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Axonal branching of medullary swallowing neurons projecting on the trigeminal and hypoglossal motor nuclei: demonstration by electrophysiological and fluorescent double labeling techniques

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Summary. The projections of ventral medullary reticular neurons on both trigeminal (Vth) and hypoglossal (XIIth) motor nucleus were studied in sheep anesthetized with halothane. In a first series of experiments, extracellular microelectrodes were used to record the activity of medullary swallowing interneurons (SINs) located in the ventral region (around the nucleus ambiguus) of the swallowing center. Antidromic activation after electrical stimulation of the Vth and XIIth nuclei was tested in 83 SINs. For 38 SINs a clear antidromic activation was observed and for 8 of them the response was triggered by stimulation of either nucleus. As confirmed by the reciprocal collision test, these 8 SINs had branched axons sending information to both nuclei tested. Average latencies for antidromic activation of branched SINs after stimulation of the XIIth and the Vth motor nucleus were 2.2 ± 0.6 ms and 2.7 ± 0.8 ms respectively. The axonal conduction velocity of these neurons was 4.4 ± 1.3 m/s for the collateral to the Vth motor nucleus and 2.7 ± 0.7 m/s for axons projecting to the XIIth motor nucleus. In a second series of experiments the double retrograde labeling technique was used to confirm the existence of neurons with branched axons in the medullary regions corresponding to the swallowing center. Small and well localized injections of Fast Blue (FB) and Diamidino Yellow (DY) fluorescent tracers were made in the Vth and in the XIIth motor nucleus respectively. A relatively large number of double-labeled cells was found in the ventral region of swallowing center (reticular formation around the nucleus ambiguus, 2–4 mm in front of obex). Such neurons (supplying both the XIIth and the Vth motor nucleus) appeared mixed with those innervating only either the XIIth or the Vth motor nucleus. Each type of neuron, i.e. single or double labeled, was shown to have bilateral distribution with an ipsilateral predominance.

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Introduction

Swallowing is a centrally programmed motor sequence resulting from the activity of hindbrain neurons (interneurons and motoneurons) belonging to the so called swallowing center (Doty 1968; Car and Roman 1970; Jean 1972a; Dubner et al. 1978; Miller 1982; Kessler and Jean 1985; Roman 1986). In sheep the swallowing neurons are mainly located in two regions of the medulla: a dorsal region including the nucleus of the tractus solitarius (NTS) and the adjacent reticular formation, and a ventral region corresponding to the nucleus ambiguus (NA) and the surrounding reticular formation. Neurons in the dorsal region are programming interneurons responsible for the spatio temporal organization of the swallowing motor sequence (Jean 1972a, 1972b); those in the ventral region are interneurons or motoneurons of the NA controlling the various muscles of the pharynx, larynx and esophagus (Roman 1986). Other motoneurons involved in swallowing are found in the Vth (Car and Amri 1982) and the XIIth motor nucleus (Sumi 1969, 1970; Car and Amri 1987). Swallowing motoneurons of the Vth nucleus supply the mylohyoïd, digastric (anterior body) and medial pterygoïd muscles (Car and Amri 1982); those of the XIIth nucleus innervate the geniohyoïd, styloglossus and hyoglossus muscles (Amri et al. 1989).

In previous works (Amri et al. 1984; Amri and Car 1988), the connections between medullary swallowing interneurons (SINs) and motoneurons of the Vth and XIIth motor nuclei were studied by means of neuroanatomical (retrograde transport of peroxydase) and elec-

trophysiological techniques (antidromic invasion of SINs). It was shown that SINs located in the ventrolateral reticular formation project to the Vth (Amri et al. 1984) or the XIIth (Amri and Car 1988) motor nuclei. These data suggest that ventral SINs are command interneurons for the two pools of motoneurons. Since the muscles innervated by the Vth and XIIth nerves contract at about the same time during swallowing (see Amri et al. 1989) there must be a synchronous activation of motoneurones. This synchronous activation could result at least in part from axonal branching of SINs. The aim of the present study was to demonstrate the existence of such branched SINs within the ventrolateral reticular formation.

Methods

This study was carried out on adult sheep (body weight: 20–30 kg). In this species reproducible swallowing can be specifically obtained by stimulating the internal branch of the superior laryngeal nerve (SLN) with pulses of low voltage (0.5 V; 0.2 ms) at 30–50 Hz (see Roman and Car 1970).

Twenty two animals were used, seventeen for electrophysiological experiments and the five others for experiments with fluorescent double labeling technique.

Surgical procedure

A short-lasting barbiturate anesthetic (sodium thiopentane, 25 mg/kg) was administered intravenously. After tracheotomy, the animal was ventilated by a respirator (Bird Mark 8) with a mixture of air and halothane (2%) to continue anesthesia. Depth of anesthesia was adjusted to maintain pupillary constriction and a heart rate typically less than 100/min.

The mylohyoïd (MH) or geniohyoïd (GH) muscle, innervated by trigeminal and hypoglossal motoneurons respectively, were exposed via a medial incision of the skin under the jaw. Synchronous activity of these suprahyoïd muscles moves the hyoïd bone upwards during swallowing (Amri et al. 1989).

The internal branch of SLN that contains laryngeal afferents involved in triggering swallowing was dissected in the laryngeal region via a ventro-medial approach. The XIIth nerve was exposed through a ventro lateral incision of the skin under the jaw. The branch of the Vth nerve innervating the MH muscle was also dissected via a transmandibular approach. Following removal of the occipital bone and cerebellum, the dorsal surface of the medulla and pons was exposed and covered with warm liquid paraffin. At the end of surgical period, the head was placed in a stereotaxic apparatus adapted to sheep. All wounds were regularly infiltrated with a 2% procaine solution. Body temperature, heart rate and arterial blood pressure were continuously monitored.

Electrical stimulation

Platinum bipolar electrodes were used to stimulate the SLN, Vth and XIIth nerves. The Vth and the XIIth motor nuclei were stimulated via steel concentric bipolar needles having a diameter of 0.4 mm and containing an axial wire (0.1 mm) such that the interelectrode distance was 1 mm. The electrodes were connected to a stimulator (WPI instruments) through a high frequency isolation unit. Stimulus parameters were 0.5–2 V, 0.02–0.2 ms pulse duration for nerves, and 2–6 V, 0.05–0.1 ms pulse duration for central stimulation.

Recordings

Electromyographic (EMG) activities of GH or MH muscle were recorded with paired copper wires, insulated except at the tip, inserted into the muscles.

The extracellular activity of medullary neurons was recorded with tungsten microelectrodes of 1–2 μm tip diameters and impedances of 5–10 MΩ at 1000 Hz.

Potentials were amplified and displayed to an oscilloscope for visual observation and filming.

Curarization

In order to prevent activation of medullary neurons by sensory feedback resulting from contraction of muscles during swallowing (see Car and Amri 1982), curarization (Flaxedil: 2 mg/kg) was sometimes used, but always after the discharge of the recorded neuron had been related to reflex swallowing (close temporal relationship between discharge and EMG of GH or MH).

Histological controls for electrophysiological experiments

Medullary stimulating and recording sites were marked by electrocoagulation (250 μA DC for 5 s) and later located from stained (Klüver and Barrera 1953) 10–25 μm thick transverse sections, or unstained frozen 50 μm thick sections.

Identification of SINs projecting to the Vth and XIIth motor nuclei

These SINs were identified by antidromic activation following stimulation of the Vth and XIIth motor nuclei. Two concentric bipolar electrodes were implanted in the posterior part of the Vth and the rostral part of the XIIth motor nucleus respectively (Fig. 1A). The electrodes were considered correctly placed when large antidromic field potentials were recorded following stimulation of the Vth and XIIth nerves. In addition, stimulation of the Vth and XIIth motor nuclei via the electrodes elicited EMG responses in MH and GH muscles respectively.

Criteria for antidromic activation of SINs: 1) spikes elicited by motor nucleus stimulation were identical to those during swallowing activity, 2) the antidromic response had a short latency and could follow high-frequency stimuli (300–500 Hz; see Fig. 2D), 3) collision between antidromic and orthodromic spikes: when orthodromic potential due to swallowing (SLN stimulation) preceded stimulation of the Vth or XIIth motor nucleus within an appropriate time interval (critical delay), no antidromic response was obtained (Fig. 2B). The critical delay corresponds to the sum of the conduction time from the cell body to the site of stimulation and the refractory period of the axon at this site (Barillot et al. 1980; Fuller and Schlag 1976; Schlag 1978; Lipski 1981).

Criteria for identification of SINs with branched axons: reciprocal collision test (Shinoda et al. 1976, 1977; Anderson and Yoshida 1980; Niijima and Yoshida 1982). When a neuron is antidromically activated by stimulations delivered at two different sites, St_1 and St_2 (XIIth and Vth motor nucleus respectively in the present experiments), it can be concluded that two axonal branches of this neuron have been stimulated if the following equation is satisfied (see Fig. 1B).

$Tc = 1/2(Ict + L_2 - L_1 - R)$ with $Tc > 0$.

Tc = conduction time between the branching point of the axon and St_2; $L_1 = Ta + Tb$ = latency of antidromic spike evoked by St_1; $L_2 = Ta + Tc$ = latency of antidromic spike evoked by St_2; R = ax-

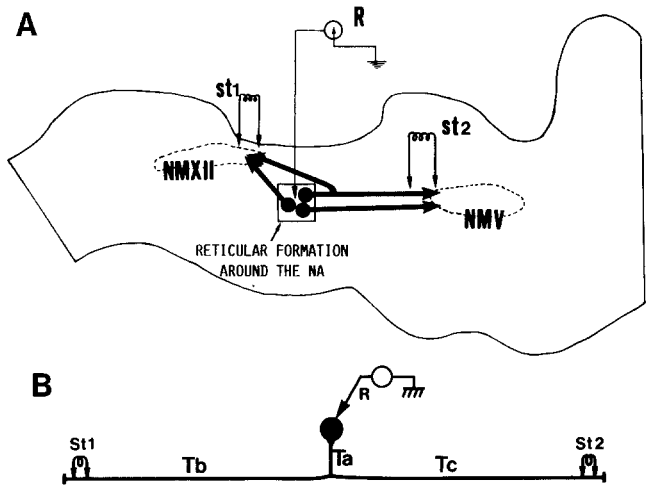


Fig. 1A, B. Experimental protocol in electrophysiological experiments. **A** Longitudinal section of the rhombencephalon showing the recording (R) and stimulation (St_1 and St_2) sites. NMXII: hypoglossal motor nucleus; NMV: trigeminal motor nucleus; NA: nucleus ambiguus. **B** Schematic representation of a neuron with two axon collaterals stimulated at St_1 and St_2 to produce antidromic potentials conducted through the unbranched axonal segment to a somatodendritic recording site. Ta = conduction time on the unbranched axonal segment; Tb = conduction time on the first collateral; Tc = conduction time on the second collateral (for further comments see Methods)

onal refractory period; *Ict* = maximum interstimulus interval (maximum collision time) producing disappearance of the second spike evoked by St_2.

$Ict = Tb + Tc + R$. Since $Ta = L_2 - Tc$ and $Tb = L_1 - Ta = L_1 - L_2 + Tc$, then $Ict = L_1 - L_2 + 2Tc + R$ and finally $Tc = 1/2(Ict + L_2 - L_1 - R)$.

Fluorescent double labeling technique

The demonstration of an axonal retrograde transport for various fluorescent substances offered the possibility of investigating axonal branching by means of a simple retrograde double labeling procedure (for a review, see Kuypers and Huisman 1984). Two substances fluorescing different colours may be injected in two different sites innervated by a same group of neurons. If the two sites are supplied by collaterals of the same axons, both substances should be visible within the individual neural cell bodies after retrograde axonal transport (Van Der Kooy et al. 1978).

The fluorescent tracers used in the present experiments were Diamidino Yellow (DY) and Fast Blue (FB) dissolved or suspended in distilled water at concentrations of 2% and 3% respectively. They were injected by mean of a micropipette (tip diameter 40–80 μm) connected to a 1 μl Hamilton microsyringue and positioned in the Vth or the XIIth motor nucleus according to stereotaxic coordinates. An electrophysiological control of these coordinates was done before positioning the micropipette by implanting bipolar concentric electrodes in the contralateral Vth and XIIth motor nuclei. When correctly placed, the electrodes recorded large antidromic potentials after stimulation of Vth and XIIth nerves. A total amount of 0.2–0.6 μl of FB solution was injected unilaterally over 2–3 micropipette penetrations into the posterior part of the Vth motor nucleus (15–16 mm rostral to obex). A similar quantity of DY solution was injected unilaterally into the rostral part of the XIIth motor nucleus (obex level). The two series of injections were both made on the same side of the brain.

After a survival time of 2–3 days, the animals were anesthetized and perfused through the two carotid arteries with a saline solution containing procaine (0.1%) and heparine (5000 UI). Perfusion was continued with 6–8 liters of 30% formalin in a cacodylate buffer 0.1 M at pH 7.3, followed by 2–3 liters of the same buffer containing 10% sucrose. The rhombencephalon was then cut transversaly into 40 μm frozen sections (cryostat) directly mounted on gelatinezed slides. Sections were examined using a Leitz Fluorescence microscope with a single filter providing excitation light of 360 nm wavelength.

Results

Electrophysiological study

We recorded the extracellular activity of 83 ventral SINs, that exhibited a burst of spikes closely linked to reflex swallowing induced by stimulation of the SLN (Fig. 2A). Thirty SINs were antidromically activated (collision test) by ipsilateral stimulation of either the XIIth ($n = 19$) or the Vth motor nucleus ($n = 11$). In addition, 8 SINs were found to be antidromically activated by stimulation of both motor nuclei (Fig. 2B). The reciprocal collision test (see methods) performed on 3 of these SINs was always significant; the Ict ranged from 7 to 9 ms, and the Tc from 3 to 5 ms (Fig. 2C). The average latency of antidromic spikes in branched SINs was 2.7 ms ± 0.8 (mean ± SEM) after stimulating the Vth motor nucleus, and 2.2 ms ± 0.6 with stimulation of the XIIth motor nucleus; coupled with estimated conduction distances of 12 and 6 mm respectively, the conduction velocities were 4.4 m/s ± 1.3 and 2.7 m/s ± 0.7 for axonal branches projecting to the Vth and XIIth motor nucleus.

The mean latencies of antidromic spikes and the mean conduction velocities were not significantly different ($P = 0.05$, Student's-t-test) for SINs only activated by one of the two nuclei tested (see Table 1).

All SINs antidromically activated by stimulation of both motor nuclei were located in the ventrolateral reticular formation, 3–4 mm rostral to the obex (Fig. 3).

Retrograde labeling

The pressure injection of FB and DY in the Vth and XIIth motor nucleus respectively provided injection sites well centred on the nuclei and extending very slightly beyond their limits (Fig. 4A). For each injection site, the tip of needle track was enlarged by necrotic tissue itself surrounded by a concentric zone displaying blue or yellow fluorescence depending on the nucleus. The fluorescent area did not extend for more than 2–3 mm in any direction.

We examined medullary regions between 1 and 4 mm rostral to the obex, in which the swallowing center is located. Neurons retrogradely labeled with only DY or FB and double labeled neurons were observed (Fig. 5). The DY single-labeled cells showed a rather diffuse golden yellow fluorescent nucleus with some yellowish fluorescence of the cytoplasm. The FB single-labeled cells displayed a uniform blue fluorescence of the cell body with weak fluorescence of the nucleus. As for the DY–FB double-labeled neurons, they exhibited a golden fluores-

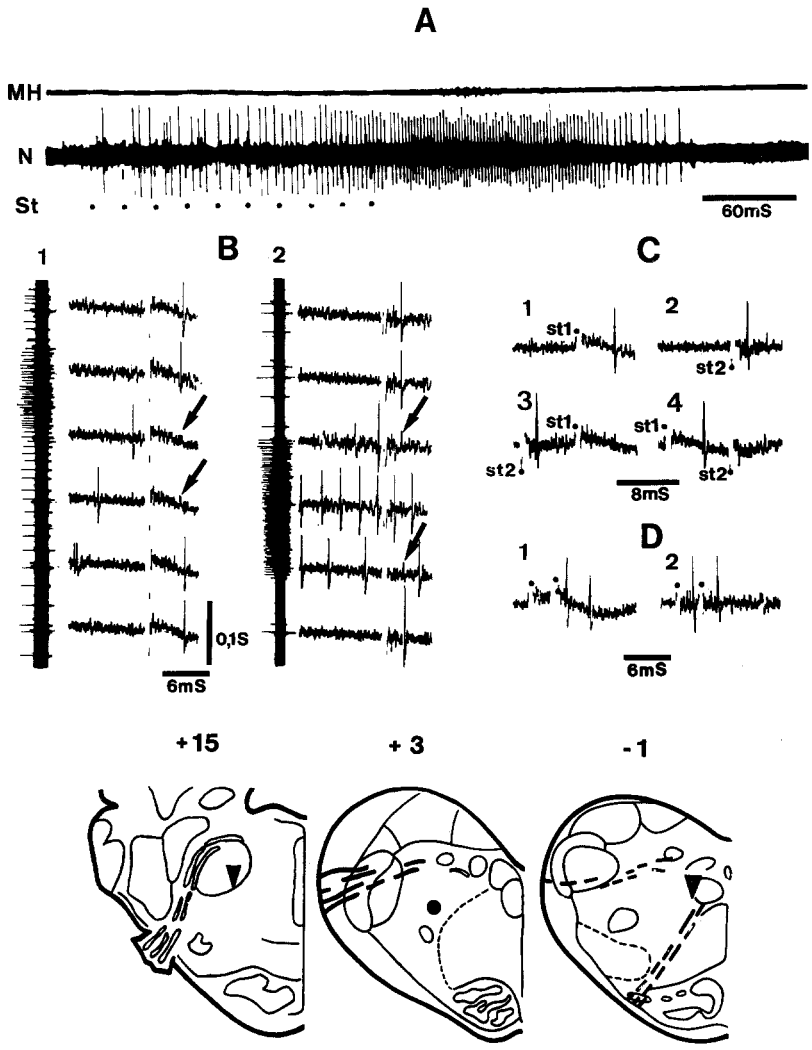


Fig. 2A–D. Swallowing interneuron sending axonal branches to the Vth and the XIIth motor nuclei. **A** Swallowing activation by stimulation of the superior laryngeal nerve at 30 Hz (0.5 V; 0.2 ms). Animal lightly curarized. MH: EMG activity of mylohyoid. N: extracellular activity of the neuron (filled circle on the medullary hemisection (+3=3 mm rostral to obex). St: stimulation. **B** Collision test between orthodromic spike of swallowing discharge and antidromic spike elicited by ipsilateral stimulation of the Vth (6 V; 0.1 ms) in 1, and the XIIth (2 V; 0.05 ms) motor nucleus in 2 (filled triangles on the pontine and medullary hemisections respectively 15 mm rostral and 1 mm caudal to obex). Antidromically evoked spikes on the horizontal traces, swallowing discharge on the vertical traces. Note the absence of antidromic spike (arrow) when a spontaneous spike precedes stimulation of motor nuclei at appropriate time interval (critical delay). **C** Reciprocal collision test. **C1** and **C2**: the test stimulation at the level of the Vth (St1) or the XIIth (St2) motor nucleus elicits an antidromic response of the neuron. **C3** and **C4**: the evoked antidromic spike fails to appear when the test stimulation is preceded by a conditionning stimulation of the other motor nucleus. The interstimulus intervals (8 ms in **C3** and 7 ms in **C4**) correspond in both cases to the maximum collision time. **D** Stimulation of the Vth (1) and XIIth (2) motor nuclei with two pulses at 300 Hz. Note that each pulse induces an antidromic spike. For identification of pontine and medullary structures see legends of Fig. 3 and 4

cent nucleus surrounded by a ring of the same fluorescence contrasting with the FB fluorescent cytoplasm in which some golden yellow fluorescent granules were visible.

All types of neurons (single or double labeled) were found, bilaterally with an ipsilateral predominance, in the parvocellular reticular formation, the adjacent gigantocellular reticular formation, the NTS, the nucleus of tractus spinalis of the Vth nerve, and in the lateral border of the XIIth motor nucleus (Fig. 4B). Still, an important concentration of labeled cells was observed in the reticular formation near the NA, an area corresponding to the ventral region of the swallowing center. Although in proportion less numerous than cells labeled only with DY, FB–DY double-labeled neurons were found to be abundant in the ventro-lateral reticular formation and also in the vicinity of the NTS (Fig. 4B).

Table 1. Latency and mean conduction velocity of SINs sending axons to the Vth (NMV) and/or the XIIth (NMXII) motor nuclei

Activation site	NMV	NMXII	NMV+NMXII
Mean antidromic spike latency	2.9±0.5 ms (n=11)	2.8±0.5 ms (n=19)	2.7±0.8 ms 2.2±0.6 ms (n=8)
Mean conduction velocity	4.1±0.7 m/s (n=11)	2.1±0.4 m/s (n=19)	4.4±1.3 m/s 2.7±0.7 m/s (n=8)

Discussion

It is now firmly established that the Vth and XIIth motor nuclei receive direct projections from neurons located in the ventral medullary reticular formation (Holstege et al. 1977; Jean et al. 1983; Travers and Norgren 1983). For example, after [3H] leucine injections in the ventral medullary region of the sheep swallowing center, Jean et al. (1983) observed silver grain aggregates within the Vth and XIIth motor nuclei on both sides; some clusters of silver grains were typical of axonal terminals abutting on motoneurones. In addition, by means of electrophysiological methods (antidromic invasion) SINs located in the ventrolateral reticular formation were found to send their axons to the Vth (Amri et al. 1984) or the XIIth (Amri and Car 1988) motor nucleus. Present data suggest that some SINs in the ventral reticular formation of the

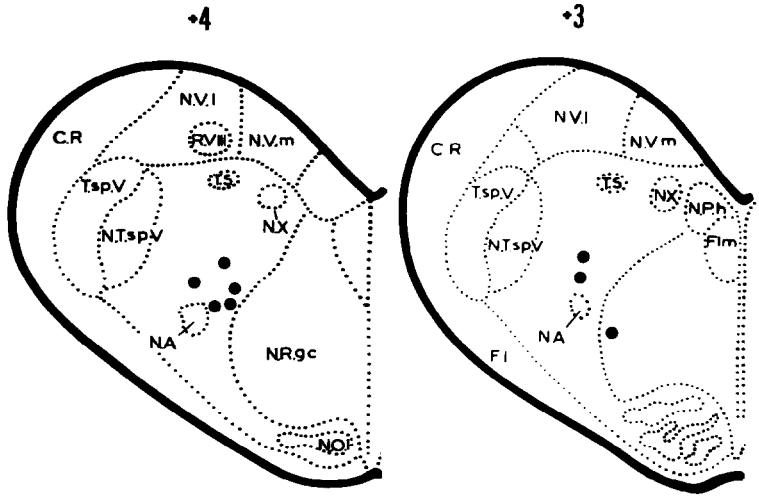
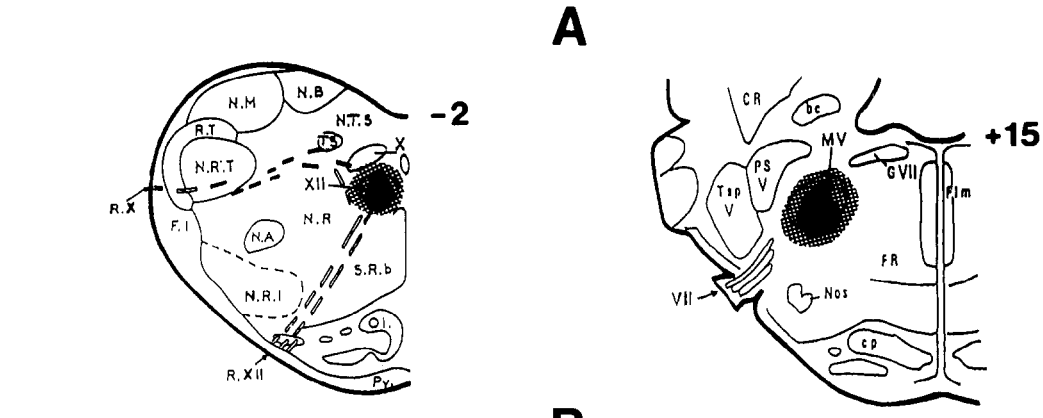


Fig. 3. Localization of medullary swallowing interneurons antidromically activated by stimulation of both the XIIth and the Vth motor nuclei. Responsive neurons are indicated by filled circles on medullary hemisections, 3 and 4 mm rostral to the obex. Note their location in the ventrolateral reticular formation. C.R.: corpus restiformis; F.L.M.: fasciculus longitudinalis medialis; Fl: fasciculus lateralis; Noi: nucleus olivaris inferior; N.P.h.: nucleus praepositus hypoglossi; N.R.gc.: nucleus reticularis gigantocellularis; N.V.l: nucleus vestibularis lateralis; N.V.m: nucleus vestibularis medialis; R. VIII: radices descendentes n. vestibuli; NA: nucleus ambiguus; T.S.: tractus solitarius; NX: nucleus dorsalis n. vagi; N.T.sp.V: nucleus tractus spinalis n. trigemini; T.Sp.V: tractus spinalis n. trigemini



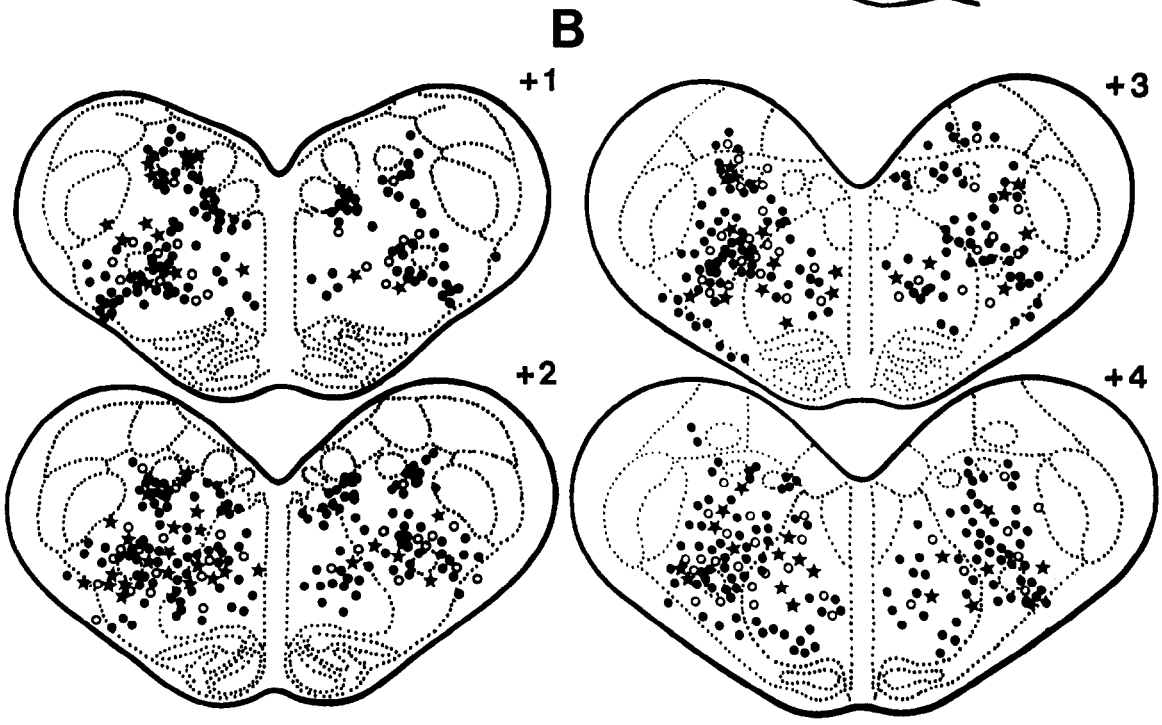


Fig. 4A, B. Localization of neurons labeled with Fast Blue (FB) and Diamidino Yellow (DY) fluorescent tracers. FB and DY were injected in the region of the Vth and XIIth motor nucleus respectively. **A** Hemisections of medulla (−2=2 mm caudal to obex) and pons (+15=15 mm rostral to obex) showing the injection sites. **B** Transverse sections of medulla (respectively 1, 2, 3 and 4 mm rostral to obex) indicating the position of labeled neurons. Each section includes cell bodies from 20 successive histological slices. Filled circles: DY single-labeled neurons; open circle: FB single-labeled neurons; filled stars: DY–FB double-labeled neurons. bc: brachium conjonctivum; cp: pedunculus cerebralis; cr: corpus restiformis; Fl: fasciculus lateralis; Flm: fasciculus longitudinalis medialis; FR: formatio reticularis; MV: nucleus motorius nervi trigemini; NA: nucleus ambiguus; NR: nucleus reticularis; NRl: nucleus reticularis lateralis; NTS: nucleus tractus solitarius; NOS: nucleus olivaris superior; PSV: nucleus principalis sensibilis nervi trigemini; RX: radices nervi vagi; RXII: radices nervi hypoglossi; TS: tractus solitarius; TSpV: tractus spinalis nervi trigemini; VII: radices nervi facialis; X: nucleus dorsalis nervi vagi; XII: nucleus nervi hypoglossi

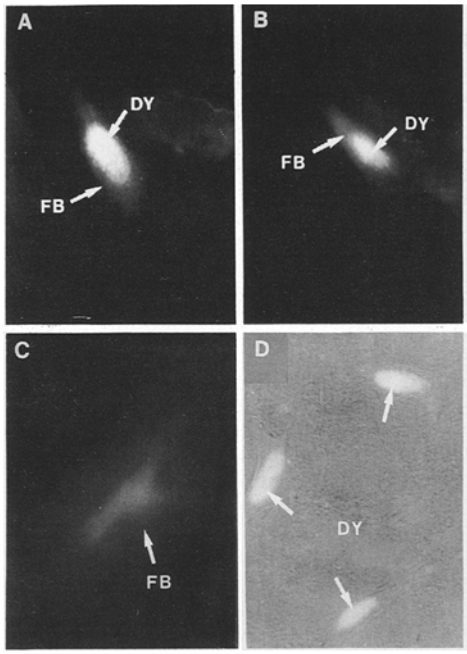


Fig. 5A–D. Photomicrographs of neurons labeled with Fast Blue (FB) and/or Diamidino Yellow (DY) fluorescent tracers, (magnification: X300). FB and DY injected in the trigeminal and hypoglossal motor nucleus respectively. Double labeling in **A, B.** Single labeling in **C, D.** All these neurons were located in the ventrolateral reticular formation, 2–4 mm rostral to obex

medulla oblongata have branched axons innervating both trigeminal and hypoglossal motor nuclei. These data are entirely new with respect to the swallowing system. However axonal branching has been repeatedly reported for other systems. For example, axons of all major descending pathways of the brain (reticulo-, vestibulo-, rubro- and cortico-spinal) send collaterals to multiple levels of the spinal cord (Abzug et al. 1974; Peterson et al. 1975; Rapoport et al. 1977; Shinoda et al. 1976 and 1977). Projections of cerebellar nuclei on the thalamus and red nucleus on the one hand, and those of substantia nigra on the pontine reticular formation, thalamus and superior colliculus on the other hand, also involve branched axons (Anderson and Yoshida 1971; Tsukahara et al. 1976; Anderson and Yoshida 1980; Niijima and Yoshida 1982).

From our data, only 8 out of 38 (21%) ventral SINs were antidromically activated by stimulating both the Vth and XIIth motor nuclei. This proportion of branched neurons is smaller than that reported for nigral (Anderson and Yoshida 1980) or reticulospinal (Peterson et al. 1975) projections. However, it is not certain that the proportion of branched cells observed in our study actually reflects that of branched SINs in ventral medulla. Our results indeed may have been biased by several factors: (1). The stimulating electrodes had fixed positions in the Vth and XIIth motor nuclei so that it was not certain that all axons innervating these nuclei were stimulated. (2). It has been suggested (Amri and Car 1988) that ventral SINs are command interneurons for the different pools of motoneurons involved in swallowing. Now, the major pool located in the NA innervating the pharynx, larynx and esophagus was not stimulated in our experiments.

In previous neuroanatomical studies, injection of horseradish peroxydase (HRP) restricted to the Vth (Jean et al. 1983) or the XIIth (Amri and Car 1988) motor nucleus resulted in cell labeling primarily in the ipsilateral parvocellular reticular formation surrounding the NA, i.e. in the ventral region of the swallowing center. Some labeled neurons were also found in the vicinity of the NTS and in the reticular formation close to the contralateral XIIth motor nucleus. Similar cell localizations have been observed in the present work with fluorescent tracers. However, it must be stressed that retrograde axonal flow of tracers may label perikarya corresponding to fibres of passage (see Payne 1987) so that some labeled neurons may send their axons rostrally to the Vth and/or caudally to the XIIth nucleus.

In addition, it is evident that the connections between the medullary regions containing the labeled neurons and the two motor nuclei are not all involved in swallowing since these regions include other neurons including for example respiratory neurons (see Bianchi and St John 1981). On the other hand, our findings with fluorescent tracers indicate a large proportion of DY single compared with that of FB single or DY–FB double-labeled neurons. There again, the experimental conditions (different distances between the sites of injection and cell bodies; survival time after injection, etc...) may account for the result. Anyway, the important point is that branched SINs do exist. Their existence could explain why the muscles of the tongue innervated by the XIIth motor nucleus and those of the jaw innervated by the Vth motor nucleus are activated roughly at the same time (Doty and Bosma 1956; Lowe 1981; Car and Amri 1982; Amri et al. 1989). The synchronous activation of the two pools of motoneurons is probably induced by branched SINs; the difference in conduction velocities on the two branches of SINs minimizing the effect of the difference in conduction distance between the cells bodies and the two motor nuclei.

Another important result is that single or double labeled neurons were shown on both sides of the medulla. This means that ventral medullary SINs are connected with eitheir ipsilateral or contralateral motor nuclei. Since the swallowing motor sequence is bilaterally symmetrical and synchronous (Doty et al. 1967), ventral medullary SINs could be responsible, at least in part, for coordination of the two half centers on each side of the medulla. This suggestion fits in with the results of previous electrophysiological and lesioning studies (Doty et al. 1967; Jean 1972b).

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