarks

The laryngeal muscles offer significant contributions to breathing, swallowing, and voicing. In recent years, a wealth of research has emerged demonstrating the sophisticated nature of these muscles and giving them a distinctive standing among the wider group of skeletal muscles.

However, select muscles within the intrinsic laryngeal muscle group have received minimal attention in physiology circles. Consequently, little is known about these muscles, their level of specialization, and their behavior relative to other laryngeal muscles. Because the laryngeal muscles work together in a delicate balance to perform the above activities, it is imperative that the biologic properties of all laryngeal muscles be appreciated. This study addressed this void in laryngeal muscle biology literature and examined the biologic properties of 2 rarely studied muscles: the IA and the CT.

Findings of the study demonstrate that the IA and CT, like other laryngeal muscles, possess a refined mechanism of sarcolemmal management which permits them to resist the effects of dystrophin deficiency. Considered in light of previous research on the IA and CT, the findings lead the author to propose these muscles as “blended” in nature, sharing the properties of highly specialized craniofacial muscles and the properties of prototypical limb muscle. The realization of this “blended” phenotype within the larynx indicates that the laryngeal muscles are a biologically diverse set of muscles uniquely equipped to perform both life-sustaining and life-enhancing functions.

As the basic sciences move to further define the features of individual laryngeal muscles, the translation of findings to the clinical arena must remain a priority. Indeed, millions suffer each year from voice conditions, a number of which include a muscular

component.[214, 216, 291-293] Clinicians working to enhance the laryngeal function of these individuals rely upon physiologically sound methods to reverse or retard pathology. As interest in laryngeal muscle biology grows, the foundation for physiologic voice therapies is being laid. However, additional work will be required and will emerge with continued discussion between the clinical and basic sciences.

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PROFESSIONAL PUBLICATIONS

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