Central Localization of the Cervical Esophageal Motoneurons in the Rabbit

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Summary: Central localization of the cervical esophageal motoneurons was investigated in the rabbit, using the intramuscular injection of retrograde labeling tracers horseradish peroxidase or nuclear yellow. The nucleus ambiguus of the rabbit comprises four subnuclei, CoG, Sgm, Sgl, and DiG, according to our previous cytoarchitectural study, of which only the ipsilateral CoG was pertinent to the esophageal motoneurons. CoG is the abbreviation for a cell grouping formed by a compact arrangement of the smallest neurons of the nucleus ambiguus, and it is situated in the rostral half of the nucleus. Labeled neurons were found in the rostral two-thirds of the CoG at levels 1.2-2.9 mm rostral to the obex; at the most rostral level, they overlapped the facial motor nucleus for about 100 µm rostrocaudally. Their mean total number was 172, and they were more numerous in the rostral one-third of CoG than in its middle one-third. In a transverse section of the brain stem, labeled neurons at the most caudal level, although a few in number, were found in the lateral portion of CoG. More rostrally, at the mid-levels of CoG, in which the CoG was divided into two subgroups, ventrolateral and dorsomedial, labeled neurons increased in number and were distributed in the entire portion of the former subgroup. Furthermore rostrally, at the rostral levels of CoG in which no further division was found, they further increased in number and were distributed caudally in the dorsal half of CoG and rostrally in its entire portion.

The muscles of the palate, pharynx, cervical esophagus and larynx are concerned with deglutition, vocalization, and respiration, etc., and the central and peripheral innervations of these groups of muscles are mainly performed by a series of nervous structures with the same ontogeny and phylogeny. Thus, their peripheral innervation is performed by a sequence of nerves included in the vago-glossopharyngeal complex: the vagus and the glossopharyngeal nerves, and the cranial root of the accessory nerve; centrally, the motoneurons supplying the respective muscles are said to be located almost solely in the nucleus ambiguus. The fact that the above groups of muscles are supplied by the same system of nervous structures is very interesting when considering what type of nervous mechanism may be responsible for the sequential series

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of muscle movements which occur in deglutition. Therefore, a detailed investigation of the central locations of the motoneurons of these groups of muscles, and that of their peripheral axon courses are very important in elucidating that nervous mechanism. However, studies on locations of motoneurons of the palatal, pharyngeal, and cervical esophageal muscles are few in number, with the details of their localization in the nucleus ambiguous unclarified as yet, although relatively numerous studies including our previous study\textsuperscript{13) have been made on the localization of the laryngeal motoneurons. As a systematic study concerning those motoneurons employing the neurological tract-tracing method, studies in the cat by Yoshida and his colleagues\textsuperscript{18,19, 20) have only recently become available.

We previously published papers\textsuperscript{6,13) on the central localization of the rabbit laryngeal motoneurons and their peripheral axon courses, and next made an oral presentation\textsuperscript{11) on the pharyngeal constrictor motoneurons. The present study, a sequel of the previous papers and presentation, examined the location of the cervical esophageal motoneurons of the rabbit, using intramuscular injections of retrograde labeling tracers, horseradish peroxidase (HRP) or nuclear yellow (NY).

Method

Eleven male rabbits weighing 1.0 to 1.5 kg were used in the present study.

1. Injection of Tracer

Subsequent to anesthetizing the animals with an injection of urethane (1.0 g/kg body weight) into the auricular vein, the larynx was exposed by a median incision through the skin of the anterior neck and the underlying muscles. The larynx, with the pharynx and trachea attached to it, was isolated from surrounding tissues and turned over to identify rostrally the cricopharyngeus muscle and caudally the cervical esophageal muscles. Then, 5 \( \mu l \) of 20 to 40\% HRP (Sigma, Type VI) solution in physiologic saline was injected unilaterally (three animals) or bilaterally (five animals) into almost the entire portion of the cervical esophageal muscles including the cricopharyngeus muscle and the proper esophageal muscles and extending caudally as far as the level of the seventh cervical vertebra. With respect to the remaining three animals, 10 to 15 \( \mu l \) of 5\% NY (a fluorescent substance) water solution was injected unilaterally into the cervical esophageal muscles. The injections were carried out using a microsyringe. A point 0.5 cm caudal to the boundary between the cricopharyngeus and cricoesophageus muscles was taken as the entrance point of the needle. The level where the pharyngeal median raphe disappears was regarded as their boundary because of a clear change in the lie of the muscle bundles at this level. The injections were carried out carefully to avoid any spread of injected tracers into muscles other than the cervical esophageal muscles, and in the case of the unilateral injection, into the contralateral side in addition. After the injection of each tracer, the injection sites were covered with a mixture of vaseline and liquid paraffin, and then the incision in the skin was sutured closed.

2. Histochemical Procedures

i) After Horseradish Peroxidase Injection. About 24 hours after the injection, the animals were reanesthetized and ultimately sacrificed by pentobarbital (25 mg/kg body weight) injected into the auricular vein, followed by perfusion through the ascending aorta with physiologic saline (500 ml), and then with a fixative composed of 1.25% glutaraldehyde and 1\% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 1500 ml),
and finally with the same buffer chilled and containing 10% sucrose (500 ml). The brain stem was immediately removed and placed overnight at 4°C in phosphate buffer containing 30% sucrose, and then cut into serial, transverse, frozen sections 60 μm in thickness. Tetramethylbenzidine (TMB) was adopted as the chromogen in the histochemical procedure for the detection of HRP activity; and the sections were counterstained with 1.0% neutral red and mounted on glass slides. The locus and number of labeled neurons were examined by light microscopy.

ii) After Nuclear Yellow Injection.

Procedures from anesthesia and perfusion of animal to sectioning of the brain stem were the same as in the HRP cases, except that adjustment for about 48 hours of post-injective survival time was made, and that 10% formalin in 0.1 M phosphate buffer (pH 7.4, 1500 ml) was adopted as a fixative. The sections were mounted on glass slides coated with gelatin and dried in air. The locus and number of the labeled neurons were then examined by fluorescent microscopy (illumination at 334 or 365 nm), and photographed. After fluorescent microscopy, sections on which labeled neurons were observed were counterstained with 0.1% toluidin blue. The loci of the labeled neurons on the fluorescent photograph were checked on the corresponding stained section.

Results

Subdivision of the nucleus ambiguus (Fig. 1) was based on our previous cytoarchitectonic study using HRP-labeled preparations of the nucleus. According to that study, the nucleus ambiguus of the rabbit comprises four subnuclei: CoG, SGm, SG1, and DiG. CoG is the abbreviation for a cell grouping formed by a compact arrangement of the smallest neurons of the nucleus ambiguus, and which is situated in the rostral half of the nucleus. The SG, situated in its rostral one-third, is a scattered cell...
group around the CoG, with a subdivision into SGm and SGI. The SGm is located medial to the CoG and is formed by slightly larger neurons, and the SGI is located laterally with neurons approximately equal in size to those of the CoG. The DiG is a diffuse cell group in the caudal two-thirds of the nucleus ambiguus and is formed by the largest neurons of the nucleus, merging rostrally into the CoG. The shift from CoG to DiG occurs gradually; the neurons of both groups are intermingled at a level between about 0.5 and 1.0 mm rostral to the obex.

1. Cases of Unilateral Injection of HRP (Fig. 2, Upper)
   Labeled neurons were found in the ipsilateral CoG, the contralateral CoG, and the ipsilateral dorsal motor nucleus of the vagus nerve. Labeled neurons in the ipsilateral CoG were approximately located in the rostral two-thirds of CoG at levels of about 1200 to 2900 μm rostral to the obex; at the most rostral level, they overlapped the most caudal portion of the facial motor nucleus for about 100 μm rostrocaudally. Their mean number was 172. They were more numerous in the rostral one-third of the CoG than in its middle one-third, and they displayed a maximum number at about 2500 μm rostral to the obex, then gradually decreased in number while being traced rostrocaudally. On the other hand, labeled neurons in the contralateral CoG were similar in the rostrocaudal distribution pattern to those in the ipsilateral CoG. However, the mean number of 35 was considerably less than that in the ipsilateral CoG, and observation of serial sections revealed caudal levels without labeled neurons intermingled with those having labeled neurons. Labeled neurons in the dorsal motor nucleus were found scattered at levels of about 500 to 2700 μm rostral to the obex. Their number was 12, extremely few in comparison with the much greater number of constituent neurons of this nucleus, and there were no sections having three or more labeled neurons.

2. Cases of Unilateral Injection of NY (Fig. 2, Lower)
   Labeled neurons after injection of NY, which is considerably superior to HRP in respect to restrained leakage from injected muscles\(^{13}\), were found only in the ipsilateral CoG. Their mean total number was 50, smaller than the number of the labeled neurons of the ipsilateral CoG of the cases of HRP injection owing to a lower ability to label by NY\(^{13}\), but they displayed a similar rostrocaudal distribution pattern to the latter labeled neurons. On the contrary, no labeled neurons were found at all in either the contralateral CoG or the ipsilateral dorsal motor nucleus, suggesting the probability that labeled neurons of these regions, small in number, in cases of HRP injection are due to a leakage of a small amount of HRP into the contralateral
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Number of labeled cells

Case of HRP- injection

Case of NY- injection
cervical esophageal muscles, and into the ipsilateral thoracic esophageal muscles or the ipsilateral mucous and submucous layers.

3. Cases of Bilateral Injections of HRP

From the results of the unilateral injection of HRP or NY, the cervical esophageal motoneurons were suggested to be ipsilaterally located in the CoG. Therefore, bilateral injections of HRP into the cervical esophageal muscles were carried out to increase the number of cases, and distribution of labeled neurons in the nucleus ambiguus was examined in detail. Scarcely any differences were found in the intranuclear distribution of labeled neurons between the cases of unilateral HRP injection and those of bilateral injections. The following four patterns of distribution were obtained in a transverse plane of the brain stem at different levels of CoG, "a to d" of figure 3 and plate 1. (i) In the caudal portion of CoG which was more caudal than a level of about 1300 μm rostral to the obex and displayed no division of any cell group, a small number of labeled neurons, one to three per section, were found adjacent to the lateral margin of the CoG at the most rostral level ("a" in Fig. 3 and Plate 1), without any labelings more caudally. (ii) In the middle portion of CoG which was located at levels ranging from about 1300 to 2100 μm rostral to the obex and was divided into two subgroups, dorsomedial and ventrolateral, labeled neurons increased in number and came to be distributed in the entire area of the ventrolateral subgroup ("b" in Fig. 3 and Plate 1). Their number was, however, not so large as in the rostral portion described below. In the rostral portion of CoG which is located more rostrally than about 2100 μm rostral to the obex and where the division into subgroups disappears again, (iii) labeled neurons reached a maximum in number at its caudal levels and came to occupy the dorsal half of CoG ("c" in Fig. 3 and Plate 1). (iv) In the most rostral portion of CoG, a small number of labeled neurons, one to three per section, were found adjacent to the lateral margin of the CoG at the most rostral level ("d" in Fig. 3 and Plate 1), without any labelings more rostrally.

Fig. 3. Diagrammatic drawing to show the intra-CoG distribution of the cervical esophageal motoneurons. The CoG is reconstructed in the rostrocaudal direction. The right lower diagrams represent the transverse plane of CoG at levels "a to d", a, The rostral half of the rostral one-third of CoG. b, The middle one-third. c, The caudal half of the rostral one-third. d, The rostral half of the rostral one-third.
and Plate 1), but (iv) more rostrally, they, though smaller in number, came to be distributed in the entire area of CoG as CoG diminished in cell number (“d” in Fig. 3 and Plate 1). Labeled neurons at the most rostral level, which overlapped the facial motor nucleus, gathered compactly to form a discrete gathering in contrast to a scattered grouping of neurons of the facial motor nucleus in this level. This gathering of labeled cells is undoubtedly continuous caudally with a zone of labeled neurons in the CoG.

Discussion

1. Site of Injection of Tracer into the Esophagus

The cervical esophageal muscles of the rabbit have been described as comprising three muscles: the proper esophageal muscles extending caudally as far as the level of the seventh cervical vertebra, the cricoesophagus muscle overlying the dorsal surface of the rostral extremity of the proper esophageal muscles, and the aryteno-esophageus muscle underlying the ventral surface of the rostral extremity. However, no specific injection into the arytenopharyngeus was possible, because this muscle could not be clearly identified owing to its scantiness and its intermixture with the proper esophageal muscles.

HRP is so highly diffusive that its leakage from the injected muscle into the surroundings becomes a problem in intramuscular injection. Therefore, the present study employed NY, too, which disperses less than HRP. Considering these properties of HRP and NY as well as the results of the respective cases of unilateral injection of these tracers, it is suggested that a small number of labeled neurons of the contralateral CoG and ipsilateral dorsal motor nucleus which appeared in the cases of HRP injection were probably due to a leakage of HRP into the contralateral cervical esophageal muscles, and a leakage into the ipsilateral thoracic esophageal muscles or the ipsilateral mucous and submucous layers. On the other hand, the probability of leakage into the laryngeal and cricopharyngeus muscles, although also conceivable from their positions adjacent to the cervical esophagus, can in all probability be denied because it has been clarified in our previous studies that the rabbit laryngeal motoneurons are located in the DiG and SGm, and that the cricopharyngeal motoneurons, in spite of their location in the CoG like the cervical esophageal motoneurons, differed from the latter both in level of appearance and in locus in transverse sections.

2. Central Location of the Cervical Esophageal Motoneurons

Previous studies offered two different opinions with regard to this subject: location only in the nucleus ambiguus, or location in both the nucleus ambiguus and the dorsal motor nucleus. Lawn approved the former opinion in the rabbit after applying electric stimulation to the medulla oblongata. However, he did not describe the intranuclear localization in as much detail as the present study. Recently, using the intramuscular injection of HRP or NY, Yoshida and his colleagues, and later Fryscak et al., affirmed the former opinion in the cat and rat, respectively, while the present study also obtained the same findings in the rabbit. On the contrary, Kosaka and Molhan proposed the latter opinion, that is, the dual location in both the nucleus ambiguus and the dorsal motor nucleus, in the dog and rabbit, respectively, both using the retrograde degeneration technique after nerve section. Hudson and Cummings also confirmed it in the dog, using an intramuscular injection of HRP. In Molhan's
study\textsuperscript{10} using the rabbit, however, there exists the possibility that degenerated neurons in the dorsal motor nucleus correspond to the thoracic and abdominal esophageal motoneurons which were reported not only in the rabbit\textsuperscript{3} but also in the cat\textsuperscript{20} and rat\textsuperscript{2} to be located in that nucleus as well, because when making experiments, he did not divide the esophagus into three portions, cervical, thoracic, and abdominal. Accordingly, with respect to the rabbit, cat, and rat, there seems to be agreement that the cervical esophageal motoneurons are located only in the nucleus ambiguus. Taking into consideration the histological findings of these animals that the cervical esophagus is formed by the striated type of muscle and that this type of muscle is gradually replaced with the smooth type at progressively more caudal levels in the thoracic and abdominal esophagus\textsuperscript{4, 17}, and also that the muscularis mucosae, formed by the smooth type of muscle, is absent in the cervical esophagus, but with a development in the thoracic and abdominal portions\textsuperscript{17}, the above observations concerning the locations of the cervical and the thoraco-abdominal esophageal motoneurons in the rabbit, cat and rat are in good agreement with a generally accepted pattern of innervation that the general visceral efferent neurons in the dorsal motor nucleus supply the smooth type of muscles and the special visceral efferent neurons in the nucleus ambiguus supply the striated type.

On the contrary, in the dog, Hudson and Cummings\textsuperscript{5}, unlike Kosaka\textsuperscript{7} who had not divided the esophagus into the three portions, reported that the cervical esophageal motoneurons were clearly located not only in the nucleus ambiguous but also often in the dorsal motor nucleus. In the dog, too, the muscular layer of the cervical esophagus consists of the striated type of muscle while the muscularis mucosae is undeveloped in that area\textsuperscript{17}. The glandular tissues, however, display a considerable development throughout the entire length of the esophagus, and the muscularis mucosae appears in the thoracic esophagus\textsuperscript{17}; therefore, the probability that a diffusion of HRP into the glandular tissues or/and into the muscularis mucosae of the adjacent thoracic esophagus caused labelings in the dorsal motor nucleus is conceivable. Hudson and Cummings\textsuperscript{5}, however, further reported that the region in the dorsal motor nucleus supplying the cervical esophagus as well as the thoraco-abdominal esophagus was segregated into two portions, rostral and caudal. The rostral portion supplied the mucous and submucous layers where the glandular tissues and the muscularis mucosae are located, and the caudal portion supplied the muscular layer which consists exclusively of striated muscle to the mid-thorax or as far caudally as the gastroesophageal junction in the dog\textsuperscript{5}. According to the results of that study, the central distribution of the cervical esophageal motoneurons in the dog is different from the generally accepted pattern of innervation of the dorsal motor nucleus and the nucleus ambiguus. The reason for the difference is unknown yet. But, the parasympathetic preganglionic neurons supplying the heart\textsuperscript{14} and those supplying and stomach\textsuperscript{1}, both previously said to be located only in the dorsal motor nucleus, were recently reported to be located also in the nucleus ambiguus, suggesting that the segregation of neurons between the two nuclei based on the histological character of muscles supplied by them is not so clear a division as has been previously claimed. The dual innervation of the dog cervical esophageal muscles by the nucleus ambiguus and the dorsal motor nucleus is thought to be a kind of evidence for this suggestion.

With respect to the localization of the cervical esophageal motoneurons within the nucleus ambiguus, there is a general
agreement that they are located in the rostral portion of the nucleus where the constituent neurons gather compactly, that is, in the rostral compact cell grouping; a similar observation in the present study helps to confirm this. On the other hand, there are differences among species of animals as regards the rostrocaudal range of their appearance. For example, in the rabbit in the present study, they were located in the rostral one-third of the nucleus at levels more rostral than 1.2 mm rostral to the obex, with a larger number in the rostral half of the range of their appearance; in the rat\(^2\), in the rostral one-fourth of the nucleus at levels from the obex to 0.9 mm rostrally; in the dog\(^3\), they were located to a larger extent from 0.3 mm caudal to the obex rostrally as far as the rostral extremity of the nucleus, but in the cat\(^19\), they were found in the caudal two-thirds of the rostral half at levels more rostral than 2.5 mm rostral to the obex. An indiscriminate comparison among these location ranges cannot be performed, because there are differences among the species in the rostrocaudal range of the rostral compact cell grouping within the nucleus ambiguus. However, the following experimental factor must be given strict attention: avoid leakage of the tracer into the pharyngeal constrictors when studying location of the cervical esophageal motoneurons with intramuscular injection of tracer because these muscles are adjacent to each other with partial overlapping, and their motoneurons are located in the same portion of the nucleus ambiguus\(^{11,19}\). With this factor in mind, it is interesting that in our previous\(^{11}\) and present researches using the rabbit and Yoshida's\(^{19}\) research using the cat, both of which described the localization of the cervical esophageal motoneurons relatively confined within a rostrocaudal range, the locations of the pharyngeal constrictor motoneurons were studied as well, and an absence of leakage was confirmed based on the difference of distribution of motoneurons in the transverse plane.

From our present and previous\(^{11}\) studies, it has become clear that in the rabbit, the cervical esophageal motoneurons and the pharyngeal constrictor motoneurons display a clear segregation in the rostral compact cell grouping (CoG), in which the former motoneurons were located rostrally and ventrolaterally, and the latter caudally and dorsomedially. Yoshida et al.\(^{19}\) also reported a similar segregation in the cat. However, the degree of segregation is larger in the rabbit than in the cat, where they considerably overlapped each other in the rostrocaudal direction in spite of segregation in the transverse plane. The cricopharyngeus muscle, the most caudal portion of the pharyngeal constrictor, can act by itself strongly in the rabbit as a pinch-cock in deglutition\(^5\). In the cat\(^5\), however, it has no strong pinch-cock action because of its weak fan-shaped development, and therefore, it carries out this action in cooperation with the cervical esophageal muscles. The different degree of segregation of the cervical esophageal motoneurons and the pharyngeal constrictor motoneurons between rabbit and cat is presumably reflected by the above different degree of cooperation of these muscles in the two animals.

References

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PLATES
Explanation of Figures

Plate I

Photomicrographs showing the loci of the cervical esophageal motoneurons at levels "a to d" of figure 3. Dorsal above, medial left. a: Labeled motoneurons are located adjacent to the lateral margin of CoG. b: They are located in the ventrolateral subgroup (VL) of CoG. "DM" represents the dorsomedial subgroup, formed by the pharyngeal constrictor motoneurons. c: They occupy the dorsal half of CoG. Subnuclei, SGm and SG1, were not found in sections of "b and c". d: They are distributed in the entire area of CoG.