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CHANGES IN NEUROTROPHIC FACTORS OF ADULT RAT LARYNGEAL MUSCLES DURING NERVE REGENERATION

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Abstract—Injury to the recurrent laryngeal nerve (RLN) leads to the loss of ipsilateral laryngeal fold movement, with dysphonia, and occasionally dysphagia. Functional movement of the vocal folds is never restored due to misrouting of regenerating axons to agonist and antagonist laryngeal muscles. Changes of neurotrophic factor expression within denervated muscles occur after nerve injury and may influence nerve regeneration, axon guidance and muscle reinnervation. This study investigates the expression of certain neurotrophic factors in the laryngeal muscles during the course of axonal regeneration using RT-PCR. The timing of neurotrophic factor expression was correlated to the reinnervation of the laryngeal muscles by motor axons. Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF) and Netrin-1 (NTN-1) increased their expression levels in laryngeal muscles after nerve section and during regeneration of RLN. The upregulation of trophic factors returned to control levels following regeneration of RLN. The expression levels of the neurotrophic factors were correlated with the innervation of regenerating axons into the denervated muscles. The results suggest that certain neurotrophic factor expression is strongly correlated to the reinnervation pattern of the regenerating RLN. These factors may be involved in guidance and neuromuscular junction formation during nerve regeneration. In the future, their manipulation may enhance the selective reinnervation of the larynx. **© 2016 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: reinnervation, recurrent laryngeal nerve, NGF, BDNF, Netrin, peripheral nerve regeneration.

INTRODUCTION

Following injury or transection of a peripheral nerve, robust axonal regeneration occurs. However, functional recovery is usually impaired due to inappropriate target selection by regenerating axons (Sunderland, 1978). This problem is rather significant in regeneration of the recurrent laryngeal nerve (RLN). As a result, vocal fold function is never restored (Crumley, 2000; Tessema et al., 2008, 2009; Pitman et al., 2011; Hernandez-Morato et al., 2013, 2014a).

RLN is a branch of the vagus nerve that innervates the intrinsic laryngeal muscles. In the rat, the laryngeal muscles are the posterior cricoarytenoid muscle (PCA), which is the unique abductor muscle of the larynx; the lateral (LTA) and the medial thyroarytenoid muscles (MTA), which are the two main adductor muscles (Fig. 1). Axonal regeneration and reinnervation of the denervated muscles is mediated by neurotrophic factors (Terenghi, 1999; Pitts et al., 2006; Gordon, 2009; Sun et al., 2011). These factors activate signaling in the axon growth cone that stimulates the axonal elongation and the synapse formation (Wheeler and Bothwell, 1992; Wang et al., 1995; Ip et al., 2001; Tomàs et al., 2011; Je et al., 2012; Harrington and Ginty, 2013).

The main group of neurotrophic factors are the neurotrophins. Those members present in mammalian muscles are Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4) (Gotz et al., 1994; Dethleffsen et al., 2003; Omura et al., 2005; Pitts et al., 2006; Allen et al., 2013). Their expression promotes neurite outgrowth and synaptogenesis during development (Lindsay, 1988; Wheeler and Bothwell, 1992; Hory-Lee et al., 1993; Koliatsos et al., 1994; Wang et al., 1995; Liou and Fu, 1997; Garcia et al., 2010b). Neurotrophins are also involved in neuromuscular junction maturation and their maintenance (Heumann et al., 1984; Wheeler and Bothwell, 1992; Funakoshi et al., 1995; Wang et al., 1995; Belluardo et al., 2001; Saka et al., 2007; Garcia et al., 2010a, 2010c, 2010d; Tomàs et al., 2011). The role of NT-3 has been implicated in the proprioceptive innervation of skeletal muscles (Ernfors et al., 1995; Wright et al., 1997; Xie et al., 1997). Neurotrophins also modulate the balance of slow and fast muscle fiber composition (Belluardo et al., 2001; Carrasco and English, 2003; Mousavi et al., 2004; Saka et al., 2007; Ogborn and Gardiner, 2010).

Netrin-1 (NTN-1) is another neurotrophic factor that mediates axonal guidance (Colamarino and Tessier-

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Abbreviations: BDNF, Brain-Derived Neurotrophic Factor; DPI, days post injury; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LTA, lateral thyroarytenoid; MTA, medial thyroarytenoid; NGF, Nerve Growth Factor; NT-3, Neurotrophin-3; NT-4, Neurotrophin-4; NTN-1, Netrin-1; PCA, posterior cricoarytenoid; RLN, recurrent laryngeal nerve; SLN, superior laryngeal nerve; TLR-4, Toll-Like Receptor 4.

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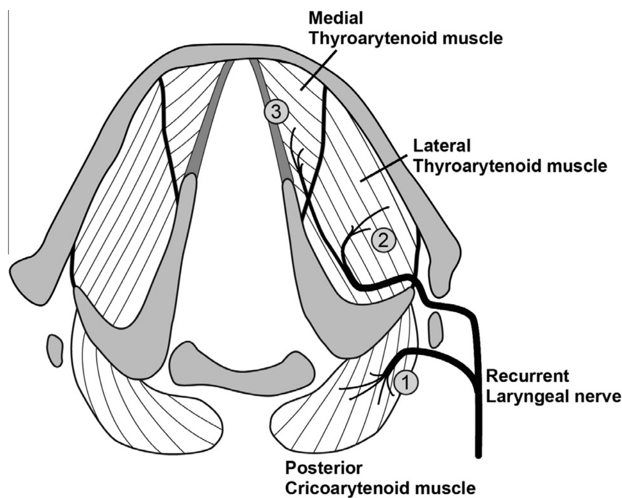

Fig. 1. Representation of transversal section of the rat larynx. The recurrent laryngeal nerve innervates the ipsilateral larynx. Its branches innervate the abductor posterior cricoarytenoid muscle first (1), followed by the adductors lateral thyroarytenoid muscle (2) and median thyroarytenoid muscle (3).

Lavigne, 1995; Mitchell et al., 1996; Lauderdale et al., 1997; Varela-Echavarría et al., 1997; Sun et al., 2011). Its depletion also affects the formation of neuromuscular synapses (Mitchell et al., 1996; Burgess, 2006).

Toll-Like Receptor 4 (TLR-4) is involved in pathogen recognition. Although not a neurotrophic factor, TLR-4 has been implicated in the maintenance of motor innervation (Radin et al., 2007; Reyna et al., 2008; Welc et al., 2013; Wu et al., 2013). Therefore, it was considered as an additional element for investigation.

The aims of this study were to evaluate the expression of NGF, BDNF, NT-3, NT-4, NTN-1 and TLR-4 in the abductor and adductor muscles following RLN transection and anastomosis in adult rats and to correlate this expression with the reinnervation of the laryngeal muscles.

EXPERIMENTAL PROCEDURES

Animals

114 adult female Sprague Dawley rats (250 g) were used in the present study. The rats were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee of New York Medical College approved the animal use protocol.

Surgery-RLN section and repair

The animals were divided into three groups (Table 1). Rats were deeply anesthetized with an intraperitoneal injection of 70 mg/kg of ketamine and 7 mg/kg of xylazine. The right RLN was exposed at the seventh tracheal ring and a piece of gelfoam was gently placed beneath the nerve. It was then transected with an iridectomy scissor. The ends were separated to confirm full transection. They were then reapproximated on the original piece of gelfoam. A second piece of gelfoam was placed over the anastomosis to create a firm connection.

In order to avoid ipsilateral collateral innervation from the superior laryngeal nerve (SLN) to the denervated intrinsic laryngeal muscles (Hydman and Mattsson, 2008), the right SLN was identified and two vascular clips were placed using a Ligaclip endoscopic clip applier (Ethicon). The nerve was transected between the clips.

Functional evaluation

While animals were still under anesthesia, a 0° 4 mm endoscope (Stoerz, Germany) was inserted transorally to evaluate vocal fold motion in normal control animals and after RLN injury. Once the nerve was transected, right vocal fold paralysis was confirmed. Food and water were provided "*ad libitum*". At the end of each time period (Table 1), the animals were anesthetized once more as described above. Table 1 Vocal fold motion was evaluated and recorded. Two glottal frames were selected as was described in earlier studies (Hernandez-Morato et al., 2013). One represented maximal adduction of vocal folds while the other a maximal abduction (Fig. 2). The angles between both vocal folds were measured using Image J software (Public Domain, created by Wayne Rasband). This procedure was repeated three times per animal. The means of maximal abduction and maximal adduction angles were calculated. Position of the paralyzed vocal fold was labeled median when the angle between both folds in maximal adduction measured less than 15°, paramedian when the angle was between 15° and 25° and lateral when the angle was greater than 25°.

Real-time quantitative RT-PCR

Animals from Group 1 (Table 1) were euthanized with isoflurane inhalation. Right PCA, LTA and MTA muscles were isolated and dissected out and were frozen in liquid nitrogen and kept at −80 °C. Total RNA was isolated from each muscle using the TRIzol method according to the manufactures instructions (Life

Table 1. Number of animals used in this study

Groups	Time periods (DPI – Days Post Injury)									
	Control	1 DPI	3 DPI	7 DPI	14 DPI	21 DPI	28 DPI	56 DPI	84 DPI	112 DPI
qRT-PCR (n = 72)	18	6	6	6	6	6	6	6	6	6
WBlot (n = 18)	6		6	6						
IHC (n = 24)		4	4	4	4	4	4			

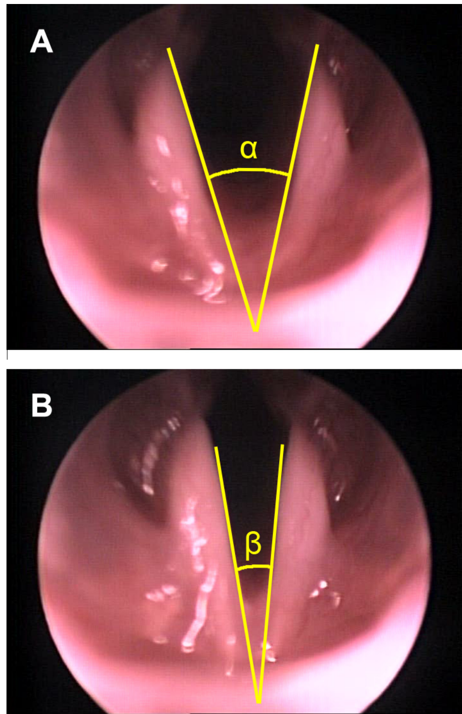

Fig. 2. Images of two glottal frames at maximal abduction (A) and maximal adduction (B) taken from a paralyzed right vocal fold of the rat.

Technologies, CA). cDNA was generated from 500 ng of total RNA in a thermo cycler PCR System 2400 (Perkin Elmer) using Trinscriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science).

cDNA amplification was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystem). The relative expression of the internal control gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the studied genes NGF, BDNF, NT-3, NT-4, NTN-1 and TLR-4 were measured using FastStart Universal SYBR Green Master (ROX) (Roche Applied Science). In the first step of the RT-PCR the cycle was 50 °C for 30 min, 85 °C for 5 min and then the samples were kept at 4 °C, and stored at −20 °C. In the second step the cycling conditions and cycle threshold values were determined by the supplied ABI software. The annealing temperature used according to SYBR protocol was 60 °C. The sequences of the primers are summarized in Table 2. The relative quantification of gene expression was calculated using the comparative Ct method [$2^{\Delta C_t}$, where ΔC_t represents the differences between the studied genes (NGF, BDNF, NT-3, NT-4, NTN-1, TLR-4) and the internal control gene (GAPDH)].

Western blot

Ipsilateral PCA, LTA and, MTA muscles of 12 animals were dissected out at 3 and 7 days following right RLN injury. In 6 control animals the right laryngeal muscles were isolated and dissected out to establish basal levels of protein expression. They were homogenized with ice-cold lysis buffer (1 M Tris–HCl, pH 7.4, 3% (v/v) Glycerol, and 3% sodium dodecyl sulfate (SDS)). Following the debris removal, samples containing proteins were mixed 1:1 with Laemmli sample buffer and boiled for 5 min at 100 °C. The samples were then loaded onto 15% sodium dodecyl sulfate polyacrylamide (SDS–PAGE) gels and electrophoresed at 100 V for 60 min. Gels were electroblotted onto polyvinylidene difluoride (PVDF) membrane at 100 V for 45 min. The membranes were then blocked with 5% (w/v) non-fat dry milk in TBS/T (20 mM Tris pH 7.5, 500 mM NaCl, 01% (v/v) Tween-20) for 2 h and then incubated at 4 °C overnight in rabbit antibodies against NGF (1:300), BDNF (1:300), and Netrin-1 (1:300) (Santa Cruz Biotechnologies, CA) in 0.1% non-fat dry milk in TBS/T. After the washes, the membranes were incubated for 2 h at room temperature with donkey anti-rabbit IgG peroxidase conjugate (Jackson ImmunoResearch, 1:10,000). Membranes were treated with ECL chemiluminescent substrate (GE Healthcare Life Sciences) for 1 min and then exposed to autoradiography film (Denville Scientific).

Immunohistochemistry

The animals from the third group (Table 1) were overdosed with isoflurane inhalation and transcardially perfused with 200 ml of Saline Phosphate Buffer (PBS) followed by fixation in 4% paraformaldehyde in PBS (250 ml). After perfusion, the larynges were dissected out, postfixed for 2 h in the same fixative solution, and were cryoprotected in 30% sucrose in PBS before sectioning in a Leica cryostat.

In order to identify the presence of axons and motor endplates in the laryngeal muscles after nerve injury, 14-μm serial sections of PCA, LTA and MTA were incubated with rabbit antibody against β-tubulin III polyclonnal antibody (1:1000, Covence) in TBS plus 0.8% BSA for 48 h at 4 °C. After a TBS wash, the sections were incubated in anti-rabbit Immunoglobulin G (IgG) Cy3 conjugated (1:400, Jackson ImmunoResearch, PA) for 1 h at 4 °C. The sections were then incubated in Alexa Fluor 488 conjugated α-bungarotoxin (1:500) for 2 h at room temperature.

Sections were mounted with glycerin PBS 1:1 solution, coverslipped and stored at 4 °C and were visualized using a Zeiss Axoskop epi-fluorescence microscope.

Statistical analysis

All results are expressed as mean ± SEM. Statistical significance was determined by paired or unpaired Student's t test where appropriate. For comparison among experimental groups, one-way ANOVA tests were used to determine statistical significance. The level of significance was set at $p < 0.05$.

RESULTS

Functional evaluation of the vocal folds

After RLN section, anastomosis and reinnervation, movement of the right vocal fold was not observed at any time points studied. Twenty percent of the animals

Table 2. Sequences of primers used in qRT-PCR

Gene	Primer sequences	
	Forward	Backward
GAPDH	TGGACCACCCAGCCCAGCAAG	GGCCCCTCCTGTTGTTATGGGGT
NGF	TGACTCCAAGCACTGGAACTCAT	GTTTGTCGTCTGTTGTCAACGC
BDNF	CGACGTCCCTGGGCTGACACTTTT	AGTAAGGGCCCGAACATACGATTGG
NT-3	ACATCACCTTGTTCACCTCTA	AGTCCACCTTTCTCTTCATGTC
NT-4	CCCCTGCGTCAGTACTTCTGAGAC	CTGGACGTCAGGCACGGCTGTTC
NTN-1	CTTCATGTTCATCTTCAGTTTTCCT	CTTGCATTAAGATTCCTGTAGCG
TLR-4	CCATGCCTTGTCTTCAATTGTC	AACCTAGATCTGAGCTTCAACC

had a paralyzed vocal fold in the median position (9.24° ± 1.23). The remaining 80% of the animals had the paralyzed vocal fold in paramedian position (18.3° ± 0.49). No difference regarding the paralyzed vocal fold position was observed among experimental groups from day 1 to 112 days post injury.

Immunohistochemistry of laryngeal muscles

α-Bungarotoxin labeled muscles showed motor endplate labeling in the middle of the muscle belly of PCA, LTA and MTA in experimental and control animals. Location of motor end plates of the normal and denervated muscles remained same as compared to the unoperated contralateral side of the same animal (Fig. 3). At 1-day post injury, labeled motor axons were observed in the ipsilateral PCA, LTA and MTA. However, in the PCA the immunoreactivity was less intense near the motor endplates when compared to the contralateral control side suggesting the presence of still intact but degenerating axons. At day 3 labeled axons were not observed in any of the ipsilateral laryngeal muscles (Fig. 3B). At 7 days post injury (DPI), a few labeled axons were observed in the PCA but not in LTA or MTA. Axons in the right PCA were randomly arranged in the muscle when compared to the control side. From 14 DPI onward, there was a progressive reorganization of axons in the PCA. At day 21 the arrangement was similar to that in the contralateral control PCA (Fig. 3D). At 14 DPI a few labeled axons were observed in the LTA and a very few axons in the MTA. Labeled axons in ipsilateral LTA and MTA at 21 and 28 days respectively, were similar to that of controls (Fig. 3H and L).

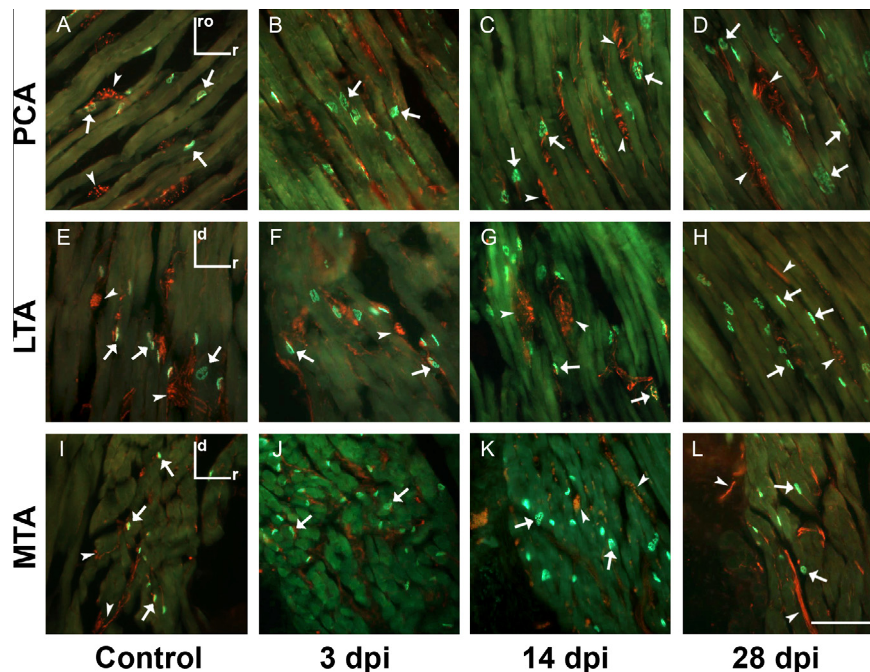

Fig. 3. Double labeling of the motor end plates (green fluorescence) and the axons (red fluorescence) in PCA (A–D), LTA (E–H), and MTA (I, J) by IHC. PCA (A), LTA (D), and MTA (I) of the left side show positive axonal staining in the motor end plates (merge in yellow). At 3 DPI of RLN in PCA (B), LTA (E), and MTA (J) motor end plates were labeled but no axonal labeling was detected. Motor axons in neuromuscular synapses were again observed in the three muscles at 28 DPI (D, H, L). The arrows point to the motor endplates and the arrowheads point to the motor axons. All photographs at each stage were taken at the same magnification. The bar at the bottom of the figure represents 100 μm. All images are presented at the same magnification. dpi, days post injury; ro, rostral; r, right; d, dorsal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mRNA expression of NGF, BDNF and NTN-1 after RLN injury

The levels of NGF, BDNF and NTN-1 mRNA in laryngeal muscles showed a significant increase post injury when compared to controls. mRNA level of NGF increased 4.2-fold at 1 DPI in PCA. This level began to decrease by three DPI and from 14 DPI onward the level of NGF further declined and stayed at this lower level throughout the experimental period. NGF expression in LTA also changed at day 3 and continued to increase up to five fold at day 14. Subsequently the mRNA expression of NGF decreased. In the MTA there was an initial increase of NGF followed by a decrease of the expression level from day 28 DPI onward (Fig. 4A).

BDNF level in all three muscles increased four fold up to day 7 and thereafter it decreased. The BDNF expression decreased to control level in PCA at 14 DPI and remained at that level in other time points. In the LTA and MTA, the expression of BDNF increased and remained elevated until day 21 and after thereafter it decreased to control level (Fig. 4B). mRNA expression of NTN-1 slowly increased and reach the peak 14 DPI in PCA and subsequently decreased at a slow rate remaining higher than control levels until 56 DPI. In MTA, NTN-1 expression decreased slightly at day 1 and remained at that low level throughout the experimental period. In LTA, NTN-1 level slightly decreased at day 1, and then increased 0.7-fold until day 28. After 28 DPI it decreased to the control levels (Fig. 4C).

The mRNA expression of NT-3, NT-4 and TLR-4 compared to other trophic factors was minimal in all muscles and these values were statistically non significant. They were excluded from further analyses (Fig. 5).

Western blot analyses

In order to confirm the changes of mRNA expression of NGF, BDNF and NTN-1 during laryngeal muscle reinnervation by regenerating RLN, the protein levels of these trophic factors were studied by Western blot analyses.

The level of NGF protein in PCA increased 1.7-fold at three DPI. It increased 2.6-fold by day 7. In the LTA, it increased 1.5-fold by three DPI and its level decreased by seven DPI. In the MTA very little increase was observed at three DPI. By seven DPI it decreased to the control values (Fig. 6A). In all three muscles, protein levels of BDNF increased by twofold at three DPI and remained elevated at seven DPI (Fig. 6B). NTN-1 protein level increased 2.5-fold by three DPI in PCA and remained elevated at seven DPI. In LTA it increased 1.5-fold and was very low at seven DPI. In MTA, NTN-1 protein level decreased by 50% at day 3 and return to control level by seven DPI (Fig. 6C).

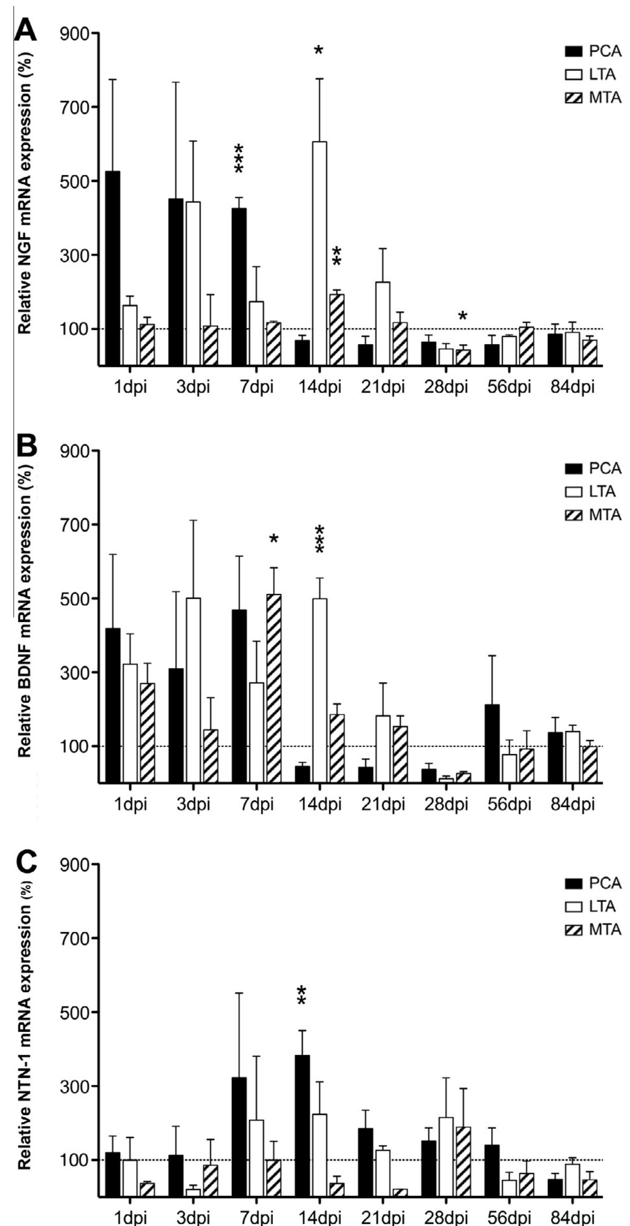

Fig. 4. mRNA expression of NGF (A), BDNF (B) and NTN-1 (C) within PCA, LTA, and MTA muscle fibers during RLN regeneration at different time points. Data are expressed as a percentage of mRNA expression (mean ± SEM) relative to control. The time course reveals differences of mRNA expression among NGF, BDNF and NTN-1 in the three laryngeal muscles. Asterisk (*) indicates results are statistically significant different (* means $p < 0.05$; ** means $0.001 < p < 0.01$; and *** means $p < 0.001$).

DISCUSSION

The present results showed differential increases in expression of mRNA of several trophic factors. In general, expression of NGF, BDNF and NTN-1 sharply increased and returned to control levels at 21 DPI onward. Specifically, NGF and BDNF mRNA increased. It is increased in the PCA before it is increased in LTA and MTA (see Fig. 4A and B). NTN-1 mRNA expression was significantly increased only in PCA, whereas, in the LTA and MTA its level increased slightly and remained near control levels (Fig. 4C). The timing of these changes and the return to baseline levels correlated with the timing of denervation and axonal reinnervation. Regarding NT-3, NT-4 and TLR-4, they displayed

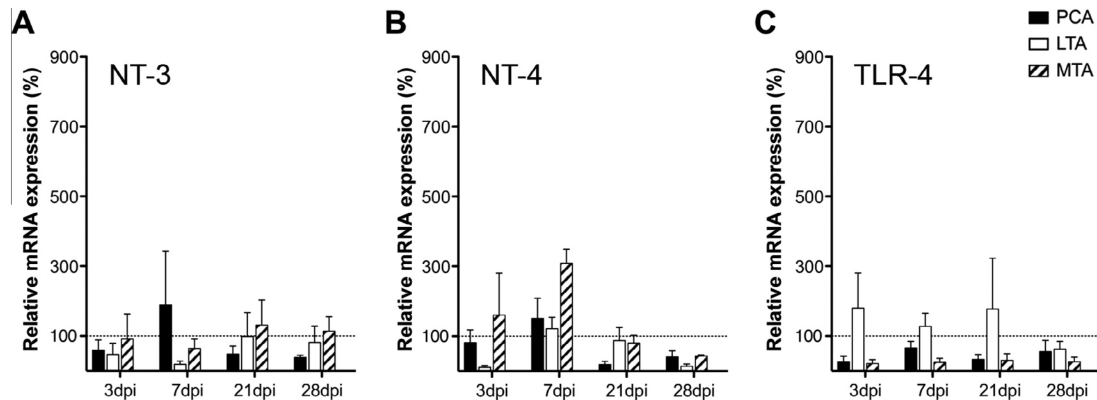

Fig. 5. mRNA expression of NT-3 (A), NT-4 (B) and TLR-4 (C) within PCA, LTA, and MTA muscle fibers during RLN regeneration at different time points. Data are expressed as a percentage of mRNA expression (mean ± SEM) relative to control. Non significant differences of mRNA expression were observed among NT-3, NT-4 and TLR-4 in the three laryngeal muscles.

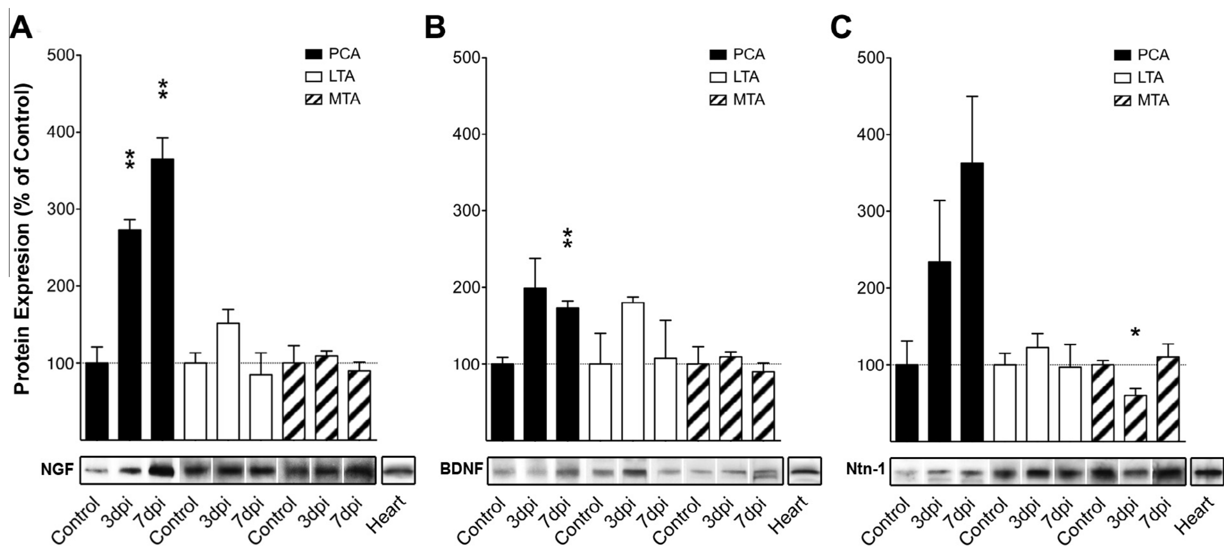

Fig. 6. Western blot analyses showing changes in NGF (A), BDNF (B) and Netrin-1 (C) protein expression at three and seven DPI of the RLN within PCA, LTA and MTA. Bars are determined by densitometry and expressed as percentage of nondenervated controls (mean ± SEM). Asterisk (*) indicates results are significantly different (* means $p < 0.05$; ** means $0.001 < p < 0.01$; and *** means $p < 0.001$).

minimal mRNA overexpression and these values were not statistically significant (Fig. 5).

We have previously shown that following RLN transection regenerating axons innervate PCA first followed by LTA and MTA (Hernandez-Morato et al., 2014b). PCA is the nearest first muscle along the path of regenerating RLN axons (Fig. 1). Regenerating axons at entered the PCA at seven DPI. LTA and MTA are more distal muscles (Fig. 1). Their regenerating axons entered LTA at 14 DPI and MTA at 21 DPI. The pattern of mRNA expression of the present study chronologically correlates with the nerve reinnervation pattern following nerve injury as it has been observed during the larynx innervation during development (Pitman et al., 2013; Hernandez-Morato et al., 2014b) (Figs. 1 and 3).

In evaluating the changes in neurotrophic factors noted in this study, it is important to consider some of the major and unique properties of the laryngeal muscles. The differences between slow and fast subtypes in skeletal muscles are well delineated (Schiaffino and Reggiani, 1996). Slow and type IIA fast muscle have high levels of oxidative enzyme, whereas type IIB fibers have increased glycolytic activity. However, there are also several other myosin heavy chain proteins present in the larynx that have not been found in the limb muscles. DelGaudio and Sciote (1997) showed that the LTA and MTA express a protein found in extraocular muscles that might be responsible for fast contraction time of the laryngeal muscles. In addition, there are elevated levels of hybrid muscle fibers in laryngeal muscles as denoted by fibers having two or more myosin heavy chain proteins (Wu et al., 2000). It has been proposed that such hybrid fibers are responsible for multiple functions in muscles, adding a level of complexity to the laryngeal muscle action. Shiotani et al. documented significant changes in these characteristics of laryngeal muscles after RLN transection in rats (Shiotani and Flint, 1998). Transition in fast subtype proteins in the laryngeal muscle was well documented. They also showed that myosin heavy chain would either decrease or increase in rat laryngeal mus-

cles during RLN regeneration. Considering these unique laryngeal muscle properties and that subsequent changes in laryngeal muscle following RLN transection may be influenced by neurotrophic factor expression, it is important to study the role of trophic factor during regeneration.

Because of the unique properties of laryngeal muscle as compared to other striated muscles of the body, our discussion will be limited to comparing the changes in neurotrophic factors found in the present study to those found in others studies specific to laryngeal muscle. Kingham et al. (2005) reported changes in pig laryngeal muscle with a fivefold increase in NT-3 protein in PCA and a two fold increase in NT-3 in TA muscles, using Western blot. Some changes were seen in NT-4 levels as well. These changes were reported in muscle that had been denervated for 2 months. The results of our study in rats may not be comparable to pig as transected rat RLN regenerates within 7–21 days and increases in the level of trophic factor protein may be species specific.

A recent study on the rat laryngeal muscle following 5-mm transection of the RLN without reanastomosis showed that the BDNF mRNA expression in TA decreased slowly over time. At three weeks BDNF expression decreased significantly. These authors further showed that BDNF expression increased at 6 weeks in PCA (Wang et al., 2015). In our present study, the nerve was transected and anastomosed, and axons began to innervate the muscles by day 7. The higher level of BDNF expression at 7 DPI could be due to this early innervation pattern. It is possible that due to the 5 mm resection in the Wang study, reinnervation occurred but was delayed, hence the increase in BDNF was delayed as well.

NGF and BDNF have been shown in postnatal animals to maintain the neuromuscular connections established during development (Wheeler and Bothwell, 1992; Wang et al., 1995; Ip et al., 2001; Je et al., 2012). Retrograde transport of NGF and BDNF to the neuron cell body has been reported (Heumann et al., 1984; McKay et al., 1996; Curtis et al., 1998; Kishino and Nakayama, 2003). In the present study, the increase of mRNA levels of NGF and BDNF occurred within a day following RLN injury in laryngeal muscles (Fig. 4A) may have been due to the accumulation of trophic factors because of the interruption of retrograde transport due to the transection of the nerve (Xie and Barrett, 1991; Ahmed et al., 2001). Increase in expression of trophic factors continued until 7 DPI and decreased thereafter. Timing of neurotrophic factors expression is correlated to the neuromuscular junction formation in the PCA at 14 days. Similarly, the downregulation of NGF occured when motor-axons established neuromuscular junctions in LTA at 21 DPI.

In the present study, BDNF mRNA upregulation occurred in MTA (Fig. 4B). There was no increase in the level of NGF in MTA except at day 14 (Fig. 4A). We are unable to provide a rational for this occurence. In other studies, peripheral nerve injury led to an increase of NGF and BDNF expression in the denervated muscle (Funakoshi et al., 1993; Sakuma et al., 2001; Michalski et al., 2008; Vega-Cordova et al., 2010). NGF involvement in axon outgrowth and less so in neuron survival has been reported (Barde et al., 1982; Henderson et al., 1993; Hory-Lee et al., 1993). NGF role in neuromuscular junction maintenance has been proposed (Heumann et al., 1984; Curtis et al., 1998). NGF is particularly involved in muscle regeneration and myoprotection (Menetrey et al., 2000; Wu et al., 2009; Ettinger et al., 2012). These studies suggest that NGF plays a critical role in the maintenance of the neuromuscular junction and myoprotection, but is less integral to the establishment of neuromuscular connections. In contrast, the formation and maturation of neuromuscular junction is mediated by BDNF (Wang et al., 1995). BDNF also enhances axonal outgrowth and the reinnervation of the motor end plates following sciatic nerve injury (Zhang et al., 2000; Boyd and Gordon, 2002). In the present experiment, the peak of BDNF mRNA expression chronologically corresponded to the arrival of regenerating axons into the denervated laryngeal muscles. Our results are in concordance with these studies.

NTN-1 plays several roles during development. In the axonal pathfinding of the cranial nerves, NTN-1 repulses the axonal extension in the floor plate of the brainstem during development (Varela-Echavarría et al., 1997). However, the expression of NTN-1 works also as an attractant to the axons during development (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995). In the periphery, a depletion of NTN-1 during development leads to the failure of axons to reach the muscles (Burgess, 2006). In the present study the peak of NTN-1 mRNA overexpression occurred in the PCA at 14 DPI. These results suggest that NTN-1 increases its expression after regenerated axons have established the synapses. Considering NTN-1's known repellent action (Varela-Echavarría et al., 1997; Murray et al., 2010), the increase in NTN-1 may have steered later arriving regenerating axons away from PCA toward the LTA whereas GDNF was overexpressed (Hernandez-Morato et al., 2014b). In LTA, supernumerary axons were less in number compared to PCA which is the first muscle where motor axons establish synapses. Finally, MTA is the last muscle to be reinnervated and relatively few axons reach this muscle. As a result, NTN-1 expression in MTA did not change.

Although numerous studies have shown the role of NT-3, NT-4 and TLR-4 following nerve injury, NT-3, NT-4 and TLR-4 expression in the present study did not change significantly in muscles after nerve transection and regeneration (Copray and Brouwer, 1997; Taylor et al., 2001, 2005). It is conceivable that the lack of such upregulation is due to the mixed and unique nature of laryngeal muscle protein in normal and RLN regenerating muscles. Further studies in laryngeal muscle of other species are needed to clarify the differences.

Regenerating axons, regardless of their origin, encounter the closest muscle first, that is PCA, and innervate it. Later arriving axons innervate LTA (Hernandez-Morato et al., 2013, 2014a, 2014b). This innervation pattern, based on axon speed, may be on reason for the inappropriate synkinetic reinnervation that leads to a paralyzed vocal fold. To influence reinnervation so that axons will reinnervate the proper laryngeal

muscles resulting in return of normal function, we must further investigate the role of neurotrophic factors in the guidance of these axons, as well as the establishment and maintenance of neuromuscular junctions. BDNF, NGF and NTN-1 appear to be most relevant for further study compared to NT-3, NT-4 and TLR-4. NTN-1 is particularly intriguing due to the significant difference in expression of NTN-1 in the PCA compared to the MTA and LTA. Further experiments, either blocking or enhancing the neurotrophic factor expression, may help to further delineate the role of these factors in axon guidance and the formation of specific neuromuscular junctions.

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REFERENCES

Ahmed FAKM, Ingoglia NA, Sharma SC (2001) Axon resealing following transection takes longer in central axons than in peripheral axons: implications for axonal regeneration. Exp Neurol 167:451–455.

Allen SJ, Watson JJ, Shoemark DK, Barua NU, Patel NK (2013) GDNF, NGF and BDNF as therapeutic options for neurodegeneration. Pharmacol Ther:1–21.

Barde YA, Edgar D, Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J 1:549–553.

Belluardo N, Westerblad H, Mudo G, Casabona A, Bruton J, Caniglia G, Pastoris O, Grassi F, Ibañez CF (2001) Neuromuscular junction disassembly and muscle fatigue in mice lacking neurotrophin-4. Mol Cell Neurosci 18:56–67.

Boyd JG, Gordon T (2002) A dose-dependent facilitation and inhibition of peripheral nerve regeneration by brain-derived neurotrophic factor. Eur J Neurosci 15:613–626.

Burgess RW (2006) Motor axon guidance of the mammalian trochlear and phrenic nerves: dependence on the Netrin receptor Unc5c and modifier loci. J Neurosci 26:5756–5766.

Carrasco DI, English AW (2003) Neurotrophin 4/5 is required for the normal development of the slow muscle fiber phenotype in the rat soleus. J Exp Biol 206:2191–2200.

Colamarino SA, Tessier-Lavigne M (1995) The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. Cell 81:621–629.

Copray JC, Brouwer N (1997) Neurotrophin-3 mRNA expression in rat intrafusal muscle fibres after denervation and reinnervation. Neurosci Lett 236:41–44.

Crumley RL (2000) Laryngeal synkinesis revisited. Ann Otol Rhinol Laryngol 109:365–371.

Curtis R, Tonra JR, Stark JL, Adryan KM, Park JS, Cliffer KD, Lindsay RM, DiStefano PS (1998) Neuronal injury increases retrograde axonal transport of the neurotrophins to spinal sensory neurons and motor neurons via multiple receptor mechanisms. Mol Cell Neurosci 12:105–118.

DelGaudio JM, Sciote JJ (1997) Changes in myosin expression in denervated laryngeal muscle. Ann Otol Rhinol Laryngol 106:1076–1081.

Dethleffsen K, Heinrich G, Lauth M, Knapik EW, Meyer M (2003) Insert-containing neurotrophins in teleost fish and their relationship to nerve growth factor. Mol Cell Neurosci 24:380–394.

Ernfors P, Kucera J, Lee KF, Loring J, Jaenisch R (1995) Studies on the physiological role of brain-derived neurotrophic factor and neurotrophin-3 in knockout mice. Int J Dev Biol 39:799–807.

Ettinger K, Lecht S, Arien-Zakay H, Cohen G, Aga-Mizrachi S, Yanay N, Saragovi HU, Nedev H, Marcinkiewicz C, Nevo Y, Lazarovici P (2012) Nerve growth factor stimulation of ERK1/2 phosphorylation requires both p75NTR and $\alpha 9\beta 1$ integrin and confers myoprotection towards ischemia in C2C12 skeletal muscle cell model. Cell Signal 24:2378–2388.

Funakoshi H, Frisen J, Barbany G, Timmusk T, Zachrisson O, Verge VM, Persson H (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. J Cell Biol 123:455–465.

Funakoshi H, Belluardo N, Arenas E, Yamamoto Y, Casabona A, Persson H, Ibáñez CF (1995) Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons. Science (New York, N.Y.) 268:1495–1499.

Garcia N, Santafe MM, Tomas M, Lanuza MA, Besalduch N, Tomas J (2010a) Involvement of neurotrophin-3 (NT-3) in the functional elimination of synaptic contacts during neuromuscular development. Neurosci Lett 473:141–145.

Garcia N, Santafe MM, Tomas M, Lanuza MA, Besalduch N, Tomas J (2010b) Involvement of brain-derived neurotrophic factor (BDNF) in the functional elimination of synaptic contacts at polyinnervated neuromuscular synapses during development. J Neurosci Res 88:1406–1419.

Garcia N, Santafe MM, Tomas M, Lanuza MA, Besalduch N, Tomas J (2010c) Neurotrophin-4 couples to locally modulated ACh release at the end of neuromuscular synapse maturation. Neurosci Lett 468:72–74.

Garcia N, Tomàs M, Santafe MM, Lanuza MA, Besalduch N, Tomàs J (2010d) Localization of brain-derived neurotrophic factor, neurotrophin-4, tropomyosin-related kinase b receptor, and p75NTR receptor by high-resolution immunohistochemistry on the adult mouse neuromuscular junction. J Peripher Nerv Syst 15:40–49.

Gordon T (2009) The role of neurotrophic factors in nerve regeneration. Neurosurg Focus 26:E3.

Gotz R, Köster R, Winkler C, Raulf F, Lottspeich F, Schartl M, Thoenen H (1994) Neurotrophin-6 is a new member of the nerve growth factor family. Nature 372:266–269.

Guthrie S, Pini A (1995) Chemorepulsion of developing motor axons by the floor plate. Neuron 14(6):1117–1130.

Harrington AW, Ginty DD (2013) Long-distance retrograde neurotrophic factor signalling in neurons. Nat Rev Neurosci 14:177–187.

Henderson CE, Camu W, Mettling C, Gouin A, Poulsen K, Karihaloo M, Rullamas J, Evans T, McMahon SB, Armanini MP (1993) Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature 363:266–270.

Hernandez-Morato I, Valderrama-Canales FJ, Berdugo G, Arias G, McHanwell S, Sañudo J, Vazquez T, Pascual-Font A (2013) Reorganization of laryngeal motoneurons after crush injury in the recurrent laryngeal nerve of the rat. J Anat 222:451–461.

Hernandez-Morato I, Berdugo-Vega G, Sanudo JR, McHanwell S, Vazquez T, Valderrama-Canales FJ, Pascual-Font A (2014a) Somatotopic changes in the nucleus ambiguus after section and regeneration of the recurrent laryngeal nerve of the rat. Anat Rec 297:955–963.

Hernandez-Morato I, Isseroff TF, Sharma S, Pitman MJ (2014b) Differential expression of glial-derived neurotrophic factor in rat laryngeal muscles during reinnervation. Laryngoscope 124:2750–2756.

Heumann R, Korsching S, Scott J, Thoenen H (1984) Relationship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. EMBO J 3:3183–3189.

Hory-Lee F, Russell M, Lindsay RM, Frank E (1993) Neurotrophin 3 supports the survival of developing muscle sensory neurons in culture. Proc Natl Acad Sci U S A 90:2613–2617.

Hydman J, Mattsson P (2008) Collateral reinnervation by the superior laryngeal nerve after recurrent laryngeal nerve injury. Muscle Nerve 38:1280–1289.

Ip F, Cheung J, Ip N (2001) The expression profiles of neurotrophins and their receptors in rat and chicken tissues during development. Neurosci Lett 301:107–110.

Je SH, Yang F, Ji Y, Nagappan G, Hempstead BL, Lu B (2012) Role of pro-brain-derived neurotrophic factor (proBDNF) to mature

BDNF conversion in activity-dependent competition at developing neuromuscular synapses. PNAS 109:15924–15929.

Kingham PJ, Birchall MA, Terenghi G (2005) Neurotrophin expression in laryngeal muscles following denervation and reinnervation [WWW Document]. J Peripher Nerv Syst.

Kishino A, Nakayama C (2003) Enhancement of BDNF and activated-ERK immunoreactivity in spinal motor neurons after peripheral administration of BDNF. Brain Res 964:56–66.

Koliatsos VE, Cayouette MH, Berkemeier LR, Clatterbuck RE, Price DL, Rosenthal A (1994) Neurotrophin 4/5 is a trophic factor for mammalian facial motor neurons. Proc Natl Acad Sci U S A 91:3304–3308.

Lauderdale JD, Davis NM, Kuwada JY (1997) Axon tracts correlate with netrin-1a expression in the zebrafish embryo. Mol Cell Neurosci 9:293–313.

Lindsay RM (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J Neurosci 8:2394–2405.

Liou JC, Fu WM (1997) Regulation of quantal secretion from developing motoneurons by postsynaptic activity-dependent release of NT-3. J Neurosci 17:2459–2468.

McKay SE, Garner A, Caldero J, Tucker RP, Large T, Oppenheim RW (1996) The expression of trkB and p75 and the role of BDNF in the developing neuromuscular system of the chick embryo. Development 122:715–724.

Menetrey J, Kasemkijwattana C, Day CS, Bosch P, Vogt M, Fu FH, Moreland MS, Huard J (2000) Growth factors improve muscle healing in vivo. J Bone Joint Surg Br 82:131–137.

Michalski B, Bain JR, Fahnestock M (2008) Long-term changes in neurotrophic factor expression in distal nerve stump following denervation and reinnervation with motor or sensory nerve. J Neurochem 105:1244–1252.

Mitchell KJ, Doyle JL, Serafini T, Kennedy TE, Tessier-Lavigne M, Goodman CS, Dickson BJ (1996) Genetic analysis of Netrin genes in *Drosophila*: Netrins guide CNS commissural axons and peripheral motor axons. Neuron 17:203–215.

Mousavi K, Parry DJ, Jasmin BJ (2004) BDNF rescues myosin heavy chain IIB muscle fibers after neonatal nerve injury. Am J Physiol Cell Physiol 287:C22–C29.

Murray A, Naeem A, Barnes SH, Drescher U, Guthrie S (2010) Slit and Netrin-1 guide cranial motor axon pathfinding via Rho-kinase, myosin light chain kinase and myosin II. Neural Dev 5:16.

Ogborn DI, Gardiner PF (2010) Effects of exercise and muscle type on BDNF, NT-4/5, and TrKB expression in skeletal muscle. Muscle Nerve 41:385–391.

Omura T, Sano M, Omura K, Hasegawa T, Doi M, Sawada T, Nagano A (2005) Different expressions of BDNF, NT3, and NT4 in muscle and nerve after various types of peripheral nerve injuries. J Peripher Nerv Syst 10:293–300.

Pitman MJ, Weissbrod P, Roark R, Sharma S, Schaefer SD (2011) Electromyographic and histologic evolution of the recurrent laryngeal nerve from transection and anastomosis to mature reinnervation. Laryngoscope 121:325–331.

Pitman MJ, Berzofsky CE, Alli O, Sharma S (2013) Embryologic innervation of the rat laryngeal musculature-a model for investigation of recurrent laryngeal nerve reinnervation. Laryngoscope 123:3117–3126.

Pitts EV, Potluri S, Hess DM, Balice-Gordon RJ (2006) Neurotrophin and Trk-mediated signaling in the neuromuscular system. Int Anesthesiol Clin 44:21–76.

Radin MS, Sinha S, Bhatt BA, Dedousis N, O'Doherty RM (2007) Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle. Diabetologia 51:336–346.

Reyna SM, Ghosh S, Tantiwong P, Meka CSR, Eagan P, Jenkinson CP, Cersosimo E, DeFronzo RA, Coletta DK, Sriwijitkamol A, Musi N (2008) Elevated Toll-Like Receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes 57:2595–2602.

Saka Y, Yoshimura O, Tahara H, Takeda Y, Moriyama H, Maejima H, Tobimatsu Y (2007) The mRNA expression of neurotrophins in different skeletal muscles of young rats. Hiroshima J Med Sci 56:23–28.

Sakuma K, Watanabe K, Sano M, Uramoto I, Nakano H, Li YJ, Kaneda S, Sorimachi Y, Yoshimoto K, Yasuhara M, Totsuka T (2001) A possible role for BDNF, NT-4 and TrkB in the spinal cord and muscle of rat subjected to mechanical overload, bupivacaine injection and axotomy. Brain Res 907:1–19.

Schiaffino S, Reggiani C (1996) Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Physiol Rev 76:371–423.

Shiotani A, Flint PW (1998) Myosin heavy chain composition in rat laryngeal muscles after denervation. Laryngoscope 108:1225–1229.

Sun KLW, Correia JP, Kennedy TE (2011) Netrins: versatile extracellular cues with diverse functions. Development 138:2153–2169.

Sunderland S (1978) Nerves and nerve injuries. 2nd ed. New York: Churchill Livingstone.

Taylor MD, Vancura R, Patterson CL, Williams JM, Riekhof JT, Wright DE (2001) Postnatal regulation of limb proprioception by muscle-derived neurotrophin-3. J Comp Neurol 432:244–258.

Taylor MD, Holdeman AS, Weltmer SG, Ryals JM, Wright DE (2005) Modulation of muscle spindle innervation by neurotrophin-3 following nerve injury. Exp Neurol 191:211–222.

Terenghi G (1999) Peripheral nerve regeneration and neurotrophic factors. J Anat 194(Pt 1):1–14.

Tessema B, Pitman MJ, Roark RM, Berzofsky C, Sharma S, Schaefer SD (2008) Evaluation of functional recovery of recurrent laryngeal nerve using transoral laryngeal bipolar electromyography: a rat model. Ann Otol Rhinol Laryngol 117:604–608.

Tessema B, Roark RM, Pitman MJ, Weissbrod P, Sharma S, Schaefer SD (2009) Observations of recurrent laryngeal nerve injury and recovery using a rat model. Laryngoscope 119:1644–1651.

Tomàs J, Santafe MM, Lanuza MA, Garcia N, Besalduch N, Tomàs M (2011) Silent synapses in neuromuscular junction development. J Neurosci Res 89:3–12.

Varela-Echavarría A, Tucker A, Püschel AW, Guthrie S (1997) Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D. Neuron 18:193–207.

Vega-Cordova X, Cosenza NM, Helfert RH, Woodson GE (2010) Neurotrophin expression of laryngeal muscles in response to recurrent laryngeal nerve transection. Laryngoscope 120:1591–1596.

Wang T, Xie K, Lu B (1995) Neurotrophins promote maturation of developing neuromuscular synapses. J Neurosci 15:4796–4805.

Wang B, Yuan J, Xu J, Xie J, Wang G, Dong P (2015) Neurotrophin expression and laryngeal muscle pathophysiology following recurrent laryngeal nerve transection. Mol Med Rep.

Welc SS, Clanton TL, Dineen SM, Leon LR (2013) Heat stroke activates a stress-induced cytokine response in skeletal muscle. J Appl Physiol 115:1126–1137.

Wheeler EF, Bothwell M (1992) Spatiotemporal patterns of expression of NGF and the low-affinity NGF receptor in rat embryos suggest functional roles in tissue morphogenesis and myogenesis. J Neurosci 12:930–945.

Wright DE, Zhou L, Kucera J, Snider WD (1997) Introduction of a neurotrophin-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous neurotrophin-3. Neuron 19:503–517.

Wu YZ, Baker MJ, Crumley RL, Caiozzo VJ (2000) Single-fiber myosin heavy-chain isoform composition of rodent laryngeal muscle: modulation by thyroid hormone. Arch Otolaryngol Head Neck Surg 126:874–880.

Wu C, Erickson MA, Xu J, Wild KD, Brennan TJ (2009) Expression profile of nerve growth factor after muscle incision in the rat. Anesthesiology 110:140–149.

Wu S-C, Rau C-S, Lu T-H, Wu C-J, Wu Y-C, Tzeng S-L, Chen Y-C, Hsieh C-H (2013) 2013 Wu_Knockout of TLR4 and TLR2 impair

the nerve regeneration by delayed demyelination but not remyelination. J Biomed Sci 20:1.
Xie XY, Barrett JN (1991) Membrane resealing in cultured rat septal neurons after neurite transection: evidence for enhancement by Ca(2+)-triggered protease activity and cytoskeletal disassembly. J Neurosci 11:3257–3267.
Xie K, Wang T, Olafsson P, Mizuno K, Lu B (1997) Activity-dependent expression of NT-3 in muscle cells in culture: implications in the development of neuromuscular junctions. J Neurosci 17:2947–2958.
Zhang JY, Luo XG, Xian CJ, Liu ZH, Zhou XF (2000) Endogenous BDNF is required for myelination and regeneration of injured sciatic nerve in rodents. Eur J Neurosci 12:4171–4180.

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