Exp Brain Res (1997) 115:513–519 © Springer-Verlag 1997

RESEARCH ARTICLE

Keisuke Shiba · Toshiro Umezaki · Yu Zheng
Alan D. Miller

The nucleus retroambigualis controls laryngeal muscle activity during vocalization in the cat

Received: 17 July 1996 / Accepted: 20 December 1996

Abstract The purpose of this study was to determine (1) whether the nucleus retroambigualis (NRA) plays an essential role in periaqueductal gray (PAG)-induced vocalization and (2) which NRA neurons are involved in the projection from the PAG to laryngeal motoneurons. Bilateral injections of the neurotoxin kainic acid into the NRA in decerebrate cats abolished PAG-induced vocalization; PAG stimulation after the injections no longer modulated vocal fold adductor or tensor activity, and only tonically, but no longer phasically, activated the abdominal muscles. In contrast, PAG-induced inspiratory excitation remained even after the injections. These results suggest that the NRA is essential for the vocal activation of the laryngeal adductor and abdominal muscles, and that an additional pathway from the PAG to respiratory motoneurons other than through the NRA is important for mediating PAG-induced inspiratory activation. Secondly, axonal projections of NRA neurons to the contralateral nucleus ambiguus (NA) were studied electrophysiologically. Five expiratory neurons, which had decrementing (n=4) or constant (n=1) firing patterns, were identified as both having axonal projections to the NA and receiving inputs from the PAG. Furthermore, following NA stimulation many constant-latency action potentials of silent cells were recorded in the vicinity of the NRA, where many silent cells were also orthodromically activated by PAG stimulation. No NRA augmenting expiratory neurons could be antidromically activated from the NA. It is suggested that the NRA and adjacent reticular formation integrate inputs from the PAG and send outputs to laryngeal motoneurons for vocalization.

K. Shiba · T. Umezaki · Y. Zheng · A.D. Miller
The Rockefeller University, 1230 York Avenue, New York, NY 10021–6399, USA

K. Shiba (✉)
Department of Otolaryngology, School of Medicine, Chiba University, 1–8–1 Inohana, Chuo-ku, Chiba City, Chiba 260, Japan;
Tel.: +81–43–2227171, Fax: +81–43–2262028,
e-mail: cum107d@med.m.chiba-u.ac.jp

Key words Vocalization · Nucleus retroambigualis · Periaqueductal gray · Nucleus ambiguus · Abdominal muscle · Cat

Introduction

Vocalization is induced by adducting and stretching the vocal fold, and opening the mouth during expiration reinforced by abdominal muscle activation. Thus, vocalization needs highly coordinated laryngeal, respiratory, and oropharyngeal muscle activities. The periaqueductal gray (PAG) has been thought to be one of the most important centers of vocalization, since electrical or chemical stimulation of the PAG induces vocalization (Bandler 1982; Jürgens 1994; Zhang et al. 1994), and lesions invading the caudal PAG or adjacent tegmentum cause mutism (Kelly et al. 1946; Adametz and O'Leary 1959). Furthermore, Larson et al. (1984, 1986; Larson 1991) showed that activities of some PAG neurons are correlated with intralaryngeal muscle activities during vocalization. However, the neuronal pathway and the mechanism of PAG-induced vocalization have not been fully clarified. Which site integrates the vocal signals derived from the PAG? How are the integrated signals transmitted to the motoneurons necessary for vocalization?

On the basis of neuroanatomical tracing studies, Holstege (1989) proposed the final common pathway for vocalization: the projections from the PAG via the nucleus retroambigualis (NRA) of the caudal ventral respiratory group (VRG) to motoneurons necessary for vocalization, including laryngeal, abdominal, and oropharyngeal motoneurons. He showed that [^{3}H]leucine injections involving the NRA label axonal terminations in the nucleus ambiguus (NA) bilaterally, but mainly contralaterally. Recently Zhang et al. (1992, 1995) showed that excitatory amino acid microinjection into the NRA causes vocalization, and that a brainstem transection at the obex level abolishes the PAG-induced vocal motor pattern of the vocal fold adductor and tensor. These results suggest that the NRA integrates the vocal signals derived from the

PAG and sends the integrated signals to laryngeal motoneurons. The purpose of the present study was to reveal whether NRA neurons are part of a vocalization pathway from the PAG to laryngeal motoneurons by determining (1) whether kainic acid injections involving the NRA abolish PAG-induced laryngeal movement, and (2) whether the area of the NRA has neurons that both project to the NA and receive inputs from the PAG.

Materials and methods

All the procedures used in this study conform to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Rockefeller University Animal Care and Use Committee.

Data were obtained from 16 adult cats of either sex. The animals were initially anesthetized with isoflurane (Aerrane; Ohmeda Caribe) vaporized in nitrous oxide and oxygen and were decerebrated at the pre-collicular level after bilateral ligation of the common carotid arteries. The trachea was cannulated by inserting the horizontal portion of a T-shaped tube into both the rostral and caudal tracheal cut ends. Cannulae were placed in the femoral artery to monitor blood pressure and in the femoral veins for drug administration. Mean blood pressure was maintained above 90 mmHg, if necessary using intravenous infusion of metaraminol bitartrate (Aramine; Merck, Sharp & Dohme). The animals were placed in a stereotaxic frame and supported using hip pins. Rectal temperature was kept at 36–37°C using a heating lamp. Anesthesia was discontinued following the completion of all surgical procedures and at least 1 h prior to data collection.

For inducing vocalization, a tungsten microelectrode (FHC, cat. #25–08–3; tip impedance 9–12 MΩ) was inserted into the PAG (Horsley-Clarke stereotaxic coordinates A 1.0–2.5, L 1.0–2.0, H +2.0–0) before paralyzation. Microstimulation (pulse duration 0.2 ms; frequency 100 Hz; intensity 30–150 μA; lasting for 5–10 s) was delivered with tracking steps of 0.5 mm to identify the call site. PAG stimulation can induce "meow" and "hiss" vocalization, defined as type A and B, respectively (Zhang et al. 1994). We analyzed only "meow" vocalization. When PAG stimulation induced "hiss" vocalization, we changed the stimulus site to one where the stimulation induced only "meow" vocalization. The electrode was fixed at the site where the stimulus threshold for vocalization was the lowest. The stimulus intensity was fixed at 1.5 times vocalization threshold for the remainder of the experiment. The vocal motor pattern was monitored by recording the activities of respiratory and intralaryngeal muscles (Figs. 1Aa, 2Aa); bipolar stainless steel wire electrodes (50 μm in diameter) were implanted into the diaphragm, rectus abdominis (or external oblique), cricothyroid (CT; vocal fold tensor), thyroarytenoid (TA; vocal fold adductor), and posterior cricoarytenoid (PCA; vocal fold abductor) muscles. A fine needle, connected to a pressure transducer (Kent Scientific, model 600D, 5 ms response time), was inserted into the trachea to record tracheal pressure. Voice was recorded with a microphone positioned 10 cm from the animal's mouth.

Kainic acid (Sigma, 2 mg/ml), dissolved in phosphate-buffered saline (pH 7.4) and saturated with fast green or methyl blue dye, was injected into the region of the NRA using a microsyringe (Hamilton, model 7001) in six non-paralyzed cats. The needle of the syringe, positioned using a micromanipulater, was directly inserted into the brainstem. Multiple injections (0.1 μl each) spaced 0.5 mm apart were made bilaterally in the rostral-caudal plane between 1.0 and 3.0 mm caudal to the obex. The extent of neuronal damage was estimated by determining the location of dye in the tissue after the end of the experiment. Furthermore, to assess that kainic acid did not invade the laryngeal motor pools, intralaryngeal muscle activity reflexly evoked by stimulation of the superior laryngeal nerve (SLN) was recorded before and after the injections. A bipolar cuff electrode was placed around the SLN for stimulation (pulse duration 0.2 ms; frequency 0.5–1 Hz, intensity 60–150 μA). Kainic acid causes functional inactivation of neuronal cell bodies within 30 min while sparing axons of passage (Coyle and Schwartz 1983). We thus waited 1 h before assessing the effects of the kainic acid injections.

Extracellular recordings were conducted in ten paralyzed cats. Before paralyzation, vocalization was induced by the same method used in the kainic acid injection experiments. In three cats in which PAG stimulation did not induce vocalization, we stimulated the pontine call site (PCS) (de Lanerolle 1990) instead of the PAG; the PCS is thought to be part of the descending pathway from the PAG to the lower brainstem, conveying the information necessary for inducing vocalization (Kanai and Wang 1962; Kobayashi et al. 1994; Wada 1994; Sakamoto et al. 1996). After a stimulating electrode was fixed in the right PAG or PCS, the animals were paralyzed with gallamine triethiodide (Sigma, initial injection of 10 mg/kg i.v., maintained by hourly injections of 5 mg/kg) and artificially ventilated with room air (24 cycles/min). End-tidal CO_2 was typically kept between 4% and 6%. Cuff electrodes were placed around the C5 phrenic and left recurrent laryngeal nerves. The caudal most 3–4 mm of cerebellum were aspirated to facilitate inserting microelectrodes into the brainstem. The tip of a second tungsten microelectrode (same type as used for stimulating the PAG) was fixed into the NA for stimulation; the location of the NA was confirmed by recording antidromic field or spike potentials in response to stimulation of the ipsilateral recurrent laryngeal nerve. Extracellular recordings were obtained using another medullary microelectrode positioned in the vicinity of the NRA caudal to the obex, where many expiratory neurons are located. The two medullary electrodes, one for stimulation and one for recording, were positioned on opposite sides of the brainstem, since Holstege (1989) has shown that NRA neurons project mainly to the contralateral NA.

For extracellular recording in the NRA and adjacent reticular formation, we used glass micropipettes (1–10 MΩ) containing 2 M NaCl saturated with fast green dye. First, we found cells that were antidromically activated by NA stimulation (pulse duration 0.2 ms; intensity <40 μA). Then, we examined whether these cells were orthodromically activated by PAG or PCS stimulation consisting of one to three pulses (pulse duration 0.2 ms; inter-pulse interval 1 ms; intensity 1.5 times threshold for inducing vocalization). Action potentials, and either muscle or nerve activities, were sampled at 50 kHz and 1 kHz, respectively, using a Cambridge Electronic Design 1401-plus data interface and Spike 2 software in conjunction with a Power Macintosh 8100/110 computer. Some recording locations were marked by iontophoretic ejection of dye from the glass micropipette. Electrical lesions (20 μA negative current, 10 s) were also made to mark PAG, PCS and NA stimulating sites.

At the end of each experiment the animal was killed with an overdose of sodium pentobarbital. The portions of brainstem including the recording and stimulating sites were fixed in 10% formalin, sectioned transversely at 100 μm, and stained with thionine. The locations of recording sites in the VRG were reconstructed with reference to dye marks. In the kainic acid injection experiments, alternate histological sections were processed with or without counterstaining, and the extent of the injection sites was estimated by determining the location of dye in the tissue.

Results

Kainic acid injection into the NRA

Bilateral injections of kainic acid into the NRA and its adjacent area abolished PAG-induced vocalization, as tested in six cats. Before the injections, PAG stimulation first increased the activity of the diaphragm and PCA, which was followed by vocalization with highly facilitated activities of vocal fold adductor and tensor, and abdominal muscles (Figs. 1Aa, 2Aa). The TA, CT and ab-

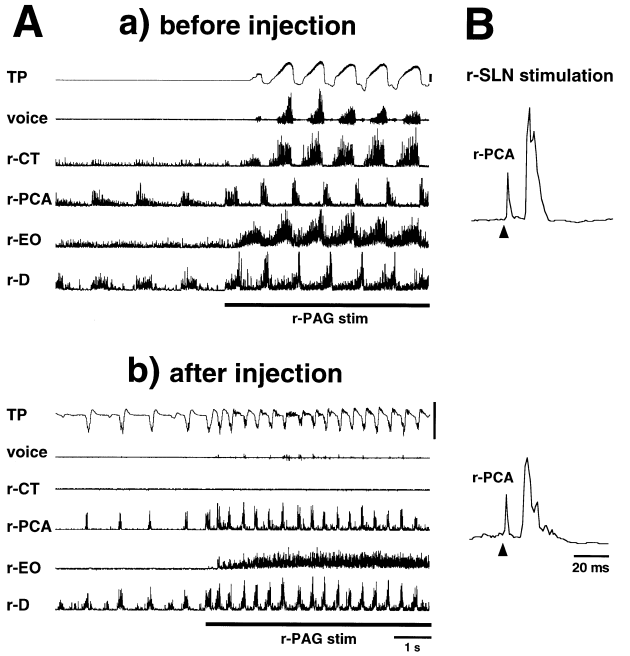

Fig. 1A, B An example of the effect of kainic acid injections into the NRA on PAG-induced vocalization (injection area #1 in Fig. 3). **A** Activities of intralaryngeal and respiratory muscles during periaqueductal gray (PAG) stimulation before (**a**) and 1 h after injection (**b**). The period of PAG stimulation is indicated by a *thick line* at the bottom of the records. **B** The reflex response of the posterior cricoarytenoid (*PCA*) muscle to stimulation of the superior laryngeal nerve (*SLN*) before (**a**) and 1 h after the injections (**b**). Time of delivery of SLN stimulation is indicated by an *arrowhead*. Number of sweeps on average was 42 in **a** and 49 in **b**. *CT* cricothyroid muscle, *D* diaphragm, *EO* external oblique muscle, *TP* tracheal pressure, *r* right. TP calibrations are 5 cm H_2O. Gain of individual electromyograms (EMGs) after kainic acid injections is the same as that before injections

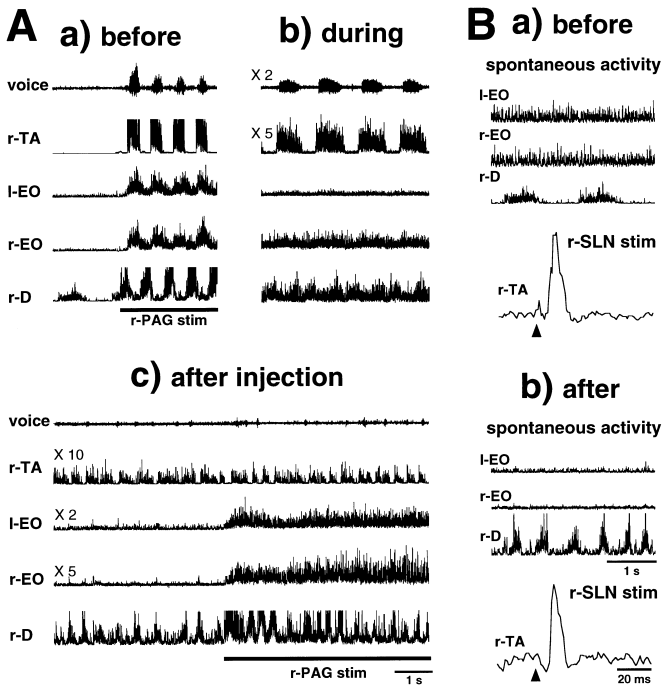

Fig. 2A, B Another example of the effect of kainic acid injections on PAG-induced vocalization (injection area # 2 in Fig. 3). **A** Activities of intralaryngeal and respiratory muscles during PAG stimulation before (**a**), during (**b**), and 1 h after injection (**c**). Part **b** was recorded during left-side injection, following right-side injection. Post-injection changes in gain are indicated on the *left* of records. **B** Spontaneous respiratory activities recorded on the diaphragm and bilateral abdominal muscles (*upper*), and reflex response on the thyroarytenoid (*TA*) muscle to stimulation of the superior laryngeal nerve (*SLN*) (*lower*) before (**a**) and 1 h after injection (**b**). Number of sweeps in average was 58 in **a** and 50 in **b**. Same *abbreviations* and *symbols* as in Fig. 1. Except for EMGs in which changes in gain are indicated, the gain of individual EMGs after kainic acid injections is the same as that before injections

dominal muscles were strongly activated during the expiratory phase with vocalization. This combination of facilitated inspiration and expiration with vocalization was induced repeatedly during PAG stimulation. As shown in Figs. 1Ab and 2Ac, kainic acid injections in four cats (#1, 2, 5 and 6 in Fig. 3) completely abolished this motor pattern of the vocal fold adductor and tensor. After the injections, PAG stimulation did not modulate TA or CT muscle activity; the CT muscle was silent during stimulation as shown in Fig. 1Ab, and the spontaneous activity of the TA muscle was not increased by PAG stimulation as shown in Fig. 2Ac. Furthermore, kainic acid injection changed the abdominal motor pattern; PAG stimulation after injection caused a bilateral tonic activation of the abdominal muscles (Figs. 1Ab, 2Ac). In contrast, PAG stimulation still increased the inspiratory activity after injections; the kainic acid injections did not change the amplitude of the PAG-induced activity of the diaphragm or PCA muscle. The respiratory frequency during stimulation was increased after the injections.

During kainic acid injections, cats usually vocalized spontaneously as a result of the initial excitation caused by the kainic acid, as shown in Fig. 2Ab. The abdominal muscles were typically activated bilaterally; however, as in the case of Fig. 2Ab in which the opposite (right) side of the brainstem had already been injected prior to the left side injection, only the abdominal muscle contralateral to the most recent injection site was activated. The motor pattern was similar to that reported by Zhang et al. (1992, 1995) in response to chemical activation of the NRA.

In two cats in which kainic acid did not completely invade into the NRA (#3 and 4 in Fig. 3), the activities of vocal fold adductor and tensor muscles were remarkably decreased in amplitude compared with before the injections and voice production did not occur. PAG stimulation no longer rhythmically, but only tonically, activated the abdominal muscles following the injections in both cats.

In the six cats, kainic acid spread between 0.2 and 4.5 mm caudal to the obex (Fig. 3); kainic acid did not invade rostrally to the obex where the laryngeal motoneurons are located. This was also confirmed by comparing the SLN-induced reflex response of intralaryngeal

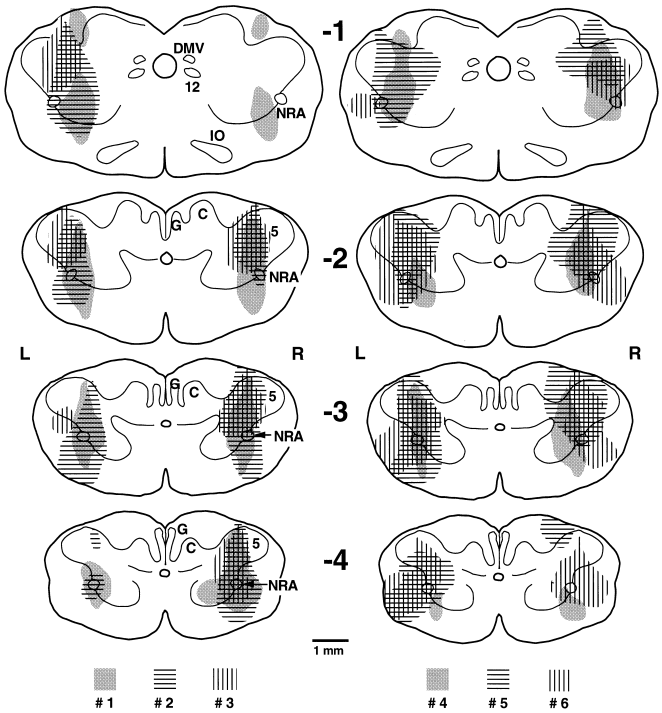

Fig. 3 Locations of kainic acid injections estimated from dye location on histological sections from six cats. Injections # 1, 2, 5, and 6 completely abolished the PAG-induced excitation of the laryngeal adductor and tensor muscles. Incomplete injections #3 and 4 did not abolish this PAG-induced motor pattern, but remarkably decreased the amplitude of vocal fold adductor and tensor activities. *C* cuneate nucleus, *DMV* dorsal motor nucleus of vagus, *G* gracile nucleus, *IO* inferior olive, *NRA* nucleus retroambigualis, *5* spinal trigeminal nucleus, *12* hypoglossal nucleus. *Number* beside each figure indicates distance (in millimeters) caudal to the obex

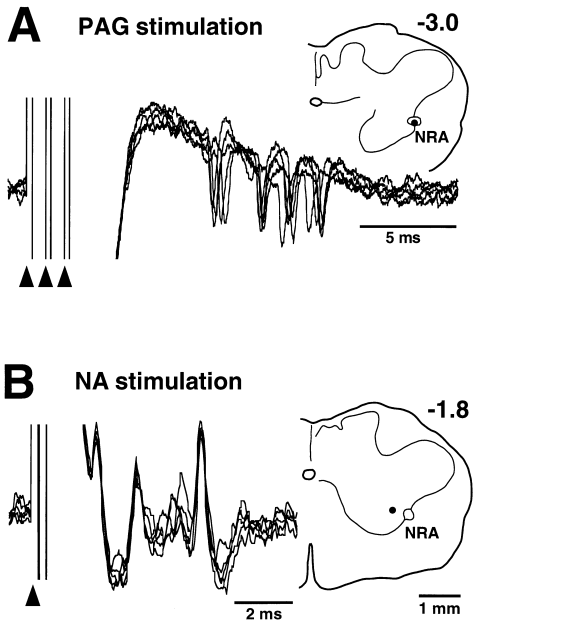

Fig. 4A, B Action potentials recorded in the vicinity of the NRA. **A** Orthodromic action potentials of a single unit evoked by a three-shock stimulus train delivered to the PAG where electrical stimulation induced vocalization (five superimposed sweeps). This unit was silent and recorded at the *small dot* indicated in the *right-hand figure*. **B** Presumptive antidromic action potentials evoked by stimulation (20 μA) of the contralateral nucleus ambiguus (*NA*) (five superimposed sweeps). These cells were recorded in the reticular formation near the NRA, indicated by a *small dot* in the *right-hand figure*. Time of delivery of stimulation is indicated by *arrowheads*

muscles before and after the injections, as shown in Figs. 1B and 2B. In the case of Fig. 1, the CT muscle response is not shown. However, since the motor pool of the CT muscle is located rostrally to that of the PCA muscle (Yoshida et al. 1982; Davis and Nail 1984), the absence of an effect on the PCA muscle response indicates that kainic acid also did not invade the CT motoneurons.

Kainic acid injection greatly reduced or abolished the spontaneous discharge of the abdominal muscle (e.g., Fig. 2Bb). Following the injections, the duration of inspiratory activity of spontaneous respiration, observed in diaphragm and PCA activities, was decreased; the respiration rate was increased (e.g., Fig. 2Bb).

Projection from the NRA to the NA

We made extracellular recordings in the vicinity of the NRA between 1 and 3.5 mm caudal to the obex. In this region, orthodromic action potentials were easily recorded following stimulation of the ipsilateral PAG or PCS (e.g., Figs. 4A, 5C and 6A), at the site where electrical stimulation induced vocalization prior to paralyzation. We could also easily record constant-latency spikes following stimulation of the contralateral NA; these presumptive antidromic potentials were recorded in the NRA and adjacent reticular formation, as indicated in Fig. 4B. The cells from which these presumptive antidromic potentials were recorded were otherwise usually silent. It was difficult to identify spikes evoked by PAG stimulation as recorded from the same cell as the presumptive antidromic spikes activated by NA stimulation because of the small amplitude of these spikes and the presence of many orthodromic spikes following PAG or PCS stimulation.

In the NRA and adjacent reticular formation, we recorded 46 cells that had constant-latency responses to contralateral NA stimulation; 40 of these were otherwise silent and six were respiratory-related neurons. For the six respiratory-related cells, the antidromic nature of the response was confirmed by the collision test (Lipski 1981). Fifteen of the 40 silent cells were tested by delivering paired short interval stimuli to the NA; 12 cells could follow interstimulus intervals of less than 3 ms. We determined whether the 40 silent cells that had a presumptive antidromic response were orthodromically activated following PAG or PCS stimulation. When an orthodromic spike seemed to be identical to a presumptive antidromic spike, the collision test was conducted by stimulating the NA following orthodromic spikes caused by PAG or PCS stimulation, as shown in Fig. 5C. We conducted this collision test to confirm that NA stimula-

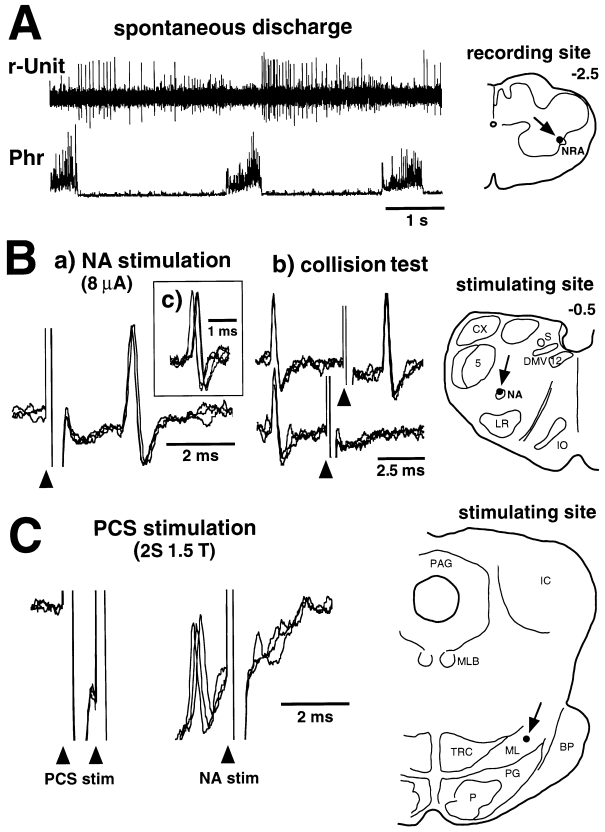

Fig. 5A, B An example of the decrementing expiratory (E) cells antidromically activated from the NA and orthodromically activated from the PAG or pontine call site (*PCS*). **A** Spontaneous activity of this unit. The recording site is indicated by a *small dot* with an *arrow*. **B a** Stimulation of the contralateral NA caused constant-latency action potentials of this unit (three superimposed sweeps). **b** NA stimulation applied after the spontaneous spikes at less than the critical delay (3.0 ms) resulted in collision of the antidromic spike (three superimposed sweeps each). **c** Slight changes in stimulus intensity, around 15 μA, caused a latency jump (three sweeps superimposed each discrete latency). The stimulating site in the NA is indicated by a *small dot* with an *arrow*. **C** Orthodromic activation of this cell in response to ipsilateral PCS stimulation (three superimposed sweeps). NA stimulation applied just after the PCS-induced spikes resulted in collision of the antidromic spike. The stimulating site in the PCS is indicated by a *small dot* with an *arrow*. *BP* brachium pontis, *CX* external cuneate nucleus, *DMV* dorsal motor nucleus of vagus, *IC* inferior colliculus, *LR* lateral reticular nucleus, *ML* medial lemniscus, *MLB* medial longitudinal bundle, *P* pyramidal tract, *PG* pontine gray, *S* solitary tract, *TRC* tegmental reticular nucleus. Other abbreviations and symbols are the same as in Fig. 3

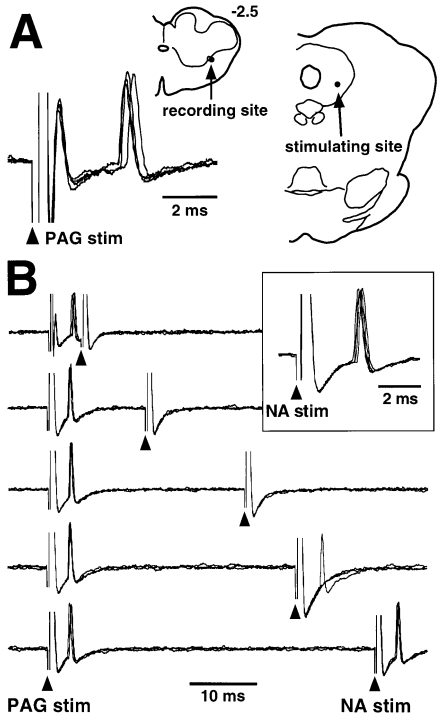

Fig. 6A, B Example of the long-term inhibition following PAG stimulation. **A** Orthodromic activation (latency at around 3 ms) of a silent cell in the NRA to ipsilateral PAG stimulation (four superimposed sweeps). Note the constant-latency response less than 1 ms after simulation. **B** NA-evoked orthodromic spike, its latency fluctuating, is shown in the *square insert* (four superimposed sweeps). NA stimuli (1.5 times threshold for inducing orthodromic action potentials) were applied 3 ms (*first trace*), 12 ms (*second trace*), 25 ms (*third trace*), 33 ms (*fourth trace*), and 45 ms (*fifth trace*) after PAG-evoked orthodromic spikes

tion evoked antidromic responses in these neurons and to determine whether the PAG- (or PCS)-evoked spike was identical to the NA-evoked antidromic spike. We could not find any silent cells in the region of the NRA that were both antidromically activated from the NA and orthodromically activated by PAG or PCS stimulation. The latencies of the presumptive antidromic responses for 34 silent units activated from the contralateral NA ranged from 0.7 to 7.0 ms (mean±SD: 2.5±1.6 ms).

Of the six respiratory-related cells antidromically activated from the NA, four had decrementing expiratory (E) firing pattern (e.g., Fig. 5A). The other two cells had E constant and weak respiratory-related firing patterns. These six cells were located within the NRA. Five of these E neurons were orthodromically activated by PAG or PCS stimulation. Figure 5 shows an example of the antidromic response of a decrementing E neuron (Fig. 5B). Stimulation of the ipsilateral PCS caused orthodromic spikes of this neuron; their latencies fluctuated (Fig. 5C). The antidromic response of this neuron was confirmed by its constant latency and the collision test (Fig. 5Ba,Bb). There were jumps in the response latency when the stimulus intensity was changed slightly at the same stimulating point in the NA, which indicated arborization of the axon (Fig. 5Bc). We also observed changes in the antidromic latency following stimuli delivered at different locations in the NA, which also indicated axonal arborization. This phenomenon was also observed in the presumptive antidromic spikes of the silent cells. The antidromic latencies for five units activated from the contralateral NA ranged from 0.8 to 2.3 ms (mean±SD: 1.9±0.7 ms). Conduction velocities, calculated from the antidromic latencies and an assumed straight-line distance between the stimulating and recording point, ranged from 3.5 to 10 m/s.

We also examined the possible projection from the NRA to the contralateral NA for more than 40 augmenting E neurons. None of these augmenting E neurons projected to the NA.

In five cells orthodromically activated by both NA and PCS stimuli, we delivered NA stimulation following PAG- (or PCS)-induced spikes. As shown in Fig. 6B, PAG stimulation caused a long-term (>10 ms) inhibition following the PAG-induced spikes in three cells (two were silent and the other one a decrementing E cell).

We also recorded constant short-latency spikes (latency <1 ms) in the NRA for three cells (two were silent and the other one a decrementing E cell) evoked by PAG stimulation. In Fig. 6A there was a constant-latency (around 0.8 ms) response just after the stimulus artifact, which was thought to be an antidromic action potential considering its short latency and the extra (and variable) time that would be required for synaptic delay. This cell both received a projection from the PAG and projected to the PAG.

Discussion

Vocalization, which can readily be elicited by electrical stimulation of the PAG, is produced by augmented expiration along with vocal fold adduction and tension, alternating with facilitated inspiration. PAG-induced vocalization was abolished by injection of the neurotoxin kainic acid into the NRA and adjacent region. Without changing the PAG-induced facilitated inspiratory activity, kainic acid injection abolished the PAG-induced motor pattern of the vocal fold adductor and tensor muscles. Furthermore, incomplete destruction of the NRA reduced the amplitude of the PAG-induced laryngeal motor pattern. Kainic acid did not invade the motoneuron pools of the CT and TA muscles in the present study. These results suggest that the NRA mediates the signals necessary for vocalization between the PAG and the laryngeal motoneuron pools. Our results are consistent with a recent report that transverse section of the brainstem at the obex level abolishes the PAG-induced laryngeal motor pattern (Zhang et al. 1995).

After kainic acid injection, PAG stimulation tonically activated the abdominal muscles bilaterally without the usual rhythmic activation. Before the injections, PAG stimulation caused the abdominal muscles to be activated not only during expiration but also during inspiration; there was an increase in background activity during the whole PAG stimulation (e.g., Fig.s 1Aa, 2Aa). The amplitude of this base activity was almost the same as that of the post-lesion tonic activity. Katada et al. (1996) showed that PAG-induced rhythmic abdominal activity was abolished by a midsagittal brainstem section between the level of the obex and the C1 root, where the axons of caudal VRG E bulbospinal neurons cross the midline before descending in the spinal cord (Merrill 1974; Arita et al. 1987; Miller et al. 1989). More than two-thirds of VRG E bulbospinal neurons increase their firing during the expiratory phase of vocalization (Katada et al. 1996); it is suspected that these neurons cause the expiratory part of PAG-induced abdominal activity. Mouton and Holstege (1994) showed that PAG neurons project to the medial parts of the intermediate zone of the thoracic and lumbar cord, where interneurons for motoneurons are located. We suggest that the PAG-induced tonic abdominal activity may be due to activation of PAG-spinal fibers.

Although kainic acid injection abolished PAG-induced excitation of the vocal fold adductor and abdominal muscles during the expiratory phase, the inspiratory activity recorded from the diaphragm and PCA muscle was not changed in amplitude. This result indicates that the PAG affects the respiratory system not only via the PAG-NRA pathway. The PAG neurons project to the region of the retrofacial nucleus (RFN) (Kobayashi et al. 1994; Sakamoto et al. 1996), which is thought to be a key region for respiratory rhythmogenesis (Smith et al. 1991; Bianchi et al. 1995; Ezure 1996). The retrofacial region contains several types of respiratory neurons, including inspiratory neurons having a constant discharge pattern (I-CON neurons) that excite other inspiratory neurons, including bulbospinal, propriobulbar, and laryngeal motoneurons (Ezure et al. 1989). Most of these I-CON neurons increase their firing during the inspiratory phase of vocalization (Sakamoto et al. 1996; Nonaka et al. 1997). Thus, the PAG-RFN projection may participate in generating the rhythm and enhanced inspiratory discharge during PAG-induced vocalization. In contrast, the PAG-NRA projection mainly controls the vocal activity during the expiratory phase, such as adducting and tensing the vocal fold, and reinforcing expiration.

We found some NRA decrementing E neurons intermediating between the PAG and the NA. These neurons may play an important role in PAG-induced vocalization. However, it seems likely that there are not sufficient of such neurons to explain the origin of the remarkable excitation of the vocal fold adductor and tensor during vocalization. Indeed, decrementing E neurons are very few in the NRA (Miller et al. 1985). We would have to record cell activity during vocalization to discuss the functional role of these E neurons in vocalization. On the other hand, NRA augmenting E neurons were never antidromically activated by NA stimulation. In addition, since virtually all NRA augmenting E neurons are bulbospinal and none have medullary axon collaterals, at least in the cat (Merrill 1974; Miller et al. 1985; Arita et al. 1987; Merrill and Lipski 1987), we think that NRA augmenting E neurons are not important for the laryngeal vocal movement.

We also recorded in the vicinity of the NRA many constant-latency action potentials of silent cells following contralateral NA stimulation. However, we could not confirm that these potentials were really antidromic or that PAG or PCS stimulation activated these same silent cells. It was difficult to identify the presumptive antidromic potentials with those activated by PAG or PCS stimulation, because these action potentials were usually small in amplitude. Since some of these silent neurons receive complex and long-term inputs from the PAG as

shown in Figs. 4A and 6, it is possible that these cells receive the PAG outputs integrated via interneurons located in or around the NRA. We suspect that the silent cells in the NRA and the reticular formation around the NRA may play an important role in PAG-induced vocalization. In addition, the NRA projection to the PAG indicates the possibility of a feedback system between the PAG and NRA in controlling vocalization. Many authors have reported that pulmonary and laryngeal afferent inputs affect PAG- or PCS-induced vocalization (Testerman 1970; Thoms and Jürgens 1981; Davis et al. 1993; Sakamoto et al. 1993; Shiba et al. 1995, 1997). Thus, several kinds of feedback, including not only external or response feedback but also feedback via internal neuronal circuits, may function in the control of voice production.

Acknowledgements This work was supported by grant NS20585 from the National Institute of Neurological Disorders and Stroke and grant DC02644 from the National Institute on Deafness and Other Communication Disorders, National Institutes of Health.

References

Adametz J, O'Leary JL (1959) Experimental mutism resulting from periaqueductal lesions in cats. Neurology 9:636–642

Arita H, Kogo N, Koshiya N (1987) Morphological and physiological properties of caudal medullary expiratory neurons of the cat. Brain Res 401:258–266

Bandler R (1982) Induction of "rage" following microinjections of glutamate into midbrain but not hypothalamus of cats. Neurosci Lett 30:183–188

Bianchi AL, Denavit-Saubié M, Champagnat J (1995) Central control of breathing in mammals: neuronal circuitry, membrane properties, and neurotransmitters. Physiol Rev 75:1–45

Coyle JT, Schwartz R (1983) The use of excitatory amino acids as selective neurotoxins. In: Björkand A, Hökfelt T (eds) Handbook of chemical neuroanatomy. Elsevier, Amsterdam, pp 508–527

Davis PJ, Nail BS (1984) On the location and size of laryngeal motoneurons in the cat and rabbit. J Comp Neurol 230:13–32

Davis PJ, Zhang SP, Bandler R (1993) Pulmonary and upper airway afferent influences on the motor pattern of vocalization evoked by excitation of the midbrain periaqueductal gray of the cat. Brain Res 607:61–80

de Lanerolle NC (1990) A pontine call site in the domestic cat: behavior and neural pathways. Neuroscience 37:201–214

Ezure K (1996) Respiratory control. In: Miller AD, Yates BJ (eds) Vestibular autonomic regulation. CRC Press, Boca Raton, Fla, pp 53–84

Ezure K, Manabe M, Otake K (1989) Excitation and inhibition of medullary inspiratory neurons by two types of burst inspiratory neurons in the cat. Neurosci Lett 104:303–308

Holstege G (1989) Anatomical study of the final common pathway for vocalization in the cat. J Comp Neurol 284:242–252

Jürgens U (1994) The role of the periaqueductal grey in vocal behavior. Behav Brain Res 62:107–117

Kanai T, Wang SC (1962) Localization of the central vocalization mechanism in the brain stem of the cat. Exp Neurol 6:426–434

Katada A, Sugimoto T, Utsumi K, Nonaka S, Sakamoto T (1996) Functional role of ventral respiratory group expiratory neurons during vocalization. Neurosci Res 26:225–233

Kelly AH, Beaton LE, Magoun HW (1946) A midbrain mechanism for vacio-vocal activity. J Neurophysiol 9:181–189

Kobayashi Y, Katada A, Myoga H, Sakamoto T (1994) A PHA-L study on descending pathway for vocalization from the periaqueductal gray to the lower brainstem in cats. Soc Neurosci Abstr 20:1407

Larson CR (1991) On the relation of PAG neurons to laryngeal and respiratory muscles during vocalization in the monkey. Brain Res 552:77–86

Larson CR, Kistler MK (1984) Periaqueductal gray neuronal activity associated with laryngeal EMG and vocalization in the awake monkey. Neurosci Lett 46:261–266

Larson CR, Kistler MK (1986) The relationship of periaqueductal gray neurons to vocalization and laryngeal EMG in the behaving monkey. Exp Brain Res 63:596–606

Lipski J (1981) Antidromic activation of neurones as an analytic tool in the study of the central nervous system. J Neurosci Methods 4:1–32

Merrill EG (1974) Finding a respiratory function for the medullary respiratory neurons. In: Bellaris R, Gray EG (eds) Essays on the nervous system. Clarendon Press, Oxford, pp 451–486

Merrill EG, Lipski J (1987) Inputs to intercostal motoneurons from ventrolateral medullary respiratory neurons in the cat. J Neurophysiol 57:1837–1853

Miller AD, Ezure K, Suzuki I (1985) Control of abdominal muscles by brain stem respiratory neurons in the cat. J Neurophysiol 54:155–167

Miller AD, Tan LK, Lakos SF (1989) Brainstem projections to cats' upper lumbar spinal cord: implications for abdominal muscle control. Brain Res 493:348–356

Mouton LJ, Holstege G (1994) The periaqueductal gray in the cat projects to lamina VIII and the medial part of lamaina VII throughout the length of the spinal cord. Exp Brain Res 101:253–264

Nonaka S, Katada A, Sakamoto T, Unno T (1997) Brainstem neural mechanisms for vocalization in decerebrate cats. Ann Otol Rhinol Laryngol Suppl (in press)

Sakamoto T, Yamanaka Y, Wada K, Nakajima Y (1993) Effects of tracheostomy on electrically induced vocalization in decerebrate cats. Neurosci Lett 158:92–96

Sakamoto T, Nonaka S, Katada A (1996) Control of respiratory muscles during speech and vocalization. In: Miller AD, Bianchi AL, Bishop BP (eds) Neural control of the respiratory muscles. CRC Press, Boca Raton, Fla, pp 249–258

Shiba K, Yoshida K, Miura T (1995) Functional roles of the superior laryngeal nerve afferents in electrically induced vocalization in anesthetized cats. Neurosci Res 22:23–30

Shiba K, Yoshida K, Nakajima Y, Konno A (1997) Influences of laryngeal afferent inputs on intralaryngeal muscle activity during vocalization in the cat. Neurosci Res 27:85–92

Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL (1991) Pre-Bötzinger complex: a brainstem region that may generate respiratory rhythm in mammals. Science 254:726–729

Testerman RL (1970) Modulation of laryngeal activity by pulmonary changes during vocalization in cats. Exp Neurol 29:281–297

Thoms G, Jürgens U (1981) Role of the internal laryngeal nerve in phonation: an experimental study in the squirrel monkey. Exp Neurol 74:187–203

Wada K (1994) Brainstem mechanisms of vocalization analyzed by electrical stimulation and chemical stimulation of sodium glutamate in cats (in Japenese). J Otolaryngol Jpn (Nippon Jibiinkoka Gakkai Kaiho) 97:1456–1463

Yoshida Y, Miyazaki T, Hirano M, Shin T, Kanaseki T (1982) Arrangement of motoneurons innervating the intrinsic laryngeal muscles of cats as demonstrated by horseradish peroxidase. Acta Otolaryngol 94:329–334

Zhang SP, Davis PJ, Bandler R, Carrive P (1994) Brain stem integration of vocalization: role of the midbrain periaqueductal gray. J Neurophysiol 72:1337–1356

Zhang SP, Davis PJ, Carrive P, Bandler R (1992) Vocalization and marked pressor effect evoked from the region of the nucleus retroambigualis in the caudal ventrolateral medulla of the cat. Neurosci Lett 140:103–107

Zhang SP, Bandler R, Davis PJ (1995) Brain stem integration of vocalization: role of the nucleus retroambigualis. J Neurophysiol 74:2500–2512