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Effects of pharmacological lesion of the nucleus retroambiguus region on the pharyngeal phase of swallowing

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ABSTRACT

Pharyngeal swallowing is controlled by synaptic interactions within a swallowing central pattern generator (sw-CPG) that is composed of a dorsal and a ventral swallowing group (VSG). Here, we used electrical stimulation (10 s) of the superior laryngeal nerve (SLN; 20 Hz; pulse width: 100 μs) to explore the role of the VSG in an arterially-perfused brainstem preparation of rats. To investigate the effects of pharmacological lesion (local microinjection of an GABA(A)-R agonist) of the nucleus retroambiguus (NRA), a designated component of the VSG, we recorded phrenic (PNA) and vagal nerve (VNA) activities. Control SLN stimulation with stepwise increasing stimulus intensities (from 20 μA to 160 μA) elicited robust suppression of PNA and evoked sequential swallowing activity in the VNA. Lesioning of the NRA had no effect on the pattern of pharyngeal swallowing, but significantly increased the sensory gating of SLN inputs. We conclude that the NRA is not part of the VSG, but appears to have important roles for the central gating of swallowing.

1. Introduction

The pharyngeal phase of swallowing is regulated by the swallowing central pattern generator (sw-CPG) that consists of a distributed neuronal network located in the brainstem (Bautista et al., 2014a; Jean, 2001; Kessler and Jean, 1985; Umezaki et al., 1998). Two major swallowing-related neuron pools have been identified in the dorsal and ventrolateral medulla. The dorsal swallowing group (DSG) is located within the nucleus tractus solitarius (NTS), whereas the ventral swallowing group (VSG) is located within the ventral reticular formation in the vicinity of nucleus ambiguus (N. Amb), which contains the laryngeal motor pool (Amri and Car, 1988; Ezure et al., 1993; Jean, 2001). VSG neurons fire before N. Amb laryngeal motoneurons during swallowing. However, the duration and frequency of VSG neuron firing is reportedly similar to the activities expressed by DSG neurons (Jean, 2001; Jean and Dallaporta, 2006). Importantly, the latency of synaptic responses to electrical stimulation of the superior laryngeal nerve (SLN) or glossopharyngeal nerve (GPN) differs between VSG and DSG neurons. VSG neurons have longer and variable synaptic latencies compared to DSG neurons. Thus, the contemporary view is that DSG neurons generate the swallowing motor pattern, whereas premotor neurons of the VSG relay the swallowing pattern to the various motoneuron pools involved in swallowing (Amri et al., 1990; Jean, 2001; Bautista et al., 2014a,b). However, studies concerned with the role of the VSG are limited to a handful of studies in a variety of mammalian species, such as cats (Ezure et al., 1993) and sheep (Amri and Car, 1988). Thus, the location and function of the VSG remains unclear in rats.

Previously, the perfused brainstem preparation of rats was established as an experimental model to study the central pattern generation of swallowing motor activities *in situ* (Bautista and Dutschmann, 2014; Bautista et al., 2014b; Hashimoto et al., 2019). These studies showed that swallowing activities are observed as discrete bursting activities in cervical vagal nerve recordings. These studies also confirmed a critical role of the DSG in the generation of pharyngeal phase of swallowing. In the present study, we applied electrical stimulation of the superior laryngeal nerve to investigate the role of the VSG in the central generation of pharyngeal swallowing motor patterns in the perfused brainstem preparation of rats. Since the VSG is located close to the laryngeal motor neurons of the N. Amb, The nucleus retroambiguus (NRA) is a brain region that overlaps with the designated location of the VSG (Amri and Car, 1988; Ezure et al., 1993; Jean, 2001). Further, the NRA was previously implicated as an important region for the integration of orofacial behaviors, such as swallowing, with breathing because of its monosynaptic connectivity with the laryngeal adductor motor neurons pools of the N. Amb (Boers et al., 2002). Thus, we microinjected the GABA(A)-receptor agonist isoguvacine in the region of

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the nucleus retroambiguus (NRA) to test the hypothesis that the NRA is a component of the VSG in rats. The present study demonstrates that pharmacological lesion of the NRA has no effect on the generation of the pattern of swallowing, but instead, contributes to the determination of the threshold for sensory gating of swallowing-related SLN inputs.

2. Materials and methods

Experiments were performed at the Florey Institute of Neuroscience and Mental Health (Australia). All experiments were approved by the Florey Animal Ethics Committee. Experimental procedures were performed in accordance with international guidelines for the care and use of laboratory animals.

2.1. Perfused-brainstem preparation

Experiments were performed using the arterially-perfused brainstem preparation as previously described (Paton, 1996). A total of twelve Sprague-Dawley rats (male and female rats; post-natal days: 15–24; Weight: 32–65 g) were used in this study. Briefly, rats were anesthetized by inhalation of isofluorane (2%) until they reached a surgical plane of anesthesia. The rats were then transected below the diaphragm, immediately decerebrated at the pre-collicular level. Next, the lungs and heart were removed (for details, see Dutschmann et al., 2009) and the descending aorta, left phrenic, and left vagal nerves were isolated for later cannulation and recording, respectively. The preparation was then transferred into a recording chamber. The descending aorta was cannulated with a double-lumen catheter for perfusion (16-22 mL/min flow rates) and measurement of perfusion pressure. The preparation was perfused with aCSF (aCSF, in mM: 125 NaCl, 3 KCl, 1.25 KH_2PO_4, 2.5 $CaCl_2$, 1.25 $MgSO_4$, 25 $NaHCO_3$, 10 D-glucose) containing 4.5×10^{-3} g/mL of sucrose for oncotic pressure. The aCSF was then continually bubbled with carbogen (95% O_2/5% CO_2), warmed to 31 °C and delivered to the preparation using a peristaltic pump (Watson and Marlow, 501). The phrenic and vagal nerves were mounted in suction electrodes to measure respiratory motor output. Upon resumption of apneustic respiratory motor output, the preparation was tuned to produce a eupnea-like respiratory pattern by administering a bolus of NaCN (0.1–0.3 mL, 0.2% w/v).

2.2. Nerve recording and superior laryngeal nerve stimulation

To determine the respiratory motor activity of the perfused brainstem preparation, we recorded PNA and VNA. The phrenic nerve was isolated from the diaphragm and pericardia. The vagus nerve was isolated from the carotid artery. PNA and VNA was amplified (10,000×, Warner Instruments, DP-311), filtered (0.01–10 kHz), digitized (AD Instruments, PowerLab 16/35) and stored on a computer using LabChart software (AD Instruments).

To elicit fictive swallowing, we stimulated the superior laryngeal nerve (SLN). The SLN was identified as a branch of cervical vagus at the level of the carotid artery bifurcation. The SLN was isolated from the surrounding connective tissue and mounted on a bipolar suction electrode. During the course of the experiments, the SLN was electrically stimulated (frequency: 20 Hz; pulse width: 100 µs; train length: 10 s) via the suction electrode. For our experiments, we used stepwise increasing stimulus intensities (20, 40, 80 and 160 µA). All stimulus trials were separated by 2 min to avoid evoking SLN stimulation-dependent sensory plasticities.

2.3. Isoguvacine microinjection

At baseline, we measured the pharyngeal swallowing motor pattern evoked by SLN stimulation at various stimulation intensities (2 min interval). Then, we used a single-barrel glass micropipette to perform local microinjections. The pipette was filled with the GABA(A) receptor agonist isoguvacine (10 mM, Sigma–Aldrich) and Chicago sky blue. Microinjections were targeted to the vicinity of the NRA at the following coordinates: 0.5–1.0 mm caudal from calamus, 1.8–2 mm lateral to midline and 1.8–2.2 mm ventral to the brainstem surface. The injected volumes ranged 50–70 nL, and were optically confirmed via the movement of the liquid meniscus. After the experiment, we histologically verified the center of the injection (Chicago sky blue). After the isoguvacine microinjections (5 min), we repeated the SLN stimulation protocol using the same stimulus intensities as in baseline measurements. At the end of the experiment, the brainstem was removed and post-fixed in 4% paraformaldehyde for histological analysis. The brainstem was cut into 50 µm thick transverse consecutive sections of the caudal brainstem using a freezing microtome. The locations of microinjections were documented on semi-schematic drawings of coronal sections of the caudal medulla oblongata.

2.4. Data analysis

Changes in respiratory parameters 1 min before or after isoguvacine microinjection were analyzed using the integrated PNA and VNA signals to determine: (i) inspiratory duration (T(i), time of PNA burst discharge) (ii) respiratory rate (PNA bursts/min), (iii) expiratory duration (T(e), the interval between the cessation of the PNA burst and the onset of the subsequent PNA burst), and (iv) post-inspiratory duration (T(pi), the duration from the peak of VNA discharge at the end of inspiratory PNA until the time point when VNA ceased).

SLN stimulation artifacts were removed by linear interpolation using the recorded stimulus train (TTL pulses from stimulus generator) to determine event times (see Fig. 1). Consistent with previous publications, SLN-stimulation evoked sequential pharyngeal swallowing activity that was identified by repetitive bursts of VNA activity and a cessation of PNA. We sometimes observed bell-shaped bursts of VNA activity of > 1 s duration, particularly at a high intensity SLN stimulations. In accordance with previous publications that defined swallowing activity in the perfused brainstem preparation, we classified these VNA bursts as gag-like activity (see Bautista and Dutschmann, 2014), and did not include these events in subsequent quantitative characterization of swallowing activity.

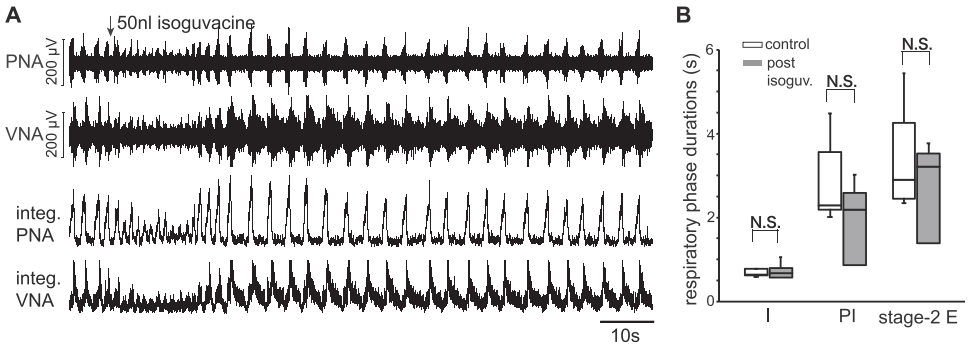

Fig. 1. **(A)** Representative activity patterns of phrenic nerve activity (PNA) and central vagus nerve activity (VNA) before and after isoguvacine microinjection. PNA and VNA was rectified and integrated at a time constant of 0.01 s. **(B)** Group data that summarize the effects of isoguvacine injection on the duration of inspiration (I), post-inspiration (PI) and late-expiration (stage-2 E) of the stationary respiratory motor pattern.

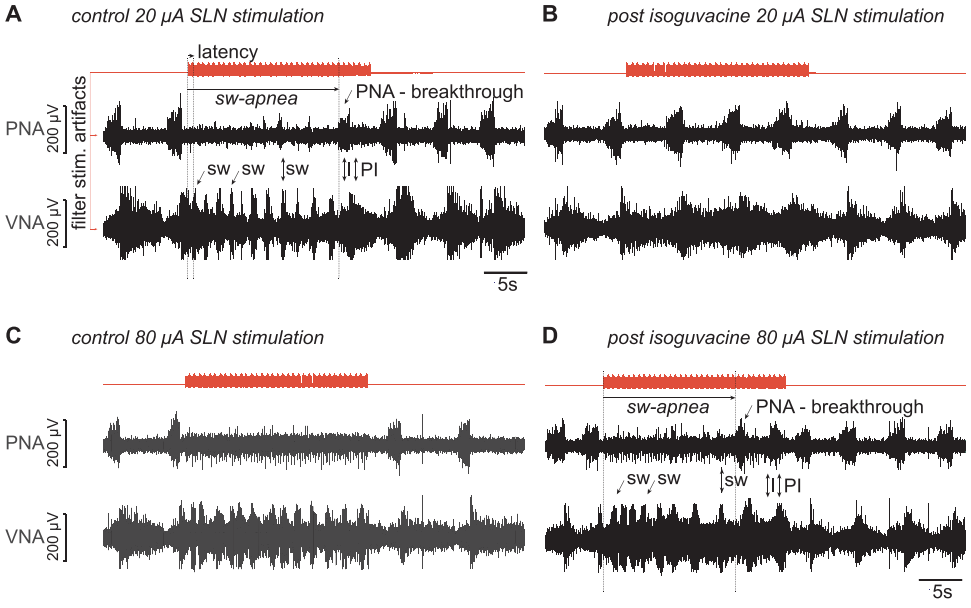

Fig. 2. Original recordings of SLN-evoked changes in respiratory and swallowing activities before and after isoguvacine microinjections. **(A)** A representative example of a 20 µA SLN-stimulation prior to isoguvacine injection is shown. SLN-stimulation (red trace of TTL pulses from the stimulus generator, upper panel) evoked sequential swallow bursts (sw) on VNA are highlighted by arrowheads. The duration of the SLN-evoked sw-apnea is illustrated. The sw-apnea was defined as the duration from the onset of stimulation unitl the onset of the first inspiratory PNA breakthrough. Note that a PNA breakthrough was associated with a VNA burst with a eupnea-like bi-phasic inspiratory and post-inspiratory discharge pattern (see double arrows that indicate inspiration (I) and post-inspiration (PI), panels A and D). **(B)** At 20 µA SLN-stimulation after injection of isoguvacine into the nucleus retroambiguus (see injection 14/2, Fig. 3), SLN-evoked swallowing was absent **(C & D)** These representative traces compare the SLN-evoked swallowing activity and sw-apnea at 80 µA stimulation intensity before and after injection blockade of the NRA in the same preparation. Please note that in baseline conditions, SLN stimulation at 40 µA triggered the same response of sequential swallowing as SLN stimulation at 20 µA (like A). After isoguvacine injection, at 40 µA, no swallowing activity was observed (like in B). Because the threshold stimulus intensity for SLN-stimulation to evoke sequential swallowing was increased to 80 µA after isoguvacine injection into the NRA, 40 µA SLN-stimulation traces are not shown.

The analysis of the potential effects of isoguvacine on swallowing activity included the following measurements: the latency to SLN-evoked swallowing activity, the interval of sequential swallows, the number of evoked swallows and the duration of the SLN-evoked apnea. The latency of SLN-evoked swallowing was defined as the time between the onset of stimulation and the peak of the first VNA burst. The interval of sequential swallowing was measured as the time between the peaks of sequential VNA swallowing bursts. In cases where the 10 s SLN stimulation did not evoke any swallowing activity, we assigned a latency of 10 s to allow for the statistical comparison of this measurement.

All statistical analyses were performed using a paired, two-tailed Student's *t*-test in Microsoft Excel. All statistical data are reported as mean ± standard error.

3. Results

We injected isoguvacine in the vicinity of the NRA ipsilaterally to the stimulated SLN in a total of 12 preparations. In N = 8/12 perfused brainstem preparations, ipsilateral isoguvacine injections evoked significant effects on SLN-evoked swallowing, whereas in the remaining 4 preparations, histologic analysis located isoguvacine microinjections either rostral or caudal to the effective injection sites (see Fig. 3).

3.1. Effects of isoguvacine microinjection on the respiratory motor pattern

Fig. 1**A** shows the time course and discharge pattern of PNA and VNA before, during, and after injection of isoguvacine into the caudal medulla oblongata (10 mM, 50–70 nl). All animals maintained a normal three-phase breathing pattern comprised of inspiration, post-inspiration, and late-expiration. The group data indicate that on average, neither the respiratory rate (pre-injection: 16.3 ± 1.7 PNA bursts/min vs. post-injection: 18.5 ± 2.4 breath/min, p = 0.32), nor the peak amplitude of PNA (pre-injection 0.30 ± 0.05 mV vs. post-injection 0.29 ± 0.06 mV, p = 0.39) showed statistically significant changes after isoguvacine injection. Similarly, other respiratory parameters including Ti (pre-injection: 0.75 ± 0.04 s vs. post-injection: 0.78 ± 0.07 s, P = 0.65), Te (pre-injection: 3.29 ± 0.46 s vs. post-injection: 2.85 ± 0.42 s, P = 0.21) and Tpi (pre-injection 2.50 ± 0.38 s vs. post-injection: 2.23 ± 0.31 s, P = 0.37) also did not show any statistically significant changes after NRA inhibition (Fig. 1**B**, **C**).

3.2. Effects of isoguvacine microinjection on swallowing

Electrical stimulation of the SLN (10 s trains, 20 Hz, at increasing stimulus intensities 20–160 µA) suppressed PNA, either completely or partially, in a stimulus-dependent manner and evoked trains of bursting VNA activity (Fig. 2**A**, **C** & **D**). According to previous publications, the VNA bursting resembled fictive swallowing in the perfused brainstem preparation (Bautista and Dutschmann, 2014; Bautista et al., 2014b). To evaluate the effects of isoguvacine on sequential pharyngeal swallowing activity, we first analyzed the amplitude and duration of a single evoked fictive swallowing bursts in VNA before and after ipsilateral isoguvacine injection. The analyses are summarized in Table 1 and show that isoguvacine injections had no significant effect on the duration of or the amplitude of individual fictive swallowing bursts in VNA at any stimulus intensity. Contrary to the pattern of fictive swallowing in VNA, isoguvacine injections into the caudal medulla had significant effects on the sensitivity of SLN-evoked fictive sequential swallowing evoked at low stimulus intensities (20 and 40 µA). In control conditions, SLN stimulation at all stimulus intensities evoked fictive swallowing in all preparations. However, after isoguvacine microinjections, SLN stimulation with a stimulus intensity of 20 µA did not evoke any fictive swallows (N = 3/8 preparations; see Fig. 1**C**). It should be noted that in these preparations, the control stimulations were apparently close to the threshold for SLN-evoked sequential swallowing, and therefore in all controls stimulation trials, PNA breakthroughs could be observed (Fig. 1**B**). At SLN stimulus intensities of 40 µA or greater, all control stimulation trials evoked fictive swallowing throughout the 10 s stimulation period (see Fig. 2**D** for a representative example at 80 µA). After isoguvacine injection, we observed a significant increase in the SLN stimulation intensity required to elicit sequential pharyngeal swallowing. The increase in the threshold stimulus intensity was accompanied by a significant increase in the latency from stimulus onset to the first SLN-evoked fictive swallow at lower stimulus intensities (20 and 40 µA; Fig. 3**A**). After isoguvacine injection, the latency to SLN-evoked swallowing was prolonged from 0.38 ± 0.04 s to 4.06 ± 1.74 s (p < 0.001) at 20 µA and from

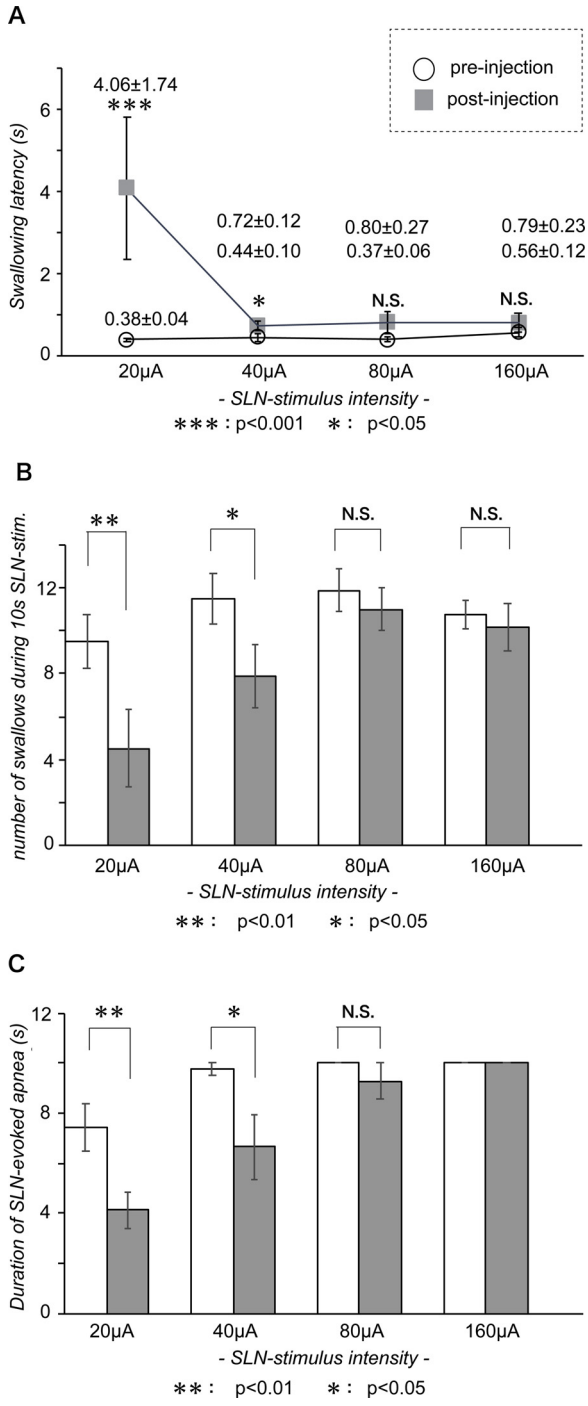

Fig. 3. Group data illustrates the changes in swallowing parameters before and after isoguvacine injection. **A**: Swallowing latency. **B**: Number of swallows. **C**: Duration of swallowing apnea. *: $p < 0.05$, **: $p < 0.01$. ***: $p < 0.001$.

0.44 ± 0.10 s to 0.72 ± 0.12 s ($p < 0.05$) at 40 μA. In addition, the number of SLN-evoked fictive swallows during the 10 s stimulus period was significantly reduced from 9.5 ± 1.22 swallows to 4.5 ± 0.18 swallows ($p < 0.01$) at 20 uA and from 11.38 ± 1.18 to 7.75 ± 1.46 ($p < 0.05$) at 40 uA stimulation (Fig. 3**B**). In correlation with the reduced numbers of fictive swallows, PNA breakthroughs were observed at the lower stimulus intensities (see Fig. 1), which consequently reduced the duration of the SLN-evoked apnea at 20 μA (pre-injection 7.44 ± 0.96 s vs. post-injection 4.13 ± 0.72 s, $p < 0.01$) and at 40 μA (pre-injection 9.75 ± 0.25 s vs. post-injection 6.68 ± 1.29 s, $p < 0.05$; Fig. 3**C**). Remarkably, when sequential fictive swallowing activity (at least 2 consecutive swallows) was triggered at lower stimulus intensities, the duration of the swallow interval showed no significant changes after isoguvacine at 20 μA ($n = 5$, pre-injection 1.15 ± 0.14 s vs. post-injection 1.14 ± 0.16 s; $p = 0.22$) and at 40 μA ($n = 7$, $p = 0.15$). Consequently, at higher stimulation intensity the duration of inter-swallow intervals also remained unchanged after isoguvacine compared to control (at 80 μA: pre-injection: 0.86 ± 0.09 s vs. post-injection: 0.85 ± 0.06 s, $p = 0.34$; at 160 μA: pre-injection 0.93 ± 0.05 s vs. post-injection 0.99 ± 0.09 s, $p = 0.50$).

3.3. Location of the injection sites

Fig. 4 depicts the anatomical location of the ipsilateral injection sites on semi-schematic drawings of the caudal medulla oblongata. All effective injections sites (black and grey dots) were placed at close vicinity of the nucleus retroambiguus (NRA) at the most caudal parts of the medulla oblongata. Injections more rostral or caudal to the NRA-hotspot were ineffective and did not alter SLN-evoked suppression of the respiratory rhythm or swallowing.

4. Discussion

In the present study, we investigated the caudal ventrolateral medulla oblongata as a potential candidate for the location of the ventral swallowing group in rodents. The study reveals that pharmacological lesion of the nucleus retroambiguus (NRA) in the caudal medulla oblongata significantly changed the threshold for superior laryngeal nerve (SLN)-stimulation evoked swallowing, but had no significant effects on the general pattern of pharyngeal swallow-related bursts on the vagus nerve.

4.1. Stimulation of the superior laryngeal nerve elicits sequential swallowing in situ

A previous study that employed the perfused brainstem preparation to investigate the coordination of respiration and swallowing used water injections into oro-pharyngeal cavity (Bautista and Dutschmann, 2014). In that study, oral water injections reliably triggered sequential swallowing superimposed on a tonic post-inspiratory motor discharge. This tonic discharge is indicative of laryngeal adduction that prevents aspiration (see Bautista and Dutschmann, 2014). Here, we used electrical stimulation of the SLN to evoke swallowing in the perfused brainstem preparation (Hashimoto et al., 2019). Supra-threshold SLN-

Table 1
A summary of changes in swallowing duration and amplitude before and after isoguvacine injection.

	Swallowing duration(s)		P value	Swallowing amplitude(mV)		%	P value
	Control	After injection		Control	After injection		
20 μA	0.33 ± 0.02	0.29 ± 0.04	P = 0.42	0.41 ± 0.04	0.42 ± 0.07	93.0 ± 3.3	P = 0.08
40 μA	0.38 ± 0.02	0.36 ± 0.02	P = 0.17	0.49 ± 0.06	0.45 ± 0.04	92.9 ± 4.1	P = 0.12
80 μA	0.35 ± 0.02	0.36 ± 0.02	P = 0.35	0.51 ± 0.06	0.45 ± 0.05	91.5 ± 4.8	P = 0.08
160 μA	0.39 ± 0.02	0.39 ± 0.02	P = 0.94	0.6 ± 0.11	0.48 ± 0.05	96.4 ± 5.3	P = 0.52

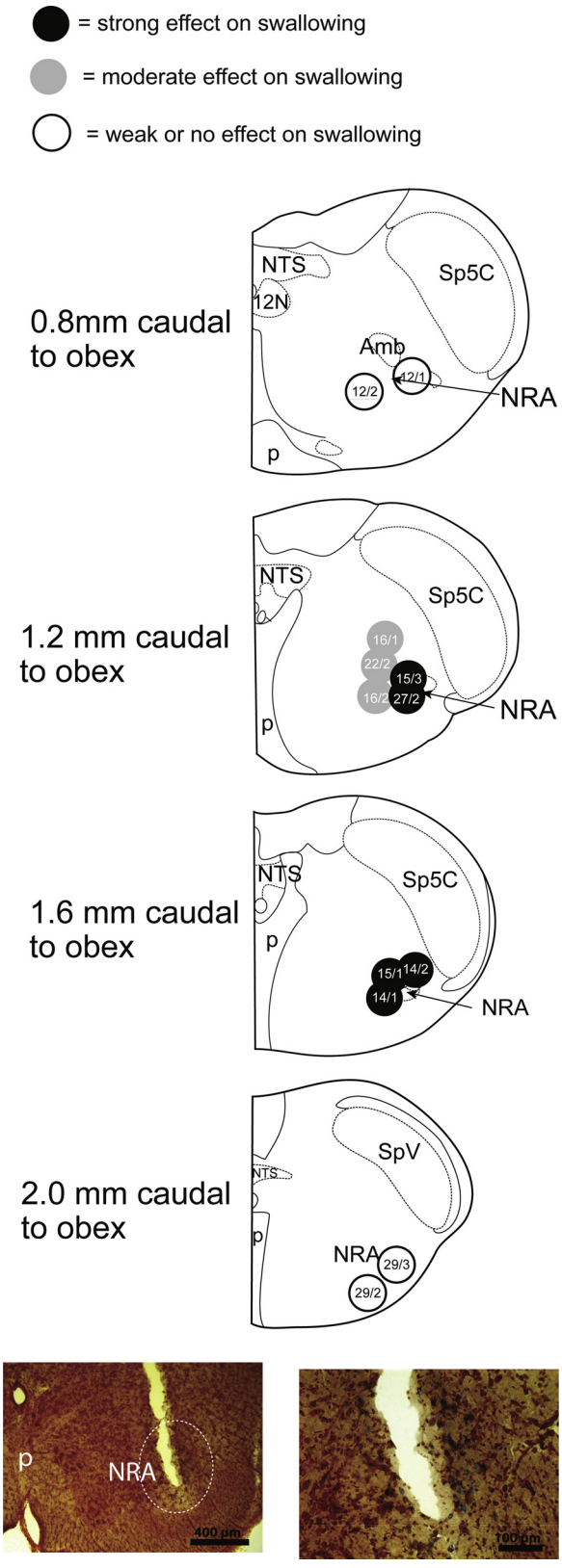

Fig. 4. Semi-schematic drawing of transverse sections of the most caudal aspects of the medulla oblongata that show the location of the Nucleus retroambiguus (NRA). White circles demark ineffective injections sites, gray circles represent sites where the injections affected the threshold of SLN-evoked swallowing only at a stimulus strength of 20 µA (a weak effect), and black circles represent sites where the isoguvacine injections consistently changed the threshold of SLN-evoked swallowing and duration of swallowing apnea at 40 µA. Photomicrographs in the bottom panel illustrate the location of a representative injection in a coronal section of the caudal brainstem. *Abbreviations:* Amb = Nucleus ambiguus, NRA = Nucleus retroambiguus; NTS = Nucleus of the solitary tract; p = pyramidal tract; Sp5C = caudal nucleus of the spinal trigeminal tract; XII = hypoglossal motor nucleus.

stimulation *in situ* evoked on average between 9–12 sequential swallow bursts during the 10 s stimulus train in a stimulus-dependent manner (e.g. 40 µA and higher). The evoked swallowing pattern was in line with previous reports of SLN-evoked swallowing activity in decerebrate or anesthetized animal preparations of various mammalian species (Dick et al., 1993; Oku et al., 1994; Umezaki et al., 1998; Harada et al., 2005; Sugiyama et al., 2011, 2015). While the tonic laryngeal discharge was evoked by SLN-stimulation (e.g. see Fig. 1**B**), the tonic activity was confounded by stimulus artifacts and was not further analyzed in the present study.

4.2. The role of the NRA in the coordination of breathing and swallowing

In accordance with previous studies, injection of the GABA(A)-receptor agonist into the area of the NRA did not significantly change the baseline breathing pattern (Jones et al., 2016). In earlier studies, the NRA area, at the transition between the caudal medulla oblongata and cervical spinal cord, was associated with a latent respiratory rhythm generator (Oku et al., 2008; Jones et al., 2012). We hypothesized that the NRA might be part of the ventral swallowing group (VSG) of the ventral reticular formation (vRF) in the vicinity of nucleus ambiguus (Amri and Car, 1988; Ezure et al., 1993; Jean, 2001). However, the present results show that pharmacological inactivation (GABA(A) receptor-mediated local hyperpolarization) of the NRA did not affect the generation nor the pattern of sequential swallows. Since the VSG contains critical premotor populations that relay the swallowing pattern to the various pools of motoneurons (Amri et al. 1990, Jean, 2001; Bautista et al., 2014a), a blockade of the VSG should have changed the pattern and/or the reliability of SLN-evoked swallowing sequences. These findings are consistent with a previous study that showed no effect of NRA inactivation on SLN-evoked swallowing (Umezaki et al., 1997). However, in the present study, we also observed that the suppression of NRA neuronal activity selectively changed the gain of the SLN input to the swallowing pattern generator. The latter was indicated by lower numbers of swallows and a reduced duration of the swallowing apnea at lower stimulus intensities compared to control stimulations. These findings suggest the presence of a classic inhibitory feedback loop between the NRA and the sensory relay neurons of the NTS. Indeed, such connectivity was previously demonstrated via the axonal projections of swallowing-related neurons of the reticular formation to NTS (Sugiyama et al., 2011), which in turn contains the primary pool of sensory relay neurons. Alternatively, identified monosynaptic projections from the NRA to laryngeal constrictor motorneurons of the nucleus ambiguus (Boers et al., 1992) could also decrease the excitability of laryngeal motor pool. Such a reduction in the excitability would in turn increase the threshold for the SLN-evoked generation of the sequential swallowing pattern at the motorneuron level.

The precise nature of the NRA-mediated gain control of sensory-evoked swallowing needs to be further explored in future experiments. Interestingly, the coordinated interaction between the coughing and swallowing pattern generators is considered to generate a protective meta-behavior that prevents aspiration (Pitts et al., 2013). It was shown that codeine, a potent cough suppressant significantly reduced cough-induced c-fos expression in the NRA (Gestreau et al., 1997). Thus, we speculate that an overarching role of the NRA is concerned with the gain control of various oropharyngeal behaviors such coughing swallowing and vocalizing.

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