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Research report

# Changes in the frequency of swallowing during electrical stimulation of superior laryngeal nerve in rats

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ABSTRACT

The aim of the present study was to investigate the adaptation of the swallowing reflex in terms of reduced swallowing reflex initiation following continuous superior laryngeal nerve stimulation. Forty-four male Sprague Dawley rats were anesthetized with urethane. To identify swallowing, electromyographic activity of the left mylohyoid and thyrohyoid muscles was recorded. To evoke the swallowing response, the superior laryngeal nerve (SLN), recurrent laryngeal nerve, or cortical swallowing area was electrically stimulated. Repetitive swallowing evoked by continuous SLN stimulation was gradually reduced, and this reduction was dependent on the resting time duration between stimulations. Prior SLN stimulation also suppressed subsequent swallowing initiation. The reduction in evoked swallows induced by recurrent laryngeal nerve or cortical swallowing area stimulation was less than that following superior laryngeal nerve stimulation. Decerebration had no effect on the reduction in evoked swallows. Prior subthreshold stimulation reduced subsequent initiation of swallowing, suggesting that there was no relationship between swallowing movement evoked by prior stimulation and the subsequent reduction in swallowing initiation. Overall, these data suggest that reduced sensory afferent nerve firing and/or trans-synaptic responses, as well as part of the brainstem central pattern generator, are involved in adaptation of the swallowing reflex following continuous stimulation of swallow-inducing peripheral nerves and cortical areas.



## 1. Introduction

Swallowing is the first stage of nutrition and serves to transport a food bolus from the oral cavity into the stomach through the pharynx and esophagus. Swallowing is also a protective reflex that prevents the bolus from penetrating the larynx or aspirating. It is generally accepted that swallowing is centrally programmed by the swallowing central pattern generator (CPG) in the lower brain stem, which is activated by either supramedullary or pharyngeal/laryngeal sensory inputs in humans and animals (Doty, 1968; Jean, 2001; Miller, 1982).

Electrical stimulation of either the superior laryngeal nerve (SLN) or recurrent laryngeal nerve (RLN) can evoke a swallowing reflex in anesthetized animals (Fukuhara et al., 2011; Tsujimura et al., 2013; Yamada et al., 2013). Initiation of the swallowing reflex and associated swallow-related motor patterns are areas of major interest. Although it is well known that repetitive swallowing can be evoked by continuous stimulation of pharyngeal/laryngeal areas, the frequency of swallowing induction gradually decreases. For example, Kajii et al. (2002) examined the effects of chemosensory inputs on occurrence of the swallowing reflex in anesthetized animals; results showed that acetic acid and citric acid (sour taste) had a stronger effect on evoking swallowing when compared with other taste solutions. Interestingly, their data also indicated a time-dependent reduction of swallowing initiation, even during acid infusion, although these phenomena were not described. Takatsuji et al. (2012) reported that swallowing reflexes were induced by repetitive electrical pulse stimulation applied to the pharyngeal mucosa in conscious humans, and that prolonged stimulation often failed to elicit multiple swallowing reflexes.

We previously examined the effects of water stimulation in the pharynx on voluntary swallowing by measuring swallowing intervals in healthy humans (Nakamura et al., 2013). We found no

*Abbreviations:* SLN, superior laryngeal nerve; CPG, central pattern generator; NTS, nucleus tractus solitarii; RLN, recurrent laryngeal nerve; Cx, cortical swallowing area.

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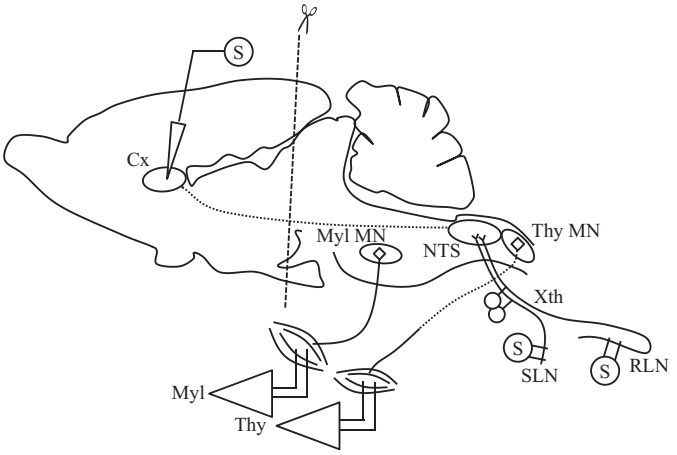


**Fig. 1.** Experimental setup. Stimulating sites (S) included the superior laryngeal nerve (SLN), recurrent laryngeal nerve (RLN), and cortical swallowing area (Cx). Recordings were mylohyoid (Myl) and thyrohyoid (Thy) electromyograms. MN, motor nucleus; NTS, nucleus tractus solitarii; Xth, vagus nerve.

time-dependent change in the swallowing interval, suggesting that the effects of peripheral input on voluntary swallowing were minimally altered, although the interval itself was different among the taste stimulants. Thus, it remains unclear how continuous pharyngeal stimulation affects the swallow-related neural network and swallowing initiation. The aim of the present study was to investigate the adaptation of the swallowing reflex in terms of reduced swallowing reflex initiation, which was evoked by SLN stimulation in anesthetized animals.

## 2. Materials and methods

### 2.1. Animals

Experiments were performed on a total of 44 male Sprague Dawley® rats weighing between 250 and 350 g. This study was reviewed and approved by the Niigata University Intramural Animal Care and Use Committee.

### 2.2. Surgical preparation

The animals were anesthetized with urethane (1.3 g/kg, i.p.), supplemented with intraperitoneal injections of urethane whenever necessary to maintain anesthesia at a level at which corneal reflex and spontaneous eye movements were absent. The trachea was cannulated for spontaneous respiration. The right femoral vein and artery were also cannulated to administer drugs intravenously and monitor blood pressure, respectively. Respiratory rhythm was recorded using a pneumatic belt. Rectal temperature was maintained between 37 and 38 °C using a thermostatically controlled heating pad. A midline incision was made along the ventral aspects from the pogonion to the rostral portion of the neck. Bipolar enamel-coated copper wire electrodes (0.18 mm diameter and 2 mm inter-polar distance) were inserted into the left mylohyoid (Myl) and thyrohyoid (Thy) muscles for electromyographic (EMG) recordings.

### 2.3. Stimulus procedure to evoke swallow

To evoke the swallowing reflex, the SLN, RLN, or cortical swallowing area (Cx) was electrically stimulated (Fig. 1). The bilateral SLN and RLN were cut and bipolar enamel-coated silver wire electrodes (0.2 mm diameter) were placed on the central severed nerve ending. The swallowing reflex was evoked by repetitive electrical stimulation of either unilateral SLN or RLN (0.2 ms pulse duration; 30 Hz) and was identified by Myl and Thy EMG bursts and visual observation of the laryngeal elevation. The stimulus threshold for evoking swallowing was determined as follows. Current intensity was increased by 0.1 μA every 1 s between 0 and 10 μA. If swallowing was not induced, the current intensity was further increased by 1 μA between 10 and 100 μA, and by 10 μA over 100 μA, until swallowing was evoked. Once swallowing was successfully evoked, the same stimulation was repeated for 10 s after a 5-min interval to ascertain that swallowing was evoked at least once.

In an additional animal, the Cx was electrically stimulated to evoke swallowing (Tsujimura et al., 2012b). The animal's head was fixed on a stereotaxic holder, and the cortical surface was exposed. A bipolar needle electrode (0.25 mm inner diameter; 0.6 mm outer diameter) was inserted into the insular cortex (1–2 mm anterior, 4.7–5 mm lateral to bregma and 4.5–5 mm depth from the cortical surface) on either side. The electrode was then moved in 0.5-mm steps, and the cortex was repetitively stimulated (0.5 ms pulse duration; 10 Hz) for 10 s. At each step, current intensity was increased by 10 μA every 10 s between 60 and 250 μA at 5-min intervals.

The cortical masticatory area, or the so called P-area, is known to be a part of the insular cortex. In a previous study, rhythmic jaw movements, but not swallowing, were induced during stimulation (Tsujimura et al., 2012a). However, in cases where both swallowing and rhythmic jaw movements were evoked by stimulation to the same cortical site, the stimulus threshold was much greater for initiation of rhythmic jaw movements than that of swallowing. Therefore, once swallowing was induced during stimulation, the electrode tip was fixed and the threshold was determined. The methods employed to identify swallowing and determine the threshold for cortical stimulation were the same as for SLN/RLN stimulation.

In all experiments, except conditioning six (see below), the stimulus intensity was 1.1 T times of threshold (T) for SLN or RLN stimulation and 1.2 T for cortical stimulation.

### 2.4. Data collection

To determine the time-dependent changes in initiation of swallowing, SLN was repetitively stimulated at 30 Hz for 50 s. Next, to investigate the effects of the resting period between repeated SLN stimulations (5 bouts of 30 Hz, 10 s), a resting interval of 30 or 50 s was allowed between each bout of stimulation. We then investigated the effect of conditioning stimulation on the following swallowing initiation (Fig. 2).

*Conditioning stimulation 1*. First, unilateral SLN was repetitively stimulated at 30 Hz for 10 s as a test stimulation. After an interval of 5 min, the ipsilateral SLN was repetitively stimulated for 30 s prior to test stimulation. The stimulus frequency for conditioning stimulation was 10, 20, 30, or 40 Hz.

*Conditioning stimulation 2*. To evaluate the effect of prior SLN stimulation on SLN-evoked swallow and as a test stimulation (pre-control), the unilateral SLN was repetitively stimulated at 30 Hz for 10 s. After a 5-min interval, either the ipsilateral or contralateral SLN was repetitively stimulated for 30 s prior to test stimulation. Again, after a 5-min interval, the ipsilateral SLN was repetitively stimulated at 30 Hz for 10 s (post-control).

*Conditioning stimulation 3*. To evaluate the effect of prior SLN stimulation on RLN-evoked swallow and as a test stimulation (pre-control), the unilateral RLN was repetitively stimulated at 30 Hz for 10 s. After a 5-min interval, either the ipsilateral or contralateral SLN was repetitively stimulated for 30 s prior to test stimulation. Again, after a 5-min interval, the

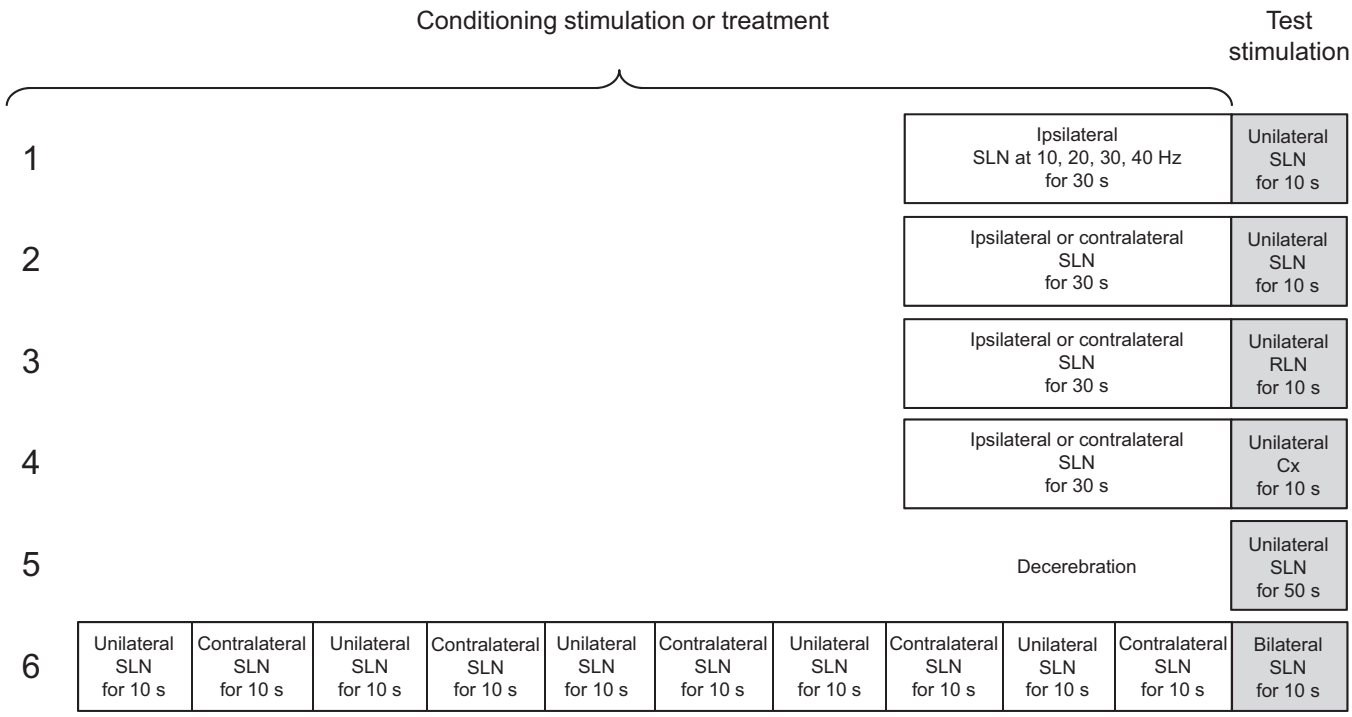


**Fig. 2.** Experimental protocol with conditioning stimulation. Cx, cortical swallowing area; RLN, recurrent laryngeal nerve SLN, superior laryngeal nerve.

ipsilateral RLN was repetitively stimulated at 30 Hz for 10 s (post-control).

*Conditioning stimulation 4*. To evaluate the effect of prior SLN stimulation on cortically evoked swallows and as a test stimulation (pre-control), the unilateral Cx was repetitively stimulated at 10 Hz for 10 s. After a 5-min interval, either the ipsilateral or contralateral SLN was repetitively stimulated for 30 s prior to test stimulation. Again, at a 5-min interval, the ipsilateral Cx was repetitively stimulated at 10 Hz for 10 s (post-control). At the termination of each experiment, a small electrolytic lesion was made by passing negative current (0.05 mA, 30 s) through the stimulating electrode. The animal was sacrificed with an intravenous overdose of urethane and then perfused through the left cardiac ventricle with 0.1 M phosphate buffer (pH 7.4), followed by 4% paraformaldehyde fixative. Serial frozen coronal sections (0.05 mm thick) were stained with cresyl violet.

*Conditioning stimulation 5*. To evaluate the effect of decerebration on reduced swallowing initiation, the unilateral SLN was stimulated at 30 Hz for 50 s before and after decerebration (Fig. 1). For decerebration, the dura mater was breached, the cerebral cortex was gently aspirated to visualize the superior and inferior colliculi, and the brain was perpendicularly and coronally sectioned. A minimum recovery period of 0.5 h after decerebration was employed prior to data collection.

*Conditioning stimulation 6*. Finally, to evaluate the effects of conditioning stimulation under the threshold for eliciting swallowing, bilateral SLN were initially stimulated at 30 Hz for 10 s as a test stimulation (pre-control). Although the stimulus intensity in this session was 0.9 T on each side, we confirmed that it was strong enough to initiate a swallowing reflex at least once during a 10-s period. Next, after a 5-min interval, a 10-s unilateral SLN stimulation at 30 Hz was reciprocally repeated five times as a conditioning stimulation, but a swallowing reflex was never evoked. This was immediately followed by 10 s of bilateral SLN stimulation at 0.9 T (test stimulation). Lastly, after a 5-min interval, the test stimulation was repeated (post-control).

### 2.5. Data analysis

EMG signals were amplified (AM-601G, Nihon Kohden, Tokyo, Japan), digitized, and stored on a computer hard disk with a sampling rate of 10 kHz. Data analysis was performed using the Spike2 analysis package (Cambridge Electronic Design, Cambridge, UK). The number of swallows was measured in each recording session to examine changes in the swallowing occurrence and effects of conditioning stimulation. Results are presented as mean ± SEM. Differences among group means were assessed using two-way repeated measures ANOVA, one-way repeated measures ANOVA, or Friedman one-way ANOVA with Tukey test following normality and equality tests where appropriate. Student *t*-tests or Mann–Whitney rank sum test were used to analyze differences between the two groups. Differences were considered significant at $P < 0.05$.

## 3. Results

Swallowing was evoked by electrical stimulation of the SLN, RLN, and Cx (Fig. 3). Stimulation of sites neighboring the Cx often evoked rhythmic jaw movements, as did high-intensity (>1.5 T) stimulation applied to the Cx, which was expected, because the insular cortex is anatomically close to the cortical masticatory area. The threshold value to elicit a swallow ranged from 1.8 to 100 μA, from 7.5 to 80 μA, and from 60 to 250 μA for the SLN, RLN, and Cx, respectively. The cortical stimulation sites ($n = 5$) were histologically identified in the dysgranular and dorsal part of the agranular insular cortex, according to the stereotaxic atlas by Paxinos and Watson (Fig. 4).

### 3.1. Reduction in swallowing initiation evoked by SLN stimulation

Swallowing initiation gradually decreased during continuous SLN stimulation (Fig. 5). The mean number of swallows was 12.0 ± 3.1 for 0 s rest (*i.e.*, 50 s continuous SLN stimulation), 19.5 ± 5.0 for 30 s rest every 10 s, and 24.5 ± 2.6 for 50 s rest every 10 s ($n = 6$) (Fig. 6A). There was no difference in

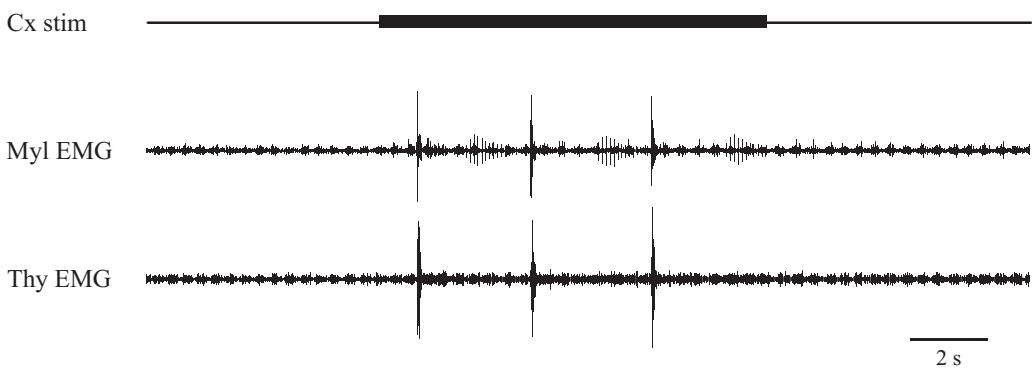


**Fig. 3.** Example of electromyographic (EMG) recordings in the mylohyoid (Myl) and thyrohyoid (Thy) muscles before, during, and after continuous cortical stimulation (Cx stim). The stimulus intensity and frequency were 100 μA and 10 Hz, respectively.

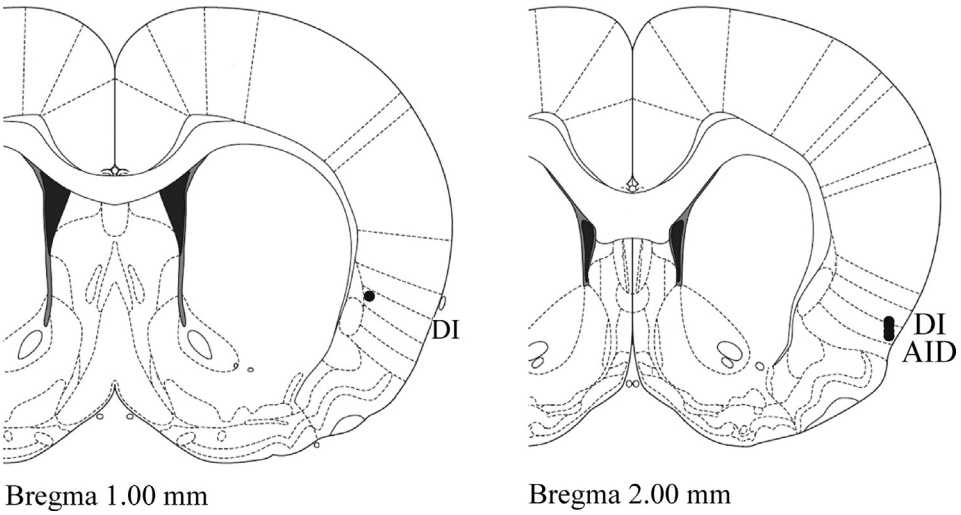


**Fig. 4.** Schematic drawing of stimulation sites in the dysgranular (DI) and dorsal part of the agranular insular cortex (AID).

the number of swallows evoked by SLN stimulation between the left ($n = 3$) and right ($n = 3$) sides, although the sample number was low (left: $11.0 \pm 4.1$ for 0 s rest, $16.0 \pm 0.6$ for 30 s rest and $24.3 \pm 4.3$ for 50 s rest; right: $13.0 \pm 7.5$ for 0 s rest, $23.0 \pm 13.2$ for 30 s rest and $24.6 \pm 14.2$ for 50 s rest). There was a significant difference in the number of swallows between the stimulation protocols. In all conditions, the number of swallows evoked by SLN stimulation decreased in a time-dependent manner, with a significant decrease in the number of swallows every 10 s between the various intervals (Fig. 6B). There was no obvious difference in the number of swallows or effect of time interval of stimulation between the left and right sides. Therefore, for the remaining experiments we did not distinguish between the left and right sides.

Prior ipsilateral SLN stimulation (conditioning stim 1) strongly affected the number of subsequent SLN-evoked swallows. The number of SLN-evoked swallows without prior stimulation was significantly larger compared with prior stimulation, and also

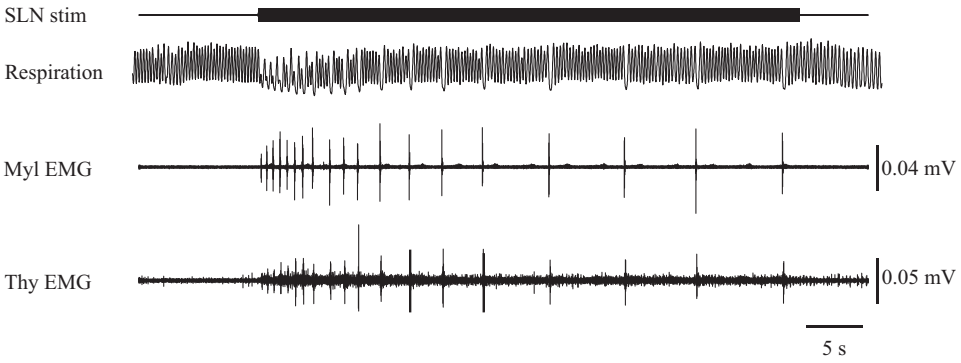


**Fig. 5.** Example of electromyographic (EMG) recordings in the mylohyoid (Myl) and thyrohyoid (Thy) muscles, and respiration before, during, and after continuous superior laryngeal nerve stimulation (SLN stim). The swallowing reflex is accompanied by an EMG burst of both muscles and transient apnea. *Note*: the number of swallows during stimulation gradually decreased.

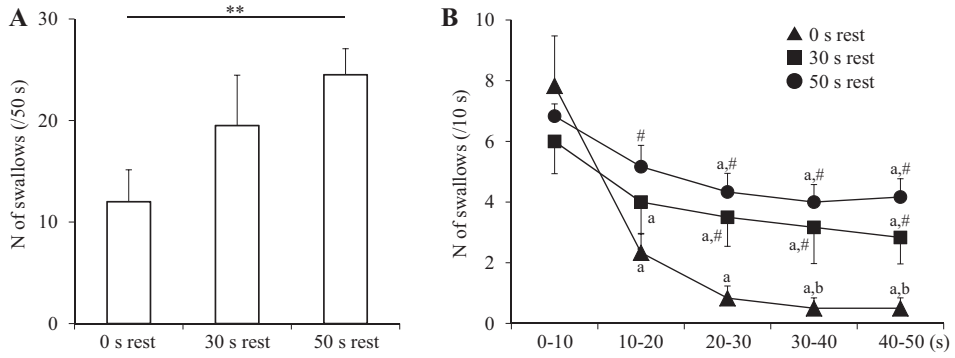


**Fig. 6.** Effect of resting time on swallowing initiation. (A) Total number of swallowing over 50 s was compared among the different resting times inserted every 10 s. The number of swallows increased with resting period duration. $^{**}P < 0.01$. (B) Number of swallows was compared among 10-s periods, and gradually decreased depending on resting time. [a] *vs.* 0–10, [b] *vs.* 10–20, [#] *vs.* 0 s rest.

negatively correlated with stimulus frequency (Fig. 7A). Prior SLN stimulation evoked a swallowing reflex, and the number of swallows positively correlated with stimulus frequency (10 Hz, 8.2 ± 1.4; 20 Hz, 12.4 ± 2.4; 30 Hz, 16.6 ± 1.9; 40 Hz, 16.4 ± 2.0, $n = 5$) (Fig. 7B). These results suggest that prior SLN stimulation induced a reduction in swallowing initiation by subsequent SLN stimulation.

### 3.2. Effect of prior SLN stimulation on SLN, RLN, or cortically evoked swallows

Prior SLN stimulation was applied to investigate whether it modulates swallowing initiation evoked by SLN, RLN, and cortical stimulation. The mean number of swallows evoked by SLN stimulation was 8.8 ± 2.2 (pre-control) and 7.3 ± 2.1 (post-control) without prior ipsilateral SLN stimulation, and 1.3 ± 0.7 with prior ipsilateral SLN stimulation ($n = 4$), which was significantly different between the conditions (Fig. 8A). In contrast, there was no significant difference during prior contralateral SLN stimulation; the mean number of swallows evoked by SLN stimulation was 8.8 ± 1.4 (pre-control) and 8.4 ± 1.8 (post-control) without prior contralateral SLN stimulation, and 10.6 ± 2.0 with prior contralateral SLN stimulation ($n = 5$) (Fig. 8A).

The mean number of swallows evoked by RLN stimulation was 6.4 ± 0.5 (pre-control) and 6.0 ± 0.7 (post-control) without prior SLN stimulation, and 3.8 ± 0.6 with prior ipsilateral SLN stimulation ($n = 5$), which was significantly different between conditions (Fig. 8B). The mean number of swallows evoked by RLN stimulation was 7.3 ± 0.7 (pre-control) and 6.6 ± 0.6 (post-control) without prior SLN stimulation, and 5.6 ± 0.6 with prior SLN stimulation on the contralateral side ($n = 7$) (Fig. 8B). In this case, a significant difference was only observed between pre-control and with prior SLN stimulation.

The mean number of swallows evoked by cortical stimulation was 3.9 ± 0.3 (pre-control) and 3.6 ± 0.4 (post-control) without prior SLN stimulation, and 2.0 ± 0.5 with prior ipsilateral SLN stimulation ($n = 7$), which was significantly different between conditions (Fig. 8C). The mean number of swallows evoked by cortical stimulation was 2.9 ± 0.4 (pre-control) and 3.3 ± 0.5 (post-control) without prior SLN stimulation, and 1.7 ± 0.3 with prior SLN stimulation on the contralateral side ($n = 7$) (Fig. 8C). In this case, the significance was observed only between pre-control and prior SLN stimulation.

The effect of prior SLN stimulation on subsequent swallowing initiation was compared among the test stimulations (SLN, RLN, and Cx), as well as between the sides. The normalized number of swallows following conditioning stimulation (number of swallows with conditioning stimulation/control number of swallows obtained from pre- and post-control) was 0.2 ± 0.1 ($n = 4$) and 1.2 ± 0.2 ($n = 5$) for ipsilateral and contralateral SLN stimulation, respectively, 0.6 ± 0.1 ($n = 5$) and 0.8 ± 0.2 ($n = 5$) for ipsilateral and contralateral RLN stimulation, respectively, and 0.5 ± 0.3 ($n = 7$) and 0.6 ± 0.4 ($n = 7$) for ipsilateral and contralateral cortical stimulation (Fig. 9A). With regard to the effect of prior SLN stimulation on ipsilateral and contralateral sides, there was a significant difference in the effect on SLN- and RLN-evoked swallowing between the sides. Among the SLN-, RLN-, and Cx-evoked swallows, there was a significant difference between ipsilateral SLN and RLN, SLN and Cx, and RLN and Cx. These results suggest that ipsilateral prior SLN stimulation has a strong inhibitory effect on subsequent

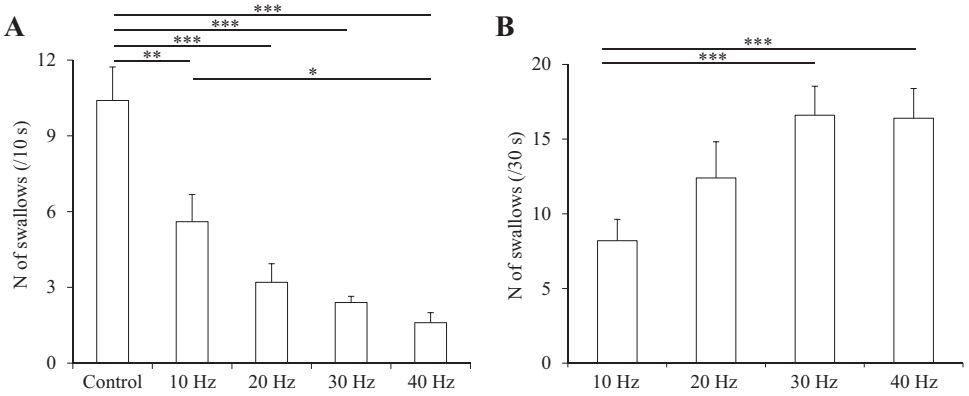


**Fig. 7.** Effect of prior superior laryngeal nerve (SLN) stimulation at different frequencies on swallowing initiation. (A) The greater the stimulus frequency of SLN stimulation, the greater the decrease in frequency of evoked swallows. (B) Number of swallows during prior SLN stimulation was also dependent on stimulus frequency. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

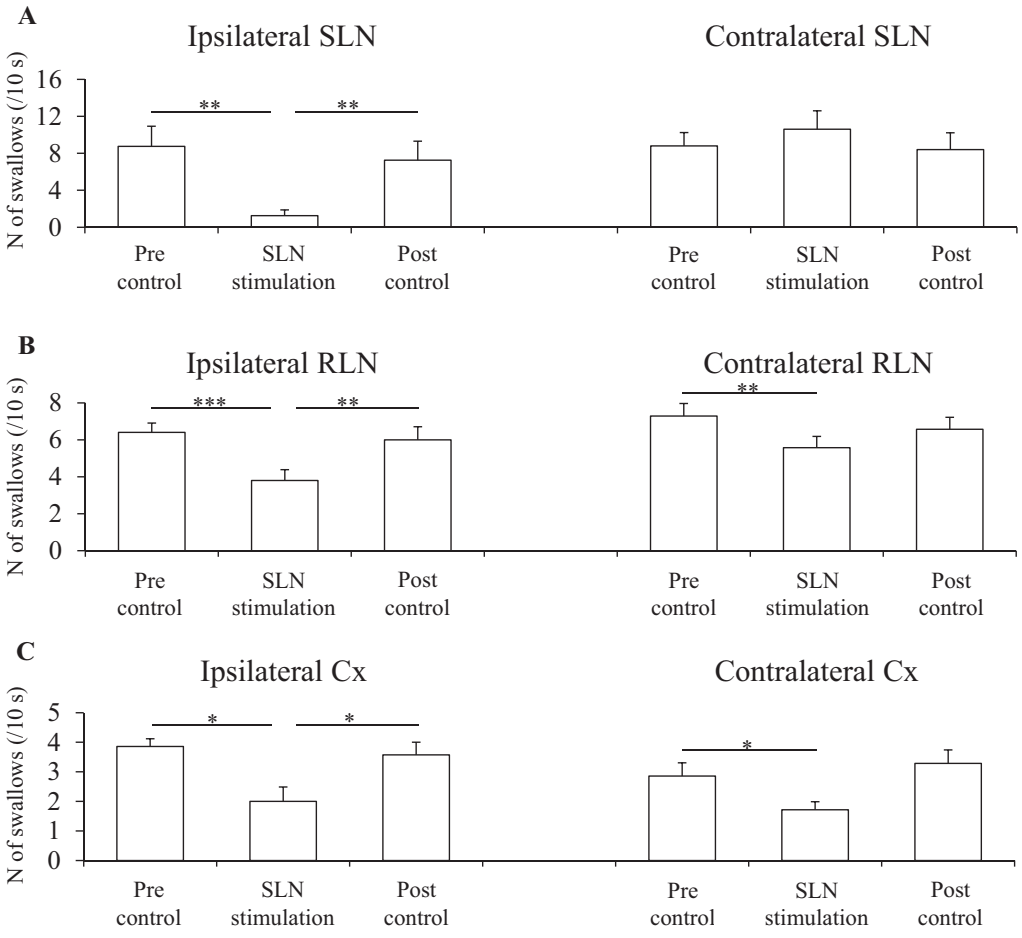


**Fig. 8.** Effect of prior superior laryngeal nerve (SLN) stimulation on subsequent SLN, recurrent laryngeal nerve (RLN) and cortically (Cx) evoked swallows. (A) The number of swallows evoked by ipsilateral SLN stimulation was smaller than in controls (without prior SLN stimulation). (B) The number of swallows evoked by ipsilateral RLN stimulation was smaller than in controls (without SLN stimulation). This was not the case in the contralateral RLN, where the number of swallows evoked by contralateral RLN stimulation was smaller than pre-control only. (C) The number of swallows evoked by ipsilateral Cx stimulation was smaller than in controls (without SLN stimulation). This was not the case in the contralateral Cx, where the number of swallows evoked by contralateral Cx stimulation was smaller than the pre-control only. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

initiation of swallowing, and that conditioning stimulation applied to the SLN has different effects on inputs into swallowing CPG among SLN, RLN, and Cx. Although the baseline swallowing number was different between conditions, the decreased number of swallows was also different between conditions; $6.8 \pm 3.2$ ($n = 4$) and $-2 \pm 0.2$ ($n = 5$) for ipsilateral and contralateral SLN stimulation, respectively, $2.4 \pm 0.2$ ($n = 5$) and $1.3 \pm 1.4$ ($n = 5$) for ipsilateral and contralateral RLN stimulation, respectively, and $1.7 \pm 0.9$ ($n = 7$) and $1.4 \pm 1.6$ ($n = 7$) for ipsilateral and contralateral cortical stimulation, respectively (Fig. 9B).

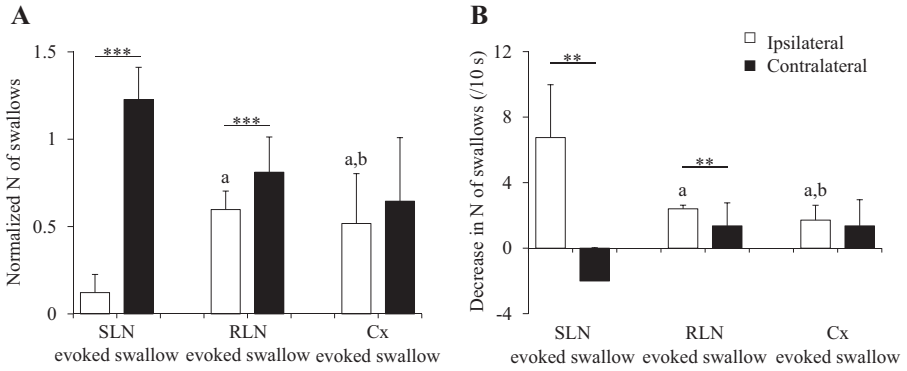


**Fig. 9.** Difference in effect of prior superior laryngeal nerve (SLN) stimulation on swallowing initiation among SLN, recurrent laryngeal nerve (RLN), and cortical swallowing area (Cx) stimulation. (A) The normalized number of swallows following conditioning stimulation was significantly larger for ipsilateral SLN and RLN stimulation than contralateral ones. Among the SLN-, RLN-, and Cx-evoked swallows, there was a significant difference between ipsilateral SLN and RLN, SLN and Cx, and RLN and Cx. (B) The decreased number of swallows was also different between sides; it was significantly larger for ipsilateral SLN and RLN stimulation than contralateral stimulation. Among the SLN-, RLN-, and Cx-evoked swallows, there was a significant difference between ipsilateral SLN and RLN, SLN and Cx, and RLN and Cx. [a] *vs.* SLN evoked swallow, [b] *vs.* RLN evoked swallow, $^{***}P < 0.001$, $^{**}P < 0.01$.

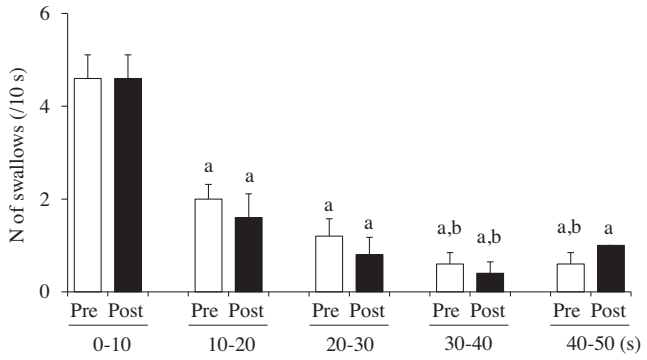


**Fig. 10.** Effect of decerebration on reduction in swallowing initiation during continuous SLN stimulation. There was no significant difference in reduction in swallowing initiation between before (pre) and after (post) decerebration. [a] *vs.* 0–10, [b] *vs.* 10–20.

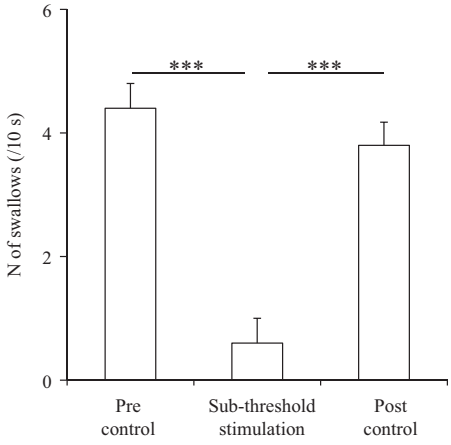


**Fig. 11.** Effect of reciprocal SLN stimulation. The number of swallows during reciprocal sub-threshold SLN stimulation was much smaller than in controls. ***$P < 0.001$.

### 3.3. Effect of decerebration on reduced swallowing initiation

The number of swallows during continuous SLN stimulation was compared before and after decerebration; before decerebration, $4.6 \pm 0.5$ for 0–10 s, $2.0 \pm 0.3$ for 10–20 s, $1.2 \pm 0.4$ for 20–30 s, $0.6 \pm 0.2$ for 30–40 s, and $0.6 \pm 0.2$ for 40–50 s ($n = 5$); after decerebration, $4.6 \pm 0.5$ for 0–10 s, $1.6 \pm 0.5$ for 10–20 s, $0.8 \pm 0.4$ for 20–30 s, $0.4 \pm 0.2$ for 30–40 s and $1.0 \pm 0.0$ for 40–50 s ($n = 5$) (Fig. 10). These data indicate there was no significant difference in the number of swallows before and after decerebration.

### 3.4. Effect of subthreshold stimulation on reduced swallowing initiation

The number of swallows evoked by bilateral SLN stimulation significantly decreased following reciprocal application of unilateral sub-threshold SLN stimulation, while there was no difference in the two control values (Fig. 11). The mean number of swallows in pre- and post-control was $4.4 \pm 0.4$ and $3.8 \pm 0.4$, respectively, and $0.6 \pm 0.4$ ($n = 5$) with prior SLN stimulation. These results strongly suggest that reduced swallowing initiation was less dependent on contralateral SLN inputs or the occurrence of repetitive swallows.

## 4. Discussion

Results from the present study showed that continuous SLN stimulation gradually decreased repetitive swallowing, and this reduction was dependent on resting time duration between test stimulations. Prior SLN stimulation also suppressed the swallowing initiation evoked by stimulation of the SLN, RLN, or Cx, while stimulus frequency of prior SLN stimulation affected the reduction in swallowing initiation. There was a strong inhibitory effect of prior SLN stimulation on the subsequent SLN-evoked swallow only on the ipsilateral side. The inhibitory effect of prior SLN stimulation on the RLN-evoked swallow was moderate when compared with the SLN-evoked swallow. Interestingly, the inhibitory effect on the subsequent RLN-evoked swallow on the ipsilateral side was larger than on the contralateral side and the SLN-evoked swallow. However, inhibition was significant on ipsilateral and contralateral sides. Prior SLN stimulation also inhibited subsequent Cx-evoked swallow on both sides, although inhibition was moderate when compared with SLN-evoked swallow (there was no significant difference in the effect between ipsilateral and contralateral sides). Decerebration did not affect swallowing reduction. Finally, our data suggest that there is no relationship between the occurrence of swallows evoked by prior stimulation and subsequent reduction in swallowing initiation, because sub-threshold SLN stimulation affected the subsequent initiation of swallowing.

Rhythmic and repetitive swallowing behavior can occur during physiological activities such as drinking. In fact, animals have been reported to exhibit up to 100 successive swallows, depending on the liquid amount (Doty, 1968). This was also shown under experimental conditions, where the rhythmic pattern is continued during long-lasting SLN stimulation, although the frequency was lower compared with natural swallowing (Jean, 2001). Few studies have examined the effect of continuous pharyngeal stimulation on the reduction in swallowing initiation or signs of fatigue, while repetitive swallowing initiation does not completely return within 50 s, as described in the present study. Electrical stimulation of SLN or RLN has been widely used to evoke swallowing in animals, and this methodology is accepted for investigating the neural mechanisms of swallowing function (Jean, 2001). However, we found a significant difference in the adaptation of swallowing initiation between experimentally evoked swallow and natural swallow. Therefore, differences between the experimental conditions, such as activity patterns of related muscles and neurons in the brain stem and higher centers, as well as changes in autonomic or respiratory responses, should be considered.

With regards to the reduction in swallowing initiation, the following five components may be involved: (1) changes in afferent activity, (2) changes in synaptic activity at primary endings, and changes in excitability of swallowing CPG caused by (3) long-lasting peripheral inputs, (4) sensory input resulting from the swallowing movement, or (5) supramedullary inputs from the higher centers. With respect to the first three components, time-dependent changes of firing discharges in the response properties of afferent SLN fibers during liquid stimulation applied to the pharynx or larynx have been reported (Bartlett and Knuth, 1992; Boushey et al., 1974; Bradley et al., 1983; Rosen et al., 2010). Boushey et al. (1974) also reported potential adaptation of responses in the SLN to natural stimulation in anesthetized cats, while single fibers recorded from the SLN did not show clear adaptation to mechanical stimulation applied to the laryngeal mucosa. Presumably, sustained responses are required to protect the upper airway from boluses such as saliva. Theoretically, one-by-one responses to the electrical single-pulse stimulation applied to the peripheral nerve are not adapted, and 30-Hz stimulation does not cause nerve fatigue within a few seconds.

In the present study, the reduction in swallowing initiation evoked by RLN and cortical stimulation was moderate when compared with SLN-evoked swallows, suggesting that synaptic activity at the level of primary endings in the SLN may be affected by long-lasting prior SLN stimulation. Numerous studies have investigated central afferent projections of the SLN and RLN to the brain stem

in the rat (Furusawa et al., 1996; Hisa et al., 1985; Pascual-Font et al., 2011; Patrickson et al., 1991). Although there are some differences in projective patterns among studies, the afferents from both the SLN and RLN predominantly project to the ipsilateral NTS. Patrickson et al. (1991) investigated central afferent projections of the SLN and RLN in a rat model and found that although the distribution of both terminals was similar, the RLN projections were solely ipsilateral. In addition, there was a marked decrease in the terminal field density compared with SLN projections. Thus, reduction in RLN-evoked swallowing initiation with prior SLN stimulation may be attributed to changes in the responses of common neural components receiving inputs from both the SLN and RLN.

It should be noted that RLN-evoked swallows following contralateral SLN stimulation were also significantly reduced, although prior ipsilateral SLN stimulation produced the greatest effect. Because the RLN projects only to the ipsilateral NTS in the rat (Hisa et al., 1985; Patrickson et al., 1991), the reduction in swallowing initiation is unlikely to be due to adaptation at the level of primary endings or secondary neurons, but rather due to neural adaptation in the swallowing CPG, which receives inputs from left and right sensory neurons.

Although the reason SLN-evoked swallow was unaffected by prior contralateral SLN stimulation remains unclear, Patrickson et al. (1991) reported that WGA-HRP labeled SLN terminal fields were located bilaterally in the NTS, with the ipsilateral being denser and richer than RLN terminal fields. Thus, neural activation in the swallowing CPG, which was evoked by ipsilateral SLN, is less dependent on contralateral SLN inputs, while the RLN-evoked swallow is readily affected by peripheral conditions. This is also the case between the SLN- and glossopharyngeal nerve (GPN)-evoked swallow. In the latter, GPN can elicit swallowing, but the effect is less constant, and swallowing is easily interrupted when compared with the SLN-evoked swallow (Sinclair, 1971). Thus, primary afferents or endings, as well as a part of swallowing CPG, may be involved in the reduction in swallowing initiation following continuous SLN stimulation.

The present study determined a positive relationship between the number of swallows evoked by prior SLN stimulation and the inhibitory rate of swallowing initiation. However, there was a trend for a reduction in the number of swallows following prior SLN stimulation at 30 Hz compared with 40 Hz, while the number of swallows evoked by prior SLN stimulation was not different between 30 and 40 Hz. Additionally, subthreshold conditioning stimulation applied to the SLN, which did not evoke a swallowing reflex, also decreased swallowing initiation. These findings strongly suggest that sensory inputs resulting from swallowing movement contribute less to the reduction in swallowing initiation. In addition, we found no effect of decerebration on swallowing reduction. The current results strongly suggest that supramedullary inputs from the higher centers, such as the motor cortex or insula, are less involved in the swallowing reduction. However, because the animals were not paralyzed in the present study, we cannot completely discard the possibility of an effect of reflexive movements in the oral and pharyngeal regions, such as elementary reflex, on modulation of swallowing initiation (Doty, 1968; Jean, 2001).

It is not surprising that ipsi- or contralateral cortically evoked swallowing was also reduced following continuous SLN stimulation *via* the swallowing CPG. Under physiological conditions, the swallowing CPG receives inputs from higher centers (Doty, 1968; Hamdy et al., 1996; Martin and Sessle, 1993; Miller, 1982) to allow continuous swallowing of a bolus, such as a liquid, for as long as possible. A number of cortical and subcortical sites exist, including the corticofugal swallowing pathway, which can trigger or modify swallowing, in particular the internal capsule, subthalamus, amygdala, hypothalamus, substantia nigra, mesencephalic reticular formation, and monoaminergic brain stem nuclei (for review, see Jean (2001)). Whether these areas act directly on the swallowing CPG in the brain stem, or involve a more complex central pathway, has not yet been clarified.

Topographical projections from the cortex to the NTS in rats have been extensively studied (Saper, 1982; Terreberry and Neafsey, 1983; van der Kooy et al., 1982, 1984). According to these studies, direct projections from the insular cortex to the NTS are bilateral with a contralateral predominance (van der Kooy et al., 1982, 1984), while those from the medial frontal cortex, including the prelimbic or infralimbic cortex, have an ipsilateral predominance (van der Kooy et al., 1982). So far, we have not found any differences in projection sites into the NTS between cortical regions and the SLN or RLN to the NTS. Further studies are required to clarify which sites in the NTS or surrounding areas are involved in the reduction in swallowing initiation.

Finally, it is interesting to speculate why we swallow repeatedly without quick adaptation. It is possible that during natural swallowing in conscious animals, oral processing always happens before swallowing. Although oral sensation itself never evokes swallowing responses (Jean, 2001), oral inputs or boluses in the oral cavity markedly improve the ease of swallowing and change the cortical activity pattern (Hiraoka, 2004) during the swallowing process. Previous physiological and neuroanatomical reports also indicate that NTS neurons and laryngeal motor neurons receive rich inputs from the trigeminal nerve (Kecskes et al., 2013; Zhang et al., 1991). Swallowing initiation may be inhibited during chewing, suggesting that a close interaction exists between oral and pharyngeal functions (Lamkadem et al., 1999; Shiozawa et al., 2012). Thus, trigeminal inputs may have a strong effect on swallowing initiation directly and indirectly through higher centers.

In conclusion, the present findings suggest that primary afferents or endings of peripheral nerves, as well as a part of the central network associated with swallowing in the brain stem, may be involved in the reduction in swallowing initiation during continuous SLN stimulation.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

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