植草学園大学研究紀要　　第10巻　145 ～ 151頁（2018）

B.Edu.Health Sci.UG Univ. vol.10, 145-151, 2018　　Original Article

Visualized Projection From the Spinal Trigeminal Nucleus to the Parabrachial Nucleus in Isolated Brainstems of Neonatal Rats: A Voltage-Sensitive Dye Imaging Study

Shun-ichi Kuwana[1], Akiko Matsumoto[2], Takeo Sugita[2] , Nobuo Umezawa[2], Yuki Kaku[3] ,Shigeki Sakuraba[2], Hitoshi Maeda[1], and Hirofumi Arisaka[2]

[1] Faculty of Health Sciences, Uekusa Gakuen University,

[2] Department of Highly Advanced Stomatology, Kanagawa Dental University Graduate School,

[3] Center for Medical Sciences, Ibaraki Prefectural University of Health Sciences

Background: Anatomical studies have shown that neurons in the spinal trigeminal subnucleus caudalis (Sp5c) in the medulla oblongata projected to the ipsilateral parabrachial nucleus (PBN) where they contribute to the autonomic component of pain. However, little is known about connection between the Sp5c and the PBN in living animals. Here, we recorded spatial and temporal expansion of afferent activity from the Sp5c to the PBN in the trigeminal nerve-attached brainstem of neonatal rats using a voltage-sensitive dye imaging technique.

Methods: Trigeminal nerve-attached brainstem preparations were isolated from 2–4 days old rats and stained with a voltage-sensitive dye, di-4-ANEPPS. The preparation was placed in a recording chamber with the dorsal side up, and the trigeminal nerve rootlet was stimulated electrically.

Results: Electrical stimulation evoked changes in fluorescence intensity of the dye in the ipsilateral Sp5c. Then, changes in the fluorescence intensity appeared in the bilateral PBN after a short delay. The optical signal from the Sp5c was composed of two phases, a fast component with a sharp peak at approximately 35 ms after the stimulation followed by a long-lasting component with the period of more than 500 ms. The peak optical signal in the PBN appeared at approximately 23 ms after the peak in the Sp5c. In low Mg^{2+} (0.8 mM) conditions, the signal amplitude of the long-lasting component increased markedly in the Sp5c but not in the PBN.

Conclusions: Voltage-sensitive dye imaging showed that excitation of the unilateral Sp5c propagated bilaterally to the PBN. Furthermore, low Mg^{2+} conditions induced hyperexcitability in the Sp5c but not in the PBN. We suggest that the synaptic plasticity of the long-lasting component in the Sp5c may contribute to chronic trigeminal pain or hyperalgesia.

Keywords: Pain, Spinal trigeminal nucleus, Parabrachial nucleus, Rat brainstem, Voltage-sensitive dye imaging

1．Introduction

Trigeminal afferents conduct nociceptive information from the orofacial area to the spinal trigeminal subnucleus caudalis (Sp5c) in the medulla oblongata. Chronic and intractable pain from the orofacial area appears frequently, including in idiopathic trigeminal neuralgia, intracranial tumors, post-herpetic neuralgia, or dental-oral diseases. It has been reported that migraine also involves the trigeminal system [1]. The plasticity of the secondary neurons in the Sp5c has been considered as the cause of chronic pain. In electrophysiological studies, activity-dependent neuronal hyperexcitability, so-called long-term potentiation, has been reported in

the Sp5c using trigeminal nerve-brainstem preparations from neonatal rats [2,3]. Hyperexcitability was induced in low Mg^{2+} conditions and suppressed by antagonists of N-methyl-D-aspartate (NMDA) receptors. Therefore, these previous studies showed that NMDA receptors contribute substantially to polysynaptic transmission in the Sp5c and to long-term potentiation.

On the other hand, many anatomical studies have shown that neurons in the Sp5c project to the contralateral thalamus where they mediate the sensory-discriminative component of pain, and the neurons project to the ipsilateral parabrachial nucleus (PBN) where they contribute to the autonomic component of pain [4-8]. However, little is known about the connection between the Sp5c and the PBN in living animals [9].

To analyze the spatial dynamics of neuronal excitation propagation in the Sp5c, it is possible to use optical imaging analysis and voltage-sensitive dyes [10, 11]. In the present study, we examined the propagation of excitation from the Sp5c to the PBN in isolated brainstems of neonatal rats using an optical imaging technique. Furthermore, we examined whether hyperexcitability in the PBN was induced by low Mg^{2+} concentration conditions or not.

2. Material and Methods

All procedures were conducted in accordance with the guidelines of the Uekusa Gakuen University Laboratory Animal Care and Use Committee. Data were obtained from six neonatal Wistar rats (2–3 days old). The isolation of brainstem-spinal cord preparations has been described in detail previously [12]. In brief, rats were deeply anesthetized with diethyl ether and the brainstem was isolated in a dissecting chamber at room temperature. The chamber was filled with mock cerebrospinal fluid (CSF) equilibrated with a gas mixture (5% CO_2 in O_2; pH 7.4). The composition of the mock CSF was as follows (in mM): NaCl, 126; KCl, 5; $CaCl_2$, 2; $MgSO_4$, 2; NaH_2PO_4, 1.25; $NaHCO_3$, 26 and glucose, 30. The cerebrum was quickly removed by transection at the upper border of the inferior colliculus. Each trunk of the bilateral trigeminal nerves that run through the craniobasal bone was isolated to a length of 1 mm, enabling it to be pulled into a sucking electrode. Subsequently, the trigeminal nerve-attached brainstem-spinal cord was cut caudally at the level of the C3 roots (Figure 1A). The preparation was placed in a recording chamber (volume 1.0 mL) with the dorsal side up and continuously superfused (flow 4–6 mL/min) at 26°C with oxygenated mock CSF.

The voltage-sensitive dye imaging technique has been described in detail previously [13]. In brief, for staining, preparations were kept for 30 min in mock CSF containing the voltage-sensitive dye Di-4-ANEPPS (7.5 mg/ml in 0.1% DMSO, Molecular Probes, Eugene, Oregon, USA), before being incubated for at least 30 min in normal mock CSF. After staining, excess dye was removed by superfusion of the preparation with dye-free solution. After 30 min of washing, optical imaging and data analysis were performed using the MiCAM02 hardware and software package (BrainVision, Tokyo, Japan). For optical imaging, we used a fixed-stage upright fluorescence microscope (Measurescope UM-2, Nikon, Tokyo, Japan) with a low magnification objective lens (XL Fluor 4×/340, Olympus, Tokyo, Japan) and with a high-resolution MiCAM02 camera.

To record the voltage-sensitive dye signals, we used light from a 150 W halogen lamp controlled by an electromagnetic shutter (Oriel Instruments, Stratford, USA). Changes in fluorescence of the dye were detected by the camera through a 510–560 nm excitation filter, a dichroic mirror, and a 590 nm absorption filter (MBE1405, Nikon). The camera captured images of 88 ×60 pixels and the size of the area was 5.4 mm × 3.7 mm (Figure 1A).

Total frame acquisition was set to 255 or 511. The sampling time was 2.2 ms/frame, so the total recording time was 561 ms or 1890 ms. Neuronal activity was evoked by square pulse electrical stimuli (1.0 ms, 0.5–1.0 mA) delivered to the trigeminal nerve rootlet via a glass suction electrode. Acquisition was triggered by the electrical stimuli. Signal amplitude was normalized using the dF/F method, where F is the total fluorescence

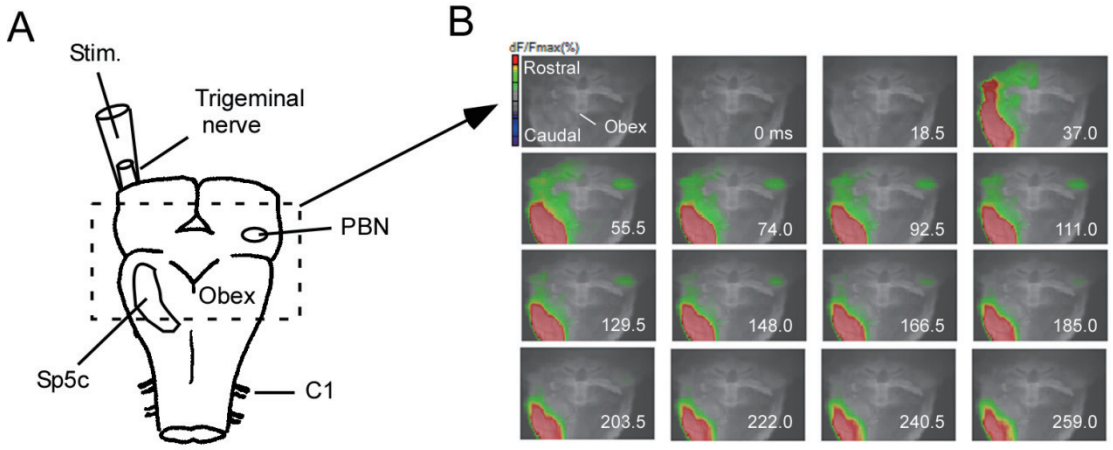

Figure 1: Optical responses in a trigeminal nerve-brainstem preparation

A: Measurement was performed on the dorsal side of the preparation. The optical recording area is indicated by a dotted square. Electric stimulation was applied by sucking the left trigeminal nerve root with a suction electrode. Sp5c: spinal trigeminal subnucleus caudalis, PBN: parabrachial nucleus, C1: 1st cervical nerve rootlet. B: Optical images after electrical stimulation. The time after stimulation is indicated in the bottom right of each image. Neural activity is represented as changes in fluorescence intensity using pseudocolor, as indicated in the color bar in the upper left corner of the image series. Excitation propagated ipsilateraly from the rostral pons to the caudal medulla, and then excitation was recognized in the contralateral PBN.

signal and d*F* corresponds to the change in fluorescence observed following evoked modification of the membrane potential. To improve the signal-to-noise ratio, we averaged signals detected in 10 consecutive trials.

It has been reported that the optical signal in the Sp5c is potentiated in low Mg^{2+} conditions due to the activation of NMDA receptors [14]. We examined whether the potentiation in the PBN was induced by low Mg^{2+} concentration conditions (in mM): NaCl, 126; KCl, 5; $CaCl_2$, 2.6; $MgSO_4$, 0.8; NaH_2PO_4, 1.25; $NaHCO_3$, 26 and glucose, 30. Optical records using electrical stimulation were taken 20 min after the start of superfusion of control mock CSF and were taken 20 min after switching to the low Mg^{2+} concentration solution.

３．Results

Figure 1 shows typical optical results obtained from the trigeminal nerve attached-brainstem preparation. Electrical stimulation of the trigeminal nerve trunk evoked a spatial propagation of membrane excitation from the rostral pons to the caudal medulla, especially to the ipsilateral Sp5c (Figure 1B). Then, optical responses appeared in the bilateral dorsal pons with a latency of approximately 20 ms. These pontine regions anatomically corresponded to the PBN. Thus, this result suggested that neurons in the Sp5c bilaterally conduct the excitation to the PBN.

Temporal changes in optical signals of the Sp5c and PBN are shown in Figure 2. In control conditions (Figure 2A), a sharp fluorescence signal peak of a fast component was noted 43.2 ± 7.64 ms after electrical stimulation of the trigeminal nerve root, and the signal then slowly declined to a slow component over approximately 500 ms during superfusion with mock CSF. In the bilateral PBN, optical signals were also composed of two phases, a fast component with a sharp peak followed by a long-lasting component. The sharp peak appeared with a time delay

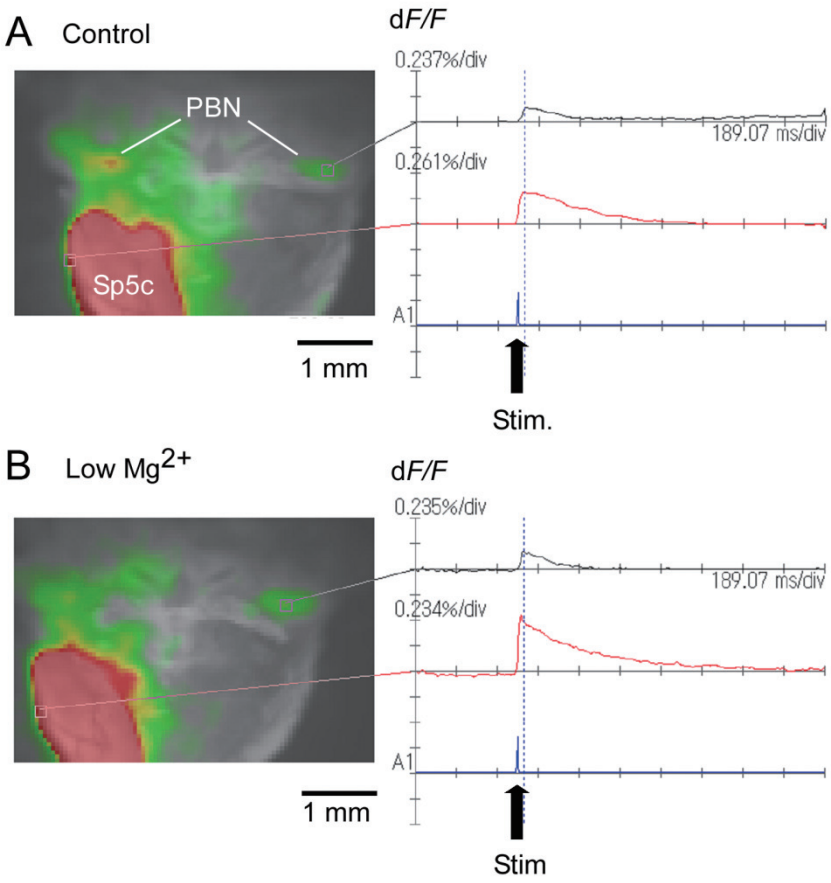

Figure 2: Temporal changes in fluorescence intensity after electrical stimulation of the trigeminal nerve

A: Record during superfusion with control mock CSF. The right panels show the course of the signal change in the Sp5c and PBN. Ordinates axes show d*F*/*F* (%) where *F* is the total fluorescence signal and dF corresponds to the change in fluorescence observed after evoked modification of the membrane potential. A1 indicates the time of the electrical stimulation. B: Record during superfusion with low Mg^{2+} concentration (0.8 mM) solution. Electrical trigeminal nerve stimulation-induced excitement of the Sp5c and the slow component increased spatially and temporally during superfusion with low Mg^{2+} concentration solution. The evoked optical signal in the PBN was not affected by low Mg^{2+} conditions.

of about 53.0 ± 3.82 ms after. However, the intensity of optical signal was weak and the long-lasting period was shorter compared with those in the Sp5c. The latencies from electrical stimulation of the trigeminal nerve rootlet did not differ between the ipsilateral and contralateral PBN.

The influence of low Mg^{2+} concentration (0.8 mM) on evoked excitation in the Sp5c and PBN was observed (Figure 2B). When electrical stimulation was applied during superfusion with low Mg^{2+} concentration solution, the sharp peak of the fast component after stimulation was similar to that in the control. The time delay from stimulation to the fast peak did not change with low Mg^{2+} concentration solution. The low Mg^{2+} concentration increased the electrical trigeminal nerve root stimulation-induced excitement of the long-lasting component in the Sp5c and prolonged the excitement. In addition, as shown in the left panel of Figure 2B, the low Mg^{2+} concentration increased the excited area of the Sp5c. Conversely, optical signals in the PBN did not change during superfusion with low Mg^{2+} concentration solution.

４．Discussion

Applying voltage-sensitive dye imaging to isolated newborn rat brainstem preparations allowed for electrical trigeminal nerve stimulation-induced excitement from the Sp5c to the bilateral PBN to be temporally and spatially visualized. Lengthy persistence of excitement of secondary neurons, and enhancement by low Mg^{2+} concentration, were observed in the Sp5c but not in the PBN.

In previous anatomical studies, when fluorogold was injected into one side of the PBN, retrograde labeled neurons were found ipsilateral in the Sp5c and in the caudal ventrolateral medulla [7,8,15]. Our results showed excitation of the PBN seen bilaterally after trigeminal nerve stimulation-induced excitement of the ipsilateral Sp5c. Optical changes in the ipsilateral PBN seemed to be larger than those in the contralateral PBN. Taken together, ipsilateral conduction from the Sp5c to the PBN may be the main pathway.

In the present study, we found that the pathway from the Sp5c to the PBN was functionally intact. Many studies have demonstrated that the PBN received the nociceptive information and regulated the autonomic function, including cardiovascular reflexes [16], respiratory reflexes [17], visceral reflex [18], etc. Using our same preparation, Kobayashi et al. [17] reported that neurons with respiratory rhythm were located in the rostral pons, and electrical stimulation of the trigeminal nerve induced inspiratory activity in the 4^{th} cervical nerve, including the phrenic nerve. We also confirmed that repetitive electrical stimulation of the trigeminal nerve induced the acceleration of rhythm in the 4^{th} cervical nerve in the same preparation (unpublished data).

It has been shown that the optical signals in the Sp5c were composed of two phases, a fast component followed by a long-lasting component using a trigeminal nerve attached parasagittal brainstem block preparation of 1-week-old rats [14]. In low Mg^{2+} conditions, the long-lasting component was potentiated and attenuated markedly by administration of an NMDA receptor antagonist. The previous study suggested that the long-lasting component, which was induced by synaptic transmission by unmyelinated afferents in the Sp5c, is mediated by NMDA glutamate receptors [14]. Although we did not examine the effect of NMDA receptor antagonist treatment in the present study, we confirmed several results of the previous study. Furthermore, we found that excitation of the PBN was not potentiated by low Mg^{2+} solution. This result suggested that NMDA receptors may not contribute to synaptic transmission in the PBN. Thus, increasing orofacial pain and hyperalgesia may occur at the Sp5c level.

Acknowledgements

This work was supported by JSPS KAKENHI grant numbers 23590719, 26460704, by the Science Research Promotion Fund of the Promotion, and by the Science Research Fund of the Uekusa Gakuen University.

References

1) Goadsby PJ. Recent advances in understanding migraine mechanisms, molecules and therapeutics Trends Mol Med. 2007; 13:39-44.
2) Hamba M, Onodera K, Takahashi T. Long-term potentiation of primary afferent neurotransmission at trigeminal synapses of juvenile rats. Eur J Neurosci. 2000; 12:1128-1134.
3) Onodera K, Hamba M, Takahashi T. Primary afferent synaptic responses recorded from trigeminal caudal neurons in a mandibular nerve-brainstem preparation of neonatal rats. J Physiol. 2000; 524:503-512.
4) Iwata K, Kenshalo DR Jr, Dubner R, Nahin RL. Diencephalic projections from the superficial and deep laminae of the medullary dorsal horn in the rat. J Comp Neurol. 1992; 321:404-420.
5) Sessle BJ. Neural mechanisms and pathways in craniofacial pain. Can J Neurol Sci. 1999; 26:S7-11.
6) Gauriau C, Bernard JF. Pain pathways and parabrachial circuits in the rat. Exp Physiol. 2002; 87:251-258.
7) Mitchell JL, Silverman MB, Aicher SA. Rat trigeminal lamina I neurons that project to thalamic or parabrachial

nuclei contain the mu-opioid receptor. Neuroscience. 2004; 128:571-582.

8) Aicher SA, Hermes SM, Hegarty DM. Corneal afferents differentially target thalamic- and parabrachial-projecting neurons in spinal trigeminal nucleus caudalis. Neuroscience. 2013; 232:182-193.

9) Jansen NA, Giesler GJ Jr. Response characteristics of pruriceptive and nociceptive trigeminoparabrachial tract neurons in the rat. J Neurophysiol. 2014;Oct 8:jn.00596.2014. doi: 10.1152/jn.00596.2014.

10) Seo K, Fujiwara N, Takeuchi K, Maeda T, Someya G. Optical imaging of excitation propagation evoked by stimulation to the trigeminal caudalis. Neuroreport. 2001; 12:3985-3988.

11) Seo K, Fujiwara N, Takeuchi K, Maeda T, Someya G. Repetitive afferent stimulation propagates excitation in the trigeminal caudalis. Neuroreport. 2003;14:1321-1325.

12) Kuwana S, Tsunekawa N, Yanagawa Y, Okada Y, Kuribayashi J, Obata K. Electrophysiological and morphological characteristics of GABAergic respiratory neurons in the mouse pre-Bötzinger complex. Eur J Neurosci. 2006; 23:667-674.

13) Okada Y, Chen Z, Yoshida H, Kuwana S, Jiang W, Maruiwa H. Optical recording of the neuronal activity in the brainstem-spinal cord: application of a voltage-sensitive dye. Adv Exp Med Biol. 2001; 499:113-118.

14) Takuma S. Effect of neonatal capsaicin treatment on neural activity in the medullary dorsal horn of neonatal rats evoked by electrical stimulation to the trigeminal afferents: an optical, electrophysiological, and quantitative study. Brain Res. 2001; 906:1-12.

15) Abdallah K, Artola A, Monconduit L, Dallel R, Luccarini P. Bilateral descending hypothalamic projections to the spinal trigeminal nucleus caudalis in rats. PLoS One. 2013; 8:e73022.

16) Boscan P, Dutschmann M, Herbert H, Paton JF. Neurokininergic mechanism within the lateral crescent nucleus of the parabrachial complex participates in the heart-rate response to nociception. J Neurosci. 2005; 25:1412-1420.

17) Kobayashi S, Onimaru H, Inoue M, Inoue T, Sasa R. Localization and properties of respiratory neurons in the rostral pons of the newborn rat. Neuroscience. 2005; 134:317-325.

18) Suzuki T, Sugiyama Y, Yates BJ. Integrative responses of neurons in parabrachial nuclei to a nauseogenic gastrointestinal stimulus and vestibular stimulation in vertical planes. Am J Physiol Regul Integr Comp Physiol. 2012; 302: R965-975.

植草学園大学研究紀要　　第10巻　145 ～ 151頁（2018）

B.Edu.Health Sci.UG Univ. vol.10, 145-151, 2018

原　著

膜電位イメージング法による三叉神経脊髄路核から傍小脳脚核への興奮伝播の可視化

――新生ラットの単離脳幹標本を用いた研究――

桑名　俊一[1]，松本安紀子[2]，杉田　武士[2]，梅澤　伸夫[2]，
角　　友起[3]，櫻庭　茂樹[2]，前田　仁士[1]，有坂　博史[2]

［1］植草学園大学保健医療学部，［2］神奈川歯科大学大学院高度先進口腔医学講座，
［3］茨城県立医療大学保健医療学部

三叉神経脊髄路尾側亜核（Sp5c）は三叉神経支配領域に出現する疼痛の中継核だけでなく片頭痛の発生源とも考えられている。三叉神経痛覚情報の上行路や中継核の興奮状態を解明することは，慢性疼痛の予防・治療法を開発する上で必須である。本研究では，膜電位イメージング法をラット脳幹単離標本に適用し，三叉神経根の電気刺激に対する Sp5c からの上行路および中継核の興奮を可視化することを目的とした。新生ラットより三叉神経付き脳幹ブロック標本を作製し，膜電位感受性色素で染色した後，標本からの赤色蛍光を特殊高速高感度光計測システムで計測・記録した。三叉神経根の電気刺激後 10ms で Sp5c に強い蛍光，すなわち興奮が認められ，その後数百 ms 持続した。さらにその後，約 10ms の遅延をもって反対側の傍小脳脚核（PBN）に蛍光変化が認められた。これは，Sp5c から反対側の PBN へ興奮が伝導したことを示している。低 Mg^{2+} 液で標本を潅流しながら三叉神経根の電気刺激を行ったところ，Sp5c の興奮は増強したのに対し，PBN の興奮は増強しなかった。低 Mg^{2+} 下において興奮が増強し長期間持続する状態は central sensitization（中枢神経における痛覚増強）と考えられている。すなわち，本研究では膜電位イメージング法を用いて central sensitization が三叉神経脊髄路尾側亜核のレベルで惹起されることを明らかにした。このことから，三叉神経脊髄路尾側亜核の central sensitization が三叉神経痛の慢性化および片頭痛の発生に深く関与していることが示唆された。

キーワード：三叉神経，三叉神経脊髄路尾側亜核，膜電位イメージング法，三叉神経付き単離脳幹標本，新生ラット