Electrical Stimulation of a Denervated Muscle Promotes Selective Reinnervation by Native Over Foreign Motoneurons

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INTRODUCTION

Laryngeal paralysis is a debilitating clinical problem. When the nerves innervating the laryngeal muscles are injured on both sides, the patient can no longer open (abduct) the vocal folds during breathing. Emergent tracheotomy followed by a partial resection of the vocal fold in case of prolonged paralysis can restore ventilation through the mouth. However, the procedure sacrifices voice and compromises the ability to swallow without aspiration. A more physiological approach to treatment involves electrical stimulation of the vocal fold abductor (posterior cricoarytenoid, PCA) muscle in pace with inspiration. Paced muscle stimulation has been studied in animals and explored more recently in preliminary clinical trials using an implantable commercial stimulator (Zealear et al. 2002). Although results have been favorable, there has been concern that early application of electrical stimulation might interrupt the natural course of reinnervation and the potential for spontaneous recovery would be lost (Kallo and Steinhardt 1983).

In a previous qualitative study of the effects of electrical stimulation on canine PCA muscle reinnervation, there was indication that stimulation preferentially repressed reconnection by foreign motoneurons, thereby promoting selective reinnervation (Zealear et al. 2000). The goal of the present study was to quantitate the effects of chronic stimulation on the magnitude of PCA muscle reinnervation, and to characterize the amount of appropriate and inappropriate reconnection using physiological and behavioral parameters.

METHODS

Anatomy

The two PCA muscles are situated on the posterior larynx (Fig. 1A). When this muscle contracts, it rocks the arytenoid cartilage in a postero medial direction to open the vocal fold (Fig. 1B). The thy roarytenoid (TA) muscle is the principal adductor of the vocal folds to close the glottic airway (Fig. 1C). Both abductor and adductor muscles are supplied by motor fibers in the recurrent laryngeal (RL) nerve. Injury to this nerve commonly results in misdirected regeneration to the PCA muscle and its antagonists, resulting in a functionally paralyzed, albeit reinnervated, larynx.

The abductor and adductor muscles are distinguished with respect to their motor unit composition. The PCA muscle exclusively contains inspiratory motor units that increase firing during hypercapnic or hypoxic conditions (Insalaco et al. 1990). In contrast, the TA muscle and its synergists exclusively contain reflex glottic closure (RGC) motor units that close the glottis reflexly on activation of sensory receptors within the laryngeal mucosa. The internal branch of the superior laryngeal nerve is a purely sensory nerve containing the afferent fibers of these receptors (Ludlow et al. 1992). In this study, electromyographic (EMG) recordings confirmed that these two motor unit pools were uniquely present in the PCA and TA muscle, respectively.
Surgery and assessment of implant stability

This study was approved by the Vanderbilt Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Under isoflurane gas anesthesia, 10 canines weighing 20–25 kg were implanted with a patch electrode array (Fig. 2). The patch was configured in a 6 × 6 matrix of electrodes to allow discrete stimulation and EMG recording at any site on either PCA muscle. A receptacle containing wire terminations from the patch was tunneled subcutaneously to the skull and anchored with bone cement. A pacing circuit was encased in a box with an interface plug constructed complementary to the skull receptacle (see Fig. 2).

Following implantation, each animal was brought to the laboratory every 3–4 wk. The animal was anesthetized with 10 mg/kg pentobarbital sodium and maintained in a moderate plane of anesthesia in a supine position. A zero degree Wolfe endoscope was inserted through a laryngoscope to videomonitor and measure spontaneous or stimulated vocal fold motion. The magnitude of abduction from the glottal midline was measured on a superimposed grid, calibrated by a ruler placed on the vocal fold. The positional stability of the patch electrode array was assessed by stimulating sequentially at each of its 36 electrode sites while monitoring the magnitude of evoked abduction, producing a "map" of the most effective stimulation sites on the PCA muscle. Normative evoked EMG (EEMG) recordings were obtained at each of these sites elicited by supramaximal stimulation of the RL nerve with a percutaneous needle electrode (Fig. 1A). Eight of the 10 animals demonstrated implant stability with stimulation and recording over 4 mo time and were randomized to the experimental or control group. During a second operative procedure, the surgeon sectioned and reanastomosed the right RL nerve 5–6 cm from the larynx in each animal without knowledge of its group assignment. A pacemaker circuit was attached to the skull receptacle of the experimental animals (*2, *3, *6, and *7). A 1-s, biphasic, charge-balanced pulse train with a frequency of 30 pps and amplitude of 2–6 mA was delivered at four PCA muscle sites to produce a moderate level of abduction on the paralyzed side (2–4 mm). This stimulus paradigm was repeated.

FIG. 1. Laryngeal anatomy and muscle actions. For the normally innervated larynx, stimulation of afferents in the superior laryngeal nerve (internal branch) reflexly activate reflex glottic closure (RGC) motor units in the recurrent laryngeal nerve and thyroarytenoid (TA) muscle to adduct the vocal fold and close the airway (arrows, C). Inspiratory motor units in the recurrent laryngeal nerve and posterior cricoarytenoid (PCA) muscle are recruited during hypercapnea to abduct the vocal fold and open the airway (arrows, B).

FIG. 2. Stimulation and recording implant system. Each Teflon-coated stainless steel lead wire was deinsulated 1.5 mm at the tip to form a patch electrode for discrete PCA muscle stimulation and recording. Nerve stimulus cuffs were included in the implant design but not used in this experiment. Circuit was composed of small outline integrated circuit components.
every 10 s and applied continuously for the entire 11-mo study. Nonstimulated animals (1, 4, 5, and 8) served as controls. The number assigned each animal corresponded to its position in the implant sequence. After RL nerve section and repair, each animal rotated through the laboratory for a monthly physiological session.

Physiological sessions

All physiological sessions were conducted under telazol anesthesia delivered intravenously at a rate of 1.4 mg·kg⁻¹·h⁻¹ to maintain laryngeal reflexes and respiratory responsivity to inhaled CO₂.

Appropriate PCA muscle reinnervation was measured in two ways. First, the change in cross-sectional area of the (glottal) airway with spontaneous vocal fold abduction was measured. Specifically, two video stillframes representing the vocal folds at rest and maximally abducted were digitized and analyzed using computer morphometry (Adobe Photoshop). A line was drawn from the anterior commissure to the posterior commissure of each frame to allow independent measurement of the hemiglottal area on each side. The percent change in hemiglottal area was determined by the change in number of pixels. Four trials were run during normal breathing or hypercapnic conditions. Second, the magnitude of appropriate PCA muscle reinnervation was based on direct recordings of spontaneous EMG activity when respiratory drive was maximized by administration of CO₂ mixed with room air. Exposure was limited to 1–2 min during which time maximum inspiratory motor unit recruitment occurred (Fig. 3A). Recordings at an electrode site were amplified, rectified, and integrated over an 8-s time interval. The mean value obtained at all muscle sites was averaged to give an overall index of its inspiratory capacity.

To quantify the level of aberrant PCA muscle reinnervation by RGC motoneurons, two different approaches were taken to activate these motor units via sensory stimulation. In the first approach, sensory nerve fibers within the vocal fold mucosa were electrically stimulated using a sponge electrode saturated with saline. In the second approach, the internal branch of the superior laryngeal nerve was stimulated with a percutaneous needle electrode (Fig. 1A). Sensory-elicited motor unit activity was recorded across the PCA muscle at the same electrode sites used previously for quantifying inspiratory activity (e.g., Fig. 3D). RGC unit activity recorded at a site was quantified by rectification and integration over a 20-ms window, positioned in time to capture the entire RGC waveform. The average across all sites gave an estimate of the incorrect reinnervation of the muscle.

The magnitude of PCA reinnervation was measured in each session. EEMG responses were recorded sequentially at each muscle site following RL nerve stimulation proximal to the anastomosis (Figs. 1A and 3B). The average EEMG response recorded from all sites across the surface of the PCA muscle gave a good index of the overall magnitude of its reinnervation. EEMG motor unit activity was rectified and integrated over a 10-ms window.

Statistical analysis

A two-tailed, unpaired Student's t-test was used to assess differences in derived data on the final outcome measures in stimulated and nonstimulated animals: percent change in hemiglottal area, inspiratory unit amplitude, RGC unit amplitude, and EEMG amplitude.

RESULTS

Appropriate reinnervation by native motoneurons

Two series of sessions were run on animals in light plane of anesthesia to estimate change in hemiglottal area (ΔGA1 and ΔGA2). Although results from the two series were similar, it was believed that animals may have differed in the respiratory drive of the PCA muscle. Therefore a third and fourth series (ΔGA3 and ΔGA4) were run under hypercapnic conditions.

In the fourth series, a short-acting neuromuscular blocking agent (pancuronium bromide) was injected into the antagonist TA muscle so that glottal opening would reflect inspiratory activity of the PCA muscle in isolation. For ΔGA3 and ΔGA4, the relative performance of animals was identical in the presence or absence of TA muscle blockade. For a given series, the ΔGA for the right reinnervated PCA muscle of each animal was normalized to the average value obtained from the left PCA muscle across all animals. In this manner, a percentile ranking of inspiratory performance on the reinnervated side of each animal was expressed in reference to a normally innervated muscle. The average percentile rankings across the four behavioral series for ΔGA is shown in column 1 of Table 1. It should be noted parenthetically that the spontaneous vocal fold
abductions observed were strikingly different for experimental and control animals irrespective of test conditions. Experimental animals *2 and *6 showed near-normal recovery of vocal fold motion on the reinnervated side. In contrast, control animals 4, 8, and 5 exhibited spontaneous adductory motion with net loss in glottal area during inspiration. The remaining three animals (*7, *3, and 1) showed intermediate levels of abdution.

A similar approach was employed to derive the percentile ranking of animals with respect to the average inspiratory EMG activity of reinnervated PCA muscles. As shown in column 2, the ranked order of animals was nearly identical to that observed for the average ΔGA series. Possibly the best estimate of appropriate PCA reinnervation would reflect both the electrical activity of the muscle (inspiratory EMG) and the mechanical activity produced by its contraction (ΔGA). The average percentile ratings obtained with these two different methods is shown in column 3. All of the experimental animals ranked higher than the control animals, and there was a significant gap between *5 and 1, the worst experimental animal and best control animal. Chronic electrical stimulation enhanced correct reinnervation of the PCA muscle (P < 0.0064).

**Inappropriate reinnervation by foreign motoneurons**

To quantify the level of aberrant reinnervation of the PCA muscle by RGC motoneurons, the two different approaches described in METHODS were taken to activate these motor units via sensory stimulation. Similar results were obtained; however, superior laryngeal nerve stimulation was believed to be more reliable. Activation of the entire internal branch of the nerve ensured maximum and consistent recruitment of RGC motor units from trial to trial. The rank and percentile ratings of the right PCA muscle during superior laryngeal nerve stimulation is shown in column 4, normalized to the average value of the innervated PCA muscle. All experimental animals demonstrated lower levels of RGC activity and ranked higher than control animals. Chronic electrical stimulation suppressed incorrect reinnervation of the PCA muscle (P < 0.0084).

**Magnitude of reinnervation**

Changes in percent reinnervation of a muscle relative to its initial (innervated) state were chronicled by the ratio of the average EEMG recording from each session to that obtained before nerve section. Ratios steadily increased in the first 5 mo and then plateaued over the remaining 6 mo of investigation. This asymptotic stage signaled the completion of the reinnervation process during which all final outcome parameter values were obtained. The rank order and percentile ratings for EEMG is shown in Table 1, column 5. The magnitude of reinnervation might also be estimated by simply summing the percentile values for correct (column 3) and incorrect (column 4) reinnervation, and expressing the sum as a percent of the sum on the normal side (column 6). There was general agreement in the ranking between the two methods with the exception of animals *7 and 1. By averaging the normative data between the two methods, a more accurate estimate of the relative ranking of animals may be gained, as shown in column 7. Regardless of the method of assessment, the four experimental animals were in the top six of the rank list and experienced a greater level of reinnervation than the control animals. Although not statistically significant (P < 0.113), chronic stimulation appeared to increase the overall magnitude of PCA muscle reinnervation.

**DISCUSSION**

This experiment demonstrates that chronic electrical stimulation of the PCA muscle promotes selective reinnervation of native over foreign motoneurons. All of the experimental animals showed significantly greater appropriate reinnervation and less inappropriate reinnervation than controls. Although significance was not demonstrated, electrical stimulation apparently increased the overall magnitude of reinnervation, presumably due to the protective effect of stimulation in preventing muscle atrophy. Preservation of the viability of muscle fibers and endplates may have enhanced overall reinnervation irrespective of motoneuron type. However, this protective effect could not explain the change in preference of the stimulated PCA muscle for native over foreign motoneurons. With striking consistency, an animal in the experimental group that had a greater level of correct reinnervation also had a lower level of incorrect reinnervation. In contrast, control animals did not show such a reciprocal relationship. In fact, just the opposite was observed: a greater level of correct reinnervation was paralleled by a greater level of incorrect reinnervation. These observations suggest that chronic stimulation induced a bias in

**TABLE 1.** PCA muscle reinnervation results

<table>
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<tr>
<th>Animal Rank</th>
<th>Correct Rein Trials</th>
<th>Average Correct Rein (C)</th>
<th>Average Incorrect Rein (I)</th>
<th>Magnitude Reinnervation Trials</th>
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In each entry of the table, the reinnervated PCA muscle of each animal is identified by its number followed by its percentile ranking in parentheses. Reinnervated muscles in the experimental stimulated group are distinguished by asterisks: *2, *6, *7, *3; reinnervated muscles in the control group: 1, 4, 5, 8. The average normally innervated muscle is identified by “N.” Raw data for outcome parameters are shown for average innervated PCA muscle and the worst ranked reinnervated PCA muscle. SL nerve = internal branch of superior laryngeal nerve. Animal *6 correct reinnervation (C) ranking determined by averaged ΔGA1 and ΔGA2 performance; incorrect reinnervation (I) ranking determined by muscular sponge stimulation. A value of 81% was derived for *6 inspiratory EMG through interpolation of column 1 with column 2 data. If the interpolated value is used, it only changes the rank of the animal with respect to C + I. Rank decreases from 1st to 3rd and percentile rating from 120% to 112%. Animal 4 (C) and (I) ranking determined by averaged recordings across 12 representative patch sites using invasive electrodes.
endplate affinity for competing motoneurons, in which the original motoneuron was favored.

Although induction of specificity in motoneuron-muscle reconnection by electrical stimulation has not been described by other investigators, there are reports that regenerating motoneurons are influenced by trophic factors in distal nerve stumps. Wigston and Donahue (1988) described selective reinnervation of surgically exchanged axolotl muscles by their native motoneurons. In the mammal, Brushart et al. (1998) observed that collaterals of single motor axons often regenerate down both sensory and motor pathways at a nerve bifurcation. Subsequently, the collaterals in the sensory pathway are pruned, while those in the motor pathway are maintained. This process termed “preferential motor reinnervation,” is believed to be triggered by Schwann cell tube neurotranspans and directed by motoneuron cell bodies. In particular, brief electrical stimulation of motoneurons has been found to accelerate the speed and accuracy of reinnervation (Al-Majed et al. 2000). Politis (1985) reported preferential regeneration of peroneal and tibial components of the sciatic nerve down their native branches. These branches each contain motor fibers. However, preferential regeneration down individual muscle pathways in the mammal cannot be inferred from these studies and did not occur in the present study. To the contrary, any neurotrophic effect on regeneration was inadequate to prevent misdirected regeneration of adductor fibers in the RL nerve and inappropriate reinnervation of the PCA muscle in the control animals. Apparently, reinnervation specificity was conferred only when muscle fibers or their reconnecting motoneurons were electrically activated.

Two postulates are offered as to how electrical stimulation could confer neuromuscular specificity. The first idea holds that muscle stimulation maintains the motoneuron-muscle fiber specificity that was established during development (Thompson 1983), and prevents its loss on denervation. The second assumes that the concept of muscle plasticity extends to the synapse, and that electrical stimulation can modulate not only contractile protein synthesis and muscle fiber properties, but receptivity of the endplate for a particular motoneuron type. Although speculative, the former concept appears more plausible at the present time: the specificity between a single neuron and its endplate is established during development with the competitive elimination of redundant innervation (Bennett and Robinson 1989; Thompson 1983). Following denervation in the adult, motoneurons revert to a more embryonic or dedifferentiated state, but retain some specificity for their original muscle target (Gordon et al. 1988; Wigston and Donahue 1988). Denervation induces formation of extrajunctional (de novo) receptors on each muscle fiber. However, the original endplate may be distinguished from these de novo receptors by its recognition and affinity for the original motoneuron (Lomo and Waerhaug 1985). Stimulation of a muscle represses the formation of extrajunctional receptors without making the original endplate refractory to reinnervation (Kallo and Steinhardt 1983). Thus stimulation may favor reconnection between an original endplate and its nerve fiber and foster restoration of the synapse that was competitively selected in development.

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REFERENCES


