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## Evidence of parasympathetic postganglionic neurons in the rat hypoglossal nerve trunk

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**Abstract** Previous studies have indicated that the geniohyoid (GH) muscle is innervated by efferent axons from both the hypoglossal nerve (CN XII) and ansa cervicalis. To clarify the physiological significance of this dual innervation of the GH muscle, we examined properties of efferent innervations in rat GH muscle using electrophysiological, horseradish peroxidase (HRP) tracing and immunohistochemical techniques. Recordings from the branch of the XII nerve that innervates the GH (GH.Br) revealed that bursts of impulses during fictitious swallowing were conducted via the XII nerve trunk, in which neuronal cell bodies were labeled in the ventrolateral subnucleus of the XII nucleus by HRP tracing. In contrast, *in vivo* experiments demonstrated that tonic discharges in GH.Br were conducted via the ansa cervicalis. However, HRP-labeled efferent neurons were observed in neither brainstem nor upper spinal cord, but sensory neurons were labeled in the most rostral cervical spinal ganglia via the ansa cervicalis. Tonic activity was abolished *in vitro* by mecamylamine, an antagonist of nicotinic acetylcholine receptors (nAChR), and by pirenzepine, an antagonist of muscarinic M<sub>1</sub> receptors. Incubation of isolated XII nerve segments with antisera to vasoactive intestinal peptide, nAChR, and muscarinic M<sub>1</sub> receptor yielded small numbers of labeled neurons with each antiserum. All labeled neurons displayed similar diameters and were located approximately 1.5 mm proximal to the bifurcation

of the XII nerve into medial and lateral branches. Our findings indicate that GH muscle in the rat is innervated by both somatic and parasympathetic nervous systems.

**Keywords** Hypoglossal nerve · Ansa cervicalis · Hyoid bone · Parasympathetic pathway · Ganglion

### Introduction

The geniohyoid (GH) muscle originates on the anterior aspect of the mandible and attaches to the hyoid bone, which moves during swallowing and respiration. Many studies have examined the central distribution of GH motoneurons in the rat (Krammer et al. 1979; Chibuzo and Cummings 1982; Uemura-Sumi et al. 1988; Aldes 1990), cat (Uemura et al. 1979; Miyazaki et al. 1981), dog (Chibuzo and Cummings 1982; Uemura-Sumi et al. 1988), rabbit (Uemura-Sumi et al. 1988), frog (Matesz et al. 1999) and monkey (Uemura-Sumi et al. 1981; Sokoloff and Deacon 1992). These studies indicate that GH motoneurons are located in the ventrolateral subnucleus of the hypoglossal (XII) nucleus. It is conceivable that human GH muscle is innervated by both motoneurons in the XII nucleus and cervical spinal motor neurons, whose axons course, for a portion, with the XII nerve trunk (Martin 1996). In rodents, topographic organization of the cervical nerves is simple and no connection exists between the XII nerve and the cervical nerves proximal to the union of the upper root of the ansa cervicalis (Müntener et al. 1980). By applying horseradish peroxidase (HRP) to GH muscle in rats, Kitamura et al. (1983) found that the GH muscle is innervated by axons in both the XII nerve and the ansa cervicalis. This finding was confirmed by O'Reilly and Fitzgerald (1990). Although that report suggested that autonomic nerve fibers were included in the XII nerve trunk, no information is available in the literature regarding the physiological significance of this dual innervation of GH muscle. We therefore used electrophysiological, HRP tracing and immunohistochemical techniques in the present study to investigate the

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functional and morphological properties of the efferent nerve supply to the GH muscle.

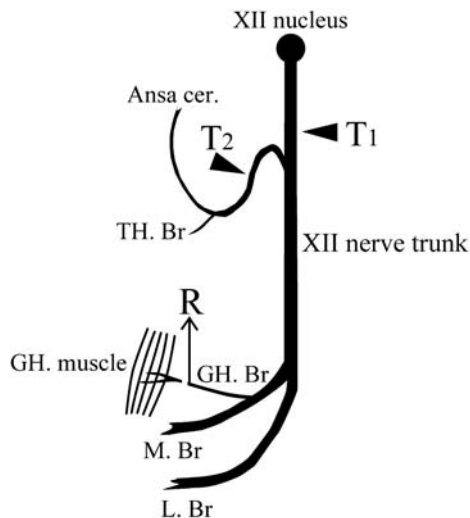
## Materials and methods

### General procedures

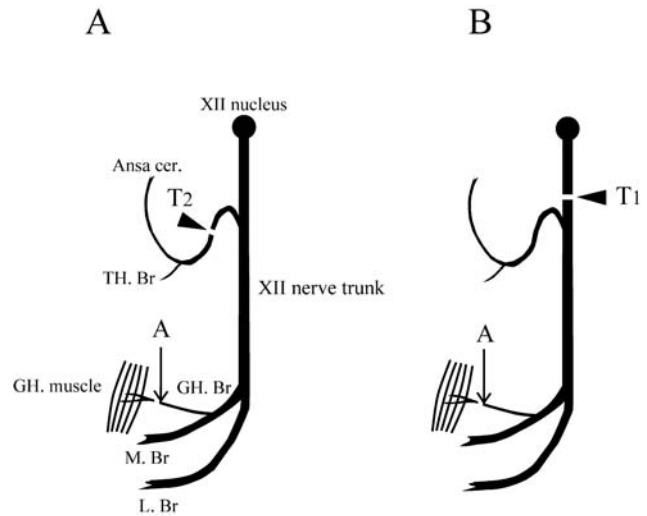
All procedures were performed in accordance with guidelines of the Animal Ethics Committee, Matsumoto Dental University. This study was performed using 43 Wistar rats (200–250 g), which were anesthetized by intraperitoneal injection of ketamine (0.05–0.1 mg/g bodyweight) without tracheal cannulation. Each rat was placed on a table in the supine position, and body temperature was maintained at 37–38°C using a heating pad. Under an operating microscope, a unilateral sagittal incision was made in the skin from the intermandibular space to the sternoclavicular bone. Posterior bellies of the digastric and stylohyoid muscles were dissected near the tympanic bulla and retracted downward, exposing the XII nerve trunk and its union with the upper root of the ansa cervicalis. GH muscle and the branch of the XII nerve that innervates this muscle (GH.Br) were exposed by retracting the anterior belly of the digastric muscle ventromedially. The GH.Br arises from the medial branch of the XII nerve, which diverges from the lateral branch at the level of the hyoid bone (O'Reilly and Fitzgerald 1990).

### Electrophysiological recordings in vivo

In five rats, the GH.Br was severed and the central cut end was placed on a bipolar tungsten hook electrode (resistance  $>2\text{ M}\Omega$ ; Fig. 1, R; Furusawa et al. 1996). To distinguish between efferent impulses in the GH.Br that traveled via the XII nerve and those arriving via the ansa cervicalis, the XII nerve was severed proximal to union with the ansa cervicalis (Fig. 1, T1). The ansa cervicalis was also severed (Fig. 1, T2). Fictitious swallowing was monitored using bipolar electromyographic (EMG) electrodes placed in the mylohyoid muscle (Yasuda et al. 2002). Neural discharges and EMG signals were amplified using a high-input-impedance preamplifier and main amplifier (AVH-10, Nihon Kohden, Tokyo), displayed on an oscilloscope (VC-11, Nihon Kohden), and stored on a data recorder (RD-100T, TEAC).



**Fig. 1** Schematic diagram of preparation used for in vivo recording. R site of recording of efferent discharges in the GH.Br, T1, T2 sites of nerve transection, Ansa cer. ansa cervicalis, TH. Br Thyroid muscle branch, M. Br, L. Br medial and lateral branches of the XII nerve, respectively

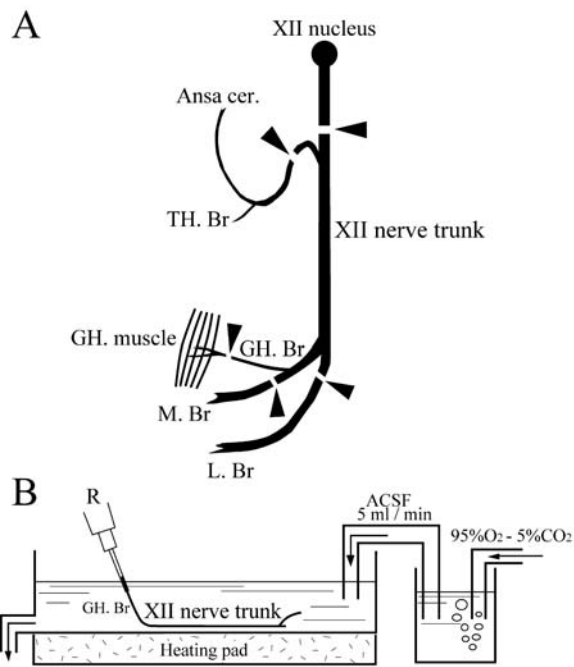


**Fig. 2** Schematic diagrams illustrating the sites of application of the HRP-WGA and transection points in the various experiments. A site of application of HRP-WGA, Arrow heads sites of the transection points, T1, T2 sites of nerve transection, Ansa cer. ansa cervicalis, TH. Br Thyroid muscle branch, M. Br, L. Br medial and lateral branches of the XII nerve, respectively

### Central distribution of neuronal cell bodies supplying the geniohyoid muscle

A total of ten rats were available for morphological experiments. In five of the ten rats, HRP conjugated with wheat germ agglutinin (HRP-WGA; Toyobo) was applied to the central cut end of the GH. Br after the ansa cervicalis was severed (Fig. 2A). In the remaining five rats, HRP-WGA was applied to the central cut end of the GH.Br after severing the XII nerve proximal to union with the ansa cervicalis (Fig. 2B). The central cut end of each nerve was suctioned into a glass micropipette (tip diameter, 50–80  $\mu\text{m}$ ), and the pipette was filled with 10% HRP-WGA in physiologic saline and left for 90–120 min (Yasuda et al. 2002, 2003). After HRP-WGA was washed away from the tissue surrounding the nerve, the wound was closed. At 24–48 h after surgery, rats were anesthetized with an intraperitoneal injection of thiopental (0.2–0.3 mg/g) and perfused transcardially with 200 ml of heparinized physiologic saline followed by 500–700 ml of 0.5% glutaraldehyde solution and 4% paraformaldehyde (pH 7.4) in 0.1 M phosphate buffer, and finally with 200–300 ml of cold (4°C) 10% sucrose solution in 0.1 M phosphate buffer. The medulla oblongata and upper cervical cord were resected as one piece, and ipsilateral cervical spinal ganglia were removed from all ten rats. These materials were refrigerated for 48 h in 30% sucrose solution with 0.1 M phosphate buffer. Serial transverse sections of the medulla and cervical cord were cut at 30  $\mu\text{m}$  on a cryostat (CM 1850, Leica, Germany) and treated for HRP-WGA application with tetramethylbenzidine (Mesulam and Brushart 1979). Two sets of alternate sections were mounted on gelatin-coated glass slides: sections in one set were covered with unstained cover slides, and those in the other were counterstained with neutral red. Sections were examined under light microscopy using both bright- and dark-field illumination. Both number and diameter of labeled motoneurons were analyzed in each counterstained section. Thus, roughly half of the labeled motoneurons were counted and measured. Serial sections of cervical spinal ganglia were cut at 20  $\mu\text{m}$  on the cryostat and treated for HRP-WGA application with tetramethylbenzidine. All sections were counterstained with neutral red. Sections were examined under light microscopy using bright-field illumination.

A camera lucida apparatus (BH-DA-LB, Olympus, Tokyo) was then used for tracing. In the description of our findings, the obex is used as a point of reference and is defined as the caudal end of the area postrema (Hamilton and Norgren 1984; Furusawa et al. 1996).



**Fig. 3A, B** Schematic diagram of preparation used for in vitro recording. **A** Arrowheads indicate sites of nerve transection. **B** The recording chamber. *R* suction electrode used for recording

The number and distribution of labeled soma were counted in sections which were counterstained. Outlines of labeled soma in each counterstained transverse section were traced using the camera lucida apparatus. Soma area and mean somal diameter (Jacquin et al. 1983; Yasuda et al. 2003) were computed using a computer graphics tablet and NIH image software (National Institutes of Health, USA).

#### Electrophysiological recordings in vitro

In ten rats, a segment of XII nerve trunk was isolated. This segment included a small portion of the ansa cervicalis and the proximal stump of the GH.Br (Fig. 3A). Isolated nerve segment was transferred to a recording chamber (Fig. 3B) containing artificial cerebrospinal fluid (ACSF), which contained 128 mM NaCl, 3 mM KCl, 0.5 mM  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 1.5 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1 mM  $\text{MgSO}_4$ , 23.5 mM  $\text{NaHCO}_3$ , and 30 mM glucose. Fluid temperature was maintained at 26–27°C and flow rate was 5 ml/min. The pH was adjusted to 7.45 by bubbling with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . Impulses in the GH.Br were recorded using a suction electrode and amplified, displayed, and stored as described above. Impulses were also analyzed by computer (VAIO, SOHY, Tokyo; LEG-1000 software package, Nihon Kohden). In five of these experiments, the nicotinic acetylcholine receptor (nAChR) antagonist mecamylamine was added to the bath. In the other five experiments, the muscarinic M1 receptor antagonist pirenzepine was added. Both antagonists were dissolved in ACSF before use and were applied at a final concentration of 10 mM.

#### Staining and immunohistochemical analysis of neurons in the XII nerve trunk

##### Hematoxylin-eosin staining

Three nerve segments isolated as described above were preserved for 24 h in Zamboni fixative solution (Zamboni and DeMartino

1967) at 4°C. Specimens were then refrigerated in 5% sucrose solution in 0.1 M phosphate buffer for 12 h, rapidly dehydrated, infiltrated with JB-4 (Polysciences, USA), and flat-embedded. Serial sections 5- $\mu\text{m}$  thick were cut along the longitudinal axis using a microtome. Sections were mounted on silanized slides, stained with hematoxylin-eosin (H-E), and examined under light microscopy. The number of neuronal cell body was counted as the number of cell bodies displaying a clear and maximal nucleus in serial sections. Somal diameter of neurons in sections was measured using a graphics tablet and computer (Apple Power Macintosh G3) and NIH image software (National Institutes of Health).

#### Immunohistochemical analysis

Fifteen nerve segments were isolated, fixed, and sectioned as described above. Endogenous peroxidase activity in sections was quenched by incubation in 0.3%  $\text{H}_2\text{O}_2$  in 100 mM phosphate-buffered saline containing 0.2% Triton X-100 (PBS-Triton) for 1 h. To block nonspecific binding sites, sections were pre-incubated with 3% sheep serum (Sigma, USA) in PBS-Triton for 1 h at room temperature. Sections were then incubated for 36 h at 4°C with rabbit polyclonal antiserum to one of the following: vasoactive intestinal peptide (VIP; Sigma); tyrosine hydroxylase (TH; Sigma); nAChR (Santa Cruz, USA); or muscarinic M<sub>1</sub> receptor (Biogenesis, UK). Antisera were diluted (1:2000) in PBS-Triton containing 3% sheep serum. Sections from three nerve segments were incubated with each antiserum. Sections from the remaining three nerve segments were not exposed to any primary antiserum and served as controls. After incubation, sections were washed in PBS-Triton and treated with biotinylated sheep anti-rabbit immunoglobulin (1:400; Sigma) for 3 h, followed by more washes and exposure to ExtrAvidin peroxidase conjugate (1:1000; Sigma) for 3 h. After three additional washes (30 min each), the peroxidase was visualized with 0.5 mg/mL 3'3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in PBS-Triton. Sections were then coverslipped using Permount (Fisher Chemical, USA) and examined under light microscopy. The number of immunoreactive-cells was counted as the number of cell bodies displaying a clear and maximal nucleus in serial sections. Somal diameter of peroxidase-labeled neurons was measured as described above.

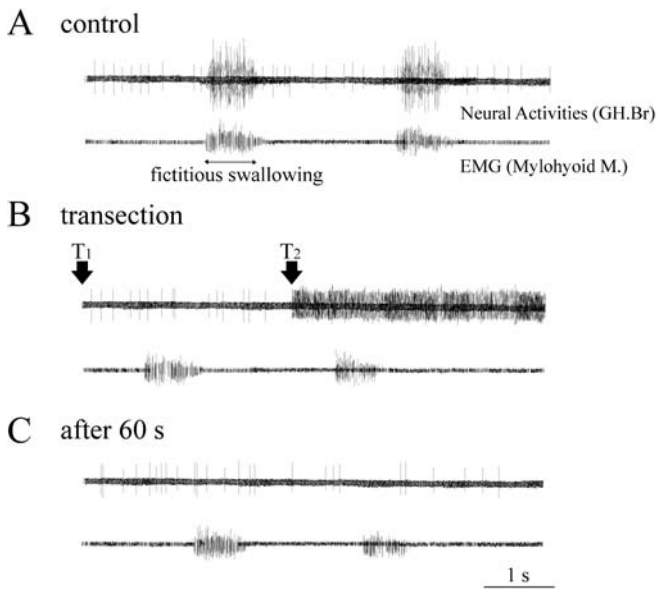
## Results

### Neuronal discharges in the GH.Br in vivo

In vivo recordings from the GH.Br exhibited background tonic discharges in addition to concentrated bursts of impulses synchronized with fictitious swallowing (Fig. 4A). Transection of the XII nerve proximal to the ansa cervicalis connection abolished these bursts, but not the tonic discharges (Fig. 4B, *top trace, left*). Frequency of tonic firing increased dramatically when the ansa cervicalis was then severed in the same preparation (Fig. 4B, *top trace, right*), but returned to initial levels after 30–60 s (Fig. 4C). These changes in GH.Br activity were observed in all five rats in which in vivo recordings were made.

### Central distribution of neuronal cell bodies of neurons supplying GH muscle

In all five rats in which HRP-WGA was applied to the GH.Br following severing of the ansa cervicalis, HRP-labeled cells were observed ipsilaterally in the ventrolateral



**Fig. 4A–C** Nerve activity in the GH.Br (*upper traces*) and mylohyoid muscle EMG activity (*lower traces*) in vivo. **A** Bursts of impulses in the GH.Br coincide with activity in the mylohyoid muscle (fictitious swallowing). **B** Bursts disappear when the XII nerve is cut proximal to the ansa cervicalis connection at T1 (see Fig. 1); severing the ansa cervicalis at T2 increases frequency of tonic firing. **C** Tonic firing has returned to original frequency as of 60 s later

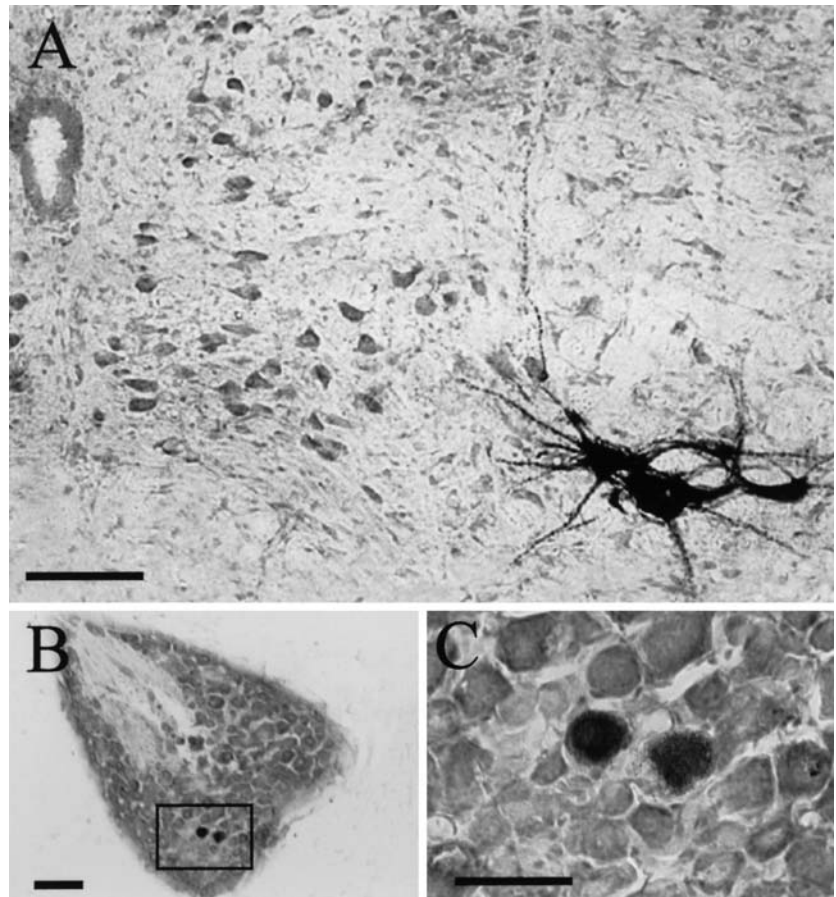
subnucleus of the XII nucleus (Fig. 5A). These labeled cells were found to form a cell column extending into the region between approximately 0.4 mm rostral and 1.3 mm caudal to the obex (Fig. 6). A total of 105 labeled cells were observed in counterstained sections from the five rats. These labeled cells were multipolar, with mean somal diameter of  $30.6 \pm 4.0 \mu\text{m}$ .

In all five rats in which HRP-WGA was applied to the GH.Br following severing of the XII nerve, no HRP-labeled cells were observed in serial transverse sections of the medulla and cervical cord. However, a total of 45 labeled cells were observed in the C2 spinal ganglion (Fig. 5B, C) of all five rats. These labeled cells displayed a mean somal diameter of  $25.4 \pm 3.2 \mu\text{m}$ .

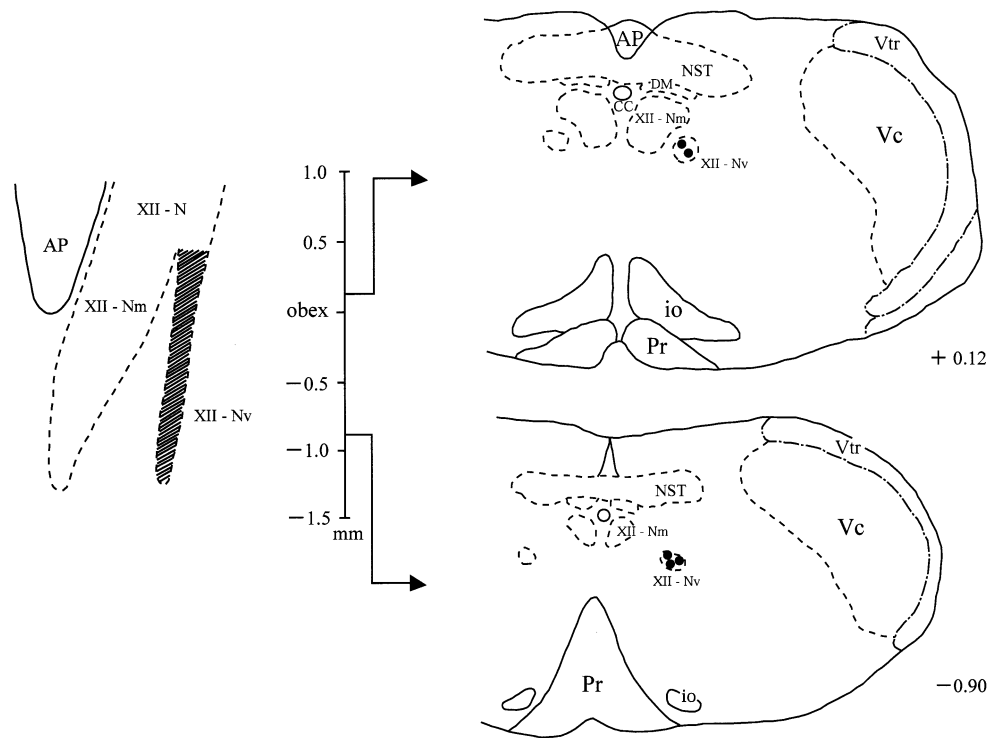
#### Effect of antagonists on neuronal discharges in the GH.Br in vitro

Recordings from the GH.Br in isolated XII nerves in vitro detected tonic discharges but no bursts (Fig. 7A1, B1, *top traces*). Tonic discharges were abolished within 10 min by 10 mM mecamlamine (Fig. 7A1, *middle trace*) and within 3 min by 10 mM pirenzepine (Fig. 7B1, *middle trace*). Frequency of tonic firing recovered to  $49 \pm 18\%$  of original value as of 15 min after wash out of mecamlamine (Fig. 7A1, *bottom trace*; Fig. 7A2;  $n=5$ ) and to 76

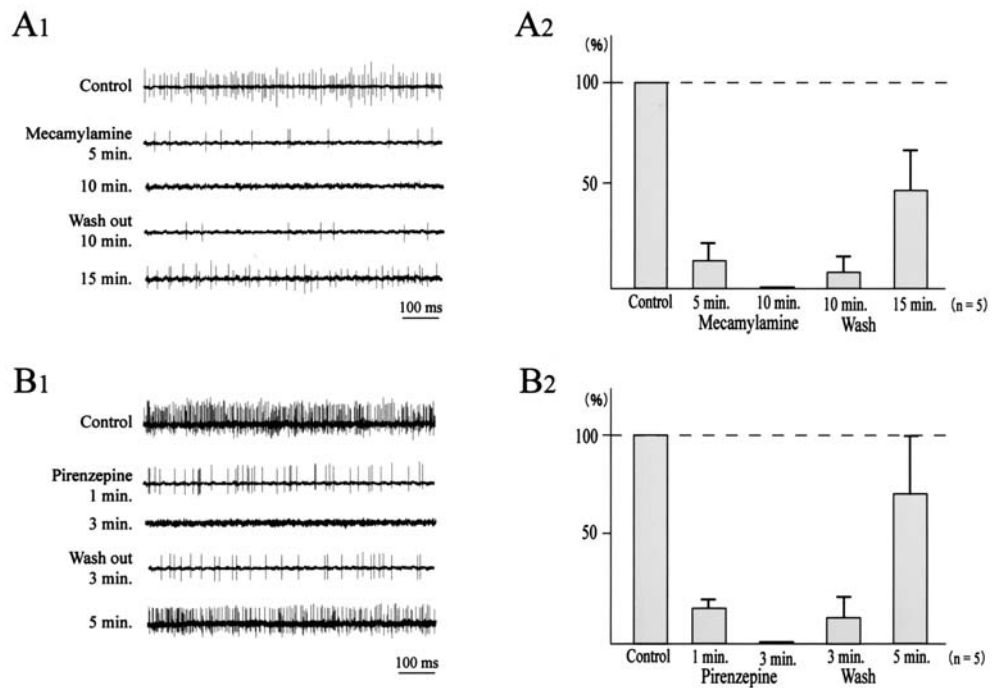
**Fig. 5. A** Brightfield photomicrographs of labeled cells localized in the XII nucleus following application of HRP-WGA to the GH.Br after severing the ansa cervicalis. **B** Brightfield photomicrographs of labeled cells localized in the most rostral cervical spinal ganglion following application of HRP-WGA to the GH.Br after severing the XII nerve. **C** Higher magnification of B. Scale bar =  $50 \mu\text{m}$  in A and C,  $100 \mu\text{m}$  in B



**Fig. 6** Location of labeled cells in the XII nucleus on a dorsal view illustration of the medulla (*left*) and camera lucida drawings at the coronal sections of the medulla (*right*), following application of HRP-WGA to the GH.Br. *Numbers* to the right of drawings represent distance rostral or caudal to the obex (in millimeters). *Hatched bar* in left panel indicates area of labeled cells. *Closed circle* in right panel indicates individual labeled cells



**Fig. 7A, B** Effect of nAChR and M<sub>1</sub>-receptor antagonists on discharges in the GH.Br in vitro. **A1** Blockage of tonic activity by 10 mM mecamlamine. **A2** Effect of mecamlamine on frequency of tonic discharges, expressed as a percentage of the frequency in normal ACSF. **B1** Blockage of tonic activity by 10 mM pirenzepine. **B2** Effect of pirenzepine on frequency of tonic discharges, expressed as a percentage of the frequency in normal ACSF



±24% of original value as of 5 min after wash out of pirenzepine (Fig. 7B1, bottom trace; Fig. 7B2; n=5).

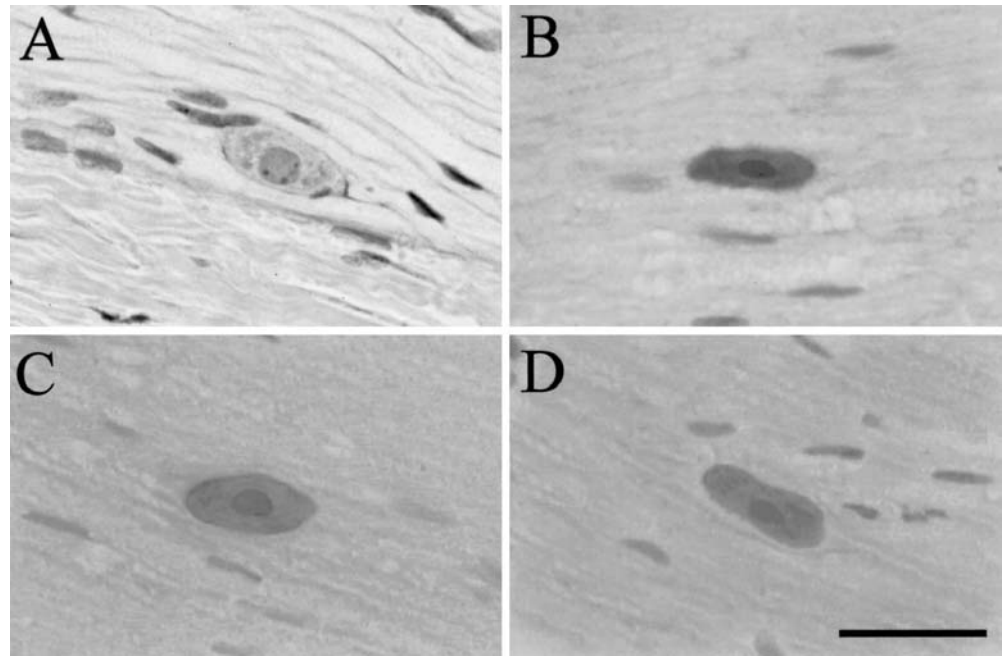
Distribution and immunohistochemical analysis of neurons in the XII nerve trunk

H-E staining of three isolated XII nerve segments revealed 28 somata that were larger than surrounding Schwann cells

(Fig. 8A). These somata were located approximately 1.5 mm proximal to the bifurcation of the nerve into medial and lateral branches.

Immunohistochemical staining revealed 31 VIP-positive cells (Fig. 8B), 33 nAChR-positive cells (Fig. 8C), and 31 M<sub>1</sub>-receptor-positive cells (Fig. 8D) in the region of the XII nerve that contained H-E-stained somata. Each number represents the total for three isolated nerve segments tested using that antiserum. In contrast, no TH-

**Fig. 8A–D** Histology and immunohistochemistry of isolated XII nerve segments. **A** H-E-stained somata. **B** VIP-positive cells. **C** nAChR-positive cells. **D** M<sub>1</sub>-receptor-positive cells. Scale bars = 50  $\mu$ m



positive cells were observed anywhere in the isolated XII nerve, and no stained cells were seen in the absence of primary antiserum. Mean diameters  $\pm$  SD of VIP-positive cells ( $30.1 \pm 1.2 \mu\text{m}$ ), nAChR-positive cells ( $29.7 \pm 1.2 \mu\text{m}$ ), and M<sub>1</sub>-receptor-positive cells ( $30.1 \pm 1.5 \mu\text{m}$ ) did not differ significantly from each other or from H-E-stained somata ( $29.8 \pm 1.4 \mu\text{m}$ ).

## Discussion

Several investigators have shown that GH motoneurons are located in the ventrolateral subnucleus of the XII nucleus (Krammer et al. 1979; Miyazaki et al. 1981; Uemura-Sumi et al. 1981, 1988; Chibuzo and Cummings 1982; Kitamura et al. 1983, 1986; Aldes 1990). Furthermore, Car and Amri (1987) reported that swallowing motoneurons, which discharge only in association with EMG activity in the GH muscle, were located in the XII nucleus. These studies indicate that GH motoneurons in the ventrolateral subnucleus of the XII nucleus are almost all swallowing motoneurons. This conclusion is supported by the finding in the present investigation that bursts in the GH.Br were synchronized with fictitious swallowing and were abolished by transection of the XII nerve proximal to the ansa cervicalis connection. Furthermore, HRP-labeled cells were observed ipsilaterally in the ventrolateral subnucleus of the XII nucleus in the present study, essentially confirming the results of previous reports. Hence, efferent impulses from the GH motoneurons related to swallowing are conducted via the XII nerve trunk.

Previous studies indicate that GH muscle is innervated via both the XII nerve and the ansa cervicalis (Kitamura et al. 1983, 1986; O'Reilly and Fitzgerald 1990). Kitamura et al. (1983) reported that most GH motoneurons extend

axons through the XII nerve, although motoneurons at the most caudal level of XII nucleus have axons in the first cervical nerve. In our study, tonic discharges in the GH.Br remained after transection of the XII nerve proximal to the ansa cervicalis connection, indicating that these impulses were conducted via the ansa cervicalis. However, no labeled cells were identified in the brainstem or upper spinal cord, despite the fact that labeled sensory neurons were observed in the most rostral cervical spinal ganglia. These facts suggest that synaptic transmissions form an efferent innervating course to GH muscle. Furthermore, in this study, frequency of discharges increased dramatically after severing the ansa cervicalis. This finding suggests that discharges are produced by neurons located in the ansa cervicalis or XII nerve trunk, which were stimulated by transection of presynaptic axons in the ansa cervicalis. The existence of such neurons is supported by the observations that tonic firing returned to original frequency 30 to 60 s after the ansa cervicalis was severed and persisted in isolated XII nerves *in vivo*. Moreover, tonic discharges in the GH.Br were reversibly blocked *in vitro* by the nAChR antagonist mecamylamine and by the muscarinic M<sub>1</sub> receptor antagonist pirenzepine.

Our anatomical observations demonstrated about 30 H-E-stained somata in the XII nerve. Immunohistochemical staining of the nerve revealed similar numbers of VIP-positive, nAChR-positive, and M<sub>1</sub>-receptor-positive cells, but no TH-positive cells. TH is usually employed as a marker of adrenergic neurons, and VIP as a specific marker of cholinergic neurons (Lundberg et al. 1981). All cells displayed roughly the same diameter and were located in the same region of the nerve trunk.

In light of our physiological, pharmacological, and immunohistochemical findings, we conclude that the XII nerve contains somata for a small number of postganglionic autonomic neurons, which are excited via the ansa

cervicalis and innervate the GH muscle. Lack of reactivity to anti-TH antiserum suggests that these neurons belong to the parasympathetic system, rather than the sympathetic. Although earlier studies have reported that the XII nerve contains parasympathetic (O'Reilly and Fitzgerald 1990) and sympathetic axons (Fukui et al. 1992), we know of no other studies showing the presence of parasympathetic somata in the XII nerve. However, parasympathetic somata have been found in the glossopharyngeal nerve (CN IX), which innervates the fungiform papillae of the tongue (Inoue and Kitada 1991).

The tongue coordinates movements that assist in swallowing, phonation, mastication, and respiration (Lowe 1981, 1984). Muscles responsible for tongue movements are innervated by neurons in the XII nucleus and include, for tongue protrusion, the genioglossus and GH muscles (Odutola 1976; Lowe 1981, 1984). The genioglossus muscle is very active during inspiration and is involved in maintenance of the upper airway (Smith et al. 1990; Greer et al. 1991; Funk et al. 1993, 1997; Johnson et al. 1994; Yasuda et al. 2001). Some investigators have reported that the relationship between the hyoid bone and mandible is constant, and the position of the hyoid bone seems determined by the musculature (Grant 1959; Yamaoka et al. 2003). Our results suggest that the GH muscle might play a significant role in the positioning of the hyoid bone under control modulated by the autonomic nervous system.

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