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# Hypoglossal premotor neurons with rhythmical inspiratory-related activity in the cat: localization and projection to the phrenic nucleus

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**Abstract** Localization and projection to the phrenic (PH) nucleus were studied in a sample of premotor neurons that directly projected to hypoglossal motoneurons (XII Mns) and showed respiratory-related patterns of activity. The experiments were carried out in cats, under pentobarbital anesthesia. In the first part of the study, the retrograde double-labeling technique was used to reveal the existence of neurons projecting to both the XII and the PH nuclei. Injection of a fluorescent dye (fast blue, FB) into the XII nucleus and another (nuclear yellow, NY) into the PH nucleus retrogradely labeled, with either FB or NY, medullary reticular neurons mainly in the regions ventrolateral to the nucleus of the tractus solitarius (vl-NTS), ventrolateral to the hypoglossal nucleus (vl-XII), and dorsomedial to the nucleus ambiguus (dm-AMB) bilaterally. In addition, some neurons in these regions were labeled with both FB and NY. In the second part of the study, unitary activity was recorded extracellularly from medullary respiratory neurons. In the regions vl-NTS, vl-XII, and dm-AMB, inspiratory neurons were found which antidromically responded to stimulation of the XII nucleus. Some of them also responded antidromically to stimulation of the PH nucleus. Averaging of rectified and integrated XII and PH nerve discharges by spontaneous spikes of single inspiratory neurons in the vl-NTS and dm-AMB regions revealed a facilitation in either XII nerve discharge or both XII and PH nerve discharges after a short latency of monosynaptic range. It is concluded that in the vl-NTS and dm-AMB regions there are inspiratory neurons that are excitatory premotor neurons projecting to XII Mns showing the respiratory-related activity. Some of them have excitatory synaptic connections to XII and PH Mns via bifurcating axons.

**Key words** Inspiratory neurons
Hypoglossal motoneuron · Phrenic motoneuron
Dual-projection neuron · Cat

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## Introduction

The tongue plays an important role in a variety of orofacial functions such as respiration, mastication, deglutition, and speech (for review, see Lowe 1981). During mastication, the tongue shows rhythmical activity in coordination with jaw movement when food is manipulated in the oral cavity, and it changes its posture rhythmically in association with respiratory movements to secure a patent airway.

A number of studies have addressed the neural mechanisms controlling tongue posture. These include: peripheral somatosensory inputs to hypoglossal motoneurons (XII Mns) from the trigeminal (V) nerve (Morimoto et al. 1972; Sumino and Nakamura 1974; Takata and Tomomune 1986), the glossopharyngeal nerve (Duggan et al. 1973; Hunter and Porter 1974), the superior laryngeal nerve (Sumi 1969), and the XII nerve (Morimoto and Kawamura 1972); central inputs from the cerebral cortex (Sumi 1970a; Lund and Dellow 1973); and central pattern generators (CPGs) for respiration (Sica et al. 1984), mastication (Dellow and Lund 1971), and deglutition (Sumi 1970a, b; Travers and Jackson 1992). Recently special attention has been paid to inputs from the respiratory CPG in relation to the pathogenesis of obstructive sleep apnea, for which the loss of genioglossal (GG) muscle tone may be responsible.

Although respiratory-related rhythmical activity has been reported in some XII Mns (Withington-Wray et al. 1988), no information is available concerning the location of excitatory premotor neurons directly projecting

to XII Mns that show respiratory-related rhythmical activity.

It was recently demonstrated tha medullary inspiratory neurons that projected to the spinal cord had axon collaterals projecting to vagal (X) Mns innervating accessory respiratory muscles (Ezure and Manabe 1989). In addition, some axon collaterals of these neurons were found in the vicinity of the XII nucleus (Sasaki et al. 1989). With regard to central mechanisms controlling the respiratory-related XII Mn activity, these reports strongly suggest the existence of respiratory neurons that possess bifurcating axons projecting to XII Mns as well as to spinal respiratory Mns, including phrenic (PH) Mns.

Takada et al. (1984) found horseradish peroxidase (HRP)-labeled neurons bilaterally in the parvocellular reticular formation and reticular regions around the XII nucleus after HRP injection into the XII nucleus of the cat. On the other hand, when HRP was injected into the PH nucleus, retrogradely labeled cells were seen bilaterally, with a contralateral dominance in the region ventrolateral to the tractus solitarius (vl-NTS), the region dorsomedial to the nucleus ambiguus (dm-AMB), and the parvocellular reticular formation (Rikard-Bell et al. 1984). On the basis of these physiological as well as morphological studies, it seems possible that in the medullary reticular formation, including the regions around the XII nucleus and the nucleus ambiguus, there might be respiratory neurons which directly project to the XII nucleus and some of them might project also to the PH nucleus with bifurcating axons.

In this study, locations of bulbar reticular neurons projecting to the XII and PH nuclei were investigated by means of retrograde axonal transport of two kinds of fluorescent dyes, one injected into the XII nucleus and the other into the PH nucleus. In addition, we averaged the rectified and integrated XII and PH nerve discharges with respect to spontaneous spikes of a single respiratory neuron recorded in the region where bulbar reticular neurons projecting to the XII and/or PH nucleus were found in our morphological study. We paid special attention to whether there were XII premotor neurons that also projected to PH Mns via bifurcating axons.

Part of this work has been reported in abstract form (Ono et al. 1989).

## Materials and methods

### Morphological studies

Experiments were performed on eight adult cats of either sex, weighing 2.4–4.2 kg. The animals were given a prophylactic dose of antibiotics for 2 days before surgical operations. Chlorpromazine hydrochloride (2.0 mg/kg i.m.) and atropine sulfate (0.1 mg/kg i.m.) were initially administered; 30 min later, ketamine

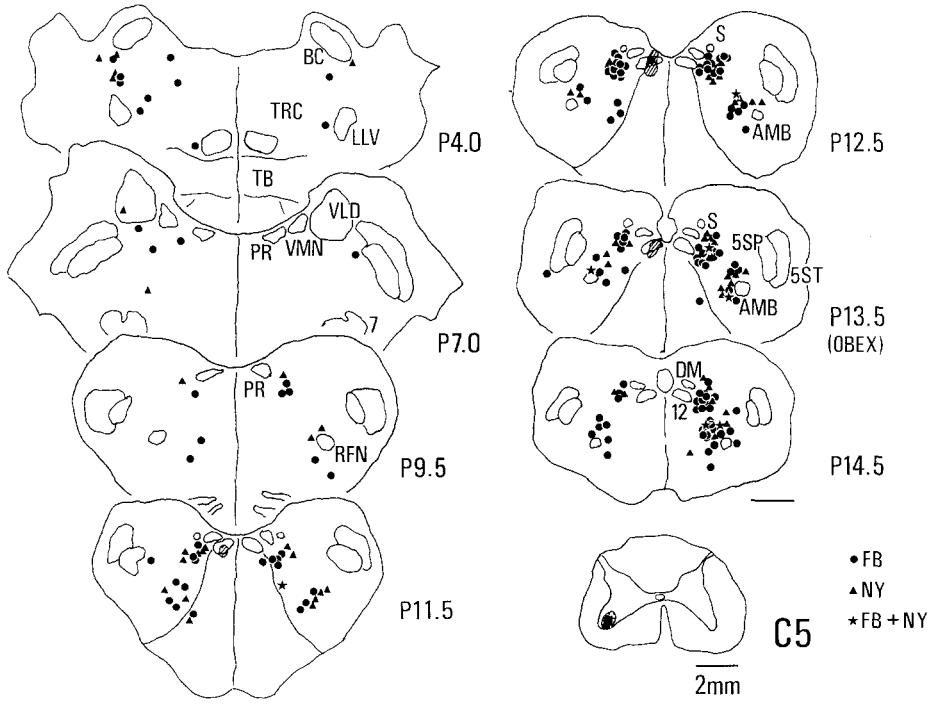


**Fig. 1** Schematic illustration of transverse sections of the medulla, from cat F71, indicating the location of neurons labeled with fluorescent dyes. Each section includes cell bodies from five consecutive histological slices. *Circles*, fast blue (*FB*)-labeled neurons; *triangles*, nuclear yellow (*NY*)-labeled neurons; *stars*, double-labeled neurons with FB and NY. *Hatching*, site of FB injection into the left hypoglossal nucleus; *stippling*, site of NY injection into the left phrenic nucleus. *Abbreviations*; *AMB*, nucleus ambiguus; *BC*, brachium conjunctivum; *DM*, dorsal motor nucleus of the vagus; IO, inferior olivary nucleus; *LLV*, ventral nucleus of the lateral lemniscus; *PR*, nucleus prepositus hypoglossi; *RFN*, retrofacial nucleus; *S*, solitary tract; *TB*, trapezoid body; *TRC*, tegmental reticular nucleus, central division; *VLD*, lateral vestibular nucleus, dorsal division; *VMN*, medial vestibular nucleus; *5SP*, spinal trigeminal nucleus; *5ST*, spinal trigeminal tract; 7, facial nucleus; 12, hypoglossal nucleus

hydrochloride was given (40 mg/kg i.m.). Anesthesia was maintained with pentobarbital sodium (40 mg/kg i.p.) throughout the surgical procedures, and all animals respired spontaneously. The electrocardiogram (ECG) was monitored, and the rectal temperature was maintained at 37–38° C by an incandescent lamp from above and by an electric heater under the abdomen.

Each animal was mounted on a stereotaxic apparatus in a prone position. A midline skin incision was made over the cranium, and the left skin flap was reflected to detach the temporal muscle from the cranium. Following a partial occipital craniotomy, the floor of the fourth ventricle around the obex was exposed by aspiration of a small part of the overlying cerebellum. A laminectomy was performed at the C5 segment, and the dura was opened to expose the dorsal surface of the spinal cord on the left side.

In the present study, we used fluorescent dyes fast blue (FB) and nuclear yellow (NY) for convenience in histological procedures. In consideration of the velocity of retrograde axonal transport and an optimal survival period (Bentivoglio et al. 1980; Keizer and Kuypers 1984), NY was injected into the PH nucleus and FB into the XII nucleus, and the animals were killed after 72 h to prevent NY leaking from neurons.

Hamilton microsyringes (0.1 µl) were filled with either FB (5.0%) or NY (2.0%), which were dissolved in distilled water. To prevent the spread of fluorescent dyes into adjacent structures from the center of the injection sites, a relatively small volume (0.05 µl) of dye was injected. FB (0.05 µl) was injected in the left XII nucleus stereotaxically. For injection of NY into the PH nucleus, an enamel-coated tungsten microelectrode was inserted into the left C5 segment to locate the point where the maximum negative antidromic field potential was evoked by stimulation of the left PH nerve by a pair of enamel-coated, fine steel wire electrodes (200 µm in diameter; interpolar distance ca. 10 mm) inserted into the left diaphragm. After the point was determined, the tungsten electrode was withdrawn and replaced with a Hamilton microsyringe filled with NY solution, and 0.05 µl of the solution was injected into the site. The dye solutions were pressure-injected into the XII and PH nuclei slowly over 2 h, after microsyringes had been left at the predetermined points for more than 10 min for stabilization. Wounds were carefully sutured and each animal was treated with penicillin and appropriate amounst of glucose.

After 72 h survival, the animals were deeply anesthetized with pentobarbital sodium (80 mg/kg i.p.), and perfused transcardially with 0.9% saline, followed by 10% formalin (pH 7.2). The brainstem and the fourth to sixth cervical segments of the spinal cord were taken out separately and soaked in 30% cacodylate-buffered sucrose (pH 7.2) for 24 h in the dark at 2–4° C (Bentivoglio et al. 1980). Then transverse frozen sections (50 µm in thickness) were made of the brainstem and the cervical spinal cord. They were collected in distilled water, immediately mounted and dried at room temperature, but not coverslipped. Sections were stored for 12 h in the dark at 4° C prior to histological studies, observed on a fluorescence microscope equipped with a filter system providing an excitation wavelength of 360 nm, and photographed. Every sixth section was counterstained by the Klüver-Barrera method, for reference purposes.

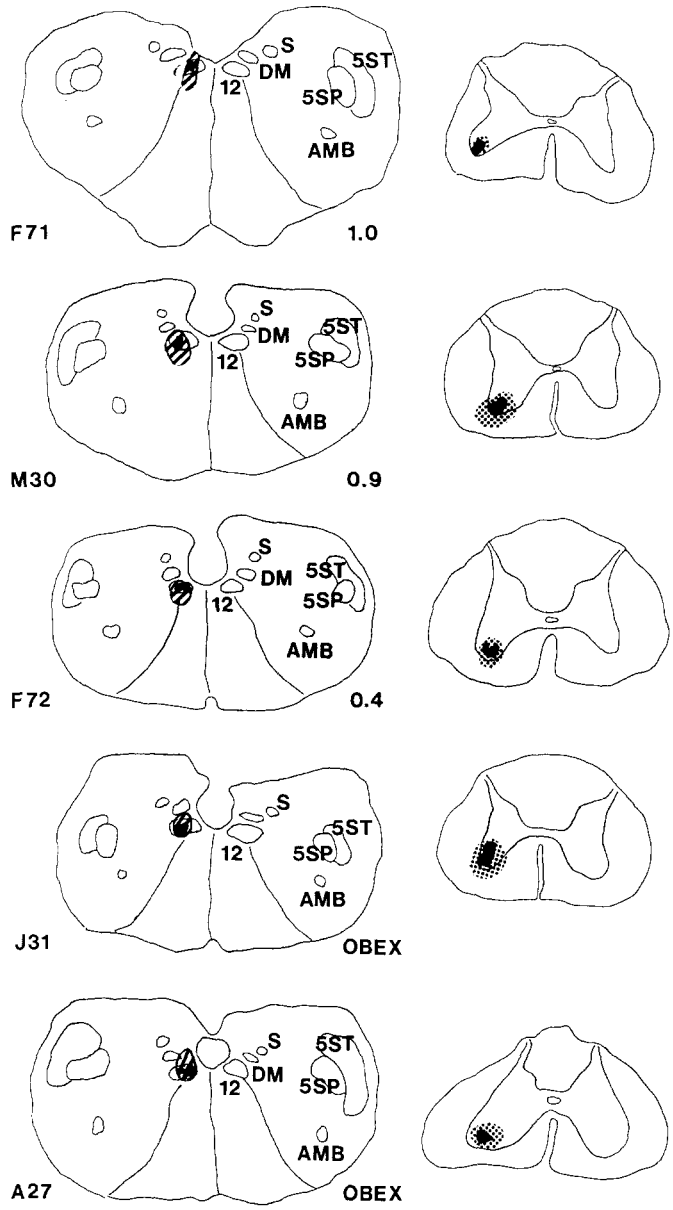


**Fig. 2** Schematic illustration of transverse setions through the center of injection site of the medulla and spinal cord, showing the largest injection sites of FB and NY in five animals. Numbers at *bottom right* of the medulla indicate the distance rostral to the obex in millimeters. *Black*, site where the necrotic tissue and neurons intensely labeled with tracers are seen; *hatching*, sites where glia and neurons are slightly labeled with FB injected into the left hypoglossal nucleus, *stippling*, sites where glia and neurons are slightly labeled with NY injected into the left C5 phrenic nucleus. *Abbreviations* as in Fig. 1

## Physiological studies

Experiments were performed on 19 adult cats of either sex, weighing 2.7–4.5 kg. They were sedated using the same procedures as used in morphological studies, and ketamine hydrochloride was given (40 mg/kg i.m.). After the trachea and right superficial radial vein were cannulated, halothane (1.5–2.0%) insufflation was started and continued throughout the surgical procedures.

A midline skin incision was made from the symphysis mentalis to the top of the sternum. The medial and lateral branches of the XII nerve were dissected free and sectioned distally on the left side. A bipolar silver-wire collar electrode (interpolar distance ca. 5 mm) was fixed to the central cut end of the medial branch for stimulation and recording, which innervates tongue-protruding muscles including the GG muscle. The C5 and/or C6 portion of PH nerve on the left side was dissected free near the diverging point of the jugular and subclavian veins and sectioned distally. After the incision was sutured and lidocaine hydrochloride (1.0%) was infiltrated to pressure points, each animal was mounted on a stereotaxic apparatus in a prone position with the head ventroflexed. The temporal muscle was stripped off from the origin on the cranium. A partial occipital craniotomy was performed, and the cerebellum was partly aspirated to expose the floor of the fourth ventricle around the obex. A laminectomy at the C5 and C6 segments was performed and the overlying dura was removed.

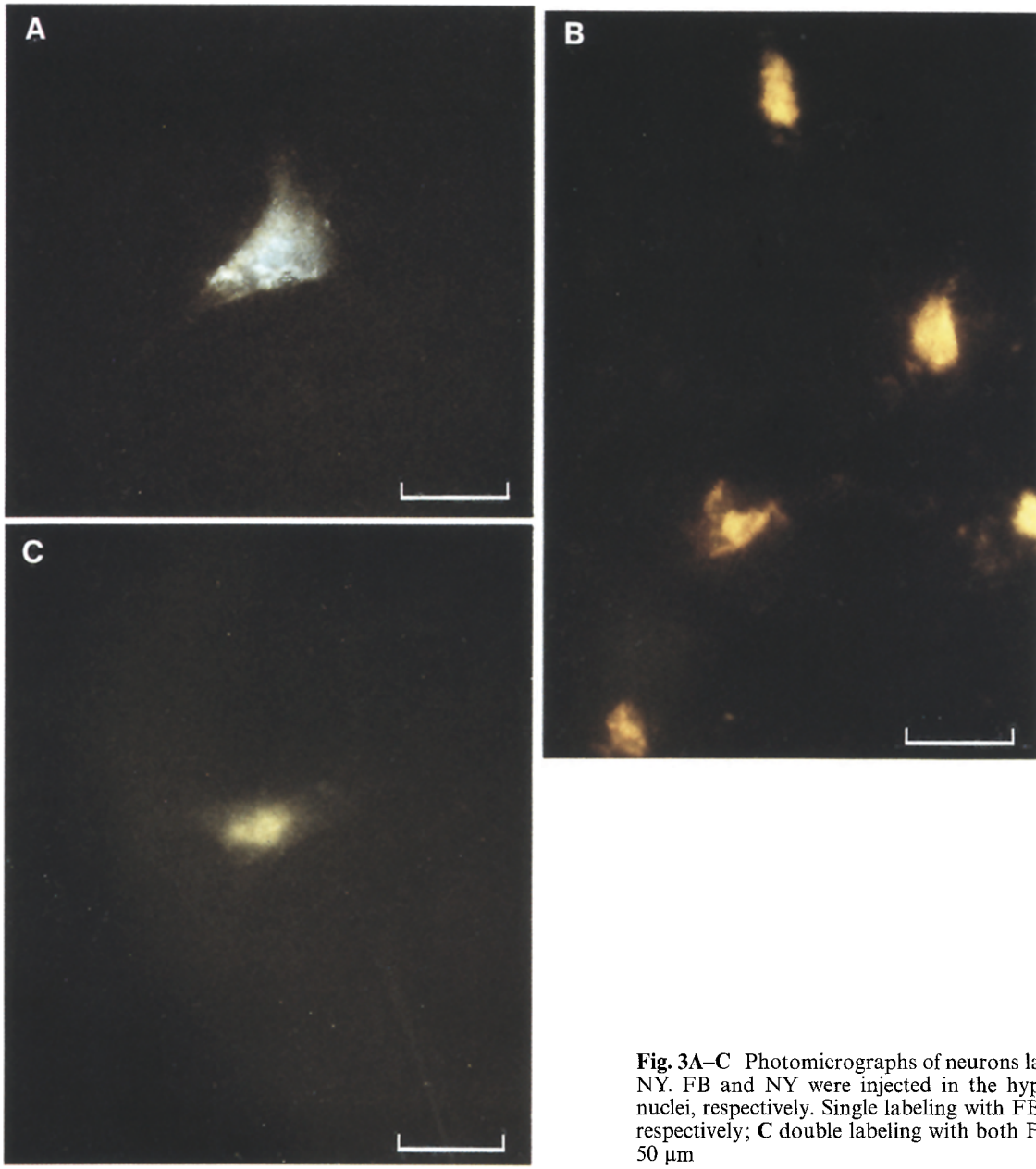


**Fig. 3A–C** Photomicrographs of neurons labeled with FB and/or NY. FB and NY were injected in the hypoglossal and phrenic nuclei, respectively. Single labeling with FB and NY in **A** and **B**, respectively; **C** double labeling with both FB and NY. *Scale bars* 50 μm

After separation of the splenius muscle from the platysma, a bipolar silver-wire collar electrode (interpolar distance ca. 3 mm) was fixed to the central cut end of the left PH nerve. In some animals, a bilateral cervical vagotomy was performed.

When surgical procedures were completed, halothane anesthesia was terminated. During recording sessions, the animal was kept under pentobarbital anesthesia (10–15 mg/kg per hour i.v.), paralyzed with pancronium bromide (0.1 mg/kg per hour i.v.), and artificially ventilated. A bilateral pneumothorax was made to improve recording stability.

An enamel-coated tungsten microelectrode, electrically etched to a tip size of 1–3 μm, was inserted into the left PH nucleus at the C5–C6 segments, for monopolar stimulation, and fixed to the point where the largest negative antidromic field potential was evoked by stimulation of the left PH nerve. To minimize stimulus artifacts, a pair of enamel-coated tungsten microelectrodes was inserted into the left XII nucleus for bipolar stimulation. Each electrode of the pair was inserted one after the other, separately. First, one of the pair was inserted into the XII nucleus at the middle level of its rostrocaudal extent, then the other into its close vicinity. The two electrodes were fixed to the points in the XII nucleus where the largest negative antidromic field potentials were evoked along each insertion track by stimulation of the medial branch of the left XII nerve (interpolar distance $\leq 1.0$ mm).

Single-unit activities were recorded from respiratory neurons by enamel-coated tungsten microelectrodes (impedance 10–20 MΩ at 1 kHz) in the lower brainstem on both sides (from 3.0 mm rostral to 3.0 mm caudal to the obex in the rostrocaudal direction; from 1.5 mm to 4.5 mm lateral to the midline). Respiratory neurons projecting to the XII nucleus or both the XII and PH nuclei were identified by (1) a rhythmical firing activity corresponding with the respiratory cycle and (2) spike responses after a fixed short-latency to microstimulation ($< 50$ μA) of the XII nucleus or both the XII and the PH nuclei, which showed collision with spontaneous spikes. During recording, single-unit activity of medullary respiratory neurons, the tracheal pressure, and discharges in both the XII and PH nerves were simultaneously displayed on oscilloscopes after amplification and recorded on a tape recorder. The XII and PH nerve discharges were full-wave rectified, integrated (time constant 10 ms), and averaged by the use of

extracellular spikes of a medullary respiratory neuron as the trigger. Usually more than 2000 sweeps were averaged with respect to trigger pulses generated by a window discriminator.

Over an entire series of experiments, end-tidal $CO_2$ was maintained at 3.5–5.0%. The tracheal pressure was monitored by a pressure change in the insufflation side of a Y-shaped tracheal cannula. ECG was monitored, and rectal temperature was maintained at about 37° C by radiating heat from above and a heating pad under the abdomen.

At the end of each experiment, the animal was deeply anesthetized with pentobarbital sodium. Locations of tips of stimulating and recording electrodes were marked by passing d.c. cathodal current of 20 μA for 20 s through the electrodes, and the animal was perfused transcardially with 0.9% saline followed by 10% formalin. Serial transverse frozen sections of 75 μm thickness were made of the brainstem, and stained by the Klüver-Barrera method for verification of electrolytically lesioned points. The locations of the lesions were used as reference points to reconstruct the sites of single-unit recording.

## Results

### Neurons retrogradely labeled with fluorescent dyes

Figure 1 shows schematically the transverse histological sections of the medulla and spinal cord of one animal, for which sites of fluorescent dye injection as well as locations of neurons labeled with the dyes are plotted. Both NY and FB were injected on the left side (FB P11.5–13.5; NY C5). NY-labeled neurons (triangles) and FB-labeled neurons (circles) were mainly found in the regions vl-NTS, ventrolateral to the XII nucleus (vl-XII), and dm-AMB bilaterally around the level of the obex. Double-labeled neurons (stars) were mainly found in the contralateral vl-NTS, vl-XII, and dm-AMB regions (the terms "ipsilateral" and "contralateral" in the text are used with respect to the side of dye injection). In this animal, 129 FB-labeled neurons were found, 57 on the ipsilateral and 72 on the contralateral sides. A total of 62 NY-labeled neurons were found, 25 on the ipsilateral and 37 on the contralateral sides. Seven neurons were double-labeled, one on the ipsilateral and six on the contralateral sides. Thus all three types of neuron were found to have a contralateral dominance. Despite

**Fig. 4A–D** Patterns of discharge and projection of a respiratory neuron. **A** Simultaneous record of extracellular spikes of a respiratory neuron (*a*), rectified and integrated hypoglossal (XII) nerve discharge (*b*), rectified and integrated phrenic (PH) nerve discharge (*c*), and tracheal pressure (*d*). **B** A portion of **A** (*underlined*) displayed on an expanded time base. **C** *a*, Fixed short-latency response of the respiratory neuron to stimulation of the contralateral XII nucleus (1.54 Hz, 0.1 ms, 40 μA); *b*, collision of the spike with a spontaneous spike. **D** Averaged of integrated discharges of XII and PH nerves by spontaneous spikes of the neuron shown in **A–C**. Note facilitation in the XII nerve, but not in the PH nerve, after a short latency. *a*, triggering spike; *b*, XII nerve discharge; *c*, PH nerve discharge. Traces *a–c* were simultaneously obtained by averaging 2000 traces. In this and following figures, negative deflection of single spikes is shown *downward*, and *upward arrows* indicate the onset of the increase in nerve activity

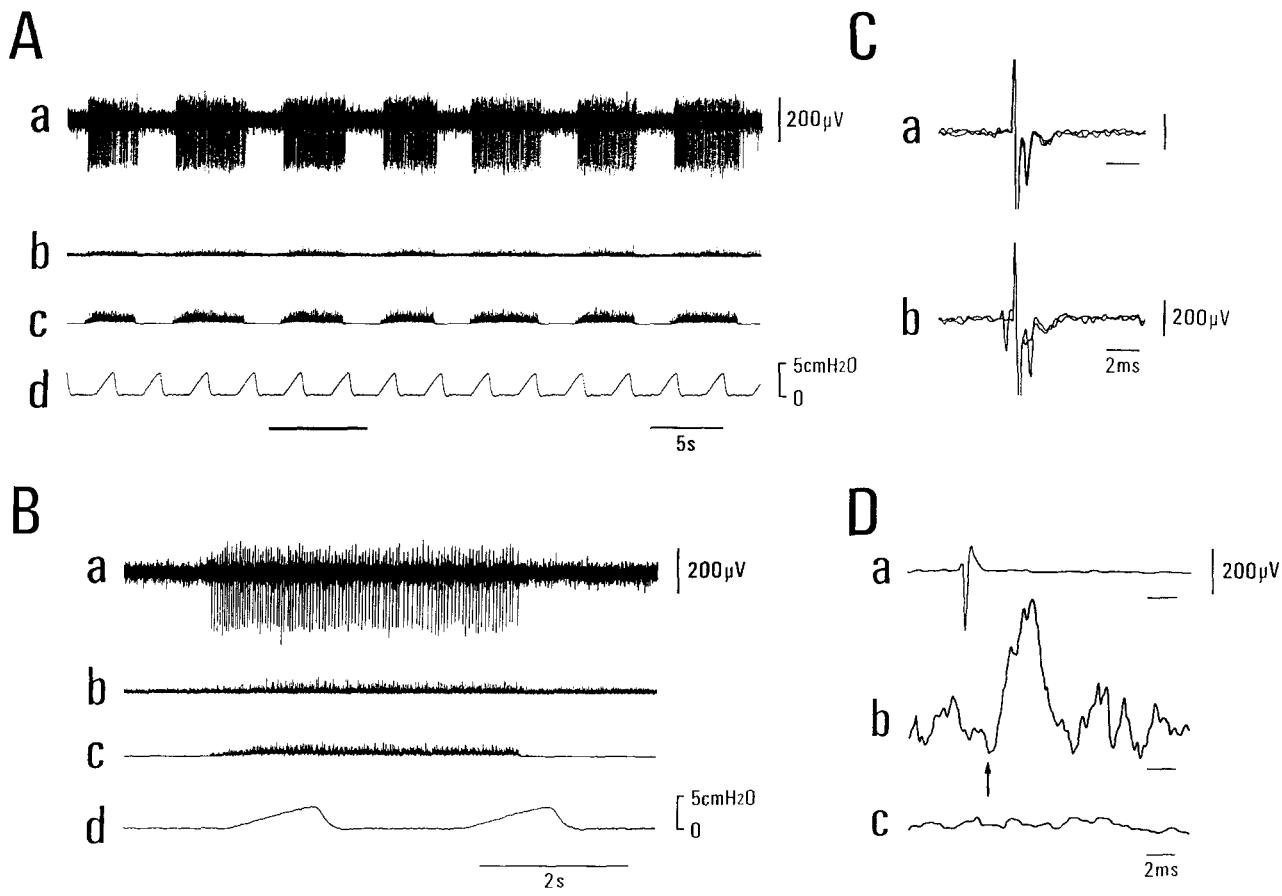

**Table 1** Locations and numbers of labeled neurons in the pons and medulla in five cats. In each animal, the top, middle, and bottom numbers show the number of neurons labeled with fast blue (FB), nuclear yellow (NY), and both FB and NY, respectively. Neurons in the regions ventrolateral to the tractus solitarius and ventrolateral to the hypoglossal nucleus are shown as "dorsal," and those in the region dorsomedial to the nucleus ambiguus are shown as "ventral". I, ipsilateral; C, contralateral

| | | Pons | | Medulla | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Dorsal | | Ventral | | Others | |
| Animal | | I | C | I | C | I | C | I | C |
| | FB | 8 | 2 | 22 | 39 | 15 | 27 | 12 | 4 |
| F71 | NY | 4 | 1 | 12 | 17 | 6 | 17 | 3 | 2 |
| | FB+NY | 0 | 0 | 0 | 1 | 1 | 4 | 0 | 1 |
| | FB | 3 | 3 | 5 | 14 | 5 | 5 | 21 | 14 |
| M30 | NY | 8 | 9 | 14 | 16 | 24 | 32 | 35 | 27 |
| | FB+NY | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | FB | 8 | 2 | 19 | 38 | 15 | 25 | 12 | 9 |
| F72 | NY | 3 | 1 | 2 | 17 | 6 | 20 | 3 | 2 |
| | FB+NY | 0 | 0 | 0 | 1 | 1 | 5 | 0 | 0 |
| | FB | 10 | 4 | 15 | 19 | 19 | 10 | 49 | 21 |
| J31 | NY | 18 | 5 | 17 | 31 | 34 | 28 | 79 | 58 |
| | FB+NY | 4 | 0 | 2 | 5 | 3 | 10 | 1 | 0 |
| | FB | 19 | 1 | 13 | 9 | 3 | 8 | 18 | 13 |
| A27 | NY | 9 | 4 | 10 | 10 | 3 | 10 | 4 | 6 |
| | FB+NY | 1 | 0 | 0 | 0 | 0 | 3 | 0 | 0 |

the injection of relatively small volume, the spread of fluorescent dyes was seen outside the XII and PH nuclei. Figure 2 schematically illustrates the transverse sections of the medulla and spinal cord, showing the central region of injection sites of NY and FB in five of eight animals into which fluorescent dyes were injected. Each section illustrates the injection site where the spread of dyes was maximum. Thus in these five animals the bulk of FB and NY were confined in the XII and PH nuclei, respectively, throughout the rostrocaudal extent. In the remaining three animals, however, a substantial amount of FB and/or NY was spread outside the XII and/or PH nucleus. Accordingly the morphological study was performed in the five animals. Table 1 shows the numbers and locations of neurons labeled with FB and/or NY in these five animals.

Figure 3A is a photomicrograph of a FB-labeled neuron found in the ipsilateral vl-NTS region in the animal, from which the plot in Fig. 1 was made. The cytoplasm of the neuron shows blue fluorescence. Figure 3B illustrates several NY-labeled neurons in the contralateral vl-NTS region in this animal. Nuclei of neurons are labeled with yellow fluorescence. Figure 3C shows a double-labeled neuron, located in the contralateral dm-AMB region in the same animal. Its cytoplasm and nucleus were labeled with FB and NY, respectively. Although retrogradely labeled neurons with either or both fluorescent dyes were found throughout the brainstem, they were most densely clustered in two distinct regions (Fig. 1). One was the vl-NTS and vl-XII region, and the other was the dm-AMB region. In these two regions, neurons were found which were retrogradely double-labeled with FB and NY.

## Inspiratory neurons directly projecting to XII Mns

Using the results of the morphological study, we searched for respiratory neurons projecting to the XII nucleus or both the XII and the PH nuclei mainly in the vl-NTS, vl-XII, and dm-AMB regions in the medullary reticular formation. In 19 animals, single-unit activity was recorded from a total of 42 putative premotor neurons which antidromically responded to stimulation of the XII nucleus or both the XII and the PH nuclei and showed rhythmical burst activities in coincidence with rhythmical PH nerve discharges. No expiratory neuron that antidromically responded to stimulation of the XII nucleus was recorded.

Figure 4 illustrates one of 33 inspiratory neurons which antidromically responded to stimulation of the XII nucleus but not to stimulation of the PH nucleus. This neuron, in the right dm-AMB region (Fig. 6, thin arrow), showed a rhythmical burst activity (Fig. 4A, B, a) synchronous with rhythmical discharges in the XII and PH nerves (Fig. 4A, B, b, c), indicating that it is an inspiratory neuron. It responded to stimulation of the left XII nucleus with spike potentials after a fixed latency of 0.6 ms (Fig. 4C, a), which showed a collision with its spontaneous spikes (Fig. 4C, b). Thus the neuron was assumed to be a possible premotor neuron projecting to XII Mns which show a respiratory-related rhythmical activity. Averaging the rectified and integrated discharges of both the XII and the PH nerves by spontaneous spikes of this inspiratory neuron revealed a facilitation in the XII nerve discharge after 1.9 ms from the negative deflection of spikes of the inspiratory neuron (Fig. 4D, b, upward arrow), but no facilitation in the PH nerve discharge (Fig. 4D, c). The peak of the facilitation was 43% greater than the mean control level of rectified

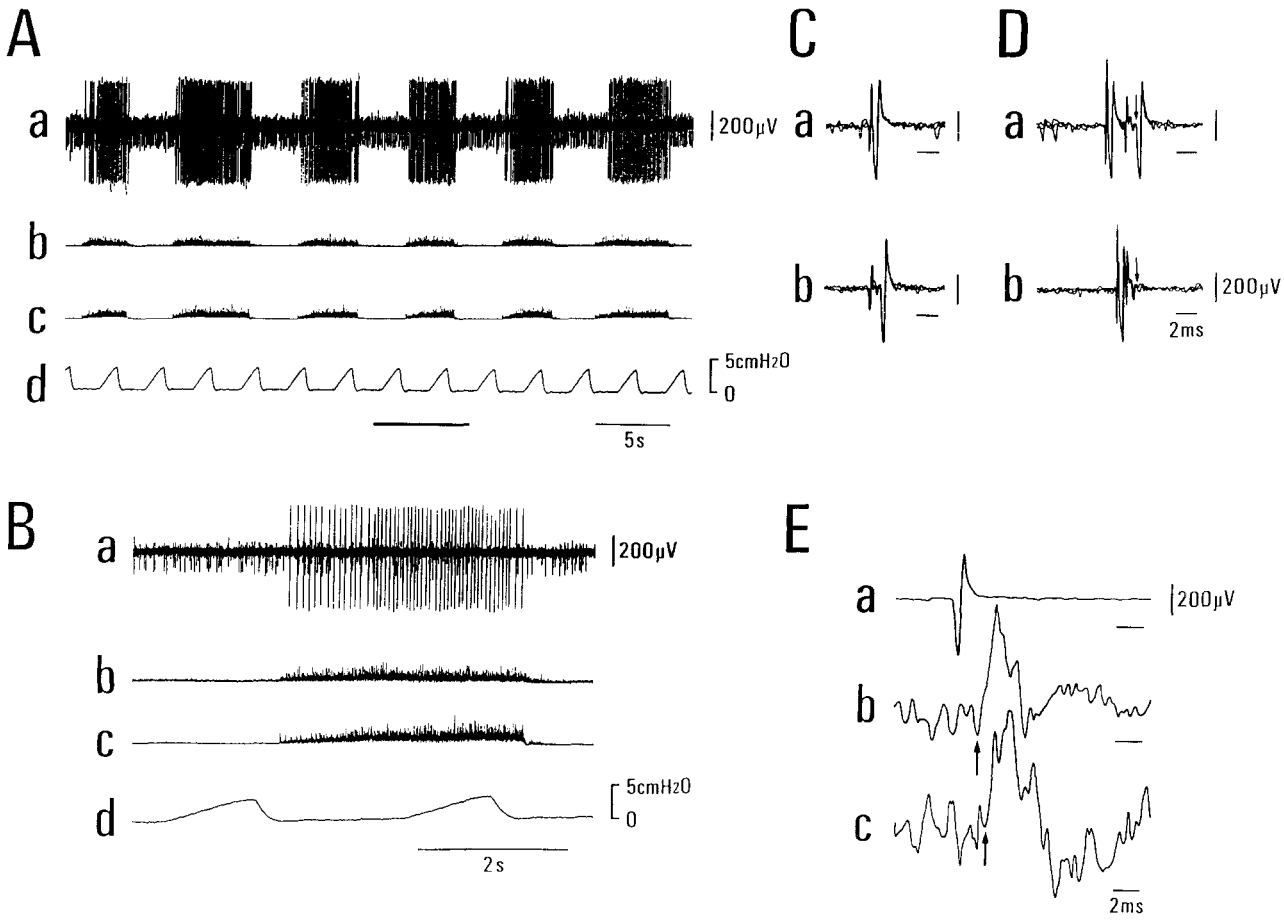


**Fig. 5A–E** Patterns of discharge and projection of a respiratory neuron. The format of **A, B, E** is the same as in **A, B, D** in Fig. 4, respectively. **C** Fixed short-latency responses of the respiratory neuron to stimulation of: *a*, the contralateral XII nucleus (1.54 Hz, 0.1 ms, 17 μA) and *b*, the contralateral PH nucleus (1.54 Hz, 0.1 ms, 40 μA). **D** Collision of spikes evoked by stimulation of the PH nucleus (*downward arrows*) eith preceding spikes evoked by stimulation of the XII nucleus. Intervals between stimulation of the XII and PH nuclei are 2.0 ms (*a*) and 1.0 ms (*b*). In **E,** note facilitation in the XII and PH nerve discharges after short latencies (*upward arrows*)

and integrated discharge for 10 ms immediately preceding the triggering spikes.

Figure 5 shows one of nine inspiratory neurons which antidromically responded to stimulation of both the XII and the PH nuclei. This neuron was found in the right dm-AMB region (Fig. 6, thick arrow) and showed a burst activity (Fig. 5A, B, a) coincident with rhythmical bursts in the XII and PH nerves (Fig. 5A, B, b, c). It responded with spike potentials after fixed short latencies of 0.4 ms to stimulation of the left XII nucleus (Fig. 5C, a) and 1.4 ms to stimulation of the left PH nucleus (Fig. 5C, b).

The neuron responded with spikes to stimulation of the two nuclei when the interval of stimulation was 2.0 ms or longer (Fig. 5D, a, downward arrow). In contrast, at intervals shorter than 2.0 ms, the spike potential evoked by stimulation of the PH nucleus collided with the spike potential evoked by stimulation of the XII nucleus (Fig. 5D, b, downward arrow). Averaging the rectified and integrated discharges in the XII and PH nerves by spontaneous spike discharges of this neuron (Fig. 5F, a) revealed a 72% facilitation after a latency of 1.9 ms in the discharge of the XII nerve, while a 42% facilitation after a latency of 2.6 ms in the discharge of the PH nerve compared with the mean level of respective nerve discharges for 10 ms immediately preceding the triggering spike (Fig. 5E, b, c, upward arrows).

The spike-triggered averaging technique revealed a prominent peak in the discharge of the XII nerve in 27 of 33 neurons which responded to stimulation of the XII nucleus, and in the discharge of the XII and PH nerves in seven of nine neurons that responded to stimulation of both the XII and the PH nuclei. Of the 27 neurons, 2 and 25 neurons were antidromically activated by stimulation of the ipsilateral and contralateral XII nucleus, respectively. Likewise, of the seven neurons, one and six neurons were antidromically activated from the XII and PH nuclei on the ipsilateral and contralateral sides, respectively.

The facilitation in the XII nerve discharge started 1.5–2.0 ms ($n=27$) after the triggering spikes in the former group of neurons, while the latencies of facilitation of the XII and PH nerve discharges were 1.7–2.3 ms and 2.2–2.7 ms ($n=7$), respectively, after spikes of the latter group of neurons.

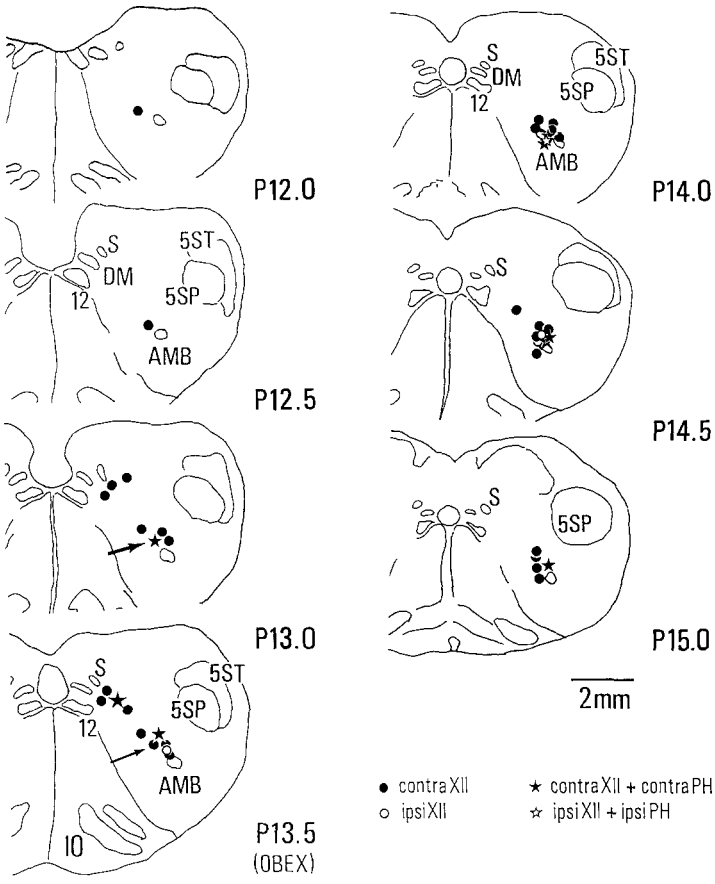


**Fig. 6** Location of inspiratory neurons activated antidromically by stimulation of the XII nucleus or both the XII and the PH nuclei. Neurons are plotted with different symbols depending on the nuclei from which they were antidromically activated: *filled circles* ($n=29$), contralateral XII nucleus; *open circles* ($n=4$), ipsilateral XII nucleus; *filled stars* ($n=8$), contralateral XII and PH nuclei; *open star* ($n=1$, section P14.0), ipsilateral XII and PH nuclei. *Thin arrow*, the neuron shown in Fig. 4; *thick arrow*, the neuron shown in Fig. 5. Locations of neurons recorded on the left side are plotted in the corresponding sites on the right side. *Abbreviations* as in Fig. 1

Locations of a total of 42 inspiratory neurons are schematically illustrated on transverse sections of the brainstem in Fig. 6. Thirty-three neurons (circles) antidromically responded to stimulation of the XII nucleus and nine neurons (stars) did so to stimulation of the XII and PH nuclei. These dual projection neurons were mainly located in vl-NTS and dm-AMB regions near the obex.

## Discussion

### Retrograde labeling of neurons with fluorescent dyes

Neurons retrogradely labeled with either FB or NY were located in a widespread region of the lower brainstem, including the area where retrogradely labeled neurons were found after injection of HRP into the XII and PH nuclei (Rikard-Bell et al. 1984; Takada et al. 1984). The most notable finding in the present study is that there are neurons retrogradely labeled with both FB and NY in the vl-NTS, vl-XII and dm-AMB regions with a contralateral dominance.

The retrograde labeling technique has been utilized by a number of studies to identify the medullary neurons which project to the XII or PH nucleus (XII: Takada et al. 1984; Amri et al. 1990; Manaker et al. 1992; PH: Rikard-Bell et al. 1984; Onai and Miura 1986; Portillo and Pásaro 1988). In comparison with these previous studies, a relatively small number of neurons was retrogradely labeled with FB and/or NY injected into the XII and PH nuclei in the present study. Several factors may be involved in this difference: (1) the kind of injected tracers–FB into the XII nucleus and NY into the PH nucleus (the present study), HRP into the PH nucleus (Rikard-Bell et al. 1984; Onai and Miura 1986), HRP into the XII nucleus (Takada et al. 1984), FB into the XII nucleus (Amri et al. 1990), diamidino yellow (DY) into the cervical spinal cord (Portillo and Pásaro 1988), DY into the XII nucleus and FB into the spinal cord (Manaker et al. 1992) – and (2) the volume of injected tracers – 0.05 µl (the present study), 0.2–0.6 µl (Amri et al. 1990), 2.0 µl (Rikard-Bell et al. 1984), 2.0–5.0 µl (Manaker et al. 1992), unclear (Takada et al. 1984; Onai and Miura 1986; Portillo and Pásaro 1988).

At the sites of dye injection into the XII and PH nuclei in five animals, the region which showed intense fluorescence was confined within respective nuclei, though slight fluorescence was seen in their close vicinity (Fig. 2). Moreover, the area with some residual fluorescent dyes observed 72 h after dye injection could not necessarily be assumed to be the same as the area from which the fluorescent dyes were taken up into the axon. The dye might have further spread from the injection site after that which was transported to the soma had been taken up from the axon terminals. Thus it could be assumed that most of the neurons retrogradely labled with dyes had projections to the XII and/or PH nucleus, though some of them may have taken up dyes from the vicinity of respective nuclei.

Inspiratory neurons in the dorsal respiratory group (DRG) and the ventral respiratory group (VRG) which responded to stimulation of the cervical cord decussate near the obex to descend in the spinal cord (Otake et al. 1989; Sasaki et al. 1989). Most of their stem axons run ventrally and then turn medially to cross the midline in the ventral portion of the brainstem near the obex. Since the spread of FB injected into the XII nucleus was confined to the most dorsal portion of the brainstem and far apart from these crossing axons, it could not be likely that FB was taken up by the fibers of the neurons projecting to the PH nucleus alone.

In the present morphological study, no analysis could be made of whether there were neurons which projected to both the XII nucleus on the one side and the PH nucleus on the other side, because fluorescent dyes were injected into the two nuclei on the same side.

## Inspiratory neurons directly projecting to XII Mns and dual-projection neurons

Two types of inspiratory neurons were found in the vl-NTS and dm-AMB regions: one antidromically responded only to stimulation of the XII nucleus, while the other did so to stimulation of both the XII and the PH nuclei. This series of experiments alone could not exclude the possibility that the current spread to the region surrounding the PH as well as XII nucleus or activated fibers passing through or near these nuclei. We accordingly averaged the discharge of XII and PH nerves with respect to spontaneous spikes of simultaneously recorded, single inspiratory neurons to reveal projections from these inspiratory neurons to the XII and PH Mns (Miller et al. 1985; Merrill and Lipski 1987).

The inspiratory neuron shown in Fig. 4 responded with antidromic spike potentials after a fixed latency of 0.6 ms to stimulation of the contralateral XII nucleus. The conduction time between the soma of the inspiratory neuron and the stimulating electrode in the contralateral XII nucleus was estimated to be 0.4 ms, after 0.2 ms was subtracted as the utilization time (Jankowska and Roberts 1972a). The conduction time from the somata of XII Mns to the recording electrode on the XII nerve was estimated to be 1.2 ms, on the basis of the latency of the negative deflection of the antidromic field potential in the XII nucleus evoked by stimulation of the XII nerve minus 0.2 ms as the utilization time. If we assume a synaptic delay to be 0.3–0.4 ms (Jankowska and Roberts 1972b), the sum of conduction times in the inspiratory neuron (0.4 ms), the XII nerve (1.2 ms), and one synaptic delay (0.3–0.4 ms) amounts to 1.9–2.0 ms. In fact, facilitation was seen in the XII nerve discharge 1.9 ms after the onset of the negative deflection of triggering spikes of the inspiratory neuron. Thus the calculated latency corresponded well with the observed latency, with a difference of 0.1 ms or less. It is therefore unlikely for another neuron to have been intercalated between the recorded inspiratory neuron and XII Mns. In contrast, no direct projection was found from this inspiratory neuron to PH Mns: neither antidromic spike response to stimulation in the PH nucleus nor facilitation in the PH nerve discharge was present. With respect to the remaining 26 inspiratory neurons which induced facilitation of the XII nerve discharge, the latency of facilitation corresponded with the calculated monosynaptic latency with a difference of 0.2 ms or less. Thus we are led to conclude that there are monosynaptic excitatory projections from the recorded inspiratory neurons to XII Mns.

As to the inspiratory neuron shown in Fig. 5, the conduction time to XII Mns was estimated to be 0.2 ms, by subtracting the utilization time (0.2 ms) from the latency of antidromic spikes evoked by stimulation of the contralateral XII nucleus (0.4 ms). The sum of 0.2 ms, 0.3–0.4 ms as a synaptic delay and 1.2 ms as a conduction time from the XII Mn to the recording electrode on the XII nerve amounts to 1.7–1.8 ms. This calculated monosynaptic latency corresponded with the observed latency of facilitation (1.9 ms) with a difference of less than 0.2 ms. Likewise, the conduction time from the stimulating electrode in the PH nucleus to the soma of the recorded neuron was 1.2 ms. The negative deflection of the antidromic field potential in the PH nucleus evoked by stimulation of the PH nerve started 1.2 ms after the stimulus artifact; the subtraction of 0.2 ms yielded 1.0 ms. If we add 1.2 ms, 1.0 ms, and a synaptic delay of 0.3–0.4 ms, we get 2.5–2.6 ms in total. In Fig. 5, the facilitation of the PH nerve discharge is seen to start 2.6 ms after triggering spikes. It is thus concluded that the recorded inspiratory neuron makes a direct excitatory projection to both the XII and PH Mns. With regard to the remaining six inspiratory neurons which induced facilitation of the discharges in both the XII and PH nerves, similar calculations revealed that the observed latencies of facilitation corresponded with the calculated monosynaptic latency from these neurons to XII and PH Mns with a difference of 0.2 ms or less. It is accordingly concluded that these inspiratory neurons make monosynaptic excitatory projections to both XII and PH Mns.

Sasaki et al. (1989) reported that some inspiratory neurons in the VRG had axon collaterals on their way to the spinal cord and sent axon terminals to the vicinity of the XII nucleus, suggesting the possibility that they were dual-projection neurons. The present study has demonstrated that collaterals of descending axons of inspiratory neurons actually make excitatory synaptic linkages with XII Mns. It is noteworthy that all of nine inspiratory neurons activated antidromically by stimulation of both the XII and PH nuclei are located in the dm-AMB region, while most inspiratory neurons in the vicinity of the DRG and VRG activated antidromically from the cervical spinal cord have been found exclusively in the vl-NTS and vl-AMB regions (Otake et al. 1989; Sasaki et al. 1989).

It was reported that medullary respiratory neurons projecting to the spinal cord descended with a contralateral dominance and made monosynaptic connections with respiratory motoneurons in the ventral horn (Cohen 1979; Feldman and Speck 1983; Davies et al. 1985a, b; Ellenberger et al. 1990) and that there were axon collaterals of these neurons making monosynaptic excitatory connections with inspiratory neurons in the contralateral VRG (Manabe and Ezure 1988) and X Mns (Ezure and Manabe 1989). In the present morphological study in five animals, a total of 252 and 293 medullary neurons were retrogradely labeled with NY injected into the PH nucleus on the ispilateral and contralateral sides, respectively (Table 1). Similarly other 9 and 30 medullary neurons were retrogradely double labeled with FB injected into the XII nucleus and NY injected into the PH nucleus on the ipsilateral and contralateral sides, respectively (Table 1). Thus our results appear to be consistent with previous reports showing a contralateral dominance of spinal projection

of medullary respiratory neurons, though all the medullary neurons retrogradely labeled with NY injected into the PH nucleus may not be respiratory neurons. In relation to this point, eight inspiratory neurons antidromically responded to stimulation of the contralateral XII and PH nuclei (Fig. 6, filled stars), while one neuron responded to stimulation of the ipsilateral XII and PH nuclei (Fig. 6, open star). Though we did not search for respitatory neurons in the medulla systematically on both sides, these findings may support a contralateral dominance of projection from medullary inspiratory neurons to the PH nucleus.

The spike-triggered averaging technique revealed no facilitation in the XII nerve discharge after spontaneous spikes of 6 of 33 inspiratory neurons which antidromically responded to stimulation of the XII nucleus. It is possible that the axons of these neurons may run within or near the XII nucleus and project to other cranial motor nuclei, including the V motor and VII nuclei. Likewise, two of nine inspiratory neurons, which were antidromically activated from the XII and PH nuclei but showed no facilitation in the discharge of either nerve, may project outside the XII and PH nuclei. Thus a great majority of neurons antidromically responding to stimulation of the XII nucleus (27/33) as well as both the XII and the PH nuclei (7/9) were found to have projections to respective nuclei.

In the initial studies, we used monopolar electrodes for stimulation in the XII nucleus. Monopolar stimulation caused, however, such large stimulus artifacts that they hid antidromic spikes due to their short latency, especially after stimulation on the ipsilateral side. In bipolar stimulation the stimulus artifacts were so small that we could easily measure the latency of antidromic spikes.

We cannot completely exclude current spread outside the XII nucleus, although tips of each electrode of bipolar electrodes were located at the points where the largest antidromic field potentials were recorded in the XII nucleus along the penetrating track after stimulation of the XII nerve and intensities used in the present study were relatively low ($< 50$ μA). However, with respect to 27 of 33 neurons that antidromically responded to stimulation of the XII nucleus, monosynaptic projections to XII motoneurons were revealed by the use of the spike-triggered averaging technique. With the same technique, monosynaptic projections were found to both XII and PH Mns in seven of nine neurons that were antidromically activated from the XII and PH nuclei. The results strongly suggest that the monopolar as well as bipolar stimulating current was mainly confined within the PH and XII nuclei.

## Functional significance of XII inspiratory premotor neurons

Various possible roles have been proposed for dual projection neurons in the corticocortical (Schwartz and Goldman-Rakic 1982), corticospinal (Shinoda et al. 1976; Keizer and Kuypers 1984, 1989), rubrospinal (Shinoda et al. 1977), reticulospinal (Peterson et al. 1975; Manaker et al. 1992), and somatosensory cortical neurons (Killackey et al. 1989). With regard to oro-facial function, Amri et al. (1990) reported that the axonal branches of some medullary swallowing neurons (8/32, 25%) projected to the V and XII motor nuclei, and suggested that its role was to synchronize the activation of muscles innervated by V and XII Mns. In the present study, 9 of 42 (21%) neurons antidromically responding to stimulation of the XII nucleus were also antidromically activated by stimulation of the PH nucleus. It was also found that one of 3 inspiratory neurons projecting to ipsilateral XII Mns and 6 of 31 neurons projecting to contralateral XII Mns were dual-projection neurons (7/34, 21%). Thus approximately 20% of inspiratory neurons directly projecting to XII Mns may be assumed to be dual-projection neurons, regardless of whether they project to the ipsilateral or contralateral XII nucleus.

Though this rather small proportion may suggest that the dual projection neurons do not play a principal role in respiration, it appears that these neurons play an important role in synchronization of activities of synergistic muscles that cocontract to smoothly perform inspiratory movements. In addition, the proportion of the inspiratory neurons with bifurcating axons projecting to the XII and PH Mns may have been underestimated in the present study, because there may possibly be a number of inspiratory neurons which project to XII Mns on the one side and to PH Mns on the other side, in addition to inspiratory neurons projecting to XII and PH Mns on the same side, which were found in the present study.

Rhythmical EMG activities have been recorded in extrinsic tongue muscles, especially in the GG muscle, in association with respiration in humans and animals. An obstruction in the upper airway due to suppression of the GG muscle activity has recently been shown to be a factor associated with obstructive sleep apnea (Lowe 1990). Insight into the pathogenesis of sleep apnea may be obtained by further studies on two types of inspiratory neurons found in the present study from two aspects. First, recent studies have revealed heterogeneity in the activity between the diaphragm (a major ventilatory muscle) and upper airway muscles, which affect the airway resistance but which do not have ventilatory functions, in response to various perturbations such as chemical, mechanical, and pharmacological stimuli (van Lunteren and Strohl 1986). Similarly, inspiratory GG muscle activity increases abruptly to reach its maximum in the early inspiratory phase in a way similar to the pharyngeal and laryngeal abductor muscle, while the diaphragm increases its activity gradually throughout inspiration (van Lunteren and Strohl 1986). The heterogeneity of subpopulations of premotor neurons of the XII and PH Mns may be involved not only in coordination of the activity of the tongue muscle and diaphragm during spontaneous breathing but also in their differen-

tial responses to various stimuli (van Lunteren and Dick 1992). Second, pharmacological studies have delineated the serotonergic control of respiratory neuronal activity (Lalley 1986; Holtman 1988; Morin et al. 1990; Arita and Ochiishi 1991; Manaker et al. 1992). The activity of two types of inspiratory neurons revealed in the present study in association with changes of sleep stage may provide a knowledge about sleep stage-dependent control of respiratory muscles.

Despite the need for further study on behavior of two types of inspiratory neurons, the presence of dual projection neurons and coupled activity between XII and PH Mns emphasizes a dual functional role of the tongue. In addition to its contribution to speech and mastication, it clearly plays an important part, in close harmony with diaphragmatic contraction, in the maintenance of a patent airway.

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