• Basic Research •

Is there a primitive reflex residue underlying Marcus Gunn Syndrome? Rat electrophysiology

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Abstract

● **AIM:** To make an electrophysiological demonstration of a possible jaw muscle afferents-oculomotor neural pathway that was proposed by our previous works on rats, which substantiates an early "release hypothesis" on pathogenesis of human Marcus Gunn Syndrome (MGS).

● **METHODS:** Extracellular unit discharge recording was applied and both orthodromic and spontaneous unitary firing were recorded in the oculomotor nucleus (III), and the complex of pre-oculomotor interstitial nucleus of Cajal and Darkschewitsch nucleus (INC/DN), following electric stimulation of the ipsilateral masseter nerve (MN) in rats.

● **RESULTS:** Extracellular orthodromic unit discharges, with latencies of 3.7±1.3 and 4.7±2.9ms, were recorded unilaterally in the III, and the INC/DN neurons, respectively. Spontaneous unit discharges were also recorded mostly in the INC/DN and less frequently in the III. Train stimulation could prompt either facilitation or inhibition on those spontaneous unit discharges. The inhibition pattern of train stimulation on the spontaneous discharging was rather different in the III and INC/DN. A slow inhibitory pattern in which spontaneous firing rate decreased further and further following repeated train stimulation was observed in the III. While, some high spontaneous firing rate units, responding promptly to the train stimuli with a short-term inhibition and recovered quickly when stimuli are off, were recorded in the INC/DN. However, orthodromic unit discharge was not recorded in the III and INC/DN in a considerable number of experiment animals.

● **CONCLUSION:** A residual neuronal circuit might exist in mammals for the primitive jaw-eyelid reflex observed in amphibians, which might not be well-developed in all experimental mammals in current study. Nonetheless, this pathway can be still considered as a neuroanatomic substrate for development of MGS in some cases among all MGS with different kind of etiology.

● **KEYWORDS:** masseter nerve; single unit discharge; oculomotor nucleus; pre-oculomotor neurons; interstitial nucleus of Cajal/Darkschewitsch nucleus; Marcus Gunn Syndrome

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INTRODUCTION

An oculomotor dysinnervation disorder known as Marcus Gunn Syndrome (MGS) is characterized by abnormal eye and eyelid movements and, most notably, eyelid retraction that is elicited by jaw movements[1-2]. This disorder is also termed trigemino-oculomotor synkinesis or Jaw-Winking. Early clinical electromyography (EMG) studies on MGS cases showed distinct co-firing of masticatory and extraocular muscles when EMG was recorded from both muscle groups simultaneously[3-4]. Moreover, stimulation of the pterygoid muscle nerve elicited ipsilateral eyelid retraction, and section of this nerve from the trigeminal motor root could relieve the eyelid activity[3-4]. These findings suggested an intrinsic linkage between the masticatory and extraocular muscle systems[3-4]. Based on these findings, a "release hypothesis" proposed[3-4] that a primitive masticatory oculomotor reflexive circuit is preserved, but is normally suppressed in humans, and it can be released and manifested whenever congenital or traumatized

disorders occur in the system. However, the existence of a primitive masticatory oculomotor reflexive pathway remains to be shown. Coincidently, a neural tract tracing study unveiled that central processes of the temporal muscle afferent mesencephalic trigeminal nucleus (Vme) neurons project directly to oculomotor (III) and trochlear nuclei (IV) in *Xenopus toad*[5]. This neuronal circuit could be useful in a hunting scenario in which amphibians could simultaneously open their eyes and mouths widely to follow and catch a prey. Interestingly, in our recent studies in rats[4-6], we also observed projections of the Vme neurons to the III/IV, and even to their premotor neurons in the interstitial nucleus of Cajal (INC) and Darkschewitsch nucleus (DN).

We considered INC and DN together as a pre-oculomotor complex because early tract tracing study on monkey showed projections from INC/DN to III/IV and termed this area as accessory oculomotor nuclei[4,6-7]. Furthermore, in our own previous studies in rats, a large number of retrograde labeled cells was constantly observed in this combined area and equally distributed in both INC and DN following injection of retrograde tracer into the III or IV[4,6-7]. In addition, more intriguing, c-Fos expression was consistently induced in this combined area either by electric stimulation of masseter nerve (MN) or after repeated and rapid down-stretching the lower jaw[6-7].

It is also noteworthy that a recent clinic study in humans without any congenital ptosis, the authors observed the same retraction of the upper eyelid by electric stimulation of pterygoid nerve as reported previously in MGS cases[8]. This report implied that this masticatory oculomotor pathway is not merely present in MGS patients, but is also expressed in some people without congenital ptosis. Nonetheless, the correlated reflex is strikingly manifested in MSG patients according to "release hypothesis". We have consistently witnessed co-firing of eyelid and jaw closing muscles during steady occlusion (isometric contraction of masseter without jaw movement) by recording of EMG from both the eyelid and jaw muscles simultaneously in healthy human volunteers[9]. All evidences aforementioned substantiate the existence of a residual of jaw muscle-oculomotor reflex pathway and supports the "release hypothesis". Therefore, in the present work, we attempt to further our early[7] and recent exploration[4,6] of a masticatory oculomotor neural pathway in the rat by electrophysiological extracellular unit recording from neurons of the III and the INC complex (namely, the INC/DN) following electric stimulation of the MN.

SUBJECTS AND METHODS

Ethical Approval 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. The experiment protocol was approved by the Medical College and Affiliated Hospital.

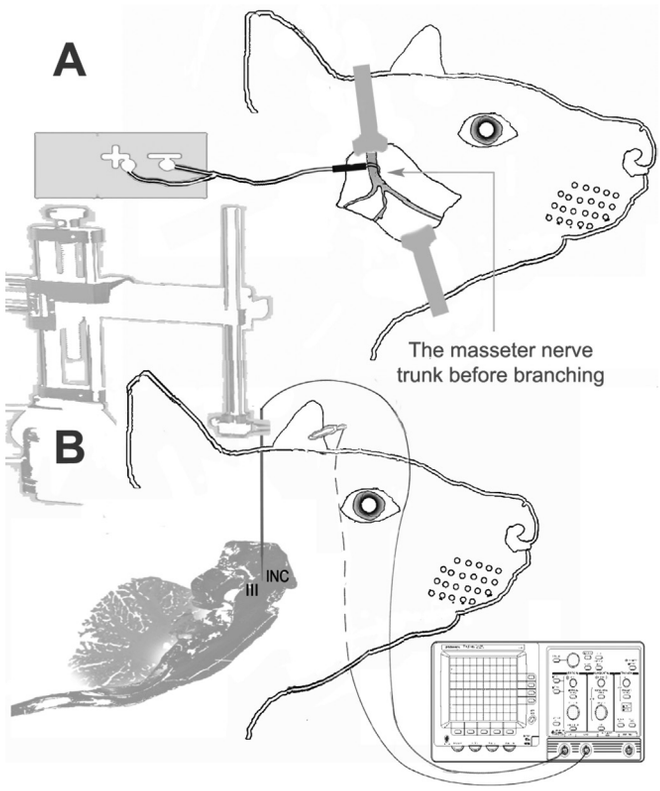

Figure 1 The location of bipolar silver-wire stimulating electrode on the trunk of MN (A) and the position of recording electrode on the III and INC/DN in the midbrain (B).

Animals Totally 37 adult male Sprague-Dawley rats (300-350 g), obtained from Animal Facilities of the Xi'an Jiaotong University Medical College, were used in these experiments.

Single Unit Recording Following Electric Stimuli of Unilateral Masseter Nerve Animals were anesthetized with urethane (1.25-1.50 g/kg, *i.p.*), and administrated with atropine sulfate (0.5 mg/kg, *i.p.*) to reduce tracheal secretion before surgery. Experiment was not started until the limb-withdrawal reflex elicited by pinching the hind paw was absent. The MN trunk was exposed and a bipolar silver-wire collar electrode with 1.5 mm inter-polar distance was placed around the nerve trunk (Figure 1A). Kwik-Cast Silicon Sealant glue (World Precision Instruments Inc., Sarasota, FL, USA) was applied to secure stimulating electrode and to isolate it from surrounding tissues. The trachea was cannulated. The animal was then placed in a stereotaxic frame (Narishige Stereotaxic Frame SR-6C, with KOPF micro-positioner mounted on Narishige SM-11 micromanipulator). A parietal craniotomy and aspiration was performed to expose the midbrain. The surface of the brain was covered with warm mineral oil (37°C). The animals were then paralyzed with gallamine triethiodide (30 mg/kg, *i.p.*) and artificially ventilated (2 cm^3, 1.7 Hz). During electrophysiological recording, threshold intensity (T) was determined, and then the nerve was stimulated at 1 to 2.5 times T. Core body temperature was maintained at 37°C

throughout the experiment *via* a thermostatically controlled heating pad.

Extracellular recording electrodes with a tip diameter of 1.0-2.0 μm were made from borosilicate glass pipettes (1.0 mm O.D., 0.7 mm I.D., WPI Inc.). The electrodes were filled with 2% pontamine sky blue in 0.5 mol/L sodium acetate (pH 7.4), and the impedances were 8-10 Mega Ohms. The electrode was advanced into the midbrain toward the III and/or the INC/DN ipsilateral to the MN stimulation (Figure 1B) *via* a KOPF micro-positioner (Hydraulic Model 662, David KOPF Instruments). The stereotaxic coordinate used for III was 0.3-0.5 mm lateral to midline; while coordinate for the INC/DN was 0.5-0.8 mm lateral to midline as described in our previous study[4]. The dorsal surface of superior colliculus was used as a landmark for localization of the III, and the INC/DN is commonly 0.5-1.0 mm rostral to the III (Figure 1B). A dual-channel stimulator (Grass, Model S88) was used to apply electric stimulation on the MN. Unit response was recorded with an Axoclamp2B amplifier (Axon Instruments, Union City, CA, USA), filtered at 10 kHz, and further amplified 100 times *via* a differential AC amplifier (Model 1700; A-M Systems, Carlsberg, WA, USA). The signals were sent to a Dell computer armed with Clampex 9.2 software (Axon Instruments) through a Digidata 1322A converter (Axon Instruments). After a successful recording, sky blue was electrophoresed by giving 70-90 μA negative current, 250/250 ms On/Off for 10-15min. The animals were finally euthanized with an overdose of sodium pentobarbital (140 mg/kg, *i.p.*). All animals were then transcardially perfused with 0.9% saline, followed by a fixative solution containing 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol/L PB (pH 7.4). The brain was removed, cryoprotected, frozen and cut into 50 μm sections. The sections marked with sky blue were examined immediately under a Nikon microscope. The rats with recording site outside of the III or INC/DN regions were excluded from data analysis. After being air-dried, these sections were counterstained with 1% neutral red (Sigma), dehydrated in ethanol, defatted in xylene, and then were sealed with permount. The counterstained sections were photographed by Nikon E-600 LM.

RESULTS

Responses of the III Neurons to the MN Stimulation A total 13 extracellular units were recorded at the III (Figure 2A, 2D, and 2G) from 11 rats and 2 types of responding properties were observed on these units. The recorded units had been carefully screened by examining if the recording site is exactly in the III, because trigeminal mesencephalic tract and occasionally Vme neuronal soma are located lateral to the III/IV at levels of the IV and the caudal III. This step eliminated a large portion of decent unit discharge recording; while, there might be a considerable number of cases without responses. The type of responding is herein classified as follow: 1) Units that did not show spontaneous discharges responded in a single discharges fashion to individual electric pulses (Figure 2B and 2C; total 8 units); 2) Units that displayed a spontaneous discharge responded only to trains of stimuli by changing their firing frequency (Figure 2E, 2F, and 2H; 5 units). The latter cell type did not show explicit single responses to the single stimuli.

In the 1st group, the intensity of 1.7 to 2.5 T evoked a stable repeated single discharge. The mean latency of the discharge was 3.9±1.8ms (average±standard deviation). In the 2nd group, two subtypes of units could be identified: subtype 1 (n=1), the unit showed spontaneous firing rate increases immediately after a train stimulation (2.5 T, 52 Hz, 200ms duration), then the firing is relaxed shortly and enhanced again (Figure 2E and 2F). Thus, this unit showed an excitatory-relax-excitatory pattern. Subtype 2 (n=4): units exhibited a slow inhibitory pattern in which spontaneous firing rate decreased further and further following repeated train stimulation, and was recovered when the stimulation was discontinued if the unit was still stable (Figure 2H).

Responses of the INC/ND Neurons to the MN Stimulation A total of 12 extracellular single units were recorded in the INC/DN area from the aforementioned 11 rats (Figure 3A, 3D, 3G) and similarly, the recorded units had been carefully screened by examining the recording sites. Also, two types of responding properties were detected: 1) Units with some spontaneous discharges (n=5) but still responding to single pulse stimuli. The response latencies had a mean of 4.7±2.9ms (Figure 3B, 3C). 2) Units that displayed spontaneous firing responding only to trains of stimuli (n=7; Figure 3E, 3F, 3H, 3I). These units lacked explicit single response to the single pulse stimulation. More spontaneously discharging units were encountered in the INC/DN area, but more than half of them did not change their firing rate after consecutive train stimulation of the MN. These units were excluded from the current analysis.

In the group of spontaneous firing units, there were also 2 subtypes, but the responding manner were different from those recorded from the III group. Subtype 1 (n=5), units with low firing rate that showed immediate increment of discharges following each train stimulation, and declined quickly back to the initial level until the next train (Figure 3E, 3F). Subtype 2 (n=2), displayed high spontaneous firing rates, and responded with short latency and short-term inhibition (200-250ms) following each episode of train stimulation (Figure 3H, 3I). The slow inhibitory pattern of response recorded in III neurons (Figure 2H) were not encountered in this group.

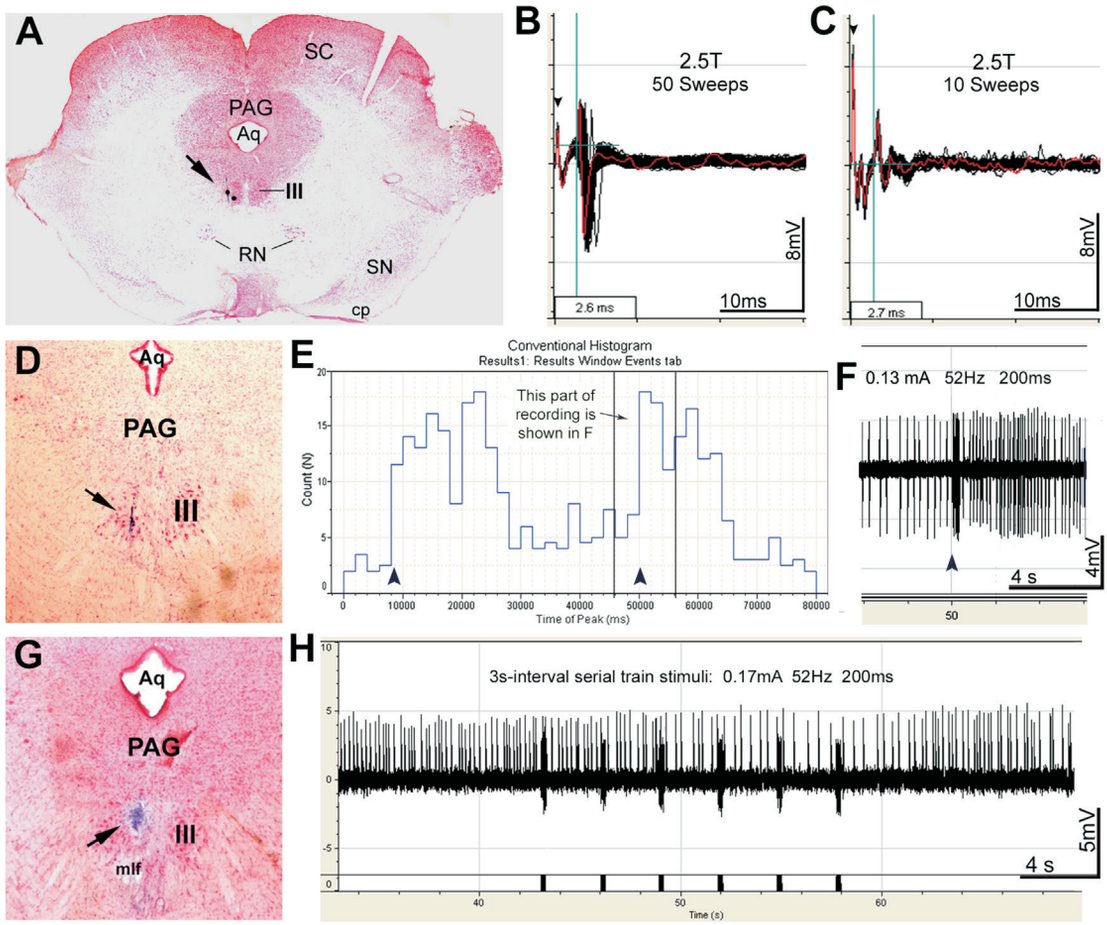

Figure 2 Unitary discharges and recording sites in III A, D, G: Recording sites marked by sky blue (arrows); B, C: Orthodromic unitary responses recorded from the III shown in the A, and downward arrowheads indicate the starting of the stimulation; E: A spontaneous discharging unit encountered in the III in D with a pattern of been excited, relaxed and further excited after each train stimuli of the MN. Upward arrowheads indicate the start of the train stimuli. F: A real time recording extracted from the framed area in E (indicating by a thin arrow; from 46 000 to 56 000ms). The arrowhead points to the start of the train stimuli. H: A real time recording from a spontaneous firing unit at III shown in G. Upper track displays gradually reduced spontaneous firing rate following train stimuli, bottom track shows the stimulation artifact. Aq: Aqueduct; cp: Cerebral peduncle; mlf: Medial longitudinal fasciculus; PAG: Periaqueductal gray; RN: Red nucleus; SC: Superior colliculus; SN: Substantia nigra.

DISCUSSION

The present work showed neurophysiological profile of a masticatory oculomotor neuronal pathway that we found in recent decade[4,6-7], which is probably the residue of a primitive jaw-eye cooperative reflex. Electric stimulation of the MN, the largest branch of the trigeminal motor root in rats and cats, has been widely used to study the central pathways of jaw muscle spindle Vme afferents in the rats and cats[10-11]. There are three principal types of somatic nerve fibers in the MN. The majority of them are efferent fibers of motoneurons in the trigeminal motor nucleus (Vmo)[12]. The second most common fibers are from primary jaw muscle spindle Vme afferents[10-11], those conduct muscle proprioception. Trigeminal ganglion afferent fibers that transmit nociception from deep jaw muscle and/or connective tissues are the smallest component[13]. Stimulation of the axons of trigeminal motoneurons innervating masticatory muscles evokes antidromic impulses that are only conducted to the Vmo[12-14]. And ganglionic afferents carrying jaw muscle nociception project restrictedly to the dorsolateral rim of the spinal trigeminal nucleus and lamina I/II of dorsal horn at upper cervical levels[13]. In contrast, the Vme afferents project broadly, but unilaterally to the brainstem through their central processes. Their terminations are found in a wide variety of areas in the brainstem[10-11], including ascending projections[10] to midbrain. Thus, the MN stimuli evoked orthodromic unitary responses that were recorded in the III and/or INC/DN in the present work are most likely elicited by inputs of jaw muscle Vme afferents. Moreover, an interesting finding in our recent study was that repeated and rapid down-stretch of the lower jaw induced bilateral c-Fos expression in pre-oculomotor neurons located in INC/DN[6]. It is known that specific sensory stimulation can induce c-Fos expression in relay neurons along a pertinent functional pathway[15]. Therefore, this observation substantiates the participation of jaw muscle spindle Vme

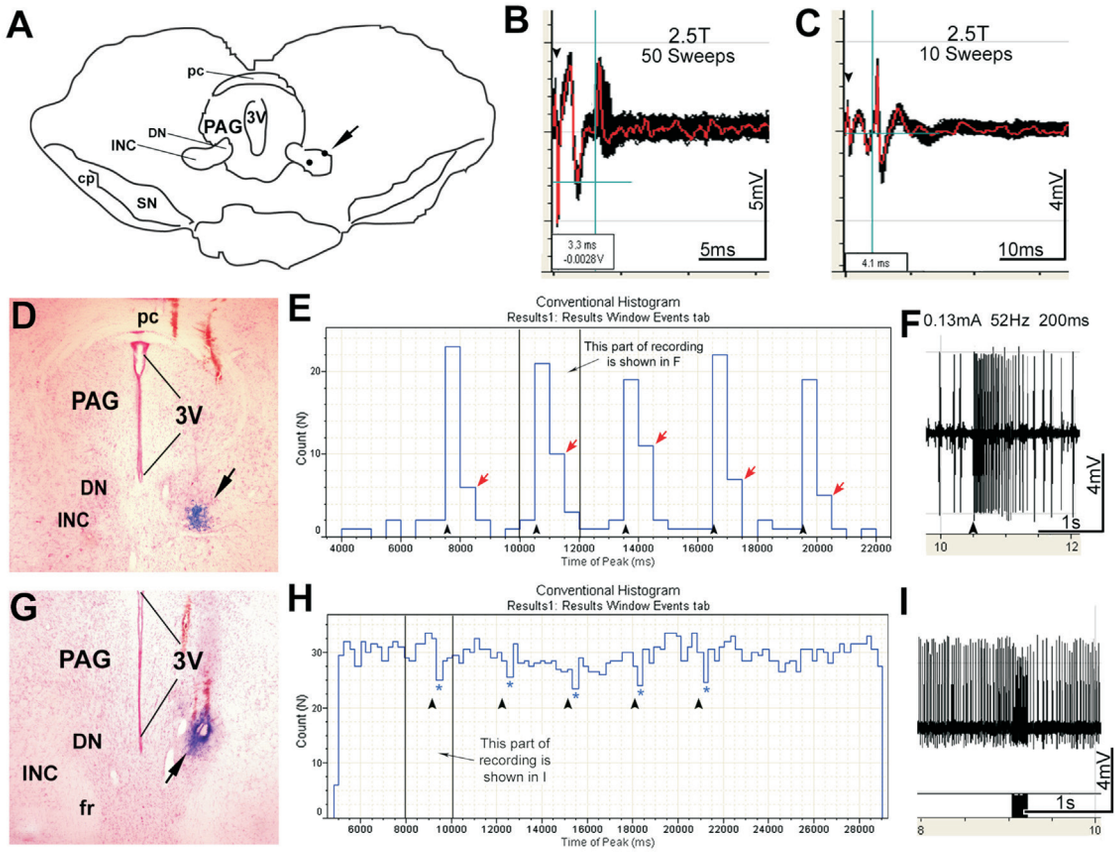

Figure 3 Unitary discharges and recording sites in the INC/DN A, D, G: Recording sites (arrows) stained by sky-blue. B, C: Orthodromic unitary discharges recorded from the INC shown in A. The downward arrowheads point the starting of the stimulation. E: Train stimuli immediately increased firing rate (pointing by head-down arrows) of a spontaneous discharging unit in the INC and the recording site is shown in D. Upward arrowheads on the bottom indicate starting of each train stimuli. F: Real time recording clipped from the selected area in E (indicating by a thin arrow; from 10 000 to 12 000ms), an upward arrowhead points to starting of the train stimuli. H: A spontaneous discharging unit recorded in the DN was immediately or rapidly inhibited after each train stimuli (pointing by upward arrowheads). Reduced firing rates are marked by asterisks. The recording site is shown in G. I: A real time recording extracted from the framed area in H (pointing by a thin arrow; from 8000 to 10 000ms), and artifact of the train stimuli (bottom line) is on the bottom. 3V: Third ventricle; fr: Fasciculus retroflexus; pc: Posterior commissure.

afferents in masticatory oculomotor pathway. It further suggests that this pathway is somehow functional even under normal conditions.

Our recent neuronal tract tracing study suggested that projections from the Vme neurons to the III/IV and INC/DN are direct projections[4], namely the monosynaptic innervations. In general, people use responsive latency to judge whether a recorded unitary discharge is conducted *via* a monosynaptic or through a multiple synaptic pathway[7,12,14,16]. However, the latencies of unitary discharges after initial of stimulation is prominently variated in the present work in units recorded from both III and INC/DN (2.1-5.7ms and 1.8-7.6ms, respectively). Nonetheless, this kind of broad variation of unitary discharge latencies following the MN stimulation was also observed in our previous studies[16-17]. On one hand, it is possible that a masseter muscle afferent impulse prompted an interneuron excitation firstly and then ensued an III motoneuron or INC/DN pre-oculomotor neuron discharge. Dual synaptic pathway from jaw muscle afferents to ipsilateral III through ipsilateral INC/DN premotor neurons can be excluded because in the rat the INC/DN premotor neurons project only to the contralateral III/IV[7]. On the other hand, if monosynaptic innervation of jaw muscle afferents to the III and/or INC/DN neurons are truly existent, some action potential transmitting may have been delayed or vanished in the bifurcations of neuronal processes. It is because all of intracellular recording followed by tracer injection studies on jaw muscle spindle Vme afferents of cats and rats have revealed that Vme neuronal processes are highly branched and the central axons commonly have 3-order branches before finally reaching the targets[10-11,14]. Considering a linear correlation between axon diameter and conduction speed[18-19], it is possible that action potential transmission is decelerated when it prompts from thicker axons to thinner offspring branches. Besides, the previous studies had displayed action potential transmission failure at axon bifurcation when an investigated nerve was repeatedly stimulated[20]. A single III

motoneuron or INC neuron may be innervated by more than one Vme neuronal axons based on previous intracellular tract tracing studies[10-21]. If action potentials carried by these axons are partially vanished at their bifurcation, fewer impulses would finally arrive to a targeted III or INC/DN neuron. In this situation, the duration from postsynaptic membrane depolarization to action potential initiation may be prolonged due to fewer spatial or weaker temporal summation[22]. That might be also a reason for fewer orthodromic unit discharge was recorded than expected.

In the present work, we observed inhibitory effects on both III and INC/DN in response to train stimulation of the MN. However, based on established chemical neuroanatomy and neurophysiology data[6,23], projections from the Vme to the III and INC/DN should be primarily excitatory. INC/DN neurons use both glutamate and GABA as neurotransmitters[24-25], and they can exert both excitatory and inhibitory effects on the III neurons[26]. In addition, bilateral INC neurons communicate each other through their projecting fibers traveling in the posterior commissure[4,6-7]. These neuroanatomical frameworks established a potential inhibitory circuits from the INC/DN to the III/IV and/or between the INC of both sides. But in the rat, the INC/DN only innervate the contralateral III[7]. Hence, in the present work, if any inhibition onto the III is from the INC/DN, the circuit of this inhibitory impulses may be from ipsilateral INC/DN that is activated by stimulation of the MN to the contralateral one, and then back to the ipsilateral III. Whereas, in front eyed mammals and humans, the pre-oculomotor INC neurons project to both contra- and ipsilateral III[25]. The existence of these neuronal circuits in humans had been suggested by a clinic phenomenon: bilateral MGS[1-2,27]. Simultaneous bilateral levator palpebrae superioris (LPS) EMG recordings from a patient suffering bilateral MGS revealed an explicit and complete inhibition of LPS activity, whenever the contralateral LPS fired. This concomitant excitation and inhibition persistent whenever the patient's lower jaw kept moving right-left horizontally[1-2,27].

Although the current electrophysiological study adds new data for the masticatory oculomotor pathway that we have been exploring, we still can't exclude involvement of other possible neuronal pathways. In recent years, a set of reports suggest that some Vme neurons innervate mechanoreceptors in Müeller's muscle in the superior tarsal plate of human and rat[28-29]. In the rat, the central axons of these tarsal plate afferent Vme neurons project to ipsilateral III, and terminate on motoneurons those innervate slow-twitch skeletal muscles in the LPS[28-29]. Hence, it is possible that these tarsal plate afferent Vme neurons may play a role in excitement of the LPS motoneuron in the III. In addition, a neural tract tracing study in Macaque monkey has also demonstrated a limited number of labeled Vme neurons following injection of retrograde tracers into the LPS and superior rectus muscles[30]. Notably, the authors, who showed tarsal plate Vme afferents projecting to the III, also discovered that tarsal plate Vme afferents connect with jaw muscle spindle and/or periodontal Vme neurons through gap junctions, since they observed spreading of gap-junction permeable dye from tarsal plate Vme afferents into abutting Vme neurons[28]. Electrotonic coupling through gap-junction between the Vme neurons was reported decades ago[31]. More importantly, somatofugal action potentials have been recorded from the Vme neurons those coupling to the other discharging Vme neurons, when their resting potential reaches the threshold to initiate action potentials[31]. It is likely that tarsal plate afferent Vme neurons may be co-fired through this electrotonic coupling mechanism when jaw muscle afferent Vme neurons are excited by either electric stimuli, or mechanic stimulation such as vigorously stretching of the lower jaw[6].

Finally, it is notable that in a considerable number of experimental rats in our current work, the single neuron unit or the recorded unitary spontaneous discharges did not explicitly respond to stimulation of the MN. Although it has been well known that the physiological condition of each animal may be largely varied, the possibility that this pathway is not well developed in some rats could not be excluded. This situation is probably parallel to the finding in genetically healthy humans that LPS retraction was evoked by stimulation of the ipsilateral trigeminal motor root in about 1/3 of subjects[8]. Also, some reported cases manifest MGS only temporally in life[1-3]. These findings suggest the neural circuit we explored may be better developed in some creatures, but not in all of them.

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