Fastigial nucleus-mediated respiratory responses depend on the medullary gigantocellular nucleus

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Xu, Fadi, Tongrong Zhou, Tonya Gibson, and Donald T. Frazier. Fastigial nucleus-mediated respiratory responses depend on the medullary gigantocellular nucleus. J Appl Physiol 91: 1713–1722, 2001.—Electrical stimulation of the rostral fastigial nucleus (FNr) alters respiration via activation of local neurons. We hypothesized that this FNr-mediated respiratory response was dependent on the integrity of the nucleus gigantocellularis of the medulla (NGC). Electrical stimulation of the FNr in 15 anesthetized and tracheotomized spontaneously breathing rats significantly altered ventilation by 35.2 ± 11.0% (P < 0.01) with the major effect being excitatory (78%). This respiratory response did not significantly differ from control after lesions of the NGC via bilateral microinjection of kainic or ibotenic acid (4.5 ± 1.9%; P > 0.05) but persisted in sham controls. Eight other rats, in which horseradish peroxidase (HRP) solution was previously microinjected into the left NGC, served as non-stimulation controls or were exposed to either 15-min repeated electrical stimulation of the right FNr or hypercapnia for 90 min. Histochemical and immunocytochemical data showed that the right FNr contained clustered HRP-labeled neurons, most of which were double labeled with c-Fos immunohistochemistry in both electrically and CO2-stimulated rats. We conclude that the NGC receives monosynaptic FNr inputs and is required for fully expressing FNr-mediated respiratory responses.

electrical stimulation; hypercapnia; kainic acid; double labeling.

SEVERAL LINES OF EVIDENCE have demonstrated the involvement of the cerebellar fastigial nucleus (FN), especially its rostral region (r) in respiratory central control. First, electrical stimulation of neurons within the FNr primarily elevates respiratory frequency in both anesthetized cats and rats (27, 28, 30). Second, investigators, using extracellular recordings, have confirmed the presence of respiratory-mediated FN neurons in alert (9) and anesthetized cats (14, 29). Third, c-Fos immunohistochemical studies in the cat have shown a remarkable increase in FNr neuronal activity in response to severe hypercapnia (33) and ablation of this region attenuated respiratory response to hypercapnia in the rat and cat (30, 31). In agreement, by using functional magnetic resonance imaging in the human, FN neurons were found to respond to hypercapnia (8), and their activity was comparatively decreased in patients with respiratory dysfunction, such as congenital central hypoventilation syndromes (10). Fourth, electrical stimulation of the FNr elevates respiratory-modulated neuronal activities recorded within the medullary dorsal and ventral respiratory groups concomitant with the changes denoted in phrenic motor output (28). Moreover, the latency required for the onset of these neuronal responses to FNr stimulation suggested an involvement of paucisynaptic rather than monosynaptic contacts onto the dorsal and ventral respiratory groups of the medullary respiratory network. Subsequent studies have examined the effects of destruction of neurons within the pontine respiratory groups, Botzinger complex, and the red and paramedian reticular nucleus on FNr-mediated respiratory response (34). These regions are believed to be critical to respiratory timing control and/or known anatomically to receive FN inputs. Unfortunately, these lesions failed to alter respiratory responses to electrical stimulation of the FNr. Therefore, the site of entry of FNr efferents into the pontomedullary respiratory control network remained unclear.

In addition to the regions described above, anatomic studies have indicated that the FN also projects to the contralateral nucleus gigantocellularis of the medulla (NGC), inferior olivary complex, vestibular nucleus, and midbrain in rats, cats, and monkeys (1, 2, 25). Further investigations demonstrated that pressure injection of phaseolus vulgaris-leucoagglutinin (an anterograde tracer) into the FNr most heavily labeled terminals in the contralateral NGC (11). In contrast, the caudal FN predominantly projected to the inferior olivary complex and midbrain (18). The potential involvement of the NGC in respiratory control is supported by several parallel studies. Neuronal pathways controlling the diaphragm and abdominal muscle have been investigated in the rat by transynaptic transport of the neuroinvasive pseudorabies virus (3, 6). Propriobulbar (third-order) neurons that project to the bulbospinal neurons innervating phrenic and transverses and rectus abdominal motoneurons were found in the NGC, which strongly suggests that NGC neurons innervate premotoneurons responsible for control of inspiration and expiration. Moreover, respiratory-
modulated neurons have been recorded in cats’ NGC, and electrical stimulation of these neurons predominantly increased respiratory neuronal activity within the nucleus tractus solitarii and ambiguous nuclei (26). Therefore, we hypothesized that the FN-mediated respiratory response depends on efferent projections that relay through the NGC.

We tested our hypothesis in anesthetized and spontaneously breathing rats by comparison of cardiorespiratory responses evoked by electrical stimulation of the FNr before and after chemical [ibotenic or kainic acid (KA)] lesions of the NGC. We found that electrical stimulation of the FNr predominantly increased or occasionally depressed respiratory frequency, and these responses disappeared after selective destruction of NGC neurons via bilateral microinjection of ibotenic or KA. In addition, KA microinjection produced an immediate significant elevation in respiratory output that approached control values 1.5 h after injection. The possibility that FNr neurons involved in respiratory modulation monosynaptically project to the NGC was tested by subsequently using a double-labeling technique. Horseradish peroxidase solution (HRP) was microinjected into the left NGC for retrograde tracing of FNr neurons. After 16 h of survival, the animal was exposed to repeated electrical stimulation of the right FNr for 15 min or hypercapnia (10% CO2) for 90 min. The histochemical and immunocytochemical data showed that the right FNr contained clustered HRP-labeled neurons, most of which were double labeled with c-Fos immunoreactivity in both electrical and CO2 stimulated rats. These results demonstrated that the NGC receives FNr monosynaptic projections and is required for full expression of FN-modulated respiratory responses.

**METHODS**

**General.** The experimental protocols described in this study were approved by the Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act. They were in accordance with National Institutes of Health policy on humane care and use of laboratory animals. The experiments were conducted in two series. The first series of experiments was performed on 13 anesthetized (chloralose and urethane; 500 and 100 mg/kg ip, respectively), tracheotomized, and spontaneously breathing Sprague-Dawley rats (300–400 g). The left femoral vein and artery were cannulated, the former for anesthetic administration and the latter for monitoring arterial blood pressure (ABP). Supplementation with 2% Chicago Sky Blue, 100 nl over 1 min). The electrical stimulation was repeated 1.5 h after KA injection. Ibotenic acid was reported to produce a much more localized lesion of cell bodies in the injection site with less neurotoxicity compared with KA (13). In two rats, the same protocols were repeated except that microinjection of ibotenic acid (100 mM, 100 nl) into the NGC was utilized instead of KA. Considering the time-course effect, FNr stimulation was given over the same time sequentially after the above protocols without lesion in three rats.

**General histological examination.** After completion of protocols, the animals were killed by administration of additional anesthetic, and the brain stem and cerebellum were removed and placed in 10% formalin. After at least 3 days of immersion fixation, the brain stem was frozen, and 50-μm sections were cut, mounted, and stained for cell bodies. The tissue sections containing sites marked with Chicago sky blue were drawn with camera lucida.

**Employment of double-labeling technique.** To morphologically confirm that FNr neurons involved in respiratory modulation monosynaptically project to the NGC, a double-labeling technique was used in the second series experiment (*n* = 8). After 16 h of survival, HRP (25–50 nl of 20% Sigma lectin conjugated) was injected into the left NGC for retrograde tracing of FNr neurons, and the animals were divided into three groups for subsequent protocols. A stimulating electrode was placed into the right FNr, as described previously, in two groups. Animals either received no electrical stimulation (group I, *n* = 2) or received electrical stimulation (group II, *n* = 3) via an electrode fixed at the site where electrical stimulation produced a significant respiratory augmentation (at least 20% greater than control). The stimulation (50 or 100 Hz for 10-s duration) was repeated every 30 s for 15 min (*n* = 2) or 1 h (*n* = 1). Three rats in group III were exposed to hypercapnia (10% CO2 + 21% O2 balanced, with N2) for 90 min. Transcardial perfusion with PBS (100 ml) and then...
Paraformaldehyde (2%, 400 ml) were performed in all three groups of animals 1.5 h after initiation of the protocols.

The brain stem and cerebellum were removed and cut into blocks with identifiable landmarks, fixed in 4% paraformaldehyde for 4–6 h, and cryoprotected overnight in 30% sucrose in PBS at 4°C. For preparation of slices, the brain stem and cerebellum were frozen and transected (50 μm) with a freezermicrotome. After transection, the cerebellar and brain stem slices were stored in PBS-azide (0.1%) at 4°C overnight. The sections were incubated with 1) 0.3% H2O2 in PBS for 30 min at room temperature, 2) PBS containing 3% normal goat serum (NGS; catalog no. S100) and 0.25% Triton X-100, 3) c-Fos antiserum (catalog no. PC38; diluted 1:1,500 in PBS-3% NGS-0.25% Triton X-100) for 48 h at 4°C, 4) biotinylated goat antirabbit secondary antibody (diluted 1:600 in PBS-3% NGS-0.25% Triton X-100; Vector, ABC kit, PK-6100, IgG) for 2 h at room temperature, and 5) alkaline phosphatase avidin-biotin complex (Vector, ABC-AP Kit, Cat. AK-5000) diluted according to the instruction of Vector blue substrate at room temperature in a dark environment until suitable blue staining developed (10–20 min). Rinses were performed after each incubation step except step 2. The sections were immersed in PBS and finally mounted on subbed slides, air dried, dehydrated, cleared, and covered with slips.

Data acquisition and analysis. ABP, VT, respiratory frequency (f) and minute ventilation (V˙E, product of VT and f) were recorded and analyzed. The control (baseline) values, expressed as absolute values, were obtained by averaging the relevant variables within five breaths just before application of electrical stimuli and 1 min immediately before microinjection of KA (ibotenic acid) into the NGC, respectively. The responses were collected and measured for 1) three breaths immediately after electrical stimulation and 2) five breaths (immediate response) and 1.5 h after KA injection. The responses were presented as percent change from control. All data are presented as means ± SE. Paired t-test was used for comparing the differences of cardiorespiratory activities before and after FNr stimulation. One-way ANOVA and the Newman-Keuls test were used to identify significance of cardiorespiratory responses immediately and 1.5 h after KA injection into the NGC. A P value of <0.05 was considered significant.

In each animal, the numbers of neurons either single (brown for HRP and blue for c-Fos protein) or double labeled were counted by eye under a microscope in two to three representative slices containing FNr. The total number of FNr neurons projecting to the NGC was the sum of the neurons both brown and double labeled. The percentage of double-labeled neurons over the total neurons projecting the NGC represents the density of FNr neurons projecting to the NGC as well as activated by electrical or CO2 stimulation.

RESULTS

The ventilatory responses to electrical stimulation of FNr were evaluated in 15 rats. Similar to previous reports (27, 28, 30), simulation of the FNr facilitated or inhibited ventilation. As shown in Fig. 1, threshold electrical stimulation markedly enhanced ventilation by increasing VT and f (Fig. 1A). On the other hand, in a few cases, stimulation inhibited ventilation primarily via slowing respiratory frequency (Fig. 1B). Compared with the respiratory responses, the associated pressor
response was not consistent, i.e., absent in some rats but present in others, as shown in Fig. 1, A and B, respectively. Electrical stimulation was applied in 19 sites of the FNr with the major response excitatory (78%). The averaged change in $V_E$ was $\sim 30\%$ for the excitatory responses and 45% for the inhibitory responses as summarized in Fig. 2, A and B. The changes in $f$ were greater than that in $V_T$, but both were significant compared with control. The placements of the stimulating electrode are illustrated in Fig. 2C. The sites where electrical stimulation produced inhibitory and excitatory ventilatory responses are also shown. No specific FNr region was found to be responsible for either excitatory or inhibitory responses.

Except for one rat in which KA injection produced an apnea leading to death, FNr stimulation was repeated after bilateral lesions of the NGC ($N = 11$). Figure 3 depicts examples showing that both ventilatory excitatory and inhibitory responses are abolished after bilateral lesions of the NGC. As shown (Fig. 3A), electrical stimulation of FNr elicited an increase in ventilation, but the responses were eliminated after KA was injected into NGC. Similar NGC lesions also abolished the inhibitory response to stimulation of FNr (Fig. 3B). Both lesioned areas were located in the vicinity of the NGC (Fig. 3C). Similar results were also observed following microinjection of ibotenic acid into NGC. Group data are illustrated in Fig. 4. In general, FNr-stimulation-induced changes, whether excitatory (9 rats) or inhibitory (2 rats), were $35 \pm 11\%$ for $V_E$, $30 \pm 7\%$ for $V_T$, and $28 \pm 8\%$ for $f$. These changes were eliminated after chemical lesions of the NGC. These data indicate that the integrity of the NGC was essential to the FN-mediated respiratory responses. To evaluate any time-related effects, FNr stimulation was given over the same experimental time frame without lesion in three rats. FN-mediated responses were not significantly altered over the time interval equivalent to that of experiments involving lesions.

The effects of KA injection into NGC on cardiorespiratory baseline values were evaluated. A typical example of cardiorespiratory activity before, immediately after, and 1.5 h after bilateral injection of KA is illustrated in Fig. 5. Tidal volume was dramatically elevated immediately after administration of KA, returning to baseline values 1.5 h after the injection. Statistically, initial increases in $V_E$ were significant primarily via an elevation in $V_T$ (Fig. 6). However, the values obtained 1.5 h after KA injection did not reach significance. These data suggest that the initial effect of KA injection is to stimulate the NGC, thereby elevating respiration. In addition, the ABP response to KA injection into the NGC was either absent, increased, or decreased. The differences of the averaged ABP values obtained from control (132.2 ± 5.5 mmHg), immediately after (141.2 ± 4.4 mmHg), and 1.5 h (135.7 ± 8.4 mmHg) after KA injection were not significant ($P > 0.05$).

With respect to morphological data, in group I (electrode implanted but without electrical stimulation), heavily HRP brown-labeled neurons were predominantly observed in the contralateral FNr, suggesting that FNr neurons synaptically project to the contralateral NGC. In group II (with electrical stimulation),
most FNr neurons were double labeled with c-Fos expression and HRP, i.e., blue coupled with brown color (Fig. 7A). Magnified examples of neurons single (blue or brown) and double labeled are presented in Fig. 7B. It should be noted that c-Fos immunoreactivity of the FNr in the rats receiving electrical stimulation for 1 h was not different from that for 15 min. The results suggest that FNr neurons presumably responsible for respiratory modulation evoked by electrical stimulation synaptically project to the NGC. Similar staining was also observed in animals of group III exposed to hypercapnia (Fig. 8). Interestingly, a much heavier staining (single and double labeling) was observed in the FN as contrasted to the interposed and lateral cerebellar nuclei (Fig. 8A). This suggests that, among the cerebellar deep nuclei, the NGC primarily receives FNr projections and that hypercapnia selectively activates FNr neurons. It is clear that not all FNr neurons showing monosynaptic projections to the NGC are activated by electrical or CO2 stimula-
tion because ~20% of the FNr neurons were brown, single labeled.

**DISCUSSION**

Chemical lesions of the NGC eliminated FN-mediated respiratory responses. The absence of FN-mediated respiratory responses 1.5 h after KA was because of selective inactivation of NGC neurons. KA is an excitotoxic agent for neurons (sparing axons of passage and surrounding terminals and glial), causing an initial transient excitation of neuronal activity followed by inactivation and destruction (15, 22). Similar results were obtained after microinjection of ibotenic acid, which has been reported to produce a much more localized lesion of cell bodies with less neurotoxicity compared with KA (12). These findings demonstrated that respiratory responses to FNr stimulation were eliminated after cell death in the vicinity of the NGC. The data provide the first experimental evidence to demonstrate that NGC neurons are essential for the full expression of FN-mediated respiratory responses. The variability in the location and extent of chemical lesions prevents completely ruling out the possible participation of structures around the NGC in the FN-mediated respiratory responses. The assumption that expression of FN-mediated respiratory responses depends on the brain stem respiratory network has been raised previously. It has been reported that FN-mediated respiratory responses primarily affect respiratory timing controlled by pontomedullary respiratory groups. Extracellular recordings have showed that electrical stimulation of the FNr alters respiratory-modulated neuronal activities within the medullary dorsal and ventral respiratory groups concomitant with changes denoted in the phrenic motor output (28). Furthermore, investigators have demonstrated that this FNr modulation of respiratory-modulated neurons within the dorsal and ventral medullary respiratory groups was achieved by paucisynaptic contacts. Similar to the previous reports (27, 28, 30, 34), electrical stimulation of different sites within the FNr produced either excitatory (major) or inhibitory respiratory responses. The mechanisms underlying these different responses are not clear. The FN contains large (major)

![Fig. 5. A typical example comparing ventilation before, immediately after, and 1.5 h after bilateral lesions of the NGC in an anesthetized rat. The injection duration is marked by a horizontal bar (bottom). KA, kainic acid.](image)

![Fig. 6. Group data comparing V̇E (A), V̇ (B), and f (C) before, immediately after, and 1.5 h after bilateral lesions of the NGC. Values are means ± SE. *Significantly different among the ventilatory values obtained before, immediately after, and 1.5 h after bilateral lesions of the NGC (P < 0.05).](image)
and small neurons; the former are excitatory and glutamnergic or aspartatergic, and the latter are inhibitory and GABAergic (13). The excitatory respiratory response evoked by stimulation in various FNr sites may result from the activation of a preponderance of large neurons compared with the small or vice versa for the inhibitory response. Interestingly, both excitatory and inhibitory respiratory responses to stimulation of the FNr were eliminated after ablation of the NGC, suggesting that the NGC is required for fully expressing both types of FNr respiratory modulation.

NGC is involved in respiratory control. Our data indicate that the immediate respiratory responses to microinjection of KA into the NGC are excitatory via dramatic elevation of VT. The initial excitatory responses to the injections are most likely due to KA excitatory effect on the NGC neurons. Our data are consistent with those reported by Foster and colleagues (personal communication). They found in awake goats that a significant hyperventilation was observed within minutes when agonist of excitatory amino acid receptor (methyl-D-aspartic acid) was injected into the NGC. Conversely, inactivation of the NGC by microinjection of neurotoxin (ibotenic acid) eventually caused hypoventilation in most goats. In addition, recent studies reported that lesions of the NGC abolished or significantly attenuated the excitatory respiratory motor responses to electrical stimulation of the vestibular nerve (17) and nucleus (32). These data demonstrate that the major effect of the NGC is to facilitate respiration, although an inhibitory influence on respiration was also observed. For example, electrical or chemical stimulation of the NGC occasionally caused respiratory inhibition via reduction of respiratory frequency (26). Moreover, the excitatory respiratory response to stimulation of hypothalamic locomotor region was significantly depressed when the stimulation was applied close to the pontomedullary border within the rostral NGC in anesthetized cats (20). Several reasons might account for the different NGC effects (excitatory or inhibitory) on respiration. First, there may be a species difference. Second, the region of the NGC that produces respiratory inhibition seems to be located in the most rostral region, whereas the region to induce respiratory excitation is toward the caudal region. Third, the roles the NGC plays in different types of respiratory modulations are not uniform. There are several lines of evidence to support NGC involvement in respiratory control. Morphologically, neuronal pathways controlling the diaphragm and abdominal muscle have been investigated in the rat by transynaptic transport of the neuroinvasive pseudorabies virus (3, 6). Propriobulbar (third-order) neurons that project to the bulbospinal neurons innervating phrenic and abdominal motoneurons were found in the NGC. Moreover, by using retrograde tracing technique (HRP), it was found that nasolabialis motoneurons of the facial nucleus driven by the respiratory central network received monosynaptic afferents from NGC neurons (4). These results strongly suggest that NGC neurons innervate premotoneurons responsible for control of inspiration and expiration. With the use of c-Fos immunohistochemical approach, brain stem neuronal pathways activated during hypercapnia and hypoxia have been mapped (24). Other than the pontomedullary nuclei known to be critical in respiratory control, such as the nucleus tractus solitarii, retrotrapezoid nucleus, locus coeruleus, pontine respiratory groups, and A1 and A5 regions, the NGC was also labeled by c-Fos immunoreactivity. These data indicate NGC linkage with central pathways involved in respiratory chemoreflexes. Functionally, respira-
tory-modulated neurons have been recorded in cats’ NGC, and electrical stimulation of these neurons predominantly increased respiratory neuronal activity within the nucleus tractus solitarii and ambiguous nuclei (26).

The NGC may not be critical for eupneic breathing control in the anesthetized rat since eupneic breathing observed 1.5 h after KA injection into the NGC was not significantly different from the control. It appears different from the NGC effect observed in the awake goat in which dysfunction of the NGC eventually caused hypoventilation (unpublished observation in Forster’s laboratory). This difference might be related to different species, experimental preparation (awake vs. anesthetized), and time course at which the respiratory responses to lesions were collected (5 h vs. 1.5 h after KA injections). On the other hand, it is possible that the facilitatory role of the rats’ NGC in respiratory motor control emerges when local neurons are stimulated during stressed breathing. In support, c-Fos immunohistochemistry studies have shown that NGC neurons are activated by exposure to either hypercapnia or hypoxia (24).

NGC is not critical for cardiovascular modulation. Previous studies have demonstrated the presence of a depressor region in the rostral NGC. Electrical or chemical stimulation of the pontomedullary border lowered ABP and heart rate in anesthetized cats (20,

Fig. 8. Cross sections of cerebellar deep nuclei in the rat exposed to hypercapnia following horseradish peroxidase (HRP) injection into the NGC. A: overview of the cerebellar deep nuclei in which heavy staining selectively exists in the FN; the horizontal bar represents 500 μm. B: magnified view (×3) of the FN area of A. C: magnified view (×9) of the FN area of A. Arrows marked by 1, 2, and 3 point to brown-, blue-, and double-labeled neurons, respectively.
Do FNr neurons responsible for respiratory modulation synaptically project to the NGC? Previous studies, using either autoradiographic (1, 2) or anteriograde tracing techniques (11) have demonstrated that the FN predominantly innervates the contralateral NGC. The double labeling used in the present study was designed to provide experimental evidence to show that FNr neurons related to respiratory modulation directly project to the NGC. The c-Fos protein product of c-fos protooncogene has been extensively utilized as an index to express the population of cells whose activity increases in response to controlled stimuli (7, 16, 21). For example, investigators have used c-Fos immunocytochemistry to label the neurons electrically stimulated (21). Investigators implanted a bipolar stimulating electrode into the rat hindlimb motor-sensory cortex to activate local neurons that were subsequently identified by using c-Fos immunoreactivity. Interestingly, c-Fos immunoreactivity was significantly increased in the stimulating sites with the labeling density showing no difference in rats receiving electrical stimulation for 15 min compared with 1 h. Similarly, electrical simulation of carotid sinus nerve over a 15-min period was adequate to increase c-Fos immunoreactivity in the nucleus tractus solitarii (7). In the present study, local electrical stimulation and hypercapnic exposure sufficient to evoke a significant respiratory augmentation were used to activate FNr neurons, and subsequent c-Fos immunocytochemistry was employed to mark these neurons. Some neurons were labeled by c-Fos immunoreactivity in the FNr with most of them double-labeled with HRP. We recognized that both electrical and chemical stimulation of the FNr could also activate nonrespiratory-related neurons. However, our data suggest that at least a population of FNr neurons responsible for respiratory modulation synaptically project to the NGC.

In summary, in the present study, three major findings were obtained. First, bilateral lesions of the NGC eliminate FN-mediated ventilatory responses, including both excitatory and inhibitory effects. Second, at least some of FNr neurons responsible for respiratory modulation monosynaptically project to the NGC. Third, chemical activation of NGC causes an excitatory influence on respiration, although the NGC seems to be not critical to eupneic breathing in anesthetized rats. We conclude that the NGC receives monosynaptic inputs from the FNr and that its integrity is essential for fully expressing the FN-mediated respiratory responses. Moreover, the NGC is likely capable of facilitating respiratory output, and this ability emerges presumably during stressed breathing.

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