The Plasticity of Denervated and Reinnervated Laryngeal Muscle

Focus on Single-Fiber Myosin Heavy-Chain Isoform Expression

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No studies have examined the effects of denervation on the single-fiber distribution of myosin heavy-chain (MyHC) isoforms in laryngeal muscle. The fast type IIB MyHC isoform represents the largest proportion of the myosin pool in the posterior cricoarytenoid (PCA) and the thyroarytenoid (TA) muscles. However, the fast type IIB MyHC isoform is distributed differently at the single-fiber level. Hence, we hypothesized that denervation would result in markedly different patterns of MyHC isoform expression at the single-fiber level. To test this hypothesis, we assigned animals to the following 3 groups: (1) control group; (2) denervation group; or (3) reinnervation group. Animals were killed 7, 14, 30, 90, and 180 days after denervation or reinnervation. Subsequently, the distribution of MyHC isoforms were electrophoretically determined in approximately 7200 single fibers. There were 4 key findings to emerge from this study: (1) The MyHC isoform profile of the PCA muscle, at both the whole-muscle and single-fiber level, is more malleable than that of the TA muscle. (2) In the PCA and TA muscles, denervation produced some similar changes, resulting in a large increase in the pool of fibers coexpressing fast type IIX and IIB MyHC isoforms. (3) Reinnervation of the TA muscle produced significant alterations in the single-fiber distribution of MyHC isoforms while having little effect on the whole-muscle MyHC isoform composition. (4) Since the transitions in MyHC isoform expression associated with denervation were limited primarily to fast type IIB to fast type IIX, we postulate that only minor reductions in muscle function would result (as defined by maximum shortening velocity and the force-velocity relationship).

Over the past 30 years, many studies have examined the roles of various factors in regulating the phenotypic properties of skeletal muscle.1-3 Most of these studies, however, have been performed primarily on the hindlimb musculature of various mammalian species. In contrast, much less is known about the cellular and molecular events that determine the phenotypic properties of more specialized muscles such as the extraocular and laryngeal muscles. This is somewhat surprising given the importance of these muscles in performing tasks such as eye movement, phonation, swallowing, and breathing.

Within this context, several groups of investigators4-12 have recently focused on the effects of altered physiologic states on the cellular and molecular systems of laryngeal muscle. Most of the interest has focused on myosin because of its central role in the contractile process and because different isoforms of myosin give rise to variations in contractile properties (eg, fast twitch, slow twitch) and fiber types. A native myosin molecule consists of 2 heavy chains, 2 essential light chains, and 2 regulatory light chains. The myosin heavy chain (MyHC) contains 2 key domains, the tail and the globular head, and it is the globu-
lar head that acts as a molecular motor. Currently, 5 different MyHC isoforms have been identified in rodent laryngeal muscle.4-12 These have been classified as slow type I, fast type IIA, fast type IIX, fast type IIB, and fast type IIL. The relative proportion of a given MyHC (eg, fast type IIB) in laryngeal muscle has been shown to be muscle and species dependent.6,7,11 Additionally, the fast type IIL MyHC isoform appears to be specific to muscles like the laryngeal and extraocular muscles, and it is not found in the rodent hindlimb musculature.

It is well recognized that the MyHC isoform compositions of some rodent hindlimb muscles (eg, the soleus) are highly malleable.13 In this context, our group has used 3 different approaches to explore mechanisms regulating the MyHC isoform composition of rodent laryngeal muscle. The first approach involved examining the MyHC isoform composition of single fibers in the posterior cricoarytenoid (PCA) and the thyroarytenoid (TA) muscles.10 The findings from this earlier study demonstrated that (1) there is a significant degree of MyHC isoform coexpression (ie, polymorphism) in single fibers of the rodent PCA and TA muscles; (2) the fast type IIB MyHC isoform represents about 60% of the total MyHC pool in both the PCA and TA muscles of rats; and (3) the distribution of the fast type IIB MyHC isoform, at the single-fiber level, is different in the different muscles. In the PCA muscle, the fast type IIB MyHC appears to be primarily expressed in IIB and IIX/IIB fibers (ie, fibers coexpressing MyHC isoforms). In the TA muscle, the fast type IIB MyHC isoform is found largely in IIB/IIL fibers and to a lesser extent in IIX/IIB/IIL fibers.

The second approach involved manipulating the MyHC isoform composition of rodent laryngeal muscle by inducing states of hypothyroidism and hyperthyroidism.10 This strategy was used because previous studies had shown that the MyHC isoform compositions of some rodent hindlimb muscles are dependent on thyroid hormone status.13,14 The findings suggest that the MyHC isoform composition of the PCA muscle is more sensitive to the thyroid state than is that of the TA muscle.

The third approach represents the basis of the present study and focuses on the effects of denervation and reinnervation on the MyHC isoform profile of the PCA and TA muscles. Studies examining the effects of denervation on the MyHC isoform expression in fast rodent hindlimb muscles have shown that denervation downregulates the expression of the fast type IIB MyHC isoform and concomitantly up-regulates the expression of the fast type IIX MyHC isoform.15-17 Shiotani and Flint6 and Shiotani et al8 examined the effects of denervation on laryngeal muscles, and their findings are consistent with those from the hindlimb studies. It should be noted, however, that these investigators were unable to accurately determine the effect of denervation on the expression of the fast type IIX MyHC isoform because in their study, the fast type IIX MyHC isoform appeared to electrophoretically comigrate with the fast type IIA MyHC isoform. Additionally, an accurate understanding of the effects of denervation on MyHC isoform expression can only be obtained by performing single-fiber analyses given (1) that the whole-muscle MyHC isoform profile is determined by the single-fiber distribution of MyHC isoforms and (2) the high degree of MyHC isoform coexpression found in the single fibers of laryngeal muscles.

The present study had 3 primary objectives. The first was to test the hypothesis that denervation produces in rodent laryngeal muscle a fast type IIB to fast type IIX MyHC isoform transition as observed in rodent hindlimb studies. The second objective was to characterize the effects of denervation on the single-fiber MyHC isoform composition. We were especially interested in determining if denervation produced a fast type IIB to fast type IIX MyHC isoform transition that resulted from an increase in the proportion of fibers that only expressed the fast type IIX MyHC isoform or if there was an increase in the proportion of fibers coexpressing both the fast type IIX and IIB MyHC isoforms. In previous studies our group observed fast type IIB to fast type IIX MyHC isoform transitions that produced significant proportions of fibers coexpressing both isoforms. Hence, we hypothesized that denervation would also produce an increase in the proportion of fast IIX/IIB hybrid fibers. Finally, our third objective was to determine the extent to which MyHC isoform transitions could be mitigated by reinnervation. We hypothesized that reinnervation would only be partially effective in preventing or reversing MyHC isoform transitions.

METHODS

ANIMAL CARE AND WELFARE

Approval was obtained from our institutional review board prior to conducting this study. Female Sprague-Dawley rats (weight, 250-300 g) were randomly assigned to 1 of 3 groups: (1) control; (2) denervated; or (3) reinnervated. Animals were humanely killed 7, 14, 30, 90, and 180 days after denervation or reinnervation. Six animals were assigned to each group and time point. Denervation of the PCA and TA muscles was surgically produced by removing about 1 cm of the recurrent laryngeal nerve at the level of the fourth tracheal ring. Reinnervation was performed by cutting the recurrent laryngeal nerve at the fourth tracheal ring and then reanastomosing the nerve using 10-0 nylon suture (Ethicon Inc, Piscataway, NJ). These techniques were similar to those reported by Marie et al.16,17

The PCA and TA muscles were harvested at the time points noted above. This was accomplished by killing each animal with an overdose of sodium pentobarbital and then removing the larynx. The PCA and TA muscles were then extirpated using a dissecting microscope, blotted dry, weighed, and then frozen using isopentane cooled by liquid nitrogen.

SINGLE-FIBER ANALYSIS

Approximately 40 single muscle fibers were randomly microdissected from each muscle sample using a dissection microscope (Ams Jena Technival microscope; Southland Instruments, Fountain Valley, Calif; original magnification ×25 to ×30) and fine Dumont tweezers. Hence, we performed MyHC isoform analyses on about 7200 single fibers. The remainder of the muscle was used for whole-muscle analyses.

SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS FOR MYHC ANALYSIS

As described previously,3,12,14 isolated single muscle fibers were placed into 30 µL of sample buffer containing 5%
**Figure 1.** Time-course changes in muscle weight for the posterior cricoarytenoid (PCA) and the thyroarytenoid (TA) muscles. Note that denervation produced a significant decrease in muscle weight at the initial time points. There was a significant recovery in muscle weight between 90 and 180 days after denervation for both the PCA and TA muscles. This may be related to the reappearance of synaptic vesicles between 90 and 180 days after denervation. The muscle weights of the reinnervated muscles were similar to those of the control muscles for most of the time points. The 2-way analysis of variance for the PCA muscle weight data demonstrated significant main effects for the group (P<.001) and the group-time interaction (P<.001). For the TA muscle, the main effects were time (P<.05), group (P<.001), and the interaction of time and group (P<.001). Abbreviations: a, significant difference from the control group; b, significant difference from the reinnervation group; c, significant difference from both the control and reinnervated groups.

**RESULTS**

**MUSCLE WEIGHT AND MUSCLE-FIBER DIAMETER**

The mean muscle weights of the 3 groups are shown in Figure 1. Note that the denervated PCA muscle weights were consistently lower than those of either the control or reinnervated PCA muscles up to 90 days after denervation. By 180 days, however, the muscle weight of the denervated PCA group was similar to that of both the control and reinnervated PCA muscle groups. Consistent with this observation, we also noted a reduction in muscle-fiber diameter in the denervated PCA muscle (P<.01; Figure 2). The muscle weights of the denervated TA muscles were lower than those of the control PCA muscles at each time point except at 180 days. Muscle-fiber diameter followed a similar trend. The recovery of muscle masses and fiber diameters of the denervated PCA and reinnervated groups was similar to that of the control muscles for most of the time points. The recovery of muscle weight between 90 and 180 days after denervation for both the PCA and TA muscles was not significantly different from the reinnervation group; c, significant difference from both the control and reinnervated groups.

**IMMUNOCYTOCHEMICAL CHARACTERIZATION OF INNERVATION STATE**

The presence of innervation state for each sample was determined by isolating single fibers or bundles of fibers and then detecting the presence of SV2, which is a transmembrane protein found in synaptic vesicles. Its absence was interpreted to reflect the lack of synaptic vesicles and innervation. The SV2 protein is currently thought to play a key role in synaptic transmission mediated by the calcium ion. Once the fiber or bundle of fibers was isolated, it was placed into a 4% paraformaldehyde solution for 10 minutes. The presence of SV2 was determined using a monoclonal antibody (MAB) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) specific for this synaptic vesicle transmembrane transporter. The presence of acetylcholine receptors was detected using a rhodamine-labeled o-bungarotoxin (Molecular Probes, Eugene, Ore). The number of fibers expressing SV2 on each slide was determined and expressed as a percentage of the total fibers.

An investigator who was blinded to experimental conditions performed all counts. Typically, 40 to 50 fibers per specimen were examined. In some instances, the fiber itself was labeled using phalloidin conjugated with fluorescein isothiocyanate (Molecular Probes). The fibers were then scanned using a BioRad MRC-1024 UV Laser Scanning Confocal Microscope (Hercules, Calif). Three-dimensional rendering was performed using Advanced Visualization Systems software, version 4.1 (Waltham, Mass).

**STATISTICAL ANALYSES**

Whole-muscle analyses for a given MyHC isoform were performed using a 2-way analysis of variance. The 2 factors were innervation state (ie, control, denervated, and reinnervated) and time (ie, 7, 14, 30, 90, and 180 days). If a significant F ratio was obtained, then supplemental analyses (Tukey honestly significant difference test) were used to determine which groups were different from one another. Differences between the single-fiber distributions of the 3 groups were determined using χ² analyses. Only those χ² test results that were statistically significant are reported herein. All statistical analyses were performed using the Systat computer program (Systat, Evanston, Ill), and findings were considered significant at P<.05.
TA muscles may be related to reinnervation that occurred between 90 and 180 days.

IMMUNOCYTOCHEMICAL DETERMINATION OF INNERVATION STATE

In the present study, we used 2 approaches to determine the innervation state of a muscle fiber. The first of these was an immunocytochemical approach to detect the presence or absence of SV2 and acetylcholine receptors (Figure 3 and Figure 4). The detection of SV2 was interpreted to indicate that a given fiber was innervated. All of the control fibers examined were positive for the presence of both SV2 and acetylcholine receptors. None of the PCA or TA denervated fibers that were examined at 7, 14, 30, and 90 days after denervation exhibited the presence of SV2. However, synaptic vesicles were detected in some (about 25%) of the PCA and TA denervated samples 180 days after denervation (Figure 4B).

The appearance of SV2 at 180 days coincided with a significant recovery of muscle weight and fiber diameter in both the PCA and TA denervated fibers. This observation is consistent with the concept that the presence and absence of key synaptic vesicle proteins can be used as a marker of innervation and denervation, respectively.

One cautionary note, the complete recovery of muscle weight may not reflect a corresponding recovery of muscle fiber cross-sectional area (Figure 2). It is entirely possible that some of the increase in muscle weight at 180 days reflects a proliferation of connective tissue in the denervated group. Clearly, more rigorous analyses of muscle fiber cross-sectional area, connective tissue content, and SV2 localization are needed.

Although it is impossible to ascertain whether the TA is innervated using laryngoscopy, each vocal cord was assessed visually to evaluate whether abduction and/or adduction was present. As expected, none of the denervated TA muscles (regardless of time point) exhibited adduction or abduction. At the later times this might have been due to synkinetic reinnervation, which is thought to result in immobility or at least in failure of abduction and adduction.

With respect to the reinnervated group, SV2 was completely absent from all PCA and TA fibers examined 7 and 14 days after denervation and reanastomosis. Thirty days after reanastomosis, however, synaptic vesicles began to reappear, and by 90 to 180 days, most fibers (>90% of the fibers examined) appeared to be innervated as defined by the presence of SV2 and synaptic vesicles (Figure 4B). As with the denervated TA muscles, however, abduction and adduction were absent in all of the reinnervated TA muscles.

PCA WHOLE-MUSCLE MyHC ISOFORM ANALYSES

The whole-muscle MyHC isoform data for the PCA muscle are shown in Figure 5. The data for the control PCA muscle are qualitatively similar to those reported by our group previously. The predominant MyHC isoform in the control PCA muscles was the fast type IIB isoform, which represented approximately 55% to 60% of the total myosin pool. The fast type IIX MyHC isoform represented the second largest pool of myosin (about 25%-35% of the total myosin pool). There was a slight but statistically significant increase in the relative content of the fast type IIX MyHC isoform with time, increasing from about 25% at 7 days to 35% at 180 days. It should also be noted that the manner in which these changes occurred was manifested in a very complex fashion at the single-fiber level.

Denervation and reinnervation had a profound and rapid effect on the expression of the fast type IIB and IIX MyHC isoforms. Thirty days after denervation, the fast type IIB MyHC isoform represented only 20% of the total myosin pool (down from a control value of about 60%), while the relative proportion of the fast type IIX MyHC isoform increased from a control value of about 25% to about 80% of the total myosin pool. Beyond 30 days of denervation, these rapid changes in the expression of the fast type IIX and IIB MyHC isoforms began to slowly reverse themselves such that there was a partial return to the normal expression pattern.
SINGLE-FIBER ANALYSES OF PCA MyHC ISOFORM EXPRESSION

For simplicity of presentation, only the 30-, 90-, and 180-day data are presented. Additionally, when we refer to a loss or increase in proportion of a specific fiber type, this is not meant to be interpreted as the disappearance or appearance of new fibers. Rather, this is meant to indicate that there was a loss or increase in the proportion of fibers expressing various combinations of MyHC isoforms. In other words, we mean that a given pool that was lost, for example, underwent a change in its phenotype such that the combinations of MyHCs were altered.

There are several key points to be made with respect to the distribution of MyHC isoforms at the single-fiber level in the control PCA muscles. First, at the early time points, there were very few fast type IIB fibers, and most of the fast type IIB MyHC isoform was found in IIX/IX-A fibers. Second, between 90 and 180 days out, many fibers in the denervated groups appeared to be undergoing reinnervation as defined by the presence of synaptic vesicles.
IIB, IIB/IIL, and IIX/IIB/IIL hybrid fibers (Figure 6). Second, the distribution of the fast type IIB MyHC isoform became consolidated at the later time points (eg, 180 days) with the fast type IIB MyHC isoform primarily expressed in fast type IIB and IIX/IIB fibers (Figure 6). These changes produced a profile that was somewhat similar (large increase in the pool of IIX/IIB fibers) to that of the denervated PCA muscle.

At the single-fiber level, the down-regulation of the fast type IIB MyHC isoform in the denervated PCA muscles appeared to occur because of a reduction in the pool of IIX/IIB and IIX/IIB/IIL fibers and a relative reduction in the expression of the fast type IIB MyHC isoform within the IIX/IIB fibers (Figures 6A and B). The large up-regulation of the fast type IIX MyHC isoform occurred primarily as a result of an expansion of the pool of fibers that expressed just the fast type IIX MyHC isoform and fibers coexpressing the fast type IIX and IIB MyHC isoforms.

The MyHC isoform profile of the reinnervated PCA muscles was very similar to that of the denervated PCA muscles at 30 and 90 days (Figure 6A and B). At 180 days, however, there were fewer fast type IIX fibers in the reinnervated group.

**TA WHOLE-MUSCLE MYHC ISOFORM ANALYSES**

The whole-muscle MyHC isoform data for the TA muscle are shown in Figure 7. The MyHC isoform profile of the control TA group is similar to that published by our group previously. The time-course data for the control TA muscle demonstrated small progressive changes in the expression of the fast type IIX and IIB MyHC isoforms over the time course examined. Denervation produced a rapid decrease in the expression of the fast type IIB and IIL MyHC isoforms, and their levels of expression appeared to stabilize between 30 and 90 days after denervation (Figure 7). Concomitantly, there was a significant increase in the expression of the fast type IIX MyHC isoform. In the reinnervated group, there appeared to be a transient effect in the expression of the fast type IIX and IIB MyHC isoforms. However, the MyHC isoform profiles of the control and reinnervated groups were very similar to one another at 180 days (Figure 7).

**SINGLE-FIBER TA MYHC ISOFORM ANALYSES**

The single-fiber data for the TA muscles are shown in Figure 8. The fast type IIB MyHC isoform represented about 60% of the total myosin pool, and most of the fast type IIB MyHC isoform was found in fibers coexpressing the fast type IIB and IIL MyHC isoforms. The expression of the fast type IIL MyHC isoform was confined primarily to these fibers. In contrast, the expression of the fast type IIX MyHC isoform (about 30% of the total pool of myosin) occurred in 3 different pools of fibers (IIX, IIX/IIB, and IIX/IIB/IIL fibers). Overall, the large proportion of fast IIB/IIL fibers represented the hallmark of the innervated state.

Denervation significantly reduced the expression of the fast type IIB and IIL MyHC isoforms and produced a large upregulation of the fast type IIX MyHC isoform (Figure 8A). In the control TA muscles, the IIB/IIL pool of fibers represented 60% of the total population of fibers sampled. Denervation resulted in a complete loss of this pool of fibers and a large increase in the pool of IIX and IIX/IIB fibers 30 days after denervation (Figure 8A). The pool of IIX fibers remained stable for up to 90 days of denervation and then exhibited a significant decrease at 180 days that corresponded to an increase in the IIX/IIB, IIB/IIL, and IIX/IIB/IIL pools of fibers.

The single-fiber data for the reinnervated TA muscles are also shown in Figure 8. Although the whole-muscle MyHC isoform data of the reinnervated TA muscles were similar to those of the control TA muscles, there were substantial differences in the single-fiber distribution of MyHC isoforms at all time points examined. Initially, there was almost a complete loss of the large pool of fast type IIB/IIL fibers that typified the control fibers (Figure 8A).
As shown in Figure 8, there was a gradual recovery of this pool of fibers. However, even at 180 days, this pool of fibers had only recovered to about 33% of that seen in the control muscles (ie, 60% vs about 20% of the total pool of fibers).

**COMMENT**

The present study had 3 key objectives. The first was to clarify the effects of denervation on the expression of the fast type IIX MyHC isoform. Our findings clearly demonstrate that denervation produces a rapid increase in the fast type IIX MyHC isoform in PCA and TA muscles. The second objective was to determine how the fast type IIB to fast type IIX transition was manifested at the single-fiber level. This MyHC isoform transition seems to occur primarily as a result of (1) an increase in the proportion of fibers coexpressing the fast type IIX and IIB MyHC isoforms and (2) an increase in the proportion of fiber expressing only the fast type IIX MyHC isoform. The third objective was to determine the extent to which MyHC isoform transitions could be prevented or reversed by reinnervation. The effects of reinnervation were muscle specific, showing the greatest magnitude in the TA muscle. Herein, we address each of these issues in detail and also explore the functional implications of the MyHC isoform transitions that were observed.
FAST TYPE IIB TO FAST TYPE IIX MyHC ISOFORM TRANSITION RESULTING FROM DENERVATION

Studies have reported that denervation of laryngeal muscles produces a decrease in the relative proportion of the fast type IIB MyHC isoform.6,8 These studies, however, were unable to accurately determine if there was a concomitant increase in the relative proportion of the fast type IIA and/or IIX MyHC isoforms. The findings of the present study demonstrate that denervation primarily affects the relative proportions of the fast type IIX (up-regulated) and type IIB (down-regulated) MyHC isoforms, with little effect on the fast type IIA MyHC isoform.

Since denervation affected the whole-muscle expression of the fast type IIX and IIB MyHC isoforms in both the PCA and TA muscles, it might be asked whether there is a stereotypical pattern of MyHC isoform transitions in denervated rodent laryngeal muscle. In general, the answer appears to be yes, but this issue can only be adequately addressed by performing single-fiber analyses because the whole-muscle MyHC isoform profile really reflects events occurring at the single-fiber level. With respect to the PCA muscle, the fast type IIB MyHC isoform appeared to be primarily distributed among 4 different types of fibers in the control group at the early time points (ie, IIB, IIX/IIb, IIb/IIL, and IIb/IIL fibres; Figure 6A). Denervation initially resulted in a large increase in the proportion of fibers that expressed the fast type IIX MyHC isoform (ie, fast type IIX fibres) and the IIX/IIB isoforms. This appears to have resulted from the complete repression of the IIB/IIb and IIX/Ib/IIL patterns of coexpression in the PCA muscle. In contrast, the large proportion of fast type IIB MyHC isoform found in the control TA muscle appeared primarily in IIB/IIL fibres, and this pool of fibres represented about 60% of the fibers sampled. Fourteen days after denervation, there was almost a complete loss of fibers coexpressing the IIB and IIL MyHC isoforms in the TA muscles. Denervation produced a large increase in the expression of the fast type IIX MyHC isoform by up-regulating the relative proportions of fast type IIX and IIX/IIB fibers in the denervated TA muscles. Therefore, although the single-fiber distributions of MyHC isoforms in the control PCA and TA muscles were different from one another at each of the time points, it should be noted that denervation subsequently produced similar pools of fibers (ie, fast type IIX and IIX/IIB fibers) in both muscles.

Collectively, these findings raise the issue of whether all laryngeal muscles exhibit similar transitions in MyHC isoform expression after denervation. Shiotani and Flint9 examined the effects of denervation on the MyHC isoform composition of the PCA, TA, cricothyroid, lateral cricoarytenoid, and vocalis muscles and observed that the MyHC isoform compositions of the PCA, TA, and lateral cricoarytenoid muscles were the most responsive to denervation. The vocalis and cricothyroid appeared to be relatively insensitive to denervation. Therefore, within the collection of laryngeal muscles studied to date, there appears to be a heterogeneity with respect to the malleability of the MyHC isoform profile as influenced by innervation state. This is an important point, given that the findings of Buller et al22 have been interpreted by some to indicate that the pattern of innervation is the primary determinant of speed-related properties (eg, time-to-peak tension, maximum shortening velocity, and MyHC isoform profile) in skeletal muscle. Clearly, the findings of Shiotani and Flint9 indicate that this is not the case in laryngeal muscle.

The observation that denervation of the PCA and TA muscles leads to the down-regulation of the fast type IIB MyHC isoform and the concomitant increase in the fast type IIX MyHC isoform is similar to that found in some rodent hindlimb fast muscles. For instance, Huey and Bodine10 examined the influence of denervation on MyHC isoform expression in the rodent tibialis anterior muscle and found that denervation led to a large reduction in the expression of the fast type IIB MyHC isoform and a concomitant upregulation of the fast type IIX MyHC isoform. Consistent with these observations, Jakubiec-Puka et al22 also observed that denervation produced an inverse effect (in the tibialis anterior muscle) with respect to the expression of the fast type IIB and IIX MyHC isoforms, with denervation reducing the expression of the fast type IIB MyHC isoform. Collectively, these results demonstrate that denervation seems to produce simi-
lar results in at least some of the hindlimb and laryngeal rodent muscles.

**DENERVATION AND POTENTIAL MECHANISMS FOR THE REGULATION OF THE FAST TYPE IIB MYHC ISOFORM**

Currently, it is not clear what underlying mechanisms are responsible for the phenotypic alterations that occur in the MyHC isoform composition of laryngeal muscle after denervation. Clearly, one of the most profound effects of denervation in rodent laryngeal muscle is the down-regulation of the fast type IIB MyHC isoform. Some investigators have explored the role of so-called muscle regulatory factors (MRFs) in controlling the expression of the fast type IIB MyHC gene. The myogenic regulatory factors (MyoD, myogenin, MRF4, and Myf5) are thought to be necessary for the determination and terminal differentiation of skeletal muscle. Factors MyoD and Myf5 appear to be important for myogenic determination, whereas myogenin and MRF4 are important for terminal differentiation and lineage maintenance. In the rat, there are at least 14 E-box elements throughout the promoter region of the fast type IIB MyHC gene, and results from a cell culture study suggest that MyoD regulates the expression of the fast type IIB MyHC gene in an E-box–dependent fashion. Di Maso et al., however, demonstrated that denervation of the rodent hindlimb musculature led to a reduction in the fast type IIB MyHC protein isoform that was accompanied by a marked up-

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Figure 8. Distribution of myosin heavy-chain (MyHC) isoforms at the single-fiber level in thyroarytenoid muscles 30 (A), 90 (B), and 180 days (C) after denervation or reinnervation. A given fiber type was identified on the basis of its MyHC isoform composition. All of the potential combinations of MyHC isoform expression are shown along the x-axis in each panel. The proportion of each type of fiber is expressed relative to the total pool of fibers. For instance, the IIX/IIB fibers shown in the control group 30 days after denervation (A) represent approximately 25% of the total population of fibers examined. Additionally, the relative content of a given isoform within a given pool of fibers (eg, control IIX/IIB fibers at 30 days) is represented by the relative shading for that isoform. For instance, for the control IIX/IIB fibers at 30 days, the fast type IIB MyHC isoform represents about 60% of the myosin pool in these fibers, while the fast type IIX represents the remainder (ie, about 40%).
regulation of MyoD expression. If MyoD is a primary factor regulating the expression of the fast type IIB MyHC protein isoform, then denervation should have also resulted in a decrease and not an increase in MyoD expression. This ambiguity demonstrates that further studies are needed to develop a sound consensus about the role of MyoD in regulating the fast type IIB MyHC isoform, especially as it may apply to laryngeal muscle.

**EFFECT OF DENERVATION ON THE FAST TYPE III MyHC ISOFORM**

It has been shown that rodent and rabbit laryngeal muscles express an isoform not typically found in the hindlimb musculature. This isoform has been classified by some as the fast type III MyHC isoform.\(^4,5\) As noted by Jung et al,\(^4\) however, the fast type III MyHC isoform was first identified in extraocular muscle and initially classified as extraocular myosin. In the present study, we have simply chosen to refer to this MyHC isoform as the fast type III. to be consistent with prior studies that focused on laryngeal muscle.

Based on the findings of the present study, the PCA muscle appears to express less of the fast type III MyHC isoform than does the TA. This is consistent with the findings of earlier studies.\(^5,6,8,10\) In both the present study and a previous one,\(^10\) our group reported that the fast type III MyHC isoform represented a relatively small proportion of the total MyHC isoform pool (about 15% to 20% in TA and about 5% in PCA). DelGaudio et al\(^4\) reported that the fast type III MyHC isoform represented about 15% to 20% of the total myosin pool in the PCA muscle and about 30% in the TA. Shiotani and Flint\(^6\) found 5% to 10% in PCA and 30% in TA. With respect to the distribution of the fast type III MyHC isoform at the single-fiber level, we have rarely observed a fiber that expressed only the fast type III MyHC isoform. It appears that the type III MyHC isoform is typically found in IIB/IIL and IIX/IIB/IIL fibers in rodent laryngeal muscle.

As shown in the TA muscle (Figure 8), denervation apparently suppresses the expression of the type IIB MyHC isoform such that IIB/IIL fibers are transformed into (1) IIX fibers, (2) IIB fibers, and/or (3) IIX/IIB fibers. Currently, it is not clear which of these pools of fibers is derived from the large proportion of IIB/IIL fibers (approximately 60% of the total population of fibers sampled) observed in the control TA muscles at the early time points. It is tempting to suggest that the relatively large proportion of IIX/IIB fibers observed at 14 days (about 30% of the total population) and 30 days (about 40% of the total population) were directly derived from the IIB/IIL pool of fibers; however, further studies will be needed to more precisely understand the transitional sequences that occur in laryngeal muscle.

**EFFECT OF REINNERVATION IN MAINTAINING OR REESTABLISHING A NORMAL MyHC ISOFORM PROFILE**

As shown in Figure 6, denervation produced large shifts in the expression of the fast type IIX (ie, up-regulated) and IIB (ie, down-regulated) MyHC isoforms in the PCA muscle. Interestingly, reinnervation of the PCA muscle had little effect on reversing these shifts at the whole-muscle level. In contrast, reinnervation of the TA muscle was highly effective in restoring a normal pattern of MyHC isoform expression at the whole-muscle level. For instance, at 180 days, the relative proportions of the fast type IIX and IIB isoforms in the reinnervated TA muscle were virtually identical to those of the control group. Consistent with these observations, Shiotani et al\(^8\) recently reported that repair of the recurrent laryngeal nerve (after ligation) did not prevent denervation-like shifts in the MyHC isoform profile of the PCA muscle but was effective in minimizing shifts in the MyHC isoform profile of the TA muscle.

Collectively, these findings might be interpreted in 2 ways. First, it could be argued that reinnervation is simply more effective in the TA muscle. For instance, a greater number of TA than PCA muscle fibers might be reinnervated. Alternatively, reinnervation might be better directed (ie, reinnervation by motor neurons vs autonomic neurons) in the TA than in the PCA muscle. In fact, the temporal reappearance of SV2 vesicles was similar for the PCA and TA muscles. Additionally, the same proportion of fibers appeared to be reinnervated in the PCA and TA muscles at 30 days and beyond. Hence, there do not appear to be striking differences that might help explain the differential response between the PCA and TA muscles. However, while the response of the reinnervated TA muscle at 14 days was similar to that of the denervated TA muscle, this early response was reversed such that at 30 days, the relative proportions of the fast type IIX and IIB MyHC isoforms were similar to those of the control TA muscle. Temporally, this corresponds to the reappearance of SV2 vesicles, raising the possibility that the patterns of reinnervation between the 2 muscles are different.

With respect to the possibility of better-directed reinnervation, studies have shown that reinnervation of laryngeal muscles can be misdirected and that autonomic nerves appear to be responsible for much of the reinnervation as demonstrated by the presence of 5-OHDA labeling of the nerve terminals after periods of denervation.\(^25,26\)

The second interpretation of our findings is that reinnervation of the TA muscle may be more effective than in PCA in reestablishing normal mechanical loading conditions. (stress, strain, shear). The joint fixation model developed by Shiotani et al\(^8\) may prove very useful in dissecting the importance of mechanical factors and innervation patterns.

The whole-muscle data from the present study must be interpreted cautiously because they might contradict the single-fiber data. With respect to the control PCA muscle, the single-fiber MyHC isoform distribution was unstable over the course of the present study, and this resulted in a relatively large pool of IIX/IIB fibers at 180 days. As a result, control PCA muscle took on a single-fiber distribution that was somewhat similar to that of the reinnervated PCA muscle. The whole-muscle MyHC isoform distribution of the reinnervated TA muscle appeared to be similar to that of the control TA muscles at
the later time points. Yet inspection of the single-fiber data demonstrates that there were clear differences between the control and reinnervated TA muscles. Hence, while it is tempting to interpret the whole-muscle data to suggest that reinnervation is more effective in the TA muscle, the single-fiber data could be interpreted to suggest just the opposite.

THE PLASTICITY OF RODENT LARYNGEAL MUSCLE

Of the 2 laryngeal muscles examined in this study, the PCA appears to be somewhat more pliable than the TA muscle. For instance, denervation produced shifts in the relative proportions of fast type IIX and type IIB MyHC isoforms in the PCA muscle that appeared to be greater than those seen in the TA muscle. Why would the MyHC isoform profile of the PCA muscle be more sensitive than the TA muscle to innervation state? The TA muscle is believed to be associated with phonation, while the PCA muscle is involved with inspiration. Since there is little vocalization in rodents, presumably the rodent PCA muscle receives a much greater amount of neural activity than does the TA muscle. This may explain why the PCA MyHC phenotype may be more dependent on neural input.

Consistent with the impression that the myosin phenotype of the PCA muscle is more malleable than that of the TA muscle, Wu et al. found that altered thyroid states had a larger effect on the myosin isoform composition of the PCA than on that of the TA muscle. Additionally, Shiotani et al. recently contrasted the effects of nerve crush, recurrent laryngeal nerve transection and repair, and joint fixation on the MyHC isoform profile of the PCA and TA muscles. These investigators observed that nerve crush and joint fixation had little effect on the MyHC isoform expression of the PCA and TA muscles, whereas transection and nerve repair resulted in significant shifts in the MyHC isoform profile of the PCA muscle. Collectively, the findings from the present study and those of previously published studies suggest that the MyHC isoform phenotype of rodent PCA muscle can be altered to a greater extent than that of the TA muscle.

RODENT LARYNGEAL MUSCLE AS A MODEL FOR MYHC ISOFORMS IN HUMANS

As indicated in the present study and others, adult rodent laryngeal muscles express 5 different MyHC isoforms (slow type I, fast type IIA, fast type IIX, fast type IIB, and fast type IIL). In contrast, human laryngeal muscle appears to express only 3 (ie, slow type I, fast type IIA, and fast type IIX MyHC isoforms). Based on differences in MyHC isoform expression, it might be argued that the study of MyHC isoform expression in rodent laryngeal muscle has little relevance to human laryngeal muscle. However, there are a number considerations that favor the continued use of rodents to understand basic mechanisms regulating laryngeal muscle phenotype. First, the use of the rat as an experimental tool offers the ability to manipulate laryngeal muscle in a manner not possible with human laryngeal muscle. Second, rodent laryngeal muscle can be transfected with plasmids to perform "promoter bashing" studies designed to identify key factors regulating the transcriptional activity of key sarcomeric genes. With respect to the present study, such procedures might be used to identify DNA binding proteins in denervated laryngeal muscle that act on negative regulatory elements of the promoter region of the fast type IIB MyHC isoform. Third, rodent laryngeal muscles can also be transfected with viral vectors designed to overexpress key proteins that might be involved in key translational pathways. Finally, rodents can be pharmacologically manipulated in a manner that would be considered unethical in humans. For example, the role of the calcineurin-NFAT pathway can be studied in rodents by administering cyclosporin, a powerful immunosuppressant and known inhibitor of calcineurin.

FUNCTIONAL CONSEQUENCES OFDENERVATION AND REINNERRATION

Maximum isometric tension (P0) and maximum shortening velocity (Vmax) are 2 key mechanical measurements that have been used to characterize skeletal muscle. Maximum isometric tension is known to be dependent on muscle fiber cross-sectional area but not on MyHC isoform composition. In contrast, Vmax is dependent on MyHC isoform composition but not muscle fiber cross-sectional area. As shown in Figure 9, these 2 measurements represent opposite ends of what is known as the force-velocity relationship. Conceptually, the force-velocity relationship represents one of the most important properties of skeletal muscle because (1) it determines the amount of force that can be generated at any given shortening velocity (and vice versa) and (2) the mathematical product of force multiplied by velocity is power.

Figure 9 provides a conceptual perspective regarding the functional consequences of a reduction in muscle cross-sectional area (Figure 9A), a fast type IIB to fast type IIX transition in MyHC isoform composition (Figure 9B), and a combination of the 2 effects (as was observed in the present study; Figure 9C) on the force-velocity relationship. As shown in Figure 9A, a reduction in muscle or muscle fiber cross-sectional area (as occurs with denervation) will alter the entire force-velocity relationship, but it will do so in a graded fashion, showing the greatest loss of function at slow shortening velocities and the least effect at high shortening velocities. A fast type IIB to fast type IIX transition in MyHC isoform composition (as occurs with denervation) will have just the opposite effect (Figure 9B). The combination of these 2 effects (ie, loss of cross-sectional area and fast type IIB to fast type IIX transition) has the potential to alter the entire force-velocity relationship in a more uniform fashion. Based on the findings of the present study, we hypothesize that the force-velocity relationships of the denervated PCA muscles (up to day 90) would be similar to those shown in Figure 9C. With respect to the reinnervated PCA muscle where muscle mass and muscle
However, these transitions occurred between the 2 fastest MyHC isoforms (fast type IIB to fast type IIX) and did not involve the slower fast type IIA and slow type I MyHC isoforms. Bottinelli et al28,29 reported that the Vmax of fast type IIX fibers was about 20% slower than that of the faster type IIB fibers. Hence, the transition from a muscle that expresses only the fast type IIB MyHC isoform (ie, 100% type IIB MyHC isoform) to a muscle that expresses only the fast type IIX MyHC isoform (ie, 100% type IIX MyHC isoform) would be predicted to result in about a 20% reduction in Vmax, and this would influence the force-velocity relationship as shown in Figure 9B.27 In the context of the present study, the fast type IIB MyHC isoform represented about 30% to 60% of the total MyHC isoform pool under normal conditions in both the PCA and TA muscles, and denervation reduced this pool to about 20% of the total myosin isoform pool. Hence, the type of MyHC isoform transition associated with denervation (of both the PCA and TA muscles) and reinnervation of the PCA muscle would probably produce a 10% to 15% reduction in Vmax. With respect to reinnervated TA muscle, the loss in Vmax would have been minimal. Therefore, the alterations in MyHC isoform profiles observed in this study would be predicted to produce moderate to minor reductions in Vmax.

One of the key points to be made in this study is that partial transitions among the fast MyHC isoforms will result in relatively small functional changes (as identified by Vmax). This should apply to both human and nonhuman species. The largest functional consequences (with respect to Vmax) will occur only when there are substantial slow to fast transitions.

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