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Brainstem Circuitry of Tracheal-bronchial Cough: c-fos Study in Anesthetized Cats

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Abstract

The *c-fos* gene expression method was used to localize brainstem neurons functionally related to the tracheal-bronchial cough on 13 spontaneously breathing, pentobarbitone anesthetized cats. The level of Fos-like immunoreactivity (FLI) in 6 animals with repetitive coughs (170±12) induced by mechanical stimulation of the tracheobronchial mucosa was compared to FLI in 7 control non-stimulated cats. Thirty-four nuclei were compared for the number of labeled cells. Enhanced cough FLI was found bilaterally at following brainstem structures, as compared to controls: In the medulla, FLI was increased in the medial, interstitial and ventrolateral subnuclei of the solitary tract ($p<0.02$), in the retroambigular nucleus of the caudal medulla ($p<0.05$), in the ambigular, paraambigular and retrofacial nuclei of the rostral medulla along with the lateral reticular nuclei, the ventrolateral reticular tegmental field ($p<0.05$), and the raphe nuclei ($p<0.05$). In pons, increased FLI was detected in the lateral parabrachial and Kölliker-Fuse nuclei ($p<0.01$), in the posteroventral cochlear nuclei ($p<0.01$), and the raphe midline ($p<0.05$). Within the mesencephalon cough-related FLI was enhanced at the rostral midline area ($p<0.05$), but a decrease was found at its caudal part in the periaqueductal gray ($p<0.02$). Results of this study suggest a large medullary - pontine - mesencephalic neuronal circuit involved in the control of the tracheal-bronchial cough in cats.

Keywords

c-fos; brainstem; tracheal-bronchial cough; cat

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1. Introduction

Cough is a three-phase expulsive motor act characterized by an inspiratory effort (inspiratory phase), followed by forced expiratory effort against a closed glottis (compressive phase) and then by opening of the glottis allowing rapid airflow (expulsive phase). This behavior is a non-ventilatory airway reflex that protects the lungs against aspiration of foreign particles and noxious challenges both in animals and humans (Korpas and Tomori, 1979; Widdicombe and Fontana, 2006; Pecova and Tatar, 2007). A hypothesis regarding the neurogenesis of tracheal-bronchial cough holds that respiratory pattern generator undergoes reconfiguration to produce this behavior (Shannon et al., 2000; Baekey et al., 2001). However, we have recently proposed a hypothesis that cough is produced by a larger *holarchical* (multilevel and multibehavioral) network (Bolser et al., 2006) that includes the respiratory central pattern generator, as well as one or more other control elements (termed *holons*; Koestler, 1967). The locations and fundamental behavioral aspects of specific neuronal populations that contribute to the respiratory central pattern generator were described. The generation of the respiratory rhythm might include some neurons with pacemaker characteristics, as well as probably two separate but coordinated rhythm generators. These generators, which produce the rhythm by interactions (primarily by reciprocal inhibition) of several neuronal populations, are located in the pre-Bötzinger complex area of the rostral ventral respiratory group and in the retrotrapezoid-parafacial respiratory group of neurons (Feldman et al., 2003; Feldman and Del Negro, 2006). On the other hand, the identity and locations of neurons that contribute to control elements in the cough network are unknown (Jakus et al., 2004a; Bolser et al., 2006; Jakus, 2007). Gestreau et al. (1997) utilized the *c-fos* method to examine the brainstem locations of neurons activated during cough induced by electrical stimulation of the superior laryngeal nerve. However, such stimulation also elicits expiration reflex, swallowing, and augmented breaths, which may have induced Fos in neurons that did not participate in the cough network. We hypothesized that in addition to the well defined medullary respiratory areas, the *c-fos* method would reveal the contribution of other important brainstem regions in the neurogenesis of tracheal-bronchial cough. Furthermore, we speculated that the neuronal network responsible for the production and control of the cough would be refined in our study by utilizing of a stimulus that only elicits coughing.

2. Material and methods

2.1. General experimental procedures

Animal care in this study was in agreement with NIH Guide for the Use and Care of Laboratory Animals, the Animal Welfare Guidelines of the University of Florida, and the ethical rules and law regulations of the Slovak Government. Experiments were performed on 13 adult cats of either sex. The animals were divided into two groups. Six cats (2.90 ± 0.23 kg) were stimulated in order to induce repetitive tracheal-bronchial coughing. The other 7 animals (3.17 ± 0.32 kg) were non-stimulated and they represent a control group. Cats were anesthetized in animal facility with pentobarbital (Vetbutal, Polfa) by an initial dose of 35-40 mg/kg i.p. and then transported to the laboratory. The proper level of anesthesia was maintained by supplemental doses of pentobarbital given intravenously (1-3 mg/kg/hour) depending on an appearance of the palpebral reflex, eye blink reflex, and a jaw tone. Skin and muscle infiltrations were performed by local anesthetics (Mesocaine, Zentiva) at the sites of surgical interventions to minimize induction of *c-fos* being related to a stimulation of nociceptors (Lykkegaard et al., 2005).

The surgical preparation, processing of the signals, brainstem tissue perfusion, fixation and immunohistochemical *c-fos* processing, completed by data analysis and statistical evaluation were the same for all cats and have been described in detail previously (Jakus et al., 2004b). Briefly, a cannula was placed to the trachea allowing the animal to breathe spontaneously and

for mechanical stimulation of tracheobronchial mucosa. Catheters were inserted into the right femoral vein and artery to inject supplemental doses of anesthetic and for monitoring of the arterial blood pressure (BP), respectively. In both groups of animals arterial BP, airflow, tidal volume (Fig. 1), and the end-tidal CO₂ concentration were monitored. Then the mean values of respiratory rate, systolic and diastolic BP, and end-tidal CO₂ concentration were taken in the pre-stimulation control, stimulation, and survival time periods (analogously for non-stimulated cats in early - control and late - survival periods). These values were not different during the course of experiment and between the control and coughing groups of animals (ANOVA). Immunohistological tissue processing was restricted only to animals that had maintained systolic / diastolic values of BP within the range 120-195 / 70-130 mmHg (16-26 / 9-17 kPa), the respiratory rate 13 - 25 breaths / min., and the end-tidal CO₂ concentration between 4% and 6%. Rectal temperature was kept at 36.5-38.5 °C using a heating lamp and a pad.

Repetitive coughs (Fig. 1) were evoked by mechanical stimulation (5-20 s) of trachea and large bronchi with a nylon fiber (diameter 0.2-0.35 mm). Several “non-stimulating” respiratory cycles and some longer (up to 3 minutes) resting periods were allowed to occur between the stimuli. In the series of stimulated cats 170±12 (140-217) tracheal-bronchial coughs were induced during the 30-40 min. period of stimulation (control cats were only monitored).

Our preliminary experiments did not reveal prominent differences in basal Fos-like immunoreactivity (FLI) between 3 or 6 hour time periods from the surgical preparation to the perfusion. Thus, following the preparatory period (approximately 1 hour from the induction of anesthesia to the completion of surgery), the cough protocol was initiated 30 min later. After the stimulation period (30-40 min), both groups of cats had the same 2 hour survival time (Dragunow and Robertson, 1988; Orendacova et al., 2000), so the perfusion of all animals occurred 3 hours after the completion of the surgical preparation. Animals were then deeply anaesthetized (by an additional dose of 10 mg/kg of pentobarbital i.v.) and a medial thoracotomy was performed.

2.2. Perfusion, fixation, and immunocytochemical tissue processing

Cats were perfused transcardially with a 2500 ml bolus of saline containing heparin (1000U/100 ml), followed by a solution of 1500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PBS) at pH 7.4. Immediately after perfusion, the brainstem was removed and *post-fixed* in the same fixative solution for 6 hours. Tissue was then processed in solutions containing 10, 20, and 30% sucrose at 4°C during a period of 48-72 hours. Transverse frozen tissue sections (40µm) were prepared using a cryostat and collected in PBS (0.9% NaCl in 0.1 M phosphate buffer, pH 7.4). In order to block peroxidase activity, free-floating sections were incubated (30 min. at room temperature) in PBS containing 0.3% H₂O₂. The slices were then rinsed twice in PBS. To prevent non-specific binding, the sections were incubated for 3 hrs in 0.1 M PBS containing 1% bovine albumin, 3% normal goat serum and 0.2% Triton X-100. An incubation in polyclonal primary Fos rabbit antibody (c-fos Ab-5 PC 38, Calbiochem, diluted 1:7000) for 24 hrs at 4°C followed. The sections were then rinsed in PBS and incubated for 2 hours in biotinylated goat-anti rabbit antiserum (BA 100, Vector Laboratories, CA, diluted 1:600 in PBS), and washed twice after incubation. Finally, the slices were incubated in avidin-biotin-peroxidase complex (1:100, 1hr, Peroxidase Vectastain Elite ABC kit VC-PK 6100, Vector Laboratories, CA). After two washes in PBS and one in Tris-HCl saline, the sections were processed using 0.05% diaminobenzidine as a chromo gene. Following two washes in distilled water, they were mounted on gelatin-coated slides, then air-dried, dehydrated, cleared, and coverslipped in Canadian balsam. Adjacent sections were counterstained with Hematoxylin-eosin staining to delineate the location of neural structures.

2.3. Data analysis and statistical evaluation

Every fifth brainstem section was processed immunocytochemically for Fos-like proteins, then it was used for identification and counting of FLI neurons (one count every 0.2 mm of rostro-caudal dimension). Some of these as well as some additional sections (3-6) with the most typical structures were pre-selected in each rostro-caudal extension before the immunocytochemical processing. Anatomical landmarks for detection of brainstem structures were generally established using adjacent counterstained sections. Sections were drawn by camera lucida. The graphic reconstructions of representative sections were performed according to the cytoarchitectonic atlas (Berman, 1968). The subdivisions of the solitary tract nucleus (NTS) are referred according to Kalia and Mesulam (1980), and that of Berman (1968), and Petrovicky (1980) for other brainstem structures. The distribution and the number of Fos positive neurons were determined with an optical Leitz microscope and video-scanning system (CCD Philips) coupled to a PC. The counted cells were inspected (and confirmed) for a presence of Fos staining under the high magnification of the microscope. Software Ellipse (ViDiTo) was also used to evaluate the grain density by automatic counting of all dots from the same pre-selected level of intensity. An increase in the number of immunoreactive neurons in coughing cats compared to control animals indicated the level of "recruitment" of neuronal activity provoked by cough. The number of FLI neurons in particular structures was averaged to obtain mean count per each area of brainstem (*the average group number of FLI neurons in particular area/hemisection - AGN/H*). Individual data were collected for comparison and averaging between the right and left sides of corresponding brainstem structures, and for comparison between the stimulated and control cats. All data were processed statistically and are given as a mean $\pm$ S.E.M. Analysis of variance (ANOVA) with Student-Newman-Keuls post tests were used for processing of cardiorespiratory parameters. For normally distributed FLI data either the unpaired t-test (when S.E.M.s were not significantly different) or Welch corrected unpaired t-test (when S.E.M.s were significantly different), and also the Mann-Whitney test for non-normally distributed data were applied in order to determine the statistical significance of the differences. $P < 0.05$ was considered significant.

3. Results

Cough (Fig. 1) was characterized by a forceful expiratory efforts preceded by deep preparatory inspirations. Fos-like expression was detected within the nuclei of immunoreactive neurons as a dark-brown staining of variable intensity. FLI was observed in both the coughing and control cats, but to a different degree. Figs. 2 and 3 show a diagrammatic reconstruction of FLI distribution within the medulla oblongata (Fig. 2; at 3 and 1.5 mm caudal to the obex and 1 and 4 mm rostral to the obex), the pons Varoli (Fig. 2; at 6 and 10.5 mm rostral to the obex), and the mesencephalon (Fig. 3; at levels 13 and 17.5 mm rostral to the obex) in both groups of cats. Table 1 summarizes the results of quantitative analysis of FLI, indicating particular AGN/H at twelve brainstem areas with significant differences found between the groups of control and coughing cats. Examples of enhanced (or reduced) FLI in the brainstem structures under control and the cough are shown on Figs. 4 and 5.

3.1. Immunostaining in control animals

In control quietly breathing cats (n=7) baseline FLI was scattered throughout the brainstem in the sensory and motor areas linked to respiratory, cardiac, vasomotor, vestibular, cochlear control and nociception. Distribution of FLI was quite similar on both sides of the transverse sections. Baseline FLI was detected throughout the rostro-caudal axis of the brainstem and was concentrated mostly at two medullary levels: 1.5 mm caudal to 2.5 mm rostral to the obex, and 3-5 mm rostrally to the obex (Table 1). Slightly below and above these levels FLI was not consistent. At the level 0.5-2.5 mm rostrally to the obex Fos-labeled neurons were distributed mainly within the medial, interstitial, and ventrolateral subnuclei of the NTS (Figs. 2, 4B), and

in the alaminar part of the spinal trigeminal nucleus (Table 1). At the same level a slightly higher density of FLI was found (Table 1) in the lateral reticular nucleus (LRN), nucleus ambiguus, ncl. paraambigualis, and in an adjacent lateral tegmental field (FTL, also referred to as the parvocellular reticular formation). At the level 3-4 mm rostrally to obex baseline FLI was detected within the ventral FTL, in the medial part of alaminar spinal trigeminal nucleus, and the infratrigeminal ncl., in ncl. ambiguus, ncl. paraambigualis, and the retrofacial nucleus (Figs. 2, 4C; Table 1). At the level 4-6 mm rostral to obex discrete FLI was found in retrofacial ncl. (Figs. 2, 4C, Table 1) in the caudal edge of the facial and retrotrapezoid nucleus, in LRN and in the ventrolateral part of FTL. Weak FLI labeling was detected in the caudal medulla (at 4 to 2.5 mm caudal to the obex) mostly confined to the retroambigual ncl. and the adjacent FTL (Figs. 2, 4A; Table 1), as well as in the raphe nuclei (ncl. raphe magnus and parvus) at the level of 5-6 mm rostral to the obex (Table 1). Higher, but inconsistent baseline FLI was also detected within the vestibular nuclei around the ponto-medullary junction (Fig. 2) with $AGN/H = 21 \pm 4$. At the pons Varoli the highest baseline density of *c-fos* neurons was found bilaterally in the rostral dorsolateral pons (levels 10-11 mm rostral to the obex), functionally known to as “pontine respiratory group”. Higher baseline FLI was detected at the level 10.5-11 mm in the brachium conjunctivum, in the coerue nucleus, in the medial (NPBM), and the lateral (NPBL) parabrachial nuclei, and within the Kölliker-Fuse nucleus (Figs. 2, 5A; Table 1). From this total value almost 70% was detected in NPBL and the Kölliker-Fuse ncl. (Table 1). A faint baseline FLI was detected at the lower (6-7 mm rostral to the obex), and the middle (7-9 mm rostral to the obex) pons, within the posteroventral cochlear nucleus and also in the raphe midline (Figs. 2, 4D; Table 1). Within the mesencephalon the highest baseline FLI was found around the periaqueductal gray (PAG) of the lower mesencephalon (at 12.5-14 mm rostral to the obex) mainly laterally to the aqueduct (Figs. 3, 5C; Table 1), and in the midline area of the rostral mesencephalon (17-18 mm), including the oculomotor ncl., medial longitudinal bundle, tegmental decussation, the central linear ncl., the interfascicular ncl. (Figs. 3, 5B; Table 1). No baseline FLI was observed in the following brainstem structures: the area postrema, medullary hypoglossal nucleus, the spinal trigeminal tract and nucleus, the subretrofacial nucleus, the accessory olive nuclei - medial, dorsal, inferior, in the medullary and pontine gigantocellular tegmental fields (FTG), the preolivary nucleus, in the trapezoid body, in the external cuneate nucleus, in the paralemniscal nuclei (tegmental field) of the mesencephalon, and in the superior colliculi.

3.2. Immunostaining in coughing animals

In coughing cats (n=6) dense FLI staining was detected bilaterally throughout the brainstem. At the medulla oblongata cough-related FLI (cough FLI) neurons extended from 4 mm caudal to 6 mm rostral to the obex (Figs. 2, 4, Table 1). Compared to control (baseline FLI), at two levels of the caudal medulla oblongata (4 and 3 mm; 2 and 1.5 mm caudal to the obex, respectively) there were significantly increased the counts of cough FLI, detected in the area of retroambigual ncl. and in an adjacent FTL ($p < 0.05$). Similarly, a higher count of immunostained neurons was found at the rostral medulla (level 0.5-2.5 mm rostral to the obex) including medial, interstitial, and ventrolateral subnuclei of NTS, and adjacent part of alaminar spinal trigeminal ncl. ($p < 0.02$), as well as in ncl. ambiguus, ncl. paraambigualis, LRN, and FTL ($p < 0.05$). At the level approx. 3-4 mm rostral to the obex, cough FLI was seen mainly in FTL, in the medial part of alaminar spinal trigeminal ncl., infratrigeminal ncl., ncl. ambiguus, ncl. paraambigualis, and retrofacial ncl. ($p < 0.05$). More rostrally (at 4-6 mm rostral to the obex), cough FLI was detected predominantly within retrofacial ncl., LRN, in the ventrolateral part of FTL, and less in the caudal edge of facial and retrotrapezoid ncl. ($p < 0.01$). The higher count of cough FLI was found around the pontomedullary junction (5-6 mm rostral to the obex) within the raphe region ($p < 0.05$). Approx at the same level a higher but non-significant cough FLI was seen within vestibular nuclei. (Fig. 2), comparing to baseline FLI. We detected also higher count of cough FLI neurons caudally to the obex (approx. 1.5 mm), mostly in the area

of commissural subnucleus of NTS (AGN/H=31±6 vs. control 17±2), however, the difference was not statistically significant ($p=0.078$). At the pons enhanced cough FLI was observed at its caudal part (level 6-7 mm rostral to the obex) in the posteroventral cochlear ncl. ($p<0.01$; Figs. 2, 4D; Table 1), as well as in the raphe midline (at 7-9 mm rostral to the obex; $p<0.05$). The highest density of cough FLI was detected within the rostral dorsolateral pons including brachium conjunctivum, coerule ncl., NPBm, NPBL, and Kölliker-Fuse ncl. (the area of pontine respiratory group) at the level 10.5-11 mm rostral to the obex ($p<0.05$; Figs. 2, 5A; Table 1). From this value 71% was detected in NPBL and Kölliker-Fuse ncl. ($p<0.01$). Lower density of cough FLI was seen slightly caudally (at the level 10-10.5 mm rostral to the obex) within the same above mentioned nuclei. Within the mesencephalon (at the level 12.5-14 mm rostral to the obex) a decrease of cough FLI was detected mainly in PAG lateral to aqueduct ($p<0.02$; Figs. 3, 5C; Table 1). Increased cough FLI was found in the rostral mesencephalon (at the level 17-18 mm rostral to the obex) including the mesencephalic midline structures ($p<0.05$; Figs. 3, 5B; Table 1). No cough FLI (or a non-significant increase in cough FLI compared to baseline FLI) was observed: in the medullary hypoglossal nuclei, the area postrema, the dorsal motor nucleus of vagus, spinal trigeminal tract, in the subretrofacial ncl., in the facial and retrotrapezoid ncl. (except their caudal edge), the medial, dorsal, and inferior accessory olive ncl., in vestibular ncl., in the medullary and pontine FTG, in the trapezoid body, in the preolivary ncl., the external cuneate ncl., in the paralemniscal ncl. of the mesencephalon, and in both inferior and superior colliculi.

3.3. Alterations of BP in coughing animals

In stimulated animals, we detected two distinct patterns of BP changes caused by coughing (Fig. 1). Cough expulsions frequently caused elevated BP of one systole (average increase in systolic pressure: 13 ± 3 mmHg, 1.7 ± 0.4 kPa; $p<0.01$; Fig. 1). During the later part of stimulation and several seconds after the coughing, BP decreased (mean BP decrease: 16 ± 3 mmHg, 2.1 ± 0.4 kPa; $p<0.001$; Fig. 1). The maximum BP depressions lasted 8 ± 1 s.

4. Discussion

The present study is the first attempt to identify brainstem circuitry of the tracheobronchial cough in spontaneously breathing cats by Fos-like immunoreactive technique. Frequent mechanical stimulation of the lower airways resulted in repetitive tracheal-bronchial coughs comprising stronger expiratory and weaker inspiratory efforts, differing from the stronger inspiratory and weaker expiratory efforts typical for the laryngeal cough (Korpas and Tomori, 1979), or from motor pattern during fictive laryngeal cough (Gestreau et al., 1997).

Although Fos expression is known as a reliable marker of neuronal activation (Dragunow and Robertson, 1988), not all neurons activated during the given motor reflex express Fos (Gestreau et al., 1997; Jakus et al., 2004b). This was also confirmed in this study, because e.g. the hypoglossal, or other motoneurons involved in tracheal-bronchial coughing, did not exhibit marked FLI. Another limitation of the method is that subthreshold stimuli do not induce detectable levels of Fos, however, manipulations such as surgery and stress can cause Fos labeling. The period of time required for FLI related to non-specific (e.g. nociceptive) stimulation to decline to minimal levels differs depending on the type, intensity, and duration of the stimuli, and also on anesthetic conditions (Bullitt et al., 1992). Pentobarbital anesthesia is known as a suppressant of *c-fos* expression (Morgan et al., 1987) and also as a depressant of the respiratory neuronal network. However, this anesthetic has been utilized for decades in the cat to study cough and this species will cough reliably under pentobarbital anesthesia (Korpas and Tomori, 1979; Jakus et al., 2004a). In pentobarbital anesthetized cats, marked reductions of FLI induced by dynamic and repetitive sneezing were observed after a 3 hour period from the stimulation to the perfusion (Wallois et al., 1995). We chose a 30 min period

between the surgical preparation and tracheal stimulation (3 hours from the end of preparation to the perfusion) because prolonged of anesthesia can reduce cough excitability and we also did not detect differences in baseline FLI in our control animals with 3 or 6 hours post-surgical periods. In control cats we found low levels of baseline FLI in most brainstem areas and these findings correspond well with the results of e.g. Wallois et al. (1995) and Gestreau et al. (1997). Limited manipulation of the animal and surgery, the use of local anesthetic in the wound margins leading to a reduction in nociception-related FLI (Lykkegaard et al., 2005), and suppression of *c-fos* expression by pentobarbital anesthesia (Morgan et al., 1987) all could account for our observations. In contrast with some studies (Miller and Ruggiero, 1994; Gestreau et al., 1997) that reported robust FLI in the vestibular nuclei and alaminar spinal trigeminal ncl. (probably as a result of stimulation of nociceptors due to more extensive surgical preparations in decerebrated animals), we found only a weak baseline FLI in those structures. In addition to the caudal trigeminal complex, other pain-related brainstem areas such the medullary and pontine raphe, caudal pontine reticular formation, olivary nuclei, superior colliculi (Bullitt, 1990; Liu et al., 1998) expressed very little FLI in our animals. Similarly, a faint baseline FLI was detected in the retrotrapezoid ncl., which is known to be related to breathing under hypercapnia (Teppena et al., 1994). However, we revealed a large baseline FLI at the rostralateral pons, and also at the caudal mesencephalon, probably indicating their complex integrative role in a control of many physiological functions, e.g. somatosensory, alimentary, cardiovascular, respiratory, arousal, behavioral reactions, vocalization, etc. Taken together, our control animals expressed FLI levels that were in line with other studies in the cat that employed longer post surgical times.

As we stated before, we successfully maintained stable cardiorespiratory parameters in our cats. However, transient alterations of BP were associated with coughing in our study and these changes have been observed before (Korpas and Tomori, 1979). The compensatory mechanisms of autonomic control activated by sustained hypotension or hypertension can induce substantial expression of *c-fos* (McAllen et al., 1992; Dampney et al., 2003). However, the magnitude and in particular the duration of the BP increases or decreases in our experiments were far smaller than those required to induce the *c-fos* by others (Dampney et al., 2003). Our cats showed scarce Fos labeling in the area postrema, which expresses high levels of FLI in both hypertensive and hypotensive animals (Dampney et al., 2003). Also dense clustering of labeled cells in the sub-retrofacial nucleus related to hypotension (McAllen et al., 1992) was not present in our brainstems.

We evoked cough by mechanical stimulation restricted to the regions of trachea and large bronchi. Other approaches (e.g. capsaicin challenge) are less efficient in production of multiple cough trials, less selective (stimulating also intrapulmonary receptors), and it is difficult to control the intensity of such stimuli. Coughing is accompanied by additional involvements, such as pleural pressure changes, glottal movement, bronchoconstriction, hyperventilation, etc. (Korpas and Tomori, 1979). “Over-stimulation” (too many coughs in the trial due to e.g. high concentration of tussive agent) could create excessive cardiovascular and respiratory effects with an increase of non-specific FLI. Conversely, weak coughing e.g. when cough is induced on paralysed animals might make cough FLI undetectable.

4.1 Involvement of the medulla oblongata structures in coughing

Tracheal-bronchial stimulation in our experiments caused a large increase of cough FLI in the caudal and rostral medulla oblongata, through the rostral, dorsolateral pons, up to the midline area of the rostral mesencephalon. There is relatively little information on the properties of the “cough” second-order interneurons in the dorsal medulla. The afferent information from the tracheal-bronchial “cough” receptors is conveyed by the vagus nerves terminating at different subnuclei of the caudal two thirds of NTS, where they make synaptic contacts on the second-

order cough interneurons (Kalia and Mesulam, 1980; Kubin and Davies, 1988; Jordan, 2001). We found dense cough FLI mostly in the medial, interstitial, and ventrolateral NTS, consistent with the fact that the second-order neurons of the tracheal-bronchial cough reflex arc are located also in these areas (Kalia and Mesulam, 1980). In addition, pulmonary stretch receptors, which are vigorously activated during coughing, project to the NTS rostral to the obex as well (Davies et al., 1987; Kubin et al., 2006). However, the primary site of the second order neurons in the afferent pathway of the cough reflex is the commissural subnucleus in the caudal NTS (Kubin and Davies, 1988; Kubin et al., 2006; Mutolo et al., 2007). We did not detect significant increases of the cough FLI in this region. Relatively high basal staining just below the obex (observed also e.g. by Wallois et al. (1995) and Gestreau et al. (1997)) presumably interfered with the level of recruitment of cough FLI. A bilateral distribution of an intense cough FLI supports the idea that during tracheal-bronchial stimulation neural activity from the second-order interneurons may transmit information to the ventral and lateral parts of medulla, spreading it farther both in ascending and descending manner through the raphe nuclei to the ventrolateral FTL, the LRN, the regions of ncl. ambiguus and ncl. paraambigualis, the retrofacial ncl., all localized in the rostral ventrolateral medulla. It is known that many of the aforementioned structures located in the rostral ventral respiratory group (e.g. those around the facial or retrofacial nuclei are closely related with the respiratory central pattern generator, and are also involved in production of the cough (Bongianni et al., 1998; Shannon et al., 2000). Furthermore, our cough FLI overlapped the region of ventral respiratory group neurons affecting both its caudal part, consisting mainly of the pre-motor E-AUG neurons with spinal projections (supplying the spinal expiratory motoneurons during breathing, coughing, vomiting, etc.; Iscoe, 1998; Bongianni et al., 2005) as well as its rostral part, containing predominantly the pre-motor I-AUG neurons, which drive the phrenic and the external intercostal motoneurons, having also collaterals to the retroambigular area (Feldman, 1986; Lipski et al., 1991). In particular, in tracheal-bronchial coughing inspiratory and expiratory neurons of ventral respiratory group fire during either the inspiratory, compressive or expiratory phases with a higher frequency than during quiet breathing, and even new expiratory units (silent during quiet breathing) were found to be regularly recruited (Jakus et al., 1985; Shannon et al., 1998). Hence, our dense cough Fos labeling detected in ncl. retroambigualis, FTL, ncl. paraambigualis, ncl. ambiguus, and retrofacial ncl. may reflect the activities of all above mentioned neurons in tracheal-bronchial coughing. Activation of these neurons subserves various components of cough reflex (preparatory inspiration, inspiratory pharyngeal dilation, inspiratory glottal opening, closure during compressive phase and dilation to allow expulsion, bronchoconstriction supporting expulsion, etc). Whether laryngeal and pharyngeal motoneurons form part of our labeled neurons in ventrolateral medulla could not be confirmed. In our coughing cats a large Fos labeling was detected in LRN, and solely at the medullary FTL, but not in the FTG, whereas both the FTL and FTG along with the medial accessory olive ncl. were clearly labeled for “fictive” laryngeal coughing (Gestreau et al., 1997). The involvement of only LRN and FTL may be specific for the brainstem circuitry of tracheal-bronchial cough, and they may be necessary for production or modulation of the cough motor pattern (see also Jakus et al., 2000; 2004b). This part of the reticular formation is not associated with the respiratory central pattern generator. However, activation of medullary and pontine reticular neurons was coupled with the contraction of respiratory-related muscles, as seen e.g. in the gasp-like aspiration reflex (Fung et al., 1994; Jakus et al., 2004b), in vomiting (Miller and Ruggiero, 1994), sneezing (Wallois et al., 1995), or in “fictive” laryngeal coughing (Gestreau et al., 1997), and the expiration reflex (our non-published data).

4.2 Involvement of pontine and mesencephalic structures in coughing

In the pons, the large cough FLI was detected primarily within the NPBL and the Kölliker-Fuse ncl., referred to as parts of the pontine respiratory group (respiratory neurons located within and around the NPBm, NPBL, and Kölliker-Fuse ncl.), which promotes inspiratory

termination (Bianchi et al., 1995). Moreover, neurons at NPBL, Kölliker-Fuse ncl. (also in NPBM) also have firing patterns consistent with their involvement in cough production (Baekey et al., 1999; Shannon et al., 2004a). However, in contrast to involvement of NPBM in fictive laryngeal coughing (Gestreau et al., 1997), our finding of the cough FLI in that nucleus indicates that NPBM probably does not have an important role in the brainstem circuitry for tracheal-bronchial coughing. Also, we found a higher density of cough Fos neurons bilaterally in the posteroventral cochlear nucleus. This finding was unexpected. However, because this nucleus processes information coming from the cochlea to the caudal pons, it is unlikely that it is directly involved in the production of coughing. Rather it may play a role of a cough modulator, similarly as has been suggested for the cerebellar interpositus nucleus (Xu et al., 1997), the raphe nuclei (Jakus et al., 1998) or the medullary FTL (Jakus et al., 2000).

In the mesencephalon enhanced cough FLI was seen bilaterally at its rostral midline structures, and surprisingly a reduced number of cough Fos neurons was detected at the caudal levels in the PAG. As it was stressed above, the function of the midbrain structures at those levels is very complex. However, as reported recently (Baekey et al., 2004; Shannon et al., 2004b) the midbrain and diencephalon structures are not crucially involved in a control of the cough or expiration reflex in cats. Nevertheless, we reported a dense Fos labeling at the caudal and the mid-mesencephalon in our cats with the aspiration reflex (Jakus et al., 2004b). Thus, it is possible that the structures located around the central tegmental field of the caudal and mid-mesencephalon may participate in a modulation of behaviors with strong inspiratory efforts (e.g. the aspiration reflex or gasping), whereas those around the midline of the rostral mesencephalon may modulate the expiratory expulsions in the cough or expiration reflex (our unpublished data).

The motor pattern of coughing can differ in various situations (different stimulation, pathological conditions, etc.). These differences in motor pattern could be due to plasticity at various level of the reflex pathways and network (receptors, NTS second order neurons, downstream central control elements, premotor neurons) resulting in altered excitability of individual neuronal populations (Bolser et al., 2006; Bonham et al., 2006). These issues could contribute to the differences of cough FLI found in our study and the study of Gestreau et al. (1997).

In conclusion, stimulation of the tracheal-bronchial mucosa evokes tracheobronchial cough that induces a selective Fos labeling in several brainstem nuclei, with either control and/or modulatory functions, being involved in the brainstem circuitry of the tracheal-bronchial cough. Selective cough FLI, similar to that being found in fictive laryngeal cough (Gestreau et al. 1997), was detected in the medial, interstitial, and ventrolateral subdivisions of the NTS, in the retroambigular, ambigular, and paraambigular area, in the region of retrofacial ncl., LRN, NPBL, and Kölliker-Fuse ncl. Nevertheless, differences in the Fos labeling for tracheal-bronchial cough in our study and fictive laryngeal cough (Gestreau et al. 1997) were clearly seen. In the present study, cough FLI neurons were found within the lateral medullary FTL, medullary and pontine raphe, and in the posteroventral cochlear ncl. Unlike the study of Gestreau et al (1997) we did not find an involvement of FTG, the medial accessory olive ncl., and NPBM. In addition, we also found cough FLI in the midbrain.

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Fig. 1.

An original record of blood pressure (BP), airflow (flow; expiration up), and tidal volume (V_T) during quiet breathing and coughing induced by mechanical stimulation (stim) of the tracheobronchial mucosa.

Fig. 2.

Diagrammatic reconstruction of individual sections through the medulla oblongata and the pons Varoli in separate transverse hemisections at six caudal-rostral levels (3 and 1.5 mm caudal to the obex and 1, 4, 6, and 10.5 mm rostral to the obex) in quietly breathing cats (CONTROL) and in cats after repeated tracheobronchial stimulation (COUGH). Black dots represent immunostained neurons as they appear under low power of a microscope. NRA, retroambigular ncl.; FTL, medullary and pontine lateral tegmental fields; NTS, solitary tract ncl.; comNTS, commissural subdivision of the solitary tract ncl.; NA, ambigular ncl.; LRN, lateral reticular ncl.; 5SP alaminar spinal trigeminal ncl.; P, pyramidal tract; VN, vestibular nuclei; FTG, medullary and pontine gigantocellular tegmental fields; RFN, retrofacial ncl.; RN, raphe nuclei; PPR, postpyramidal ncl.; CVP, posteroventral cochlear ncl.; VII, facial ncl.; BC, brachium conjunctivum; NPBL, lateral parabrachial ncl.; NPBM, medial parabrachial ncl.; KF, Kölliker–Fuse ncl.; COE, coeruleus ncl.; 5M, motor trigeminal ncl.; TB, trapezoid body.

Fig. 3.

Diagrammatic reconstruction of transverse mesencephalic hemisections at the level of inferior colliculi (13 mm rostral to the obex) and superior colliculi (17.5 mm rostral to the obex) with baseline FLI found in quietly breathing cats (CONTROL) and in coughing cats (COUGH). ICC, central nucleus of the inferior colliculus; PAG, periaqueductal gray; AQ, aqueduct; COE, coeruleus ncl.; MLB, medial longitudinal bundle; FTC, central tegmental field of the mesencephalon; CS, superior central ncl.; FTP, paralemniscal tegmental field; SCI, superior colliculus; 3, oculomotor ncl.; TX, tegmental decussation; LC, central linear ncl.; IF, interfascicular ncl.; RRN, retrorubral ncl.

Fig. 4.

Examples of cough-related FLI on right panels (COUGH) compared to baseline FLI in quietly breathing cats shown on the left panels (CONTROL), taken from the transversal sections at the medullary levels: A (3 mm caudal to the obex), B (0.5 mm rostral to the obex), C (3.5 mm rostral to the obex), and the pontine level: D (6.6 mm rostral to the obex). For explanation see the text (Results). Arrows indicate dorsal and lateral directions. Scale bar 0.2 mm. NRA, retroambigular ncl.; FTL, medullary lateral tegmental field; mNTS, iNTS, medial and interstitial subdivisions of the solitary tract ncl.; S, solitary tract; DMNV, dorsal motor nucleus of the vagus; RFN, retrofacial ncl.; CVP, posteroventral cochlear ncl.

Fig. 5.

Examples of cough-related FLI (COUGH) compared to baseline FLI (CONTROL), taken from the transversal sections at the pontine level A (10.7 mm rostral to the obex), and at the rostral mesencephalon B (17.5 mm rostral to the obex) and caudal mesencephalon C (13 mm rostral to the obex), respectively. For explanation see the text (Results). Arrows indicate dorsal and lateral directions. Scale bar for A 0.5 mm, for B and C 0.2 mm. BC, brachium conjunctivum; COE, coeruleus ncl.; NPBL, lateral parabrachial ncl.; NPBM, medial parabrachial ncl.; KF, Kölliker–Fuse ncl.; 3, oculomotor ncl.; TX, tegmental decussation; LC, central linear ncl.; PAG, periaqueductal gray; AQ, aqueduct.

Table 1**Unilateral FLI counts in brainstem structures in a group of the control and coughing cats, respectively**

Twelve brainstem areas with the average group number of immunoreactive neurons in particular area/hemisection (AGN/H), and significant differences (Signif) between the quietly breathing (Control) and coughing cats (Cough), are shown. Mann-Whitney test (M), Welch corrected unpaired t-test (W) or unpaired t-test were used for statistical processing. 3, oculomotor ncl.; 5SP alaminar spinal trigeminal ncl.; BC, brachium conjunctivum; COE, coerule ncl.; CVP, posteroventral cochlear ncl.; FTL, medullary lateral tegmental fields; IF, interfascicular ncl.; IFT, infratrigeminal ncl.; iNTS, interstitial subnucleus of NTS; KF, Kölliker–Fuse ncl.; LC, central linear ncl.; LRN, lateral reticular ncl.; MLB, medial longitudinal bundle; mNTS, medial subnucleus of NTS, NA, ambiguous ncl.; NPA, paraambiguous ncl.; NPBL, lateral parabrachial ncl.; NPBM, medial parabrachial ncl.; NRA, retroambiguous ncl.; NTS, solitary tract ncl.; PAG, periaqueductal gray; RFN, retrofacial ncl.; RTN, retrotrapezoid ncl.; TX, tegmental decussation; VII, facial ncl.; vNTS, ventrolateral subnucleus of NTS.

Level	Nuclei and regions	AGN/H		Signif
to the obex		Control	Cough	p
MEDULLA OBLONGATA				
-4 – -3 mm	NRA and adjacent FTL	3 ± 1	13 ± 3	< 0.01 M
-2 – -1.5 mm	NRA and adjacent FTL	15 ± 3	25 ± 3	< 0.05
0.5 – 2.5 mm	mNTS, iNTS, vNTS and adjacent 5SP	17 ± 3	45 ± 8	< 0.02 W
0.5 – 2.5 mm	LRN, NA, NPA, FTL	24 ± 6	55 ± 12	< 0.05
3 – 4 mm	FTL, medial 5SP and IFT, NA, NPA, RFN	23 ± 9	54 ± 5	< 0.05 M
4 – 5 mm	RFN, LRN, ventral part of FTL	15 ± 5	52 ± 8	< 0.01
5 – 6 mm	LRN, caudal VII and RTN, medial IFT, ventral part of FTL	7 ± 3	36 ± 6	< 0.001
5 – 6 mm	Raphe nuclei	5 ± 2	21 ± 10	< 0.05 M
PONS VAROLI				
6 – 7 mm	CVP	1 ± 1	27 ± 10	< 0.01 M
7 – 9 mm	Raphe nuclei	7 ± 1	12 ± 2	< 0.05
10 – 10.5 mm	BC, COE, NPBM, NPBL, KF	23 ± 3	72 ± 7	< 0.001
	Only NPBL and KF	11 ± 1	34 ± 5	< 0.01 M
10.5 – 11 mm	BC, COE, NPBM, NPBL, KF	113 ± 8	252 ± 49	< 0.05 W
	Only NPBL and KF	79 ± 8	179 ± 24	< 0.01 W
MESENCEPHALON				
12.5 – 14 mm	PAG lateral to Aqueduct	78 ± 15	30 ± 6	< 0.02
17 – 18 mm	Midline area (3, MLB, TX, LC, IF)	54 ± 13	94 ± 10	< 0.05