Inhibitory modulation of the cough reflex by acetylcholine in the caudal nucleus tractus solitarii of the rabbit

Elenia Cinelli¹, Ludovica Iovino¹, Fulvia Bongianni, Tito Pantaleo, Donatella Mutolo⁷

Dipartimento di Medicina Sperimentale e Clinica, Sezione Scienze Fisiologiche, Università degli Studi di Firenze, Viale G.B. Morgagni 63, 50134 Firenze, Italy

A R T I C L E   I N F O

Keywords:
- Acetylcholine receptors
- Scopolamine
- Cough reflex
- Nucleus tractus solitarii
- Control of breathing

A B S T R A C T

A cholinergic system has been described in the nucleus tractus solitarii (NTS). However, no information is available on the role played by acetylcholine (ACh) in the modulation of the cough reflex within the caudal NTS that has an important function in cough regulation. We addressed this issue making use of bilateral micro-injections (30–50 nl) of 10 mM ACh combined with 5 mM physostigmine as well as of 10 mM mecamylamine or 10 mM scopolamine into the caudal NTS of pentobarbital sodium-anesthetized, spontaneously breathing rabbits. Microinjections of ACh/physostigmine caused depressant effects on the cough reflex induced by mechanical and chemical stimulation of the tracheobronchial tree. They also elicited transient increases in respiratory frequency and decreases in abdominal activity. These effects were prevented by scopolamine, but not by mecamylamine. The results show for the first time that ACh exerts an inhibitory modulation of the cough reflex through muscarinic receptors within the caudal NTS. They also may provide hints for novel antitussive approaches.

1. Introduction

Cough is one of the most important defensive reflex brought into action by actually or potentially damaging events applied to the airways (Korpás and Tomori, 1979). Peripheral and central mechanisms underlying nociception and cough share similar features; neuroactive agents involved in the central control of pain sensation (Millan, 2002; Yan et al., 2017) and concomitant reflex responses play a role also in the downregulation of the cough reflex (see Mutolo, 2017 also for further Refs. Mutolo et al., 2008, 2012, 2014; Cinelli et al., 2013, 2016).

Several brainstem areas appear to contribute to the generation and regulation of cough responses in mammals (e.g. Greer et al., 1997; Bongianni et al., 1998; Jakus et al., 2008; Mutolo et al., 2002b; Poliacek et al., 2004, 2005, 2014; Shannon et al., 2004; Simera et al., 2013; see also Mutolo, 2017). Recent findings obtained mainly in the rabbit (for review see Mutolo, 2017) have led to the proposal that two medullary structures play a prominent role in the control of cough reflex responses and are sites of action of antitussive or protussive drugs, i.e. the caudal nucleus tractus solitarii (NTS), the first relay medullary station of the cough reflex, and the caudal ventral respiratory group (cVRG), where neurons responsible for the expiratory component of the reflex are located (for the role of the cVRG see also Poliacek et al., 2007, 2010, 2015). Of note, afferent inputs from peripheral chemoreceptors and pulmonary rapidly adapting receptors converge on neurons of the caudal NTS (Mifflin et al., 1988; Mifflin, 1992; Machado, 2001; Kubin et al., 2006). However, recently evidence has been provided of the existence in the cat of important control mechanisms within the rostral NTS (Poliacek et al., 2017a,b; for review see Mutolo, 2017) very similar to those described for the caudal NTS in the rabbit. This could be possibly related to marked differences in the animal species.

Besides its modulatory quality, acetylcholine (ACh) also acts as one of the most prominent neurotransmitters in the peripheral and central nervous system. Interestingly, cholinergic transmission profoundly affects the perception of pain via both nicotinic (nAChRs) and muscarinic (mAChRs) receptors (for review see Naser and Kuner, 2017). There is considerable direct and indirect evidence that ACh is widely distributed in the region of NTS (Kobayashi et al., 1978; Criscione et al., 1983; Ernsberger et al., 1988; Ruggiero et al., 1990; Zoccal et al., 2014) where both mAChRs and nAChRs are present. Muscarinic receptors are located in more caudal regions, including the commissural subnucleus, while nicotinic receptors are predominantly located at more rostral levels, i.e. in the medial, ventrolateral and ventral subnuclei (Maley, 1996; see also Furuya et al., 2014).

ACh contributes to autonomic regulation. In particular, it is involved in the regulation of both cardiovascular (e.g. Shihara et al., 1999; Furuya et al., 2014, 2017; Zoccal et al., 2014) and respiratory activity (e.g. Haxhiu et al., 1984; Bianchi et al., 1995; Shao et al., 1999).
Feldman, 2000, 2001, 2002, 2009; Shao et al., 2008; Boutin et al., 2017) through both nAChRs and mAChRs. However, only scanty knowledge is available on its contribution to the modulation of the cough motor pattern. Recently, it has been shown that (~)-nicotine administered via brainstem circulation or directly applied to the cVRG causes mecamylamine-insensitive inhibitory effects on mechanically-induced cough (Poliacek et al., 2015). In the present research, the possible role of ACh in the modulation of the cough reflex at the level of the caudal NTS of pentobarbital sodium-anaesthetized, spontaneously breathing rabbits was investigated making use of microinjection techniques.

2. Materials and methods

2.1. Ethical approval

Animal care and experimental procedures were conducted in accordance with the Italian legislation and the official regulations of the European Community Council on the use of laboratory animals (Decreto Legislativo 4/3/2014 no. 26 and Directive 2010/63/UE). The study was approved by the Animal Care and Use Committee of the University of Florence. All efforts were made to minimize both the number of animals used and their suffering. Details about the methods employed have been described in our previous studies on the NTS region and will be concisely reported here (Cinelli et al., 2013, 2016; Mutolo et al., 2007, 2008, 2009, 2012, 2014; Mutolo, 2017).

2.2. Animal preparation and recording procedures

Experiments were carried out on 15 male New Zealand White rabbits (2.7–3.3 kg) anaesthetized with pentobarbital sodium (40 mg/kg i.v., supplemented by 2–4 mg/kg every 30 min; Sigma–Aldrich, St. Louis, MO, USA). Atropine (0.15 mg/kg i.m.) was administered to reduce mucosal secretion in the airways. The adequacy of anesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to noxious pinching of the hindpaw. Additional criteria were the presence of a stable and regular pattern of phrenic bursts and the absence of fluctuations in arterial blood pressure. The trachea was cannulated and polyethylene catheters were inserted into a femoral artery and vein for monitoring arterial blood pressure and drug delivery, respectively. The C2 or C3 phrenic root on one side was prepared for recordings. The animal was placed in a prone position and fixed by a stereotaxic head holder and vertebral clamps. The head was ventroflexed for optimal exposure of the dorsal surface of the medulla by occipital craniotomy. Body temperature was maintained at 38.5–39 °C by a heating blanket controlled by a rectal thermistor probe.

Effert phrenic nerve activity was recorded with bipolar platinum electrodes. Abdominal muscle electromyographic (EMG) activity was recorded by wire electrodes. Phrenic and abdominal activities were amplified, full-wave rectified, and “integrated” (low-pass RC filter, time constant 100 ms). Arterial blood pressure was recorded by a strain-gauge manometer and end-tidal CO2 partial pressure by an infrared CO2 analyzer (Capnocheck Plus, Smiths Medical PM, Waukesha, WI, USA). Cardiorespiratory variables were analyzed using a personal computer, supplied with an appropriate interface (Digidata 1440, Molecular Devices, Sunnyvale, CA, USA) and software (Axoscope, Molecular Devices).

2.3. Microinjection procedures

Bilateral microinjections were performed at two different sites along the rostrocaudal extent of the caudal NTS. The first was at the level of the caudal-most end of the area postrema that approximately corresponds to the opening of the central canal of the IV ventricle, 0.6–0.8 mm lateral to the midline and 0.7–0.8 mm below the dorsal medullary surface. The second was 0.5 mm more caudal, 0.4–0.5 mm lateral to the midline and 0.7–0.8 mm below the dorsal medullary surface. Owing to the spread of the 50 nl-injectate, < 400 μm in any direction (for the spread of the injectate see also Results and Discussion), injections at the first sites affected a NTS area probably including the most caudal extent of the medial subnucleus (see Mutolo et al., 2007). The stereotaxic coordinates were selected according to the atlas of Meesen and Olzewska (1949).

Microinjections (30–50 nl) were performed as described in our previous reports via a single-barrel glass micropipette (tip diameter 10–25 μm). The volume of the injectate was measured directly by monitoring the movement of the fluid meniscus in the pipette barrel with a dissecting microscope equipped with a fine reticule. The following drugs were used: 10 mM ACh chloride (endogenous neurotransmitter at cholinergic synapse; Sigma–Aldrich), 5 mM physostigmine salicylate (an acetylcholinesterase inhibitor; Sigma–Aldrich), 10 mM mecamylamine hydrochloride (a noncompetitive nAChR antagonist; Sigma–Aldrich), 10 mM (~)-scopolamine hydrobromide trihydrate (a nonselective mACh antagonist; Sigma–Aldrich). Each drug was dissolved in 0.9% NaCl solution. Drug concentrations were in the same range as those previously used in in vivo preparations (e.g. Furuya et al., 2014; Zhang et al., 2016; Boutin et al., 2017). ACh at 10 mM was injected in combination with 5 mM phystostigmine to obtain relative more pronounced and lasting effects. Control injections of equal volumes of the vehicle solution at the responsive sites were also performed. Fig. 1 illustrates the localization of injection sites that was confirmed in some preparations by injecting green fluorescent latex microspheres (LumaFluor, New City, NY, USA) added to the drug solution (three for ACh/phystostigmine and two for scopolamine).

2.4. Stimulation procedures

Mechanical stimulation was delivered by a custom-built device recently described and validated (Mutolo et al., 2014) using a 0.5-mm diameter nylon fibre with a smoothed tip inserted through a lateral port of the tracheal cannula. The device allowed to set the number of forth and back movements or cycles (1–3 cycles), shaft velocity (10–20 mm/s), and shaft displacement (10–20 mm). Mechanical stimulation was set at 1 cycle, 15 mm/s velocity, and 15 mm displacement to produce a bout of 2–4 coughs. The stimulation protocol comprised three stimulation trials performed in succession (at 1–2 min interval) before drug administration, repeated ≥10 min after the completion of all the microinjections and at appropriate intervals (at least 5 min) to follow the recovery process for a maximum of 90 min.

Chemical stimulation of the tracheobronchial tree was performed by means of citric acid inhalation (for details see Mutolo et al., 2009). Citric acid (1 M, Sigma–Aldrich) was freshly dissolved in 0.9% NaCl solution and nebulized. The opening of the tracheal cannula, through which the rabbits were spontaneously breathing, was exposed to a steady stream of the nebulized citric acid solution for ~3 s. Chemical stimulation was always applied 2–3 min after mechanically-induced cough and caused a bout of several coughs usually immediately followed by a tachypneic response. As a rule, chemical stimulation was performed both before and ~10 min after the completion of the injections and repeated at appropriate intervals (~10 min) to follow the recovery process.

2.5. Histology

The histological control of pipette tracks and injection sites was performed as previously described (for details, see Mutolo et al., 2007, 2012; Cinelli et al., 2016). Frozen 20-μm coronal sections stained with Cresyl Violet were used. Coronal sections of the medulla in which injection sites were marked by fluorescent microspheres were examined in a light and epifluorescence microscope (Eclipse E400, Nikon, Japan) equipped with the Nikon Intensilight C-HGFI mercury-fibre illuminator. A Nikon DS-F11 digital camera was used to take photomicrographs.
Illustrations were prepared in Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, USA).

2.6. Data collection and analysis

Respiratory variables were measured during eupneic breathing and reflex responses (Mutolo et al., 2014; see also Poliacek et al., 2011, 2017). The inspiratory (Ti) and expiratory (Te) times, as well as the total duration of the respiratory cycle (Tc) were measured. The respiratory frequency was subsequently calculated (breaths/min). Peak amplitude (arbitrary units) of the phrenic nerve activity and abdominal EMG activity were measured on integrated traces. They were normalized by expressing them as a fraction (or percentage) of the highest achievable amplitude observed in each animal (relative units, RU). Breathing pattern variables were measured for an average of five consecutive breaths prior to and following drug microinjections. Furthermore, systolic and diastolic blood pressures were measured at 2 s intervals and mean arterial pressure was calculated as the diastolic pressure plus one-third of the pulse pressure. The measurement periods of cardiorespiratory variables were the same selected for cough-related variables (see below). Owing to the small variations in respiratory and cardiovascular variables within each measurement period, average values were taken as single measurements for the purpose of analysis.

The cough motor pattern in response to mechanical or chemical stimulation of the tracheobronchial tree is characterized by repeated coughs. Cough-related variables included the cough-related Ti, Ti and Te, peak phrenic amplitude (RU), peak abdominal activity (RU) and the cough number, i.e. the number of coughs following each stimulation. The cough Te was defined as the interval from the onset of phrenic activity to its peak activity. The cough Tf was defined as the interval from peak phrenic amplitude to the onset of the subsequent phrenic burst. Cough-related variables were measured and averaged before and after drug administration at the time when the maximum response and the complete recovery were observed (three trials for mechanical stimulation and a single trial for citric acid inhalation). The average values of cough-related variables were taken as single measurements for subsequent statistical analysis (GraphPad Prism 5, GraphPad Software, Inc., La Jolla, CA, USA). An expiration reflex could occur as the first motor event in a cough epoch (Korpáš and Tomori, 1979; Widdicombe and Fontana, 2006; for further details and comments see Refs. Mutolo et al., 2007, 2008, 2009, 2012; Tatar et al., 2008; Cinelli et al., 2013). Expiration reflexes were not considered for data analysis. Comparisons were performed by using one-way repeated-measures ANOVA followed by Student-Newman-Keuls tests. Paired t-tests were also used when appropriate. Reported values are means ± SEM; P < 0.05 was taken as significant.

3. Results

Bilateral microinjections (n = 6) of 10 mM ACh (300–500 pmol) and 5 mM physostigmine (150–250 pmol) at the two selected caudal NTS sites caused within 1 min significant increases in respiratory frequency (from 54.5 ± 1.7 to 70.9 ± 4.1 breaths/min; +30.8 ± 7.0%; P < 0.001) that faded out within 5 min. No significant changes in mean arterial pressure occurred (Table 1). These effects were accompanied by decreases or even complete suppression of expiratory activity.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>ACh/Physio</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT, s</td>
<td>1.10 ± 0.04</td>
<td>0.86 ± 0.06</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>Ti, s</td>
<td>0.37 ± 0.01</td>
<td>0.36 ± 0.02</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>Te, s</td>
<td>0.73 ± 0.03</td>
<td>0.52 ± 0.05</td>
<td>0.73 ± 0.02</td>
</tr>
<tr>
<td>PPA, RU</td>
<td>0.53 ± 0.01</td>
<td>0.55 ± 0.03</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>PAA, RU</td>
<td>0.06 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>98.3 ± 4.7</td>
<td>97.3 ± 5.4</td>
<td>98.4 ± 5.1</td>
</tr>
</tbody>
</table>

Values are means ± SEM; n, number of animals; TT, cycle duration; Ti, inspiratory time; Te, expiratory time; PPA, peak phrenic activity in relative units (RU); PAA, peak abdominal activity in relative units (RU); MAP, mean arterial blood pressure.

*** P < 0.001, compared with controls as well as with recovery.
that persisted for longer time period (10–15 min) and recovered within 30 min. Progressive depressant effects on the cough reflex reached a maximum within 10 min. At that time cough responses induced by mechanical stimulation of the tracheobronchial tree were completely abolished, while citric acid inhalation-induced cough responses were only strongly reduced both in the cough number and in the peak abdominal amplitude (see Fig. 2 and Table 2). Cough-related variables resumed control values within 60 min.

To disclose whether ACh exerts its effects through nAChRs or mAChRs, the corresponding specific antagonists were employed. We injected 10 mM mecamylamine (n = 3; 300–500 pmol) or 10 mM scopolamine (n = 4; 300–500 pmol) and after an interval of ∼5 min 10 mM ACh/5 mM physostigmine into the same sites. Neither mecamylamine nor scopolamine caused obvious and significant effects on baseline respiratory activity (paired t-tests). Indeed, in both cases respiratory frequency remained fairly constant with variations lower than ±1% in each preparation. ACh-induced changes in baseline respiratory activity as well as in the cough reflex were not counteracted by mecamylamine, but were prevented by scopolamine (not shown).

In two additional preparations, bilateral control microinjections of 10 mM ACh/5 mM physostigmine were performed at different medullary locations (4 trials) sufficiently far (>0.8 mm) from the responsive sites (see e.g. Nicholson, 1985; Lipski et al., 1988; Mutolo et al., 2007, 2012, 2014; Sykova and Nicholson, 2008; Cinelli et al., 2013, 2016). With respect to the responsive sites, they were performed into the NTS region 1 mm rostral and 1 mm lateral to the midline (1 trial), into the adjacent reticular formation (2 trials) and into the reticular formation located 1 mm more caudal at the same depth (1 trial). Control microinjections (2 trials for each location) were also performed into the nucleus cuneatus medialis and the nucleus tractus spinalis nervi trigemini. All these microinjections failed to induce changes in the breathing pattern as well as suppressant effects on the cough reflex.

Table 2
Changes in some cough-related variables approximately 10 min following bilateral microinjections of 10 mM acetylcholine/5 mM physostigmine (n = 6) into the caudal NTS.

<table>
<thead>
<tr>
<th></th>
<th>CN</th>
<th>T1, s</th>
<th>T2, s</th>
<th>T2, s</th>
<th>IEMG, RU</th>
<th>Abd EMG, RU</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechanical stimulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.1</td>
<td>± 0.1</td>
<td>0.57</td>
<td>± 0.03</td>
<td>0.39</td>
<td>0.18</td>
</tr>
<tr>
<td>ACh/Physo</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.37</td>
<td>0.21</td>
</tr>
<tr>
<td>Recovery</td>
<td>3.05</td>
<td>± 0.1</td>
<td>0.58</td>
<td>± 0.04</td>
<td>0.37</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Citric acid inhalation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.7</td>
<td>± 0.4</td>
<td>0.52</td>
<td>± 0.03</td>
<td>0.35</td>
<td>0.16</td>
</tr>
<tr>
<td>ACh/Physo</td>
<td>1.7</td>
<td>± 0.2</td>
<td>± 0.03</td>
<td>0.36</td>
<td>0.15</td>
<td>0.65</td>
</tr>
<tr>
<td>Recovery</td>
<td>3.6</td>
<td>± 0.3</td>
<td>0.51</td>
<td>± 0.02</td>
<td>0.34</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Values are means ± SEM; n, number of animals; CN, cough number; T1, cycle duration; T2, inspiratory time; T2, expiratory time; PPA, peak phrenic activity in relative units (RU); PAA, peak abdominal activity in relative units (RU); Abd EMG, abdominal electromyographic activity.

**P** < 0.01, **P** < 0.001, compared with control cough as well as with recovery.

Fig. 2. Depressant effects on cough reflex responses induced by 10 mM acetylcholine (ACh) combined with 5 mM physostigmine (Physo) microinjected into the caudal NTS in one anesthetized spontaneously breathing rabbit. Original recordings illustrating suppressant effects on cough responses about 10 min after bilateral microinjections. Note the decreases in abdominal muscle activity. Recovery of cough responses was taken – 60 min after the injections. The onset of mechanical stimulation is indicated by arrows while chemical stimulation is marked by filled bars. Phr IN, phrenic integrated neurogram; Phr N, phrenic neurogram; Abd IEMG, abdominal integrated electromyographic activity; Abd EMG, abdominal electromyographic activity.
4. Discussion

This study shows for the first time that ACh microinjected into the caudal NTS causes strong depressant effects on the cough reflex through the activation of mAChRs. Present findings not only confirm that the caudal NTS is one of the most important sites involved in the modulation of the cough reflex in the rabbit, but also extend our previous results showing that the central mechanisms subserving nociception and cough share similar features (for review see Mutolo, 2017).

We have fully discussed in our previous reports the reliability of microinjection procedures, the spread of the injectate and the localization of injection sites that were selected using stereotaxic coordinates according to the atlas of Meessen and Olszewski (1949) and confirmed by the histological control (Mutolo et al., 2007, 2008, 2009, 2012, 2013, 2014; Cinelli et al., 2013, 2016). The absence of changes in the ongoing respiratory activity and especially in cough reflex responses following drug microinjections at sites sufficiently far from the responsive sites (> 0.8 mm) as well as following vehicle microinjections into the responsive sites of the caudal NTS supports the specificity of drug-induced effects. The distance of control microinjections from the responsive sites derives from previous observations on the spread of the injectate (see e.g. Nicholson, 1985; Lipski et al., 1988; Mutolo et al., 2002a, 2005; Bongianni et al., 2008, 2010; Sykova and Nicholson, 2008). Given the very short lasting effects of ACh due not only to diffusion, but especially to acetylcholineserase-induced degradation, we injected in combination 10 mM ACh and 5 mM physostigmine.

ACh-induced changes in respiratory frequency could be of interest. They may reveal an important mechanism of respiratory modulation at the caudal NTS level. ACh-induced increases in respiratory frequency within this region have also been reported by Furuya et al. (2014). Changes in respiratory timing are typically associated with the function of a rhythm-generating mechanism (Von Euler, 1986; Feldman and Del Negro, 2006). It seems plausible that neurons in the caudal NTS are embedded in a ponto-medullary circuit implicated in the control of the respiratory timing (Budzinska et al., 1985a; Von Euler, 1986; Bianchi et al., 1995). On the other hand, it has been recently proposed that the NTS region has an important role in the neural control of breathing (Bautista and Dutschmann, 2014; Jones et al., 2015). Accordingly, it has also been reported that respiratory neurons of the caudal NTS project to the inspiratory neurons located in the preBötzinger complex, the recognized central pattern generator of inspiratory activity, as well as to the rostral respiratory portion of the ventral respiratory group (Alheid et al., 2011) and to the pons (Takakura et al., 2006; Song et al., 2012, 2013, 2014; Cinelli et al., 2013, 2016). The absence of changes in the ongoing respiratory activity and especially in cough reflex responses following drug microinjections at sites sufficiently far from the responsive sites (> 0.8 mm) as well as following vehicle microinjections into the responsive sites of the caudal NTS supports the specificity of drug-induced effects. The distance of control microinjections from the responsive sites derives from previous observations on the spread of the injectate (see e.g. Nicholson, 1985; Lipski et al., 1988; Mutolo et al., 2002a, 2005; Bongianni et al., 2008, 2010; Sykova and Nicholson, 2008). Given the very short lasting effects of ACh due not only to diffusion, but especially to acetylcholineserase-induced degradation, we injected in combination 10 mM ACh and 5 mM physostigmine.

As already mentioned, present results are in keeping with the proposal of similarities between neural mechanisms underlying nociception and cough. For instance, there is a large body of evidence pointing to the importance of muscarinic signaling in pain control at the level of the spinal dorsal horns. In particular, ACh receptors profoundly regulate nociceptive transmission in the spinal cord via pre- and post-synaptic mechanisms and the direct activation of mAChRs reduces pain in rodents and humans, while their inhibition induces hypersensitivity (for review see Naser and Kuner, 2017).

An important issue is the source of cholinergic inputs to the caudal NTS. A functional cholinergic system has been described in the NTS region as well as a possible role of local cholinergic interneurons modulated by vagal afferent signals. While primary afferent fibers of the solitary tract use glutamate as the primary neurotransmitter, they could activate cholinergic interneurons (Kobayashi et al., 1978; Helke et al., 1983; Ruggiero et al., 1990; Maley, 1996; Shiibara et al., 1999; Furuya et al., 2014; Zoccal et al., 2014). Furthermore, it has also been suggested that in addition to glutamate other neurotransmitters, including ACh, may contribute to the neurotransmission of cardiorespiratory signals in the NTS (Criscone et al., 1983; Andresen and Kunze, 1994; Tsukamoto et al., 1994; Machado, 2001; Machado and Bonagamba, 2005; Braga and Machado, 2006; Abdala et al., 2006; da Silva et al., 2008; Furuya et al., 2014; Zoccal et al., 2014). Cholinergic fibers may also derive from the dorsal motor vagal nucleus and the nucleus ambiguus (Farkas et al., 1997). In addition, there are cholinergic projections to the brainstem from the ponto-mesencephalic tegmental cholinergic complex. However, their role in the modulation of arterial blood pressure and respiration within the NTS is not clear (Woolf and Butcher, 1989; Woolf, 1991). Further studies are needed to assess the source of ACh for the NTS and to disclose details on synaptic cholinergic mechanisms. In this context, it can be recalled that ACh microinjected into the caudal NTS of the rat causes increases in respiratory frequency through both mAChRs and nAChRs (Furuya et al., 2014). These results are in partial agreement with present findings and the discrepancy may be related to differences in the animal species employed.

The results show that a tonic action of ACh on respiration is lacking. However, it is clear that muscarinic cholinergic mechanisms can modulate both eupneic breathing and the cough reflex. When these mechanisms are brought into action is obscure and only tentative proposals can be advanced. ACh in the caudal NTS may facilitate respiratory responses to peripheral chemoreflex activation, in agreement with previous findings in the rat (Furuya et al., 2014; Zoccal et al., 2014). In addition, not only afferent inputs from chemoreceptors, but also from pulmonary rapidly adapting receptors and C-fibers, that are well known to be involved in tachypneic reflex responses (Sant’Ambrogio and Widdicombe, 2001), converge onto caudal NTS neurons (e.g. Machado, 2001; Kubin et al., 2006). Finally, somatosensory afferent signals, including those conveyed by nociceptive afferents, reach the caudal NTS relayed via the dorsal horn neurons (e.g. Kalia et al., 1981; Craig, 1995; Boscan et al., 2002; see Potts and Waldrop, 2005 for further Refs.). Cardiorespiratory changes and in particular tachypnea induced by somatic afferent stimulation (Duranti et al., 1991 also for further Refs.) may be mediated by the caudal NTS and its functional cholinergic system. In this context, it seems also relevant to mention that cough sensitivity is downregulated by exercise-induced hyperventilation as well as by voluntary isocapnic hyperpnea at similar level (Lavorini et al., 2010).

Present results confirm that the caudal NTS, the main central terminus of bronchopulmonary afferents, has an important role in cough regulation and is a site of action of several neurotransmitters and neuromodulators. However, recent findings in the cat support a similar role for more rostral portions of the NTS (Poliakec et al., 2017a,b; see also Canning and Mori, 2010; Mutolo, 2017), thus suggesting marked species differences in the anatomical and functional organization of
NTS subnuclei. On the other hand, we have to admit that AChs may also act at the level of the different brainstem neural structures involved in the control of this defensive reflex (Mutolo, 2017), as recently observed by Poliacek et al. (2015) by using nicotine microinjections into the cVRG. The observed absence of an apparent functional involvement of nAChRs following ACh microinjections could be due to barbiturate-inhibition of the nicotinic receptor channels (Morin-Surun et al., 1984; Yost and Dodson, 1993; for review see Arias et al., 2006). However, it seems that this inhibitory mechanism does not contribute very much to conceal nicotinic effects, at least in some animal species (Poliacek et al., 2015).

In conclusion, the results provide substantial evidence that mAChRs modulate both respiration and cough reflex responses. They may also provide hints for further studies not only on details of caudal NTS cholinergic mechanisms, but also on the development of novel anti-refl

### References


Andresen, M.C., Kunze, D.L., 1994. Nucleus tractus solitarius−gateway to neural circula-


Furuya, W.I., Colombari, E., Ferguson, A.V., Colombi, D.S., 2017. Effects of acetyl-
choline and cholinergic antagonists on the activity of nucleus of the solitary tract nu-
clears. Brain Res. 1659, 136–141.


### Funding

This study was supported by grants from the University of Florence. E.C was supported by a postdoctoral fellowship from the Fondazione Internazionale Menarini.

### Disclosures

No conflict of interest, financial or otherwise, are declared by the Author(s).

### Author contributions

E.C., L.I., F.B., T.P. and D.M. performed experiments; E.C., L.I., F.B. and D.M. analyzed data; E.C., L.I., F.B. and T.P. designed projects and interpreted results of ex-
periments; E.C. and L.I. prepared figures; E.C. and D.M. drafted manuscript; E.C., F.B., T.P. and D.M. edited and revised manuscript; E.C., L.I., F.B., T.P. and D.M. approved final version of the manuscript.

### References


Alheid, G.F., Jiao, W., McCrimmon, D.R., 2011. Caudal nuclei of the rat nucleus of the solitary tract differentially innervate respiratory compartments within the ven-

Andresen, M.C., Kunze, D.L., 1994. Nucleus tractus solitarius-gateway to neural circula-


Bongianni, F., Mutolo, D., Cinelli, E., Pantaleo, T., 2010. Respiratory responses induced by blockades of GABA and glycine receptors within the Bötzinger complex and the parvocellular nu-
clears of the rabbit. Brain Res. 1344, 143–147.


Maley, B.E., 1996. Immunohistochemical localization of neuropeptides and neuro-
Mifflo, S.W., Spyer, K.M., Withington-Wray, D.J., 1988. Baroreceptor inputs to the nu-
icrus tractus solitarii in the cat: modulation by the hypothalamus. J. Physiol. 399, 369–387.
Mifflo, S.W., 1992. Arterial chemoreceptor input to nucleus tractus solitarii. Am. J.
Physiol. 263, R368-R375.
Mutolo, D., Bongianni, F., Carlo, M., Pantaleo, T., 2002a. Respiratory changes induced by
Mutolo, D., Bongianni, F., Nardone, F., Pantaleo, T., 2005. Respiratory responses evoked
by blockades of ionotonic glutamate receptors within the Bötzinger complex and the
acids and substance P in the mediation of the cough reflex within the nucleus tractus solitarii of the rabbit. Brain Res. Bull. 74, 284–293.
Mutolo, D., Bongianni, F., Cinelli, E., Pantaleo, T., 2009. Role of excitatory amino acids in the
Mutolo, D., Cinelli, E., Bongianni, F., Pantaleo, T., 2014. Inhibitory control of the cough
Nasr, P.V., Kuner, R., 2017. Molecular, cellular and circuit basis of cholinergic mod-
Nicholson, C., 1985. Diffusion from an injected volume of a substance in brain tissue with
piration and aspiration reflexes following kainic acid lesions to the pontine re-