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Functional Electrical Stimulation following nerve injury in a Large Animal Model

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ABSTRACT:

Introduction: Controversy exists over the effects of functional electrical stimulation (FES) on reinnervation. We hypothesized that intramuscular FES would not delay reinnervation after recurrent laryngeal nerve (RLn) axonotmesis.

Methods: RLn cryo-injury and electrode implantation in ipsilateral posterior cricoarytenoid muscle (PCA) were performed in horses. PCA was stimulated for 20 weeks in eight animals; seven served as controls. Reinnervation was monitored through muscle response to hypercapnia, electrical stimulation and exercise. Ultimately, muscle fiber type proportions and minimum fiber diameters, and RLn axon number and degree of myelination were determined.

Results: Laryngeal function returned to normal in both groups within 22 weeks. FES improved muscle strength and geometry, and induced increased type I:II fiber proportion ($p=0.038$) in the stimulated PCA. FES showed no deleterious effects on reinnervation.

Discussion: Intramuscular electrical stimulation did not delay PCA reinnervation after axonotmesis. FES can represent a supportive treatment to promote laryngeal functional recovery after RLn injury.

Key words: larynx; posterior cricoarytenoid muscle; functional electrical stimulation; cryo injury; reinnervation; equine

Introduction

Functional recovery of muscles after denervation caused by peripheral nerve injury can be compromised by several factors including axons failing to reach the muscle fibers and inability of muscle fibers to recover from denervation atrophy.[1] Following peripheral nerve injury, muscle mass decreases significantly even after immediate nerve repair, and significant muscle changes occur after 1 month of denervation precluding full recovery.[2] Functional electrical stimulation (FES) has proved to have beneficial effects in improving mass and ultrastructural characteristics in long term denervated muscles.[3,4] Moreover, evidence of the FES positive effect on muscle apoptosis modulation,[5] muscle receptivity to regenerating axons,[6] and recovery of size, functional and histochemical muscle properties during reinnervation,[7] have been recently described. However, despite the literature supporting the positive effect of FES, a number of studies describe detrimental effects of FES following peripheral nerve injury.[8–10] Additionally, it has been shown that increased neuromuscular activity of partially denervated muscle prevents Schwann cell bridge formation resulting in a decrease of terminal axonal sprouting (though not nodal sprouting) which is often incorrectly considered to represent a detrimental effect of FES on reinnervation.[11–14] Currently, there is limited information regarding the appropriate timeline or the effects of stimulating a muscle prior to and during the reinnervation period. Zealear et al. demonstrated that chronic stimulation of the canine *posterior cricoarytenoid* muscle (PCA) increases reinnervation magnitude and significantly promotes selective appropriate reinnervation after recurrent laryngeal nerve (RLn) section and anastomosis.[15] Others have shown that laryngeal pacing based

on neuroprosthetic devices restores effective ventilation in patients with vocal cord paralysis (VCP).[16–22]

Impairment of laryngeal functions following RLn injury can lead to devastating consequences including VCP, dyspnea and dysphagia. The PCA is the sole arytenoid abductor, responsible for maintaining glottic patency, and preservation or restoration of its function is essential to avoiding the need for tracheostomy if bilateral VCP is present. In a previous study, we demonstrated that electrical stimulation can arrest and reverse the atrophic consequences of denervation on the equine PCA muscle after RLn transection.[23] The horse has been used as model for studying laryngeal paralysis as the neuroanatomy, physiology and outcome measures are well described.[24,25] Furthermore, PCA electrical stimulation maintains airway patency during strenuous exercise in horses with induced transient laryngeal paralysis.[26]

Given the number of contradictory studies in small animal models, we investigated the effects of daily FES on PCA reinnervation following injury to the RLn. We hypothesized that intramuscular electrical stimulation of the PCA would not delay its rate of reinnervation after axonotmesis.

METHODS

This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, federal and state regulations, and was approved by the Cornell University Institutional Animal Care and Use Committee (IACUC).

Experimental design. Fifteen adult thoroughbred horses (8 females, 7 castrated males; age 7.3 ± 2.5 years; weight 518 ± 34 kg) with normal laryngeal function (Havemayer grade ≤ II at rest, grade A during exercise)[27] were instrumented with two intramuscular electrodes in the right PCA, connected to a customized stimulator implanted subcutaneously in the cervical area. The system allowed daily electrical activation to the PCA. After right RLn freezing injury, spontaneous muscle function and response to stimulation were evaluated over 22 weeks to monitor the reinnervation process.

Surgical procedure. Horses were maintained under general inhalatory anesthesia in left lateral recumbency. A videoendoscope was placed through the right nostril into the nasopharynx to monitor arytenoid movement. The laryngeal area was clipped and prepared for aseptic surgery. A longitudinal 5cm incision was made ventral to the linguofacial vein. The caudo-lateral aspect of the cricoid cartilage was exposed through blunt dissection and electrodes with a spiraling cathode at their tip were positioned in the right PCA using an insertion cannula (Pajunk 18G needle, Pajunk GmbH, Germany). An insulated wire (passed through the cannula) was used to stimulate (1mA, 2Hz, 0.15ms pulses) the muscle with a portable device (Stiwell, MEDE-EL Elektromedizinische Geräte Gesellschaft m.b.H., Innsbruck, Austria) to determine the location at which arytenoid abduction was achieved most effectively.

Two quadripolar electrodes (K5-P4, Osypka AG, Rheinfelden, Germany), each with two leads, were then inserted in the right PCA; one electrode was placed in the lateral neuro-muscular compartment and the other more rostrally in the medial compartment.[24]

The PCA muscle is approximately 5cm long, 3cm wide and 1.2cm thick in adult horses.[28] Each electrode was secured to the closest laryngeal cartilage border with 2/0 nylon suture. Electrodes leads were tunneled subcutaneously and connected to the implanted stimulation device. One lead of one electrode was connected to a second customized implant (MEDE-EL, Innsbruck, Austria) to monitor electrode impedance throughout the study.

The right RLn was approached through a 10cm mid cervical incision dorsal to the jugular vein, and directly stimulated (1mA, 1Hz, 0.1ms) with an insulated needle (Stimuplex Insulated Needle; Braun Medical, Bethlehem, PA) to evoke arytenoid abduction twitches.

The distal right RLn was then isolated about 2cm caudally to the cricoid cartilage and exposed to freezing injury (-60ºC for 2 minutes), using a non-traumatic stainless-steel probe precooled in liquid nitrogen, to produce axonotmesis.[29,30] Activity of the right PCA evoked by stimulating the most proximal exposed RLn was monitored endoscopically prior to freezing injury and 10 minutes after its completion to ensure denervation. As expected, no arytenoid twitches were recorded after the injury in all horses. Broad-spectrum antibiotics (trimethoprim-sulfamethoxazole 30mg/kg, PO, BID) and the non-steroidal anti-inflammatory phenylbutazone (1mg/kg, PO, BID), were administered for 7 days postoperatively; horses were examined daily for any signs of complications or illness.

Electrical Stimulation. FES began two weeks after nerve injury to allow resolution of tissue swelling and electrode stabilization. One of the electrodes was used as cathode, the other as anode. The right PCA was stimulated in eight randomly selected horses

(referred to as the FES+ group); seven horses were implanted, underwent axonotmesis, but were not stimulated (controls, referred to as the FES- group). FES+ horses were stimulated 60 minutes twice-daily for twenty weeks (22Hz, 20ms, 10mA, 3.5sec ON, 6.5sec OFF) to produce 55,440 impulses/day (daily frequency equivalent to 0.64Hz or 2.9% daily activation). These parameters were based on prior work on equine larynx and a pilot trial (Ducharme and Cheetham, 2011, unpublished material). The right *lateral cricoarytenoid* muscle (LCA), also denervated following the RLn injury, was not stimulated and served as internal control. Muscle response to FES was monitored monthly through endoscopic and ultrasonographic exam.

Outcome measures- Overview

The effects of denervation, reinnervation and stimulation were determined immediately after surgery, two weeks from the nerve injury, and monthly thereafter through assessments of PCA contraction judged indirectly by arytenoid abduction response to hypercapnia, electrical stimulation and incremental exercise. PCA size was measured by ultrasound. After six months, quality of RLn reinnervation was estimated through response to hypercapnia before and after RLn anesthetic block. At the end of the study, immunohistochemistry of the right PCA and LCA muscles was performed; axon number and degree of myelination of the right RLn were determined distally to the injury site.

Arytenoid abduction. With the horse standing and sedated (detomidine 0.01mg/kg, IV), a videoendoscope (Olympus GIF-140) was placed in the right ventral nasal meatus and laryngeal endoscopy was recorded for subsequent analysis as previously

described.[25,26] Maximal arytenoid abduction was measured to assess PCA activation during hypercapnia and electrical stimulation.

Transient hypercapnia. Return of arytenoid function with reinnervation was assessed by inducing transient hypercapnia to stimulate ventilatory drive and PCA contraction.[31] The rebreathing test is routinely used in horses to induce hyperventilation: the horse muzzle is enclosed in a 30 liter plastic bag, so the horse will re-breath expired air loaded with CO_2. The CO_2 inside the bag increases progressively at each breath, consequently causing blood CO_2 to rise, enhancing respiratory rate and depth. The test was terminated when the horse reached maximal glottic opening or demonstrated intolerance.

At the end of study, to confirm absence of synkinetic reinnervation from axonal misdirection at the site of nerve injury, the rebreathing test was performed before and after a right RLn anesthetic block. With the animal under sedation, a stimulating injectable 22G needle (UniPlex Nanoline cannula, PAJUNK® GmbH, Germany) was advanced dorsal to the jugular vein, perpendicular to the skin, in the caudal portion of the neck and its tip located near to the RLn. Supramaximal stimulation (10mA, 100usec) was applied to stimulate all axons within the RLn and the corresponding arytenoid twitch was assessed on endoscopy. After confirmation of right arytenoid response to the RLn stimulation, 5ml lidocaine 2% was injected through the needle to anesthetize the RLn and the rebreathing test was repeated.

Acute stimulation. Muscle responsiveness and contraction force were determined by measuring arytenoid abduction evoked by brief PCA electrical stimulation. Stimuli were applied continuously for a few seconds, starting in expiratory hold (the short hesitation

at the end of expiration), using fixed pulse frequency (40Hz) and duration (10ms). These parameters were chosen as 40Hz is above the fusion frequency for the equine PCA.[26] The sequence of amplitudes used was randomized and varied from 0.5mA to a maximum of 10mA, with at least 30sec interval between each stimulation session to permit muscle recovery. Baseline data were obtained within 48h from nerve injury, prior to degeneration of the remaining axons. [32]

Standardized Exercise Protocol. Fatigue resistance under increasing inspiratory negative pressure loads was determined by incremental exercise on high-speed treadmill. The horses were trained 5 days/week on a high-speed treadmill. A standardized exercise test was performed monthly to evaluate horses at exercise intensities corresponding to 50, 80, 90 and 100% of their maximum heart rate.[33] During the exercise trial, each speed was maintained for 1 minute while simultaneously recording heart rate, upper airway videoendoscopy and pharyngeal airway pressures. Laryngeal function was recorded using a wireless videoendoscope (Optomed) placed into the nasopharynx via the right ventral nasal meatus. Nasopharyngeal pressure was measured using a Teflon catheter (1.3mm ID, Neoflon; Cole-Parmer, Chicago, IL) inserted through the left ventral nasal meatus and attached to differential pressure transducers (Celesco LCVR; Celesco Transducers Products, Canoga Park, CA) referenced to atmospheric pressure and calibrated from -70 to 70 mmHg. [34,35]

Muscle size. PCA size was monitored by transesophageal ultrasound under sedation as previously described.[28] A pediatric transesophageal echocardiography probe (9T,

pediatric TEE probe, diameter 7.5mm, 3.3–10.0MHz, Vivid 7) was placed transnasally into the esophagus to image the right and left PCA and to measure the dorsal-ventral thickness of the midbody (60 mm caudal to the palatopharyngeal arch) and caudal portions (caudally to the cricoid sagittal ridge) of the muscles.

PCA volume was estimated immediately post-implantation, 13 and 22 weeks later, using a quantitative Computed Tomography (QCT) technique.[28] At the end of the study, the laryngeal muscles were excised and weighed.

Muscle immunohistochemistry. Following euthanasia, the right PCA and LCA muscles were isolated and mid-body transverse sections embedded in cutting medium (Tissue-Tek OCT Compound, Sakura Finetek, Netherlands) and frozen in melting isopentane precooled in liquid nitrogen (-156°C). Thereafter, 7µm cryosections were air-dried onto glass slides and stored at -80°C and processed as previously described.[23] All mature skeletal muscle fiber types (I, IIa and IIx), hybrid fibers and collagen V were identified in single cryosections using a multiple immunofluorescence labelling technique with 4 different primary antibodies.[36] Fibers (n > 400) were manually measured, to obtain the minimal fiber diameter (MFD) and assigned a fiber type or hybrid fiber designation by relative fluorescence.

Nerve histomorphometry. Sections of the right RLn taken 5mm distal to the site of freezing injury were fixed in 10% formaldehyde, embedded in epoxy resin, sectioned transversely and stained with azure II/methylene blue/safranin. Photomicrographs of fascicules were taken using a digital microscope (Carl Zeiss, Jena, Germany),

magnified to 40X and viewed in Axiovision (Axiovision 4, Carl Zeiss, Jena, Germany). Automated analysis of the nerve fibers was performed with Volocity (version 6.1.1, PerkinElmer) using a customized bespoke modular program. Number of axons, diameter of the nerve fibers and axons, myelin sheath thickness and the g-ratio (minimum axon diameter divided by the minimum fiber diameter) were calculated.

***Data analysis*.** Endoscopic images of the *glottis* were captured from the digital recordings using editing software (Video Wizard, Womble Multimedia, CA, USA) to measure arytenoid abduction. Image frames corresponding to the expiratory holding phase while the PCA was stimulated, and the inspiratory phase during rebreathing test and exercise were evaluated. Breathing phases during exercise were identified using synchronized airway pressure traces overlying the video recordings. The degree of arytenoid abduction was measured as previously described.[23,25,26] MFD obtained for each fiber type within each muscle were allocated into 5µm bins and plotted as histogram envelopes. For continuous outcome measures (arytenoid abduction, PCA volume and thickness, muscle weight), a mixed effect model was fitted to the data to determine the relationship between the outcome variable and relevant fixed effects, using horse as a random effect. Morphometric data of muscle and nerve fibers were fitted in a mixed effect model with muscle and nerve nested within horse identity. Tukey's *post hoc* tests and linear contrasts were used as appropriate. Statistical analysis was performed using JMP (SAS Institute, Cary, North Carolina, USA). Significance was set at $p<0.05$ throughout.

RESULTS

All horses recovered uneventfully from surgery. Minimal post-operative swelling resolved in few days. No evidence of discomfort or complications associated with the surgery, the electrical stimulation or the implants were detected throughout the study.

Impedance. A small increase in impedance was recorded starting 4 weeks post-implant (Suppl. Fig). Thereafter no significant changes and no difference between FES+ and FES- groups were observed.

Function during transient hypercapnia. Progressive reinnervation of the PCA, determined by right arytenoid abduction in response to hypercapnia, began 14 weeks after the nerve injury, and full recovery was reached at 18 weeks in both FES+ and FES- horses, with no difference between groups (Fig. 1A). Full recovery also supported the absence of synkinetic reinnervation resulting from axonal misdirection at the site of nerve injury. After 22 weeks, all horses responded to right RLn brief electrical stimulation with abduction, confirming right PCA reinnervation from the abductor branch fibers; then the right RLn anesthetic block induced complete transient right arytenoid paralysis so that all horses showed only left arytenoid abduction in response to the rebreathing test.

Response to acute electrical stimulation. The response to stimulation decreased significantly within two weeks of nerve injury (Fig. 1B), and returned more rapidly in the FES+ group beginning after 4 weeks of FES training, indicating a greater excitability and

strength of the stimulated PCA. There was no difference between the responses at time 0 and after 22 weeks suggesting full recovery in both groups following reinnervation.

Function under inspiratory load. PCA function under increasing levels of negative inspiratory pressure load was markedly reduced after denervation ($p<0.001$, Fig. 1C). Both groups presented a concurrent significant compensatory increase in left arytenoid abduction ($p=0.037$). A similar gradual but incomplete return to function on the right side was detected in both groups.

Muscle size. PCA thickness and the right:left (R:L) ratio of muscle thicknesses were significantly greater in the FES+ group 6 weeks after injury indicating a positive training effect of electrical stimulation ($p<0.05$, Fig. 2A-B). This effect was most pronounced in the caudal (inferior) portion of the PCA muscle which undergoes more rapid atrophy during denervation.[28]

CT-determined PCA volume remained stable throughout the study, with no significant difference between the two groups (Fig. 2C). No significant difference was observed in right:left muscle mass ratio at the end of the study (Fig. 2D).

Muscle immunohistochemistry. Representative images of the PCA and LCA immunohistochemistry are shown in Figure 3. Fiber type I:II ratio was increased in the stimulated PCA ($p=0.038$, one-tail t-test, Fig. 4). The opposite effect was found in the unstimulated LCA of FES+ animals (Figure 4, $p<0.01$). No significant difference between PCA and LCA fiber type proportion was found in the FES– group (Fig. 4).

MFD distributions were significantly altered by FES (Fig. 5) that induced a bimodal distribution in the PCA fiber type I (two-tailed Mann–Whitney rank sum test, $p=0.0014$), and a left shift of the fiber type II distribution (two-tailed Mann–Whitney rank sum test, $p<0.0001$). Fiber type I and IIa distributions were unaltered in the unstimulated LCA muscle.

Nerve histomorphometry. Reinnervation following injury was accompanied by reduced axon diameters ($p=0.0003$), decreased overall fiber diameter ($p<0.001$), myelin thickness ($p<0.0001$) and myelin/axon thickness ratio, and increased g-ratio ($p=0.0052$) ($p<0.0001$, Fig. 6). No significant differences were found between stimulated and unstimulated animals.

DISCUSSION

Our data demonstrate that intramuscular electrical stimulation improves PCA responsiveness and geometry after axonotmesis, and increases the proportion of type I:II fibers. Because type I fibers are more sensitive to muscle wasting after denervation, leading to a slow-to-fast fiber type shift,[37] the higher type I:II ratio suggests that FES supports reinnervation. Improved muscle responsiveness during reinnervation could be a consequence of improved preservation of fiber size and function during the period of denervation. When regenerating axons reconnect with stimulated muscle fibers, the excitability of the fibers is enhanced compared to fibers that did not receive stimulation during the denervation period. FES improved muscle strength as shown from the electrically-induced arytenoid abduction test, however, it did not ameliorate fatigue

resistance as shown by arytenoid collapse during exercise.[38,39] As fatigue resistance depends not only on myosin isoform composition but also on oxidative capacity (number of mitochondria and oxidative enzymes, capillary density)[40] it is possible that the overall oxidative capacity of the muscle was not yet adequate.

We were unable to detect any negative impact of FES on reinnervation following freezing nerve injury in this large animal model. The lack of deleterious effect on reinnervation confirms a previous report using a rat lower limb model.[41]

The effect of electrical stimulation on sprouting has been often cited as one factor for limiting the use of FES during the reinnervation period. Some authors have argued that electrical stimulation might inhibit the reconnection of the regenerating axons with the denervated muscle fibers[9,10]. This objection is largely based on experiments in which intermittent high frequency stimulation, delivered 24 hours per day, reduced the amount of terminal sprouting in denervated or partially denervated rodent muscle[13,14]. Neuromuscular activity of partially denervated muscles reduces terminal sprouting by preventing Schwann cell bridging between innervated and denervated end-plates[11,12], but no reduction of nodal sprouting has been recorded[14] which may be more relevant to the regrowth of whole axons after axonotmesis. After RLn cryo injury, axonal sprouting starts within the first week near the injury site.[42] Our outcome measures suggest that FES applied starting 2 weeks after injury has no negative effects on sprouting progression and myelination or that terminal sprouting contributed minimally to RLn reinnervation in our model.

Myosin heavy chain gene expression is influenced by innervation, with upregulation of type IIx genes in denervated muscles and complete loss of type I fiber from the PCA muscle after RLn transection.[37,43] This dependence of fiber type expression upon neural input is also evident in the equine PCA that shows an increase in type IIx fibers after complete denervation and a marked reduction in type I fiber after progressive denervation induced by idiopathic recurrent laryngeal neuropathy.[23,44] The specific morphometric changes of laryngeal muscles after denervation and reinnervation seem to be species-specific, with a reduction of type I fibers in rats and an increase in dogs.[45–47] The innervated equine PCA has 30-40% type I fibers,[21] while in this study the reinnervated PCA after RLn axonotmesis demonstrated a higher proportion of type I fibers (>50%), with the highest value in the stimulated group suggesting a positive effect of FES on these fibers. These findings confirm a preferential denervation atrophy of type II fibers and increase in type I fibers after reinnervation possibly as a result of sprouting from slow axons as reported after other peripheral nerve injuries in different species.[48,49]

The higher type I fiber proportion in the stimulated group confirms previous findings by Carraro et al,[50] of an upregulation of slow fibers after chronic electrical stimulation. Downregulation of type I fibers has been induced in the innervated PCA by non-use after joint fixation, suggesting that absence of mechanical stretch alters the trophic factors locally in the muscle.[51] The high type I fiber content also in the unstimulated group could have resulted from a combination of reinnervation and muscle passive stretching during treadmill training.[52]

In a similar population of horses,[23] the innervated PCA showed 37.4 ± 4.2µm MFD, that significantly decreased after RLn transection (23.6 ± 4.2µm). In the present study, 6 months after RLn axonotmesis, MFD was higher than normal in both FES+ and FES- horses (43.20 ± 0.23 µm) indicating that reinnervation occurred in both groups.[48,53] Interestingly, the right LCA muscle in the FES+ group, which underwent denervation but not electrical stimulation, showed a decreased type I fiber proportion and mean MFD (31.4 ± 0.14 vs 40.4 ± 4.2 µm). This suggests delayed or reduced reinnervation, indicating a preferential reinnervation of the abductor muscle after RLn injury and direct intramuscular FES.

Reinnervation after freezing injury induced an increased number of myelinated axons with smaller diameter, as already reported.[54,55] Considering that axon diameter and degree of myelination depend on the axon maturation, the axonal growth rate seems lower than in other studies where the RLn morphologic appearance was comparable to control nerve 20 weeks after injury.[56] Rate of reinnervation is determined by many factors like species, age, type of nerves, nature of injury, and distance from target organ and neuronal cell body.[42] The horse is a domestic species with the longest RLn so the reinnervation rate could be delayed due to the long distance between the cell body and lesion site.[57] The adequate return to function despite the differing axonal morphology raises the possibility that in the horse, axon size and myelination will never return to pre-damage values, or that in this species there is need for a much longer interval before completion of the maturation process.[42,57,58]

Acute short-term electrical nerve stimulation accelerates nerve regeneration via enhancement of cell body response to nerve injury[59]. The positive effect of electrical stimulation on axonal regrowth was recorded in the cited study after continuous stimulation of the nerve stump for 1 hour to two weeks after acute repair. The electrical field created by the intramuscular electrodes during FES can spread beyond the muscle border, raising the possibility of an effect of FES directly on the nerve stump. The FES protocol in our study was started more than two weeks after injury, and the earliest exposure of the muscle to electrical stimulation was 24-48h after injury, when the muscle was subjected to a transient short burst of stimulation to record the baseline response of the muscle. We did not directly investigate the extension of the electrical field in our study, but the response to FES was assessed monthly on endoscopic and ultrasonographic examinations. During these examinations, we did not detect activation of tissues surrounding the PCA, such as the cricopharyngeous muscle laying directly over it, or activation of the contra-lateral PCA muscle, suggesting an insubstantial spread of current beyond the PCA.

The main limitations of this study include the lack of histomorphometry data of the muscles before the RLn injury and of the regenerating nerve at an earlier time point. Those data would help in a more complete assessment of the denervation and reinnervation effects on the equine PCA muscle as well as in calculating the axonal re-growth rate, to establish if more than six months are required to complete nerve maturation. However, this study gives support to the principle that stimulation of a muscle during the period of denervation improves the eventual neuromuscular recovery

and that . 60 minutes of twice-daily stimulation appears to achieve a useful preservation of muscle function without inhibition of reinnervation.

Abbreviations: CT, computed tomography; FES, functional electrical stimulation; LCA, *lateral cricoarytenoid* muscle; MFD, minimum fiber diameter; PCA, *posterior cricoarytenoid* muscle; RLn, recurrent laryngeal nerve; VCP, vocal cord paralysis.

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Figure legends

Figure 1. Arytenoid response to transient hypercapnia, acute *posterior cricoarytenoid* muscle (PCA) electrical stimulation and incremental exercise. In (A), nerve injury caused right arytenoid collapse below resting angle within the first 6 weeks. Reinnervation induced progressive return of the right PCA response and consequent increased abduction. At 22 weeks, complete reinnervation is indicated by a similar response of right and left arytenoids to hypercapnia. No significant difference between stimulated (FES+) and control (FES-) subjects was detected at any timepoint. Resting angles are cumulative of right and left arytenoids before the test from both groups. In (B), FES promoted an earlier response to acute electrical stimulation of the muscle. The significant difference between groups (asterisks), observed by 6 weeks after injury, persisted to 18 weeks and it was lost by 22 weeks. Resting angles are cumulative of the right arytenoid from both groups. In (C), nerve injury produced ipsilateral marked arytenoid collapse at exercise with recovery over 22 weeks. Compensatory hyperabduction of the contralateral arytenoid cartilage decreased during the recovery period. Left arytenoid angles are combined from both groups. Asterisks indicate significant difference between pre-injury and post-injury arytenoid abduction. Data are mean ± SEM. * ($p<0.05$), ** ($P<0.01$), *** ($p<0.001$).

Figure 2. *Posterior cricoarytenoid* (PCA) muscle geometry and volume. Functional electrical stimulation (FES+) increased PCA muscle mid-body (6, 10, 18, 22 weeks) (A) and caudal (6, 10, 22 weeks) (B) right to left muscle thickness ratio. Minimal changes in PCA muscle volume (C) or mass (D) right:left ratio were identified. Muscle volumes were determined using computed tomography reconstruction. Cricoid cartilage volume was used as an internal control and showed no changes in volume over time. Data are mean ± SEM. Asterisks indicate significant difference between FES+ and FES- subjects. * ($p<0.05$), ** ($p<0.01$).

Figure 3. Representative immunohistochemistry images of laryngeal muscles. *Posterior cricoarytenoid* (PCA) and *lateral cricoarytenoid* (LCA) muscles labeled simultaneously with antibodies specific for type I fibers (blue), type IIa fibers (red) and collagen V (green) (Tulloch et al, 2011)[36]. (+, muscle that received FES)

Figure 4. Fiber type proportions of the laryngeal muscles. Functional electrical stimulation (FES+) induced an increase in type I to type II ratio in stimulated *posterior cricoarytenoid* (PCA) muscle but a reduction in type I:II ratio in unstimulated *lateral cricoarytenoid* (LCA) from the same animals. Immunohistochemistry was performed on mid-body muscle sections (7 um) and fibers were identified with different mouse monoclonal antibodies labelled with fluorescent fragments designed for different emitting wavelengths (Tulloch et al, 2011)[35]. Data are mean ± SEM. * ($p<0.05$), ** ($p<0.01$)

Figure 5. Fiber diameter distribution of the laryngeal muscles. Functional electrical stimulation (FES+) increased the proportion of small diameter type I and type II muscle fibers in the stimulated right *posterior cricoarytenoid* (PCA) muscle (upper panels). No changes were observed in the *lateral cricoarytenoid* (LCA) muscle (internal control, lower panels). FES was only applied to the right PCA of the FES + group. Dotted lines represent animal in which FES was not applied (FES-). Bin size is 5um. Asterisks indicate significant difference in diameter distribution between the FES+ and FES- groups. * ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)

Figure 6. Distal Recurrent Laryngeal nerve (RLn) morphometry in uninjured condition and after freezing injury and reinnervation. The mean axon count is higher after injury and reinnervation, but not statistically significant. The other morphometric characteristics of the RLn were significantly different from control after injury and reinnervation, but no significant difference was found between FES+ and FES- group regarding axon count and diameter, myelin thickness and myelin/axon ratio, fiber diameter and g ratio indicating a similar degree of reinnervation between control horse and horses underwent *posterior cricoarytenoid* muscle (PCA) electrical stimulation. Data are mean ± SEM. * ($p<0.05$), ** ($p<0.01$), ***($p<0.001$)