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Role of the dorsomedial medulla in suppression of cough by codeine in cats

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Abstract

The modulation of cough by microinjections of codeine in 3 medullary regions, the solitary tract nucleus rostral to the obex (rNTS), caudal to the obex (cNTS) and the lateral tegmental field (FTL) was studied. Experiments were performed on 27 anesthetized spontaneously breathing cats. Electromyograms (EMG) were recorded from the sternal diaphragm and expiratory muscles (transversus abdominis and/or obliquus externus; ABD). Repetitive coughing was elicited by mechanical stimulation of the intrathoracic airways. Bilateral microinjections of codeine (3.3 or 33 mM, 54 ± 16 nl per injection) in the cNTS had no effect on cough, while those in the rNTS and in the FTL reduced coughing. Bilateral microinjections into the rNTS (3.3 mM codeine, 34 ± 1 nl per injection) reduced the number of cough responses by 24% ($P < 0.05$), amplitudes of diaphragm EMG by 19% ($P < 0.01$), of ABD EMG by 49% ($P < 0.001$) and of expiratory esophageal pressure by 56% ($P < 0.001$). Bilateral microinjections into the FTL (33 mM codeine, 33 ± 3 nl per injection) induced reductions in cough expiratory as well as inspiratory EMG amplitudes (ABD by 60% and diaphragm by 34%; $P < 0.01$) and esophageal pressure amplitudes (expiratory by 55% and inspiratory by 26%; $P < 0.001$ and 0.01, respectively). Microinjections of vehicle did not significantly alter coughing. Breathing was not affected by microinjections of codeine. These results suggest that: 1) codeine acts within the rNTS and the FTL to reduce cough in the cat, 2) the neuronal circuits in these target areas have unequal sensitivity to codeine and/or they have differential effects on spatiotemporal control of cough, 3) the cNTS has a limited role in the cough suppression induced by codeine in cats.

Keywords

Brainstem; Solitary tract nucleus; Lateral tegmental field; Respiratory motor output; Airway defense

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1. Introduction

There have been significant advances in our understanding of pathological cough, its symptoms, classification, diagnostic procedures and treatment (Morice et al., 2007; Diczpinigaitis et al., 2009; Plevkova and Song, 2013). Some progress has been made in our knowledge of cough neuronal circuits (Haji et al., 2013; Pitts et al., 2016) from the introduction of the revolutionary computational model of the cough generating neuronal network (Shannon et al., 1998) and the first complex description of cough brainstem neuronal circuitry in cat (Oku et al., 1994; Gestreau et al., 1997; Shannon et al., 2004; Jakus et al., 2008). Further significant knowledge has been gained during last decade about cough-related neurotransmission and neuromodulation at the central level (Bolser, 2009; Cinelli et al., 2015) including the mechanisms and sites of action of antitussive drugs (Mutolo et al., 2010; Poliacek et al., 2010). However, significant gaps remain in our understanding of the central regulatory mechanisms for coughing. Therefore, the treatment of excessive or insufficient cough is mostly empirical and not based on specific knowledge of neuronal mechanisms responsible for alterations in the cough reflex.

Codeine is very effective to reduce the number and expiratory efforts of mechanically induced cough from the tracheobronchial tree in cat by an action on the cough central neuronal circuitry within the brainstem (Bolser et al., 1995; Bolser, 2006). Among brainstem areas with significant neuronal components of cough control, central anti-tussives act within the nucleus of the solitary tract (NTS; Ohi et al., 2005; Mutolo et al., 2008) and particularly codeine works in the reticular formation of the lateral tegmental field (FTL; Kito et al., 1977) and in the caudal ventral respiratory column (Poliacek et al., 2010). The caudal ventral respiratory column contains a high concentration of expiratory premotor neurons. However, this population of expiratory pre-motoneurons, transmitting expiratory motor drive from the brainstem circuits to the spinal motoneurons (Iscoe, 1998), is relatively insensitive to codeine and unlikely to be responsible for cough suppression (Poliacek et al., 2010). In response to challenge of the medullary raphé with codeine, the excitability and motor output of tracheobronchial cough was depressed by a small amount (Poliacek et al., 2012). Considering that codeine is one of the most widely prescribed drugs to treat cough, there is still a poor understanding of its mechanisms (Haji et al., 2012, 2013). In fact, the antitussive efficacy of codeine in humans with airway pathology has been questioned (Freestone and Eccles, 1997; Smith et al., 2006; Bolser and Davenport, 2007).

The NTS, where codeine has been proposed to act as an antitussive (Kito et al., 1977; Ohi et al., 2005), is a primary projection site of vagal afferents, including cough-related afferents, and the location of 2nd order interneurons of the cough reflex arc (Kalia and Mesulam, 1980; Kubin et al., 2006). However, older microinjection experiments (Kito et al., 1977; Ohi et al., 2005) employed doses of codeine (10 and 1 µg) that were large and not restricted to the immediate region of the injection site (Nicholson et al., 2000). Therefore, the surrounding regions of the reticular formation e.g. the FTL, that contain important cough control elements (Jakus et al., 2000, 2008; Haji et al., 2012), may include cough-related neuronal populations that are sensitive to codeine and could have contributed to the antitussive effects that have been observed by others (Kito et al., 1977). In addition, we re-examined the NTS for its role in the responsiveness, motor drive and temporal features of

cough using kynurenic acid and showed very different contributions of the NTS regions caudal vs. rostral to the obex in cat (Poliacek et al., 2017).

We hypothesized that codeine administered locally in the NTS caudal to the obex (cNTS), in the NTS rostral to the obex (rNTS) and in the FTL would have suppressive effect on cough and the extent of this suppression would differ between these target areas. We also hypothesized that codeine microinjections in these brainstem locations would elicit a specific mode of action that is similar to that observed after systemic administration i.e. no changes in the cough temporal features and cough inspiratory motor drive.

2. Materials and methods

The experiments were performed on 27 cats (3.77 ± 0.20 kg; 22 males and 5 females) under pentobarbital sodium anesthesia (Morbital Polfa or Pfannenschmidt GmbH; 38 mg/kg iv initially). Supplementary doses were administered (1–3 mg/kg, i.v.) as needed (depending on the presence of reflex withdrawal of the hind limb, corneal and palpebral reflexes, jaw tone, respiratory rate and blood pressure). Atropine (HBM Pharma s.r.o., Martin, Slovakia; 0.1 mg/kg, iv) and hydrocortisone (Valeant Czech Pharma s.r.o., Praha, Czech republic; 2 mg/kg iv) were given to reduce secretions and brain swelling, respectively. The trachea, femoral artery and vein were cannulated. A balloon catheter was inserted into the esophagus for the measurement of esophageal pressure (EP). All animals were allowed to spontaneously breathe an oxygen-enriched air (30–40%). Body temperature was maintained at 38.0 ± 0.5 °C by a heating pad and continuously monitored together with blood pressure, EP, respiratory rate and end-tidal CO₂ concentration (ETCO₂). Samples of arterial blood were periodically removed to monitor blood gases and pH to maintain these values within appropriate ranges. Animal care as well as all procedures were performed in accordance with the Animal Welfare Guidelines of the Comenius University and the legislation of animal use and welfare of Slovak Republic and European Union (the Directive 2010/63/EU of the European Parliament) as well as U.S.A.

Electromyograms (EMGs) were recorded from expiratory abdominal muscles (transversus abdominis and/or obliquus externus; ABD), and an inspiratory muscle (sternal diaphragm; DIA), with bipolar teflon insulated fine steel wire hook electrodes (peeled off 3 mm of electrode tip). Tracheobronchial cough was elicited by mechanical stimulation of the intrathoracic airways with a soft polyethylene catheter. The stimulation catheter was moved periodically back and forth in the trachea 6–8 times for periods of 10 s to elicit repetitive coughing (the same experimenter conducted all stimulations).

Animals were placed prone in a stereotaxic frame and the dorsal surface of medulla was exposed. The surface of the brainstem was covered by warm paraffin oil. Microinjections of codeine (3.3 mM or 33 mM; Dr. Kulich Pharma s.r.o. Hradec Králové, Czech Republic) were performed in the NTS or FTL. The drug was dissolved in artificial cerebrospinal fluid that was prepared in our laboratory (aCSF; ALZET Osmotic Pumps, California, USA; Poliacek et al., 2012). Single barrel glass micropipettes (tip diameter 15–60 µm) were used for pressure microinjection of solutions. We did not employ neuronal recordings as a guidance for our microinjections because a presence of similar neurons in all target areas

(King and Knox, 1984; Haji et al., 2012) and large difficulty of combining recording and microinjection procedure when bilateral delivery of codeine that has to be accomplished quickly (Poliacek et al., 2010). The micropipette was driven ventrally from the dorsal approach under stereotaxic control until the tip was positioned: in the cNTS (1.0–1.2 mm caudal to the obex, 1.1–1.2 mm lateral to the midline, 1.3–1.5 mm below the dorsal medullary surface), in the rNTS (1.2 mm rostral to the obex – for aCSF microinjections at 0.7 and 1.6 mm rostral to the obex, 2.4–2.5 mm lateral to the midline, 1.7–1.8 mm below the dorsal medullary surface) and in the region of FTL (2.1–2.4 mm rostral to the obex, 2.3–2.5 mm lateral to the midline, 2.2–2.5 mm below the dorsal medullary surface). The injected volume was monitored by observation of movement of the meniscus in the micropipette barrel with a microscope (the shift in the position of meniscus determines the volume of cylinder ejected from the pipette). Average microinjected volumes are reported for each target area in the results section. Injection sites were labelled by fluorescent latex beads (Eugene, Oregon, U.S.A.) that were suspended in the injectate (Poliacek et al., 2007). The positions of the micropipette tips were confirmed by the presence of a labelled spot in or near the required medullary area (commissural sub-nucleus of the NTS for the cNTS, ventrolateral sub-nucleus of the NTS and/or close proximity of the tractus solitarius for the rNTS and reticular formation approximately in the middle between the NTS and the nucleus ambiguus for the FTL (Fig. 1)).

Cough was defined by a large burst of inspiratory related DIA EMG activity, immediately followed by a burst of expiratory ABD EMG activity, and by a related negative to positive EP change. These criteria separate cough from other defensive airway behaviors such as expiration reflex (Poliacek et al., 2008) or swallow (Pitts et al., 2013).

All EMGs were amplified, filtered (100–3000 Hz) and recorded, then rectified and integrated (moving average with the time constant 200 ms). The number of coughs (average number of coughs per 10 s stimulation) in response to mechanical stimulation of the tracheobronchial airway (cough number = CN) was recorded. For each cough, the maximum amplitude of DIA and ABD EMG moving averages and inspiratory and expiratory amplitudes of EP during the appropriate phase of cough were analyzed. The durations of: cough related DIA and ABD activation (TDIA, TABD); the augmenting segment of cough DIA activity (CTI); the active cough expiratory period (from the peak of DIA activity to the end of cough related ABD bursting; CTE1); the passive quiescent period of cough expiration (from the end of the cough related ABD activity to the beginning of the next cough/respiratory cycle; CTE2); the total expiratory duration (CTE = CTE1 + CTE2; from the peak of cough DIA activity to the next cycle); the overlapping of DIA and ABD burst (Over); the time between maxima of DIA and ABD activity (Dif); the duration of all cough related EMG activity (Tactive = CTI + CTE1); and the total cough cycle duration (CTtot) were analyzed (Poliacek et al., 2017). The maxima were determined using moving average signals, the onsets and offsets of muscle activation using raw EMG signals.

Monitored cardio-respiratory parameters were measured during 3 consecutive breathing cycles before the first microinjection (for the control pre-microinjection conditions) and approximately 1 min after the last microinjection (for the post-microinjection conditions)

before the first stimulus occurred in order to minimize the effects of airway stimulation on these data.

2.1. Protocols

Thirty-two sequences of microinjections under 5 protocols were included into the analysis (6 cNTS, 6 rNTS codeine, 6 rNTS aCSF, 7 FTL codeine and 7 FTL aCSF microinjections series). In the 27 animals 1 protocol was conducted in 22 animals, 2 different protocols were conducted independently in 5 animals.

Fifteen to 20 cough stimulation trials separated by approximately 1 min were executed to establish a stable cough baseline. Then 3–5 control pre-injection trials were made (within a 4 min time interval). Another sequence of trials was performed in the period 1–5 min after the microinjection followed by additional trials at later time points: at 7–12 or 15–20 min, > 30 min, > 60 min, > 90 min post microinjections, as cough recovery times were highly variant among different animals.

Magnitudes of the moving averages during coughing were normalized relative to the mean intensities of control pre-injection coughs (the average magnitudes of all control coughs for each particular EMG). All parameters were averaged over each group of related 3–5 trials. We often observed a reduced cough response during the first trial after a long interval in the protocol when mechanical stimuli were not applied to the trachea e.g. in the recovery period. We eliminated this cough trial from the analysis due to unstable and reduced coughing. This transient reduction in cough excitability after a delay or gap between tracheobronchial stimulation trials occurred regularly even in vehicle treated animals (Poliacek et al., 2010, 2012, 2017).

After the experiment, the caudal medulla was removed for histological processing. The tissue was fixed in 4% paraformaldehyde followed by 30% sucrose solution. The frozen medulla was then cut into transverse slices (thickness 100 or 150 μ m) by a freezing microtome. Sections were examined under light and UV microscopy for detection and localization of injection sites. The evaluation process included: 1) the detection of fluorescent marker within required area, 2) the determination of the position of the micropipette tip (based on intensity of “staining” within the area of spread of fluorescent marker – typically 0.1–0.2 mm; Fig. 1), 3) the evaluation of the tip position (the center of microinjection) relative to the anatomical landmarks – tractus solitarius, nucleus ambiguus, NTS (primarily its ventrolateral area), FTL, hypoglossal, intercalatus and lateral reticular nuclei, area postrema, raphé, 4) evaluation of expected spread and diffusion of microinjected solution relative to the target area and their overlap (Fig. 1). The structures and coordinates are consistent with published data (Berman, 1968; Dietrich et al., 1982; Claps and Torrealba, 1988; Maley, 1996) and our own adjustments (Poliacek et al., 2017). We identified (by the occurrence of fluorescent marker) all 12 microinjection locations in the cNTS, 10 out of 12 with codeine, all 24 with aCSF within the rNTS (4 microinjections were accomplished on each of these cats at 0.7 mm and 1.6 mm rostral to the obex bilaterally), 12 out of 14 with codeine and all 14 with aCSF microinjections within the FTL (Fig. 1).

Results are expressed as mean values $\pm$ SEM. For statistical analysis, repeated measures ANOVA with Student-Neuman Keuls post-tests or, when the data did not pass the normality test, the Friedman test with Dunn post-test were applied. An unpaired *t*-test or Mann-Whitney test was employed in statistical comparisons of codeine vs. aCSF microinjection data. Cardio-respiratory parameters before and after the codeine microinjections and the cough data of vehicle (aCSF) micro-injections were analyzed using Student's paired *t*-test. The differences of variables were considered significant if $P < 0.05$.

3. Results

Microinjections of 3.3 mM codeine in the rNTS (68 ± 4 nl for both microinjections, the total dose 0.22 ± 0.02 nmol, 6 cats) resulted in reduced CN, expiratory amplitudes of ABD EMG and EP and amplitudes of DIA EMG during cough (Figs. 2 and 3). Recovery of coughing close to the pre-injection values (only partial recovery was seen for the expiratory cough amplitudes of EP) was observed typically 15–90 min post-microinjections with the exception of 1 animal where we saw the return of coughing close to the pre-injection control in the time interval 7–12 min post-microinjections and no further recovery was observed. Control microinjections of the vehicle in this location (4 microinjections in each cat, total 121 ± 2 nl for all 4 doses, 6 cats) resulted in no significant alteration of coughing (all $P > 0.05$). The cough parameters post-microinjection of codeine into the rNTS were significantly reduced when compared to post-microinjection data from animals in which aCSF was microinjected (CN $122 \pm 9\%$, $P < 0.01$; DIA $106 \pm 6\%$, $P < 0.05$; and ABD EMG $111 \pm 8\%$, $P < 0.001$; as well as expiratory EP amplitudes $91 \pm 15\%$, $P < 0.05$). There was no significant change in the duration of cough phases in response to microinjection of codeine into the rNTS. The only altered cough related time interval was the prolongation of the period between the peak activity of DIA and ABD (from 0.24 ± 0.01 s to 0.31 ± 0.03 s, $P < 0.05$, recovery 0.25 ± 0.01 s, $P < 0.05$ compared to post-injection value). Some (exceeding 25%) prolongation, but statistically not significant, was seen for CTE (control, post-injection, recovery: 1.37 ± 0.23 , 1.73 ± 0.40 , 1.35 ± 0.16 s; ANOVA $P > 0.1$) and for CTE2 (0.78 ± 0.22 , 1.09 ± 0.41 , 0.76 ± 0.18 s; $P > 0.2$).

Codeine microinjections into the area of cNTS (108 ± 33 nl for both microinjections, total dose 1.56 ± 0.58 nmol, 3 cats with 3.3 mM and 3 cats with 33 mM codeine) had no significant effect on tracheobronchial cough (all $P > 0.1$; Figs. 2 and 3). The data obtained with 3.3 and 33 mM codeine concentrations were pooled as the higher codeine concentration used (33 mM) resulted in no significant differences in coughing relative to the lower dose of codeine.

Microinjections of 33 mM codeine in the FTL (66 ± 6 nl for both microinjections, the total dose 2.18 ± 0.19 nmol, 7 cats) reduced cough related amplitudes of DIA and ABD EMGs, inspiratory and expiratory EP amplitudes (Figs. 2 and 3). Recovery of coughing (partial for the DIA EMG and expiratory EP, complete for the inspiratory EP and almost complete for ABD EMG amplitudes) was seen within time intervals of 7 to 60 min. Comparable microinjections of aCSF (93 ± 14 nl for both microinjections, 7 cats) in the FTL (Fig. 1) resulted in no significant alterations of cough (all $P > 0.45$, paired test). A comparison of the cough data after microinjections of codeine to those after microinjections of aCSF in the

FTL confirmed lower amplitudes of DIA EMG (aCSF value $97 \pm 8\%$, $P < 0.05$); inspiratory EP ($114 \pm 17\%$, $P < 0.05$); ABD EMG ($129 \pm 47\%$, $P < 0.05$, Mann-Whitney test) and expiratory EP ($109 \pm 19\%$, $P < 0.01$) following the codeine administration. There was no significant effect of codeine microinjections into the FTL on the temporal characteristics of cough, with the exception of the time interval between peaks of DIA and ABD EMG activity (prolongation to 0.37 ± 0.03 s vs. control 0.30 ± 0.02 s, $P < 0.05$). The duration of this time interval did not return close to pre-injection values in recovery coughs (0.36 ± 0.04 s, $P < 0.05$ vs. control). An apparent shortening of the activation of cough-related ABD activity was observed (control, post-injection, recovery: 1.24 ± 0.23 , 0.78 ± 0.18 , 1.19 ± 0.19 s; ANOVA $P = 0.05$).

Microinjections of codeine into the cNTS, rNTS and FTL produced no alterations in monitored cardio-respiratory parameters: respiratory rate, respiratory amplitudes of EP, mean arterial blood pressure, heart rate, ETCO_2 , arterial pH (all $P > 0.09$).

4. Discussion

The major findings of this study are that 1) codeine microinjected into the NTS rostral to the obex in cats reduced the cough reflex induced by mechanical stimulation of the tracheobronchial airways, 2) similar microinjections into the NTS caudal to obex had no effect on cough and 3) cough suppressant effects of codeine also were observed following microinjections into the reticular formation of the FTL.

Codeine administered by various routes of delivery reduces CN and expiratory cough efforts (Bolser et al., 1999; Bolser and Davenport, 2002; Poliacek et al., 2010). Lower cough expirations after micro-injections of codeine in the rNTS and FTL are consistent with an anti-tussive action of this drug on structures located in these regions. Codeine delivered into the NTS of guinea pigs reduced cough induced by stimulation of superior laryngeal nerve, but this study employed a large volume of injectate (Ohi et al., 2005). The efficacy of antitussive drugs in the NTS (Mutolo et al., 2008) has been confirmed in rabbits. Indeed, both the FTL (Jakus et al., 2000, 2008) and NTS (Ohi et al., 2005; Mutolo et al., 2008; Haji et al., 2013; Poliacek et al., 2017) contain pools of neurons critically involved in the expression of cough. Specifically, various inspiratory and non-respiratory neurons in and around ventrolateral NTS, including the dorsal FTL, likely contribute to the control of cough in the cat (Haji et al., 2012, 2013; Poliacek et al., 2017). The rNTS overlap with and the dorsal FTL where our micro-injections were placed is somewhat rostral to the locations examined by Haji et al. (2012, 2013) for the presence of cough-related neurons. Codeine suppressed the activity of cough-related inspiratory modulated and non-respiratory modulated units with depolarization present during both phases of cough (Haji et al., 2013). These neurons were proposed to be cough-gating units (Haji et al., 2012). A proposed cough-gating mechanism controls CN and the magnitude of cough expiratory efforts (Bolser and Davenport, 2002; Bolser et al., 2006). However, additional neuronal populations that are specific for FTL and modulated by codeine (Kito et al., 1977) and which participate on control of cough have not yet been identified.

Codeine and other agonists of μ -opioid receptors may mediate neuronal inhibition in the several subdivisions of the NTS (Poole et al., 2007) by both pre- and post-synaptic mechanisms (Endoh, 2006; Ohi et al., 2007; Boxwell et al., 2013) including modulation of glutamatergic neurotransmission (Andresen et al., 2013) and local GABA activity (Herman et al., 2012). The antitussive activity of codeine in cat is likely mediated also via non-opioid receptor mechanisms (Nosalova, 1998; for the discussion see Poliacek et al., 2010). Opioids may act in the NTS to affect processing of primary afferent signals at the 2nd order neurons (Aicher et al., 2000; Zhang et al., 2011). Our data do not allow differentiation between pre- and post-synaptic actions of codeine in reducing the activity and/or activation of neurons. However, our data do not support a significant effect of codeine on cough primary afferent drive. Partial blockade of the cough afferent pathway (Simera et al., 2016) as well as non-specific blockage of glutamate neurotransmission in the rNTS (Poliacek et al., 2017), resulted in significant prolongation of the temporal features of the cough cycle. The latter study likely affected both codeine sensitive as well as insensitive elements in the NTS. Data from the present study show very little alterations in cough cycle timing. The only interval modified and prolonged was the time between maxima of DIA and ABD activity during coughing. The magnitude of this change suggests that the physiological significance of the prolongation may be limited. Similar changes in temporal characteristics of cough motor pattern (that included also prolongations of CTE and C-TE2) were seen when codeine was microinjected in the medullary raphé (Poliacek et al., 2012).

Systemic and intra-vertebral delivery as well as local application of codeine in other brainstem regions resulted in no significant modulation of cough inspiratory activity (Bolser et al., 1999; Bolser and Davenport, 2002; Poliacek et al., 2010; Poliacek et al., 2012). However, codeine microinjections into the rNTS and mainly in the FTL reduced cough amplitudes of DIA activity. Our primary target area in the NTS, the ventrolateral subdivision, contains a high density of inspiratory neurons of various properties (Bianchi and Gestreau, 2009; Haji et al., 2012). Their activity is altered consistently during the cough inspiratory phase (Gestreau et al., 1996; Shannon et al., 1998). Lesion experiments in the NTS are consistent with a significant contribution of these neurons in the generation of inspiratory motor drive during cough as well (Kito et al., 1977; Haji et al., 2013; Poliacek et al., 2017). The FTL also contains inspiratory neurons, but unlike those in the NTS, they have a limited function in breathing (Gebber and Barman, 1985; Haji et al., 2012). However, they are considered to be a crucial component for the execution of powerful inspiratory motor acts of sniff, gasp and aspiration reflex (Fung et al., 1995; Jakus et al., 2000). In this context, our results support the concept that the FTL contains neuronal structures involved in the development of all ballistic-like inspiratory activities (including those during cough) and also that they might be suppressed by codeine.

The existence of neuronal clusters inhibited only by high concentrations of codeine is suggested by the facts that expiratory pre-motor units within the caudal ventral respiratory column are not inhibited during breathing by clinical concentrations of opioids in the dog (Stucke et al., 2008) and that a high dose of codeine was required to reduce ABD motor activity induced by microinjections of DLH in the caudal ventral respiratory column in cat (Poliacek et al., 2007). A large dose of codeine affected power spectra and discharge characteristics of phrenic nerve activity during cough in anesthetized dogs (Hosokawa et al.,

1982). The most likely explanation for the reduction of cough inspiratory motor drive induced by our codeine microinjections is that there are codeine sensitive inspiratory neurons in the rNTS and FTL, but their sensitivity to codeine is low. Codeine is effective to suppress cough when administered by systemic, intra-vertebral or intra-cerebroventricular routes. However, we estimate (see also Poliacek et al., 2010) that the brainstem concentrations of codeine attained via these routes are far below the concentrations temporarily present within the restricted area of microinjections (Lipski et al., 1988; Xie and Hammarlund-Udenaes, 1998). Codeine delivered into other brainstem areas with low numbers of inspiratory neurons and/or different types of cells was ineffective in modulation of cough inspiratory efforts (Poliacek et al., 2010, 2012). Opioids and particularly agonists of μ -receptors can modulate the motor pattern and activity of respiratory neurons during breathing (Lalley, 2003; Stucke et al., 2008; Zhang et al., 2011). However, our codeine microinjections had no significant effect on cardiorespiratory characteristics, even though others have suggested that the rNTS is fundamentally involved in generation of the respiratory motor pattern (e.g. Bianchi and Gestreau, 2009). These findings are consistent with limited respiratory and cardiovascular changes induced by the doses of codeine that significantly reduce coughing (Yanaura et al., 1983; Bolser et al., 1999; Poliacek et al., 2010, 2012) and limited respiratory alterations due to local NTS delivery of opioid receptor agonists (e.g. Hassen et al., 1982).

The concentrations and doses of codeine used in the present study were comparable to those employed in similar experiments affecting the cough response (Ohi et al., 2005; Poliacek et al., 2010, 2012). Concentrations of codeine that were lower than those employed in this study effectively reduced excitatory post-synaptic currents induced by electrical stimulation of the solitary tract in guinea pigs (Ohi et al., 2007). Higher codeine concentrations e.g. 16.5 mM (Poliacek et al., 2010, 2012) or 33 mM (injected in 3 out of 6 cats in the cNTS in present study) did not reduce cough to a greater extent than the lower doses. Our findings support the concept that high sensitivity receptors on neurons were “saturated” by codeine. We did not attempt to conduct a dose response and we worked with fixed codeine concentrations of lower doses in the rNTS (3.3 mM) and higher concentrations in the FTL (33 mM). We employed higher concentrations of codeine in the FTL in order to ensure effective coverage of larger volume of tissue because neurons in this area are not as densely concentrated as in the NTS. Note, that even at low concentration that we employed, the local concentration of the active substance during microinjection can briefly reach high levels before the process of diffusion has completed (Poliacek et al., 2010). Given that we did not use a lower dose of codeine in the FTL, there is a risk that the effects that we observed in this area were due to nonspecific actions of the drug.

The complexity of the actions of codeine on various neuronal populations and the dynamic diffusion process associated with micro-injection likely contributed to variable times for recovery of coughing. A variable recovery time is consistent with our other studies that employed codeine or other neuroactive drugs (Poliacek et al., 2007, 2010, 2017).

The modulation of neuronal functions in the area of the commissural sub-nucleus of the NTS by codeine did not result in an alteration of tracheobronchial cough in anesthetized cats (Figs. 2 and 3). This area includes 2nd order neurons that are part of the afferent pathways

from rapidly adapting receptors and C-fibers (Davies and Kubin, 1986; Kubin et al., 1991). However, the role of RARs in the production of cough has recently been called into question (Canning and Mori, 2010). A similar outcome was observed after reducing the excitability of neurons in the caudal NTS by microinjections of kynurenic acid (Poliacek et al., 2017). The cNTS is a site of action of antitussive drugs in rabbits and is an important area for regulation of this behaviour by neuromodulators (Mutolo et al., 2007, 2008, 2009) and our results support significant species differences with regard to the action of cough suppressant drugs within the subnuclei of the NTS. Haji et al. (2012, 2013) also reported the presence of cough related neurons in the cat caudal to obex, however it seems that most of these neurons were located in the NTS caudally and laterally to the commissural NTS, but not directly in this sub-nucleus. In the present study, microinjections of codeine that were located within 0.5 mm medial to the sites shown by Haji et al. (2012, 2013) were ineffective to modulate cough. The result also supports the view that the volumes and concentrations of injectate used in this study can efficiently modulate neurons located no further than few hundreds μm ($< 500 \mu\text{m}$) from the micropipette tip (Lipski et al., 1988; Poliacek et al., 2010).

The rNTS significantly contributes to the regulation of CN by a codeine sensitive mechanism. Taking into account recent findings of the role of the NTS in cough control (Haji et al., 2012, 2013; Poliacek et al., 2017) the rNTS is unlikely to simply transmit cough related afferent information to higher levels of cough circuitry (Shannon et al., 1998).

Amplitudes of EP (an integral measure of respiratory motor output) during coughing corresponded in general to the EMG activities of DIA and ABD. However, there was some variance between EMGs and the mechanical output of EP (Fig. 2) suggesting uneven effects of our codeine microinjections on DIA and ABD vs. intercostal motor output and/or changes in the rate of inspiratory and expiratory co-activation during cough (e.g. pre-expulsive inspiratory motor drive, Bolser et al., 2000).

In conclusion, unlike the cNTS, the rNTS and adjacent region of the reticular formation (rostral and dorsal FTL) contains codeine sensitive complex neuronal circuits involved in control of cough reflex in the cat.

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Fig. 1.

Reconstruction of microinjection sites.

Stars represent highlighted positions of the micropipette tip during bilateral microinjections of codeine (code) and vehicle artificial cerebrospinal fluid (aCSF) as determined by fluorescent marker. Inset photographs demonstrate the process. The arrows 'injection' point out light spots of a spread of fluorescent marker (typically 0.1–0.2 mm). A: All 12 microinjection locations were found within or near the commissural sub-nucleus of the comNTS. Reference points: comNTS, 12, central canal, medullary surface. B: In the rostral NTS all 24 aCSF (for this figure the spot shown was estimated to be located one-half way between corresponding microinjections at 0.7 and 1.6 mm rostral to the obex) and 10 out of 12 codeine microinjection locations were identified near the solitary tract (TS). Reference points: TS, NTS, 12, Int, the bottom of the 4th ventricle, medullary surface. C: All 14 aCSF and 12 out of 14 microinjection locations were identified in the FTL between NTS and the nucleus ambiguus (NA). Reference points: TS, NTS, 12, NA, LRN, midline, medullary surface.

12: hypoglossal ncl., comNTS: commissural subnucleus of the NTS, GR: gracile ncl., Int: ncl. intercalatus, LRN: lateral reticular ncl., NA: ncl. ambiguus, NTS: ncl. Tractus solitarius, TS: tractus solitarius.

Fig. 2.

Changes in tracheobronchial cough induced by microinjections of codeine into the caudal NTS (cNTS), the rostral NTS (rNTS) and the lateral tegmental field (FTL).

Open bars represent percentages of control (100%) within 5 min post-microinjections, gray bars recovery data (7–90 min following microinjections depending on the time when the cough recovery occurred). CN: number of coughs; DIA: amplitudes of the diaphragm EMG, EP I: inspiratory amplitudes of esophageal pressure; ABD: amplitudes of abdominal EMG; EP E: expiratory amplitudes of esophageal pressure during cough. *, **, ***: $P < 0.05$, 0.01 , 0.001 compared to the control data; +, ++, +++: $P < 0.05$, 0.01 , 0.001 compared to the recovery data, respectively.

Fig. 3.

Representative examples of coughing changes induced by microinjections of codeine into the caudal NTS (cNTS), the rostral NTS (rNTS) and the lateral tegmental field (FTL).

∫: moving average; DIA: the diaphragm EMG, ABD: the abdominal muscles EMG; EP: esophageal pressure; BP: arterial blood pressure. No significant cough changes were induced by microinjections of codeine into the cNTS. Microinjections of codeine in the rNTS and the FTL resulted in reduction of expiratory and inspiratory cough efforts. Microinjections of codeine into the rNTS also decreased CN. Post-microinjection cough trials were executed within 2–5 min post-injection time window.