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**Abstract**

We previously demonstrated that during nasal air-jet stimulation, both the activities of intrinsic laryngeal adductor and abductor muscles persistently increase, whereas the respiratory cycle prolongs and the activity of diaphragm decreases [7, 19]. The purpose of this study was to clarify the neuronal circuit underlying the augmentation of intrinsic laryngeal muscles evoked by nasal air-jet stimulation. The immunohistologic analysis of Fos-expression was reported to determine the distribution of activated neurons in cat brainstem evoked by sneeze-inducing air puff stimulation of the nasal mucosa [27]. In sneezing cats, immunoreactivity was evoked in projection areas of the ethmoidal afferents, e.g. the subnuclei caudalis, interpolaris and in interstitial islands of the trigeminal sensory complex. Immunoreactivity was also enhanced in the solitary complex, the nucleus retroambiguus, the pontine parabrachial area and the lateral aspect of the parvocellular reticular formation [27].

In the present study, we focussed on the parvocellular reticular nucleus (PRN) as a relay of the neural circuit contributed to the augmentation of intrinsic laryngeal muscles evoked by nasal air-jet stimulation. We recorded the neuronal behavior of PRN during the nasal air-jet stimulation in precollicular-postmammillary decerebrate cats. As the results, twenty-four percent (17/71) of recorded neurons which were activated orthodromically by the electrical stimulation to anterior ethmoidal nerve, increased their firing rates in response to the nasal air-jet stimulation. Furthermore, spike-triggered averaging method revealed that four out of these 17 PRN neurons activated intrinsic laryngeal muscles, suggesting that such neurons have excitatory projections to the intrinsic laryngeal muscle motoneurons in the nucleus ambiguus. These results suggest that the some of PRN neuron play a role in augmentation of the intrinsic laryngeal muscles activities during nasal air-jet stimulation.

Classification terms

Theme G: [Motor Systems and Sensorimotor Integration]

Topic: [Reflex function] or [spinal core and brainstem]

Keywords:

Nasal air-jet stimulation, Parvocellular reticular nucleus,

Intrinsic laryngeal muscle, decerebrate cats,

Spike-triggered averaging method

## 1. Introduction

Various types of nasal stimulation such as chemical, mechanical, and air-jet stimulation elicit apnea as an airway defensive reflex [1,2,20,27]. We reported that nasal air-jet stimulation prolongs the respiratory cycle and decreases diaphragmatic activity [19] to prevent entry of the offending materials into the upper airway. Furthermore, we also clarified that nasal air-jet stimulation persistently increases the activities of both the intrinsic laryngeal adductor muscle, thyroarytenoid; TA, and abductor muscle, posterior cricoarytenoid muscle; PCA (Fig.1: [7]). From these results, it is plausible that functional projections from trigeminal nerve to laryngeal motoneurons might exist. The bulbospinal augmenting inspiratory neurons in the dorsal respiratory group (DRG) near the nucleus of solitary tract (NST) are known to make mono- or oligosynaptic excitatory connections with phrenic motoneurons [5,6]. During nasal air-jet stimulation, the DRG inspiratory neurons decrease their firing rates, consistent with the finding of decreased diaphragmatic activity [7,19]. However, our data also showed that change in behavior of brainstem respiratory neurons does not explain the augmentation of intrinsic laryngeal muscle activity [7]. Although it may be still possible that the spontaneous activity of recorded neurons did not have respiratory rhythm, brainstem respiratory premotor neurons known to have excitatory or inhibitory connections with laryngeal motoneurons may not relay the tonic excitatory afferents during the period of nasal air-jet stimulation. The question remains which respiratory and/or non-respiratory neurons are involved in relaying the tonic excitatory inputs to laryngeal motoneurons during nasal air-jet stimulation.

After cutting the anterior ethmoidal nerve, the change of diaphragmatic and intrinsic laryngeal muscle activities during nasal air-jet stimulation disappeared, indicating that afferents evoked by air-jet stimulation transmitted via the anterior ethmoidal nerve [7]. The ethmoidal nerve afferents mainly terminate in the subnucleus interpolaris and caudalis of spinal trigeminal nucleus, which has been confirmed as the first central relay by means of horseradish peroxidase (HRP) as a neuronal tracer [12]. On the other hand, it has been demonstrated that the nucleus ambiguus (NA) receives the efferent

projections from NST by distribution of the terminations of degenerating fibers in cats using the Nauta and the Fink-Heimer methods [26]. It has been also reported that the NA receives the projections from bilateral NST, the contralateral NA, and the adjacent reticular formation to contralateral NA by means of the anterograde and retrograde axonal transport of wheat germ agglutinin-HRP (WGA-HRP) [28]. Although the existence of functional projections from trigeminal afferents to laryngeal motoneurons in NA is easily predicted, the detailed neuronal circuit underlying this phenomenon is still unclear.

*C-fos* is an immediate early gene that encodes transcription factors. The immunohistological detection of Fos, the protein product of *c-fos*, has been used as a trans-synaptic marker for neuronal activation within the central nervous system [17]. Recently, the immunohistological investigation for Fos-expression to determine the distribution of activated neurons in cat brainstem evoked by sneeze-inducing air puff stimulation of the nasal mucosa was reported [27]. In cats stimulated by air puff, immunoreactivity was evoked in projection areas of the ethmoidal afferents, e.g. the subnuclei caudalis, interpolaris and in interstitial islands of the trigeminal sensory complex. Moreover, immunoreactivity was also enhanced in the solitary complex, the nucleus retroambiguus, the pontine parabrachial area and the lateral aspect of the parvocellular reticular formation [27]. Because our previous data suggested that most of respiratory premotor neurons of laryngeal motoneurons may not relay the tonic excitatory inputs during nasal air-jet stimulation [7], the lateral aspect of the parvocellular reticular formation is one of the most involved regions to relay tonic inputs to laryngeal motoneurons.

The goals of this study are to clarify neuronal circuit underlying the augmentation of intrinsic laryngeal muscle activities during the nasal air-jet stimulation and to clarify neurons relaying tonic inputs to the laryngeal motoneurons. To do this, we planned two experimental designs. Firstly, we aimed to clarify the neuronal behavior during the nasal air-jet stimulation of the brainstem neurons within the parvocellular reticular nucleus (PRN), which received orthodromic afferents from the anterior ethmoidal nerve. We

recorded the activities of these neurons during the nasal air-jet stimulation in precollicular-postmammillary decerebrate cats. Secondly, we examined the functional connections from these detected neurons to the intrinsic laryngeal muscles using spike-triggered averaging method.

## 2. Materials and Methods

The present studies were carried out in accordance with the Guidelines for Animal Experiments of Asahikawa Medical College.

*1. Neuronal behavior in augmentation of intrinsic laryngeal muscle activity during nasal air-jet stimulation.*

The activities of neurons evoked by nasal air-jet stimulation were recorded in 7 adult (B.W. 2.1-3.7 kg) male or female cats. These cats were precollicular-postmammillary decerebrated and tracheotomized as described previously [7,19]. The surgical procedures including decerebration were performed under halothane, nitrous oxide and oxygen anesthesia. The superior laryngeal nerves were sectioned bilaterally to eliminate the influences of laryngeal reflexes. The right side of anterior ethmoidal nerve was exposed by complete enucleation of the eyeball. A pair of thin (50μm) stainless steel wires which were insulated except for an approximately 1 mm portion at the tips was attached to the ethmoidal nerve to stimulate by single-shock (duration; 0.2 ms). After the completion of all surgical procedures anesthesia was discontinued at least 1hour prior to data collection. The cat was placed in a stereotaxic frame and supported with a clamp placed on an upper thoracic vertebra and a rubber hammock. Rectal temperature was maintained at 36-38°C using a radiant heating lamp.

Electromyograms (EMGs) were recorded from the laryngeal adductor muscle (thyroarytenoid, TA) and diaphragm (DIA). EMGs were recorded using a pair of thin (50 μm) stainless steel wires which were insulated except for 1 mm portion at the tips. EMGs were amplified, low-cut filtered with a 10 ms time constant and high-cut filtered with 3 kHz. In order to identify brainstem PRN

neurons projecting from the anterior ethmoidal nerve, neuronal activities evoked by single-shock stimulations to ethmoidal nerve were recorded extracellularly with a tungsten microelectrode (tip diameter 10 μm, tip impedance 10-12 MΩ, FHC Co., Brunswick, ME, USA). Single-cell activity was amplified with an extracellular preamplifier (DAM-80E, World Precision Instruments, New Haven, CT, USA). Air-jet stimulation (20°C, 10 L/min) was delivered continuously for 10 seconds to the right side of the nasal cavity through a metal catheter (tip diameter 2 mm). EMGs and brainstem neuronal recordings were simultaneously stored on a DAT data recorder (DC-10 kHz; RD-135T, TEAC, Japan) for off-line analysis using AD Instruments MacLab and software (Scope) with a Power Macintosh 7300/166 computer. Statistical analysis was assessed by Wilcoxon signed rank test. P-value less than 0.05 was considered to be statistically significant.

*2. Functional connections of the neurons determined by spike-triggered averaging technique*

EMGs of intrinsic laryngeal muscles were orthodromically averaged using extracellular spikes of the isolated brainstem neuron within PRN as triggers (spike-triggered averaging method). Averaging of 100 sweeps using 512 points of 10 msec bin width was employed. The trigger pulses were produced via a time-amplitude window discriminator. These recordings were stored on a same system as above experiment. At the end of data collection, electrolytic lesions were made by passing 40 μA of anodal DC current for 20 s through the recording electrode to identify the location of selected neurons in the brainstem. Two separate electrolytic lesions were also made along each recording track. At the end of the experiment, the animals were euthanized with an overdose of sodium pentobarbital. The portion of brainstem encompassing the electrode tracks and electrolytic lesions were fixed in 10% formalin. Tissue was sectioned at 50 μm and stained with 1% neutral red. The locations of recorded neurons that were not marked by electrolytic lesions were estimated by the reading of the micromanipulator. The locations of the

electrolytic lesions were referred to the stereotaxic atlases of Berman [3].

## 3. Results

*1. Behavioral change of the brainstem neurons evoked by nasal air-jet stimulation*

Neuronal activities were recorded in the vicinity of PRN which were activated orthodromically by single-shock stimulation via the ipsilateral anterior ethmoidal nerve in seven decerebrate cats. Fig. 2 shows the representative location of these neurons, activated orthodromically by electrical stimulation to the ipsilateral anterior ethmoidal nerve, obtained from 3 decerebrate cats. From seven decerebrate cats, 71 neurons were recorded in the vicinity of PRN, which mainly distributed 0-2.0 mm rostral to the obex and 2.0-4.5 mm lateral to the midline. The latency of these neurons ranged from 2.55 to 9.05 ms (5.63±1.57 ms; mean±S.D.). Fluctuation in the latency was usually less than 2 ms.

The recorded neurons were divided into two types according to the behavior during the nasal air-jet stimulation; one group was the neurons that did not change their firing rates during the stimulation, the other group was the neurons that increased their firing rates. Fifty-four (76%) out of 71 recorded neurons did not change their activities during the air-jet stimulation (38±30.6 Hz (mean±SD) before stimulation vs. 43±29.7 Hz during stimulation: p=0.3943). Fig. 3A shows an activity of representative neuron that did not change its firing rate during the nasal air-jet stimulation; the firing rate of the neurons before the nasal air-jet stimulation was 14.4 Hz and that during the stimulation was 15.6 Hz.

On the other hand, the remaining 17 neurons (24%) fired at frequency of 40±38.0 Hz before stimulation and increased their firing rates to 95±58.6 Hz throughout the nasal air-jet stimulation (p=0.0003). Fig. 3B shows an activity of representative neuron that increased its firing rate during the nasal stimulation; the firing rate of the neuron before the nasal air-jet stimulation was about 8 Hz and the firing rate increased up to 88 Hz during nasal stimulation. The firing rate returned to its prestimulus level just after cessation of nasal

stimulation. There were no differences of either latency or brainstem distribution between these two types of neurons.

*2. Functional connections of the neurons determined by spike-triggered averaging technique*

The increased discharges of isolated neurons in the vicinity of PRN during nasal air-jet stimulation were used as trigger signals to average the evoked EMG of intrinsic laryngeal TA muscle and to measure latencies. Seventeen units, which were activated by both single-shock electrical stimulation of the anterior ethmoidal nerve and the nasal air-jet stimulation, were subject to test whether these neurons have functional connections to the intrinsic laryngeal TA motoneurons or not. In four (24%) out of the 17 neurons, orthodromically evoked activations of TA muscle were recorded, suggesting such PRN neurons may have excitatory oligosynaptic projections to TA motoneurons (Fig. 4A)

On the other hand, in the remaining 13 (76%) out of 17 neurons, there was no apparent evoked potentials in the activity of TA muscle, suggesting that these neurons may have no excitatory connections with the motoneurons of TA muscles (Fig. 4B).

## 4. Discussion

It is known that respiratory neurons play important roles not only in producing respiration but also in producing non-respiratory movements such as vomiting [9,14,15,16] and vocalization [11]. Most of the brainstem respiratory neurons are known to control the activities of respiratory and laryngeal muscles directly and/or indirectly [5,6,8,10,13]. We previously reported that nasal air-jet stimulation prolonged the respiratory cycle and decreased the activity of diaphragm in decerebrate cats [7, 19]. During the nasal air-jet stimulation, the bulbospinal inspiratory neurons in the DRG decreased their firing rates to diminish diaphragmatic activity [7]. On the other hand, the nasal air-jet stimulation increased both the activities of intrinsic laryngeal adductor and abductor muscles. These augmentations of the activities of intrinsic laryngeal

muscles lasted throughout the period of nasal air-jet stimulation. However, no brainstem respiratory premotor neurons, which are known to make excitatory or inhibitory connections with the intrinsic laryngeal motoneurons, discharged appropriately for initiating the augmentation of intrinsic laryngeal muscle activities during stimulation [7]. Namely, no brainstem respiratory premotor neurons relay the tonic excitatory afferent signals to intrinsic laryngeal motoneurons during nasal air-jet stimulation. The afferent signals resulting from nasal air-jet stimulation via trigeminal nucleus may transmit to the intrinsic laryngeal motoneurons by way of pathway(s) other than respiratory neurons, for example, via non-respiratory neurons.

Recently, the Fos-expression was analyzed to determine which brainstem neurons were activated during sneeze-inducing air puff stimulation of the nasal mucosa to evoke sneezing using cats [27]. As the results, immunoreactivity was enhanced in projection areas of the ethmoidal afferents, such as the subnucleus caudalis and interpolaris. Immunoreactivity was also evoked in the areas related to the respiratory neurons such as the solitary complex, the nucleus retroambiguus, and the pontine parabrachial area. Moreover, Fos-expression was also observed in the lateral aspects of the parvocellular reticular formation [27]. Because subnuclei caudalis and interpolaris are the first central relay of the ethmoidal nerve afferents, it is plausible that Fos–positive cells are rich in those nuclei. As the most of bulbospinal inspiratory neurons in the DRG, which did not increase their firing rate throughout the period of the nasal air-jet stimulation [7], are located in the NST, these NST neurons do not relay the excitatory afferent signals to the intrinsic laryngeal motoneurons. Therefore, we assumed that the neurons in the PRN probably play an important role to relay the excitatory afferent signals for augmenting the activities of intrinsic laryngeal muscle during nasal air-jet stimulation.

In the present study, we investigated neuronal behavior at PRN in the augmentation of the intrinsic laryngeal muscle activities during nasal stimulation. As the results, seventy-one PRN neurons were activated orthodromically by the electrical stimulation to anterior ethmoidal nerve. Moreover, twenty-four

percent (17/71) of recorded neurons also increased their firing rate in response to the nasal air-jet stimulation. These neurons increased their firing rates continuously throughout the nasal air-jet stimulation in phase with the augmentation of intrinsic laryngeal muscle activities. This result suggests that these neurons can be candidates to relay the afferent signals activated by nasal air-jet stimulation to the intrinsic laryngeal motoneurons. On the other hand, seventy-six percent (54/71) of recorded neurons did not change their firing rates during nasal air-jet stimulation. The PRN neurons projected by ethmoidal nerve may be divided into two groups. One is a group that responds to the air-jet stimulation and the other is a group that does not respond. There was no apparent difference in location within brainstem and latency between the two groups. It has been reported that nasal 'flow' receptors which were sensitive to cold air were clearly present in the trigeminal nerve, and that fifty-five percent of anterior ethmoidal nerve endings were identified as 'flow' receptors [24]. We have reported that cold air-jet stimulation is more effective than warm air-jet stimulation for decreasing the amplitude of diaphragmatic activity [19]. Therefore, it is possible that the nasal air-jet simulation evokes the phenomena by activating the 'flow receptor' located in the nasal mucous membrane. In present study, we found the PRN neurons that did not respond to the nasal air-jet stimulation. These PRN neurons might receive afferents from the other nerve endings such as mechanoreceptor.

As the next step, we have investigated whether the activated PRN neurons by the nasal air-jet stimulation have functional projections to the laryngeal motoneurons. As the results, it was clarified that four out of these 17 PRN neurons have excitatory projections to intrinsic laryngeal TA muscle motoneurons by spike-triggered averaging method. Although the ratio of the neuron which has an excitatory projection to the TA motoneuron is very small, it is certain that some of the neurons within PRN play a role in augmentation of the intrinsic laryngeal muscles activities throughout the period of nasal air-jet stimulation. In the present study, we have recorded 71 neurons, which were responded to the electrical stimulation to the anterior ethmoidal nerve, in the vicinity of PRN. These neurons mainly distributed 0-2 mm rostral to the obex

and 2-4.5 mm lateral to the midline. The distribution of recorded PRN neurons was almost similar to that of c-Fos expression in the reticular formation of sneezing cats, which was reported by Wallois et al. [27]. They inferred that these parvocellular reticular neurons having c-Fos expression have a pivotal role in functional adaptation of the central respiratory drive for expulsion, observed in sneezing and vomiting [27]. Because the glottis closes tightly during both behaviors [9,18], it is apparent that some of these parvocellular reticular neurons may have an important role in controlling intrinsic laryngeal muscles.

The larynx is multifunctional organ involved in respiration, vocalization, swallowing, and the airway reflex. Recently, it was reported that the laryngeal motoneurons change their patterns of membrane potential in response to the various laryngeal functions mentioned above [23]. It indicated that central drives to laryngeal motoneurons, consisting of complex combinations of excitation and inhibition, vary during the various laryngeal functions [23]. It is also easy to speculate that a variety of sensory inputs may converge to the laryngeal motoneurons via unknown relays for accomplishing this multifunction of the larynx. In general, it is well known that a variety of sensory inputs converge in the PRN neurons [4,21,22,25]. In present study, it was clarified that a part of PRN neurons have an excitatory projection to the motoneurons of laryngeal TA muscles. Consequently, the convergence of afferent signals to the PRN neurons from a variety of central nervous system may play an important role for accomplishing the multifunction of the larynx.

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**Figure legends**

Fig. 1.

Effects of nasal air-jet stimulation on intrinsic laryngeal and respiratory muscle activities in a decerebrate tracheotomized cat. Horizontal thick solid line at bottom: period of nasal air-jet stimulation (10 L/min). PCA, posterior cricoarytenoid muscle (vocal fold abductor); TA, thyroarytenoid muscle (vocal fold adductor); DIA, diaphragm; EA, external abdominal muscle (Reprinted from Neuroscience Research, 31, Enomoto et al.; “The augmentation of intrinsic laryngeal muscle activity by air-jet stimulation of the nasal cavity in decerebrate cats137-146, 1998, with permission from Elsevier Science.)

Fig. 2.

Anatomic distribution of the parvocellular reticular nucleus (PRN) neurons located from 0 to 2 mm rostral to the obex. The data were obtained from three decerebrate cats. The neurons were divided into two groups according to the behavior during nasal air-jet stimulation. One group is neurons that did not change their firing rate during the stimulation, represented by open circles. The other group is neurons that increased their firing rate during the stimulation, represented by closed circles. Abbreviations: STN, spinal trigeminal nucleus; S, solitary tract; DMV, dorsal motor nucleus of vagus; 12, hypoglossal nucleus; IO, inferior olive; P, pyramidal tract.

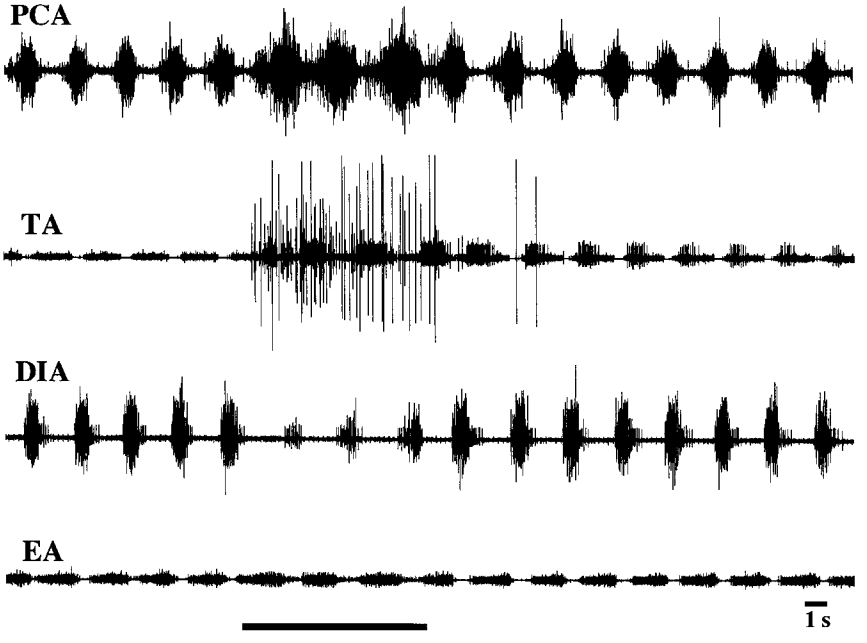
Fig. 3.

A: Representative behavior of a parvocellular reticular nucleus (PRN) neuron which did not change its firing rate during the nasal air-jet stimulation.

Horizontal solid lines indicate periods of air-jet stimulation to the ipsilateral nasal cavity. B: Representative behavior of a PRN neuron which increased its firing rate during the stimulation. The firing rate of this neuron before the nasal air-jet stimulation was about 8 Hz and it increased up to 88 Hz throughout the air-jet stimulation. Abbreviations: PRN neuron, parvocellular reticular nucleus neuron; DIA, diaphragm; TA, thyroarytenoid muscle

Fig. 4.

Representative evoked EMGs recorded from thyroarytenoid (TA) muscle using spike-triggered averaging method. Fig. 4A indicates a representative parvocellular reticular (PRN) neuron whose action potential induced evoked EMGs of intrinsic laryngeal thyroarytenoid (TA) muscle. Fig. 4B indicates a PRN neuron whose action potential did not induce any evoked EMGs of TA muscle. (a): An activity of PRN neuron which was used as trigger pulse to average the activity of TA muscle during the nasal air-jet stimulation, (b): superimposed and (c): averaged activity of TA muscle. For each averaging, 100 of triggers were employed.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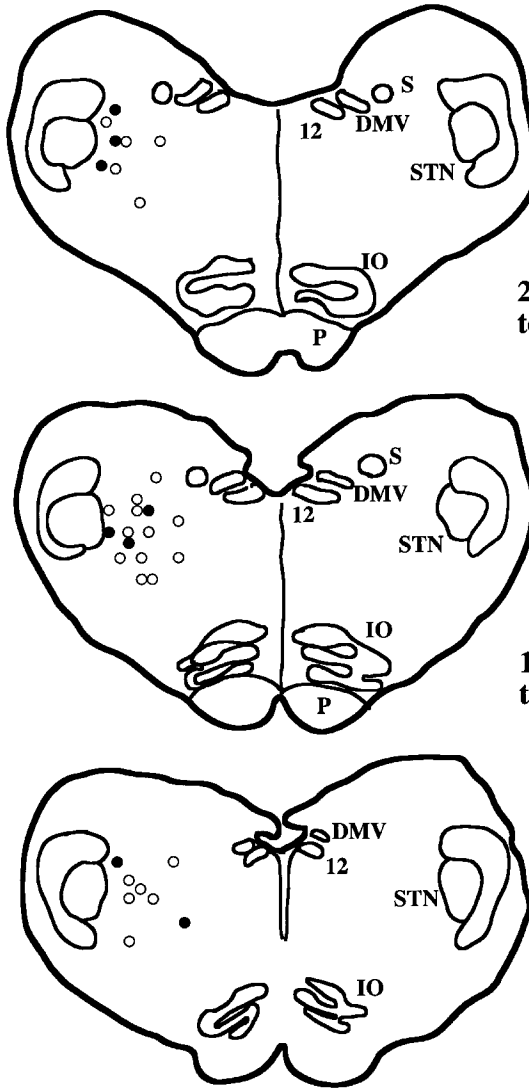
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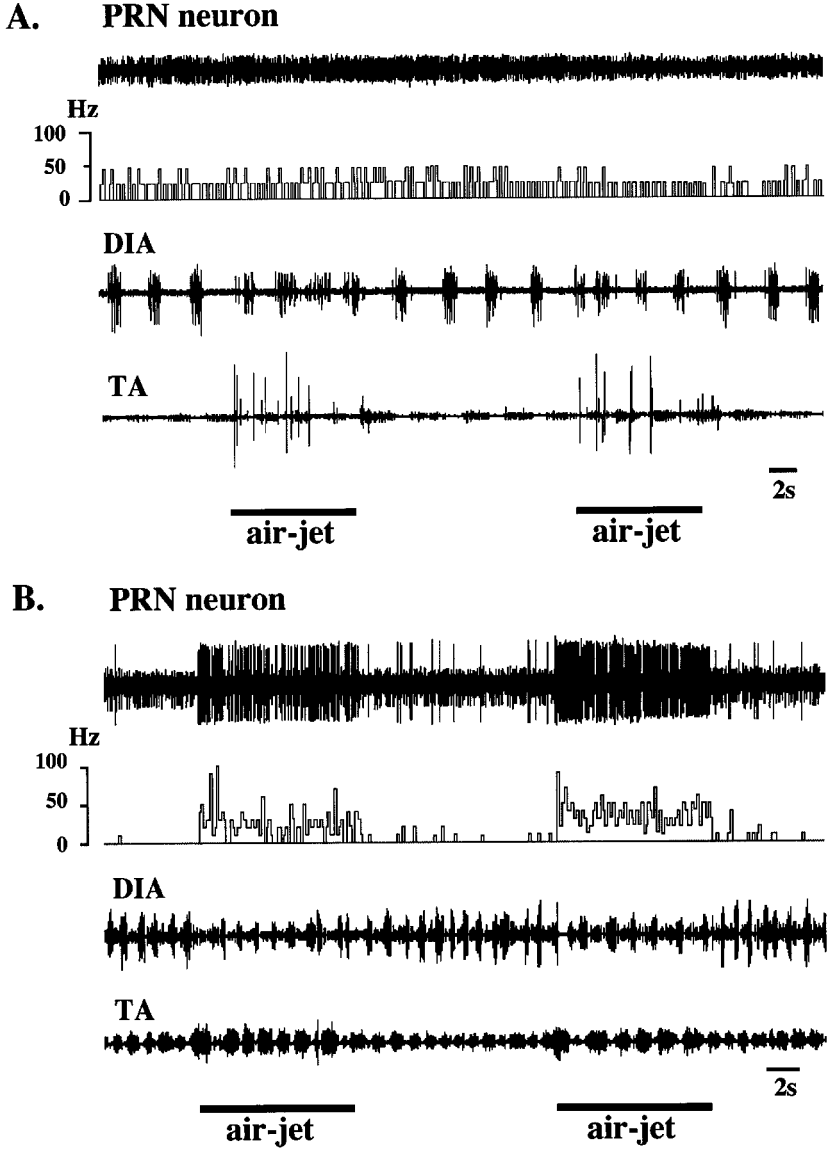
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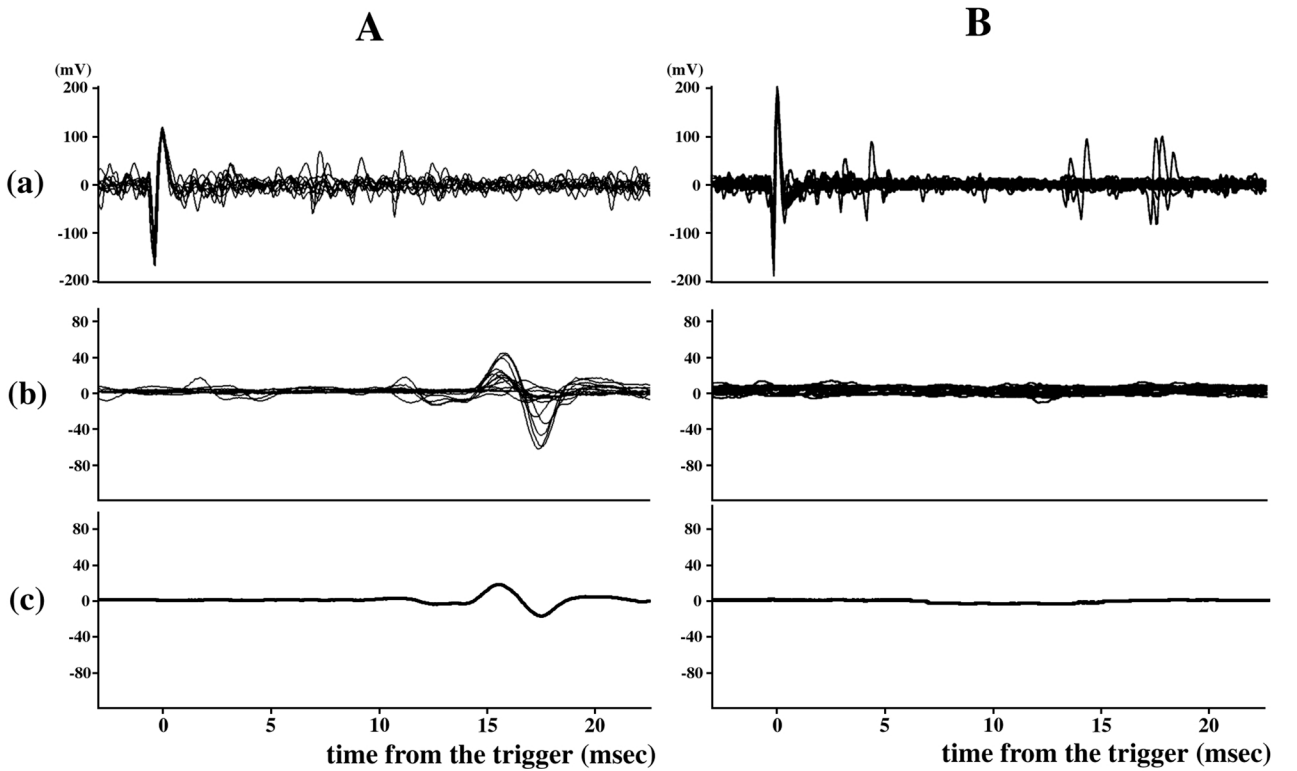
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