

ABSTRACT OF DISSERTATION

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The Graduate School
University of Kentucky

2008

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

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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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Lexington, Kentucky

Co-Directors: Dr. Joseph C. Stemple, Professor of Communication Sciences and
Disorders
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

Lisa Beth Thomas

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TABLE OF CONTENTS

Acknowledgments.....	iii
List of Tables	viii
List of Figures.....	ix
List of Files	x
Chapter One: Introduction	1
Chapter Two: Review of the Literature	
Introduction	5
Craniofacial Musculature	5
Laryngeal Musculature.....	6
Overview of the Intrinsic Laryngeal Muscles.....	7
Intrinsic Laryngeal Muscles in Voice Production.....	8
Intrinsic Laryngeal Muscle Deviation from Limb Skeletal Muscle.....	9
Morphogenesis.....	9
Background	9
General Craniofacial Muscle Development	10
Tongue and Laryngeal Muscle Development	10
Limb Muscle Development	11
Summary	11
Motor Innervation.....	11
Sensory Mechanisms	12
Histologic and Immunocytochemical Studies of Laryngeal Proprioception.....	12
Clinical Studies of Laryngeal Proprioception	13
Summary of Laryngeal Proprioception	14
Fiber Size.....	14
Contractile Properties	15
Myosin Isoforms.....	15
Laryngeal Myosin Expression.....	15
Metabolism.....	17
Regenerative Capacity and Ability to Recover Post Insult.....	18
Patterns of Aging	20
Summary of Laryngeal Muscle Specializations	21
Diversity with the Intrinsic Laryngeal Musculature	21
The Interarytenoid.....	22
Morphogenesis.....	22
Function	22
EMG Studies	22
In Vivo Studies.....	24

In Vitro Studies	24
Summary of Function Studies	25
Innervation	25
Sensory Mechanisms	25
Contractile Properties	27
Summary of Interarytenoid Literature: Strengths, Limitations, and Future Directions	27
The Cricothyroid	28
Morphogenesis	29
Function	29
EMG Studies	29
In Vivo Modeling	30
In Vitro Modeling	32
Summary of CT Function Studies	32
Innervation	33
Sensory Mechanisms	33
Histological Studies	33
Clinical Studies	33
Contractile Properties	34
Myosin Isoform Profile	34
Fiber Size and Arrangement	35
Sensitivity to Disease	36
Summary of CT Literature: Strengths, Limitations, and Future Directions	37
Summary of IA and CT Muscles	38
The Model	38
The Rodent Larynx	38
The Rat Larynx: Gross Anatomy and Myology	39
The Mouse Larynx	40
Rodent Larynx: Summary	40
History and Features of the <i>mdx</i> Mouse	40
Pathophysiology of DMD	41
The <i>mdx</i> Strain	42
Assays Used in the Study of Dystrophin Deficiency	42
Histologic Assays	43
Histologic Staining	43
Vital Dyes	43
Immunocytochemical Assays	44
Background	44
Monoclonal Antibodies	44
Polyclonal Antibodies	45
Species Selection with Primary Antibodies	45
Secondary Antibodies	45
Fluorescence Microscopy	46
Immunocytochemistry in the Study of Dystrophin Deficiency	46

Summary.....	46
Previous Model Use in the Laryngeal Muscles.....	47
Potential Mechanisms of Laryngeal Muscle Sparing	47
Muscle Fiber Types and Utrophin Expression	47
Reduced Mechanical Strain.....	49
Sarcoplasmic Reticulum Development and Mechanisms of Calcium Homeostasis.....	50
Regenerative Capacity.....	50
Summary.....	51
Translational Extensions from Use of the <i>mdx</i> Mouse	51
Purpose Statement	53
Hypotheses	53
 Chapter Three: Methodology	
Animals.....	56
Preliminary Investigation of Mouse Laryngeal Anatomy	56
Primary Investigation	57
Histology and Immunocytochemistry	58
Morphology	58
Sarcolemmal Integrity.....	59
Immunocytochemistry	59
Data Analysis	61
Overall Morphology	61
Central Nucleation	61
Sarcolemmal Integrity.....	63
Immunocytochemistry	63
 Chapter Four: Results	
Preliminary Investigation	64
Framework	64
Musculature.....	65
Additional Observations.....	66
Primary Investigation	66
Histology.....	66
Overall Morphology	66
Central Nucleation.....	66
Inter-rater Reliability.....	67
Sarcolemmal Integrity	67
Immunocytochemistry.....	68
Dystrophin	68
Utrophin	68
Results in Relation to Hypotheses.....	69
 Chapter Five: Discussion	
The Superior Cricoarytenoid (SCA).....	91
Implications Regarding the Nature of the SCA	92

Ambiguity of “Classic” vs. “Specialized” Muscle	92
Generalization across Species.....	93
The Cricothyroid (CT).....	93
Implications Regarding the Nature of the CT	95
Mechanisms of Sparing and Protection	97
Utrophin Upregulation	97
Utrophin Upregulation in Affected Muscles	98
Utrophin Expression in Spared and Protected Muscle Groups.....	98
Utrophin Complexity and Implications for Study	99
Future Studies of Utrophin in Laryngeal Muscles.....	100
Allotype-Based Perspectives on Sparing	100
Embryological Links to Sparing	101
Summary Remarks on Sparing.....	101
Implications	102
Laryngeal Muscle Diversity and Implications for Laryngeal Function ...	102
Dystrophin-Glycoprotein Complex of Laryngeal Muscles.....	103
Murine Model in Laryngeal Study	104
Clinical Implications	105
Voice and Swallowing Concerns Associated with Duchenne	
Muscular Dystrophy	105
Laryngeal Muscle Physiology and Vocal Rehabilitation	106
Mechanisms of Vocal Aging	106
Limitations.....	107
Muscle Sections	107
Utrophin Antibodies.....	108
Use of the Animal Model.....	109
Concluding Remarks	110
References.....	112
Vita.....	129

LIST OF TABLES

Table 4.1, Percentage Centrally Nucleated Fibers by Muscle	71
Table 4.2, Wilcoxon Rank Sum Statistics for Control- <i>mdx</i> Muscle Comparisons.....	72

LIST OF FIGURES

Figure 2.1, Model of the Dystrophin-Glycoprotein Complex	55
Figure 4.1, Hematoxylin and Eosin Staining of Mouse Larynx Viewed from Superior Aspect.....
Figure 4.2, Hematoxylin and Eosin staining of Mouse Larynx Viewed from Superior Aspect.....
Figure 4.3, Superior View of C57BL Mouse Larynx
Figure 4.4, Hematoxylin and Eosin Staining of Mouse Larynx Viewed along a Sagittal Cut.....
Figure 4.5, Posterior View of Laryngeal Inlet
Figure 4.6, Mid-sagittal Image of C57 Mouse Larynx
Figure 4.7, Sagittal View of C57BL Mouse Larynx.....
Figure 4.8, Anterior Larynx Viewed from Sagittal Cut.....
Figure 4.9, Posterior View of Larynx Showing Bilateral Arytenoid Cartilages
Figure 4.10, Mid-sagittal Image of SCA
Figure 4.11, Hematoxylin and Eosin Staining of Mouse Larynx Viewed from Sagittal Cut
Figure 4.12, Hematoxylin and Eosin Staining of Gastrocnemius Muscles
Figure 4.13, Hematoxylin and Eosin Staining of Control and <i>mdx</i> Laryngeal Muscles
Figure 4.14, Central Nuclei Counts for Control and <i>mdx</i> Muscles of the Hindlimb and Larynx
Figure 4.15, Results of Evans Blue Dye Testing.....
Figure 4.16, Dystrophin Distribution.....
Figure 4.17, Utrophin (Polyclonal) Distribution.....
Figure 4.18, Utrophin (Monoclonal) Distribution

LIST OF FILES

1.	Figure4_1	tiff	977 KB
2.	Figure4_2	tiff	977 KB
3.	Figure4_3	tiff	1.31 MB
4.	Figure4_4	tiff	999 KB
5.	Figure4_5	tiff	886 KB
6.	Figure4_6	tiff	818 KB
7.	Figure4_7	tiff	897 KB
8.	Figure4_8	tiff	597 KB
9.	Figure4_9	tiff	548 KB
10.	Figure4_10	tiff	295 KB
11.	Figure4_11	tiff	998 KB
12.	Figure4_12	jpeg	2.34 MB
13.	Figure4_13	jpeg	4.87 MB
14.	Figure4_15	jpeg	1.58 MB
15.	Figure4_16	jpeg	2.71 MB
16.	Figure4_17	jpeg	2.42 MB
17.	Figure4_18	jpeg	2.41 MB

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- μ m-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- μ m-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 group¹ 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 manipulations¹² 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 from 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 al¹⁴ 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 Jennekens⁸¹ 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- μ m 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 al¹⁹ examined the motor and sensory innervation of 4 intrinsic laryngeal muscles in humans: TA, PCA, LCA, and CT. The group incubated 60- μ m 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 al⁸² 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 al¹⁶ 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 al⁵⁰ examined the size of human CT, PCA, LCA, and vocalis muscles and reported an average diameter of 40-50µm. Rodeno

et al⁸⁴ found similar results in their examination of the TA and PCA muscles. In their study, TA diameter ranged from 38 μ m to 39 μ m, while PCA fibers ranged from 43 μ m to 47 μ m, pending fiber type. Finally, Perie et al¹⁹ reported slightly smaller fibers than the above authors, with mean diameters of 20-35 μ m for the TA, LCA, PCA, and CT. These fiber size estimations suggest that laryngeal muscles are small relative to limb muscle (35-75 μ m)⁷² but comparable to that of extraocular muscle (20-50 μ m).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 isoforms⁸⁹; 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 al⁹⁷ 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 Dulhunty¹⁰⁰ 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 al⁴¹ 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 al³

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 Shinnars et al⁶ 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 endurance¹²⁶ 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 II⁸¹ 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 al¹⁴⁰ 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, Hillel³¹ 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 these studies, Nasri et al¹³⁸ 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 al¹³⁹ 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 al¹³⁷ 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 act 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 al⁷⁷ 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 al³² 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.⁹ The IA's MyHC composition was most similar to that of the LCA, a partner vocal fold adductor. Tellis et al³² 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

⁹ 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 al³² 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 Leden¹⁴³ 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 colleagues¹⁴⁰ 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. Hillel³¹ 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 al¹⁴⁴ 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 al¹⁴⁵ 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 colleagues¹⁴² 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 al¹⁴⁶ 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 al¹⁴⁸ 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 al⁷⁹ 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 al¹⁶ 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 colleagues⁹⁴ 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 Hoh³⁵ 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 al¹⁷ 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 Flint²² 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 al²³ 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 al⁹⁶ 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 al⁵⁰ 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 (60 μ m). 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 (38.19 μ m to 43.25 μ m) were found in the CT, whereas the smallest fibers (29.38 μ m to 32.05 μ m) 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 15-30 μ m.^{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 al¹³⁵ 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 al⁴ 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 family¹⁵⁹⁻¹⁶¹ 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 Stromberg¹⁵⁹ 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 al¹⁶⁰ 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 colleagues²⁵ identified TA and PCA muscles in the mouse, while Marques et al⁴ 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 al¹⁷⁸ 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 fluorescence 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 fluorescent 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 by 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 fluorescent label to the primary antibody and indirect methods which apply a fluorescent secondary antibody against the primary antibody. Fluorescence 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

