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# GABAergic Synaptic Input to Mesencephalic Trigeminal Neurons in Rat

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**The mesencephalic trigeminal nucleus (Mes V) contains cell bodies of primary afferent sensory neurons that relay proprioceptive information from the periodontium and masticatory muscles and function as typical sensory neurons or potentially as integrative interneurons. In the present study, we studied these two potential functions using combined experimental approaches of retrograde labeling and whole cell patch clamp recording. Mes V neurons that presumably originate from periodontal nerve fibers in subsets of Mes V nucleus were identified by retrograde labeling with a fluorescent dye, DiI, which was applied onto inferior alveolar nerve. These cells were elliptical perikarya shaped cells about 40 μm in diameter. In these neurons, we measured high voltage-activated calcium channel (HVACC) currents. $GABA_B$ agonist, baclofen, inhibited calcium currents, and the HVACC currents inhibition by baclofen was voltage-dependent, exhibited prepulse facilitation, indicating that it was mediated by $G_i/G_o$ protein. Taken together, our results demonstrate that Mes V neurons not only have cell bodies originating from periodontium, but also receive synaptic inputs including GABAergic neurons suggesting that Mes V neurons function as both primary sensory neurons and integrative interneurons.**

**Key words: mesencephalic trigeminal nucleus, retrograde labeling, DiI, whole-cell patch clamp recording, calcium currents**

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## Introduction

The mesencephalic trigeminal nucleus (Mes V) consists of a narrow band of pseudo-unipolar cells extending from the rostral border of the trigeminal main sensory nucleus in the pons to the superior colliculus in the mid-brain and is known to contain the first-order somata of afferents from jaw-muscle spindles and mechanoreceptors in the periodontal ligament (Lazarov, 2002). Previous studies have indicated that approximately 20% of the Mes V neurons in the cat (Gottlieb *et al.*, 1984; Nomura & Mizuno, 1985) and about 10~15% in the monkey (Hassanali, 1997) have afferent connections to the periodontal mechanoreceptors while the remainder projects to the jaw-closing muscles. Axon collaterals from Mes V neurons course into the region of the trigeminal motor nucleus (Dessem *et al.*, 1997). Primary afferent Mes V neurons are functionally similar to other proprioceptors in the body. However, unlike these neurons, which are encapsulated and located in discrete ganglia outside central nervous system (CNS), the Mes V neurons are non-encapsulated and are retained within the brainstem (Liem *et al.*, 1991; Linden *et al.*, 1994).

In view of the central location of these neurons, some studies have examined the possibility that Mes V neurons may receive synaptic contacts. Synapses can readily be identified on these somata (Hinrichsen & Larramendi, 1968, 1970; Witkovsky & Roberts, 1976; Liem *et al.*, 1991, 1992; Zhang *et al.*, 1997), a feature which contrasts with the reported rarity of synapses on somata of neurons in the dorsal root ganglia (Kayahara *et al.*, 1981; Kayahara *et al.*, 1984). However, electrophysiological studies have failed to identify synaptic currents in Mes V neurons despite the fact that electron microscopy studies show that Mes V neurons receive a sparse synaptic innervation to their somata (Liem *et al.*, 1991). On the basis of such data, it seems likely that

Mes V primary afferent terminals in the periphery (jaw ligaments and periodontal layers) and their central terminals (in the trigeminal mesencephalic motor nucleus, the supratrigeminal nucleus, trigeminal principal sensory nucleus and part of the spinal trigeminal subnucleus oralis) are important in the detection, relaying and central processing of proprioceptive information (Luo & Dessem, 1996; Dessem *et al.*, 1997). Besides, because Mes V receives a variety of synaptic inputs on their somata, Mes V neurons can play functional role as integrative interneurons.

In the present study, we examined whether Mes V neurons can act as both potentially integrative interneurons and typical sensory neurons. We found that Mes V neurons receive GABAergic synaptic inputs and have cell bodies originating from periodontium. These results suggest that Mes V neurons function as both integrative interneurons and primary sensory neurons.

## Materials and Methods

### Surgery and Identification of Mes V nucleus

All procedures for animal were reviewed and approved by the Animal Care and Use Committee of the Seoul National University prior to the experiments.

Sprague Dawley rats, weighing 150~200 gm, were deeply anesthetized with ethyl ether and perfused transcardially with 10% neutral formalin. The tissue block containing trigeminal mesencephalic nuclei was sectioned transversely at 30 μm thick and stained with 1% neutral red (Sigma, U.S.A.). Mes V nucleus was identified under light microscope (Fig. 1A).

The Mes V neurons that originate from periodontal ligament were localized by retrograde labeling with a fluorescent dye, DiI (Molecular Probes, U.S.A.). Under anesthesia with pentobarbital (50 mg/kg, i.p., supplemented as necessary), an incision was made in the buccal skin to reveal the buccal surface of the mandibular bone, and a small amount of the bone covering the mandibular canal was removed. The inferior alveolar nerve (IAN) was exposed, and then transected by fine scissors. The cut ends of the IAN were returned into the mandibular canal, and DiI was applied to the cut end of IAN, then which was sealed with Para film to prevent DiI from spreading to the surrounding tissue.

The wound was sutured, after 48 hours, the brainstem area was frozen-sectioned (14 μm thick) and Mes V neurons were identified under a fluorescent microscope (Fig. 1B)

### Isolation of Mes V neurons for experiments

Mes V neurons were acutely isolated with modification of methods described previously (Yang *et al.*, 2003). Briefly, 5~10 days old Sprague Dawley rats were anesthetized with ether and the tissue block including brainstem was rapidly removed. Transverse slices (3006 μm thickness) including Mes V nucleus were prepared using Vibratome (Technical Products International, St. Louis, MO) in ice-cold oxygenated artificial cerebrospinal fluid (aCSF; NaCl 126, KCl 3, $NaH_2PO_4$ 1, $NaHCO_3$ 26.2, $MgSO_4$ 1.5, $CaCl_2$ 2.5 and glucose 10, pH 7.4, 310 mOsm.) gassed with 95% $O_2$, 5% $CO_2$ and kept in a holding chamber filled with 35°C aCSF gassed with 95% $O_2$, 5% $CO_2$. 3~4 slices were usually obtained from one animal. Slices were enzymatically treated in a conical tube with 15 U/ml papain (preactivated in a $Ca^{2+}$ and $Mg^{2+}$ free aCSF with 3mM EDTA and 0.16 mg/ml L-cysteine for 30 min.) at 35°C for 30 min, and subsequently rinsed twice with aCSF containing trypsin inhibitor (Ovamucoid, Worthington Biochemical Corporation, Lakewood, NJ). The slices were then kept in a holding chamber containing aCSF bubbled with 95% $O_2$, 5% $CO_2$ for at least 1 hour. When needed, slices were placed on Sylgard-coated petri dish, and Mes V neurons were micropunched under the dissecting microscope. The cells were then mechanically dissociated in plating media (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1% 10,000 μg streptomycin/10,000 U penicillin, pH 7.4, 300 mOsm.) on poly-l-lysin coated coverslips using a series of fire-polished glass pipette, and allowed to settle for at least 1 hour on a coverslip at 37°C in a water saturated atmosphere with 5% $CO_2$.

### Electrophysiology

Whole cell patch clamp technique (Hamill *et al.*, 1981) was performed to record barium currents ($I_{Ba}$) from trigeminal mesencephalic neurons. The patch electrodes were made of soft, soda-lime capillary glass and had resistances of 2~5 MΩ with pipette solution before seal formation. Pipette solution was composed of (mM): CsCl 100, $MgCl_2$ 1, HEPES 10, BAPTA 10, Mg-ATP 3.6, Phosphocreatine 14, GTP 0.1, creatine phosphokinase 50 units/ml, adjusted to pH 7.4 with CsOH. Extracellular solution contained (mM): Tetraethylammonium chloride (TEACl) 151, HEPES 10, $BaCl_2$ 5, $MgCl_2$ 1, glusose 10, adjusted to pH 7.4 with TEAOH. The osmolarity of the extracellular solution and internal standard solution was adjusted to 310~320 mOsm and 290~300 mOsm with sucrose, respectively. The $I_{Ba}$ was obtained by a test pulse to 0 mV from the holding potential (-80 mV). When $I_{Ba}$ was depressed by baclofen, double-pulse protocol was employed, in which $I_{Ba}$ was evoked by the application of 0 mV depolarization (5 msec) from a holding potential of -80 mV either without (-prepulse) or 5 msec from a strong depolarizing prepulse (90 mV, +prepulse) every 20 sec. Whole-cell currents were recorded with Axopatch-1C amplifier (Axon Instruments, USA). Partial series resistance compensation was employed and currents lowpass was filtered at 2 kHz, and sampled at 10 kHz. The pClamp6 (Axon Instruments, USA) software was used during experiments and analysis.

### Drugs

$GABA_B$ agonist baclofen was purchased from Sigma (St. Louis, MO). 25 μM baclofen was used in this experiment. The drugs were applied by gravity using a continuous bath perfusion system at a flow rate of 1 ml/min.

## Results

### Identification of Mes V neurons

When the transverse slices were observed under light microscope following staining with 1% neutral red, Mes V nucleus was found from transverse brainstem slices at 3~5 mm rostral to obex (Fig. 1A). Because Mes V neurons were compactly localized in a defined area, it was not difficult to discriminate them from other brainstem areas. DiI labeled neurons, presumably derived from periodontal nerve fibers, were found in the subpopulation of Mes V nuclei. When observed under fluorescent microscope, DiI labeled neurons were elliptical perikarya shaped cells with a diameter about 40 μm (Fig. 1B).

### Measurement of voltage-activated calcium currents

Whole-cell recording of $Ca^{2+}$ currents was performed under experimental conditions that suppress other voltage-dependent currents, such as $Na^+$ and $K^+$ currents (Fig. 2). 5 mM $Ba^{2+}$ was used as charge carrier and the currents were measured during depolarizing pulse (200 msec) to 0 mV from a holding potential, -80 mV. We found that HVACC $I_{Ba}$ was activated in all Mes V neurons tested (n=5) (Fig. 2). Upon application of command potentials ranging from -60 to +40, calcium currents were activated above -30 mV, indicating $I_{Ba}$ being mainly HVACC currents.

### $I_{Ba}$ inhibition by baclofen in Mes V neurons

Since GABAergic neurons constitute the most prevalent inhibitory synapses in the central nervous system, we tested whether GABAergic synaptic input exists in Mes V neurons.

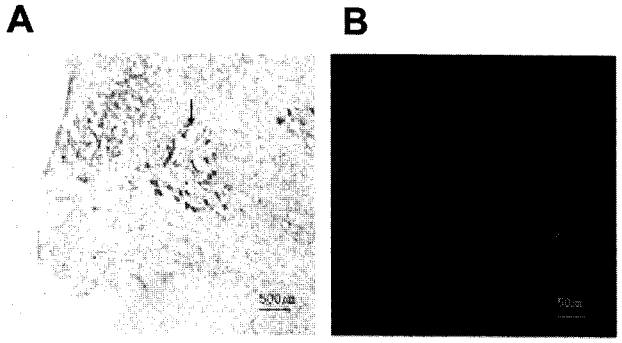


**Fig. 1.** Identification of mesencephalic trigeminal nucleus neurons (Mes V). A. Illustrated is Mes V nucleus (see arrow) stained with 1% Neutral red. (×40) B. The Mes V neurons were localized by retrograde labeling with a fluorescent dye, DiI (×400). Cells were visualized under fluorescent microscope using fluorescent filter which can detect DiI.

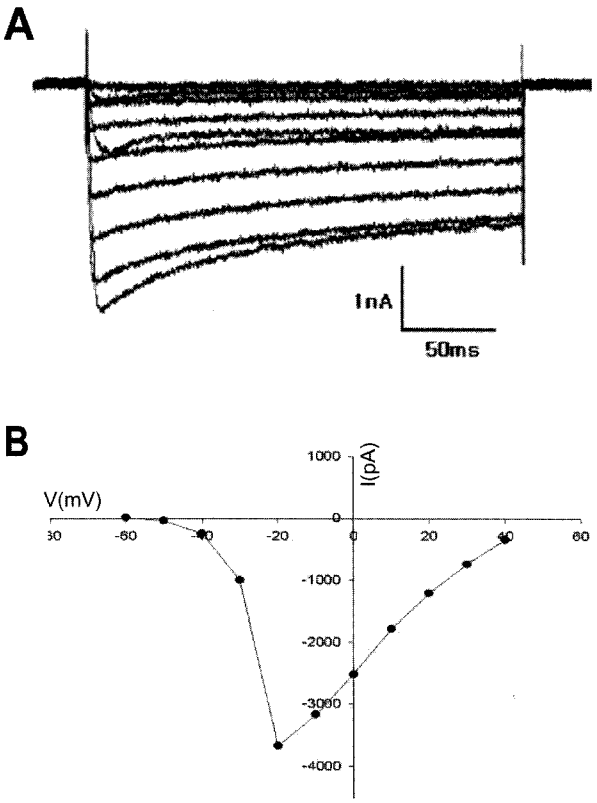


**Fig. 2.** A. Representative calcium current traces recorded from Mes V neuron. Currents were evoked by voltage steps between -60 mV and +40 mV with 10 mV increments from -80 mV. B. Current-voltage (I-V) relationships for the calcium currents.

$GABA_B$ receptor, out of two GABA receptor -i.e. $GABA_A$ and $GABA_B$ receptor, is well known to inhibit calcium currents, $GABA_B$ agonist baclofen was applied to the Mes V neurons.

Baclofen (25 μM) inhibited reversibly calcium currents (n=4) (Fig. 3), indicating the expression of $GABA_B$ receptors in Mes V neurons.

### $G_i/G_o$ protein mediates the $I_{Ba}$ inhibition by baclofen

To determine the underlying mechanisms for the $I_{Ba}$ inhibition by baclofen, we used a double-pulse protocol (Ikeda, 1991; Hille *et al.*, 1995), in which the $I_{Ba}$ was evoked by the application of a depolarizing command potential to 10 mV (50 msec) from a holding potential of -80 mV every 20 sec either without (-prepulse) or following (+prepulse) a strong depolarizing prepulse (+90 mV) (Fig. 4A). Fig. 4 illustrates the effect of baclofen (25 μM) on the $I_{Ba}$ in Mes V neurons. As shown in Fig. 4B, the inhibition of $I_{Ba}$ by baclofen was readily reversible and the inhibition of the $I_{Ba}$ after prepulse significantly decreased compared to that without prepulse, exhibiting prepulse facilitation (36±2 versus 16±2) (Fig. 4B and 4C). This result suggests that $G_i/G_o$ protein is involved in the HVACC currents inhibition by baclofen.

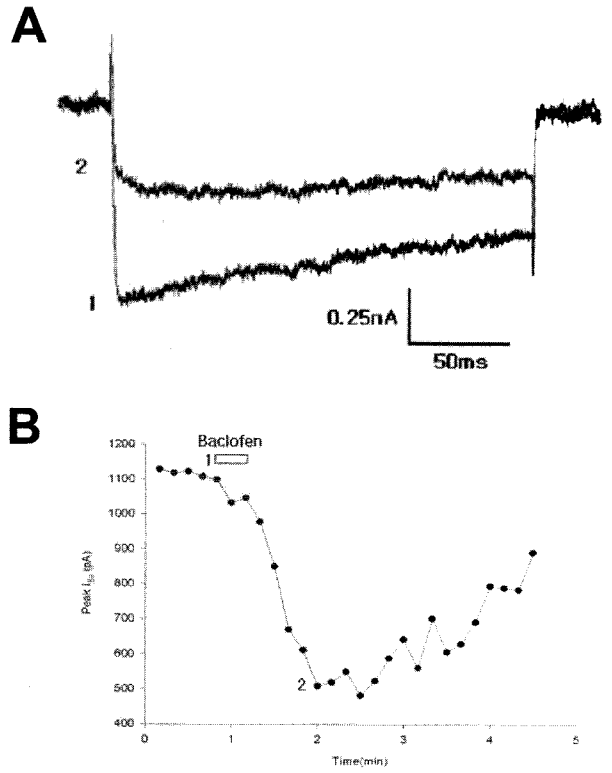


**Fig. 3.** A representative recording of the $I_{Ba}$ inhibition by 25 μM baclofen in Mes V neurons. A. Peak currents of $I_{Ba}$ were evoked by voltage test potential to -20 mV from holding potential of -60 mV. Before (1) and after (2) the application of baclofen. B. Time course of the effect of baclofen.

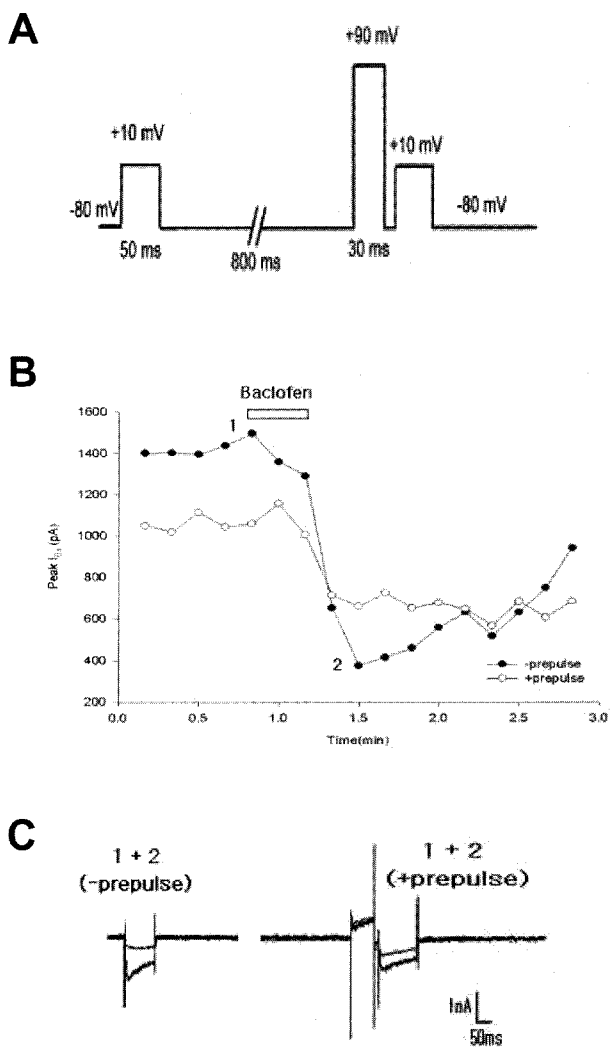


**Fig. 4.** The effect of baclofen on $I_{Ba}$ in Mes V neurons A. Illustration of the double-pulse protocol employed in our experiments. The current was first recorded with a 50 msec test pulse to +10 mV (prepulse); then, after 800 ms, the second test pulse following a 30 msec conditioning prepulse to +90 mV was applied (+prepulse). B. Time course of the effect of baclofen. C. Superimposed $I_{Ba}$ evoked by test pulse with (+prepulse) and without prepulse (prepulse) at the points indicated in (B).

## Discussion

The trigeminal sensory system has a striking peculiarity that the cell bodies of trigeminal primary afferents are located in trigeminal ganglion (TG) as well as in the brainstem, Mes V (Lazarov, 2002). Although primary sensory neurons, whose cell bodies normally lie in ganglia in peripheral nervous system, transmit somatosensory information to central neurons from a variety of sensory receptors in the periphery, the Mes V is the only known nucleus situated within the CNS that contains the cell bodies of primary afferent neurons. It is also unique in that these constitute one distinct functional class of trigeminal sensory neurons, i.e. proprioceptive neurons. Since Mes V is located in CNS and receives synaptic inputs from other brain areas including sensory innervations, it could act as an integrative interneuron as well as primary sensory neurons. In this study, we examined these two potential functions with combined experimental approaches that utilize retrograde labeling and electrophysiological methods

In this study, we located Mes V neurons that presumably originate from periodontal nerve fibers in subsets of Mes V nucleus using retrograde labeling with a fluorescent dye, DiI, which was applied onto inferior alveolar nerve. These cells were elliptical perikarya shaped cells about 40 μm in diameter (Fig. 1). This result clearly demonstrates that Mes V neurons serve as primary sensory neurons which innervate periodontal ligament.

We also found that baclofen inhibits HVACC through the activation of $GABA_B$ in a subpopulation of Mes V neurons by a voltage-sensitive, G-protein-dependent mechanism (Fig. 3 & Fig. 4). Neuronal $Ca^{2+}$ channels have been subdivided on the basis of electrophysiological and pharmacological properties into LVACC or T-type channels and HVACC, a class that includes L-, N-, P/Q-, and R-types. The HVACC inhibition by baclofen exhibited voltage dependence and prepulse facilitation (Fig. 4), indicating that it is mediated by $G_i/G_o$ proteins (Zamponi & Snutch, 1998). Thus, the Ca

channel inhibition produced by baclofen in Mes V neurons closely resembles that produced in trigeminal motor nucleus neurons (Oh *et al.*, 2003). We also found that the effects of baclofen were relieved, although incompletely, by a depolarizing prepulse. Such an effect is usually interpreted as indicating that Ca channel inhibition is mediated by a rapid "membrane delimited" pathway, probably involving the interaction of G protein $\beta, \gamma$ subunits with the Ca channel $\alpha_1$ subunit (Hille *et al.*, 1995).

From these data, we identified that Mes V neurons receive GABAergic synatic input. Unlike TG neurons, no synapses between immunoreactive pericellular baskets and primary afferent neuronal somata have been detected, except for the report of Yamamoto and Kondo (Yamamoto & Kondo, 1989), Mes V neurons seem to receive profound synaptic inputs. The prominent innervation of Mes V neurons by nerve fibers containing a variety of putative neurotransmitters supports the notion that Mes V cell activity is under the influence of various synaptic inputs at the level of the primary afferent cell soma rather than at the nerve terminals as in the TG. It has been demonstrated that both amino acid neurotransmitters are present in separate types of trigeminal neurons, i.e. most large neurons are glutamatergic while certain small neurons are GABAergic (Lazarov & Chouchkov, 1995; Lazarov, 2002). GABA is probably the transmitter released at axo-axonic synapses onto primary afferent terminals (Levy, 1977). Currently, it has also been shown that presynaptic axon terminals on labeled boutons from Mes V periodontal afferents in the cat supratrigeminal nucleus are immunoreactive for GABA (Bae *et al.*, 1997) and, moreover, both $GABA_A$ and $GABA_B$ receptors might be involved in the presynaptic inhibition of Glu-mediated transmission (Peng & Frank, 1989). However, it seems unlikely that GABA mediates the predominantly inhibitory modulation on Mes V neurons reported by Kolta *et al.* (1990), since their responses are mediated by receptors having properties appropriate for $GABA_A$ subtype (Hayar *et al.*, 1997). Hence, it is tempting to speculate that the GABAergic system may play an indirect role in the proprioceptive information processing in Mes V by interactions of GABA-IR neurons with the systems that control the discharge of large Mes V neurons and, in this way, the transmission of selected sensory information.

In summary, in Mes V neurons that are located in brainstem, we found that there exist sensory neurons from periodontium via retrograde labeling with a florescent day. It was also found that HVACC currents were inhibited by $GABA_B$ agonist, baclofen. The HVACC currents inhibi-tion by baclofen was voltage-dependent, exhibited prepulse facilitation, indicating that it was mediated by $G_i/G_o$ protein.

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