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Quantitative analysis of GABA-, serotonin-, enkephalin-, and substance P- immunoreactive axons that make contact with jaw opener and closer motoneurons in the trigeminal motor nucleus of the rat.

Osamu Takahashi

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INTRODUCTION

Previous anatomical studies have revealed the presence of trigeminal premotor neurons that send their axons into the trigeminal motor nucleus of the cat[1-5] and of the rat[6,7]. These premotor neurons seem to exert strong excitatory and inhibitory effects on the activities of the trigeminal motoneurons[8,9]. Electrophysiological studies showed that disynaptic inhibition of the trigeminal motor neurons was evoked by the contralateral supratrigeminal nucleus, indicating that some of the trigeminal premotor neurons contain inhibitory neurotransmitters[10]. In addition to immunocytochemical studies showing the existence of various types of afferent inputs, that is, noradrenergic terminals[3,7], and serotoninergic[11-15], peptidergic[14] and cholinergic terminals[3] in the trigeminal motor nucleus, various types of γ-aminobutylic acid (GABA)-immunoreactive terminal boutons have been found to make synapses on the trigeminal motoneurons[16]. Moreover, it has been reported that the GABAergic afferent to the Vm neurons arises mainly from the ipsilateral parvocellular reticular formation of the lower brain stem[17,18]. However the neuromodulator responsive terminal boutons that make contact with the jaw opener and jaw closer motoneurons in the trigeminal motor nucleus have still not been identified.

The present study was, therefore, undertaken to identify trigeminal motor nucleus connected terminal boutons showing GABA-, serotonin (5HT)-, enkephalin (ENK)- and substance P (SP)-like immunoreactivity. The study was carried out using a double labeling method, one based on the retrograde transport of cholera toxin subunit B (CTB) injected into both the jaw opener and jaw closer motoneuron, and the other on immunofluorescence for GABA, 5HT, ENK and SP.

Department of Oral Histology, Kanagawa Dental College, Inaoka 82, Yokosuka, Kanagawa 238-8580, Japan

MATERIALS AND METHODS

The experiments were carried out on 31 female Wistar rats weighing 220 to 265 grams. For all surgical procedures, animals were initially anesthetized with sodium pentobarbital (35 mg/kg) and maintained, when necessary, on small doses of the same anesthetic.

In these rats, a single injection of 1% cholera toxin B subunit (CTB) dissolved in 0.05M Tris buffer was injected into the unilateral masseter (Ma) or the anterior digastric (AD) muscle via a glass micropipette with a tip diameter of 25 to 35 μm. After a survival period of 36 to 48 hours, the animals were again anesthetized deeply and fixed by transcardial perfusion with 200 ml of 2.0% paraformaldehyde and 0.25% glutaraldehyde in 0.1M phosphate buffered saline (PBS, pH 7.4). Subsequently, the brains were removed and saturated with a cold solution of the same fixative with 25% sucrose in PBS (pH 7.4) at 4°C. Transverse frozen sections through the lower brainstem were cut serially with a thickness of 30 μm, and sections were separated into four groups.

Immunocytochemical detection of CTB was carried out by first incubating free-floating sections in primary antiserum against CTB (1:1,000, goat anti-GABA, Chemicon Co. Inc., USA) for 24-48 hours at 4°C[19], and then in secondary antiserum labeled with TRITC (donkey anti-rabbit IgG, TRITC-labeled, 1:50, Chemicon Co. Inc.) for one hour at room temperature. Primary and secondary antiserums were diluted with 1% normal goat serum (NGS) containing 0.75% Triton-X 100. All incubation steps were

preceded by 2 washes in 0.1M PBS (10 min. each) and 3% NGS (30 min.). Fluorescent microscopy (Nikon Optiphoto) of the TRIPC-labeled cells revealed a punctuated pattern of labeled granules distributed throughout the cytoplasm, indicating CTB-like immunoreactivity (CTB-LI) (Fig. 1).

Subsequently, the same sections were incubated in primary antiserum against GABA (1:1,000, rabbit anti-GABA, INCSTAR), 5HT (1:1,000, rabbit anti-serotonin, INCSTAR), ENK (1:1,000, rabbit anti-leu-enkephalin, INCSTAR) and SP (1:1,000, rabbit anti-substance P, INCSTAR), for 24-48 hours at 4°C, and subsequently incubated in secondary antiserum labeled with FITC (goat anti-rabbit IgG, FITC-labeled, 1:400, Sigma Chemical Co.) for one hour at 4°C. Primary and secondary antiserum were diluted with 1% NGS containing 0.75% Triton-X 100. All incubation steps were preceded by 2 washes in 0.1M PBS (10 min. each) and 3% NGS (30 min.). Fluorescent microscopy (Nikon Optiphoto) revealed FITC-labeled terminal boutons distributed around the TRITC-labeled trigeminal motoneurons throughout the motor nucleus, indicating GABA-, 5HT-, ENK-, SP- LI association with trigeminal motoneurons (Fig. 1, 2).

RESULTS

The distribution of retrogradely CTB-labeled cell bodies in the trigeminal motor nucleus (Vm) localized well with the target masticatory muscles (Fig. 1). Motoneurons that send their axons to the masseter muscle (Ma) were observed within the dorsolateral subnucleus of the Vm (dl in Fig. 1A). On the other hand, the cell bodies labeled by an injection into the anterior digastric muscle (AD) were localized in the confines of the ventromedial subnucleus (vm in Fig. 1A) of the Vm. The average *long* diameter of the cell bodies after Ma injection was greater than those seen after AD injection.

Numerous GABA-LI axon terminals were encountered in the Vm. First of all, they were identified as a pattern of small, punctuated FITC-labeled granules making contact with the TRITC-labeled cell bodies (arrow heads in Fig. 2-B, 3-B). They were attached not only to the cell bodies but also to the proximal dendrites (Fig. 3-B). A greater number of GABA-LI axons made contact with Ma motoneurons than with AD motoneurons (Fig. 4). The average number of GABA-LI terminal boutons on a Ma motoneuron was 1.4, and these made contact with the cell body (Table). On the other hand, the average number of immunoreactive axonal terminal boutons making

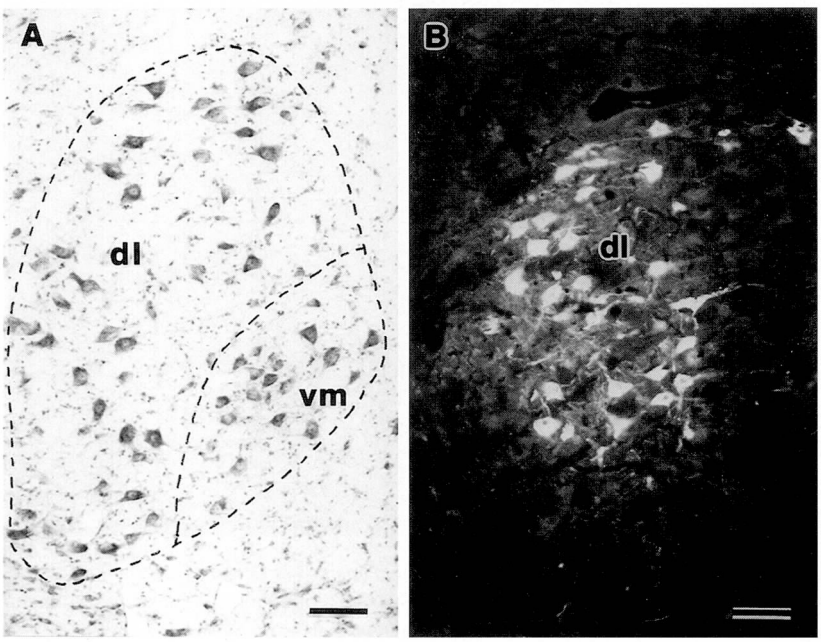

Fig. 1 Photomicrographs of the left trigeminal motor nucleus (Vm) on the transverse section of the pons (A), and TRITC-labeled motoneurons after an injection of CTB into the masseter muscle on a adjacent section (B). dl; the dorsolateral subnucleus of Vm, vm; the ventromedial subnucleus of Vm. Scale bars = 100 μm.

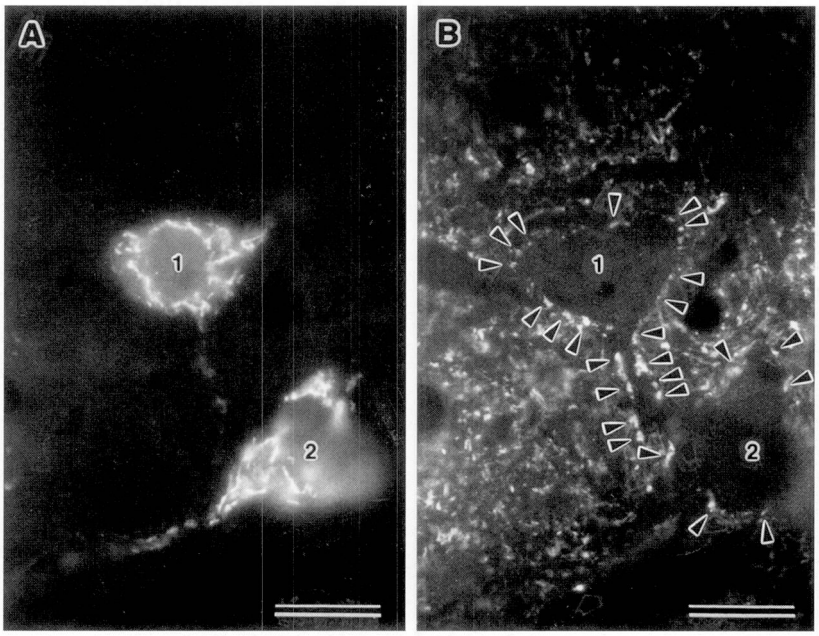

Fig. 2 Photomicrographs showing TRITC-labeled motoneurons in Vm (1, 2 and 3) after a injection of CTB into the masseter muscle (A), and FITC-labeled GABA-like immunoreactive axon terminals (arrow heads) making contact with the same group of motoneurons (1, 2 and 3) of the section shown in A (B). Scale bars = 20 μm.

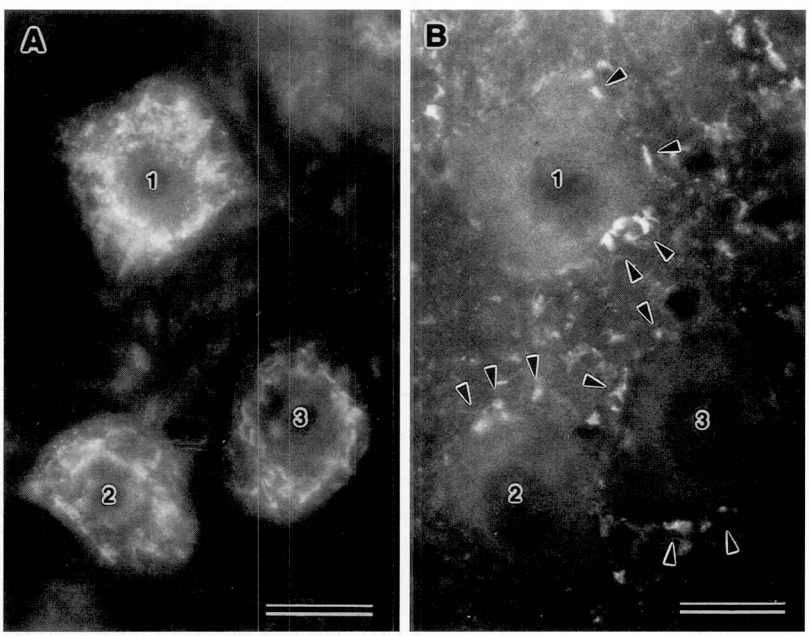

Fig. 3 Photomicrographs showing TRITC-labeled motoneurons in Vm (1 and 2) after an injection of CTB into the anterior digastric muscle (A), and FITC-labeled GABA-like immunoreactive axon terminals (arrow heads) making contact with the same motoneurons shown in A (1 and 2). Scale bars = 20 μm.

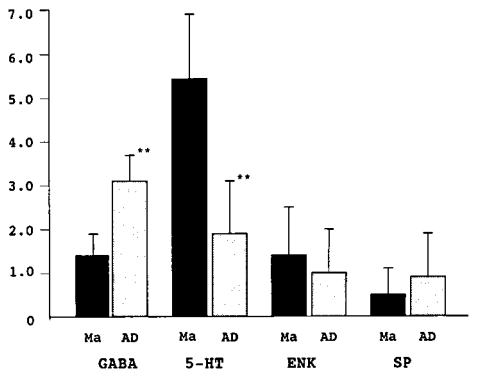

Fig. 4 Histograms showing the average numbers of immunoreactive terminal boutons on a motoneuron. Ma; motoneurons sending their axons to the masseter muscle, AD; motoneurons sending their axons to the anterior digastric muscle.

contact with AD motoneurons was 3.1 (Table).

Many 5-HT-LI axon terminals were observed in the Vm. First of all, they were identified as FITC-labeled small to medium axonal terminal boutons making contact with the TRITC-labeled cell bodies. They were attached not only ~~on~~ to cell bodies but also to the proximal dendrites. A greater number of 5-HT-LI axons were in contact with the AD motoneurons than with the Ma motoneurons (Fig. 4). On average, 5.2 5HT-LI terminal boutons made contact with the cell body of a Ma motoneuron (Table). On the other hand, 1.9 immunoreactive axonal terminal boutons were observed to make contact with an AD motoneuron (Table).

ENK-LI and SP-LI axon terminals were observed to make axonal contacts with cell bodies that send their axons to both Ma and AD motoneurons. As compared to GABA-LI and 5HT-LI, the ENK-LI and SP-LI terminal boutons were smaller. Moreover the mean number of ENK and SP immunoreactive axons was smaller than the average number of GABA-LI and 5HT-LI immunoreactive axons. These findings should be noted because they are relevant when making comparisons of the numbers of axons that make contact with a single motor neuronal cell body.

DISCUSSIONS

The present study demonstrated that the Vm motoneurons receives substantial GABAergic and serotoninergic afferent input. Moreover, the distribution of these afferent terminal boutons depended on the peripheral target of the postsynaptic motoneurons, that is, the jaw closer masticatory muscle (Ma) or the jaw opener (AD). Vm motoneurons innervating AD (jaw opener) received heavier GABAergic input than those innervating Ma (jaw closer). On the other hand, cell bodies innervating Ma received more serotoninergic afferents than those unnervating AD. There was no significant difference between the two groups of terminal boutons with enkephalin (ENK)-like and substance P (SP)-like immunoreactivity with regard to their motoneurons target. These results seem to show functional heterogeneity in afferent inputs and selectivity by their neurotransmitters or neuromodulators for postsynaptic motoneuron target.

Trigeminal premotor neurons are considered to receive direct projections from the primary afferents[7, 20] of the cerebral cortex[2], from the central nucleus of the amygdala[21, 22], and from other regions of the central nervous system. The results of this investigation have shown that a population of GABAergic interneurons is located in the parvocellular reticular nucleus, and that these cells project their axons into the trigeminal motor nucleus. The presence of GABA-synthesizing neurons

Table:

	Masseter Muscle Motoneurons		Anterior Digastric Muscle Motoneurons	
	mean number of labeled cells	immuno-reactive bouton/cell	mean number of labeled cells	immuno-reactive bouton/cell
GABA	159.2	1.4	81.8	3.1**
5-HT	114.3	5.2	58.4	1.9**
ENK	138.5	1.4	42.6	1.0
SP	142.4	0.5	40.2	0.9

($P < 0.01$**)

within the pons and the medulla reticular formation was previously been shown by immunocytochemistry of glutamic acid decarboxylase (GAD)[23].

GABA neurons are considered to act primarily as interneurons within the brain. Electrical stimulation of the parvocellular reticular nucleus in the medulla induced inhibitory postsynaptic potentials (IPSPs) in jaw-closer motoneurons with latencies shorter than 1.5 m sec.[4]. Our observation that premotor neurons in this area contain inhibitory neurotransmitters lends further support to this conclusion by furnishing morphological data. The neurons in the supratrigeminal nucleus and in the reticular formation medial to the oral subnucleus of the spinal trigeminal nucleus are thought to be involved in the cortically evoked inhibition and excitation of the masticatory motoneurons and in the central generation of the rhythmical masticatory movement[9]. It is noteworthy that ultrastructural analysis demonstrated that GABA-immunoreactive terminal boutons in the rat trigeminal nucleus formed mainly axo-dendritic synapses and contained flattened vesicles. Therefore, GABAergic premotor neurons in the parvocellular reticular nucleus exert inhibitory effects on trigeminal motor neurons.

Numerous axons from the cerebral cortex terminate in the medulla and the pons reticular formation[2, 24]. These GABAergic neurons for the trigeminal motor nucleus may act as inhibitory premotor neurons, relaying masticatory signals from the cerebral cortex.

CONCLUSION

The present study demonstrated that the Vm motoneurons receives substantial GABAergic and serotonergic afferent input. Moreover, the distribution of these afferent terminal boutons depended on the peripheral target of the postsynaptic motoneurons, that is, the jaw closer masticatory muscle or the jaw opener. Vm motoneurons innervating the anterior digastric muscle (AD) (jaw opener) received heavier GABAergic input than those innervating the masseter muscle (Ma) (jaw closer). On the other hand, cell bodies innervating Ma received more serotonergic afferents than those innervating the AD. There was no significant difference between the two groups of terminal boutons with enkephalin (ENK)-like and substance P (SP)-like immunoreactivity with regard to their targets among the subpopulations of motoneurons. These results seem to show functional heterogeneity in afferent inputs and selectivity of the neurotransmitter or the neuromodulator for the postsynaptic target.

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