Original Article

Electromyography and Fos immunostaining study establish a possible functional link between trigeminal proprioception and the oculomotor system in rats

Houcheng Liang[1,2,3], Jingdong Zhang[1,✉], Pifu Luo[4], Hongna Zhu[2], Ying Qiao[2], Anle Su[2], Ting Zhang[2]

[1] *Xi'an BRIGHT Eye Hospital, Xi'an, Shaanxi 710000, China;*
[2] *Shaanxi Provincial Eye Research Institute and Xi'an Eye Hospital Xi'an, Shaanxi 710002, China;*
[3] *Department of Ophthalmology, 2nd Affiliated Hospital of Xi'an Jiao-Tong University, Xi'an, Shaanxi 710002, China;*
[4] *Department of Pathology, KingMed Diagnostics, International Bioisland, Guangzhou, Guangdong 510050, China.*

Abstract

The objective of this study was to explore whether there was a functional link between trigeminal proprioception and the oculomotor system mediated through jaw muscle afferents. Electromyography (EMG) was undertaken of the levator palpebrae (LP) and superior rectus (SR), and Fos expression was detected in the brainstem following consecutive down-stretching of the lower jaw at 2-4 Hz in rats. Retrograde tracing was undertaken of the interstitial nucleus of Cajal and Darkschewitsch nucleus (INC/DN) pre-oculomotor neurons. EMG-like responses were recorded from the LP/SR during down-stretching of the lower jaw at 2-4 Hz in 3 out of 11 rats. Fos expression was induced by consecutive down-stretching of the lower jaw at 2-4 Hz for 20-30 seconds. Interestingly, Fos expression was distributed mainly in the bilateral INC/DN area. We also examined Fos-like immunoreactivity in central mesencephalic and paramedian pontine reticular formation that harbors premotor neurons controlling horizontal eye movement, but no Fos-like staining was observed therein. By injection of retrograde tracers into the oculomotor nucleus combined with Fos immunostaining, double labeled pre-oculomotor neurons were visualized to distribute in the INC/DN. In conclusions, there may exist a trigeminal proprioceptive – oculomotor system neural circuit through jaw muscle afferents in rats. Judging from Fos distribution pattern, this pathway might be related to vertical and torsional eye movements.

Keywords: Marcus Gunn syndrome, down-stretching lower jaw, levator and superior rectus, electromyography, interstitial nucleus of Cajal and Darkschewitsch nucleus, Fos expression

Introduction

Marcus Gunn syndrome (MGS) is an oculomotor disorder characterized by abnormal eye movements, most notably eyelid retraction that is elicited by jaw movements[1-3]. The MGS is a congenital synkinetic eyelid disorder with a prevalence of 4%-6% among cases of congenital ptosis[1-3]. Congenital miswiring is

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considered to be a pathogenic mechanism of the MGS because aberrant innervation of the lateral rectus by an oculomotor nerve branch has been demonstrated in cases of Duane syndrome[4-6]. Furthermore, mutation of a gene named *TUBB3* in humans and mice resulted in innervation of the lateral rectus by the oculomotor branch and severe underdevelopment of brain white matter[7]. Congenital miswiring might have occurred in both the brainstem level and peripheral nerve system[4,8-9]. For example, magnetic resonance imaging (MRI), tracing nerve bundles or white matter tract in cases of MGS including familiar MGS unveiled brainstem structural abnormality instead of oculomotor nerve miswiring[8-9]. In addition, more than one gene has been reported to be involved in congenital ptosis including MGS[10-12], suggesting pathogenic mechanism underlying the MGS to be more variated than we have expected. However, direct evidence for miswiring of the trigeminal motor branch to the levator palpebrae (LP) or superior rectus (SR) is still lacking.

The idea of aberrant innervation of LP and/or SR by trigeminal motor branch is based on early clinical studies on the MGS cases. In these studies, the authors showed distinct co-firing of masticatory and eyelid muscles when both muscle activities were recorded simultaneously[13-14]. Sona *et al.* found that stimulation of the pterygoid muscle nerve elicited ipsilateral ptotic eyelid retraction, which was relieved by section of this nerve from the trigeminal motor root[15]. Sona and Wartenberg proposed a "release" hypothesis arguing that jaw-winking is probably a primitively normal reflex but becomes highly controlled and undetectable during phylogenetic development[13-15]. This primitive reflex might be released or manifested when brain networks are congenitally disordered or injured by trauma[1,3,13]. More interestingly, the same phenomenon of retraction of the upper eyelid by electric stimulus of the pterygoid nerve was also observed in humans without congenital ptosis in a recent clinical study[16]. Moreover, a neural tract tracing study in *Xenopus laevis*[17] showed that the central axons of the masticator afferent mesencephalic trigeminal nucleus (Vme) neurons project directly to the oculomotor nucleus (III) and trochlear nucleus (IV). This neuronal circuit may help an amphibian to stare at its prey while opening its mouth widely[17]. These findings seem to add substantiation to the "release hypothesis".

On the other hand, we also observed in rats that some Vme neurons project to the III by either introducing tracers to the Vme or intracellularly injecting tracers into the Vme neuron when we studied the trigeminal proprioceptive pathway[18-19]. We also observed that the Vme projected not only to the III/IV but also to pre-oculomotor neurons[20-21] in interstitial nucleus of Cajal (INC), stimuli of the masseter nerve evoked discharges in the III and the INC, and train stimuli even induced Fos expression in the INC and Darkschewitsch nucleus (DN) areas[19]. We hypothesized that the pterygoid muscle or masticatory muscles may elicit eyelid movement through their muscle spindle Vme afferent inputs but not through masticator motor neuron efferent impulse. To test our hypothesis that the Vme neurons projecting to the III and INC/DN are masticator muscle spindle afferents, we attempted to evoke some LP/SR muscle activities and examined Fos expression through this potential pathway by vigorous lower jaw stretching in this study.

Materials and methods

Animals

Twenty-three Sprague-Dawley rats (200-300 g, both female and male) from Animal Facilities of the Shaanxi Provincial Eye Research Institute and Eye Hospital were used in the experiment. All surgical procedures and animal care were carried out in accordance with the Guidelines for the Care of Laboratory Animals in Research issued by The Chinese Academy of Sciences, which is equivalent to the European Union guideline for Animal Used for Scientific Purpose. The experimental protocol was approved by the Shaanxi Provincial Eye Research Institute and Eye Hospital.

Preparation for LP/SR electromyogram (EMG) recording

Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and administered with atropine (0.15 mg/kg, i.p.). Surgery to expose LP/SR started until no limb-withdrawal reflex was elicited by pinching the hind paw. An incision was made unilaterally along the upper eyelid in one side after shaving and sterilizing the skin. The superficial rim of the LP and/or SR was exposed by removing connective and fatty tissues. Then, the animal was mounted on a rat stereotaxic frame in the prone position with its head fixed through ear bars and the upper jaw fixed on a tooth bar firmly. Unipolar stainless electrodes coated with Teflon were placed into the middle of exposed LP/SR (*Fig. 1*) and the incision was then closed by instant bio-glue. A reference electrode was placed through an alligator clip on the earlap opposite to the EMG recording site. The animal became mildly anesthetized after surgery and was mounted on a stereotaxic frame. EMG-like response was elicited by repeatedly down-stretching the lower jaw at about 2-3 Hz (*Fig. 1*). An NTS-2000 EMG-EEG multi-channel amplifier (Pukang Electronic Tech. Ltd, Shanghai,
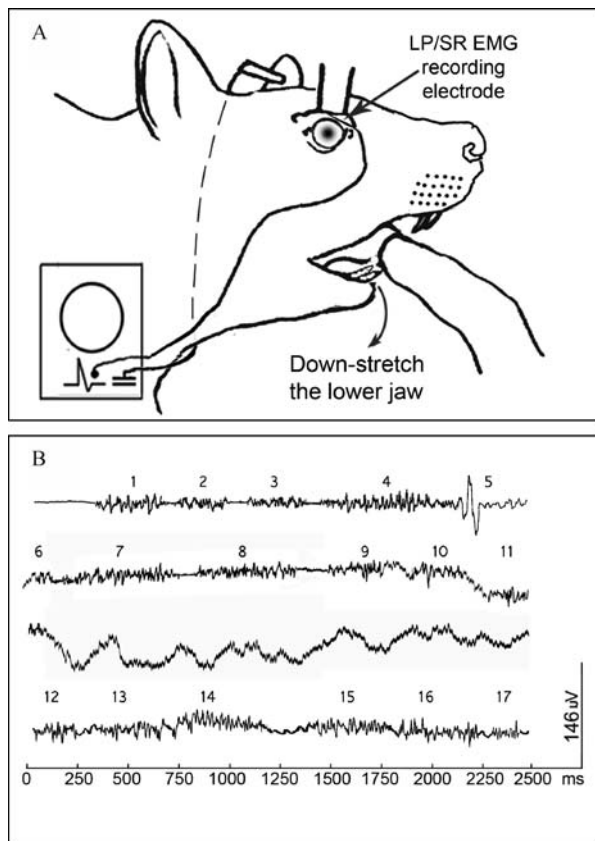
***Fig. 1* Schematic drawing for down-stretching the lower jaw and EMG recording from the levator palpebrae/superior rectus (LP/SR).** A: EMG recording electrode onto the LP/SR with a reference electrode on the ear of the contralateral side, and down-stretching the jaw. B: Representative EMG-like muscle activities are recorded from the LP/RS; however, the 3rd trial was on a period of resistance to down-stretching the jaw. The numbers above the trials indicate down-stretching stimulations; while, responses to probable 6-10 stimulations during the period of resistance were not well recorded.

China) was used to record EMG and the signals were filtered at 1 KHz.

Induction of Fos expression by repeated down-stretching of the lower jaw

In two rats of this group (a female and a male), the masseter nerve was exposed and electric train stimuli were applied to induce Fos expression in the same way as described in our previous work[19] as positive control. In another six animals of this group, mechanical stimulation by down-stretching of the lower jaw was used to induce Fos expression. Negative control rats were not included herein because that was performed in our previous work[19]. To determine the time-course of Fos expression after stimulation, the rats were allowed to survive for 1.0 hour (2 rats), 1.5 hours (2 rats) and 2.0 hours (2 rats). Then, the rats were euthanized by giving overdose sodium pentobarbital (100-120 mg/kg, i.p.)

and transcardially perfused with saline and 4% paraformaldehyde, followed with 5% sucrose.

The brain was removed and placed in 20% sucrose overnight. Coronal frozen sections (30 μm in thickness) were cut through the whole length of the midbrain and pons. All sections were incubated in a rostro-caudal sequence with rabbit anti-Fos antibody (1:300, Santa Cruz Biotech, Santa Cruz, CA, USA) followed by biotinylated goat anti-rabbit serum (Vector Laboratories, USA). Finally, ABC kit (Vector Laboratories; 1:50 A:B) and DAB-NAS (3,3′diaminobenzidine-nickel ammonium sulfate) histochemical protocol were applied to visualize Fos immunoreactivity. Distribution of Fos expression in all midbrain and pons was examined, including the pre-oculomotor area of INC/DN and central mesencephalic reticular formation and the paramedian pontine reticular formation (CMRF/PPRF) that harbors premotor neurons to the abduces nucleus[22].

Retrograde and Fos double labeling of pre-oculomotor neurons

In the last four rats, cholera toxin B (CTB) was delivered into the III to retrogradely label contralateral INC/DN pre-oculomotor neurons. The animals were anesthetized and mounted onto a stereotaxic frame and the same coordinate parameters as described in our previous work[19] were used to perform injection of CTB into the III. After five days, the animals were anesthetized and fixed onto the stereotaxic frame again with only the lower jaw free as described above. When the animal became lighter and anesthetized, the lower jaw was repeatedly stretching down at 23 Hz for 20-30 seconds. The rats were euthanized 2 hours after mechanical stimulation by giving an overdose of sodium pentobarbital (100-120 mg/kg, i.p.). The negative control rats suffered stereotaxic frame mounting, craniotomy and saline injection alone without down-stretching the jaw[19]. Then, coronal frozen sections (30 μm in thickness) were cut through the midbrain and pons. The sections were incubated with rabbit anti-Fos antibody (Santa Cruz Biotech) and goat anti-CTB serum (1:500, List Biological Laboratories, USA) overnight. On the second day, donkey anti-rabbit-Alexa Fluor 488 and anti-goat-Alexa Fluor 568 were applied to localize the tracer injection center and to visualize possibly double labeled pre-oculomotor neurons in the INC/DN. The sections were examined and photographed with Nikon E-600 fluorescent microscope and Bio-Rad 1024 Laser Scan Confocal microscope when necessary.

The number of controlled rats for examining Fos expression after all procedures of CTB vehicle injection

without down-stretching of the jaw were not included in this work because these controls had been done in our early work[19].

Results

EMG-like activities in the LP/SR evoked by repeated down-stretching of the lower jaw

EMG-like activities were recorded from LP/SR in 3 out of 11 rats and *Fig. 1* displays a representative response from one rat. During repeatedly down-stretching the lower jaw, we kept stretching for 10 seconds and the number of stretches was consistently 23 to 27, namely at 2.3-2.7 Hz. We judged whether or not our recording was an EMG-like response by a criterion that a gap between the burst discharge and the base line could be observed. Furthermore, we considered a recording as an EMG-like response if the burst frequency was about 2.3-2.7 Hz, as shown in *Fig. 1*. But notably, some hardness would suffer in many cases during down-stretching stimulation of the lower jaw, which often broke the line of recording, though this kind of resistance generally lasted a short time.

Fos expression neurons in the midbrain and pons

Fos expression was evidently identified in the midbrain and pons after 1.5 hours and 2.0 hours of survival following 20 seconds of down-stretching jaw stimulation. Fos positive cells were observed bilaterally, in sequence of density, in the INC/DN (*Fig. 2*, *3*), Edinger-Westphal nucleus (*Fig. 2*, *3*), supratrigeminal areas including the dorsolateral rim of the trigeminal motor nucleus, the reticular formation dorsal to the superior olive nuclei group, lateral deep mesencephalic reticular formation and reticular formation medial to the Kölliker-Fuse nucleus (*Table 1*). Comparing to Fos expression induced by electric stimuli of the masseter nerve (*Fig. 2B*), the density of Fos-like immunoreactivity was lower in cases of down-stretching jaw stimulation (*Fig. 2A*).

We also paid specific attention to the areas reported to harbor premotor neurons to the abduces nucleus or horizontal eye moving premotor neurons[22], namely, the areas of CMRF/PPRF[22]. There was no Fos immunoreactivity in the least observed or in the CMRF/PPRF regions. In our negative control animals, Fos-like immunoreactivity was not observed in all of coronal sections from the midbrain and pons[19].
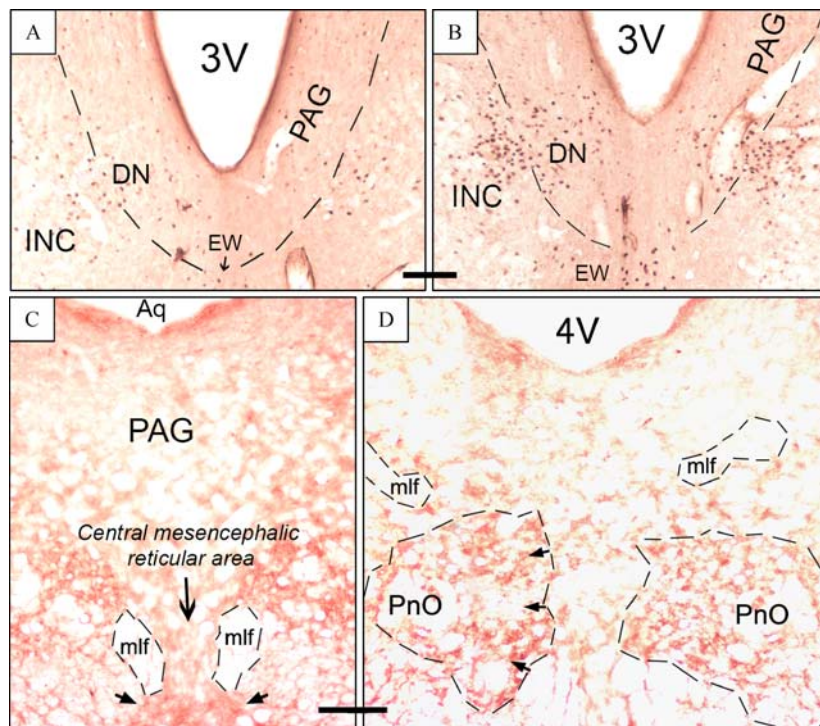

Fig. 2 **Distribution of Fos-like immunoreactivities in the midbrain and pons following down-stretching jaw stimulation.** A: Fos-like immunoreactivities are located in neuronal nuclei in interstitial nucleus of Cajal and Darkschewitsch nucleus (INC/DN) areas and in Edinger-Westphal nucleus (EW) induced by repeatedly down-stretching the jaw at 2-4 Hz. B: Fos expression in the INC/DN and EW neurons evoked by electric train stimuli of the masseter nerve, as a positive control (negative control was described in our early work). C and D: Fos-like immunoreactivity was not observed in regions of central mesencephalic and paramedian pontine reticular formations, where premotor neurons to the abduces nucleus were harbored. 3V, third ventricle; 4V, fourth ventricle; Aq, aqueduct; mlf, medial longitudinal fasciculus; PAG, periaqueductal gray; PnO, pontine reticular nucleus, oral part. Scale bars = 200 μm.
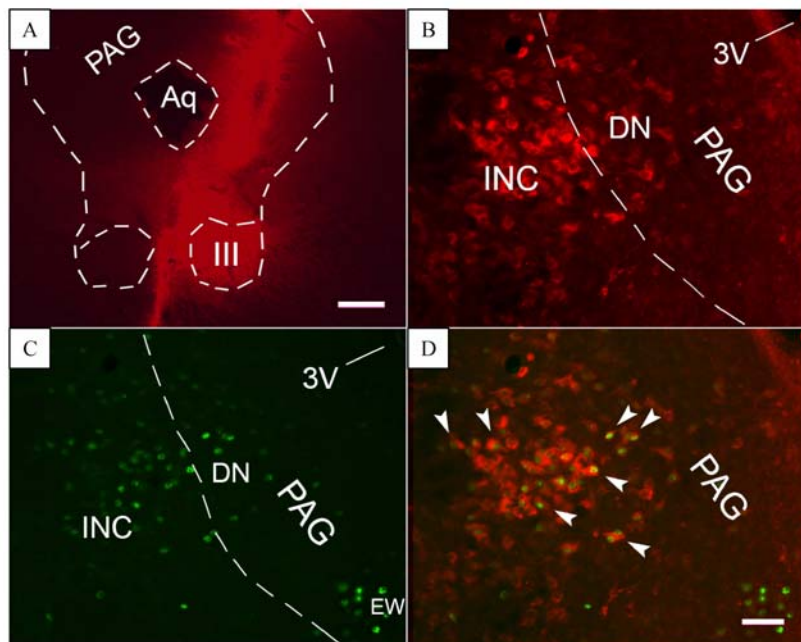
Fig. 3 **Fos expression in tracer-labeled INC/DN pre-oculomotor neurons.** A: Chloride toxin B (CTB) injection site in the III. B: CTB retrograde labeled premotor neurons in the INC/DN contralateral to the injection. C: Fos-like immunoreactivities at neuronal nuclei in the INC/DN and the EW, induced by down-stretching the jaw at 2-4 Hz. D, Double labeling of the INC/DN pre-oculomotor neurons by both CTB and Fos immunostaining (arrowheads). Scale bars: 200 μm in A; 50 μm in B-D.

Fos expression in tracer-labeled INC/DN pre-oculomotor neurons

In this group, retrograde-tract tracer CTB was successfully delivered into the III (***Fig. 3A***) and premotor neurons in the contralateral INC/DN were clearly labeled in three rats (***Fig. 3B***). In addition, these rats were effectively stimulated by down-stretching the lower jaw and Fos expression was induced (***Fig. 3C***). The distributive pattern of green-fluorescent Fos immunoreactivity positive neurons in the midbrain and pons was the same as Fos immunostaining by immunoperoxidase techniques (***Fig. 2*** and ***Fig. 3C***; ***Table 1***). CTB and Fos double labeled cells were observed in the INC/DN areas (***Fig. 3D***). The double labeling of pre-oculomotor neurons was explicit (***Fig. 3D***), although the ratio of double labeled cells to single labeled pre-oculomotor neurons was lower than that in cases of electric stimuli of the masseter nerve[19] (***Fig. 2B***). By counting every other section under a Nikon fluorescent microscope, the percentage of CTB-Fos (Red-Green; ***Fig. 3B-D***) double labeled neurons to CTB single labeled pre-oculomotor neurons in the INC/DN was 15%, 13% and 16%, respectively, in these rats. In our control animals, Fos-like immunoreactivity was not encountered in all coronal sections from the midbrain and pons[19].

Discussion

A clinical fact that has been overlooked for a long time is that some MGS cases showed trigeminal oculomotor synkinesis only temporally in life and some other MGS exhibit a pattern of alternative healing and relapse[13-14,23-24]. How could we explain these phenomena by hypothesis that trigeminal oculomotor synkinesis is caused by congenital miswiring of trigeminal motor branch into oculomotor nerve? Interestingly, a group of authors demonstrated that eyelid

Table 1 **Distribution of Fos positive cells in midbrain and pons**

Midbrain and Pons Areas	Fos Labeling
INC/DN Areas	+ + +
Edinger-Westphal Nucleus	+ + +
Supratrigeminal and Dorsolateral Rim of Trigeminal Motor Nuclei Regions	+ +
Reticular Formation Dorsal to Superior Olive Nucleus	+
Lateral Deep Mesencephalic Reticular Formations	±
Reticular Formations Medial to Kölliker-Fuse Nucleus	±

retraction was elicited by electrical stimulation of the ipsilateral trigeminal motor root in humans that suffered trigeminal neuralgia without any history of congenital ptosis[16]. We observed in healthy human volunteers that static jaw occlusion evoked EMG-like activities and the waveform and frequency of muscle activities were unequivocally similar to the EMG recorded from the same electrode when the volunteer actively looked upward with the head fixed[25], namely, when the eyelid was retracting. This data suggests an intrinsic linkage between masticatory and oculomotor system even in healthy humans[16,25]. A neuronal tract tracing study in *Xenopus laevis* unveiled that the Vme neurons project their central axons to the III/IV, and meanwhile send peripheral processes to the temporalis, the largest masticator in *X. laevis*[17]. This anatomic data suggests masticatory muscle spindle afferent Vme neurons project to the III/IV directly in amphibians. Meanwhile, a trigeminal oculomotor reflex with widely opening the mouth and eyes simultaneously is a normal reflex in *X. laevis* that helps this creature to target the prey and shoot its tongue[17]. Is it possible that this reflex has been functionally distinguished during phylogenetic development but a residue of reflexive neural pathway still exists to some extent? To explore this question, we selected a rat as a mammal to investigate whether there is a functional link between masticator proprioception and oculomotor eyelid control system. Our current results indeed imply a functional association between these two systems but not in all of rats: down-stretching lower jaw induced EMG-like activity in LP/SR was recorded in about 27% (3/11) of tested rats, and Fos expression was induced in 75% (3/4) of these rats.

As we know, passively elongating jaw closing muscles will activate their muscle spindles and in-turn their afferent Vme neurons, even under systematic anesthesia in cats and rats[26-27]. The jaw displacement approach has been generally used in studies of the jaw muscle spindle afferents pathway[27,28]. Stretching the lower jaw by pressing down the rat's lower teeth achieves similar effect as displacement apparatus was performed; however, squeezing periodontal ligaments of the lower teeth might be another possibility to excite the Vme neurons. In other words, the Vme afferent neurons innervating periodontal ligament mechanoreceptors might also be involved in generating excitatory afferent impulse. Based on a previous study[28], not all of jaw muscle spindle afferents are stretching sensitive neurons; hence, it is possible that distribution of stretching sensitive neurons might not be the same in different animals or different physiologic status. Is this the reason for negative results from most of the rats in our current work? We assumed the predominant reason

might be the extent of structural residue preserved for the masticator oculomotor reflex. Furthermore, it is known that following functional related stimulation, *c-Fos* oncogene and Fos proteins are usually expressed along the functional pathway[29]. For example, taste related stimulation induced Fos expression in neurons along the visceral sensation central pathway[30-31]; however, nociceptive stimulation evoked Fos production among neurons of the nociception conveying central pathway[32-33]. As aforementioned, down-stretching of the lower jaw predominantly motivates jaw muscle spindle afferents[26-27]; therefore, Fos expression should be associated with trigeminal proprioception conductance. Consequently, this pathway seems to not be completely without function. It has been well documented that the INC is a pre-oculomotor center that controls vertical-torsional eye and head movements and the DN is a member of accessory pre-oculomotor nuclei reported in early studies[20-21,34]. It was also known that premotor neurons modulate horizontal eye movements and are situated in the CCRF/PPRF[21-22]. Intriguingly, Fos was expressed exclusively in the INC/DN following the lower jaw stretching stimulation but was not observed in areas of CCRF/PPRF, suggesting that this pathway be probably related to vertical-torsional eye and head movements. In fact, modulation of the eyelid action is involved in controlling vertical eye and head movement[5,20-21].

On the other hand, a group of scholars reported that some Vme neurons innervate mechanoreceptors of Mueller's muscle in the superior tarsal plate and the central axons of these Vme neurons project to the III and terminate on the LP motoneurons that innervate slow-twitch skeletal muscle part of the LP in rats[35-36]. A neural tract tracing study in Macaque monkey also revealed a limited number of labeled Vme neurons following injection of retrograde tracers into the LP and SR[37]. Those authors had demonstrated that these tarsal plate Vme afferents somata form a gap-junction connecting to adjacent periodontal and/or jaw muscle spindle Vme neurons because gap-junction-permeable dye spread from tarsal plate Vme afferents to adjacent Vme neurons[35]. These findings give rise to another possible interpretation for our current results: following injection of tracers into the Vme in our previous work[18,19], anterograde labeled projections to the III/IV and INC/DN might be from tarsal plate Vme neurons[35,36] or maybe not; or fewer jaw muscle spindle afferents as we had expected. Electrotonic coupling through gap-junction between the Vme neurons had been well documented decades ago[38]. Moreover, somatofugal action potentials were recorded from those been coupled Vme neurons when the electric

tone reaches the threshold to discharge action potentials[38-39]. Therefore, if jaw muscle spindle afferent Vme neurons, or probably some periodontal afferents were fired by repeated jaw stretching or pushing against periodontal ligaments, it was possible that the neighboring tarsal plate afferent Vme neurons were excited through electrotonic coupling mechanism[38-39]. Then, LP motoneurons in the III might be excited by the input from the tarsal plate afferent Vme neurons, those being coupled with jaw muscle spindle or periodontal proprioceptive Vme neurons.

Acknowledgements

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