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Review

Brainstem mechanisms underlying the cough reflex and its regulation

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ABSTRACT

Cough is a very important airway protective reflex. Cough-related inputs are conveyed to the caudal nucleus tractus solitarii (cNTS) that projects to the brainstem respiratory network. The latter is reconfigured to generate the cough motor pattern. A high degree of modulation is exerted on second-order neurons and the brainstem respiratory network by sensory inputs and higher brain areas. Two medullary structures proved to have key functions in cough production and to be strategic sites of action for centrally active drugs: the cNTS and the caudal ventral respiratory group (cVRG). Drugs microinjected into these medullary structures caused down-regulation or upregulation of the cough reflex. The results suggest that inhibition and disinhibition are prominent regulatory mechanisms of this reflex and that both the cNTS and the cVRG are essential in the generation of the entire cough motor pattern. Studies on the basic neural mechanisms subserving the cough reflex may provide hints for novel therapeutic approaches. Different proposals for further investigations are advanced.

1. Introduction

Cough is a very important airway defensive act that functions to expel foreign or endogenously produced materials from the lower airways (Macklem 1974; Korpáš and Tomori 1979). It is usually an adequate reflex response to nociceptive stimuli, i.e. actually or potentially tissue damaging events (e.g. Woolf and Ma 2007; Loeser and Treede 2008) applied to the airways, but can also be initiated on voluntary command. Cough is one of the most common and troubling symptoms for which patients seek medical attention. It is usually purposeful and useful, but in case of persistent or chronic cough is without benefit or even with clear physical and psychological complications that considerably impair the quality of life. Available antitussive therapies have limited efficacy and unacceptable side effects. Thus further research is needed to find better antitussive drugs (Dicpinigaitis et al., 2014). On the other hand, an impairment of airway protective reflexes, including cough and swallowing, in some neurodegenerative diseases such as Parkinsonism, Alzheimer's disease and fronto-temporal dementia, or following ictus could lead to high risk of aspiration and consequent life-threatening conditions (e.g. Dicpinigaitis et al., 2014; Dutschmann et al., 2014; Bolser et al., 2015; Pitts et al., 2016; Cinelli et al., 2016). Admittedly, studies on animal models of chronic cough or neurodegenerative diseases could be more appropriate to disclose novel therapeutic approaches. Nevertheless, investigations on the basic neural mechanisms subserving the cough reflex performed on healthy preparations can provide useful hints for the development of antitussive or protussive strategies (see e.g. Cinelli et al., 2016; Mutolo et al., 2016).

The central mechanisms controlling breathing movements depict a respiratory cycle divided in three phases: inspiration, postinspiration and expiration (for review see e.g. Richter and Smith 2014). Post-inspiratory activity provides a mechanism to mechanically brake the expiratory airflow. It is of great importance for the mediation of various protective reflexes, such as glottal closure, sneeze, cough and swallowing, that ensure protection from penetration of potentially harmful foreign substances into the airways (Dutschmann and Herbert 2006; Dutschmann et al., 2014 also for further Refs.). Cough consists of a modified respiratory act that includes three phases: inspiratory or preparatory, post-inspiratory or compressive (glottal closure) and expiratory or expulsive (e.g. Korpáš and Tomori, 1979; Widdicombe 1986).

A distinctive aspect of human cough motor responses in acute and chronic conditions is the "urge-to-cough" caused by tickling sensations in the upper airways that lead to the behavioral act of coughing (Davenport 2008; Muroi and Undem 2011). Reflex and voluntary coughing present similarities, but differ in very important respects. The cough reflex is subject to a high degree of voluntary control, so that all possible cough motor patterns can be mimicked and the frequency and intensity of spontaneous cough can be modulated up to complete suppression (see Magni et al., 2011; Mazzone et al., 2011; Ando et al., 2014; Brandimore et al., 2015 also for further Refs.). Interestingly, the habitual voluntary suppression of cough may lead to pathological

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processes in poorly draining lung regions and has been implicated in the Lady Windermere syndrome (see e.g. Reich and Johnson 1992). This review is focused on reflex cough evoked by stimulation of the tracheobronchial tree and the brainstem neuronal mechanisms that generate or modulate the cough motor pattern.

2. Cough afferent pathways

2.1. Cough-related afferent fibers

Cough-related receptors (for review see Sant'Ambrogio and Widdicombe, 2001; Canning et al., 2014) mainly located in the large extrapulmonary airways (trachea, carina, main bronchi) belong to the wide family of pulmonary Aδ rapidly adapting receptors (RARs), possibly including the so-called "cough receptors" described in the guinea pig larynx and rostral trachea (Canning et al., 2004, 2006a, 2006b). RARs are a heterogeneous family of polymodal receptors and some of them are particularly sensitive to mechanical stimulation of the airway mucosa and to chemical irritant stimuli such as citric acid, ammonia and cigarette smoke. RARs in the intrapulmonary airways very rarely evoke cough, but usually provoke other reflexes, such as hyperpnea/tachypnea, augmented breaths, bronchoconstriction and laryngeal closure. Cough-related afferents may also originate from bronchopulmonary C-fibers (Coleridge and Coleridge 1986; Widdicombe, 1998; Lee and Pisarri 2001; Canning et al., 2006b, 2014; Canning and Mori 2010, 2011; Chung 2015). In anesthetized animals, C-fiber stimulation has consistently failed to evoke coughing. In addition, the cough reflex can be inhibited owing to the activation of a subset of pulmonary C-fibers in dogs, cats and guinea-pigs (Tatar et al., 1988, 1994; Hanacek et al., 2006; Widdicombe and Singh, 2006; Canning and Mori, 2010; Canning et al., 2014; but see also Mutolo et al., 2008a). However, some studies suggested that in anesthetized guinea pigs (Mazzone and Canning, 2002b; Mazzone et al., 2005) C-fiber activation does not evoke cough, but produces changes in the cough reflex and airway autonomic outflow by amplifying the action of other airway afferent nerves, namely mechanically-sensitive vagal afferents. Thus, C-fibers may be especially relevant to coughing associated with airway inflammation and inhalation of environmental irritants. Further support for the inhibitory role of vagal C-fibers on mechanically-induced cough in anesthetized cats has recently derived from the results of the study by Simera et al., 2016. The "cough receptors" (Canning et al., 2004, 2006a,b) are innervated by slowly conducting Aδ-fibers and are sensitive to punctate mechanical stimuli and acid, but unresponsive to changes in luminal pressure, to capsaicin, bradykinin or hypertonic saline and, like Aδ RARs, do not express transient receptor potential vanilloid type 1 (TRPV1) channels under normal healthy conditions. Recent results on the antitussive effects of long-acting muscarinic receptor antagonists (LAMAs) are consistent with the possible role of this type of receptors in cough production both in awake and anesthetized rabbits (Mutolo et al., 2016). However, their presence in the tracheobronchial tree of this animal species remains to be ascertained. As a consequence, other receptors in addition to the TRPV1, as suggested by others (Birrell et al., 2014), should be taken into consideration in the mediation of LAMA antitussive effects, such as acid-sensing ion channels and mechanoreceptors of cough-related airway sensory afferents.

Despite the larynx being often disregarded in cough studies since the trachea is in most cases cannulated, laryngeal receptors are a very important source of airway defensive reflexes and, in particular, of the cough reflex. Cough-related laryngeal receptors apparently display analogies with those located in the tracheobronchial tree (Korpáš and Tomori, 1979; Widdicombe 1986, 2003; Sant'Ambrogio, 1993; Sant'Ambrogio and Widdicombe, 2001 also for further details). Cough-related laryngeal receptors innervated by myelinated Aδ-fibers are activated by mechanical and chemical stimuli and are often called "irritant receptors". They possibly include "cough receptors" (Canning et al., 2004, 2006a,b). A large proportion of "irritant" type receptors respond to water or water solutions lacking chloride ions. C-fiber activation has also been reported to have a role in cough production. It seems plausible that specific second-order neurons for cough-related laryngeal afferents exist. In this regard, note the putative model (Fig. 7) by Widdicombe (2003) on the central pathways for the cough reflex, where laryngeal RARs project to their own laryngeal relay neurons, that have distinct connections with the cough generating mechanism. However, although the central pathways have not been investigated in detail, they are probably similar to those displayed by tracheobronchial cough afferents (Kalia and Mesulam, 1980; Nomura and Mizuno, 1983; Mifflin, 1993). In addition, "irritant" receptors may evoke many other airway protective reflexes such as glottal closure, apnea, bronchoconstriction, mucus secretion, the expiration reflex, the swallowing reflex and cardiovascular reflexes (Korpáš and Tomori, 1979; Widdicombe, 1986; Mutolo et al., 1995; Sant'Ambrogio and Widdicombe, 2001; Jean, 2001; Nishino et al., 2004; Mazzone and Undem, 2016). The expiration reflex closely resembles cough responses, but it consists of a pure expiratory effort evoked by the mechanical stimulation of the vocal fold mucosa in the absence of a preparatory inspiratory phase (Korpáš and Tomori, 1979; Korpas and Jakus, 2000; Sant'Ambrogio and Widdicombe, 2001). The expiration reflex can be also evoked by the stimulation of the tracheobronchial tree (Widdicombe 1954; Tatar et al., 2008; Poliacek et al., 2008b). Glottal closure and the expiration reflex may be regarded as the first level of airway defense since they prevent penetration of foreign bodies into the airways. Other laryngeal afferents display a marked respiratory modulation and are the source of different respiratory reflexes (see e.g. Widdicombe 1986; Sant'Ambrogio, 1993). A comprehensive review on laryngeal afferents and related reflexes is beyond the scope of the present report.

2.2. Central cough pathways

Both neuroanatomical and electrophysiological studies in both cats and rats have demonstrated that the main central termination sites of RAR primary afferents are the medial subnucleus of the NTS and, especially, the lateral aspect of the commissural subnucleus, where also RAR second-order neurons (RAR cells) are located (Kubin and Davies, 1988; Ezure et al., 1991, 1999; Lipski et al., 1991; Kubin and Davies, 1995; see for review Kubin et al., 2006). RAR cells were excited by ammonia inhalation and displayed monosynaptic EPSP in response to low-intensity vagal stimulation. Other studies in the rabbit (Mutolo et al., 2007, 2009) using excitatory amino acid (EAA) receptor antagonists implicate that cough-related afferents activated by the stimulation of the tracheobronchial tree terminate in caudal portions of the NTS consistently with previous data in the cat. The results obtained in the guinea pigs both by anatomical tracing studies and by microinjections of EAA receptor antagonists (Canning and Mori 2010, 2011) led to the suggestion that afferent fibers activated in response to stimulation of the tracheal mucosa "cough-receptors" terminate mainly in more rostral and lateral NTS sites (lateral to the commissural subnucleus and, perhaps, in the medial subnuclei). The difference with previous findings in the cat, rat and rabbit was attributed by Canning and Mori (2010) mainly to the different site of tussigenic stimulation (trachea vs. tracheobronchial tree).

Interestingly, no evidence was found by Canning and Mori (2011) for a permissive effect of slowly adapting receptors (SARs) in the cough reflex, as suggested by other studies (Sant'Ambrogio 1982; Hanacek et al., 1984, 2006). The role of pulmonary stretch receptors and, in particular, of volume-related feedback in the regulation of the cough reflex is controversial (Nishino et al., 1989b; Javorka et al., 1994; Romaniuk et al., 1997; Bolser and Davenport 2000). In a recent study, Poliacek et al. (2016) found that modified lung inflations during coughing and/or additional expiratory airflow resistances altered the spatio-temporal characteristics of the cough motor pattern through

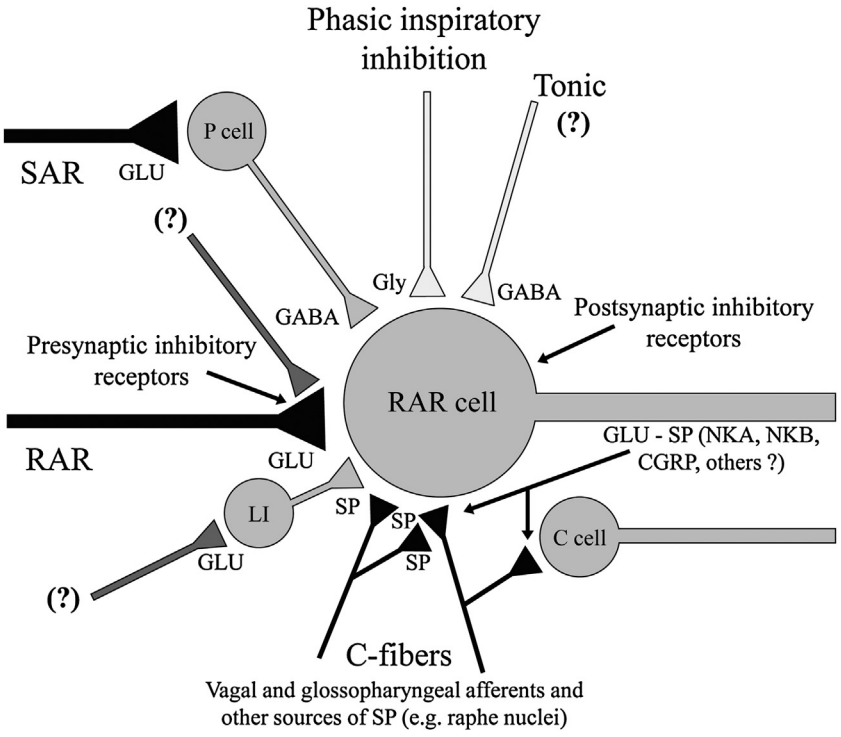


Fig. 1. Schematic representation of relevant synaptic inputs to RAR relay neurons (RAR cells), a non-homogenous population of second-order neurons in the RAR afferent pathway, located mainly within the caudal NTS (commissural subnucleus). SAR, RAR and bronchopulmonary C-fiber inputs are shown to modulate RAR cell activity through excitatory and inhibitory connections. P cells (largely localized in the medial NTS) represent a subset of the entire non-homogeneous population of second-order neurons in the SAR afferent pathway. C cells are relay neurons of the bronchopulmonary C-fiber afferent pathway located primarily in the medial portion of the commissural subnucleus. A certain degree of overlapping exists between the sites of termination of RAR and C-fiber afferents. Convergence of C-fibers onto RAR relay neurons has also been suggested. Abbreviations: CGRP, calcitonin gene-related peptide; GABA, γ-aminobutyric acid; GLU, glutamate; Gly, glycine; LI, local interneuron; NKA, neurokinin A; NKB, neurokinin B; NTS, nucleus tractus solitarii; RAR, rapidly adapting receptor; SAR, slowly adapting receptor; SP, substance P; (?), unknown sources that perhaps in certain cases may include also vagal and glossopharyngeal afferents. Reported data are mainly derived and/or inferred from the studies listed below, i.e., they are to some extent also based on suggestions or hypothesis: Thor and Helke 1987; Wilson et al., 1996; Ezure et al., 1998, 1999, 2000, 2004; Miyazaki et al., 1999; Potts et al., 1999; Mutoh et al., 2000; Canning, 2002; Colin et al., 2002; Mazzone and Canning 2002a, b, 2005; Sekizawa et al., 2003; Widdicombe 2003; Bonham et al., 2006; Mutolo et al., 2007, 2008b; Cinelli et al., 2015.

volume-related feedback mechanisms similar to those operating during eupneic breathing. In the same report, an interesting discussion on lung volume feedback during coughing and the related contribution of both SARs and RARs has also been provided. It has been shown that lung inflation (Ezure and Tanaka 2000) inhibits RAR cells and that SARs are responsible for inhibitory inputs to RAR cells via second-order neurons in the NTS, the so-called P cells (Berger 1977; Pantaleo and Corda 1986; Davies et al., 1987), that are GABAergic or may corelease GABA and glycine (Ezure et al., 1999; Ezure and Tanaka 2004; see also Cinelli et al., 2016). Second-order neurons in the cough afferent pathway have been shown to project to pontine and medullary respiratory groups (Ezure et al., 1991; Otake et al., 1992; Kubin and Davies 1995; Shannon et al., 1996, 2004; Pantaleo et al., 2002; Kubin et al., 2006; Haji et al., 2012).

A schematic representation of relevant synaptic inputs to RAR cells, originating especially from airway afferent fibers, is reported in Fig. 1. In this connection, it can also be recalled that several afferent inputs to the NTS include the release of substance P: baro- and chemoreceptor afferents, conveyed by vagus and glossopharyngeal nerves, trigeminal afferents, skeletal muscle afferents and raphe nuclei projections (e.g. Lindefors et al., 1986; Thor and Helke 1987; Zhang et al., 1991; Gatti et al., 1995; Potts et al., 1999; see also Mutolo et al., 2008b). Of note, convergence of C-fibers onto RAR cells has also been suggested (Mazzone and Canning 2002a,b, 2005). Indeed, the effects of substance P on NTS neurons are fairly complex. Substance P displays not only postsynaptic excitatory effects, but also presynaptic depressant effects on glutamatergic transmission between bronchopulmonary afferent fibers and second-order NTS neurons (Sekizawa et al., 2003; Kubin et al., 2006; Bonham et al., 2006). In addition, functional evidence has been provided that local interneurons release substance P in response to glutamatergic inputs (Colin et al., 2002; see also Sekizawa et al., 2003 for further Refs.). For a recent interesting, comprehensive review on vagal afferent innervation of the airways, its functional characteristics, and related central pathways under healthy or pathological conditions see Mazzone and Undem (2016).

3. Brainstem structures involved in the generation of reflex cough

3.1. Neuronal recordings, c-fos immunohistochemistry and lesion experiments

Several studies on the behaviour of medullary respiratory neurons during coughing have led to the conclusion that the same respiratory neurons involved in the generation of the eupneic pattern of breathing also participate in the production of the cough motor pattern (e.g. Oku et al., 1994; Bongianni et al., 1998; Shannon et al., 1996; 2004; Bolser and Davenport 2002; Pantaleo et al., 2002; Baekey et al., 2003; Ott et al., 2011). These findings support the existence of multifunctional neural networks in the mammalian brainstem and, accordingly, of neurons that contribute to different functions, such as respiration, coughing, vomiting and sneezing. Neuronal recordings performed during cough in the ventral respiratory column showed that laryngeal premotoneurons shape the firing pattern of laryngeal motoneurons located in the nucleus ambiguus (see Baekey et al., 2001 also for further Refs.).

Several brainstem structures have been found to have a respiratory function. They include the pontine respiratory group, the dorsal respiratory group, the ventral respiratory column comprising the

retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), the Bötzinger complex (BötC), the pre-Bötzinger complex (preBötC), the inspiratory portion of the ventral respiratory group (iVRG) and the caudal expiratory component of the ventral respiratory group (cVRG). For comprehensive reviews on the brainstem respiratory network see e.g. Von Euler 1986; Bianchi et al., 1995; Feldman et al., 2013; Cui et al., 2016; Ikeda et al., 2017. A recently discovered brainstem region, named "Postinspiratory Complex" (PiCo), is located rostral to the preBötC, dorsal to the BötC and caudal to the facial nucleus (Anderson et al., 2016). The PiCo is characterized by rhythm-generating properties and is considered necessary and sufficient for generating postinspiratory activity *in vivo*. On the basis of these results, a "triple oscillator model" has been proposed in which inspiration, postinspiration, and active expiration are generated by three distinct excitatory rhythmogenic microcircuits, i.e. the preBötC, PiCo and pFRG, respectively (see below).

The respiratory network appears to be reconfigured to generate the cough motor pattern when cough-related afferent inputs activate NTS second-order neurons. This hypothesis was advanced by Shannon et al. (1998) who devised a neuronal network that undergoes a process of "reconfiguration" to produce both breathing and coughing. However, a recent report dealing with a tentative computational model of coughing (Pitts et al., 2016) suggests that the "reconfiguration" hypothesis does not completely account for the differences in sensory regulation between breathing and coughing as well as the contribution of neurons to either functions. In fact, breathing relies primarily on chemoceptive feedback from peripheral and central receptors, while the main sensory drive for coughing arises from receptors located in the upper airway. On the other hand, second-order neurons in the cough afferent pathway are likely quiescent during breathing, but recruited during coughing. Probably, the brainstem population of neurons involved in cough genesis is underestimated.

Recently, recordings from respiration-related neurons have been performed also in the RTN/pFRG region of the guinea pig during coughing and swallowing (Sugiyama et al., 2015). This structure functions as a chemosensitive center that detects environmental CO_2 and expresses a paired-like homeobox 2b gene (Phox2b). Neurons located in this area in neonatal rats are intrinsically CO_2-sensitive (Mulkey et al., 2004; Guyenet and Mulkey 2010; Guyenet et al., 2016; Ikeda et al., 2017). Interestingly, Phox2b mutations have been described in most human cases of central congenital hypoventilation syndrome (Amiel et al., 2003). The RTN/pFRG has been implicated in the generation of the entire respiratory pattern (Onimaru and Homma 2003) or exclusively in the generation of active expiratory activity, i.e. it has been considered an expiratory central pattern generator (Janczewski and Feldman 2006). In more detail, expiratory muscle activation is caused by neurons of this area under hypercapnic conditions (Janczewski and Feldman 2006; Smith et al., 2007; Abdala et al., 2009; Pagliardini et al., 2011) or following peripheral chemoreceptor stimulation (Moraes et al., 2012). Furthermore, serotoninergic or cholinergic muscarinic mechanisms contribute to the appearance of neuronal expiratory activity within the RTN/pFRG region and promote the recruitment of expiratory motoneurons and active expiration (Lemes et al., 2016; Boutin et al., 2017). Neuronal recordings within this structure (Sugiyama et al., 2015) showed that the majority of recorded neurons changed activity in synchrony with coughing and swallowing. However, on the basis of brainstem transection experiments, they concluded that RTN/pFRG neurons may modulate expiratory activity during normal eucapnic breathing, but are not essential elements of the neural network responsible for coughing and swallowing. Recording experiments have been confirmed by studies on the expression of the immediate early gene c-fos during coughing (Gestreau et al., 1997; Jakus et al., 2008) as well as by lesion experiments (Jakuš et al., 1998, 2000; Mutolo et al., 2002; Poliacek et al., 2004, 2005, 2014; Simera et al., 2013 also for further Refs.). It has been shown that several brainstem regions either with or without respiration-related neuronal activities may have a role in the production of the cough motor pattern. These regions include the BötC, the raphe nuclei and other midline structures, the periaqueductal gray, the lateral tegmental field, the pontine respiratory group, and the reticular nuclei. The periaqueductal gray and the nucleus raphe magnus have also been reported to have suppressive influences on the reflexes of coughing and swallowing (Sessle et al., 1981).

3.2. Some specific neural mechanisms involved in the generation or modulation of the cough reflex

Modulation or, in some instances, even generation of the cough reflex depend on the cerebellar nucleus interpositus (Xu et al., 1997), nasal mucosa trigeminal afferents (Plevkova et al., 2009; Poussel et al., 2012), esophageal vagal afferents (Hennel et al., 2015), afferents from the external acoustic meatus (auricular branch of the vagus nerve, Arnold's or Alderman's nerve) that mediates the Arnold's ear-cough reflex (e.g. Todisco 1982; Canning et al., 2014; Murray et al., 2016), pharyngeal afferents (for review see Canning et al., 2014), chemoreceptor (Tatar et al., 1986, 1987; Nishino et al., 1989a; Hanacek et al., 2006) and baroreceptor (Poliacek et al., 2011) afferents. Pharyngeal afferents can activate not only the gag reflex (Bassi et al., 2004), but also coughing or the "urge-to-cough"; the receptors implicated are probably innervated by vagal and glossopharyngeal nerves or by trigeminal branches (Canning et al., 2014). Central and peripheral mechanisms involved in exercise and voluntary isocapnic hyperventilation may downregulate the cough response (Lavorini et al., 2010; Poussel et al., 2014). However, exercise (electrically induced hindlimb muscular contractions) in ovalbumin-sensitized rabbits fails to produce similar effects (Tiotiu et al., 2017). An important characteristic of the cough reflex is its very strong dependency on the sleep-wakefulness state as well as on anesthesia (Nishino et al., 1988; Widdicombe and Singh 2006). This reflex, in addition to a potent voluntary control, has also sensory, affective and cognitive components. In fact, the cough afferent pathway extends beyond a simple pontomedullary reflex to impinge on neuronal networks widely distributed throughout subcortical and cortical brain areas (Mazzone et al., 2011; Ando et al., 2014; Driessen et al., 2016). For further details on the central mechanisms subserving generation or modulation of the cough reflex see also Mazzone and Undem (2016). Fig. 2 provides a tentative bloc diagram summarizing the main central and peripheral neural mechanisms that generate or modulate the cough reflex. Some of the possible reciprocal connections between the brainstem structures involved in cough production have been reported or discussed later on in this review. Despite the intense research efforts of the two last decades, our knowledge on the central organization of the cough reflex is far from complete.

3.3. Central sites of action of antitussive or protussive drugs

According to earlier suggestions (Bongianni et al., 2005; Mutolo et al., 2007), at least two medullary structures appear to play a prominent role in cough production, i.e. the first relay medullary station of the reflex pathway and the medullary neuronal aggregate responsible for the expiratory drive component of the reflex: they are the caudal NTS (cNTS) and the cVRG, respectively. This latter region corresponds, to a great extent, to the nucleus retroambigualis (NRA). The following presentation is mainly focused on these medullary regions and deals largely with results obtained by our research group making use of bilateral microinjections of neuroactive agents in pentobarbitone anesthetized, spontaneously breathing rabbits. Cough was induced by mechanical (0.5-mm diameter nylon fiber with a smoothed tip inserted through a lateral port of the tracheal cannula) or chemical (1 M citric acid inhalation) stimulation of the tracheobronchial tree. In some studies, mechanical stimulation was delivered by a custom-built device recently described and validated (Mutolo et al.,

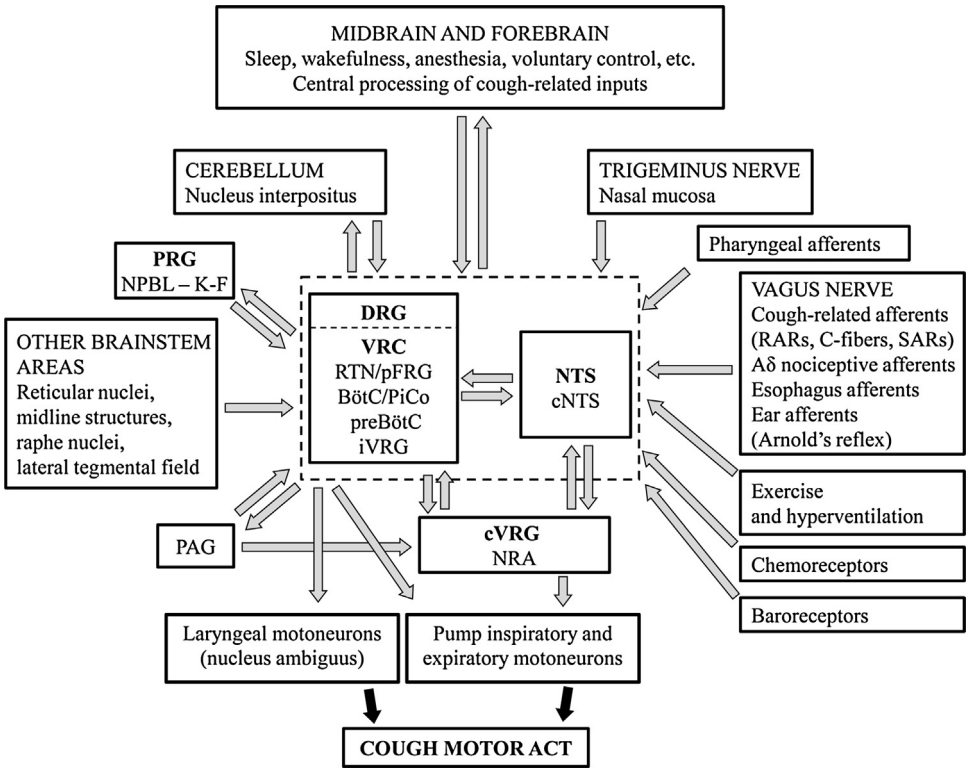


Fig. 2. Tentative block diagram summarizing the main central and peripheral neural mechanisms involved in the generation and regulation of the cough motor act. Abbreviations: BötC, Bötzinger complex; cNTS, caudal aspect of the nucleus tractus solitarii; cVRG, caudal ventral respiratory group; DRG, dorsal respiratory group; iVRG, inspiratory portion of the ventral respiratory group; K-F, Kölliker-Fuse nucleus; NPBL, nucleus parabrachialis lateralis; NRA, nucleus retroambigualis; NTS, nucleus tractus solitarii; PAG, periaqueductal grey; PiCo, Postinspiratory complex; preBötC, preBötzinger complex; PRG, pontine respiratory group; RARs, rapidly adapting receptors; SARs, slowly adapting receptors; RTN/pFRG, retrotrapezoid nucleus/parafacial repiratory group; VRC, ventral respiratory column.

2014). Each cough was identified by a preparatory phrenic burst immediately followed by a burst of abdominal activity. These features separated cough from other airway defensive reflexes such as the expiration reflex (e.g. Poliacek et al., 2008b). This topic along with the techniques employed and, in particular, recordings from medullary neurons and drug microinjections have been fully described in our reports (see e.g. Mutolo et al., 2008b, 2010, 2012). The localization of injection sites is illustrated in Figs. 3 and 4.

4. Caudal nucleus tractus solitarii

4.1. Mediation of excitatory inputs to RAR cells

As suggested by Bonham et al. (1993), the excitatory input from RARs and other vagal receptors is mediated by glutamate that accordingly is the primary neurotransmitter at the synapse between RAR afferents and RAR cells (Ezure et al., 1999). Blockade of *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors within the rabbit cNTS by 50 mM kynurenic acid, a broad-spectrum EAA receptor antagonist, abolished the cough reflex in response to mechanical or chemical stimulation, but did not affect the Breuer-Hering inflation reflex and the pulmonary chemoreflex (Mutolo et al., 2007, 2009). This is consistent with the fact that central terminations of lung RARs, SARs and bronchopulmonary C-fibers have been found in largely nonoverlapping regions of the cNTS (Kubin and Davies 1995; Kubin et al., 2006). These effects were mainly due to blockade of non-NMDA receptors as revealed by microinjections of 10 mM CNQX. Blockade of NMDA receptors by 10 mM D-AP5 strongly reduced, but did not completely suppress cough responses. An essential contribution of EAA receptors in the mediation of cough afferent inputs within the NTS has been observed in guinea pigs, but in regions more rostral and lateral to the commissural subnucleus and with a predominant role of NMDA receptors (Canning and Mori 2011). Recently, Poliacek et al. (2017) found in the cat that bilateral microinjections of kynurenic acid into the cNTS were without effects, whilst similar microinjections into the rostral NTS caused marked alterations in both baseline respiratory activity and cough motor pattern. They suggested the existence of important cough control mechanisms within the rostral NTS distinct from those processing the primary cough afferent signals. These mechanisms appear to be involved in the regulation of cough excitability, cough expiratory thrusts and phase timing as well as in the production of both rhythmic breathing and coughing. As it is noted under Section 6 "Concluding remarks and future directions", several medullary regions, including the different NTS subnuclei, may deserve further investigations for their involvement in the cough reflex. However, the lack of effects of kynurenic acid microinjected into the cat cNTS is inconsistent with the bulk of available literature providing evidence that the central terminations of RAR afferents are in that region in the cat as well as in other animal species (Ezure et al., 1991; Lipski et al., 1991; Kubin and Davies 1995; Kubin et al., 2006; Mutolo et al., 2007). The reasons of this discrepancy are obscure. For further discussion see Poliacek et al. (2017).

Interestingly, the local application of 1 mM substance P to the rabbit cNTS (Mutolo et al., 2007) potentiated cough responses by increasing the peak abdominal muscle activity (expiratory thrusts) and the cough number, i.e. the number of coughs following each stimulation. These results are in accordance with those showing that substance P microinjected into the cNTS of guinea pigs or C-fiber activation sensitize the cough reflex evoked by electrical stimulation of the trachea through the central involvement of tachykinins, with a process similar to the central sensitization described in somatosensory pathways (Mazzone et al., 2005). Importantly, the results in the rabbit also reveal that substance P effects are not limited to certain animal species and concern also coughing evoked by more natural (mechanical) stimuli. According to

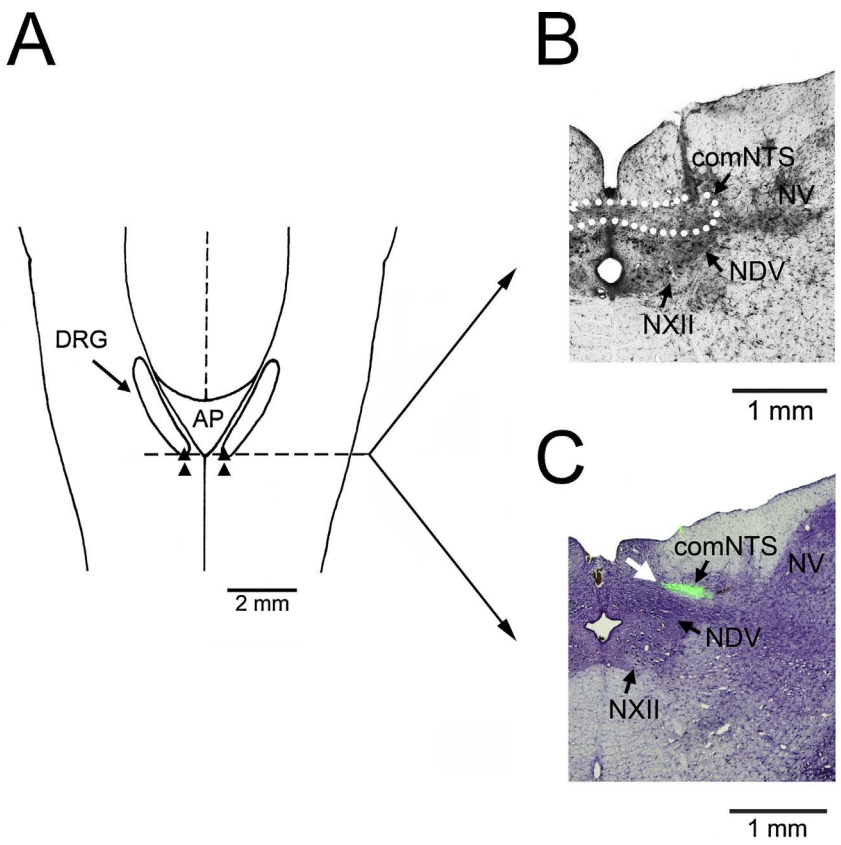


Fig. 3. Localization of injection sites within the caudal aspect of the nucleus tractus solitarii (cNTS) and histological controls. A: diagram of the dorsal view of the rabbit medulla oblongata showing the four injection sites where bilateral microinjections (▲) of drugs have been performed in each preparation. AP, area postrema; DRG, dorsal respiratory group. B: photomicrograph of a coronal section of the medulla oblongata at the level indicated in A (horizontal dashed line) showing the track along which a microinjection has been performed. The dotted line delineates the approximate edges of the commissural NTS. C: photomicrograph of a coronal section of the medulla oblongata at approximately the same level as in B showing the location of fluorescent beads (green, indicated by the white arrow) microinjected into the commissural subnucleus of the caudal NTS. These microinjections were performed only in some experiments. The section is counterstained with Cresyl Violet. Light-field and fluorescence photomicrographs have been superimposed. Microinjections were made according to stereotaxic coordinates derived from the atlas of Meessen and Olszewski (1949). The more recent atlas of Shek et al. (1986) was used for comparison. Abbreviations: comNTS, commissural subnucleus of the nucleus tractus solitarii; NDV, nucleus dorsalis nervi vagi; NV, nucleus tractus spinalis nervi trigemini; NXII, nucleus nervi hypoglossi. Modified from Mutolo et al. (2012) and Cinelli et al. (2015). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Mazzone et al. (2005), the effects of C-fiber activation could be mimicked by microinjections of capsaicin or substance P into the commissural NTS, and could be reversed by centrally administered neurokinin (NK) receptor antagonists (see also Mutolo et al., 2007, 2008b). Canning and Mori (2011) confirmed all these findings and provided evidence of processes that could facilitate or inhibit the cough reflex. The issue concerning potentiation or sensitization of airway defensive reflexes has been addressed later.

4.2. Modulation of the cough reflex by antitussive agents

Previous studies led to the conclusion that some antitussive drugs act centrally since they downregulate cough when administered by intravertebral arterial or intracerebroventricular route in cats and guinea pigs (Bolser et al., 1997, 1999). However, the central responsive structures involved and the mechanism of action of the employed drugs were obscure. The hypothesis was advanced (Mutolo et al., 2007) that the cNTS could be a site of action of some centrally acting antitussive agents. Local applications of the μ-opioid receptor agonist [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) and the $GABA_B$ receptor agonist baclofen (5 and 1 mM, respectively) decreased baseline respiratory frequency because of increases in inspiratory time, and completely suppressed the cough reflex (Mutolo et al., 2008b). DAMGO and baclofen at lower concentrations (0.5 and 0.1 mM, respectively) decreased the cough number, peak abdominal activity and increased cough-related total cycle duration. These effects were counteracted by microinjections of the corresponding antagonists, i.e. by 10 mM naloxone and 25 mM CGP-35348, respectively. Microinjections of the NK_1 receptor antagonist CP-99,994 (10 mM) abolished cough responses while those of the NK_2 receptor antagonist MEN 10376 (5 mM) were without effects (Mutolo et al., 2008b). The antitussive action of baclofen microinjected into the NTS was confirmed by Canning and Mori (2011) in anesthetized guinea pigs. These results not only provide evidence that the NTS is a site of action of some antitussive drugs, but they also indicate that substance P release in the cNTS may be involved in the mediation of cough reflex responses (Mutolo et al., 2008b). Part of these results have been summarized in Fig. 5.

4.3. Cough downregulation by drugs relevant to chronic pain treatment

A few years ago, the similarities between neural mechanisms underlying cough and pain both at the peripheral and the central level were noted. Since cough can be considered a defensive response to nociceptive stimulation it is not surprising that peripheral and central mechanisms underlying nociception and cough share similar features (Barnes 2007; Canning 2009; Mutolo et al., 2012, 2014; Lavinka and Dong 2013; O'Neill et al., 2013; Dicpinigaitis et al., 2014; Niimi and Chung 2015). Some of the main similarities between cough and pain have been reported in Table 1. In this context, it seems appropriate to mention that an interesting parallel has been made between pain and itch as well as between itch and cough (Lavinka and Dong 2013; Liu and Ji 2013; Hoon 2015). Of note, many recent outcomes have shown

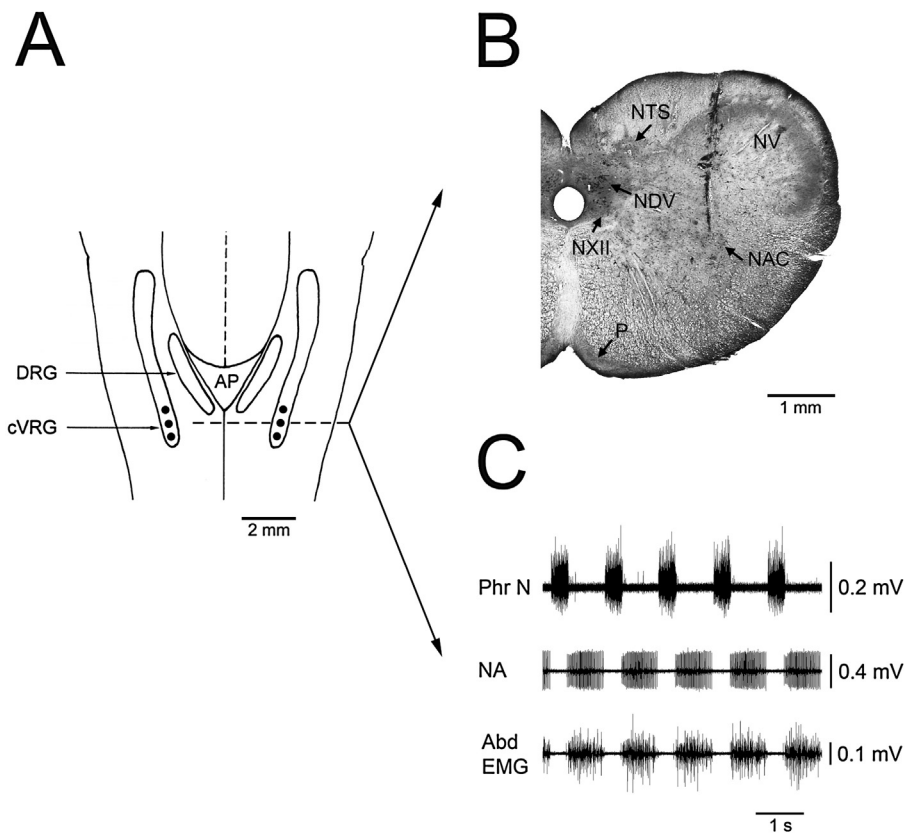


Fig. 4. Localization of injection sites within the caudal ventral respiratory group (cVRG) and histological control. A: diagrammatic view of the dorsal medulla of the rabbit showing where drug bilateral microinjections (●) have been performed in each experiment. AP, area postrema; cVRG, caudal ventral respiratory group; DRG, dorsal respiratory group. B: photomicrograph of a coronal section of the medulla oblongata at the level indicated in panel A (horizontal dashed line) showing the track along which a microinjection was made at a site where multiunit expiratory neuronal activity was recorded. Abbreviations: NAC, nucleus ambiguus caudalis; NDV, nucleus dorsalis nervi vagi; NTS, nucleus tractus solitarii; NV, nucleus tractus spinalis nervi trigemini; NXII, nucleus nervi hypoglossi; P, tractus pyramidalis. The atlas of Meessen and Olszewski (1949) was used for comparison. C: an example of neuronal expiratory activity recorded in the cVRG. Phr N, phrenic neurogram; NA, neuronal activity; Abd EMG, abdominal electromyographic activity. Modified from Cinelli et al. (2012).

that itch displays specificity not only at the level of peripheral sensory neurons, but also within the central nervous system. Thus, itch and pain display striking differences under acute conditions, but appear to have more similarities under chronic conditions, including peripheral and central sensitization (Liu and Ji 2013; Hoon 2015). Cough, pain and itch obviously have a very important protective role and serve as warning signals for actual or potential body damage, i.e. they are appropriate responses to nociceptive stimuli and clearly provide an evolutionary advantage. However, they also characterize debilitating diseases under chronic pathological conditions (O'Neill et al., 2013; Liu and Ji 2013; Dicpinigaitis et al., 2014; Hoon 2015; Niimi and Chung 2015). The protective role of cough and pain are rather obvious. The lack of a cough reflex would expose humans to higher risk of aspiration pneumonia and death, while pain insensitivity is well known to be associated with a reduced life expectancy since it predisposes to debilitating or even fatal physical injuries. On the other hand, itch causes the protective mechanism of scratching that may dislodge parasites, insects or irritant plant poisons from the skin (Lavinka and Dong 2013; O'Neill et al., 2013; Liu and Ji 2013; Hoon 2015). Conceivably, neuroactive agents involved in the central control of reflex responses to nociceptive stimuli and associated pain sensation may also have a role in the regulation of the cough reflex. Accordingly, drugs of such type were employed in an attempt to downregulate the cough reflex (see Fig. 6).

Local application of U0126, an inhibitor of ERK1/2 activation, revealed for the first time that the mitogen-activated protein kinase (MAPK) contributes to the processing of tussive inputs (Mutolo et al., 2012). Bilateral microinjections of 50 mM U0126 into the cNTS suppressed cough responses without affecting the Breuer-Hering inflation reflex, the pulmonary chemoreflex, and the sneeze reflex. Not surprisingly, these results are consistent with previous observations on the involvement of ERK1/2 in the central effects on both acute pain behaviour and neuronal plasticity underlying pain hypersensitivity (e.g. Woolf and Salter 2000; Kawasaki et al., 2004; Obata and Noguchi 2004; Ji et al., 2009). Also the activation of α_2-adrenergic receptors by microinjections of 5 mM clonidine and 0.5 mM tizanidine, two agonists that may have analgesic effects (for review see Eisenach et al., 1996; Pertovaara 2006; Chan et al., 2010), strongly downregulated cough responses (Cinelli et al., 2013). The depressant effects on the cough reflex exerted by clonidine and tizanidine were completely reverted by microinjections of the α_2-adrenergic receptor antagonist yohimbine (10 mM) performed into the same sites. The hypothesis was that clonidine- and tizanidine-induced effects were due, at least in part, to reductions in glutamate release by presynaptic inhibition operated through α_2-adrenergic receptors at the level of the central terminals of cough-related vagal afferents. On the other hand, α_2-adrenergic receptor agonists may have other functions in the nervous system through the regulation of the release of other neurotransmitters or by the activation of postsynaptic α_2-adrenergic receptors (e.g. Ono et al., 1991 and Cinelli et al., 2013 for further Refs.). Another study (Mutolo et al., 2014) investigated the regulation of the cough reflex by galanin (a neuropeptide implicated in pain control) at the level of the NTS, where galanin receptors are known to be present (Endoh et al., 2008). Bilateral microinjections of 1 mM galanin or 10 mM galnon (a nonpeptide agonist at galanin receptors) into the cNTS markedly affected cough responses by decreasing the cough number, peak abdominal activity and by increasing total cycle duration. To explain galanin antitussive effects, it should be recalled that galanin antagonizes

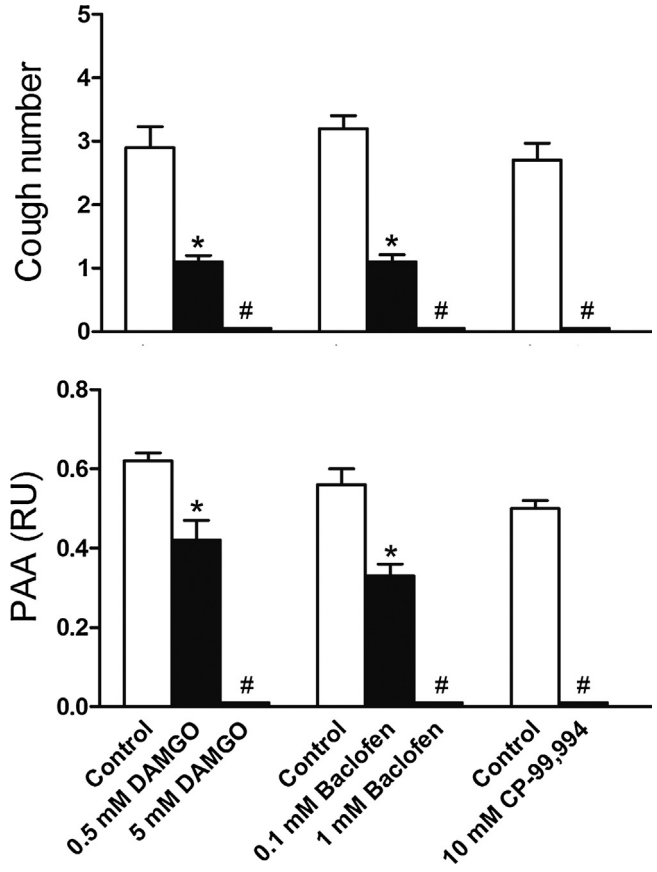


Fig. 5. Downregulation of the cough reflex induced by mechanical stimulation of the tracheobronchial tree in response to bilateral microinjections of some centrally acting antitussive agents performed into the caudal NTS of anesthetized, spontaneously breathing rabbits. Histograms illustrating maximum changes in the cough number and peak abdominal activity (PAA), i.e. the intensity of expiratory thrusts, induced by DAMGO (a μ-opioid receptor agonist), baclofen (a $GABA_B$ receptor agonist) and CP-99,994 (an NK_1 receptor antagonist). RU, relative units. Values are means ± SEM. * $P < 0.05$; # $P < 0.001$ compared with control. Data derived from Mutolo et al. (2008b).

Table 1
Some of the main similarities between cough and pain.

Afferent fibres	
Aδ, C	Cough, pain
Receptors on sensory afferents	
$TRPV_1$,$TRPA_1$, ASICs	Cough, pain
ATP and adenosine receptors	Cough, pain
Bradykinin and prostaglandins receptors	Cough, pain
Histamine receptors	Cough, pain
Serotonin receptors	Cough, pain
Peripheral sensitization	Cough, pain
Central sensitization	Cough, pain
Corresponding clinical features	
Upper airway tickling sensation	Cough
Paresthesia	Pain
Hypertussia	Cough
Hyperalgesia	Pain
Allotussia	Cough
Allodynia	Pain

substance P-evoked hyperalgesia and that its antinociceptive effect involves opioid receptors and displays positive interactions with NMDA receptor antagonists (for details see Mutolo et al., 2014). This interpretation is consistent with the above reported protussive role of substance P and the antitussive action of opioids and NMDA receptor antagonists.

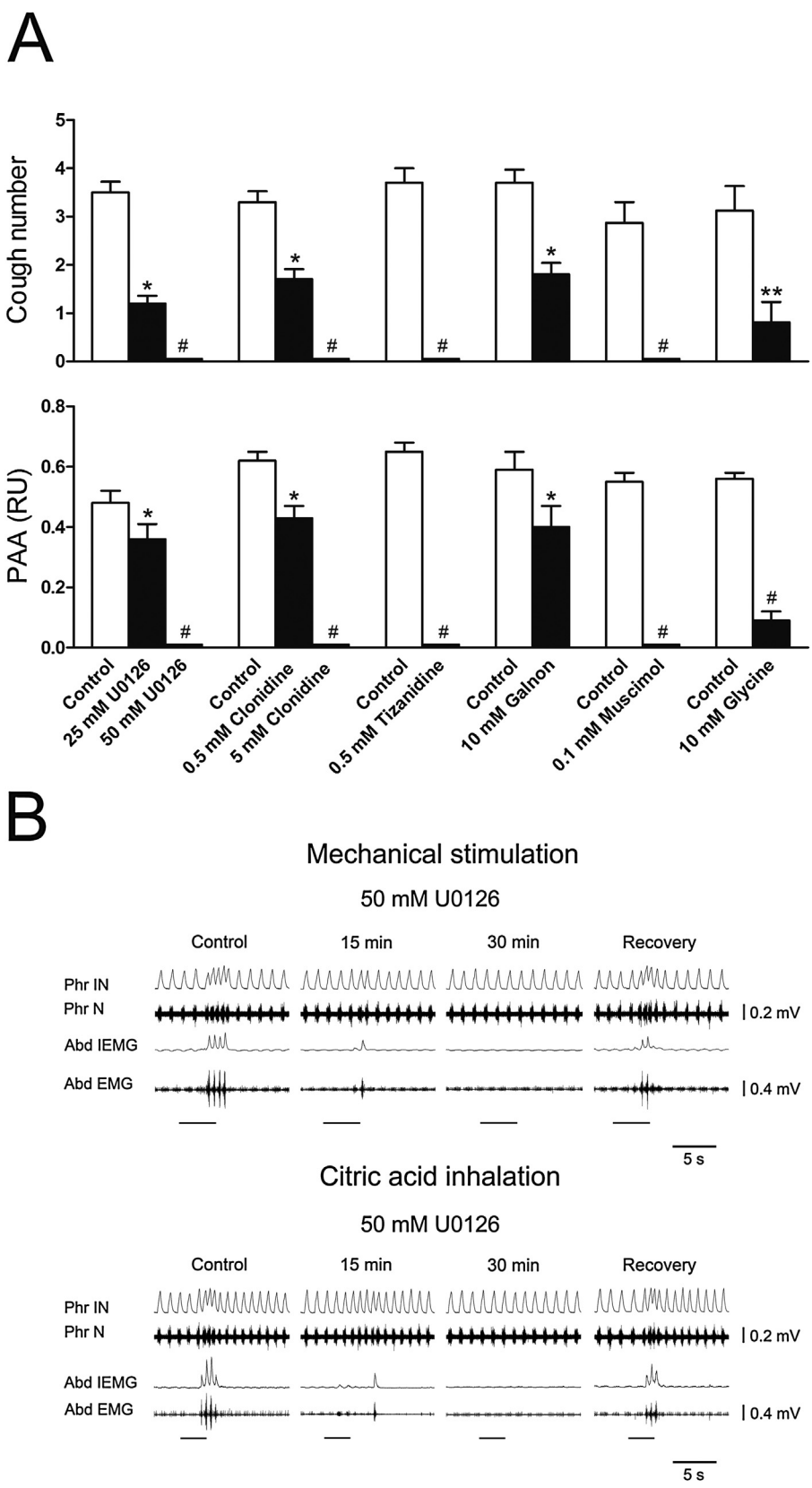
In this context, it should be also recalled that an impairment of the activation of $GABA_A$ and glycine receptors can be the neural substrate of neuropathic pain (Kami et al., 2016; for review see Prescott 2015; Zeilhofer et al., 2015). RAR cells located in the cNTS have been proven to receive both phasic glycinergic and tonic $GABA_A$ receptor-mediated inhibitory inputs (Ezure et al., 1998, 1999; Ezure and Tanaka 2000, 2004; see also Fig. 1). Evidence has been provided (Cinelli et al., 2016) that both $GABA_A$ and glycine receptors mediate at the cNTS level a potent inhibitory modulation of the pattern of breathing and, importantly, also of cough reflex responses. Bilateral microinjections of bicuculline (0.1 mM) and strychnine (1 mM) caused strong decreases in expiratory activity and marked increases in respiratory frequency. Of note, these antagonists potentiated the cough reflex mainly via increases in the cough number. The effects caused by muscimol (0.1 mM) and glycine (10 mM) were in the opposite direction to those produced by the corresponding antagonists. These results are only in part consistent with those reported by Canning and Mori (2011) in anesthetized guinea pigs, owing to the great variability of their results. It is worth mentioning that NTS microinjections of $GABA_A$ agonists and antagonists in the rat produced very pronounced disrupting effects on the baseline respiratory pattern and/or the swallowing reflex (Bautista and Dutschmann 2014). Taken together, our results strongly suggest that inhibition and disinhibition are prominent regulatory mechanisms of ongoing respiratory activity and cough reflex responses, specifically affecting the cough number.

4.4. Central protussive action of lisinopril

Cough is widely considered one of the major side effects of the angiotensin-converting enzyme (ACE) inhibitors (Berkin and Ball 1988), a group of drugs used for hypertension, heart failure and post-infarction treatment (Paul et al., 2006). ACE inhibitors have been hypothesized to have a central action on the cough reflex since they cross the blood-brain barrier (Ranadive et al., 1992; Pediconi et al., 2005; Tan et al., 2005). A study in the rabbit by Cinelli et al. (2015) showed that microinjections of the ACE inhibitor lisinopril (1 mM) into the cNTS caused changes in the cough motor pattern characterized only by increases in the cough number, thus demonstrating a central protussive action of this drug. As already proposed for cough-related receptors at the peripheral level, the results strongly suggested that lisinopril effects were due to a central accumulation of bradykinin and substance P. In fact, the complete blockade of lisinopril-induced cough potentiation was obtained either by bradykinin B_2 or NK_1 receptor antagonism at the same injection sites. An important finding of this study was that 0.05 mM bradykinin microinjections into the cNTS induced a clear cough potentiation, that was completely abolished by preceding microinjections of the NK_1 receptor antagonist CP-99,994 (1 mM) at the same sites. These findings are consistent with the view of a presynaptic action of bradykinin on sensory endings of bronchopulmonary C-fiber afferents capable of inducing the release of substance P (Cinelli et al., 2015). Data related to drug-induced cough potentiation are illustrated in Fig. 7.

4.5. Cough-gating mechanism

Bolser and colleagues (Bolser et al., 1999, 2006; see also Canning and Mori 2010) have proposed the presence of a cough-gating mechanism mainly based on evidence derived from studies on the differential effects of antitussive drugs on the cough reflex and the breathing pattern (Bolser et al., 1999). These Authors proposed that a cough-gating mechanism accounts for the fact that antitussive drugs do not alter breathing at doses that inhibit cough, suggesting the presence of a neural component important for cough that does not participate in breathing pattern generation. In addition, they supported this hypothesis with the finding that antitussive drugs do not exert a generalized cough suppression, rather they have very specific effects only on some components of the cough motor pattern, i.e. the cough number and the intensity of the expiratory thrusts. Our results agree, to a large extent,



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with the assumptions of Bolser et al. (1999, 2006). However, at variance with the hypothesis advanced for the gating mechanism, some antitussive drugs have been found to greatly influence also cough-related timing probably affecting neurons unrelated to vagal tussigenic inputs, but implicated in the control of respiratory timing and intensity (Budzinska et al., 1985a; Von Euler 1986; Bianchi et al., 1995). This obviously implies that NTS ascending projections provide synaptic interactions with neurons responsible for respiratory pattern formation

Fig. 6. Suppressant effects on the cough reflex following bilateral microinjections of different neuroactive drugs into the caudal NTS in anesthetized, spontaneously breathing rabbits. A: histograms showing maximum changes in the cough number and peak abdominal activity (PAA) induced by U0126 (an inhibitor of ERK1/2 activation), clonidine and tizanidine (α_2-adrenergic receptor agonists), galnon (a nonpeptide agonist at galanin receptors), muscimol (a $GABA_A$ receptor agonist) and glycine (endogenous glycine receptor agonist). Cough reflex responses were evoked by mechanical stimulation of the tracheobronchial tree. RU, relative units. Values are means ± SEM. $^{*}P < 0.05$; $^{\#}P < 0.001$ compared with control. B: original recordings showing the effects of U0126 microinjections into the caudal NTS on cough reflex responses evoked by mechanical stimulation of the tracheobronchial tree and by the inhalation of 1 M citric acid in one anesthetized, spontaneously breathing rabbit. Reduction and complete suppression of cough responses at different times after bilateral microinjections of 50 mM U0126. Recovery of cough responses was taken after ~3 h. Stimulation period marked by filled bars. Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity. Data and recordings adapted from Mutolo et al. (2012, 2014) and Cinelli et al. (2013, 2016).

(see Jones et al., 2016 also for further Refs.). All these results support the notion that the cNTS is not a mere relay station, but plays more extensive functions in cough motor pattern generation and, in addition, that it is an important location of cough-gating neurons (see Haji et al., 2013; Bolser et al., 2015; Pitts et al., 2016; also for further Refs.). However, it cannot be at present completely excluded that other respiration-related regions, such as the raphe nuclei, the cVRG or the BötC, may contribute to the cough-gating mechanism.

5. Caudal ventral respiratory group (nucleus retroambigualis)

5.1. Possible role of the caudal ventral respiratory group in respiratory and cough pattern formation

Another important site of action of neuroactive substances affecting the cough reflex is the cVRG where bulbospinal expiratory neurons are located intermingled with other types of respiratory and non-respiratory neurons (e.g. Arita et al., 1987; Iscoe 1998). Reasonably, this could be a neural structure very important in the control of the cough reflex since the expulsive expiratory phase represents the main purpose of this defensive reflex. Thus, bulbospinal expiratory neurons should be of crucial importance in the genesis of expiratory thrusts as well as in cough regulation (see e.g. Jones et al., 2016 also for further Refs.). The respiratory network involved in the generation of the eupnic pattern of breathing is located in the brainstem (Von Euler 1986; Bianchi et al., 1995; Janczewski and Feldman 2006; Smith et al., 2007; Abdala et al., 2009; Pagliardini et al., 2011; Feldman et al., 2013; Cui et al., 2016), however the neural structures located caudal to the obex are thought to be unnecessary for such a function. The vast majority of morphological and electrophysiological lines of evidence suggest that cVRG expiratory neurons probably are not involved in the respiratory rhythmogenesis since they do not have axon collaterals and therefore connections with other medullary respiratory neurons (Merrill 1979; Von Euler 1986; Huang and St John 1988; Bianchi et al., 1995; Iscoe 1998). However, activation of cVRG neurons through microinjections of EAA receptor agonists caused transient inhibition of inspiratory activity in cats (Bongianni et al., 1994), rats (Bonham and Jeske 1989; Chitravanshi and Sapru 1999) and rabbits (Bongianni et al., 2005). Actually, microinjections of the broad-spectrum EAA receptor agonist D,L-homocysteic acid (DLH) into the cVRG of the cat provoked the activation of expiratory motoneurons and a corresponding silent period in phrenic nerve activity (Bongianni et al., 1994). Similar findings have been obtained by Poliacek et al. (2007). Respiratory modulation triggered by EAA receptor stimulation of the NRA region, comprising the cVRG, has been confirmed and analyzed in more detail by recent studies (Subramanian and Holstege 2009; Jones et al., 2016). The hypothesis was advanced that cVRG neurons may affect the pattern of breathing, possibly through axon collaterals when strongly activated (Bongianni et al., 1994). In the interpretation of DLH-induced effects in the cVRG, it should also be taken into account that the population of neurons there located is not homogeneous (e.g. Lipski et al., 1984; Arita et al., 1987; Smith et al., 1989; Iscoe 1998). Chemical stimulation probably brings into action not only respiratory, but also non-respiratory neurons and high-threshold respiration-related neurons which may have axon collaterals (Iscoe 1998). Axon collaterals could either be relatively rare or characteristic of only a subgroup of neurons, so that they have weak interactions, if any, during normal eupnic breathing, but may disclose their whole effects when intense or massive activation of the parent neurons occurs. From this point of view, present data do not completely refute the earlier conclusion that the caudal expiratory neurons are not essential for respiratory rhythm generation. Rather, they support the possibility that these neurons can alter or shape the pattern of breathing when strongly activated. This could be relevant to some physiological conditions, such as airway defensive reflexes including coughing and sneezing (Bongianni et al., 1994; Iscoe 1998). This hypothesis is corroborated by anatomical studies showing cVRG projections to other brainstem respiration-related regions, such as the rostral VRG, the parabrachialis medialis/Kölliker Fuse nuclei and the NTS (Smith et al., 1989; Gerrits and Holstege 1996; Iscoe 1998; Zheng et al., 1998; Jones et al., 2016).

The afferent drive inputs to the NRA are not completely unraveled. They may arise from more rostral expiration-related regions such as the BötC (Bongianni et al., 1998; Shannon et al., 2000; for review see also Iscoe 1998; Pantaleo et al., 2002; Shannon et al., 2004) or the RTN/pFRG (Onimaru and Homma 2003; Mulkey et al., 2004; Janczewski and Feldman 2006; Guyenet and Mulkey 2010; Guyenet et al., 2016; Ikeda et al., 2017) as well as from the limbic system and the periaqueductal gray (Subramanian and Holstege 2009; Jones et al., 2016). Some recent findings are in contrast with the current concept that structures caudal to the obex are unnecessary for eupneic breathing. The discovery of the high cervical respiratory group (Oku et al., 2008) and the results of complete transverse transections at different levels caudal to the obex (Jones et al., 2012) have suggested the existence of an area overlapping the NRA, that provides ascending inputs to the ponto-medullary respiratory network critical for respiratory pattern generation (see also Ikeda et al., 2017).

5.2. Mediation of excitatory inputs to caudal ventral respiratory group neurons

An important issue concerns the type of neurotransmitters involved in the excitatory drive transmission to cVRG expiratory neurons. Bilateral microinjections of 50 mM kynurenic acid and of the non-NMDA receptor antagonist CNQX (10 mM) have been shown to completely suppress spontaneous rhythmic abdominal activity and reflex expiratory responses either to tracheal occlusion at end-inspiration (Breuer-Hering inflation reflex) or to expiratory threshold loading (Bongianni et al., 2005). More interestingly, they also suppressed both the inspiratory and expiratory components of the cough reflex. The NMDA receptor antagonist D-AP5 (10 mM) strongly reduced, but did not completely abolish spontaneous abdominal activity and all the studied reflex responses. One of the most significant findings of this study is that neurons located in the cVRG are not merely elements of the expiratory output system, but are essential for the production of the complete cough motor pattern. The interpretation of these results is rather complex (see Bongianni et al., 2005). Here it seems sufficient to point out that the effects of EAA antagonism on the inspiratory components of the cough reflex cannot be explained by the suppression of the excitatory output from cVRG bulbospinal neurons. Newsom Davis and Plum (1972) demonstrated that bilateral lesions of descending bulbospinal expiratory pathways impair spontaneous rhythmic abdominal activity and interfere only with the expiratory components of the

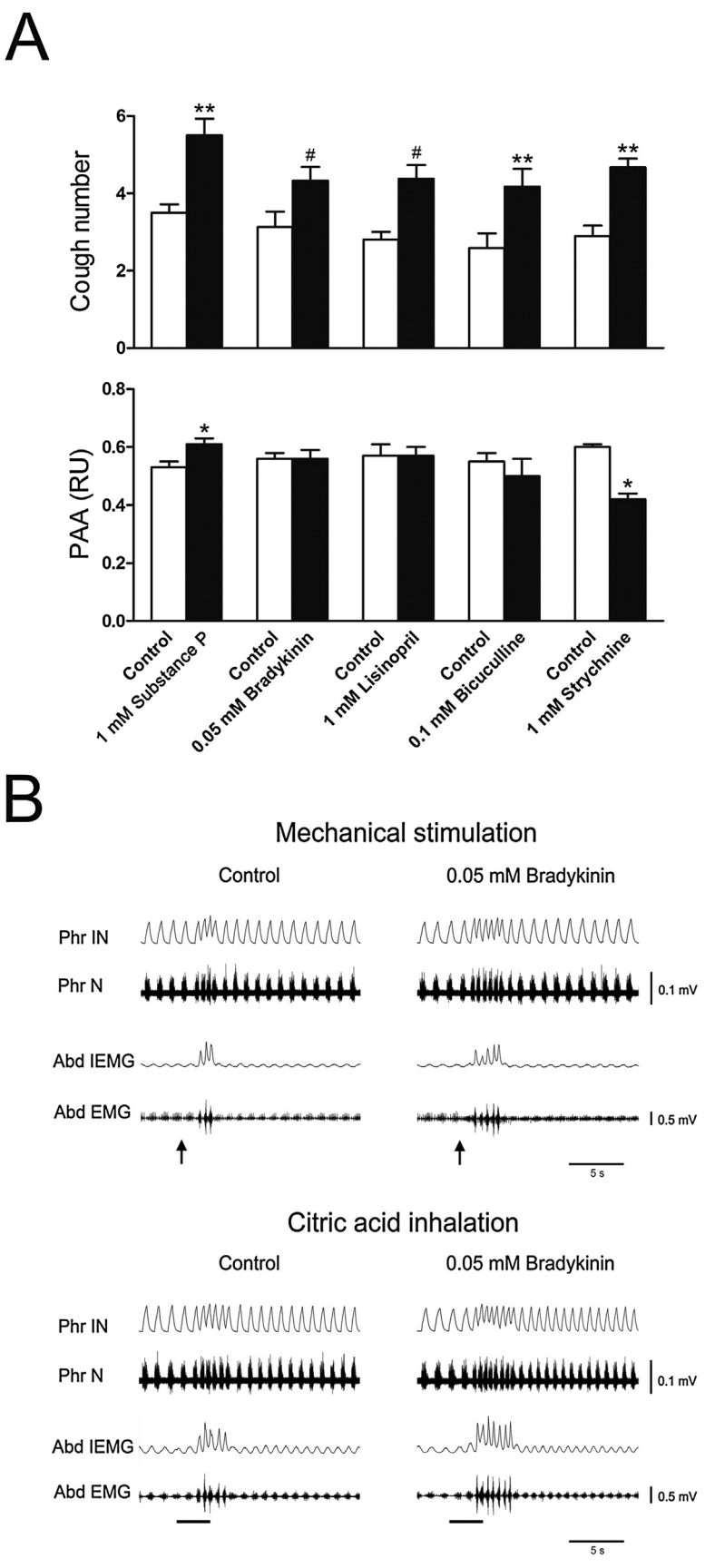

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Fig. 7. Upregulation of cough reflex responses in response to bilateral microinjections of some centrally acting agents performed into the caudal NTS in anesthetized, spontaneously breathing rabbits. A: histograms illustrating maximum changes in the cough number and peak abdominal activity (PAA) caused by substance P (an NK receptor agonist), bradykinin (a BK receptor agonist), lisinopril (an ACE inhibitor), bicuculline (a $GABA_A$ receptor antagonist) and strychnine (a glycine receptor antagonist). The cough reflex was induced by mechanical stimulation of the tracheobronchial tree. RU, relative units. Values are means ± SEM. * $P < 0.05$; ** $P < 0.01$; $^{\#}$ $P < 0.001$ compared with control. B: original recordings illustrating bradykinin-induced potentiation of the cough reflex in response to mechanical and chemical stimulation of the tracheobronchial tree in one anesthetized, spontaneously breathing rabbit. Onset of mechanical stimulation delivered via a custom-built device (arrows) and periods of citric acid inhalation (filled bars) are indicated. Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity. Data and recordings adapted from Mutolo et al. (2007) and Cinelli et al. (2015, 2016).

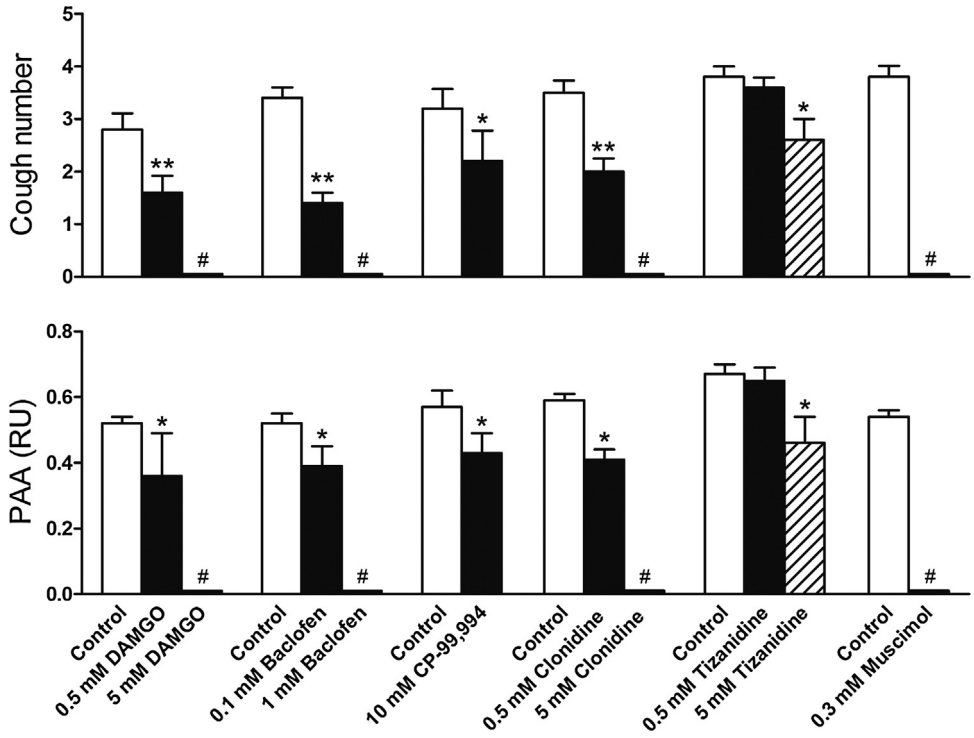

Fig. 8. Inhibitory effects on the cough reflex evoked by mechanical stimulation of the tracheobronchial tree in response to bilateral microinjections of some neuroactive agents into the caudal VRG (anesthetized, spontaneously breathing rabbits). Maximum changes in the cough number and peak abdominal activity (PAA) caused by DAMGO (a μ-opioid receptor agonist), baclofen (a $GABA_B$ receptor agonist), CP-99,994 (an NK_1 receptor antagonist), clonidine and tizanidine (α_2-adrenergic receptor agonists), and muscimol (a $GABA_A$ receptor agonist) are illustrated by histograms. RU, relative units. Values are means ± SEM. * $P < 0.05$; ** $P < 0.01$; $^{\#}$ $P < 0.001$ compared with control. Data derived from Mutolo et al. (2010) and Cinelli et al. (2012, 2013).

cough reflex. Neurons located in the cVRG should be taken into consideration in devising new computational models of the brainstem neural mechanisms underlying coughing and, probably, other defensive reflexes. In this context, it is worth noting that Poliacek et al. (2007) revealed the presence of a cough-suppressant neuronal circuit within the cVRG by showing that unilateral microinjections of DLH reduced the cough number and associated abdominal thrusts. Interestingly, the application of codeine to the cVRG proved to have an antitussive effect without a direct action on bulbospinal expiratory neurons (Poliacek et al., 2010). In this context, it could be mentioned that at variance with previous findings (Bongianni et al., 1998; Mutolo et al., 2002) on the BötC, Poliacek et al. (2008a) found that suppressant effects on the cough reflex were induced by the activation of neurons in the region of the BötC by means of unilateral microinjections of low DLH doses. Their results are in agreement with the general inhibitory role of BötC expiratory neurons (Merrill et al., 1983; Bongianni et al., 1988; Jiang and Lipski 1990; Ezure 1990; see Poliacek et al., 2008a for further Refs.) and suggest that neural mechanisms similar to the cough-suppressant circuits of the cVRG could be present in this area. The reasons for the discrepancy with previous findings is not clear and could, in part, be ascribed to differences in the preparation and microinjection procedures, including the localization of the injection site. It should be considered that the population of BötC neurons is far from homogeneous and that both excitatory and inhibitory subgroups of neurons projecting to the cVRG may exist.

5.3. Modulation of the cough reflex by some antitussive drugs and GABA

Studies were undertaken to investigate whether some antitussive drugs, already tested at the cNTS level, were active also within the cVRG. Bilateral microinjections of both DAMGO and baclofen (Mutolo et al., 2010) at relatively low concentrations (0.5 and 0.1 mM, respectively) decreased the cough number and peak abdominal activity, while increased cough-related total cycle duration. At higher concentrations (5 and 1 mM, respectively) they completely abolished this defensive reflex. Moreover, also microinjections of the NK_1 receptor antagonist CP-99,994 (10 mM) decreased the cough number and peak abdominal activity. No effects were observed in response to the local application of the NK_2 receptor antagonist MEN 10376 (5 mM). Only DAMGO and baclofen also affected baseline respiratory activity, thus suggesting, as already discussed, the presence in the cVRG of neurons that can directly or indirectly influence more rostral respiration-related areas and therefore the respiratory timing. Furthermore, bilateral microinjections of clonidine (5 mM) and tizanidine (0.5 mM) performed into the cVRG (Cinelli et al., 2013) completely suppressed both the expiratory and inspiratory components of the cough reflex, thus supporting the notion of the essential role of this region in the production of the overall cough motor pattern.

Caudal expiratory neurons are known to receive a potent bicuculline-sensitive GABAergic inhibitory modulation (McCrimmon et al., 1997; Tonkovic-Capin et al., 2001, 2003). Accordingly, bilateral

microinjections of bicuculline (1 mM) into the cVRG affected baseline respiration by increasing abdominal activity and respiratory frequency, and potentiated cough reflex responses. On the contrary, muscimol (0.3 mM) not only abolished expiratory activity and decreased respiratory frequency, but also induced progressive reduction and subsequent complete suppression of the entire cough reflex (Cinelli et al., 2012). These results show that $GABA_A$ receptors within the cVRG mediate a very strong inhibitory control not only of the pattern of breathing, but also of airway defensive reflexes involving intense expiratory efforts. They further highlight the role of inhibition and disinhibition in the central regulation of both eupneic breathing and coughing. For a more extensive discussion see Cinelli et al. (2012). Depressant effects on the cough reflex have also been obtained with microinjections of codeine and nicotine into the cVRG of the cat (Poliacek et al., 2010, 2015). Part of the above reported results is presented in Fig. 8.

6. Concluding remarks and future directions

The central organization of the cough reflex is fairly complex and involves the brainstem respiratory network ("reconfiguration" hypothesis) and many modulatory influences that may shape its motor pattern. The main conclusion that arises from this review is that, although several brainstem structures contribute to the regulation of this reflex, at least two of them, i.e. the cNTS and the cVRG, are important sites of action of antitussive or protussive drugs. Interestingly, in this review the attention has been focused not only on neuroactive agents that downregulate, but also on those that upregulate the cough reflex, emphasizing the importance of inhibition and disinhibition phenomena. In addition, an essential role of the cVRG has been suggested in the genesis of the overall cough motor pattern. The results of drug microinjections into the cVRG, along with those obtained by Sugiyama et al. (2015) with transverse brainstem sections, suggest that the neuronal network essential for coughing is rather caudal and could mainly involve the cNTS and the neighboring inspiratory and expiratory regions of the ventral respiratory column.

An issue that deserves some comments is the reason why the different cough-related variables changed according to the various experimental conditions. A general feature of the effects obtained in response to antitussive drugs microinjected into the cNTS and the cVRG was either the complete suppression of the reflex or a reduction in the cough number accompanied by decreases in peak abdominal activity and increases in cough-related total cycle duration. On the contrary, protussive drugs caused increases in the cough number and, only on some occasions, changes in peak abdominal activity or cough-related cycle duration. Obviously, the effects depended upon the brainstem structure injected as well as the type of drug used and related membrane receptors with their post- and/or presynaptic actions. Furthermore, since the neuronal populations affected by the injectate are not homogeneous, the type of neurons inhibited or activated may contribute to determine the final outcome. Some effects on the expiratory component of the cough motor pattern appear to be rather obvious when microinjections were performed into the cVRG, where expiratory premotoneurons are located, or into the NTS that is known to contribute to the regulation of the expiratory motor output (Budzinska et al., 1985b). Part of the affected neurons may be involved solely or prevailingly in the cough reflex, while others may also have a role in the regulation of timing with their projections to other brainstem respiration-related regions. However, an exhaustive interpretation of the observed microinjection-induced effects is still lacking.

Further investigations on the basic physiological and pathophysiological mechanisms underlying cough, pain and itch, and the analysis of their similarities and differences, could lead to novel therapeutic strategies. In addition, studies are needed on the different brainstem areas subserving cough motor pattern formation (Fig. 2) making use of lesion and stimulation experiments, recordings of cough-related neuronal activities as well as local applications of neurotransmitters and neuromodulators (including antitussive or protussive agents). Lesion or stimulation experiments could be performed as usual by applying neurotoxins (kainic or ibotenic acids) or EAA agonists, respectively. However, more recently developed optogenetic approaches could be employed to induce targeted photostimulation or photoinhibition of neuronal activities located in selected areas of the brainstem. Optogenetic techniques have already been used in transgenic mice to investigate the functional role of the preBötC in the control of breathing (see e.g. Alsahafi et al., 2015; Cui et al., 2016; Koizumi et al., 2016; Vann et al., 2016). Conceivably, some brainstem areas should be considered in more detail: for instance, the RTN/pFRG, that may be important in the generation of the expiratory thrusts, and the PiCo that may be involved in the control of postinspiratory behaviours, including coughing and swallowing (Anderson et al., 2016). Other central regions considered to be largely associated with the origin of the postinspiratory motor drive, such as the NTS, the BötC and the pontine respiratory nuclei, especially the Kölliker Fuse nucleus, may deserve further investigations (Dutschmann and Herbert 2006; Dutschmann et al., 2014). Further, the periaqueductal gray, that is the source of one of the major afferent input to the NRA, could be relevant for cough research (e.g. Iscoe 1998; Subramanian and Holstege 2009; Jones et al., 2016). Interestingly, reciprocal connections between the periaqueductal gray and the preBötC have been reported (for review see Ikeda et al., 2017). Despite the preBötC being considered the core of the central mechanism generating the inspiratory rhythmic activity, its contribution to the production of the cough motor pattern has not yet been unraveled.

The paratrigeminal nucleus receives primary afferent projections from the pharynx, larynx and tracheobronchial tree. In addition, visceral and somatic primary afferent inputs may converge in the paratrigeminal nucleus that has been suggested to be involved in the mediation of viscero-visceral and somato-visceral reflexes through efferent connections with autonomic centers in the brainstem (see e.g. Mazzone and Undem 2016). This nucleus is certainly involved in respiratory reflexes other than cough, such as respiratory slowing and apnea (Chou et al., 2008; Driessen et al., 2015). However, its possible role, if any, in the genesis of the cough motor pattern or of the "urge-to-cough" remains to be ascertained.

Finally, a recent aspect of the physiology of the central nervous system is the great contribution of glial cells, especially astrocytes, to the functional characteristics of neuronal activities (e.g. Fellin 2009). It has been proposed that astrocytes in the respiratory network may contribute to the characteristic of inspiratory activity (Hulsmann et al., 2000; Schnell et al., 2011; Okada et al., 2012; Oku et al., 2016) and play a crucial role in central chemoreception within the RTN (Gourine et al., 2010; Guyenet et al., 2016). The finding that ozone-induced pulmonary inflammation results in a specific activation of vagal afferents that induces astroglial cellular alterations in the NTS (Chounlamountry et al., 2015 also for further Refs.) suggests a possible involvement of astrocytes in cough regulation. The contribution of astrocytes to the modulation of the cough motor pattern at the brainstem level remains to be investigated.

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