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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.

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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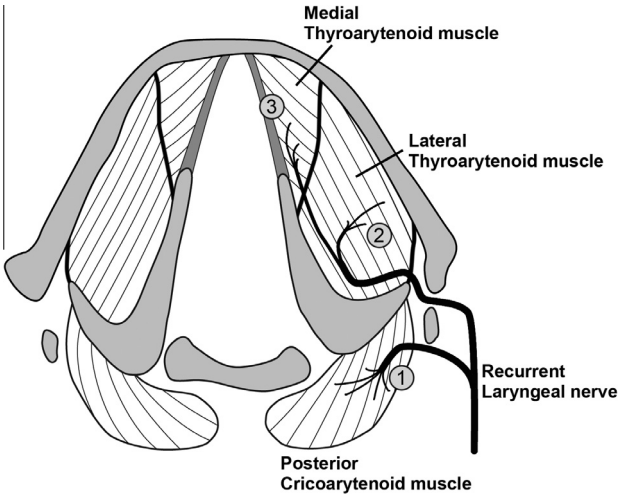

Fig. 1. Representation of transversal section of the rat larynx. The recurrent laryngeal nerve innervates the ipsilateral larynx. Its branches innervate the abductor posterior cricoarytenoid muscle first (1), followed by the adductors lateral thyroarytenoid muscle (2) and median thyroarytenoid muscle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