Role of the retrotrapezoid nucleus/parafacial respiratory group in coughing and swallowing in guinea pigs

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Sugiyama Y, Shiba K, Mukudai S, Umezaki T, Sakaguchi H, Hisa Y. Role of the retrotrapezoid nucleus/parafacial respiratory group in coughing and swallowing in guinea pigs. J Neurophysiol 114: 1792–1805, 2015. First published July 22, 2015; doi:10.1152/jn.00332.2015.—The retrotrapezoid/parafacial respiratory group (RTN/pFRG) located ventral to the facial nucleus plays a key role in regulating breathing, especially enhanced expiratory activity during hypercapnic conditions. To clarify the roles of the RTN/pFRG region in evoking coughing, during which reflexive enhanced expiration is produced, and in swallowing, during which the expiratory activity is consistently halted, we recorded extracellular activity from RTN/pFRG neurons during these fictive behaviors in decerebrate, paralyzed, and artificially ventilated guinea pigs. The activity of the majority of recorded respiratory neurons was changed in synchrony with coughing and swallowing. To further evaluate the contribution of RTN/pFRG neurons to these nonrespiratory behaviors, the motor output patterns during breathing, coughing, and swallowing were compared before and after brain stem transection at the caudal margin of RTN/pFRG region. In addition, the effects of transection at its rostral margin were also investigated to evaluate pontine contribution to these behaviors. During respiration, transection at the rostral margin attenuated the postinspiratory activity of the recurrent laryngeal nerve. Meanwhile, the late expiratory activity of the abdominal nerve was abolished after caudal transection. The caudal transection also decreased the amplitude of the coughing-related abdominal nerve discharge but did not abolish the activity. Swallowing could be elicited even after the caudal end transection. These findings raise the prospect that the RTN/pFRG contributes to expiratory regulation during normal respiration, although this region is not an essential element of the neuronal networks involved in coughing and swallowing.

Address for reprint requests and other correspondence: Y. Sugiyama, Dept. of Otolaryngology-Head and Neck Surgery, Kyoto Prefectural Univ. of Medicine, 465 Kaji-cho, Kamigyo-ku, Kyoto City 602-8566, Japan (e-mail: yoichiro@koto.kpu-m.ac.jp).
rons that can control active expiration, are in synchrony with cough-related nerve activity, the removal of this region should attenuate the cough reflex and in particular reduce the cough-related abdominal activity. We assumed that if the activity of these neurons is altered during swallowing, the motor activity during swallowing could be influenced by their removal (Abdala et al. 2009). We therefore examined the effect of transverse brain stem transection at the rostral- and caudal-most levels of the RTN/pFRG on these behaviors.

**Glossary**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABD</td>
<td>Abdominal nerve</td>
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<tr>
<td>E</td>
<td>Expiratory phase</td>
</tr>
<tr>
<td>E-aug</td>
<td>Expiratory neurons with an augmenting firing pattern</td>
</tr>
<tr>
<td>E-dec</td>
<td>Expiratory neurons with a decrementing firing pattern</td>
</tr>
<tr>
<td>EI</td>
<td>Expiratory-inspiratory phase-spanning neurons</td>
</tr>
<tr>
<td>I</td>
<td>Inspiratory phase</td>
</tr>
<tr>
<td>I-aug</td>
<td>Inspiratory neurons with an augmenting firing pattern</td>
</tr>
<tr>
<td>IE</td>
<td>Inspiratory-expiratory phase-spanning neurons</td>
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<tr>
<td>Inst Freq</td>
<td>Instantaneous frequency</td>
</tr>
<tr>
<td>ML</td>
<td>Lateral to the midline</td>
</tr>
<tr>
<td>NA</td>
<td>Nucleus ambiguus</td>
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<tr>
<td>PH</td>
<td>Nucleus prepositus hypoglossi</td>
</tr>
<tr>
<td>PHR</td>
<td>Phrenic nerve</td>
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<tr>
<td>PON</td>
<td>Preolivary nucleus</td>
</tr>
<tr>
<td>PPR</td>
<td>Postpyramidal nucleus of the raphe</td>
</tr>
<tr>
<td>RB</td>
<td>Restiform body</td>
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<tr>
<td>RLN</td>
<td>Recurrent laryngeal nerve</td>
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<tr>
<td>RLN stim</td>
<td>Stimulation of the RLN</td>
</tr>
<tr>
<td>RTN/pFRG</td>
<td>Retrotrapezoid/parafacial respiratory group</td>
</tr>
<tr>
<td>SLN</td>
<td>Superior laryngeal nerve</td>
</tr>
<tr>
<td>SLN stim</td>
<td>Stimulation of the SLN</td>
</tr>
<tr>
<td>SO</td>
<td>Superior olive</td>
</tr>
<tr>
<td>SOM</td>
<td>Medial nucleus of the superior olive</td>
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<tr>
<td>Trachea stim</td>
<td>Stimulation of the tracheal mucosa</td>
</tr>
<tr>
<td>VLD</td>
<td>Lateral vestibular nucleus, dorsal division</td>
</tr>
<tr>
<td>5SP</td>
<td>Spinal trigeminal nucleus</td>
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<tr>
<td>5ST</td>
<td>Spinal trigeminal tract</td>
</tr>
<tr>
<td>7N</td>
<td>Facial nucleus</td>
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</table>

**MATERIALS AND METHODS**

All experimental procedures conformed to the Physiological Society of Japan Principles for the Care and Use of Animals and were approved by the University Committee for the Use of Animals in Research.

**General Surgical Procedures**

Experiments were performed on 37 purpose-bred adult male guinea pigs (Hartley, Shimizu Laboratory Supplies, Kyoto, Japan) weighing 500–800 g. Animals were anesthetized with isoflurane (4% for induction, 1.0–2.0% for maintenance) vaporized in 100% O₂. The level of anesthesia was titrated such that the mean blood pressure, which was monitored using a catheter inserted into the common carotid artery, was maintained at <100 mmHg. Sufficient anesthesia was given to prevent spontaneous and reflexive movements. The trachea was intubated, and a cannula was placed in a femoral vein for drug administration. Dexamethasone (1 mg/kg) and atropine (0.1 mg/kg) were injected intramuscularly to minimize brain edema and to decrease airway secretions, respectively. Rectal temperature was maintained at 37–38°C using a DC-powered heating pad. Bipolar silver cuff electrodes were placed around the L1 abdominal nerve (ABD) and phrenic nerve (PHR) for recording, and the superior laryngeal nerve (SLN) and facial nerve for stimulation, and the recurrent laryngeal nerve (RLN) for both recording and stimulation on both sides, and covered with Vaseline and mineral oil.

The animals were placed in a stereotaxic frame and were decerebrated at the precollicular level. An occipital craniotomy was then performed to expose the dorsal aspect of the brain stem. The caudal portion of the cerebellum was gently retracted and partially aspirated to visualize the obex and the rostral part of the medulla. After the surgery was completed, anesthesia was discontinued. Subsequently, animals were paralyzed using intravenous injections of vecuronium bromide (Fuji Pharma, Tokyo, Japan; initial injection of 0.3 mg/kg, maintained by hourly injections of 0.15 mg/kg) and artificially ventilated with room air. A bilateral pneumothorax was performed to reduce ventilation-related brain movements. End-tidal CO₂ was monitored using an end-tidal CO₂ analyzer (Capstar-100 CO₂ Analyzer, CWE, Ardmore, PA). Tidal volume and ventilation frequency were adjusted to maintain end-tidal CO₂ at 3–5% (i.e., normal conditions) throughout the experimental period, except during recordings. The optimal end-tidal CO₂ level at which “active expiration” was produced was determined before a series of recording sessions was started. The ventilator setting was kept constant during a series of recording sessions. The mean blood pressure was kept above 80 mmHg using an intravenous infusion of epinephrine in saline at the rate of 0.005–0.01 mg·kg⁻¹·min⁻¹, if hypotension occurred. At the end of the experiment, animals were perfused transcardially with 300 ml of physiological saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain stem was removed and postfixed in the same solution.

**Recording Procedures**

Penetrations of the electrode for recording were conducted 3–5.5 mm rostral to the obex and 1.5–3.0 mm lateral to the midline, using a glass micropipette filled with 3 M KCl (tip impedance 5–10 MΩ). Activity recorded extracellulary by the microelectrode was amplified (MEZ-8301, Nihon Kohden, Tokyo, Japan) and sampled at 15,000 Hz using a Power 1401 mk 2 data collection system and Spike 2 version 6 software (Cambridge Electronic Design, Cambridge, UK). Activity of each nerve recorded was amplified by a factor of 10,000, filtered with a band pass of 100-10,000 Hz, integrated with a 1-ms time constant, full-wave rectified, and sampled at 2,000 Hz.

Since the RTN/pFRG region is positioned ventral to the facial nucleus (Connelly et al. 1989; Onimaru and Homma 2003), we used antidromic field potentials of facial motoneurons as a physiological landmark for identification of RTN/pFRG neurons. To identify the location of the facial nucleus, we inserted the electrode with a 0.1 mm step in the rostrocaudal and mediolateral direction and identified the antidromic field potentials evoked by the stimulation of the facial motor nerve at the intensity of 0.08–2 mA. When the amplitude of the field potential decreased to <10% of the maximal value, we considered that the electrode was outside of the facial nucleus. Collision testing was performed in some neurons, whose activities were recorded in the area where the antidromic evoked potential induced by the facial nerve stimulation was simultaneously recorded.

We recorded spontaneous activity along with the phrenic nerve discharges to determine whether the neuron in this area had respiratory-related activity. Subsequently, we recorded the neuronal activity during fictive coughing and swallowing. Fictive coughing was evoked by stimulating the afferent fibers of the RLN electrically (pulse...
duration, 0.2 ms; frequency, 10–20 Hz, intensity 40–80 μA), or by
irrigating the tracheal mucosa with a silicon tube through the trache-
ostoma, and identified by a series of neural activities consisting of the
preceding phrenic nerve discharge followed by the RLN and abdomi-
nal nerve burst, as described elsewhere (Bolser 1991; Grélot and
Milano 1991; Shiba et al. 1999; Sugiyama et al. 2014).

Fictive swallowing was identified by bursting activity of the RLN,
which was elicited by electrical stimulation of the SLN (pulse dura-
tion, 0.2 ms; frequency, 10–20 Hz, intensity, 50–80 μA) (Nishino et
al. 1985; Sugiyama et al. 2011, 2014). The activity of the hypoglossal
nerve was not recorded in the present study. Some recording sites
were marked by electrolytic lesions through the recording electrode (a
constant current of 12 μA for 30 min). Spike detection and sorting
feature of the Spike2 software were used such that the accuracy of
detection of each unit activity was secured. We calculated the max-
mum firing frequency of respiratory-related RTN/pFRG neurons in
each phase by averaging the occurrence of spikes within 0.2 s during
which the instantaneous frequency reached maximum. We then com-
pared the peak firing rate of each neuron during control respiration
with that during coughing and swallowing using a Wilcoxon signed-
rank test with a theoretical median of 1. The control trial rate data
were taken from cycles just prior to the stimulation that induced
cooking or swallowing. When continuous infusion of epinephrine
was needed, the data were discarded because of the possible epineph-
rine-induced influence on respiratory regulation during the nonres-
piratory behaviors as well as during respiration (Arata et al. 1998;
Viemari 2008; Yamanishi et al. 2010).

**Table 1**

<table>
<thead>
<tr>
<th>Neuron Type</th>
<th>Number</th>
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<tbody>
<tr>
<td>E-aug</td>
<td>15/18</td>
</tr>
<tr>
<td>E-dec</td>
<td>3/18</td>
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</tbody>
</table>

**Histological Procedures and Data Analyses**

Transverse tissue sections of the brain stem of animals, for which
unit recordings and lesioning were performed, were made using a freezing
microtome at 50 μm, and were stained with neutral red. Meanwhile, the brain stem of animals used in the transection exper-
iments was cut sagittally at 100 μm, and were stained with cresyl
violet. As such, we confirmed that the brain stem was precisely
transected at the rostral- and caudalmost levels of the facial nucleus.
Photographs of brain stem sections were captured using a CCD digital
camera (DP21, Olympus) mounted on an upright microscope (BX51,
Olympus, Tokyo, Japan) and were assembled using PTGui-Pro pho-
tostitching software (New House Internet Services BV, The Nether-
lands). Adobe Illustrator software (Adobe systems, San Jose, CA) was
used for drawing these sections. Recording sites were reconstructed
on these drawings with reference to the locations of electrical lesions,
the relative positions of electrode tracks, and microelectrode depths.
Statistical analyses were performed using Prism 5 software (Graph-Pad
Software, San Diego, CA). The data were analyzed using the nonpara-
metric one-way ANOVA with Dunn’s multiple comparison test after the
Kruskal-Wallis test. Pooled data are presented as means ± 1 SE. A P
value of <0.05 was considered significant.

**RESULTS**

**Activity of the Respiratory-Related Neurons in the
RTN/pFRG During Coughing and Swallowing**

Extracellular neuronal activity was recorded from 109 RTN/
pFRG cells in 25 animals, which consisted of 29 expiratory, 55
inspiratory, and 25 phase-spanning neurons. The expiratory
neurons were subdivided into the following two types based on their firing patterns: augmenting (E-aug) (n = 18) and decre-
menting (E-dec) (n = 11) firing patterns. All 55 recorded inspiratory neurons displayed an augmenting discharge pattern
(E-aug). The phase-spanning neurons were subdivided into the
inspiratory-expiratory (IE) (n = 5) and expiratory-inspiratory
(EIF) (n = 20) neurons. Maximal firing frequencies of these
neurons are listed in Table 1. Of all the neurons recorded, 31
(30%) neurons were tested for collision by stimulating the
dermal nerve. The maximal amplitudes of the phrenic
nerve activity, the initial burst of the cough-induced RLN activity,
and the abdominal nerve activity were calculated. In addition, we
measured the duration and changes in the amplitude of the swal-
low-induced RLN activity. The values were averaged for three
trials.

The RLN exhibited a bimodal activity during the expiratory
phase of coughing. The RLN began to burst at the transition
from the inspiratory to expiratory phase (C1 phase). After this
bursting activity, the RLN activity was transiently decreased
(C2 phase) and then it burst again (C3 phase). As indicated in
Table 1, most 15/18) of the E-aug neurons were activated
during the expiratory phase of coughing, all but one of which
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(EIF) (n = 20) neurons. Maximal firing frequencies of these
neurons are listed in Table 1. Of all the neurons recorded, 31
(30%) neurons were tested for collision by stimulating the
face nerve. The maximal amplitudes of the phrenic
nerve activity, the initial burst of the cough-induced RLN activity,
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from the inspiratory to expiratory phase (C1 phase). After this
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(C2 phase) and then it burst again (C3 phase). As indicated in
Table 1, most 15/18) of the E-aug neurons were activated
during the expiratory phase of coughing, all but one of which
fired prominently during the C2 phase (Fig. 1A). Their firing frequency during the C2 phase was significantly higher than that of control expiration ($P < 0.01$) (Table 1). During swallowing, approximately three-quarters of the E-aug neurons (13 of 18) ceased firing (Fig. 1B), and the remaining one-quarter were activated (5 of 18) (Fig. 1C). The firing rate of the swallow-active E-aug neurons during swallowing did not show a significant increase compared with that during control respiration.

The majority of E-dec neurons (9 of 11) were activated during coughing, in which 7 and 2 cells were active during the C1 (Fig. 2B) and C2 phases, respectively; the firing frequency during coughing was not significantly higher than that of control expiration. Most (9 of 11) of the E-dec neurons fired during the swallow-related RLN burst (Fig. 2C); the frequency was not significantly different from that during control respiration.

During coughing, about half (27 of 55) of the I-aug neurons were activated only during the inspiratory phase (Fig. 3A). Meanwhile, the remaining I-aug neurons exhibited biphasic activity during coughing, which consisted of augmented inspiratory activity followed by the expiratory discharge with a relatively higher activity in the C1 (Fig. 3B) or C2 (Fig. 3C) phase. During swallowing, the firing of all I-aug neurons was ceased.

Most (22 of 25) of the phase-spanning neurons were activated during the expiratory phase of coughing. About one-half of them fired predominantly during the C1 phase (3 of 5 IE, and 9 of 20 EI neurons) (Figs. 4, A and D), while the other half fired predominantly during the C2 phase (2 of 5 IE, and 8 of 20 EI neurons) (Fig. 4, B and E). In the latter EI neurons, the firing frequency during the C2 phase was significantly higher than that of control respiration ($P < 0.05$). More than half of the phase-spanning neurons discharged during swallowing (Fig. 4, C and F). Only one neuron, represented in Fig. 4C, was orthodromically excited to electrical stimulation of the ipsilateral SLN. There was no significant difference between the firing rates of these neurons during swallowing and those during control respiration. Meanwhile, the comparison across the neuron types during each phase of coughing and swallowing did not show any significant differences.

Figure 5 illustrates the locations of a total of 93 neurons in the RTN/pFRG whose activity was analyzed during coughing and swallowing. The symbols of different shapes indicate the 16 E-aug units, 8 E-dec units, 48 I-aug units, 4 IE units, and 17 EI units. These neurons were located 1.9–2.91 mm (average of 2.3 ± 0.02) from the midline, 0.09–1.0 mm (average of 0.4 ± 0.02) from the ventral surface, and −1.45 to 0.35 mm (average of −0.66 ± 0.04) anterior to the most caudal portion of the superior olive. The E-aug neurons were significantly distributed more caudally than the I-aug neurons ($P < 0.01$). Other types of neurons were intermingled in the RTN/pFRG. In the area ventromedial to the facial nucleus presumably corresponding to the RTN (Connelly et al. 1990) and its vicinity, where CO2-chemosensitive neurons, such as the Phox2b and the vesicular glutamate transporter 2 immunoreactive neurons are located (Guyenet 2008; Mulkey et al. 2004), 32 respiratory neurons were recorded (11 E-aug, 1 E-dec, 13 I-aug, 1 IE, and 6 EI). A total of about 60% of the E-aug neurons was found to be located in this portion of the RTN/pFRG, showing a significantly higher distribution compared with the other portion (Fisher’s exact test, $P < 0.01$).

Influences of Brain Stem Transections on Motor Nerve Activities During Respiration, Coughing, and Swallowing

Discharge patterns of the laryngeal, abdominal, and phrenic motor nerves during respiration, coughing, and swallowing were recorded before and after transection at the rostral- or caudalmost level of the RTN/pFRG region (Figs. 6 and 7). Of 12 animals, 10 were tested for the effect of removal of the pons by brain stem transection at the rostralmost level of the RTN/pFRG region. After the test of rostral-end transection, the influences of the transection along the caudalmost border were further analyzed in 8 animals. In the other 2 animals, only caudal level transection was examined. The end-tidal CO2 level at which active expiration was induced was 5.5–7%.

The respiratory rates before transection, after rostral section, and after caudal section were 15.7 ± 1.0, 10.5 ± 1.4, and 6.27 ± 0.9 breaths/min, respectively. The durations of the inspiratory phase of respiration before, after rostral, and after caudal transection were 0.77 ± 0.1, 2.40 ± 0.4, and 1.54 ± 0.6, while the expiratory durations were 3.24 ± 0.2, 4.31 ± 0.6, and 11.9 ± 2.6 s, respectively. The transection at the rostralmost level of the RTN/pFRG induced a significant decrease in respiratory rates, and the caudalmost transection exhibited a further decrease ($P < 0.01$). The expiratory duration was significantly prolonged by the cau-

Table 1. Maximal firing frequency and activities of the respiratory-related neurons in the retrotrapezoid/parafacial respiratory group

<table>
<thead>
<tr>
<th>Neuron type</th>
<th>Maximal Firing Frequency, spikes/s</th>
<th>Coughing</th>
<th>Swallowing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1 phase</td>
<td>C2 phase</td>
<td>Expiratory phase</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>Silent</td>
<td>Active</td>
</tr>
<tr>
<td>E-aug (n = 18)</td>
<td>34.4 ± 5.2</td>
<td>82.9 (1)</td>
<td>94.4 ± 20.3* (14)</td>
</tr>
<tr>
<td>E-dec (n = 11)</td>
<td>20.8 ± 2.3</td>
<td>53.5 ± 19.7 (7)</td>
<td>24.6 ± 9.2 (2)</td>
</tr>
<tr>
<td>I-aug (n = 55)</td>
<td>47.1 ± 3.3</td>
<td>(13)</td>
<td>(15)</td>
</tr>
<tr>
<td>IE (n = 5)</td>
<td>24.3 ± 3.4</td>
<td>40.4 ± 16.4 (3)</td>
<td>40.3 ± 12.9 (2)</td>
</tr>
<tr>
<td>EI (n = 20)</td>
<td>22.5 ± 3.6</td>
<td>40.6 ± 10.3 (9)</td>
<td>55.2 ± 9.6* (8)</td>
</tr>
<tr>
<td>Total (n = 109)</td>
<td>(33)</td>
<td>(41)</td>
<td>(35)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. Regarding the cough-related activity, the values in parentheses indicate the number of neurons whose peak firing rates were in phase with C1 and C2 during coughing, and those that did not discharge throughout the expiratory phase. For I-aug neurons, the term “active neurons” indicates neurons that discharged during both the inspiratory and expiratory phases, while “silent neurons” designates neurons that fired only during the inspiratory phase. Meanwhile, the number of neurons that were active and silent during swallowing are designated in parentheses. For expiratory and phase-spanning neurons, the maximal firing rates during each phase of coughing or during swallowing are indicated. C1 phase, the period during which the recurrent laryngeal nerve (RLN) strongly discharged just after the cough-related inspiration; C2 phase, the duration of the transient attenuation of the RLN activity during the expiratory phase of coughing. *Significant deference from control respiration. See Glossary for additional abbreviations.

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before transection were 79.7 ± 4.7 and 74.7 ± 6.2, whereas the percentages of amplitudes of the RLN activity were 57.3 ± 3.1 and 56.3 ± 2.7, respectively. The amplitudes of the abdominal nerve activity were decreased to 93.8 ± 4.1 and 15.6 ± 2.4% of control levels by rostral- and caudal-level sectioning. The activity of the phrenic nerve and RLN was significantly decreased by the rostralmost sectioning, while the abdominal nerve activity showed a significant decrease after the transection at the caudalmost level of the RTN/pFRG (P < 0.01). As such, during respiration, the rostral-level sectioning attenuated the postinspiratory activity of the RLN, with its inspiratory activity decreased in all tested animals (Fig. 6D). Meanwhile, the enhanced activity of the abdominal nerve in the late expiratory phase induced by hypercapnic ventilation was preserved after the rostral level section, but was abolished after the caudal level section in all tested animals (Fig. 6D). The abdominal nerve activity was not enhanced by an increase of CO₂ concentration of up to 10% after caudal transection.

In all animals tested, a series of cough-related discharge patterns of the RLN, abdominal, and phrenic nerves was preserved after the rostralmost transection (Fig. 6D). The caudal-level sectioning preserved the cough-related activity pattern in the RLN in 3 animals (middle column in Fig. 6D) but altered its bimodal discharge pattern to a single peak one by eliminating the C1-specific activity (right column in Fig. 6D) in 5 animals. The durations of the inspiratory phase of coughing before and after rostral, and caudal transections were 0.69 ± 0.04, 1.65 ± 0.3, and 0.86 ± 0.08 s, whereas the durations of the C1 phase were 0.28 ± 0.01, 0.37 ± 0.05, and 0.46 ± 0.14 s, respectively. The durations of the cough-related abdominal burst before the transection, and after rostral, and caudal transections were 0.57 ± 0.03, 0.88 ± 0.07, and 1.21 ± 0.17 s, respectively. On the other hand, the amplitudes of the phrenic nerve activity before rostral and after caudal transections were decreased to 74.0 ± 7.7 and 75.2 ± 10.6% relative to those before the transection, whereas those of the RLN activity during the C1 phase were reduced by 95.3 ± 3.8 and 74.3 ± 5.9, respectively. The amplitudes of the abdominal nerve activity during coughing were reduced by 92.0 ± 5.0 and 71.0 ± 3.7% due to the rostral and caudal transections, respectively. The phrenic nerve activity showed no significant changes in either duration or amplitude. The activity of the RLN was significantly reduced by the caudalmost transection of the RTN/pFRG (P < 0.01), albeit no statistical difference was observed regarding the duration. Meanwhile, the period of the cough-related abdominal burst was significantly prolonged in 5 animals. The durations of the inspiratory phase of coughing before and after rostral, and caudal transections were 0.40 ± 0.02, 0.42 ± 0.03, and 0.46 ± 0.04 s, respectively. The percentages of amplitudes of the RLN activity after rostral- and caudal-most transections compared with those before transection were 98.4 ± 5.6 and 83.0 ± 4.5, respectively. The RLN had a tendency to decrease in amplitude as a result of the caudalmost transection, although there was no significant difference between before and after the transection.
In the animals tested for only the caudalmost sectioning, the effects of the transection were the same as the results described above. The precise location of sectioning relative to surrounding regions such as the caudal tip of the RTN/pFRG and the rostral pole of the retrofacial nucleus seemed to yield uncertain results, since the cutting lines identified on the consecutive sections were likely to deteriorate due to brain tissue damage and bleeding.

**DISCUSSION**

The major finding of the present study is that although the activity of all types of the respiratory-related RTN/pFRG neurons was changed in synchrony with coughing and swallowing, the removal of this area had only a slight influence on the generation of these nonrespiratory behaviors. In particular, the cough-related abdominal bursting activity was preserved after the removal of RTN/pFRG neurons essential for the generation of the hypercapnia-related active expiration. These data imply that the RTN/pFRG is of critical importance for the generation of hypercapnia-induced active expiration, but it plays a minor role in the generation of coughing-induced active expiration.

The cough-related exhalation event can be divided into three phases with regard to vocal cord movement: compressive, expulsive, and narrowing phases (Korpáš and Tomori 1979). The vocal cords are adducted in the compressive phase with powerful expiratory muscle activation resulting in an abrupt rise in tracheal pressure. The vocal cords are then adducted transiently at the peak of the tracheal pressure, releasing an explosive expiratory airflow during the expulsive phase. The vocal cords are then adducted again in the narrowing phase. Considering that adductor motoneurons are activated during the compressive and narrowing phases and inhibited during the expulsive phase (Shiba et al. 1999) and that the RLN efferents consist mainly of adductor fibers, it is safe to say that the C1, C2, and C3 phases correspond to the compressive, expulsive, and narrowing phases, respectively. As an analogy, the RTN/pFRG neurons that were mainly activated during the C1 phase could be related to motor activity during the compressive phase of coughing including the glottal adduction, whereas those that strongly discharged during the C2 phase may have a close relation to the cough-associated muscle activity during the expulsive phase including cough-induced abdominal constriction.

**Activities of the RTN/pFRG Neurons During Coughing and Swallowing**

_E-aug neurons_. E-aug neurons, which were mainly distributed in the caudal part of the RTN/pFRG, tended to fire during the expulsive phase of coughing, whereas these activities ceased during swallowing. This firing characteristic is similar to that of caudal ventral respiratory group E-aug neurons, some of which correspond to the abdominal premotor neurons (Miller et al. 1985). The RTN/pFRG is generally recognized as the central site of chemosensitivity to CO₂, thereby contributing to pH homeostasis by regulating respiratory control (Feldman et al. 2003; Guyenet 2008; Ritucci et al. 2005), such as by active expiration produced by the increased activity of expiratory muscle groups including the abdominal muscles in the late expiratory phase under hypercapnic conditions (Abdala et al. 2009; Iizuka and Fregosi 2007; Pagliardini et al. 2011).

Some investigators (Abdala et al. 2009; Moraes et al. 2012) have reported that an increase in the level of end-tidal CO₂ facilitates activity of the RTN/pFRG E-aug neurons. Previous studies have also indicated that inhibition of GABAergic or glycinergic suppression to the RTN/pFRG region, possibly by the Bötzinger or pre-Bötzinger inhibitory neurons, increases the late expiratory activity of the RTN/pFRG neurons and thus induces active expiration (Cream et al. 2002; Morgado-Valle et al. 2010; Pagliardini et al. 2011; Rosin et al. 2006). On the other hand, the RTN/pFRG neurons project to the region of the caudal ventral respiratory group which includes abdominal muscle premotor neurons (Smith et al. 1989). In adult animals, Phox2b immunoreactive neurons in the RTN/pFRG, especially those ventromedial to the caudal end of the facial nucleus, exhibit the pronounced expiratory modulation during hypercapnia (Stornetta et al. 2006). The E-aug neurons recorded in the ventromedial portion of the RTN/pFRG may therefore...
The bulbospinal premotor neurons of the abdominal motor system, including the Phox2b-expressing neurons, could provide the excitatory drive to the bulbospinal premotor neurons of the abdominal motor system, although there is no direct evidence (Abdala et al. 2009). Additional studies are needed to determine whether the abdominal premotor neurons in the ventrolateral medulla receive monosynaptic excitatory input from the RTN/pFRG E-aug neurons. In the present study, the removal of the RTN/pFRG attenuated the abdominal nerve activity during coughing, as well as respiration, suggesting that the E-aug neurons in the RTN/pFRG cause active expiration via abdominal premotor neurons in the caudal ventral respiratory group not only during breathing but also during coughing (Janczewski and Feldman 2006; Miller et al. 1985; Pagliardini et al. 2011).

One concern is that the activity of respiratory neurons could be altered under hypercapnic conditions. This reconfiguration of the respiratory neuronal network under hypercapnic conditions may influence the activity of each type of respiratory neuron in the RTN/pFRG during the nonrespiratory behaviors as well as during respiration. For example, the changes in activity of the E-aug neurons during coughing may be masked under hypercapnic conditions, since these neurons are activated according to an increase in arterial CO₂ during respiration (Abdala et al. 2009).

A large population of neurons in the caudal end of the RTN/pFRG was located in the area ventromedial to the facial nucleus, where the chemosensitive respiratory neurons are densely distributed (Guyenet 2008; Mulkey et al. 2004; Stornetta et al. 2006). Indeed, many E-aug neurons in this region were in synchrony with the abdominal nerve activity not only during respiration but also during coughing under hypercapnic conditions. However, we should consider the potential implications that the caudal end of the RTN/pFRG may include the rostral extended column of the Bötzinger complex.

**E-dec neurons.** E-dec neurons in the RTN/pFRG were activated according to the period of the RLN activity during the compressive phase of coughing and during swallowing. The bursting activity of the RLN during the compressive phase of coughing and during swallowing tended to be attenuated after removal of the RTN/pFRG. Neurons that control the transient glottic closure during coughing and swallowing have been found in the dorsal and ventral respiratory columns in the medulla (Grélot and Bianchi 1996; Oku et al. 1994; Shiba et al. 2007; Sugiyama et al. 2011). On the other hand, the dorsolateral pons is the area where excitation or inhibition activates laryngeal postinspiratory activity (Dutschmann and Herbert 2006), and the occurrence of coughing is inhibited (Poliaček et al. 2004), and the activity of the E-dec neurons is changed in synchrony with coughing (Shannon et al. 2004a). Meanwhile, Bautista and Dutschmann (2014) pointed out that the pontine respiratory group participates in the generation of swallowing. Furthermore, there appears to be extensive interconnections among the RTN/pFRG, pontine respiratory group, and medullary respiratory group neurons (Chamberlin and Saper 1994; Dobbins and Feldman 1994; Herbert et al. 1990; Núñez-Abades et al. 1993; Rosin et al. 2006). Therefore, it seems likely that E-dec neurons in the RTN/pFRG may serve to coordinate the glottal adduction during coughing and swallowing.

**I-aug neurons.** Approximately one-half of the recorded RTN/pFRG I-aug neurons were activated not only during the inspiratory but also during the expiratory phase of coughing. This paradoxical biphasic activity has not been previously observed in the other medullary inspiratory neurons (Gestreau

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**Fig. 3.** Firing patterns of the I-aug neurons during respiration and coughing. Augmenting firing pattern of inspiratory neurons (I-aug) during respiration (left panel) and coughing (right panel). A: activity of a neuron that exhibited the same firing pattern during coughing as that during breathing. In A, the unit activity during the inspiratory phase of coughing increased compared with that during respiration, with the phrenic nerve discharge enhanced during coughing. B and C: activity of the neurons which fired throughout the coughing period, showing the predominant activity during the C1 (B) or C2 (C) phase in addition to the inspiratory discharge. See Glossary for additional abbreviations.
et al. 1996; Haji et al. 2012; Oku et al. 1994; Shannon et al. 1998; Sugiyama et al. 2014). One possibility is that this expiratory activity during coughing may be responsible for disinhibition of inhibitory respiratory neurons in the Bötzinger complex (Bongianni et al. 1998; Cream et al. 2002), whose activation suppresses the activity of phrenic motoneurons, expiratory muscle premotor neurons, and laryngeal adductor motoneurons (Ezure and Manabe 1988; Ono et al. 2006).

Fig. 4. Firing patterns of the phase-spanning neurons during respiration, coughing, and swallowing. Examples of firing patterns of the inspiratory-expiratory (IE) (A–C) and the expiratory-inspiratory (EI) (D–F) phase-spanning neurons during coughing (A, B, D, and E) and swallowing (C, F). A and B: activity of the neurons whose peak firing rate occurred during the C1 (A) and C2 phase (B) of coughing. C: activity of a neuron which fired during swallowing. In C, despite the responses of the neuron to orthodromic activation elicited by the SLN stimuli, as indicated in the bottom panel in C (five superimposed sweeps), the more robust activation was observed during the swallow-related RLN burst. Thus the activity of this neuron was deemed to be modulated with the RLN burst. The neurons in D and E exhibited strong activity during the C1 (D) and C2 phase (E) of coughing. A neuron in F was activated during swallowing. The filled triangle indicates the SLN stimulus. The second trace in C shows the temporal fluctuations in spike amplitude responsible for the pulsations of the brain stem. See Glossary for additional abbreviations.
However, because the characteristics of RTN/pFRG I-aug neurons, in particular their axonal projections and transmitters, are not well understood, here we cannot discuss the details of their possible roles in coughing. During swallowing, all I-aug neurons recorded in the present study were silent as with inspiratory motoneurons of the upper airway muscles (Gestreau et al. 2000; Grélot et al. 1989; Shiba et al. 1999). On the contrary, some inspiratory neurons in the dorsal (Gestreau et al. 1996; Saito et al. 2002) and ventral respiratory group (Oku et al. 1994; Sugiyama et al. 2014) were discharged in synchrony with a weak activation of the phrenic nerve during swallowing, referred to as “swallow-breath” (Dick et al. 1993). The result that removal of the RTN/pFRG region did not affect this minor phrenic nerve activity indicates that I-aug neurons in this area are not involved in the generation of the swallow-breath. Consequently, these distinct discharge patterns of the I-aug neurons during respiration, coughing, and swallowing may be involved in the coordination of these behaviors to prevent aspiration (Pitts et al. 2013).

Phase-spanning neurons. Many phase-spanning neurons in the RTN/pFRG were typically activated during the expiratory phase of coughing and during swallowing. Although the physiological role of the phase-spanning neurons regarding respiratory rhythm regulation have been discussed in previous studies (Connelly et al. 1990; Guyenet et al. 2005), their significance for the generation of coughing and swallowing has not been fully explored. Guyenet et al. (2005) have suggested that these phase-spanning neurons receive inhibitory inputs from either inspiratory or expiratory neurons in the medullary respiratory centers. It is therefore possible that these neurons participate in the pattern-generating processes of coughing or swallowing as do other medullary respiratory neurons (Bongianni et al. 1998; Shannon et al. 1998; Sugiyama et al. 2014).
swallow-related IE neuron orthodromically activated by the SLN was recorded in the present study, which implies that some phase-spanning neurons in the RTN/pFRG do receive laryngeal afferent information via the nucleus tractus solitarius and could act as a key center involved in coughing and swallowing (Jean 2001; Ohi et al. 2005), such that they may play a regulatory role in these behaviors. Further studies will be necessary to determine the exact role of the phase-spanning neurons involved in coughing and swallowing.

Oropharyngeal swallow is elicited by providing bolus into the pharynx, during which the physiological signals are conveyed to the nucleus tractus solitarius via the glossopharyngeal nerve as well as the SLN (Ootani et al. 1995). The activities of the RTN/pFRG neurons, a part of which receive inputs from laryngeal afferents, during swallowing elicited by electrical stimulation of the SLN might be distinct from those elicited by physiological stimuli, since the integration of afferent inputs from the upper alimentary tract could influence the elicitation of swallowing and the processing of sensory information during swallowing.

Some neurons located in the area where the facial motoneurons are distributed, may possibly include the facial motoneurons that exhibit respiratory-related activity. However, the results obtained from the collision test suggest that the neurons recorded in this area are mainly interneurons in the RTN/pFRG which project to the brain stem respiratory center (Li et al. 2004).

**Influence of Brain Stem Sectioning on Respiratory and Nonrespiratory Regulations**

Respiration. The specific discharge patterns of the laryngeal and abdominal nerves during breathing were altered depending on the transection levels. The microinjection and transection studies have strongly suggested that the rostral dorsolateral pons and their descending pathways are indispensable for the generation of the postinspiratory activity of the RLN (Abdala et al. 2009; Dutschmann and Herbert 2006; Smith et al. 2007). We also showed that the postinspiratory activity is abolished by brain stem transection at the rostral level of the RTN/pFRG.
The present study showed that the removal of the RTN/pFRG region from the medulla reduces the late expiratory activation of the abdominal nerve. Although the medullary respiratory networks could vary, to some extent, in different species (Ezure et al. 1988; Richerson and Getting 1992), the functional role of the RTN/pFRG area in the regulation of respiration in guinea pigs was similar to that in the other animals previously reported (Abdala et al. 2009; Janczewski and Feldman 2006). Some authors (Abdala et al. 2009; Moraes et al. 2012; Pagliardini et al. 2011) have shown that RTN/pFRG neurons are activated in synchrony with enhanced activity of the abdominal nerve during active expiration. Several lines of evidence such as the above-mentioned results have indicated that the RTN/pFRG area is essential for the generation of active expiration under hypercapnic conditions (Abdala et al. 2009; Janczewski and Feldman 2006; Pagliardini et al. 2011; Ritucci et al. 2005; Sato et al. 1992). This theory can explain the etiology of a congenital functional disorder of central chemoreception; patients with congenital central hypoventilation syndrome, in whom the functional impairment of the RTN/pFRG develops, suffer from fatal respiratory failure attributed to insusceptibility to hypercapnia (Dubreuil et al. 2008). On the other hand, the neuronal circuitry between the Bötzinger complex and pre-Bötzinger complex may also be responsible for the generation of the late expiratory activity in the neonatal period (Molkov et al. 2010; Smith et al. 2007). However, the influence of this area on the regulation of active expiration is attenuated in the growth process, and eventually disappears in the adult (Pagliardini et al. 2011).

The removal of the RTN/pFRG markedly reduced the respiratory rate, and in particular prolonged the expiratory phase, providing supportive evidence that the RTN/pFRG regulates respiratory rhythm, especially expiratory duration (Janczewski and Feldman 2006; Onimaru and Homma 2003). The changes in the respiratory rate due to the rostralmost transection tended to be smaller than those shown by Abdala et al. (2009), possibly because vagal intact animals, in which the pulmonary afferent inputs could affect the respiratory rhythm, were used in the present study (Bianchi and Gestreau 2009). Furthermore, the influence of age on the respiratory rhythm regulation should be considered when interpreting the effects of the transections. In juvenile animals, the pre-I neurons in the RTN/pFRG act as a respiratory pacemaker; however, in adult animals this specific activity is not present, which supports the view that juvenile and adult animals have distinct regulatory mechanisms regarding respiratory rhythogenesis (Guyenet et al. 2005).

Coughing. The parabrachial and Kölliker-Fuse nuclei, a part of the pontine respiratory group, have been suggested to contribute to the generation of coughing (Gestreau et al. 1997; Shannon et al. 2004b). Poliaček et al. (2004) have reported that bilateral chemical lesions of the rostral dorsolateral pons suppress the cough reflex evoked by mechanical stimulation of the laryngotracheal mucosa. Conversely, the present study showed that, regardless of the removal of the pons rostral to the RTN/pFRG, coughing can be evoked by stimulation of the laryngeal afferent nerve or the tracheal mucosa, and the sequential pattern of cough-related nerve activities is preserved.

One possibility is that this difference in evoking coughing may be attributed to the species differences. Although the influence of rostralmost sectioning on the respiratory-related nerve activity was similar to that in other animals (Abdala et al. 2009; Smith et al. 2007), the cough-generating neuronal networks including the pontine region are probably distinct between species. The other possibility is that the elimination of the descending inputs from higher centers by the transverse brain stem sectioning affected the sensitivity of the cough reflex. Since the medullary neuronal circuits involved in cough generation can be suppressed by the inhibition of the pontine respiratory group, this region is certainly important for evoking coughing (Poliaček et al. 2004). Our findings raise the possibility that the sensitivity of the cough reflex could be suppressed from higher centers (Widdicombe 1995). This is physiologically appropriate since, when the bolus passes over the larynx during swallowing, an inappropriate cough reflex may interfere with bolus transit. Consequently, our data indicate that the parabrachial and Kölliker-Fuse nuclei are not a source of the RLN activity during the compressive phase of coughing.
although the neurons in this region participate in the cough-generating neuronal network (Gestreau et al. 1997; Jakus et al. 2008).

As discussed above, the E-aug neurons in the RTN/pFRG are thought to be necessary for the generation of the late expiratory activity during hypercapnia (Pagliardini et al. 2011). In the present study, the E-aug neurons recorded under hypercapnic conditions were broadly distributed in this area, and tended to be activated according to the cough-related abdominal burst. Thus it is also feasible that the slight attenuation of the cough-related abdominal activity, following the caudalmost transection, could be due to the removal of the RTN/pFRG E-aug neurons. However, in contrast to the hypercapnia-induced abdominal activation, the cough-related abdominal bursting activity was not largely affected by the caudalmost RTN/pFRG transection. This finding implies that the excitatory drive to the abdominal motoneuron pool during coughing is generated within the brain stem circuitry caudal to the RTN/pFRG.

The phenomenon of the cough-related abdominal activity which started before cessation of the phrenic activity occurred sporadically during the series of recording sessions (see the middle right column of Fig. 6D as an example). This is not unexpected, since the activity of the abdominal muscles during the inspiratory phase of coughing can possibly be observed (Bolser et al. 2000).

The effects of caudal level transection on the cough-related RLN varied from animal to animal; the transection did not change the activity pattern in 3 animals but sometimes altered the activity from the bimodal discharge pattern to the single peak pattern in 5 animals. A caveat should be considered when interpreting these results. Slight pulsation of the brain stem due to the arterial pulsation may be attributed to the brain damage in the immediate vicinity of the cutting blade positioned in contrast to the perfused model. It thus seems likely that the variations of the effects of transection were attributed to variations of spread of brain damage induced by mechanical section, because the Bötzing complex, which is located adjacent to the RTN/pFRG, is a strong candidate site for the generation of the compressive phase (Shiba et al. 2007). This may be due to the elimination of the compressive phase as observed in the present study.

Swallowing. The medullary swallowing center generates sequential rhythmic movements of the upper aerodigestive tract (Jean 2001; Kessler and Jean 1985; Sugiyama et al. 2011; Umezaki et al. 1998a). The present study showed that, although the activity in the majority of RTN/pFRG neurons is changed in synchrony with swallowing, the patterned motor activity of swallowing remains unchanged after the removal of these RTN/pFRG neurons. While the spatiotemporal activity patterns of the upper airway and alimentary tract muscles involved in swallowing are always constant (Miller 1972; Umezaki et al. 1998b), the magnitude of muscular contractions could be altered by changes in the bolus volume to support its transport (Perlman et al. 1999). Some investigators have proposed that a subset of neurons in the pons functions as a relay station conveying laryngeal afferent inputs to higher brain centers, such that these neurons contribute to the regulation of swallowing (Jean et al. 1975; Perlman et al. 1999). The RTN/pFRG neurons involved in swallowing may participate in the sensory feedback regulation of swallowing, since some of them receive sensory inputs from the larynx (see Fig. 4C).

The motor patterns of the other nerves, such as the hypoglossal nerve, may be needed for a more detailed analysis of the swallow-related motor activities due to the brain stem transections (Jean 2001), although fictive swallowing induced by electrical stimulation of the SLN can be identified by the specific bursting activity of the RLN (Umezaki et al. 1998b). Our data would also suggest that there may be other influences, caused by the caudalmost sectioning, on the swallowing behavior, including the activities of the myohyoid, geniohyoid, and thyrohyoid muscles innervated by the hypoglossal nerve.

Perspectives and Significance

The present findings raise the prospect that although various types of respiratory-related neurons in the RTN/pFRG exhibit highly synchronous activity during the nonrespiratory behaviors including coughing and swallowing, these neurons are not a critical element in the pattern generation of these behaviors. The RTN/pFRG contributes to maintaining homeostasis by regulating respiratory rhythm and forced expiratory effort in response to acidemia (Feldman et al. 2003; Guenet 2008; Onimaru and Homma 2003; Ritucci et al. 2005). Likewise, the data provided in this study suggest that these neurons may also participate in the feedback system involved in coughing and swallowing, as well as respiration to achieve the appropriate movements, although there are no previous studies to support this hypothesis. Future studies are necessary to reveal the functional contributions of the ascending or descending inputs from different sites of the brain stem, possibly including the central pattern generators for those behaviors, as such studies may provide insight into the highly sophisticated optimization of sequential movements of respiratory muscles during the airway protective reflexes.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: Y.S. and K.S. conception and design of research; Y.S., K.S., and S.M. performed experiments; Y.S. and K.S. analyzed data; Y.S., K.S., and T.U. interpreted results of experiments; Y.S. prepared figures; Y.S. drafted manuscript; Y.S. and K.S. edited and revised manuscript; Y.S., K.S., S.M., T.U., H.S., and Y.H. approved final version of manuscript.

REFERENCES


