c-Fos expression in the central nervous system elicited by phrenic nerve stimulation

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Malakhova, O. E., and P. W. Davenport. c-Fos expression in the central nervous system elicited by phrenic nerve stimulation. J Appl Physiol 90: 1291–1298, 2001.—Phrenic nerve afferents (PNa) have been shown to activate neurons in the spinal cord, brain stem, and forebrain regions. The c-Fos technique has been widely used as a method to identify neuronal regions activated by afferent stimulation. This technique was used to identify central neural areas activated by PNa. The right phrenic nerve of urethane-anesthetized rats was stimulated in the thorax. The spinal cord and brain were sectioned and stained for c-Fos expression. Labeled neurons were found in the dorsal horn laminae I and II of the C3–C6 spinal cord ipsilateral to the site of PNa stimulation. c-Fos-labeled neurons were found bilaterally in the medial subnuclei of the nucleus of the solitary tract, rostral ventral respiratory group, and ventrolateral medullary reticular formation. c-Fos-labeled neurons were found bilaterally in the paraventricular and supraoptic hypothalamic nuclei, in the paraventricular thalamic nucleus, and in the central nucleus of the amygdala. The presence of c-Fos suggests that these neurons are involved in PNa information processing and a component of the central mechanisms regulating respiratory function.

afferents; control of breathing; cerebral cortex

The purpose of the present study is to provide a functional map of central structures activated by stimulation of the phrenic nerve afferents (PNa) using c-Fos immunohistochemical techniques. Neurons expressing c-Fos are identified by nuclear staining of antibodies that react with the c-Fos protein. c-Fos techniques have been widely used as a method to functionally map polysynaptic neuronal pathways activated by specific afferent stimuli. The use of c-Fos has allowed for the mapping of central neuronal pathways involved in seizure propagation (11) and pain (16). Dean and Seagard (10) used c-Fos techniques to demonstrate the brain stem sites of activation in response to carotid sinus baroreceptor stimulation. Solano-Flores et al. (30) used c-Fos to determine the forebrain, brain stem, and spinal cord structures activated by renal nerve afferent stimulation. Dun et al. (12) recommended c-Fos as a metabolic marker to identify a network of neurons responding to a specific cardiovascular challenge. Thus c-Fos activation may be utilized as a functional neuroanatomic marker for neurons activated by afferent stimulation. Identification of c-Fos-expressing neurons in response to specific afferent stimulation allows for the identification of specific afferent polysynaptic pathways in the brain and spinal cord. However, although positive signals provide valuable insights into the functional neuroanatomic organization of brain pathways involved in afferent and efferent processing of nerve afferent information, the absence of c-Fos expression does not prevent participation of a structure in these pathways. Hence, c-Fos immunohistochemistry provides a tool for mapping neuronal pathways linked to afferent stimulation, with positive identification of c-Fos expression providing

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evidence for sensory pathway connection. In the present study, it was hypothesized that electrical stimulation of PNa elicits c-Fos expression in specific nuclei in the central nervous system of rats.

METHODS

Isolation and stimulation of PNa. Experiments were performed in Long-Evans rats (300–350 g). The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida. The animals were anesthetized with urethane (1.2 mg/kg ip), tracheotomized, and artificially ventilated. The chest was opened from the right side, and the right phrenic nerve was isolated ∼1 cm cranial to the diaphragm. The phrenic nerve was freed from the caudal vena cava by blunt dissection. Placement of a bipolar sliding jaw electrode (Harvard Apparatus, Holliston, MA) on the right phrenic nerve insulated the electrode from the surrounding tissue. The electrode was connected to the stimulator (Grass Instrument). The phrenic nerve was physiologically identified by movement of the diaphragm with brief electrical stimulation of the intact nerve. Phrenic nerve efferents were eliminated by crushing and cutting the nerve distal to the electrode. Denervation of the efferents was verified by the lack of movement of the diaphragm during electrical stimulation of the phrenic nerve. Two stimulus protocols were used: 1) single pulses of 1-ms duration were presented at 0.3 Hz continuously for 30 min (n = 4); and 2) pulse trains were presented at a frequency of 0.3 Hz, the duration of the pulse trains was 100 ms, the duration of the pulses was 7 ms at a frequency of 30 Hz, and the stimulus trains were applied for 30 min (n = 4). The animals were maintained under urethane anesthesia, mechanically ventilated, and continuously monitored during the 30-min stimulation period and for 2.5 h after the cessation of stimulation. Both stimulus protocols activated myelinated and nonmyelinated phrenic afferents.

Two groups of control animals were tested. The first control group was experimentally prepared as described above. The phrenic nerve was isolated and placed over the stimulating electrodes, but the nerve was not stimulated (n = 2). The second control group of rats was only anesthetized, tracheostomized, and mechanically ventilated (the thorax was not opened) for the same time period as the experimental animals (n = 4).

c-Fos immunohistochemical procedures. After the completion of the poststimulation period (2.5 h), all the animals were overdosed with anesthesia and perfused transcardially with 0.9% saline solution. Then the animals were perfused transcardially with 4% paraformaldehyde in 0.4 M PBS (pH 7.2) at room temperature. The brain and spinal cord in T2 were removed. The neural tissue was postfixed overnight in 4% paraformaldehyde. The tissue was then blocked and placed in 30% sucrose in PBS at 4°C before it was processed for c-Fos immunohistochemistry. Frozen coronal sections (40 μm thick) of the forebrain, brain stem, and spinal cord were cut and mounted on glass slides. The slides were placed in a humidity-incubation chamber, and blocking solution (1:30 goat serum in PBS + Triton X-100) was applied for 1 h. The primary antibody, α-c-Fos antibody (Jackson ImmunoResearch Laboratory, West Grove, PA), was then applied at a dilution of 1:1,000 with PBS containing 0.4% Triton X-100. After 16–18 h, the sections were processed using Biotin-SP-Aff pure goat anti-rabbit IgG for 4 h at a dilution of 1:500. Avidin-Texas red (Jackson ImmunoResearch Laboratory) was then applied for 30 min. Mounted sections were air-dried overnight, dehydrated, and coverslipped.

Internal staining control sections were also prepared. Tissue sections were processed without the c-Fos primary antibody for each group of animals (stimulated and control). The absence of the primary antibody to c-Fos staining would eliminate specific c-Fos labeling in the neuronal nucleus. Sections from control groups were stained using the same protocol. The presence of staining in the absence of the c-Fos primary antibody would indicate nonspecific labeling by the staining procedure. No c-Fos labeling was evident in any of the brain sections treated in this internal staining control procedure.

Data analysis. Brain and spinal cord sections were analyzed using fluorescent microscopy for avidin-Texas red-stained neuronal nuclei. c-Fos-labeled neurons were identified if the neuronal nucleus was labeled (Fig. 1). The c-Fos-labeled neurons were identified, and their location in the spinal cord and brain structures of each animal was determined by Nissl staining of adjacent sections and a standard stereotaxic atlas of the rat brain (23). The magnitude of c-Fos expression in a tissue section was categorized as described in Table 1.

RESULTS

The electrical stimulation of PNa elicited c-Fos-labeled neurons (Fig. 1A) in the forebrain, brain stem,
and spinal cord in all animals ($n = 8$). Both stimulus protocols elicited a similar distribution of c-Fos-labeled neurons within the central nervous system. Few c-Fos-labeled neurons were observed in the control animals (Fig. 1B). No c-Fos labeling was observed in the internal control sections that were processed without the c-Fos primary antibody.

**Cervical spinal cord c-Fos expression.** In the spinal cord, c-Fos-labeled neurons were found in superficial laminae of the dorsal horn at the C_{3–5} spinal level ipsilateral to the side of PNa stimulation (Fig. 2, A and B). c-Fos-labeled neurons were not observed (Fig. 2C) in the contralateral dorsal horn. There were no c-Fos-labeled neurons in the spinal cord in control animals.

**Brain stem c-Fos expression.** The most prominent structure in the brain stem (Fig. 3) containing c-Fos-labeled neurons in the stimulated animals was the nucleus of the solitary tract (Sol). These labeled neurons (Table 1) were found within the dorsomedial subnucleus of the Sol (SolDM; Fig. 3) and within the intermediate subnucleus (SolIM). The number of labeled cells in the SolIM diminished (Table 1) caudal and rostral to the area postrema. Scattered c-Fos labeling was observed (Fig. 3) in the subnucleus centralis.
of the Sol. No labeled neurons were observed in other subnuclei of the Sol complex. There were very few c-Fos-labeled neurons in subnuclei of the Sol complex of control animals.

c-Fos-labeled neurons were identified bilaterally (Fig. 3) in the rostroventral respiratory group (RVRG) of the PNa-stimulated animals. These neurons were concentrated in the middle of the anteroposterior extent of the nucleus. The central region of the RVRG contained c-Fos-labeled neurons (Table 1), with a few neurons scattered around this central region. The RVRG of the control animals contained very few c-Fos-labeled neurons.

The ventrolateral medullary reticular formation (VLM) contained well-defined c-Fos-labeled neurons (Table 1) located ventrolaterally to the nucleus ambiguus (Fig. 3). c-Fos-labeled neurons were absent in control animals. A few neurons expressing c-Fos were observed in the nucleus ambiguus, raphe pallidus, lateral reticular nucleus, and area postrema (Fig. 3).

Forebrain c-Fos expression. c-Fos-labeled neurons (Table 1) were found (Fig. 4) throughout the medial division of the central nucleus of the amygdala (CeM). Labeled neurons were also observed scattered bilaterally (Fig. 4) within the medial components of the anterodorsal part of the medial amygdaloid nucleus (MeAD). No other subnuclei of the amygdaloid complex contained PNa-elicited c-Fos-labeled neurons.

A high density (Table 1) of c-Fos-labeled neurons was observed bilaterally in the supraoptic nucleus (SO) in stimulated animals (Fig. 4). There were no labeled neurons in the control animals.

c-Fos-labeled neurons were observed bilaterally in the parvocellular (Table 1) and magnocellular subdivision (Fig. 4) of the paraventricular nucleus (PAP) of the hypothalamus in the PNa-stimulated animals. The magnocellular subdivision (PaAM) contained few labeled cells (Table 1) compared with the parvocellular subdivision (PaAP). Control animals had few c-Fos-labeled neurons in both subdivisions of the PAP.

The paraventricular nucleus of the thalamus (PV) contained c-Fos-labeled neurons throughout its extent (Fig. 4). Labeled neurons were scattered bilaterally within the PV. There was no apparent regional difference in the number of c-Fos-labeled neurons from the anterior and the posterior aspect of the nucleus. Control animals exhibited a few neurons with c-Fos labeling. A few c-Fos-staining cells were observed in the arcuate nucleus, lateral hypothalamic area, dorsomedial hypothalamic nucleus, and central medial thalamic nucleus.

DISCUSSION

Immunohistochemical detection of c-Fos protein in neurons has been used as a method to identify neuronal systems that are activated by specific sensory stimulation (10, 11, 28, 30, 33, 37). The expression of c-Fos provides immunohistochemical evidence of neurons that increase their activity in response to stimulation of afferents or structures in the sensory pathway. The present study demonstrated that electrical stimulation of PNa elicited c-Fos expression in neurons from the spinal cord to the cerebral cortex. The neurons expressing c-Fos are part of the central neural system for phrenic afferent and efferent information processing. Although the expression of c-Fos demonstrates activation initiated by PNa stimulation, the lack of c-Fos expression does not indicate a lack of other central neural nuclei involvement in phrenic information processing. It is possible that some neuronal systems were inhibited and c-Fos would not have been detected. It is also likely that some of the central projection nuclei in phrenic afferent/efferent pathways are too small or too diffuse to allow detection by the induction of c-Fos (28, 30). Thus the absence of this protein expression does not preclude participation of a structure in phrenic information processing pathways.
This study used multiple methods to control for non-PNa c-Fos expression. c-Fos expression (background staining) in nonstimulated animals is very common when the animal is exposed to stress (15, 29, 36). Physical handling of unconditioned, awake rats during injection of anesthetics is a stressor and has been suggested to elicit c-Fos expression, particularly in the affective pathways (5, 16, 19). In the present study, the rats were handled daily for several months before their use, which resulted in docile behavior by the rats when they were handled for anesthesia induction. Thus one control for background c-Fos expression in the present study was stress reduction by routine handling of the rats.

The control group of animals was treated identically in surgical preparation, including thoracotomy and mechanical ventilation, to the PNa-stimulated animals. This provides a measure of the c-Fos expression elicited by the experimental preparation. Although this control elicited c-Fos in some neurons, the staining was diffuse, and relatively few neurons expressed c-Fos. Another control excluded the thoracotomy, which differentiated between the two control groups for the effect of surgical exposure of the phrenic nerve on c-Fos expression. Again, this control resulted in only scattered background c-Fos expression. Thus c-Fos expression was greater in the PNa-stimulated group than in these control groups.

Another important control was for staining unrelated to the c-Fos protein. This internal control was applied to the stimulated and control groups. Alternate spinal cord and brain sections were prepared without the application of the primary c-Fos antibody required to bind to the c-Fos protein. The fluorescent marker attached to a secondary antibody binds with the primary antibody for visualization of c-Fos expression. If fluorescent labeling occurs in the absence of the primary antibody, then the secondary antibody is binding to a molecule that may not be c-Fos and is, therefore, nonspecific fluorescence. In the course of the study, this artifact was encountered with one batch of fluorescent secondary antibody, and the results of all tissue treated with this label were unreliable. These tissues were discarded and replaced with new tissues treated with secondary antibody that did not bind in the absence of the primary antibody. The results presented in the present study include only tissue that did not have background staining with this internal control. Thus, in the present study, no fluorescent staining was found in the absence of the c-Fos primary antibody, scattered c-Fos expression was observed with experimental control animals, and increased c-Fos expression was observed (Table 1) when the phrenic nerve was electrically stimulated.

The phrenic nerve has been shown to contain myelinated and nonmyelinated afferents, but a systematic analysis of the transsynaptic central projection of PNa in a single animal has not been done (4, 17, 18, 27). Electrical stimulation of the phrenic nerve intrathoracically below the heart eliminates pericardial and mediastinal afferents. Mechanical motion of the diaphragm and associated mechanical stimulation of the attached thoracic structures were eliminated by severing the phrenic nerve distal to the stimulating electrodes; hence, the electrical stimulation activated only phrenic nerve fibers (motor and sensory). Stimulation of phrenic motor fibers would activate the cell bodies in the phrenic motor nucleus in the central horn of the cervical spinal cord, but stimulation of these neurons would not be involved in transsynaptic activation of central neurons. The absence of c-Fos expression in the ventral horn of the cervical spinal cord indicates that antidromic activation of phrenic motoneurons does not elicit c-Fos expression.
c-Fos-labeled neurons were found in the dorsal horn superficial laminae of C₃–C₅ segments of the spinal cord, ipsilateral to the side of PNa stimulation. This staining demonstrates that the stimulated PNa entered only the ipsilateral cervical spinal cord. The staining of superficial laminae dorsal horn neurons is consistent with small afferent fiber activation.

Within the brain stem, c-Fos-labeled cells were found bilaterally in Sol, VLM, and RVRG nuclei. This c-Fos expression pattern indicates that unilateral phrenic afferent stimulation has bilateral connections in the brain stem. One of the most prominent structures containing c-Fos-labeled neurons after PNa stimulation was the Sol complex, SolDM and SolIM. The reticular formation of the lower brain stem contains neuronal circuits for the generation of sympathetic tone, respiratory rhythm, and muscle tone. Langhorst et al. (20) recorded single neuron activity in the medial two-thirds of the reticular formation (VLM). They demonstrated that single neurons received information from somatosensory afferents of skin, joints, and muscles together with afferents from baroreceptors, chemoreceptors, and lung inflation receptors and lung deflation receptors in dogs. The VLM, in its caudal aspects, contained clusters of c-Fos-labeled neurons after PNa stimulation in the present experiment. The labeled clusters of neurons corresponded well with the location of the A₁ noradrenergic cells (2). The neurons within the caudal VLM project directly to the SO, PV, and PAP nuclei, suggesting that these neurons act as relays for afferents to forebrain areas, participate in hypothalamic respiratory reflexes (35), and may be correlated with the tonic involuntary descending pathway originating in the VLM. The presence of PNa-related neurons in these regions suggests a relay and integrative function of this portion of the pathway.

c-Fos-labeled neurons were found in the para-ambigual portion of the RVRG, which contains mostly inspiratory neurons. The RVRG is one of the clusters of medullary respiratory neurons that lies in the ventrolateral reticular formation between the first cervical segment and the retrofacial nucleus (13, 22). The Botzinger complex is the aggregation of respiratory neurons at the level of the retrofacial nucleus. The inspiratory neurons concentrated at the rostral pole of the RVRG and associated with the Botzinger complex provide widespread connections to several populations of respiratory neurons. Phrenic motoneurons receive inspiratory drive from neurons located in dorsal and para-ambigual portions of the RVRG (6, 9). The presence of PNa-related neurons in the RVRG suggests that these neurons may be a component mediating respiratory muscle reflexes.

c-Fos-labeled neurons were observed in forebrain structures, i.e., PaAP, PaAM, SO, PV, and CeM, after PNa stimulation. Tanaka et al. (34) used morphofunctional methods on rats to investigate projections to PaAP from the medial amygdaloid nucleus and CeM. Pickel et al. (25) showed reciprocal connections between the amygdala and medial nuclei of the solitary tract of the rat and found that the SolIM at the level of the area postrema received mainly cardiorespiratory visceral afferents. In the present study, c-Fos-labeled cells were found in CeM and medial division of Sol and PaAM and PaAP nuclei. Marson (21) used a transneuronal tracing method, injection of a pseudorabies virus into the bladder of rats, to identify labeled cells in the lateral hypothalamus, PaAP, and medial preoptic area. Similarly, using Fos immunohistochemical methods after electrical stimulation of the renal nerve afferent in rats, Solano-Flores et al. (30) found labeled cells in the SO, PaAM, and PV. Labeled neurons were also observed in the arcuate nuclei and ventrolateral medullary and pontine reticular formation, as well as in the commissural and medial subnuclei of the Sol and lamina I–V of the dorsal horn of the thoracolumbar spinal cord. These areas are also known to have connections with respiratory centers and to participate in respiratory reflexes (35). These results indicate that visceral trunk afferent information is conveyed to a number of central neural regions known to be involved in the regulation of homeostasis. The activation of these nuclei by electrical stimulation of phrenic afferents suggests that this respiratory afferent information is an important component of central mechanisms regulating these homeostatic functions.

Petrov et al. (24) used a combination of anatomic tracing with c-Fos after stimulation of the CeM. They found c-Fos activation in PaAP, Sol, and VLM nuclei as well as reciprocal connections between these structures. Their results indicate that direct and indirect inputs from the amygdala may influence the activity of autonomic neurons in the brain stem. The CeM receives projections from the Sol (1). The CeM integrates somatic and visceral responses during various adaptive behaviors and plays a role in mediating the autonomic and neuroendocrine responses to stressful environmental conditions. The PAP nucleus, via its direct projections to catecholaminergic and noncatecholaminergic neurons, may participate in activation of brain stem neurons. Using anterograde tracing in rats, Zheng et al. (40) observed descending fibers bilaterally, from PaAP to the spinal cord, through three pathways: dorsal longitudinal fasciculus, medial forebrain bundle, and lateral funiculus. The pathway for PNa activation of the amygdala and PV of the thalamus and hypothalamic structures (PAP and SO nuclei) also has outputs to brain stem respiratory centers (Sol and RVRG). Most of these structures have reciprocal connections. This circuit provides a link between phrenic afferents, cortical pathways, and output pathways to the respiratory pump.

Electrical stimulation of myelinated phrenic afferents has been shown to elicit evoked potentials in the somatosensory region of the cat cerebral cortex (8). Bolsel et al. (4) recorded phrenic afferent-activated spinothalamic tract neurons in the cervical spinal cord. These reports suggest a lemniscal and/or spinothalamic tract projection pathway to the somatosensory cortex. Retrograde fluorescent tracers were injected (38) at the site of the primary somatosensory cortical activation site. Labeled neurons were found in the
ventroposterior oralis of the thalamus nucleus. Little else is known about phrenic afferent pathways to the sensorimotor cortex. Two separate respiratory muscle afferent pathways to higher brain centers have been proposed (7): 1) respiratory muscle afferent information ascends from the spinal cord to the sensorimotor cortex via the lemniscal pathway, and 2) respiratory muscle afferents ascend to the affective cortex via the amygdala and then project to the mesocortex. Strauss et al. (32) reported phrenic afferent activation of the limbic cortex in humans. Respiratory sensations may rely, in part, on cortical integration of respiratory muscle afferent information. Phrenic activity may be one of the neural substrates involved in optimization of respiratory and nonrespiratory behaviors via adaptive and/or behavioral control of breathing (14, 39). The finding of c-Fos-labeled neurons in our study provides anatomic evidence for this higher brain center modulation of respiration.

On the basis of the results of the present study, two cortical systems may influence respiratory movements: somatosensory and limbic. Phrenic afferent information ascends from the spinal cord C3–C5 in the superficial laminae of the dorsal horn, through the lateral funiculus, to brain stem structures (Sol, VLM, and RVRG), which modulate and integrate this information. This information may be transmitted to diencephalic structures (PAP, PV, and SO), which coordinate various inputs and transmit them to the cortex through a thalamocortical pathway. The function of this pathway could relate to the proprioceptive control of respiratory muscles and the integration of movements originating in the motor cortex.

In addition, the limbic system, via the hypothalamus and thalamus, may modulate the activity of brain stem centers responsible for the coordination of respiratory, vegetative, and somatomotor reactions to phrenic afferent information. These results suggest that diaphragm afferents in the phrenic nerve are involved in the activation of the central nervous system pathways that participate in somatomotor, affective, and homeostatic respiratory functions. Further studies are needed to explore the role of these afferents in the control of breathing.

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