Somatotopic Changes in the Nucleus Ambiguus After Section and Regeneration of the Recurrent Laryngeal Nerve of the Rat

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ABSTRACT

Changes in motoneurons innervating laryngeal muscles after section and regeneration of the recurrent laryngeal nerve (RLN) are far from being understood. Here, we report the somatotopic changes within the nucleus ambiguus (Amb) after the nerve injury and relates it to the resulting laryngeal fold impairment. The left RLN of each animal was transected and the stumps were glued together using surgical fibrin glue. After several survival periods (1, 2, 4, 8, 12, 16 weeks; at least six rats at each time point) the posterior cricoarytenoid (PCA) and thyroarytenoid (TA) muscles were injected with fluorescent-conjugated cholera toxin and the motility of the vocal folds evaluated. After section and subsequent repair of the RLN, no movement of the vocal folds could be detected at any of the survival times studied and the somatotopy and the number of labeled motoneurons changed. From 4 wpi award, the somatotopy was significantly disorganized, with the PCA motoneurons being located rostrally relative to their normal location. A rostrocaudal overlap between the two pools of motoneurons supplying the PCA and TA muscles was observed from 2 wpi onwards. Hardly any labeled neurons were found in the contralateral Amb in any of the experimental groups. An injury of the RLN leads to a reinnervation of the denervated motor endplates of PCA and TA. However, misdirected axons sprout and regrowth from the proximal stump to the larynx. As a result, misplaced innervation of muscles results in a lack of functional recovery of the laryngeal folds movement following a RLN injury. Anat Rec, 297:955–963, 2014. © 2014 Wiley Periodicals, Inc.

Key words: peripheral nerve injury; somatotopy; motoneurons; larynx

Abbreviations used: Amb = nucleus ambiguus; CT = cricothyroid muscle; CtB = cholera toxin subunit B; LCA = lateral cricoarytenoid muscle; PCA = posterior crocoarytenoid muscle; RLN = recurrent laryngeal nerve; SLN = superior laryngeal nerve; TA = thyroarytenoid muscle.

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The recurrent laryngeal nerve (RLN) and the superior laryngeal nerve (SLN) are branches of the vagus nerve that supply a motor innervation to the larynx. All laryngeal muscles except the cricothyroid muscle (CT) are innervated by RLN. Hence, the sole abductor muscle, the posterior cricoarytenoid (PCA), the adductor muscles, lateral cricoarytenoid (LCA) and the interarytenoid muscles, and the thyroarytenoid (TA) muscle, are all innervated by the RLN (Sasaki, 2006; Standring, 2008). An injury of the RLN causes a paralysis of the laryngeal vocal fold that can be permanent (Crumley, 1982, 2000). Attempts to restore the vocal fold impairment have been unsuccessful because the processes of laryngeal reinnervation following a nerve injury are not fully understood.

The cell bodies that innervate the laryngeal muscles are located in the nucleus ambiguus (Amb), a column of motoneurons located within brainstem. Previous studies have demonstrated that motoneurons of the larynx are distributed in several pools with defined rostrocaudal locations within the Amb (Szentagothai, 1943; Yoshida et al., 1982; Hisa et al., 1984; Okubo et al., 1987; Portillo and Pasaro, 1988; Hirasugi et al., 2007; Hernández-Morato et al., 2013a).

Nerve injury leads to morphological changes, in both the peripheral and the central nervous systems (Ramón y Cajal, 1914; Seddon, 1942; Sunderland, 1978; Angelov and Neiss, 1994). The effect of peripheral nerve injury has been extensively studied in several cranial nerves, such as facial, hypoglossal, femoral, and oculomotor nerves (Thomander, 1984; Aldskogius and Thomander, 1986; Fernández et al., 1985, 1987; Mizuno et al., 1980; Irintchev, 2011), but there is a paucity of information about the consequences of injury to the laryngeal nerves regarding organization within the Amb (Nahm et al., 1990, 1993; Flint et al., 1991; Hydman and Mattsson, 2008).

The process of regeneration and reinnervation in the peripheral nervous system is a function of the extent of the nerve injury (Seddon, 1942; Sunderland, 1978). Following a crush injury (axonotmesis) of RLN, the continuity of the axons in the nerve is disrupted but the integrity of the perineurium and epineurium remains relatively unaffected. Hence, axonal regeneration and functional recovery can occur within a defined period postinjury (Tessema et al., 2008, 2009; Hernández-Morato et al., 2013b). However, somatotopy of the motor nuclei of the individual intrinsic laryngeal muscles within the Amb is dramatically changed after the crush injury and the previous pattern is never fully restored (Flint et al., 1991; Nahm et al., 1990, 1993; Hernández-Morato et al., 2013b). In contrast, much less is known about the effects of complete section of the RLN (neurotmesis) that causes a complete disruption of the continuity of the nerve both of the axons themselves, their myelin sheaths and of the connective tissue within the nerve. The few experiments that have been performed to study the effects of complete RLN section report that the complete restoration of laryngeal fold mobility did not take place (Nahm et al., 1993, Guérout et al., 2011) and that somatotopic organization of the motoneurons within the Amb was not restored (Flint et al., 1991; Nahm et al., 1993).

The present work was undertaken to provide a comprehensive study of the changes in the topographic distribution of motoneurons innervating the intrinsic muscles of the larynx following section (neurotmesis) of the RLN in the rat. The importance of this study in comparison to those based on axonotmesis is that it is closer to the clinical situation of nerve lesion after, for example, complications following neck or chest surgery, especially thyroid tumor surgery, and may serve as a future basis for effecting successful laryngeal nerve repair following damage after surgery or in laryngeal transplantation.

MATERIAL AND METHODS

Animals

The present research was undertaken in accordance with the laws relating to the care and handling of animals in research of the European Union (2010/63/EU) and of Spain (Royal Decree 1201/2005), and was approved by the Committee of Animal Experimentation of the Complutense University of Madrid. Experiments were carried out on 51 adult male Sprague-Dawley rats (*Rattus norvegicus*) of 250–350 g body weight. Animals were maintained in the central facilities of the Complutense University, and all the surgical procedures were performed in its animal operating room. During the first 2 days following surgery, the animals were treated with an analgesic protocol consisting of a dose of buprenorphine (0.05 mg/kg) plus meloxicam (1.0 mg/kg) administered every 8 hr.

Surgical Procedure: RLN Section and Repair

The surgery was performed as described in earlier studies (Hernández-Morato et al., 2013b). Briefly, animals were anesthetized with a mixture of xylazine (Rompun, Bayer, Spain, 8 mg/kg) plus ketamine (Imalgene, Merial, France, 90 mg/kg), a midline neck incision was made, the larynx exposed and the RLN identified. At the level corresponding to the transverse plane of the seventh tracheal ring, the RLN was cut with fine scissors. After the section, the stumps were glued together using surgical fibrin glue (*Tissucol®*, Baxter, Viena, Austria). The surgical wound was closed in layers. Immediately after surgery and before the animal recovered consciousness, the completeness of the injury was confirmed in each animal using a laryngoscope to assess the extent to which the left vocal fold had been immobilized in a manner similar to that described in previous work (Hernández-Morato et al., 2013b).

Animals were allowed to survive for periods of 1 ($n = 7$), 2 ($n = 6$), 4 ($n = 8$), 8 ($n = 7$), 12 ($n = 7$), and 16 ($n = 7$) weeks (wpi: weeks postinjury).

Surgical Procedure: Muscle Tracing

Three days before the survival time was reached, the animals were reanesthetized and the surgical field was exposed as described previously in order to inject retrograde tracers into the posterior cricoarytenoid (PCA) and thyroarytenoid (TA) muscles. Each muscle was injected with 0.5 μL of 10 μg/mL (1%) cholera toxin (CtB) conjugated to either alexa fluor 488 (AF488) (green fluorescence) or alexa fluor 594 (AF594) (red fluorescence), using a 30-μm tip glass electrode attached to a 1-mL Hamilton syringe. In order to control for the possibility that the order in which the muscles were injected

TABLE 1. Number of motoneurons labeled following the injection of retrograde tracer into the posterior cricoarytenoid muscle (PCA) and the thyroarytenoid muscle (TA)

Group		Control ($n = 9$)	1 wpi ($n = 7$)	2 wpi ($n = 6$)	4 wpi ($n = 8$)	8 wpi ($n = 7$)	12 wpi ($n = 7$)	16 wpi ($n = 7$)
No. of neurons	PCA	41 ± 7	28 ± 14	50 ± 13	52 ± 14	130 ± 30	105 ± 32	116 ± 18
	TA	31 ± 4	0 ± 0	12 ± 5	8 ± 3	43 ± 12	44 ± 19	42 ± 11

Data expressed as mean ± SEM.

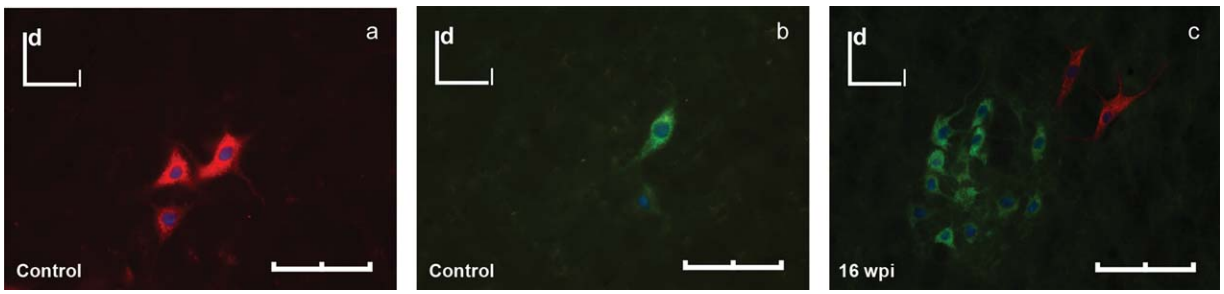

Fig. 1. Labeled motoneurons within nucleus ambiguus: (**a**) following an injection of CtB-AF594 into the thyroarytenoid (TA) muscle in normal control animal; (**b**) following an injection of CtB-AF488 into the posterior cricoarytenoid muscle in normal control animal; (**c**) following an injection of CtB-AF488 (green) into the PCA muscle and of CtB-AF594 (red) into the TA muscle, 16 weeks after section and regeneration (wpi) of the recurrent laryngeal nerve. d, dorsal; l, lateral. Scale bar: 100 μm.

could affect the results, in half the experiments, the PCA was injected before the TA, whereas in the remaining animals the TA was the first muscle to be injected. For a similar reason and as a second control, the fluorescent conjugate of cholera toxin injected into either the PCA or the TA was varied between different animals at each survival time. The injection procedure was the same as we reported previously (Hernández-Morato et al., 2013a,b). Briefly, CtB conjugated to either AF488 or AF594 were injected into PCA after the constrictor muscle was reflected. The CtB conjugated to the fluor not injected into PCA was injected into the TA after opening a window in the thyroid cartilage. At the end of the procedure, the surgical field was cleaned with saline and the wound closed in layers.

In order to determine the normal distribution of the PCA and TA motoneurons within the Amb, nine animals, in which the laryngeal nerves had been left intact, were injected with tracer (control group).

Histological Procedures

At the end of the survival period prior to the animals being killed, laryngeal fold mobility was once more examined laryngoscopically The rats then received a lethal intraperitoneal injection of pentobarbital (200 mg/kg). Samples were collected as previously described (Pascual-Font et al., 2011; Hernández-Morato et al., 2013a,b). Briefly, animals were perfused with saline (250–300 mL, 37°C) followed by paraformaldehyde (400 mL, 4% in 0.1 M phosphate buffer); the brainstems were removed and embedded for sectioning with a cryostat (serial 50-μm transverse sections from pyramidal decussation to the facial nucleus, about 6 mm). Finally, sections were stained with DAPI (Roche, Basel, Switzerland), mounted onto slides and analyzed with a fluorescent microscope (Nikon Eclipse 800M).

The neuron counting and the rostrocaudal location of the labeled motoneurons was carried out with reference to specific landmarks that have been selected previously (Hamilton and Norgren, 1984; Paxinos and Watson, 2005; Pascual-Font et al., 2011; Hernández-Morato et al., 2013a,b). The counting of the labeled neurons was always undertaken by two independent observers. In each animal, the total number of neurons was recorded, and the mean (± SEM) was calculated for each studied muscle. The sections were examined at high magnification (40×) with the appropriate filter. The following criteria were used to identify cholera toxin-labeled fluorescent structures as a neuronal soma: the presence of a granular fluorescent product confined to the cytoplasm with a nucleus distinguishable as an area relatively clear of fluorescence. Once the neuronal perikaryon was recognized, and the nucleus was focused, the filter was changed in order to confirm the DAPI-labeled nucleus and to identify the nucleolus. Only the neurons in which the nucleolus was present were counted. All numerical data are expressed as mean ± SEM. Statistical comparisons between means were made by analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests. Differences were considered statistically significant at the $P < 0.05$ level.

RESULTS

Control Group

The number of labeled motoneurons in the Amb following the injection of CtB into the PCA was 41 (± 7) and following the injection of the TA was 31 (± 4) (Table 1). All the motoneurons exhibited a multipolar morphology, as in the experimental groups (Fig. 1).

Neuronal labeling was observed ipsilateral to the injected muscle. The pool of motoneurons innervating the PCA muscle was located rostrally to the pool of

motoneurons innervating TA, as two discrete and separate columns in the majority of control animals. However, in two control animals, there was a small area of rostrocaudal overlap between the pools. The PCA muscle motoneuron pool was located between 907 (± 48) and 1985 (± 87) μm rostral to the obex whereas the TA pool was located between 335 (± 67) μm caudal to the obex and 720 (± 56) μm rostral to it (Fig. 2).

Experimental Group

Functional Evaluation of the Vocal Folds. After section and regeneration of the left RLN, all experimental animals had an ipsilateral paralysis and complete immobility of the vocal folds.

Number of Motoneurons. At 1 wpi following injection of tracer into the TA muscle, no labeled neurons were found in the Amb in four of the six animals of the group, while in the remaining two animals only a single labeled neuron was seen in each animal. In contrast, the number of labeled motoneurons following injection into the PCA muscle varied widely from 1 to 32 (mean 28 ± 14) (Table 1).

From 2 wpi onward, the number of labeled neurons following injection into TA and PCA increased in all groups. At 2 and 4 wpi, the number of labeled motoneurons in the Amb following injection into TA muscle was less than in control animals, but at 8, 12, and 16 wpi, the numbers observed were closer to those found in the control group. The number of labeled motoneurons in the Amb labeled from PCA injection increased from 2 wpi onward. From 8 to 16 wpi, the number of labeled neurons was observed to be much higher in comparison to control animals (Table 1).

Location of Motoneurons. Labeled motoneurons were observed ipsilaterally to the injected muscle from 1 to 16 wpi. However, the somatotopic organization of the motoneurons was completely altered in all experimental animals at all survival times, when compared to the control groups (Fig. 2). In addition, labeling of neurons in the contralateral Amb was also found in several animals in all experimental groups (Fig. 3).

At 1 wpi, no labeled neurons were found after the injection of tracer into the TA muscle. Following injection of the tracer into PCA, labeled motoneurons were located in a similar region of the Amb to that seen in the control group.

At 2 wpi, the labeled neurons from TA muscle were found to be located in the same region as that of the control group. In contrast, motoneurons labeled following injection into the PCA muscle were found along the entire rostrocaudal extent of the Amb.

From 4 wpi, the somatotopy of the motoneurons was more disorganized. In animals from these survival periods, the rostrocaudal distribution of labeled neurons was more extensive than in the control group. In control animals, neurons labeled following injections into the TA and PCA muscles were confined to the caudal region of the Amb corresponding to the territory of RLN. In experimental groups from 4 wpi survival onward, neurons that innervated the PCA were additionally found rostral to the cranial end of RLN region of the Amb.

In addition, from 2 wpi onward, there was an increasing degree of rostrocaudal overlap between the two pools of motoneurons supplying the TA and PCA muscles. The length of the rostrocaudal overlap between the two motoneuron pools varied in extent from 0.5 to 1.7 mm, and it increased with the survival time (Fig. 2; Table 2). Double-labeled neurons were found in all groups, that is, labeled with CTb-AF488 injected in one muscle and with CTb-AF594 injected in the other one (Fig. 4; Table 3).

In all experimental groups, in some animals, some labeled motoneurons were found in the contralateral Amb (Fig. 3). Following injection of CtB in the PCA muscle, the proportion of animals in which contralateral neurons were found to be labeled varied between 60% and 80% from 1 to 8 wpi. The proportion decreased in animals examined following longer survival periods (12 and 16 wpi). The mean number of contralateral neurons found from 1 to 4 wpi was similar. At 8 and 12 wpi, the number increased dramatically but thereafter the numbers declined. Following the injection into the TA muscle, the proportion of animals with contralateral neurons were found was approximately 50%. However, at 12 wpi, no contralateral-labeled neurons were found in any animal. After injection of CtB into the TA muscle, the number of labeled neurons observed seldom exceeded 18 in animals from any survival period though in one animal, injected at 2 wpi, there were 40 contralateral-labeled neurons.

DISCUSSION

In this study, we have shown that an injury to the RLN involving total section of the nerve (neurotmesis) followed by varying survival periods to allow regeneration results in complete paralysis of the ipsilateral laryngeal fold at all survival times studied up to 16 weeks after injury. It also leads to a progressive disorganization of somatotopic distribution of motoneurons innervating the PCA and TA muscles.

When comparing the present results with those of our previous study of RLN crush injury (axonotmesis) (Hernández-Morato et al., 2013b), differences in the effects of the two types of nerve injury are clearly observed. This is because in comparison to neurotmesis, an axonotmesis affects the continuity of the axons but not the integrity of epineurium, perineurium, or endoneuirum.

As we reported in previous studies, we have retrogradely labeled neurons innervating the larynx using cholera toxin conjugated to one of two different alexa fluorochromes (Hernández-Morato et al., 2013a,b). CtB is a tracer that has been used in a large number of neuroanatomical studies related to the Amb (Bieger and Hopkins, 1987; Saxon et al., 1996; Yoshida et al., 1998; Berkowitz et al., 1999; Hayakawa et al., 1999; Walbaum et al., 2001). In our present study, animals were sacrificed 3 days after injection of the tracer because this time period is within the range that has been used by most authors (Bieger and Hopkins, 1987; Saxon et al., 1996; Yoshida et al., 1998; Berkowitz et al., 1999; Hayakawa et al., 1999). In our previous experiments, we found that volumes of greater than 0.5 μL when injected into muscles did not result in higher numbers of neurons being labeled within Amb suggesting that leakage of tracer to adjacent muscles is not a significant problem in our experiments. In addition, we also reported that

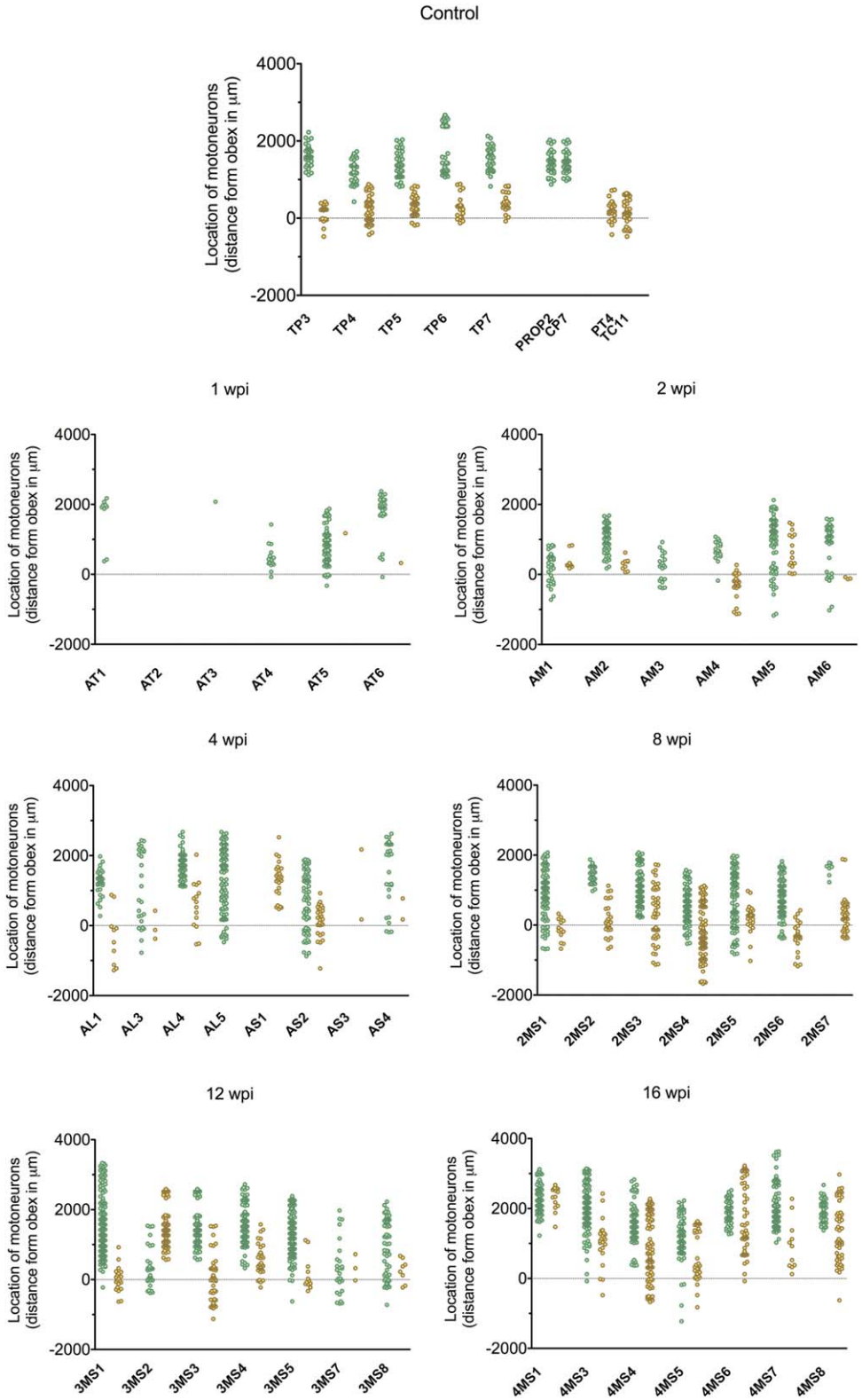

Fig. 2. Rostrocaudal distribution of labeled motoneurons within the brainstem following a CtB injection into the posterior cricoarytenoid (green spots) and thyroarytenoid (yellow spots) muscles. (**a**) Location of labeled motoneurons in control group. In five animals, both muscles were injected at the same time. In remaining four animals, tracer was just injected into one muscle. No differences can be seen between these distributions in either case. (**b–g**) Location of labeled motoneurons following an injury to the RLN; 0 represents obex level; wpi, weeks postinjury.

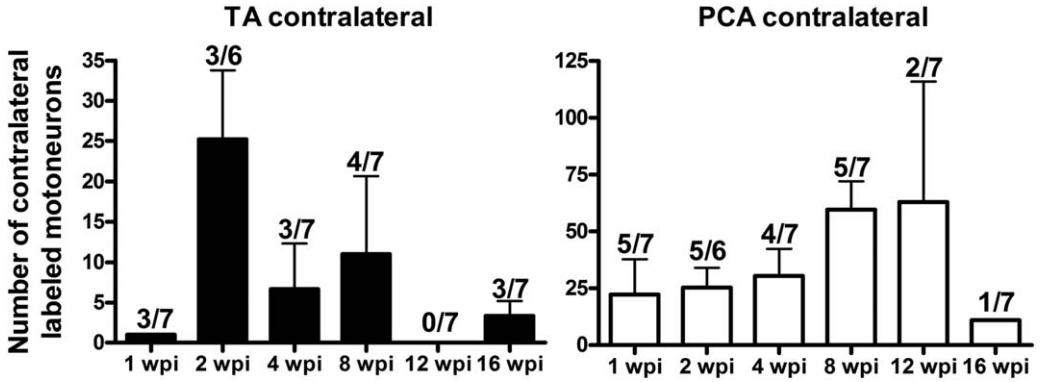

Fig. 3. Number of motoneurons labeled on the contralateral side to the injection site after recurrent laryngeal nerve section and repair. The numbers on the bars represent the ratio of animals that showing contralateral labeled motoneurons with respect to the total number of animals in each group. Error bars: standard error of mean. PCA, posterior cricoarytenoid; TA, thyroarytenoid; wpi, weeks postinjury.

TABLE 2. Degree of overlap between the pools of motoneurons supplying the posterior cricoarytenoid muscle and the thyroarytenoid muscle

Group	Control	1 wpi	2 wpi	4 wpi	8 wpi	12 wpi	16 wpi
Region of overlap (μm)	0	0 ± 0	610 ± 230	840 ± 145	1150 ± 250	950 ± 75	1735 ± 210

The rostrocaudal length of the overlap between the two motoneuron pools varied in extent between 615 and 1735 μm in all experimental groups, except in the control group, in which there is barely any degree of overlap, and in 1wpi group, in which there were no labeled motoneurons after the injection of tracer into the thyroarytenoid muscle. Data expressed as mean ± SEM.

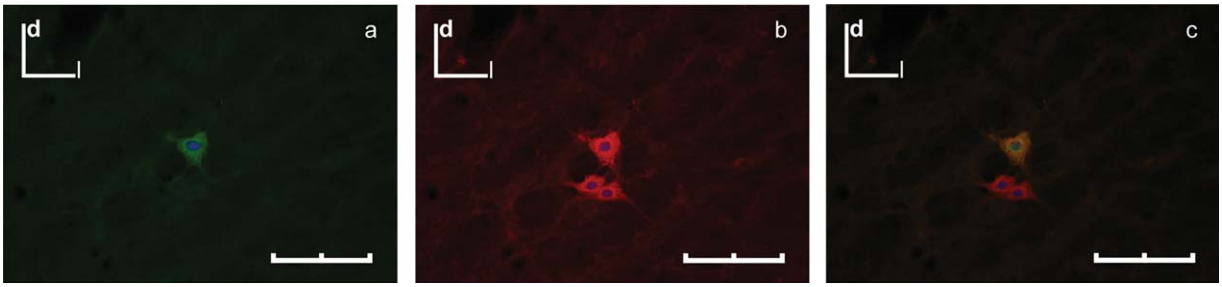

Fig. 4. Labeled neurons within the Amb 8 weeks after section and regeneration of the recurrent laryngeal nerve, following an injection of CtB-AF488 (green) into the TA muscle (**a**) or CtB-AF594 (red) into the cricoarytenoid muscle (**b**). (**c**) Merge of a and b. d, dorsal; l, lateral. Scale bar: 100 μm.

TABLE 3. Number of double-labeled motoneurons after an injection of CtB into the posterior cricoarytenoid muscle and the thyroarytenoid muscles, with CTb-AF488 injected in one muscle and with CTb-AF594 injected in the other one, at different interval times after the section and repair of the recurrent laryngeal nerve

Group	Control	1 wpi	2 wpi	4 wpi	8 wpi	12 wpi	16 wpi
No. of double-labeled motoneurons	0	1 ± 0	6 ± 0	4.5 ± 4.9	7.2 ± 2.5	1.5 ± 0.7	3.25 ± 1.5
No. of rats	0/9	1/7	2/6	2/8	5/7	2/7	4/7

The bottom row of the table shows the number of animals in which double-labeled motoneurons were found with respect to the total number of animals of each group. Data expressed as mean ± SEM.

spread of tracer to muscles that are innervated by the SLN does not occur under our experimental conditions further supporting this conclusion. When the CtB was spread randomly over the larynx, only a few labeled neurons could be observed and these were located in cervical segments of the spinal cord, but never in the brainstem. Taken together, all these data lead us to believe that, using this tracing method, there is no tracer diffusion from the site of injection (Hernandez-Morato et al., 2013a,b).

Functional Evaluation of the Vocal Folds

No movement of the vocal folds was seen immediately after section and repair of the RLN and was never restored at any of the survival times studied. These observations are in agreement with other studies of complete transection and repair of the RLN, where signs of laryngeal fold movement were either never (Nahm et al., 1993; Miyamaru et al., 2009) or rarely observed (McRae et al., 2009; Pitman et al., 2011). Although restoration of detectable laryngeal fold movements after complete nerve section have been observed only inconsistently, electromyographic analyses have shown a progressive restoration of the normal preinjury potential firing pattern (Nahm et al., 1993; Pitman et al., 2011). The latter evidence indicates that, after a section and repair of the RLN, a regeneration of the affected axons does occur, but the reinnervation process fails to produce any significant functional recovery.

In contrast to these functional findings following neurotmesis, after a RLN crush injury (axonotmesis), functional laryngeal fold movements were restored from 6 to 8 wpi (Tessema et al., 2008, 2009; Hernández-Morato et al., 2013b). This difference may be explained by the severity of the injury made in the nerve following complete nerve section preventing sufficient reinnervation necessary to restore functional movements. The reason for this remains to be explained but is clearly related to the greater disruption of the structural integrity of the nerve.

Location of Laryngeal Motoneurons

The results obtained in this study show that following complete section of the laryngeal nerve during regeneration there is a disorganization of the topography in the Amb. This disorganization persists at all survival times and was similar to that observed in previous studies of neurotmesis where the location of the laryngeal neuron pools appeared to be distributed randomly (Flint et al., 1991; Nahm et al., 1993). In addition, this disorganization has also been reported as a result of an axonotmesis of the nerve following crush (Hernández-Morato et al., 2013b) or cryoinjury (Nahm et al., 1990). One can speculate that the regenerating axons grow back randomly from the proximal stump to the laryngeal muscles.

Disorganization of the topography following a peripheral nerve injury have been described not only in the Amb but also in other cranial nuclei including; the facial nucleus (Thomander, 1984; Aldskogius and Thomander, 1986; Nakao et al., 1992; Angelov and Neiss, 1994; Angelov et al., 1996; Streppel et al., 1998; Choi and Raisman, 2002; Guntinas-Lichius et al., 2001, 2002, 2005; Franchi et al., 2006; Grosheva et al. 2008), extraocular nuclei (Fernández et al., 1985, 1992; Scherer, 1986; Sibony et al., 1986), hypoglossal nucleus (Mizuno et al., 1980), and in the spinal cord after a lesion in the sciatic nerve (Aldskogius et al., 1987; Wasserschaff, 1990; Fernández et al., 1992; Katada et al., 2006; de Ruiter et al., 2008; Landegren et al., 2011). These reports indicate that following a transection and repair of any peripheral nerve in the adult, the reinnervation of the denervated structures is not as selective as that occurring during development and the resulting disorganization of the somatotopic distribution of the denervated motoneurons persists. However, several studies demonstrate that despite this disorganization a partially functional motor recovery can occur (Thomander, 1984; Sibony et al., 1986; Asahara et al., 1999; Choi and Raisman, 2002; Guntinas-Lichius et al., 2001, 2002, 2005; Grosheva et al., 2008; de Ruiter et al. 2008; Hernández-Morato et al., 2013b) indicating that an altered somatotopic map is not a limiting factor in producing some functional recovery. It remains unclear if accurate regrowth of afferent connections onto motoneurons after a nerve injury is a prerequisite for a functional recovery. Different degrees of disorganization of the somatotopy in Amb have been found in animals following an axonotmesis or a neurotmesis of the NLR (Namh et al., 1993; Hernández-Morato et al., 2013b). The type of injury made on the nerve affects the rate of the reinnervation of the laryngeal muscles. As a transection leads to a more severe outcome than a crush of the RLN, the absence of laryngeal fold movement following an injury of the nerve must be related to the greater loss of somatotopy of neurons within the Amb.

From 4 to 12 wpi, the PCA motoneuron pool was found to extend more rostrally in comparison to the control population, and this rostral region corresponds to the region occupied by the motoneurons whose axons travel within the SLN (Pascual-Font et al., 2011). This fact suggests that there has been terminal reinnervation from neighboring nerves including the SLN. Following crush injury, we found motoneurons in this rostral region of the Amb from 1 to 4 wpi only, which is in complete contrast to the results of transection experiments described in the present study in which the rostral-most extension persisted until 12 wpi. These differences might be related to proximal sprouting following the injury in the nerve. We suggest that after a transection of the RLN collateral branching from SLN persists longer. Hence, the possibility of mature collateral branching that might affect the movement of laryngeal folds must be considered. In addition, collateral branching after surgery could also be partially responsible for the differences in time in the functional recovery of laryngeal fold mobility. Future strategies to decrease collateral branching should be considered for functional recovery after neurotmesis and repair of the RLN nerves.

In addition, in the present study we have found more double-labeled motoneurons than after crush injury (Hernández-Morato et al., 2013b). This would imply that some motoneurons innervated both an abductor and adductor muscle at the same time.

Number of Motoneurons

The number of labeled motoneurons that innervate PCA and TA muscles in control groups shows hardly any variation between animals within each group. These numbers are consistent with those previously published: in a study of the somatotopy of the neurons innervating the rat laryngeal muscles (Hernández-Morato et al., 2013a). We have previously reported that the number and location of labeled neurons after the injection of tracer into the PCA and TA muscles in a control group was similar to that of the group in which the SLN was cut immediately after the tracer injection, showing that during the injection procedure there was no spread to the CT muscle or any structure supplied by the SLN (Hernández-Morato et al., 2013b). In the experimental series, there were

significant variations in motoneuron number between animals. During the regeneration process, the number of neurons labeled following injection of the PCA was much higher than those labeled after injection of the TA. In the rat, as in man, the branch of the RLN that supplies the PCA is the first to exit the main trunk, which means, the distance needed for axonal regrowth from proximal stump is shorter. Consequently, when larynx is reinnervated, axons may reach PCA sooner and in greater numbers than they can for the other intrinsic laryngeal muscles including the TA, resulting in a high number of PCA-labeled motoneurons in comparison with TA (Crumley and McCabe, 1982; Crumley, 2000).

There are also differences over time in the numbers of labeled motoneurons seen in the Amb following injection into the PCA and TA muscles. The number of labeled neurons from PCA increases until 8 wpi and then slightly decreases but remains significantly higher than control. However, the number of TA motoneurons increases slowly from almost zero after 1 wpi to a number at 8 wpi slightly higher than the control number after which time the numbers stabilize. These findings are in agreement with other studies of section and repair of RLN (Flint et al., 1991; Nahm et al. 1993) and section and repair of other nerves, where the number of labeled neurons is higher than control (Aldskogius and Thomander, 1986; Fernández et al., 1992; Streppel et al., 1998; Choi and Raisman, 2002; Guntinas-Lichius et al., 2005).

Contralateral-Labeled Motoneurons

In the control group, all neurons projected ipsilaterally and no muscle studied received bilateral innervation. However, following RLN section and regeneration, some contralateral motoneurons in the corresponding Amb were labeled at all survival times studied. Bilateral innervation is observed at all recovery times after an injection of CtB into PCA or TA, with the number of contralateral-labeled motoneurons greater in the case of the PCA. Contralateral innervation during regeneration has been described following RLN crush injury, but the main difference with both types of injury is that after a RLN crush injury the number of contralateral-labeled motoneurons is higher at 1 wpi but decreased at longer survival times (Hernández-Morato et al., 2013b), whereas after the section and repair of the RLN, the number of contralateral-labeled motoneurons increases at 8 wpi and at 12 wpi and then declines thereafter at 16 wpi. This was especially pronounced in PCA motoneurons. Bilateral innervation has been described after injury in other nerve regeneration studies, including those looking at oculomotor, trochlear, facial and sciatic nerves (Fernández et al., 1985, 1992; Scherer, 1986). During regeneration, uninjured axons apparently produced sprouts that were able to cross the midline and innervate the denervated muscles (Fernández et al., 1992). These contralateral connections were lost at longer survival times following nerve injury: 3 months after crush RLN injury (Hernández-Morato et al. 2013b).

In conclusion, section and of the RLN followed by regeneration paralyzed the laryngeal fold. Misdirected regrowth of the injured axons leads to changes in the somatotopic distribution and the number of motoneurons compared with control group. These changes showed some similarities to those previously described after RLN crush injury, although there are also significant differences in both the distribution and number of neurons labeled at different survival times following both types of injury. Although the regenerated axons reached the intrinsic laryngeal muscles following both types of injury, the movement of the affected laryngeal fold failed to recover after section and repair of the RLN up to 16 weeks. The recovery of movement after crush injury could be observed as soon as 4 wpi.

After section and regeneration, the somatotopic organization of the Amb change and no functional recovery was observed but a partial reinnervation of the motor end plates in the TA and PCA muscles was confirmed. These findings are interpreted as being the result of the misdirected regrowth of axons in the postlesion nerve stump and of collateral sprouting, suggesting that the established neuromuscular circuits are being incorrectly regulated impairing the correct coordinated contraction of the different laryngeal muscles, in contrast to what occurred following crush injury.

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