Modulation of excitability of trigeminal neurons during electrical stimulation of the superior laryngeal nerve in anesthetized rabbits

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Abstract

The present study investigated the effect of electrical stimulation of the superior laryngeal nerve (SLN) on evoked potentials from trigeminal interneurons. Experiments were carried out on 19 rabbits anesthetized with urethane. Single-unit responses evoked by electrical stimulation of the inferior alveolar nerve were recorded in the trigeminal nucleus. To evoke swallowing reflex, the SLN was electrically stimulated (train pulses; 0.2 ms, 30 Hz). Current intensity of SLN stimulation was set at 2, 4, or 8 times the threshold for evoking swallowing reflex at least once for 10 s. Initiation and change in latency were compared among the periods (before, during, and after SLN stimulation) or among stimulus intensities. Inhibition of evoked responses and delay in latency was observed in 15 of 27 identified neurons (55.6%) and 26/27 (96.3%) neurons, respectively, either during or after SLN stimulation. The rate of inhibition of evoked responses was significantly different among SLN stimulus intensities. Modulated neurons were divided into two groups based on latency, short (< 3 ms) or long (>= 3 ms). Longer latencies resulted in longer delays. Eighteen neurons (66.7%) were found to project to the digastric motor nucleus. Most neurons were distributed in the main sensory trigeminal nucleus or subnucleus-γ of the oral nucleus of the spinal trigeminal tract. These results suggest that sensory information originating from the orofacial region, possibly related to the jaw reflex, was inhibited during or after SLN stimulation possibly by input from the swallowing central pattern generator.

Highlights

Most trigeminal interneurons were inhibited during or after SLN stimulation.

The rate of inhibition of responses was dependent on SLN stimulus intensity.

Inhibition of oral sensation by the swallowing CPG is a possible mechanism.

Keywords: trigeminal interneuron, swallow, superior laryngeal nerve

Abbreviations

CPG, central pattern generator; Dig, digastric muscle; IAN, inferior alveolar nerve; JOR, jaw opening reflex; MH, mylohyoid muscle; NTS, nucleus tractus solitarii; AVint, intertrigeminal area; NVmes, mesencephalic trigeminal nucleus; NVmot, trigeminal motor nucleus; NVsnpr, main sensory trigeminal nucleus; NVspo-γ, subnucleus-γ of the oral nucleus of the spinal trigeminal tract; SLN, superior laryngeal nerve; V, trigeminal tract

1. Introduction

Ingestion is the early stage of nutrition in most mammals. It is widely accepted that masticatory movements, including chewing and swallowing, are programmed by the central nervous system, the so-called central pattern generator (CPG) in the brainstem, and are modulated by sensory inputs from the mouth, pharynx, larynx, or esophagus (Jean, 2001; Miller, 1982; Nakamura and Katakura, 1995). Numerous studies have investigated the modulation of sensory transmission of trigeminal nerves, where stimulation of trigeminal afferents results in inhibition of presynaptic terminals (Darian-Smith and Yokota, 1966; Dubner and Sessle, 1971; Nakamura et al., 1977) and trigeminal interneurons (Browne and Goldberg, 1978) in the brainstem. During chewing, mastication-triggered or phase-linked modulation was observed in the terminals of primary afferents (Kurasawa et al., 1988), and in the neuronal activity of the trigeminal nucleus (Olsson and Landgren, 1980; Olsson et al., 1988; Westberg et al., 2001) and face primary somatosensory cortex (Yamamura, 2007). It has been reported that elementary jaw reflexes, such as the jaw opening reflex (JOR), evoked by trigeminal stimulation (Lund et al., 1981; Lund et al., 1983) are tonically and phasically suppressed during chewing. The fact that paralysis did not change such modulations, where excitability of the digastric (Dig) reflex pathway is modulated during chewing, strongly suggests that these processes are not dependent on the sensory feedback system to function (Lund et al., 1983). Such processes may be required to prevent undesirable jaw movements caused by weak stimulation during chewing. Conversely, the JOR evoked by high-threshold afferents, i.e., by noxious stimulation, is not highly modulated, likely to protect the orofacial tissue from damage by noxious inputs (Lund et al., 1984).

Swallowing can be triggered by mechanical, chemical, or electrical stimulation in the oropharynx or larynx in humans and animals (Jean, 2001; Kajii et al., 2002; Shingai and

Shimada, 1976; Shingai et al., 1989). Electrical stimulation of the superior laryngeal nerve (SLN), which contains the pharyngeal/laryngeal sensory nerve, is one of the most common methods of triggering swallowing (Jean, 2001). Our previous studies revealed that the JOR was suppressed during chewing and swallowing. Yamada et al. (Yamada et al., 2013) reported that the JOR evoked by intra-oral innocuous stimulation was suppressed during natural chewing and swallowing in conscious animals. Fukuhara et al. (Fukuhara et al., 2011) investigated the effects of swallowing responses evoked by electrical stimulation of the SLN on JORs, and found that JORs evoked by low-threshold trigeminal afferents were strongly inhibited during SLN stimulation. In addition, the inhibitory effect continued after SLN stimulation. Thus, we hypothesized that excitability of low-threshold trigeminal transmission is modulated, and that trigeminal neurons potentially mediating the JOR are inhibited by pharyngeal/laryngeal sensory input. The present study aimed to investigate whether trigeminal interneurons in the brainstem are modulated during electrical stimulation of the SLN, and how SLN stimulus intensity is related to the modulatory mode of neural excitability **(Fig. 1)**.

2. Results

2.1. JOR

The mean threshold for initiation of JOR was 0.103 ± 0.093 mA (mean ± SD, n=19), and the mean latency was 6.10 ± 0.30 ms (mean ± SD, n=19). In addition, JOR was evoked bilaterally regardless of left or right stimulus application. There was no difference in threshold or latency between the stimulus and non-stimulus sides.

2.2 Effects of SLN stimulus current intensity on swallowing

Stimulation of the SLN evoked swallows in all animals. The mean threshold for triggering swallowing was 0.29 ± 0.63 mA (mean ± SD, n=19). Stimulus current intensity was set at 2, 4, and 8 times (T) the threshold for eliciting swallowing at least once for 10 s. The number of evoked swallows was relatively consistent throughout recording, and was dependent on stimulus intensity and increased as the current in the SLN increased. There was a significant difference ($P < 0.05$) in the number of swallows between 2T and 8T current intensities **(Fig. 2A)**. The mean latency of the onset of swallowing evoked by stimulation of the SLN tended to decrease as current in the SLN increased, but no significant differences were found **(Fig. 2B)**.

2.3. Identification of trigeminal neurons

Histological locations of interneurons recorded in the present study are shown in **Fig. 3**. Out of 27 neurons identified, 23 were trigeminal interneurons; 6 were located in the main sensory trigeminal nucleus (NVsnpr), 12 in subnucleus-γ of the oral nucleus of the spinal trigeminal tract (NVspo-γ), 3 in the mesencephalic trigeminal nucleus (NVmes), and 2 in the intertrigeminal area (AVint). The remaining 4 neurons were not histologically identified because of technical errors.

The mean threshold for initiation of neural responses evoked by inferior alveolar nerve (IAN) stimulation was 0.060 ± 0.040 mA (mean ± SD, n=27). Except for 3 neurons, the threshold for initiation of evoked responses was lower than that needed to evoke JOR, suggesting that most neurons recorded in the present study, at least, received input from low-threshold afferents. Mean latency of initiation of neural responses was 2.76 ± 0.17 ms in the control period (mean ± SE; range, 1.59–4.63 ms; n=27). Latency was not dependent on location; 3.27 ms for NVsnpr, 2.08 ms for NVspo-γ, 3.33 ms for the NVmes, and 3.83 ms for

the (AVint)

We were unable to find the receptive fields of all neurons recorded in the present study. Of 27 neurons, 2 had receptive fields in the periodontal membrane, 2 in the buccal mucosa, and 1 in the gums. Regarding spontaneous activity, 8 neurons were spontaneously active, of which 1 had receptive fields in the anterior periodontal membrane.

2.4. Effect of SLN stimulation on evoked neural responses

Previously, we examined changes in JOR responses evoked by IAN stimulation (Fukuhara et al., 2011; Yamada et al., 2013). We found that JOR was suppressed during SLN stimulation, and the degree of suppression increased and latency for the JOR was delayed when SLN stimulus current intensity increased, suggesting that excitability of mediating interneurons was dependent on SLN stimulation current intensity.

In all 27 neurons recorded in the current study, we investigated the frequency of evoked responses (the number of responses per 20 IAN stimulations at 1.2T threshold) in each period. Further, depending on the inhibitory effect, all neurons were divided into 3 groups, non, lightly, and heavily modulated. Non-modulated represents no inhibition of evoked responses, and lightly and heavily modulated neurons represent, out of 20 IAN stimulations, 1–5 responses inhibited and more than 5 responses inhibited, respectively **(Fig. 4)**. During 2T SLN stimulation, 5 neurons (4 lightly and 1 heavily modulated) were inhibited in the stimulation (Stim) period, and 3 neurons (2 lightly and 1 heavily modulated) were inhibited in the post-stimulation (Post-stim) period. One neuron was inhibited (heavily modulated) in both the Stim and Post-stim periods. During 4T SLN stimulation, 7 neurons (6 lightly and 1 heavily modulated) were inhibited in the Stim period, and 5 neurons (4 lightly and 1 heavily modulated) were inhibited in the Post-stim period. Of these neurons, 4 were

inhibited (3 lightly and 1 heavily modulated) in both the Stim and Post-stim periods. During 8T SLN stimulation, 5 neurons (4 lightly and 1 heavily modulated) were inhibited in the Stim period, and 9 neurons (lightly modulated only) were inhibited in the Post-stim period. Of these neurons, 4 neurons were inhibited in both the Stim (3 lightly and 1 heavily modulated) and Post-stim (lightly modulated) periods. We compared the percentage of modulated neurons between the Stim and Post-stim periods and among 2, 4, and 8T SLN stimulation. The percentage of non-modulated neurons in the Control, Stim, and Post-stim periods was 100%, 81.5%, and 88.9%, during 2T SLN stimulation, 100%, 74.1%, and 81.5% during 4T SLN stimulation, and 100%, 77.3%, and 59.1% during 8T SLN stimulation, respectively. There was no significant relationship between Stim and Post-stim periods. The percentage of modulated neurons was not related among the stimulus intensities in the Stim period but was significantly related among the stimulus intensities in the Post-stim period ($P < 0.05$) **(Fig. 4)**. These results suggest that the greater SLN stimulation was the more evoked responses were inhibited after SLN stimulation.

Similar to experiments assessing the probability of inhibition of evoked responses, we compared mean latency for the response in each neuron among the 3 periods (Control, Stim, and Post-stim). During 2T SLN stimulation, the mean latency of 10/27 neurons in the Stim period and 14 neurons in the Post-stim period was significantly longer ($P < 0.05$) compared with the Control period. Of these neurons, 9 were significantly different in both the Stim and Post-stim periods. During 4T SLN stimulation, the mean latency of 16/27 neurons in the Stim period and 21 in the Post-stim period was significantly longer compared with the Control period. Of these neurons, 14 were significantly different in both the Stim and Post-stim periods. Only 22 neurons could be examined during 8T SLN stimulation. During 8T SLN stimulation, the mean latency of 16 neurons in the Stim period and 18 in the Post-stim period

was significantly longer compared with the Control period. Of these neurons, 14 were significantly different in both the Stim and Post-stim periods. The occurrence of evoked response from all these neurons was also inhibited.

All neurons recorded were divided into two neuron groups based on latency (>= 3 ms, or < 3 ms in the Control period), and change in latency was compared between the groups, between Stim and Post-stim periods, and among the stimulus intensities. Accordingly, there was a significant difference in latency between the 2 neuron groups. Specifically, the increase in latency in the longer-latency (>= 3 ms) neuron group was significantly greater compared with the short-latency (< 3 ms) neuron group **(Fig. 5)**.

2.5. Projections to the Dig motor nucleus

Finally, we investigated whether the neurons identified projected to Dig motoneurons. Of 27 neurons, 18 were found to possibly project to Dig motor nucleus. Mean latency for EMG responses in the Dig muscle was 2.64 ± 1.56 ms (mean ± SE; range, 1.0–5.5 ms; n=18).

3. Discussion

3.1. Identification of trigeminal neurons

In this study, we demonstrated changes in trigeminal neural responses evoked by electrical stimulation of IAN low-threshold afferents in anesthetized rabbits. We previously found that JOR responses evoked by IAN stimulation were inhibited during natural swallowing in freely behaving animals (Yamada et al., 2013), and during SLN stimulation in anesthetized animals (Fukuhara et al., 2011). As the reduction in JOR amplitude occurred before the first swallow during SLN stimulation and remained after the end of stimulation (Fukuhara et al.,

2011), it can be hypothesized that the swallowing reflex and activation of swallow-related neural networks, in the absence of swallowing movements, may be involved in the modulation of the JOR network.

It is widely accepted that modulation of oral input during chewing is controlled by brainstem neural networks at the level of terminal primary afferents (Kurasawa et al., 1988), or by activity of the trigeminal nucleus (Olsson and Landgren, 1980; Olsson et al., 1988; Westberg et al., 2001). Olsson et al. examined the excitability of trigeminal neurons during chewing and found that most neurons were inhibited during chewing, mostly throughout the cycles, and phase-dependently in some neurons (Olsson et al., 1986). As we used the same methodology of identification of single neurons as the previously mentioned study, most interneurons recorded in the current study are likely trigeminal, and may be controlled by the masticatory CPG.

There was a wide variation in latency of initiation of evoked responses ranging from 1.63 to 4.63 ms in the present study. This suggests that some neurons were not secondary neurons, which directly receive input from primary afferents.

Olsson et al. (Olsson et al., 1988) recorded trigeminal mechanoreceptor neurons in rabbits and showed that response latency to IAN stimulation was 1.2–3.4 ms. Considering that the conduction velocity range of the IAN is 25–90 m/s, with most in the 45–50 m/s range, and the minimum synaptic delay is approximately 0.4–0.6 ms (Olsson et al., 1988), it is likely that evoked responses are monosynaptic if latency is < 3 ms. Thus, in the present study, we divided all neurons identified into two groups; short-latency (< 3 ms, likely secondary neurons), and long-latency (>= 3 ms) to evaluate the effect of SLN stimulation on the modulation of evoked responses in each group (see below).

3.2. Modulation of evoked responses during SLN stimulation

To our knowledge, this is the first study to demonstrate trigeminal interneuron inhibition during SLN stimulation, which suggests that sensory information originating from the oral region is modulated during swallowing. Of 27 neurons identified, 96.3% were modulated during or after SLN stimulation. In addition, evoked response was inhibited in 55.6% of neurons, and latency of evoked response was delayed in 96.3%.

Similar modulation has been previously reported at the spinal segment level. Ghez and Pisa (Ghez and Pisa, 1972) and Coulter (Coulter, 1974) found that the amplitude of evoked responses of cuneothalamic fibers in the medial lemniscus reduced before movement was initiated. Further, this was caused by both pre- and post-synaptic inhibition (Ghez and Pisa, 1972). Similar to cuneothalamic neurons, pre- and post-synaptic inhibition occurs at the level of trigeminal nucleus during chewing (Kurasawa et al., 1988; Olsson et al., 1986; Olsson et al., 1988; Westberg et al., 2001). Thus, it is possible that inhibitory activity also occurs during SLN stimulation.

Possible mechanisms of modulation include involvement of inhibitory input from the sensory feedback pathway, swallowing neural network including CPG in the lower brainstem, or descending inhibitory input from higher centers. As rabbits were not paralyzed in the present study, inhibition may have resulted from sensory input from peripheral organs after swallowing. However, this possibility is unlikely for the following reasons. Fukuhara et al. (Fukuhara et al., 2011) demonstrated that there were no differences in amplitude or latency of the JOR evoked by low-threshold IAN stimulation, with and without swallowing during SLN stimulation. Ono et al. (Ono et al., 1999) demonstrated that modulation of JOR during SLN stimulation was caused by input from the swallowing CPG, because modulatory effects on the responses evoked during swallowing persisted in paralyzed animals. Our results

showed that there were no differences in modulation of evoked responses among the conditions: Differences in the number of swallows with differing SLN stimulation intensities did not affect inhibition of evoked responses, although the percentage of modulated neurons in the Post-stim period was related to SLN stimulus amplitude. In addition, aftereffects, i.e., inhibition of evoked responses upon discontinuation of SLN stimulation, were recorded. Doty (Doty, 1968) showed that swallowing reflexes persist upon discontinuation of SLN stimulation. Further, inhibition of JOR responses after SLN stimulation was also observed (Fukuhara et al., 2011). While the swallowing CPG is located in two main brainstem areas, in the dorsal medulla within the nucleus tractus solitarii (NTS) and in the adjacent reticular formation (Jean, 2001), where they form the dorsal swallowing group, the precise location of other swallow-related neurons has not been histologically identified. It is possible that sustained inhibition was attributable to persistent excitation of the swallowing CPG.

In the present study, we electrically stimulated the SLN. Centrally, all SLN afferent fibers project to the NTS (Jean, 2001). The NTS is the main afferent central structure involved in the initiation of swallowing. Indeed, electrical lesioning of the NTS results in reduction of swallowing initiation (Tsujimura et al., 2009), and decerebration has no effect on SLN-evoked swallows (Tsuji et al., 2014). It has also been reported that NTS neurons extensively project to the insular cortex (Cechetto and Saper, 1987; Hanamori et al., 1997a; Hanamori et al., 1997b; Hanamori et al., 1998a; Hanamori et al., 1998b; Yamamoto et al., 1989; Zhang et al., 1999). Hanamori et al. (Hanamori et al., 1998b) revealed that electrical stimulation of the NTS evoked neural responses in the insular cortex, of which most neurons responded to either gustatory, visceral, or nociceptive stimuli. In addition, it has also been reported that the NTS receives direct projections from the insular cortex (Saper, 1982; van der Kooy et al., 1984), and stimulation of the middle rostrocaudal agranular insular cortex

elicits jaw movements (Tsuji et al., 2014; Zhang and Sasamoto, 1990). We cannot exclude the possibility that such an insular cortex network loop was involved in the modulation of JOR during SLN stimulation in the current study.

In the present study, there was a significant difference in the inhibitory effect between short- and long-latency neuron groups, and longer latency resulted in larger inhibition. In contrast, no differences were found among the different SLN stimulus intensities, although the number of swallows differed among the stimulus conditions. This suggests that swallowing sequence did not affect trigeminal excitability, and that pre- and post-synaptic inhibition occurred not only at primary endings, but also at other endings. Thus far, few studies have shown direct evidence of projections from the NTS to the trigeminal nucleus (Zerari-Mailly et al., 2005), except for projections to the trigeminal motor nucleus (Oka et al., 2013). Further studies are necessary to clarify if NTS fibers project to in the trigeminal nucleus and hence are involved in the modulation of excitability of trigeminal interneurons during SLN stimulation.

3.3. Functional implications

Numerous studies have shown that during chewing and swallowing, the JOR evoked by weak stimulation is inhibited (Fukuhara et al., 2011; Lund et al., 1983; Lund et al., 1984; Thexton and McGarrick, 1987; Yamada et al., 2013). In the present study, 66.7% of neurons recorded were found to project to the trigeminal motor nucleus. Regarding latency of the JOR, there is at least 1 interneuron in involved in the JOR pathway, and the JOR is regarded as a disynaptic reflex (Kidokoro et al., 1968). Cell bodies of all trigeminal primary afferents that could give rise to the JOR are in the Gasserian ganglion, except for some located in the NVmes (Larson et al., 1980). We previously revealed that the latency of NVmes-evoked

monosynaptic trigeminal muscle activity was approximately 2.5 ms (Inoue et al., 2001). Although the exact coordinates of the trigeminal nucleus in the brainstem were not the same among all rabbit nuclei, most neurons recorded in the present study projected directly to the Dig motor nucleus, because mean latency was 2.6 ms and the neurons were interneurons of the JOR pathway. Once the swallowing CPG is excited, the upper and lower lips and jaw are closed, and the tongue is elevated to propel the food bolus or liquid into the pharynx. At this point, any unnecessary or weak sensory transmission from the oral cavity needs to be inhibited.

3.4. Limitations

In the present study, we successfully identified interneurons in several trigeminal nuclei and found that most neurons recorded were modulated during or after SLN stimulation. However, because the number of neurons in each trigeminal nucleus was low, the functional role of each nucleus in such modulation remains undetermined. In addition, although we posited that the swallowing CPG affected the excitability of trigeminal neurons during SLN stimulation, it is undetermined whether the swallowing CPG directly inhibits transmission of peripheral inputs, or whether long-loop pathways via the cortex or subcortical regions are involved in modulation. Further, we did not evaluate the effect of SLN stimulation on excitability of neurons receiving input from high-threshold afferents, because previously; we showed that JORs evoked by high-threshold afferents were also inhibited during SLN stimulation. Whether the neural network that contributes to modulation of high-threshold evoked JORs is the same as that for low-threshold afferents must be determined in future studies.

4. Experimental protocol

4.1. Animals

Experiments were performed on 19 adult male Japanese white rabbits, weighing between 2.0–2.5 kg, following the Guide for the Care and Use of Laboratory Animals (NIH Publication #86-23, revised 1996). The experimental procedure was reviewed and approved by the Niigata University Intramural Animal Care and Use Committee (73-7).

4.2. Surgical procedure

Animals were anesthetized with urethane (1 g/kg body weight) administered intravenously via the marginal ear vein, supplemented with urethane whenever necessary to maintain anesthesia at a level where the corneal reflex and spontaneous eye movements were absent. Physiological saline was administered by intravenous infusion (10 ml/kg body weight/h). Tracheal cannulation was performed, and heart rate, arterial pressure, respiratory rate, and rectal temperature were monitored continuously. Temperature was maintained between 38–40°C with a feedback-controlled heating pad.

To record EMG activity of the Dig muscle, two urethane-coated copper wire electrodes with bared tips (diameter, 0.18 mm; length, 2 mm) were inserted either side of the Dig muscle. To stimulate the IAN, two urethane-coated silver wire electrodes with bared tips (diameter, 0.2 mm; length, 2 mm) were inserted into the mandibular canal on the same side as for the Dig EMG. The whole IAN was stimulated (single pulse; duration, 0.2 ms) to evoke the JOR in the Dig muscle. The recording site was the same as the stimulus site, although the JOR was evoked on both sides. Another two urethane-coated copper wire electrodes with bared tips (diameter, 0.18 mm; length, 2 mm) were inserted either side of the MH muscle. To stimulate the SLN, the nerve was isolated from surrounding tissue in the

laryngeal region. Two urethane-coated silver wire electrodes with bared tips (diameter, 0.2 mm; length, 5 mm) were placed on the SLN. Swallowing was triggered by stimulation of the SLN with repeated electric pulses (train pulses; 0.2 ms, 30 Hz), and was monitored by EMG activity of the MH muscle. The threshold of stimulus intensity was determined as 1T when swallowing was induced at least once during 10–s SLN stimulation.

4.3. Identification of single trigeminal neurons

After the head had been fixed in a stereotaxic holder, in which lambda was 1.5 mm lower than bregma according to previous studies (Inoue et al., 2002; Tsujimura et al., 2012), parylene-coated tungsten monopolar electrodes (2–5 MΩ) were then guided to the trigeminal nuclei at an angle of 30° behind the vertical (P6.8–7.4 mm and L2.4–4.0 mm to lambda). Extracellular action potentials of neurons in the trigeminal nucleus were recorded, and a diagram of the experimental preparation is shown in Fig. 1.

As the microelectrode was advanced into the trigeminal nucleus, electrical stimulation was repeatedly applied to the IAN (train pulses; 0.2 ms, 2 Hz). Stimulus intensity was 2T the threshold for evoking the JOR. Once a single neuron, which responded to IAN stimulation, was identified using the criteria of Landgren and Olsson (Landgren and Olsson, 1976), the threshold and latency for evoking neural responses were determined.

In the recording session, the IAN was regularly stimulated to evoke responses from identified neurons at 2 Hz over 30 s. Current intensity for IAN stimulation was 1.2T the threshold for evoking neural responses. In the middle 10 s, conditioning stimulation was additionally applied to the SLN (train pulses; 0.2 ms, 30 Hz) on the same side as for the IAN. Current intensity of SLN stimulation was 2, 4, or 8T the threshold for evoking swallow at least once during 10-s SLN stimulation. The first, second, and third 10-s periods were

named the Control, Stim, and Post-stim periods, respectively. Between trials (2, 4, and 8T SLN stimulation), the time interval was set at 2 min. Finally, microstimulation was applied through the electrode to the neuron (single pulses, 0.2 ms duration, up to 50 μA) for 5 s to examine whether time-locked Dig responses were evoked

EMG signals were amplified (AM-601G, Nihon Kohden, Tokyo, Japan) with time constant, 0.03 s, low-pass filtered, with a 10 kHz sampling rate. Neural activity was amplified (Microelectrode 1800, A-M systems Inc., Tokyo, Japan) with band-pass, 0.3 Hz–10 kHz. Signals were digitized on a personal computer at a sampling rate of 10 kHz and were analyzed using the Spike2 analysis package (Cambridge Electronic Design, Cambridge, UK).

4.4. Data analysis

Statistical analysis was performed using SigmaPlot software (SigmaPlot 12.0, Systat Software Inc., CA, USA). The mean number of swallows and onset latency of the first swallow in the Control period was compared among the SLN stimulus intensities (2, 4, and 8T) using one-way analysis of variance and Tukey's post hoc test. For the analysis of identified neurons, we determined the physiological properties of neurons according to previous studies (Inoue et al., 2002; Olsson et al., 1986). Briefly, we first tested if the neurons had spontaneous activity and receptive fields. Next, we calculated the latency of evoked responses for each neuron, and the percentage of evoked responses in each period (Control, Stim, and Post-stim). The percentage was always 100% in the Control period, because the stimulus threshold was 1.2T for initiation of neural responses. Occasionally, the number of spikes evoked by IAN stimulation was more than two per single pulse IAN stimulation, but we did not analyze such spikes in the present study. The percentage of

responses was compared using the chi-square test to compare between Stim and Post-stim periods, and among stimulus intensities. The mean latency of each neuron was calculated for each period (Control, Stim, and Post-stim), and was compared between Control and Stim or between Control and Post-stim using t-tests or the Mann-Whitney U test. If the occurrence of evoked neural response was inhibited (less than 100%), or if the mean latency was significantly longer in either the Stim or Post-stim periods compared with the Control period, then neurons were deemed modulated neurons **(Fig. 6)**.

All neurons recorded were divided into two neuron groups based on latency (long-latency >= 3 ms, or short-latency < 3 ms, in the Control period). The change in latency in the Stim and Post-stim periods was compared between the groups and among stimulus intensities using t-tests or the Mann-Whitney U test with Tukey's post hoc test and using one-way analysis of variance and Tukey's post hoc test, respectively. All values are mean ± SE, except mean ± SD for mean number of swallows and onset latency of the first swallow. $P < 0.05$ was considered statistically significant.

4.5. Histology

At the termination of each experiment, a small electrolytic lesion was made by passing negative current (50 μA, 30 s) through recording and stimulating microelectrodes. The animal was killed with an intravenous overdose of urethane, and then perfused through the left cardiac ventricle with 0.1 M phosphate buffer at pH 7.4, followed by 4% paraformaldehyde fixative. Serial frozen coronal sections (50 μm thick) were stained with cresyl violet. Both the recording and stimulating sites in the brain stem were reconstructed (Donga and Lund, 1991).

Acknowledgment

This study was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture and Science of Japan (#20592170 to M.I.).

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Figure legends

Fig. 1. Composite figure showing experimental setup. Stimulation sites included the IAN and SLN. EMGs of digastric (Dig) and mylohyoid (MH) muscles were recorded to monitor the JOR and swallowing, respectively. Single-pulse stimulation was applied as a test stimulation in the IAN to evoke a single trigeminal neural response. A conditioning stimulus was applied to the SLN (2, 4, or 8 times the threshold for evoking a swallowing reflex at least once for 10 s) to evaluate the effect of SLN stimulation on neuronal evoked responses.

Fig. 2. The number of swallows and latency to the first swallow during SLN stimulation. The number of swallows during SLN stimulation for 10 s was measured, and was 2.92 ± 1.95, 3.75 ± 1.98, and 4.50 ± 2.04 (n=19) for the 2, 4, and 8T SLN stimulation intensities, respectively. Latency to the first swallow following SLN stimulation was 2.66 ± 2.19, 2.15 ± 1.93, and 1.90 ± 1.49 s (n=19) for the 2, 4, and 8T SLN stimulation intensities, respectively. All values are mean ± SD. *P < 0.05.

Fig. 3. Diagram showing the distribution of 23 neurons in the trigeminal nucleus in the transverse plane at 4 levels, from which recordings were made. Upper left panel is the most rostral and lower right panel is the most caudal. Interneurons were classified based on the pattern of the property of responses to SLN stimulation. Closed circles (●) represent interneurons that exhibited delayed latency of neural responses to IAN stimulation, and changed the probability of neural responses during/after SLN stimulation. Open circles (○) represent interneurons that exhibited delayed latency of neural responses to IAN stimulation during/after SLN stimulation, and did not change the probability of neural responses. Open rectangles (□) represent interneurons that had unchanged probability and latency.

Fig. 4. Rate of modulated neurons present in each stimulus period for each stimulus intensity. Based on the probability of neural response to 20 IAN stimulations, all neurons recorded were classified into heavily (< 14/20 responses), lightly (15–19/20 responses), or non- (20/20 responses) modulated neurons. The rate of the number of non- (□), lightly (■), and heavily (■) modulated neurons in the Control, Stim, and Post-stim periods is shown for each stimulus intensity. The rate of modulated neurons was not related between Stim and Post-stim periods (DF = 1, χ^2 = 0.15, P = 0.70 for 2T; DF = 1, χ^2 = 0.11, P = 0.74 for 4T; DF = 1, χ^2 = 0.94, P = 0.33 for 8T). The rate of modulated neurons was not related among the stimulus intensities in the Stim period (DF = 2, χ^2 = 0.43, P = 0.81), but was significantly related among the stimulus intensities in the Post-stim period (DF = 2, χ^2 = 6.556, P = 0.04). The total number of neurons recorded in each stimulus condition was 27 (2T), 27 (4T), and 22 (8T).

Fig. 5. Correlation between onset latency of neural response and delay of latency in Stim and Post-stim periods. All neurons identified were divided into short- (< 3 ms) and long-latency (>= 3 ms) groups, and were compared in each condition. They were also compared among the stimulus conditions; 2, 4, and 8T SLN stimulation. ***P < 0.001, **P < 0.01, *P < 0.05.

Fig. 6. Examples of evoked responses from one neuron. These neurons were located in NVsnpr. Left: Two waveforms superimposed. Evoked responses were inhibited during 4T SLN stimulation. Right: The latency of response was delayed during 4T SLN stimulation. Arrow heads represent the IAN stimulation on both left and right figures.

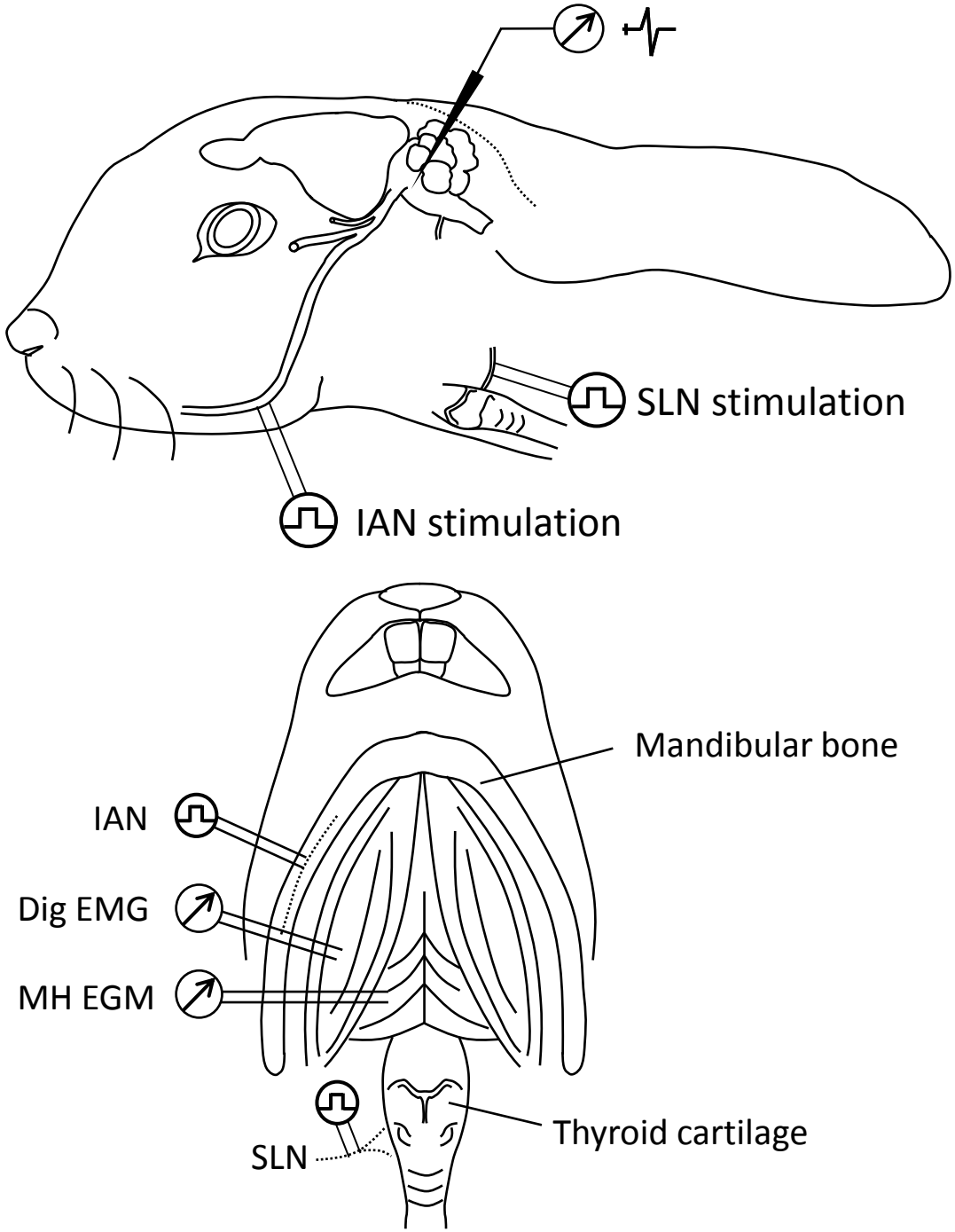

SLN stimulation
IAN stimulation
Mandibular bone
IAN
Dig EMG
MH EGM
SLN
Thyroid cartilage

Fig. 1

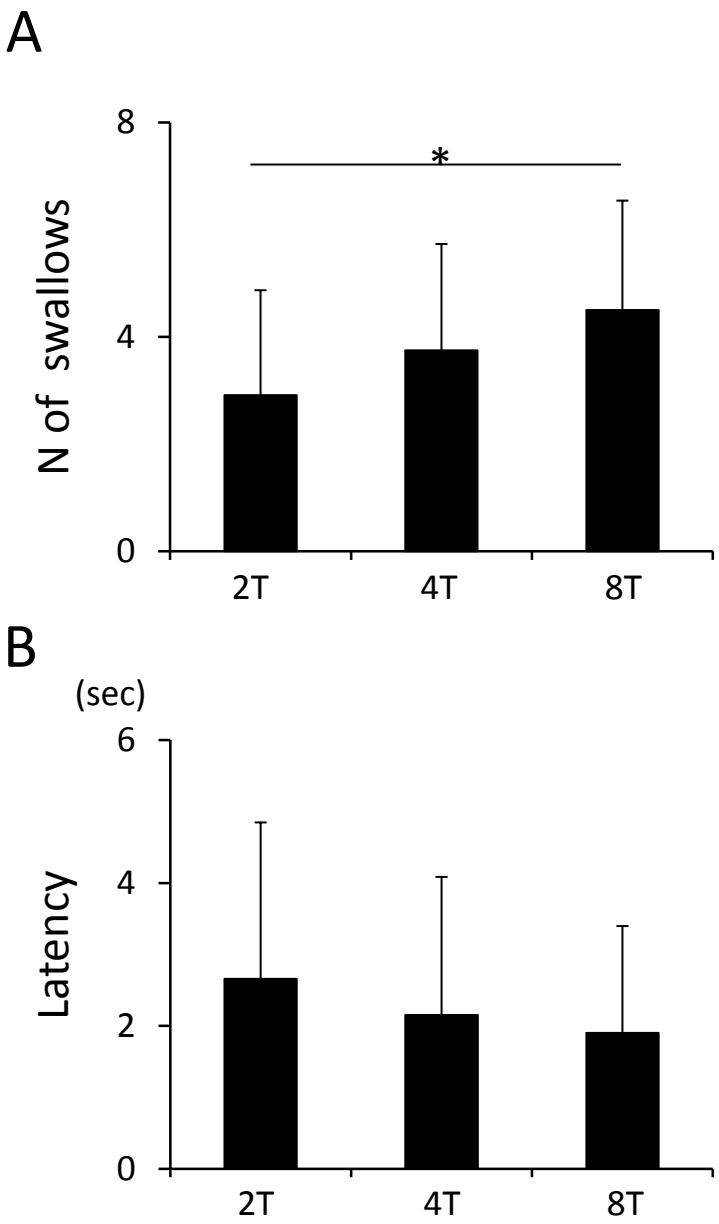
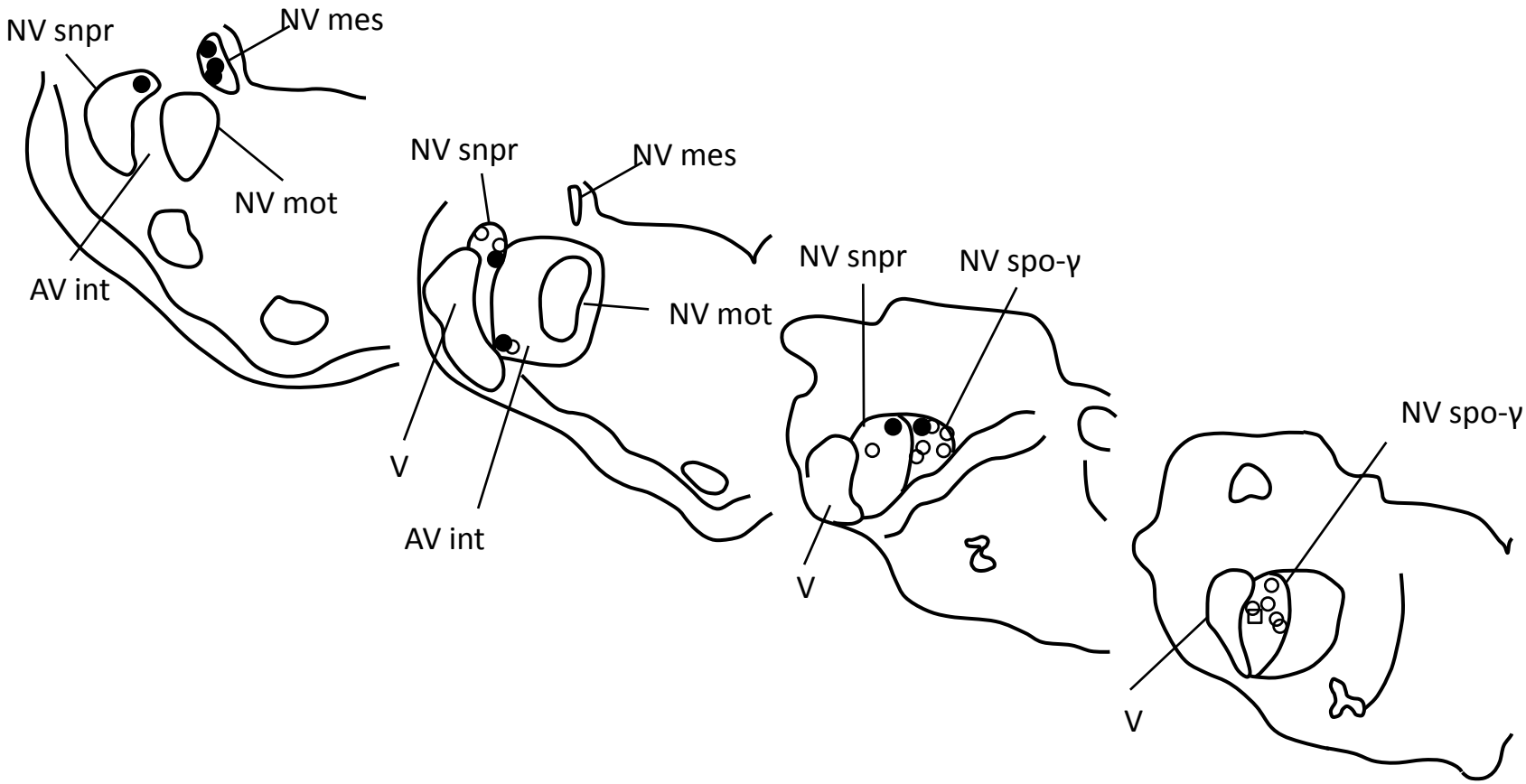


Fig. 2


NV snpr
NV mes
NV mot
AV int
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NV spo-γ
V
NV spo-γ
V

Fig. 3

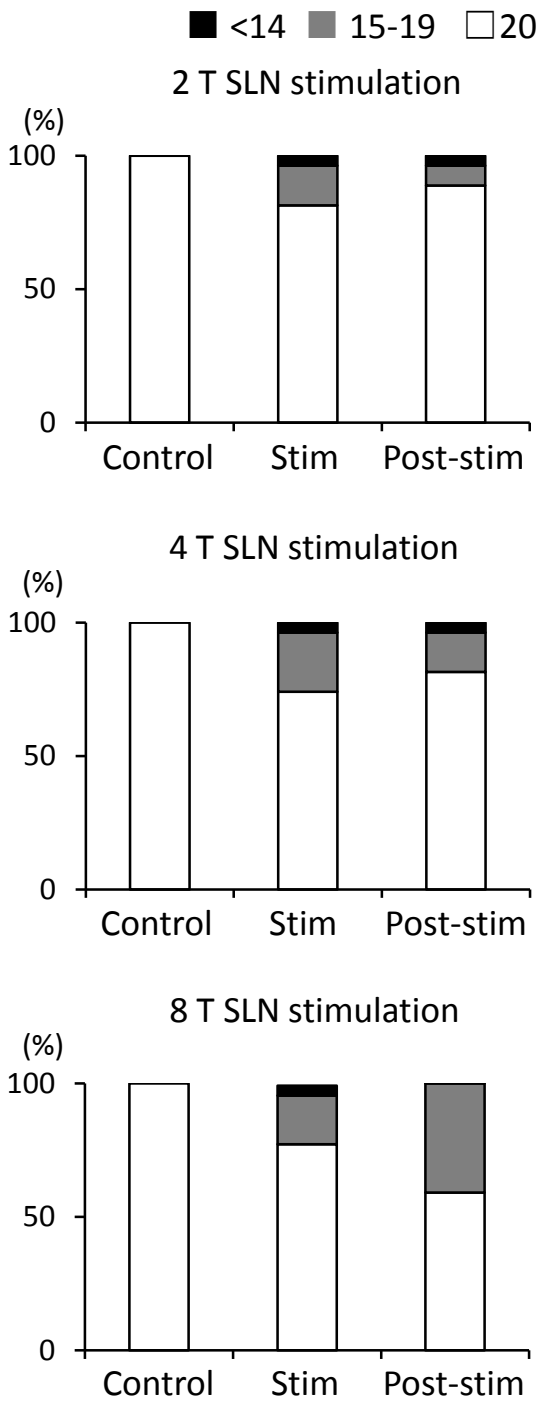
■ <14 ■ 15-19 □20
2 T SLN stimulation
(%)
100
50
0
Control
Stim
Post-stim
4 T SLN stimulation
(%)
100
50
0
Control
Stim
Post-stim
8 T SLN stimulation
(%)
100
50
0
Control
Stim
Post-stim

Fig. 4

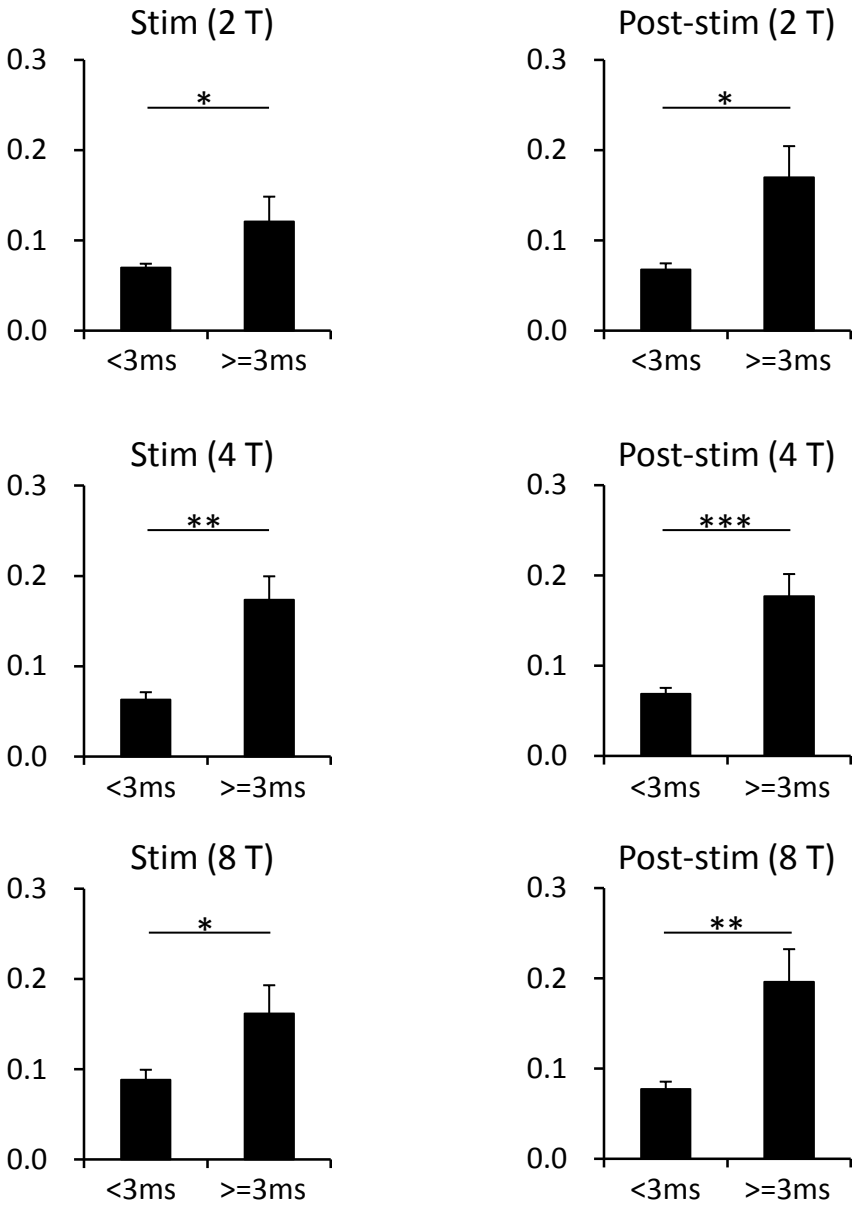
Stim (2 T)
Post-stim (2 T)
Stim (4 T)
Post-stim (4 T)
Stim (8 T)
Post-stim (8 T)
0.3
0.2
0.1
0.0
*
**
***
<3ms
>=3ms

Fig. 5

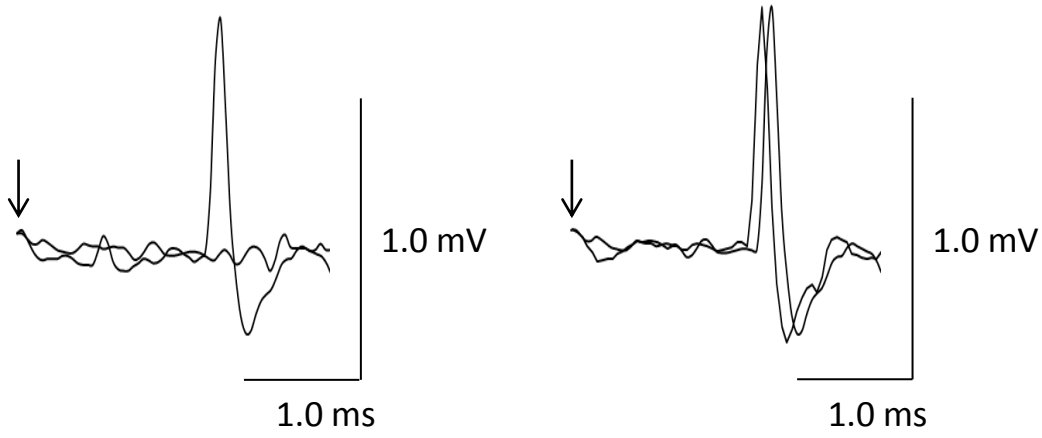

Fig. 6