J. Anat. (1988), **159**, pp. 11–18
With 2 figures
Printed in Great Britain

Location of bulbospinal neurons and of laryngeal motoneurons within the nucleus ambiguus of the rat and cat by means of retrograde fluorescent labelling*

F. PORTILLO AND R. PÁSARO

Department of Animal Physiology, Faculty of Biology, University of Sevilla, Spain

(*Accepted* 8 *September* 1987)

INTRODUCTION

The nucleus ambiguus is composed of vagal motoneurons, most of them projecting to intrinsic laryngeal muscles (Lawn, 1966*a*, *b*). The precise location of these laryngeal motoneurons within the nucleus ambiguus has been demonstrated by morphological (Lobera, Pásaro, González-Barón & Delgado-García, 1981; Pásaro, Lobera, González-Barón & Delgado-García, 1981, 1983) and electrophysiological (Delgado-García, López-Barneo, Serra & González-Barón, 1983; Yayima & Hayashi, 1983) techniques. In the same general area as the nucleus ambiguus, but outside its morphological limits, there is a pool of neurons related to respiratory activity called the nucleus retroambiguus (Long & Duffin, 1984) which extends caudally to the first cervical roots of the medulla. Some of these neurons are bulbospinal interneurons which send axonal projections by way of the lateral reticulospinal tract to the motor respiratory nuclei of the spinal cord (Merrill, 1970), preferentially contralaterally. Neurons within the nucleus ambiguus also send efferent projections to the spinal cord, mostly contralaterally (Delgado-García *et al.* 1983; Portillo & Pásaro, 1986). The precise location of each neuronal pool has been studied only by electrophysiological techniques (Delgado-García *et al.* 1983; Merrill, 1970) and no work has been done on their anatomical relationships.

The aim of the present investigation was to study the morphological relationships between bulbospinal neurons and laryngeal motoneurons within the nucleus ambiguus. The use of fluorescent dyes is a useful morphological technique for the discrimination of two neuronal pools within the same nucleus because the two pools can be visualised with the same fluorescing wavelength at the same time (Diamidino Yellow (DY) and Fast Blue (FB) in cats (Keizer, Kuypers, Huisman & Dann, 1983) and DY and True Blue (TB) in rats (Kuypers, Bentivoglio, Catsman-Berrevoets & Bharos, 1980; Kuypers & Martin, 1982)). DY labels the nucleus, fluorescing yellow; FB and TB label the soma and proximal dendrites, fluorescing blue (Bentivoglio, Kuypers, Catsman-Berrevoets & Dann, 1980; Kuypers *et al.* 1980).

MATERIAL AND METHODS

Experiments were carried out on 14 albino rats weighing 250–300 g and 8 adult cats weighing 1·5–3·5 kg. Under general anaesthesia (Ketolar, 35 mg/kg, i.m.) the animals were injected with one of the selected markers into the intrinsic laryngeal muscles and

* Reprint requests to Dr R. Pásaro, Departamento de Fisiología Animal, Facultad de Biología, Avda. Reina Mercedes, 6, Sevilla-41012, Spain.

with the other one into the lateral and ventral reticulospinal tracts of the cervical spinal cord. In the rat both injections were made in the same experimental session; in the cat two experimental sessions were needed. Firstly, multiple injections of the selected marker (FB in cats and DY in rats) were made, using a dissecting microscope, into the intrinsic laryngeal muscles of the right side (cricothyroid muscle, posterior cricothyroid muscle, lateral crico-arytenoid muscle and thyro-arytenoid muscle) with the help of a Hamilton microsyringe of 5 μl volume. Secondly, using stereotaxic coordinates, a microinjection of the marker was made, using a glass micropipette, into the left cervical spinal cord (C5–C6), including both the lateral and ventral reticulospinal tracts (DY in the cat and TB in the rat) because of the predominantly contralateral projections of bulbospinal interneurons (Delgado-García *et al.* 1983; Merrill, 1970). In the rat, the total survival time was 6 days; in the cat the period was 17 days from the first to the second surgical procedure, and 11 days following the second injection (Table 1). The appropriate survival periods have already been described for TB and DY injected in the spinal cord (Keizer *et al.* 1983; Portillo & Pásaro, 1986). The appropriate survival period after the injection of the markers peripherally was determined for these experiments for both rat and cat (Table 1). Finally, the animals were deeply re-anaesthetised (sodium pentobarbital, 50 mg/kg, i.p.) and perfused transcardially with saline followed by 10% formalin (pH 7·2). The brainstems were removed and cut serially in a freezing microtome at 50 μm. Sections were mounted on gelatin-coated slides and air dried. Every third section was counterstained with cresyl violet for reference purposes (Berman, 1968; Pellegrino, Pellegrino & Cushman, 1979). Sections between spinal level C1 and the facial nucleus were studied with a Leitz Ploemopack fluorescence microscope equipped with a filter system A (360 nm wavelength). The sections in which both types of labelled neurons appeared were charted onto outline coronal drawings of the brainstem with the help of a camera lucida. The labelled neurons were photographed with high speed film (400 ASA).

RESULTS

Firstly, the transit time for the retrograde transport from the muscle to the medulla was studied in the rat and cat (Table 1). This was 6 days for DY in the laryngeal motoneurons of the rat and 28 days for FB in the laryngeal motoneurons of the cat.

Laryngeal motoneurons and bulbospinal neurons of the rat

Following the injection of DY into the right intrinsic laryngeal muscles and TB into the left spinal cervical cord (C4–C5) both types of labelled neurons were found along the nucleus ambiguus. Only those sections where both types of labelled neurons were found were studied and a quantitative study of these was made. For the bulbospinal neurons only those around the nucleus ambiguus were counted. The location of bulbospinal neurons was preferentially ventral and ventrolateral with respect to laryngeal motoneurons at nucleus ambiguus levels rostral to the obex (from 0·5 mm caudal to 1·1 mm rostral to the obex). Around the obex (from 0·5 mm caudal to 0·5 mm rostral with respect to the obex) the bulbospinal neurons were located preferentially ventrolaterally and some of them ventromedially to the motoneurons, but always within the range of spread of the laryngeal motoneurons' dendritic trees (Fig. 1 and Table 2). In the caudal part of the nucleus ambiguus the bulbospinal neurons occupied a greater area than the motoneurons from 300 to 500 μm in diameter (Fig. 1B, C).

Table 1. *Summary of results following the injection of fluorescent retrograde markers into intrinsic laryngeal muscles of the rat and cat*

Fluorescent marker	Injection	Animal number	Survival time	Labelled neurons (nucleus ambiguus)	
DY	10 μl (2%)	2 rats	7	41*	(33–47)†
	15 μl (2%)	2 rats	6	64	(53–75)
	15 μl (2%)	2 rats	5	33·5	(28–39)
	20 μl (2%)	2 rats	3	0	
FB	30 μl (6%)	2 cats	10	0	
	30 μl (6%)	2 cats	18	Some very faint	
	30 μl (6%)	2 cats	28	312	(287–337)

DY, Diamidino Yellow; FB, Fast Blue. *, Mean; †, range.

Table 2. *Summary of results following the simultaneous injection of two retrograde fluorescent markers into intrinsic laryngeal muscles and cervical spinal cord of the rat and cat*

Fluorescent marker	Mns no.	Ins no.	Location with respect to the obex (mm)	Location of Ins with respect to mns
Injection site: right larynx and left cervical spinal cord (C3–C4) (4 rats)				
DY	24·5* (19–27)†	18 (6–23)	+1·1 to +0·5	Mostly ventrolateral
	8·7 (2–13)	36 (18–40)	+0·5 to 0	Ventrolateral
TB	26·2 (13–36)	70·7 (52–86)	0 to −0·5	Mostly ventrolateral and some dorsal
	28 (10–32)	120·2 (93–142)	−0·5 to −1·1	Around motoneurons
Injection site: right larynx and left cervical spinal cord (C5–C6) (2 cats)				
FB	18 (14–22)	16 (10–22)	+1·5 to 0	Ventrolateral
DY	38·5 (36–41)	50·5 (32–69)	0 to −1·7	Ventrolateral and ventromedial

Mns, motoneurons; Ins, interneurons; DY, Diamidino Yellow; TB, True Blue; FB, Fast Blue. *, Mean; †, range; +, rostral to the obex; −, caudal to the obex.

Laryngeal motoneurons and bulbospinal neurons in the cat

After the injection of FB into the right intrinsic laryngeal muscles and DY into the left cervical spinal cord, both types of labelled neurons appeared in the nucleus ambiguus, but the overlapping of the motoneuronal pool occupied a lesser extent of the nucleus than in the rat. The bulbospinal neurons were located ventrally with respect to laryngeal motoneurons at all levels in which both types of neurons appeared (from 1·7 mm caudal to 1·5 mm rostral with respect to the obex) (Fig. 2). The bulbospinal neurons were ventrolateral in the rostral part of the nucleus and ventrolateral or ventromedial in the caudalmost part of the nucleus. But in all cases they were found within the spread range of the laryngeal motoneurons' dendritic trees (Fig. 2 and Table 2).

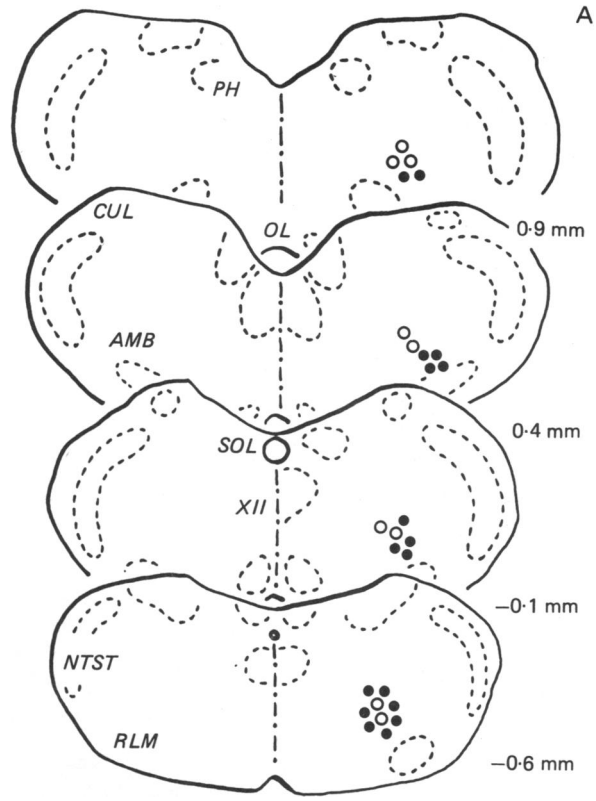
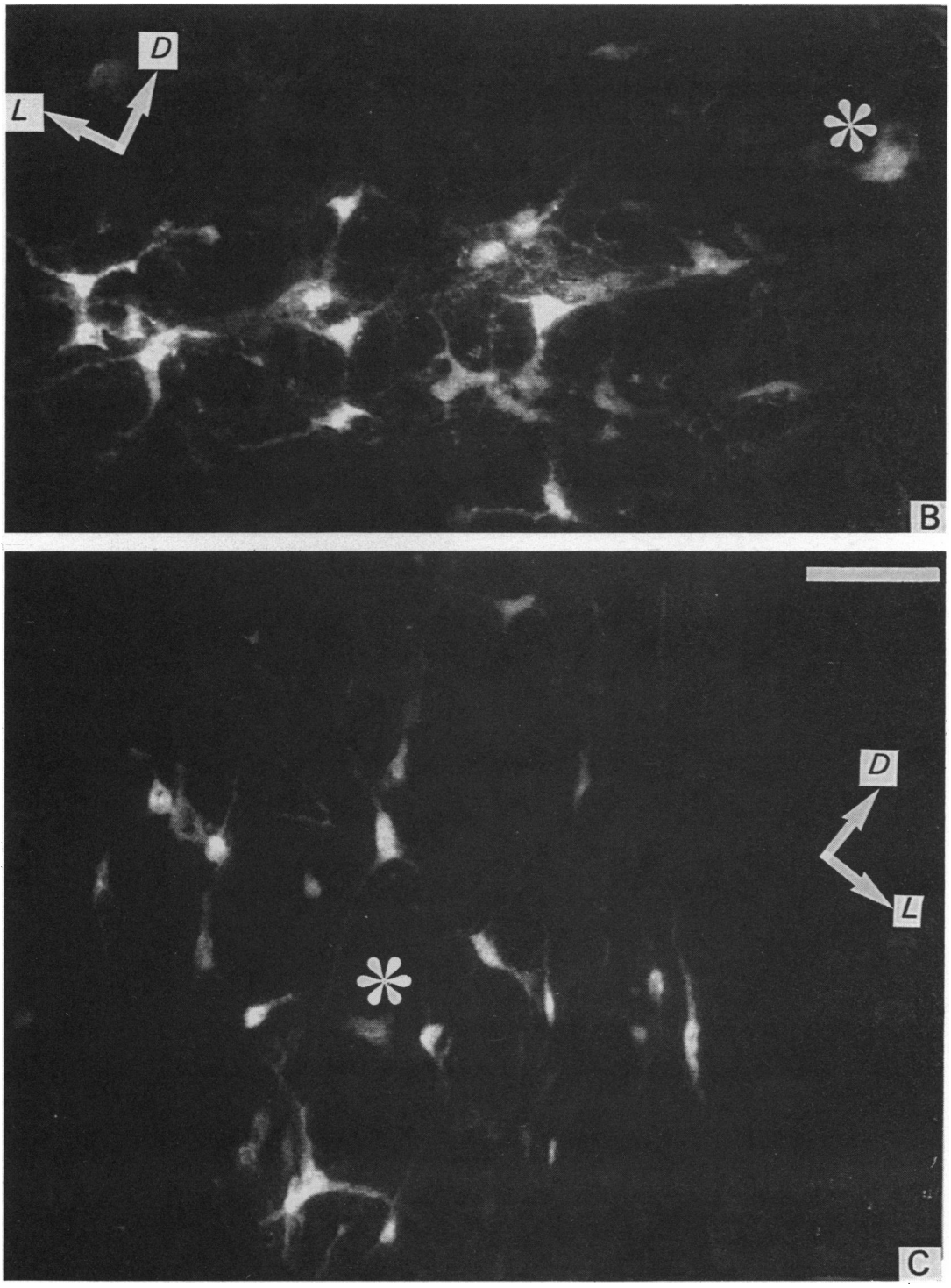


Fig. 1 (A–C). (A) Composite diagram of camera lucida drawings of coronal sections (150 μm thick) of the brainstem of the rat showing the location of True Blue (TB) labelled bulbospinal neurons (black dots) in relation to Diamidino Yellow (DY) labelled laryngeal motoneurons (circles). Each dot represents 5 neurons at the most. Coordinates: distance in mm with respect to the obex: +, rostral; −, caudal. (B and C) Microphotographs of coronal sections of brainstem of the cat showing TB-labelled bulbospinal neurons (labelled soma and dendrites) and DY-labelled laryngeal motoneurons (asterisks), the nucleus and the cytoplasm surrounding it is labelled. (B) Coronal section 0·4 mm rostral to the obex, where bulbospinal neurons are located ventrolaterally with respect to motoneurons. (C) Coronal section 0·5 mm caudal to the obex, where the bulbospinal neurons are located around the motoneurons. *AMB*, nucleus ambiguus; *CUL*, lateral cuneate nucleus; *D*, dorsal; *L*, lateral; *NTST*, nucleus of the spinal tract of the trigeminal nerve; *OL*, inferior olivary nuclei; *PH*, nucleus of the prepositus hypoglossi; *RLM*, lateral reticular nucleus, magnocellular part; *SOL*, solitary tract nucleus; *XII*, nucleus of the hypoglossal nerve. Bar, 70 μm.

DISCUSSION

The nucleus ambiguus efferent axonal projections to the spinal cord have been identified from morphological (Kuypers *et al.* 1980; Portillo & Pásaro, 1986; Rickard-Bell, Bystrzycka & Nail, 1984, 1985) and electrophysiological (Bianchi, 1971; Davies, Kirkwood & Sears, 1985; Merrill, 1970) points of view. However, the differential location of these bulbospinal neurons with respect to laryngeal motoneurons within the nucleus has not been studied.

In the present report, the overlapping of two different neuronal populations within the same area of brainstem has been demonstrated and this corroborates some of the previous electrophysiological findings (Delgado-García *et al.* 1983; Merrill, 1970). This fact, together with the relationship between the neuronal respiratory populations


D
L
B
D
L
C

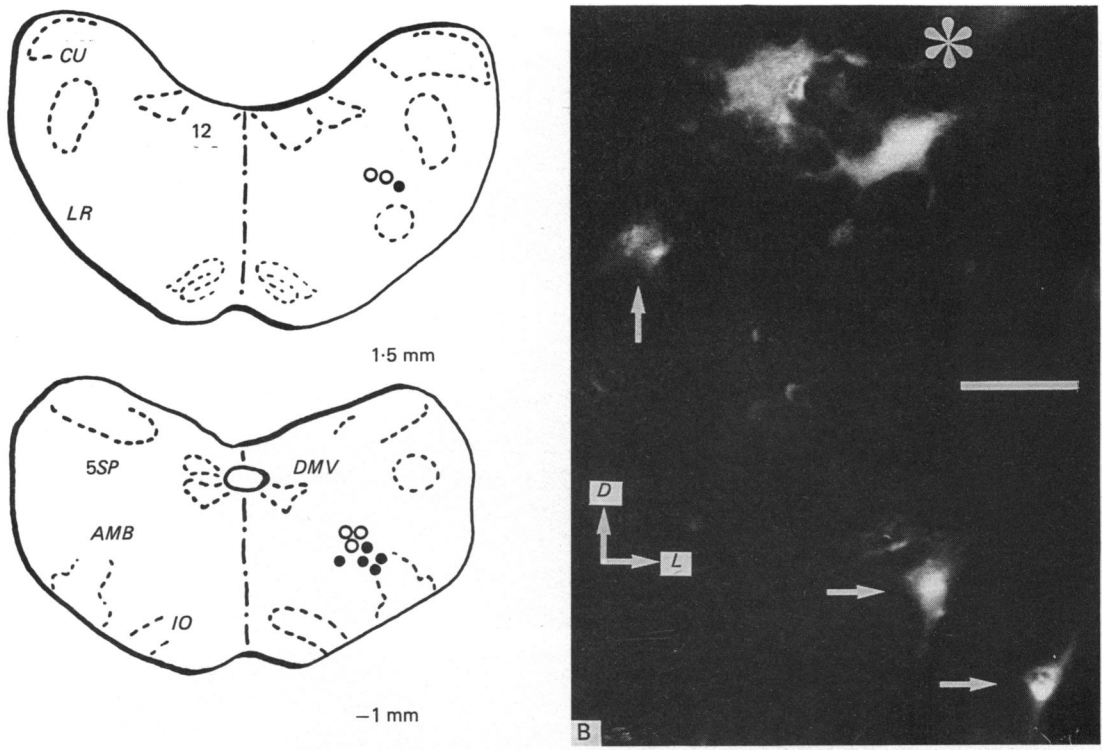


Fig. 2 (A–B). (A) Composite diagram of coronal sections of the brainstem of the cat (each section 150 μm thick) showing the location of Diamidino Yellow (DY)-labelled bulbospinal neurons (black dots) in relation to Fast Blue (FB)-labelled laryngeal motoneurons (circles) within the nucleus ambiguus. Each dot represents 5 neurons at the most. Coordinates: distance in mm with respect to the obex; +, rostral; −, caudal. (B) Microphotograph of DY-labelled bulbospinal neurons (arrows) located ventrolaterally with respect to FB-labelled laryngeal motoneurons (asterisk) within the nucleus ambiguus, in a coronal section of brainstem of the cat (1·2 mm caudal to the obex). *5SP*, alaminar spinal trigeminal nucleus, parvocellular division; 12, hypoglossal nucleus; *AMB*, nucleus ambiguus; *CU*, cuneate nucleus; *D*, dorsal; *DMV*, dorsal motor nucleus of the vagus nerve; *IO*, inferior olive; *L*, lateral; *LR*, lateral reticular nucleus. Bar, 70 μm.

of the nucleus ambiguus and nucleus retroambiguus in the respiratory rhythm (Delgado-García *et al.* 1983; Merrill, 1970), indicates the possibility of these being common discharges from both types of neuronal pool because of the need for a synchronisation in the opening and closing of the glottis with the respiratory rhythm.

Batsel (1964) located inspiratory and expiratory neurons in the reticular formation around the nucleus ambiguus in the cat, pointing out that these neurons occupy an area extending beyond the limits of the nucleus ambiguus in the rostrocaudal, dorsoventral and lateromedial directions. Later, Merrill (1970) modified the concept of the nucleus retroambiguus of Olzewski & Baxter (1954) to that of a nucleus of bulbospinal respiratory neurons overlapping the nucleus ambiguus rostral to the obex. These neurons did not project to the larynx and none of the laryngeal motoneurons projected to the spinal cord. In the present experiments no double-labelled neurons were found, in agreement with the electrophysiological findings of Merrill (1970). At the levels of overlapping of the nucleus ambiguus and the nucleus retroambiguus, Merrill (1970) was able to distinguish a more ventrolateral location of bulbospinal interneurons with respect to motoneurons, with some intermingling. Delgado-García

et al. (1983), by means of simultaneous stimulation of the superior laryngeal nerve and/or the recurrent laryngeal nerve and the cervical spinal cord, distinguished bulbospinal interneurons within the electrophysiological limits of the nucleus ambiguus. This is in agreement with the present study in which the bulbospinal interneurons were found within the spread range of the laryngeal motoneurons' dendritic trees. These results provide the anatomical information needed to complement the electrophysiological findings of several authors (Batsel, 1964; Delgado-García *et al.* 1983; Merrill, 1970) in the cat. In the rat, the anatomical distribution of bulbospinal interneurons and laryngeal motoneurons is different from that described by Merrill (1970) and Delgado-García *et al.* (1983) and follows a distribution more in accordance with that described by Batsel (1964).

SUMMARY

The location of bulbospinal neurons within the nucleus ambiguus with respect to laryngeal motoneurons has been studied by means of retrograde fluorescent neuronal markers (True Blue and Diamidino Yellow in rats, and Fast Blue and Diamidino Yellow in cats). One marker was injected into the cervical spinal cord, and the other into the intrinsic laryngeal muscles. Afterwards, the precise location of each neuronal pool was observed with the fluorescent microscope. The bulbospinal neurons in the rostral part of the nucleus were located ventrolaterally with respect to motoneurons, both in cats and rats; at more caudal levels of the nucleus the bulbospinal neurons were arranged ventromedially and ventrolaterally with respect to motoneurons in cats, and around the motoneurons in rats.

The authors wish to acknowledge the helpful advice of Dr Delgado-García in the preparation of this manuscript. This work has been supported by grants from the F.I.S.S.S. and Areces Foundation.

REFERENCES

BATSEL, H. L. (1964). Localization of bulbar respiratory center by microelectrode sounding. *Experimental Neurology* **9**, 410–426.

BENTIVOGLIO, M., KUYPERS, H. G. J. M., CATSMAN-BERREVOETS, C. & DANN, O. (1980). Two new fluorescent retrograde neuronal tracers which are transported over long distances. *Neuroscience Letters* **19**, 25–30.

BERMAN, A. L. (1968). *The Brain Stem of the Cat: A Cytoarchitectonic Atlas with Stereotaxic Coordinates.* Wisconsin: University of Wisconsin Press.

BIANCHI, A. L. (1971). Localisation et étude des neurones respiratoires bulbaires. Mise en jeu antidromique par stimulation spinale ou vagale. *Journal de physiologie* **63**, 5–40.

DAVIES, J. G. MCF., KIRKWOOD, P. A. & SEARS, T. A. (1985). The distribution of monosynaptic connexions from inspiratory bulbospinal neurons to inspiratory motoneurons in the cat. *Journal of Physiology* **368**, 63–87.

DELGADO-GARCÍA, J. M., LÓPEZ-BARNEO, J., SERRA, R. & GONZÁLEZ-BARÓN, S. (1983). Electrophysiological and functional identification of different neuronal types within the nucleus ambiguus in the cat. *Brain Research* **277**, 231–240.

KEIZER, K., KUYPERS, H. G. J. M., HUISMAN, A. M. & DANN, O. (1983). Diamidino Yellow dihydrochloride (DY 2HCl); a new fluorescent retrograde neuronal tracer which migrates only very slowly out of the cell. *Experimental Brain Research* **51**, 179–191.

KUYPERS, H. G. J. M., BENTIVOGLIO, M., CATSMAN-BERREVOETS, C. E. & BHAROS, A. T. (1980). Double retrograde neuronal labelling through divergent axon collaterals, using two fluorescent tracers with the same excitation wavelength which label different features of the cell. *Experimental Brain Research* **40**, 383–392.

KUYPERS, H. G. J. M. & MARTIN, G. F. (1982). Descending pathways to the spinal cord. *Progress in Brain Research* **57**, 145–175.

LAWN, A. M. (1966*a*). The localization of the nucleus ambiguus of the rabbit, of the cells of origin of motor nerve fibers in the glossopharyngeal nerve and various branches of the vagus nerve by means of retrograde degeneration. *Journal of Comparative Neurology* **127**, 293–306.

LAWN, A. M. (1966*b*). The nucleus ambiguus of the rabbit. *Journal of Comparative Neurology* **127**, 307–320.

LOBERA, B., PÁSARO, R., GONZÁLEZ-BARÓN, S. & DELGADO-GARCÍA, J. M. (1981). A morphological study of ambiguus nucleus motoneurons innervating the laryngeal muscles of the rat and cat. *Neuroscience Letters* **23**, 125–130.

LONG, S. E. & DUFFIN, J. (1984). The medullary respiratory neurons: a review. *Canadian Journal of Physiology and Pharmacology* **62**, 161–182.

MERRILL, E. G. (1970). The lateral respiratory neurons of the medulla: their association with nucleus ambiguus, nucleus retroambigualis, the spinal accessory nucleus and the spinal cord. *Brain Research* **24**, 11–28.

OLZEWSKI, J. & BAXTER, D. (1954). *Cytoarchitecture of the Human Brain Stem.* Philadelphia: J. P. Lippincott.

PÁSARO, R., LOBERA, B., GONZÁLEZ-BARÓN, S. & DELGADO-GARCÍA, J. M. (1981). Localization of laryngeal motoneurons in the rat. *Revista española de fisiología* **37**, 317–322.

PÁSARO, R., LOBERA, B., GONZÁLEZ-BARÓN, S. & DELGADO-GARCÍA, J. M. (1983). Cytoarchitectonic organization of laryngeal motoneurones within the nucleus ambiguus of the cat. *Experimental Neurology* **82**, 623-634.

PELLEGRINO, L. J., PELLEGRINO, A.S. & CUSHMAN, A. J. (1979). *A Stereotaxic Atlas of the Rat Brain.* New York: Plenum Press.

PORTILLO, F. & PÁSARO, R. (1986). Spinal projections of brainstem respiratory related neurons in the cat as revealed by retrograde fluorescent markers. *Revista española de fisiología* **42**, 483–488.

RICKARD-BELL, G. C., BYSTRZYCKA, E. K. & NAIL, B. S. (1984). Brainstem projections to the phrenic nucleus: a HRP study in the cat. *Brain Research Bulletin* **14**, 25–37.

RICKARD-BELL, G. C., BYSTRZYCKA, E. K. & NAIL, B. S. (1985). The identification of brainstem neurones projecting to the thoracic respiratory motoneurones in the cat as demonstrated by retrograde transport of HRP. *Brain Research Bulletin* **14**, 25–37.

YAYIMA, Y. & HAYASHI, Y. (1983). Identification of motoneurons in the nucleus ambiguus by antidromic stimulation of the superior and the recurrent laryngeal nerves in rats. *Brain Research* **288**, 302–306.