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# LOCALIZATION OF BRAIN STEM MOTONEURONS INNERVATING THE LARYNGEAL MUSCLES IN THE RUFOUS HORSESHOE BAT, *RHINOLOPHUS ROUXI*

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## SUMMARY

The motoneurons innervating the laryngeal muscles were localized in the rufous horseshoe bat, *Rhinolophus rouxi*, using the HRP method. HRP was applied to the cricothyroid muscle and to the cut end of the recurrent laryngeal nerve. Labeled motoneurons were found in two completely separated regions of the nucleus ambiguus. The motoneurons innervating the cricothyroid muscle via the superior laryngeal nerve (SLN) are located within the ventrolateral portion of the nucleus reaching the caudal pole of the motor nucleus of the facial nerve. The motoneurons innervating the other intrinsic laryngeal muscles via the recurrent laryngeal nerve (RLN) are situated in the caudal half of the nucleus ambiguus. The innervation is strictly homolateral.

## INTRODUCTION

Insectivorous bats forage at night with the help of an active sonar system. They emit ultrasonic orientation calls produced by the larynx and listen to the echoes, which are analyzed by a highly differentiated auditory system. The orientation sounds emitted by hunting bats are adapted to specific situations and may be altered in response to their echoes. Horseshoe bats, for instance, emit an orientation call consisting of a long constant frequency portion followed by a short downward frequency sweep (e.g., *Rhinolophus rouxi* emits a tone of 85 kHz and 30–60 ms duration). The constant frequency portion of the orientation call serves as a carrier for small frequency modulations in the echoes induced by the wing beats of prey[9]. As the horseshoe bat flies toward a stationary target, the frequency of each emitted pulse is lowered to compensate for Doppler shifts due to the bat's flight speed[14]. The frequency

of the constant frequency (CF) component is thus fixed in a narrow band to which the bat's cochlea is sharply tuned[3]. The CF-component of the echo frequency is maintained with a precision of 100–300 Hz or 0.1–0.3 %, demonstrating a precise control of frequency emission[15]. In addition the duration of the echolocation sound is successively shortened as the bat approaches its prey.

Both frequency and time control of the echolocation sounds require sophisticated and precise command systems for the laryngeal muscles with feedback from the auditory system.

In bats and other mammals the intrinsic laryngeal muscles are innervated by two branches of the vagus nerve, the motor or external branch of the superior laryngeal nerve (SLN) and the inferior or recurrent laryngeal nerve (RLN). Nerve fibers of the external branch of the SLN terminate on the cricothyroid muscle (CTm). The RLN innervates the remaining intrinsic laryngeal muscles. In some mammals the cricothyroid muscle receives innervation from both RLN and SLN but in bats and insectivores the superior and the recurrent laryngeal nerves remain strictly separated[1,2].

In greater horseshoe bats (*Rhinolophus ferrumequinum*) action potential recordings from the SLN and the cricothyroid muscles have disclosed that they control the frequency of the emitted echolocation sounds[16,17]; multiunit recordings from the RLN during vocalization indicated that the RLN determines the time course of each echolocation sound[12]. Apparently the SLN and RLN control two different aspects of sound production. This suggested to us that in the brain stem the two vagus nerve branches may originate from two distinct cell aggregations. In mammals it is known that motoneurons contributing fibers to SLN and RLN are located within the nucleus ambiguus situated in the lateral portion of the brain stem reticular formation. Within the nucleus ambiguus of the cat and the dog the motoneurons innervating different intrinsic laryngeal muscles are orderly arranged in a caudorostral representation[19]. A slightly different arrangement of motoneurons supplying different intrinsic laryngeal muscles has been reported in the cat by using the HRP method[4]. In the nucleus ambiguus of the cat the motoneurons innervating the laryngeal adductor muscles (cricothyroid m., lateral cricoarytenoid m., thyroarytenoid m., interarytenoid m.) are located dorsally and the motoneurons supplying the laryngeal abductor muscles (posterior cricoarytenoid m.) are ventrally placed. In addition, motoneurons innervating the cricothyroid and the posterior cricoarytenoid muscles have also been found within the retrofacial nucleus, a cell aggregation situated just caudal to the motor nucleus of the facial nerve.

Nothing is known about the functional organization of the nucleus ambiguus in bats, especially in horseshoe bats, in which control of vocalization is of utmost importance[12,16]. We, therefore, have begun neuroanatomical investigations of the central nervous system control of sound production in horseshoe bats. Here we report on the origin of the SLN and RLN in the brain stem as revealed by the HRP-tracer method.

## MATERIALS AND METHODS

Six rufous horseshoe bats, *Rhinolophus rouxi*, from Southern India were used for this investigation. The animals were anesthetized with Halothane and placed in a special head and body holder. Surgery was done under a Zeiss operating microscope.

For marking the cell bodies of SLN the nerve was exposed at its entrance into the cricothyroid muscle; this involved cutting the sternohyoid muscle and removing the thyroid gland. The cricothyroid muscle was slightly damaged close to the SLN, and HRP (Sigma type VI) in solid form was repeatedly inserted into this small damaged area. Spread of HRP into neighboring tissues was carefully avoided by covering it with a thin layer of vaseline. After 30 min the wound was closed with tissue glue (Histoacryl).

For exposing the RLN only the sternohyoid muscle was cut. The RLN was sectioned caudal to the larynx and the proximal nerve stump was inserted into a small piece of plastic tubing closed at both sides with vaseline. Within this closed chamber HRP in solid form was applied to the cut end of the RLN for half an hour. Then the tubing with surplus HRP was carefully removed.

Both methods, insertion of solid HRP into the muscle close to the SLN and the insertion of the RLN into closed tubing, effectively prevented the spread of HRP into the surrounding tissues. HRP was applied to the SLN (two bats), to the RLN (two bats) and in one bat to the SLN on the left and to the RLN on the right side. In a control experiment HRP was applied to the injured sternohyoid and other muscles adjacent to the larynx.

The animals were allowed to survive for 24 h. Then they were deeply anesthetized with pentobarbital and perfused with saline followed by fixative (1.25% glutaraldehyde plus 1% paraformaldehyde in phosphate buffer, pH 7.2, for 45 min) and a washing solution (phosphate buffer, pH 7.2, plus 10% sucrose for 30 min)[10]. The brains were removed and covered with a thin shell of egg yolk. The egg yolk was postfixed for 30 min in the fixative and washed for 1 h. On a freezing microtome the brains were cut into sections of 50 $\mu$m thickness in a frontal or sagittal orientation and were reacted the same day with tetramethylbenzidine (TMB) and hydrogen peroxide[7]. In one experiment alternative sections were reacted with diaminobenzidine (DAB) according to the method of Streit and Reubi[18]. The sections were mounted on gelatinized slides and counterstained with neutral red. For identifying labeled neurons the sections were examined under bright and dark field illumination and between crossed polarizers[6]. Drawings of the brain stem sections were made with a Zeiss drawing apparatus and the exact position of labeled neurons was plotted at higher magnification onto these drawings with the aid of a x-y plotter-system attached to the microscope stage.

## RESULTS

In brain sections of all 5 bats (excluding the control animal), labeled cells were only found within the nucleus ambiguus in the lateral portion of the reticular formation of the lower brain stem. The nucleus ambiguus in its caudal third and its

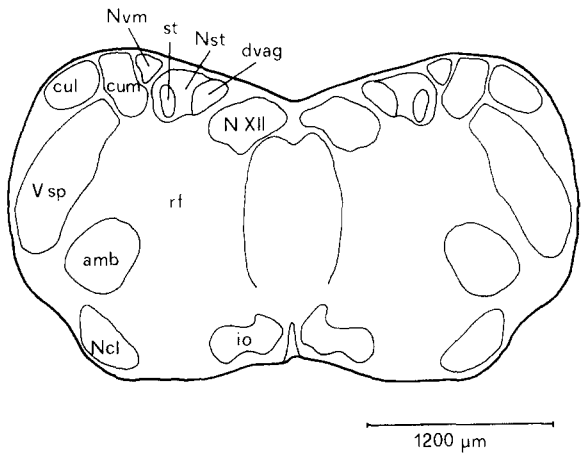


Fig. 1. Schematic line drawing of a transverse brain stem section showing the position of the nucleus ambiguus in its spatial relation to other brain stem nuclei. Abbreviations: amb, nucleus ambiguus; cul, lateral cuneate nucleus; cum, medial cuneate nucleus; dvag, dorsal motor nucleus of the vagus nerve; io, inferior olive; Ncl, lateral nucleus of the medulla oblongata; Nst, nucleus of the solitary tract; Nvm, medial vestibular nucleus; NXII, nucleus of the hypoglossal nerve; rf, reticular formation; st, solitary tract; Vsp, nucleus of the spinal tract of the trigeminal nerve.

relationship to other brain stem nuclei is illustrated in a schematic drawing of a transverse section in Fig. 1. The nucleus forms a continuous elongated cell band reaching from the level of the obex to the caudal pole of the motor nucleus of the facial nerve. It is composed of large and medium-sized multipolar shaped neurons (20–25 $\mu$m in diameter). In the rostral half of the nucleus a dorsal portion is distinguishable from a ventrolateral portion by a regicn of low cell density. The cells in the rostrodorsal and rostroventral portions of the nucleus are slightly smaller than in the caudal half. In some regions the boundaries of the nucleus are difficult to distinguish from the surrounding parvocellular reticular formation, since some large multipolar neurons are scattered in the vicinity.

After application of HRP to the SLN and RLN, the labeled neurons are concentrated in the caudal half of the nucleus and in the ventrolateral portion of the rostral half (Fig. 2). Labeled cells were found only on the homolateral side. No labeled cells were found in any other nucleus of the brain stem. The whole region of labeled cells was about 1500 $\mu$m long in its caudorostral extent. Within the nucleus ambiguus, labeled neurons were in two distinctly separated regions when HRP was applied either to the cricothyroid muscle, i.e. to the SLN, or when it was applied to the RLN. In the control experiment reaction product was found bilaterally in neurons of the cervical spinal cord. A few weakly labeled cells were also found in the dorsal motor nucleus of the vagus nerve. No labeled cells were found in this experiment in the nucleus ambiguus.

When HRP was applied to the cut end of the RLN, labeled motoneurons (Fig. 3A and Fig. 4A) occurred at the level of the caudal pole of the hypoglossal nucleus.

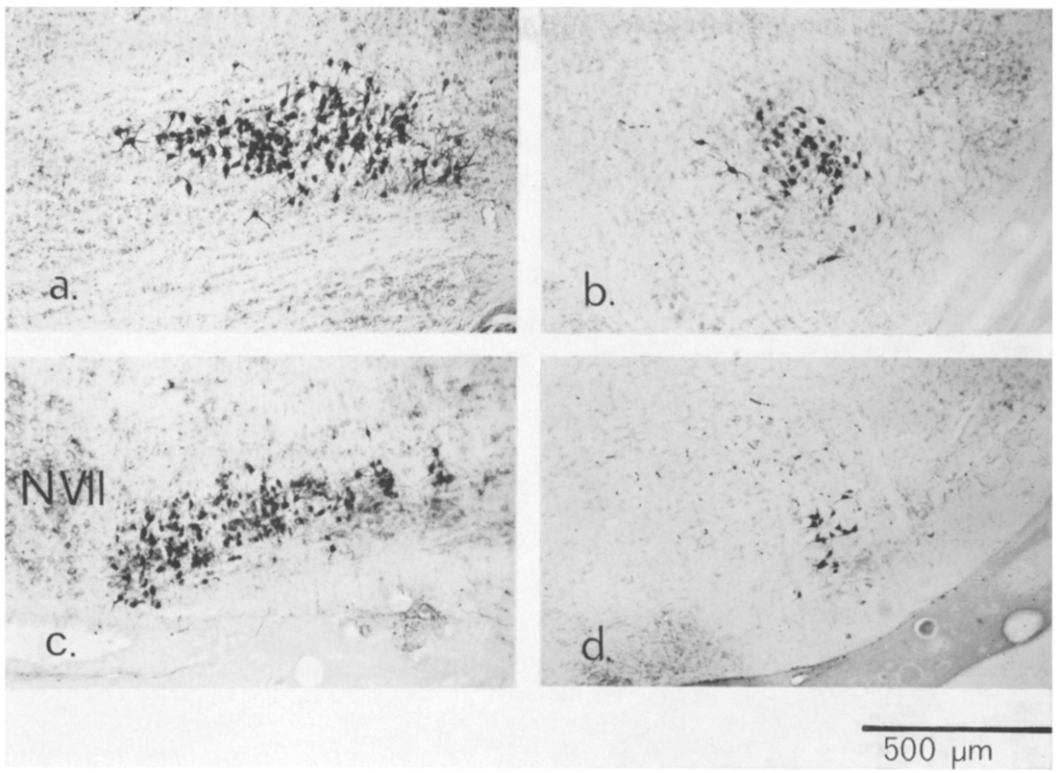


Fig. 2. Labeled RLN (a, b) and SLN (c, d) motoneurons in sagittal (a, c) and transversal (b, d) sections. The large unlabeled neurons on the left side in c are neurons of the motor nucleus of the facial nerve (NVII).

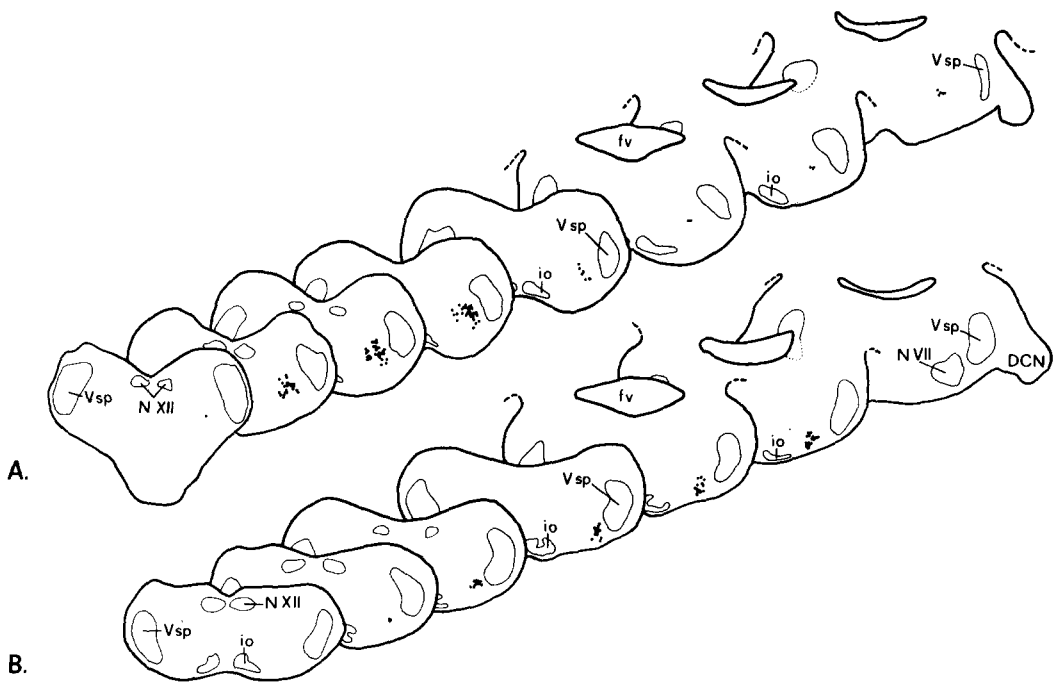


Fig. 3. Series of transversal sections through the brain stem from the level of the obex (left) to the level of the facial nerve nucleus (right) showing the distribution of labeled RLN (A) and SLN (B) neurons. The sections are spaced at 150 $\mu$m. Abbreviations: DCN, dorsal cochlear nucleus; fv, fourth ventricle; io, inferior olive; NXII, nucleus of the hypoglossal nerve; Vsp, nucleus of the spinal tract of the trigeminal nerve.

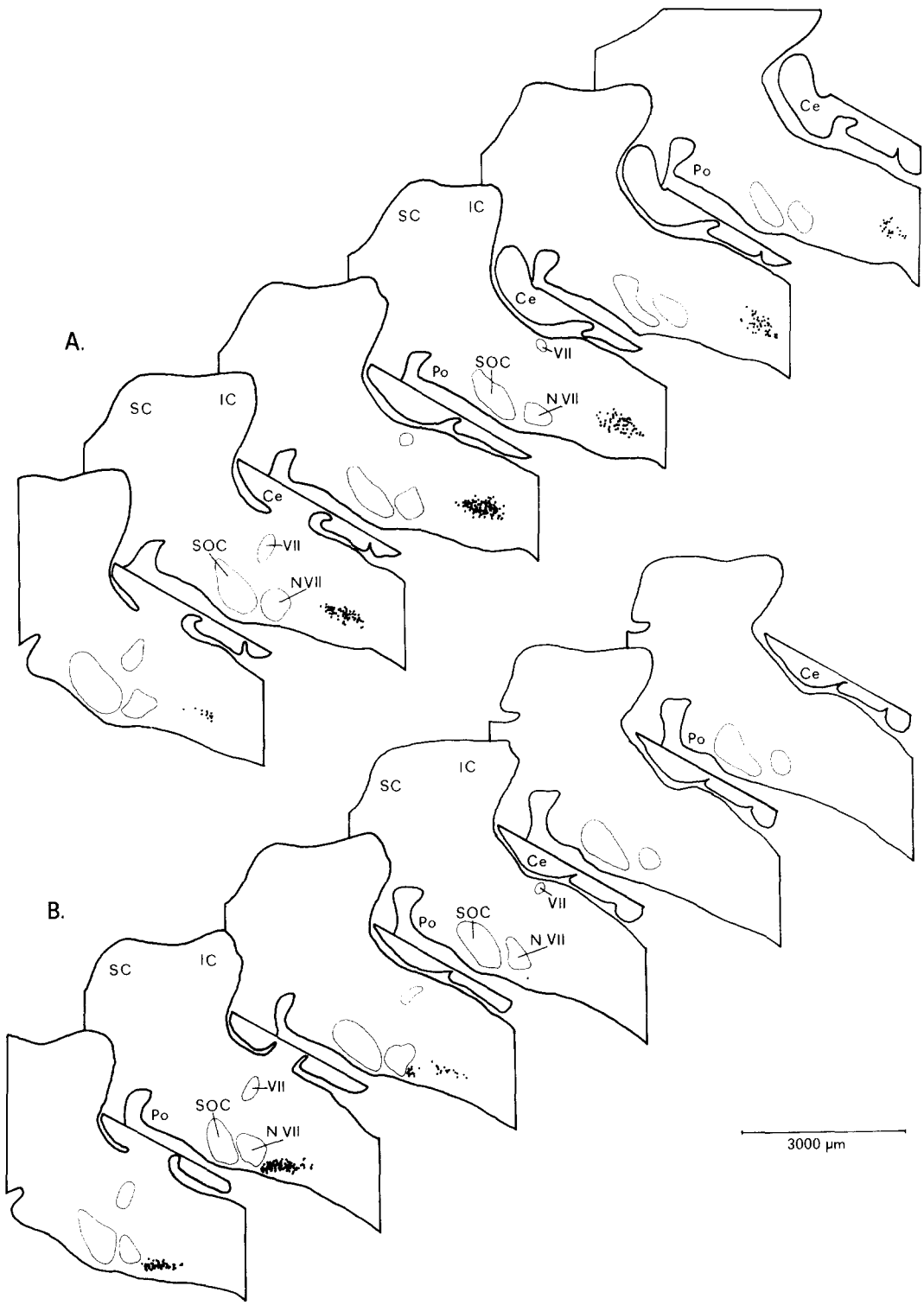


Fig. 4. Sagittal sections, showing the distribution of labeled RLN (A) and SLN (B) neurons in the brain stem. Note that the RLN neurons are located more dorsally and caudally and that only SLN neurons reach the facial nerve nucleus. The sections are spaced at 50 $\mu$m. Abbreviations: Ce, cerebellum; IC, inferior colliculus; NVII, facial nerve nucleus; Po, pontine nuclei; SC, superior colliculus; SOC, superior olivary complex; VII, facial nerve fibers.

From this region the labeled cell group extends about 1100 $\mu$m rostrally. The rostral end was situated at the level of the rostral third of the inferior olive. The dorsoventral and mediolateral extent of the labeled cell group varied considerably from section to section. Most labeled neurons had a multipolar shape with a nearly spherical cell body (about 20 $\mu$m in diameter) and seemed to be clustered in different cell groups. Unlabeled cells were located between clusters of labeled RLN neurons. In three animals an average of about 700 labeled RLN neurons was counted per bat. The axons of the motoneurons, visible under polarized light, course dorsally and at the ventral border of the nucleus of the solitary tract they turn sharply in a ventral direction. They form several distinct fiber bundles and leave the medulla at the ventrolateral border.

When the SLN was marked by HRP the labeled motoneurons in the nucleus ambiguus formed a distinct band ventrolateral to the RLN region (Fig. 3B and Fig. 4B). The caudal end of the cell band was situated about 300 $\mu$m more rostral than the most caudal RLN neurons. Rostrally the SLN neurons extend up to the caudal border of the motor nucleus of the facial nerve where the most ventral positioned neurons reach the ventral brain surface (Fig. 2c). The caudorostral extent is about 1200 $\mu$m. The cells are slightly smaller (about 15 $\mu$m in diameter) than the RLN neurons and are of multipolar shape. In three animals an average of 500 labeled motoneurons were counted per bat. Labeled axons coursed dorsally for only a short distance, then they turned sharply ventrally and left the medulla in several small fiber bundles.

The results show that the RLN neurons are not intermixed with SLN motoneurons. However, in the middle part of the nucleus ambiguus, in a region with a rostrocaudal extent of about 800 $\mu$m, the aggregation of labelled cells from both nerves overlapped, with the RLN neurons oriented dorsally and SLN neurons oriented ventrolaterally.

## DISCUSSION

After application of HRP to the SLN and to the RLN, labeled neurons were found in the lateral region of the lower brain stem reticular formation, a region we consider to be the nucleus ambiguus. The ventrolateral portion of this nucleus seems to be homologous to the retrofacial nucleus in the cat. However, since the nucleus forms a continuous cell aggregation from the region of the obex to the caudal pole of the motor nucleus of the facial nerve we did not differentiate a retrofacial nucleus in *Rhinolophus rouxi*. No labeled cells were detected in other brain stem nuclei including the dorsal motor nucleus of the vagus nerve or the hypoglossal nucleus. From all the external laryngeal muscles which are innervated by cranial nerves only the hyopharyngeal, thyropharyngeal and cricopharyngeal muscles receive fibers from the vagus nerve[21]. None of these muscles were injured in our preparations. The only muscle which was cut was the sternohyoid muscle, which is innervated by cervical nerves. Since we are sure from the results of the control experiment that no tissue was injured, innervated by other branches of the vagus nerve or by the accessory or glossopharyngeal nerve which also have their motoneurons in the nucleus ambiguus, and since

we carefully avoided spreading of HRP into surrounding tissue, we conclude that all labeled motoneurons in the brain are SLN or RLN motoneurons. An important result of this investigation is that, unlike the conditions in cats[4], the SLN motoneurons of *Rhinolophus* are clearly separated from the RLN motoneurons. The RLN motoneurons are located more dorsocaudally in the nucleus ambiguus, the SLN neurons in the ventrolateral portion of the rostral half. These results agree with results of similar experiments in the rat where a dorsoventral separation of RLN and SLN motoneurons has also been reported[20]. The difference between our findings in *Rhinolophus rouxi* and the findings in the rat is that in the latter also RLN neurons reach the caudal pole of the motor nucleus of the facial nerve and that in most cases a contralateral labeling of motoneurons was found.

The clear separation of motoneurons of SLN and RLN in the nucleus ambiguus of the horseshoe bat may have phylogenetical and/or functional explanations. In bats and insectivores the cricothyroid muscle (CTm) is exclusively innervated by the SLN whereas in 'higher mammals' interconnections between the RLN and SLN have been reported. In cats and other carnivores and in rats a small bundle of fibers of the RLN terminates on the cricothyroid muscle, so that the CTm receives a double innervation[21]. This may be the reason that SLN motoneurons in cats are not restricted to the retrofacial nucleus but also are intermixed with RLN motoneurons in more caudal parts of the nucleus ambiguus. Furthermore, this may explain the additional representation of the posterior crycoarytenoid muscle in the retrofacial nucleus[4].

The arrangement of motoneurons in the brain supplying different laryngeal muscles seems to reflect the phylogenetic and/or ontogenetic origins of different laryngeal cartilages and muscles. The thyroid cartilage is derived from both the second and third visceral arches whereas the cricoarytenoid complex develops from the fifth visceral arch[13]. Among vertebrates the thyroid cartilage has became a functional part of the larynx only in Marsupialia and Eutheria. In Monotremata there is no articulation between the cricoid and the thyroid and the CTm is poorly developed[13]. Since the muscles of different visceral arches are innervated by different nerve branches, it seems reasonable to expect that motoneurons contributing to these branches would have separate origins in the medulla. Certainly this is what we found in *Rhinolophus*. Further evidence comes from an investigation in the cat[22], in which the motoneurons supplying the cricopharyngeal muscle, which has the same phylogenetic origin as the CTm, were located within the retrofacial nucleus and the rostral part of the nucleus ambiguus.

Our experiments do not differentiate the innervation via the RLN of different intrinsic laryngeal muscles. The larynx of *Rhinolophus rouxi* is too small (about 2 mm long) for injection of HRP into different muscles without spreading of the enzyme into the neighboring muscles. However, the clustering of motoneurons in the RLN region of the nucleus ambiguus suggests that motoneurons innervating different muscles may be separated.

The nucleus ambiguus of the horseshoe bat is relatively large. It has a caudorostral extent of about 1.5 mm whereas the whole brain from the obex to the olfactory bulbs measures only about 12 mm. About 500 motoneurons of the nucleus

ambiguus innervate the cricothyroid muscle and about 700, the other intrinsic laryngeal muscles. The number of 700 RLN motoneurons corresponds well with fiber counts in the RLN of *Rhinolophus ferrumequinum* by Ruebsamen[11] who counted 750 myelinated fibers in the RLN. No counts are available from the SLN. In comparison with other mammals, however, the number of RLN motoneurons in *Rhinolophus* is high. Gacek and Lyon[5] found about 550 myelinated fibers in the RLN of cats and Murray[8] about 450 in the cat and 300 in the rabbit. The high number of RLN and SLN motoneurons in horseshoe bats suggests that a high number of muscle fibers compose the laryngeal muscles and/or that single muscle fibers have a high innervation density. Both would enable the animal to realize very precise and graduated muscle contractions for sound production.

The control of frequency and the control of the time course of horseshoe bat echolocation calls are two independent mechanisms. Depending on the situation, bats are able to change the frequency of the calls, without changing the time course, and they are able to shorten or to prolong their echolocation calls without changing the frequency. The SLN controls the fine frequency adjustment and the RLN the time structure of the echolocation calls[12,16,17]. Our investigation shows that the motoneurons which are responsible for the control of these two sound parameters are completely separated within the nucleus ambiguus.

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