UNIVERSITÀ DEGLI STUDI DI FIRENZE
Dipartimento di Scienze Fisiologiche

SCUOLA DI DOTTORATO IN SCIENZE FISIOLOGICHE E NUTRIZIONALI

Dottorato di Ricerca in Scienze Fisiologiche e Nutrizionali

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Settore disciplinare BIO/09

Control of respiratory activity by α7 nicotinic acetylcholine receptors within the paratrigeminal respiratory group of the lamprey

Tutore:

Prof. Fulvia Bongianni

DIPARTIMENTO DI SCIENZE FISIOLOGICHE
UNIVERSITA' DEGLI STUDI DI FIRENZE

Coordinatore del Dottorato:

Prof. Corrado Poggesi

Candidato: *Dott.ssa Elenia Cinelli*

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SUMMARY

1. Respiratory rhythm in adult mammals probably results from synaptic interactions between respiratory neurons located in the lower brainstem, and particularly in the medulla oblongata. The basic neural mechanisms responsible for respiratory rhythm generation are still not fully elucidated and remain highly debated. Further insights into the mechanisms underlying the genesis of rhythmic activities may arise from studies on lower vertebrates, such as the lamprey which diverged from the main vertebrate line around 450 million years ago and has proved to be highly useful to identify the cellular mechanisms underlying neural control of locomotion.

2. The isolated brainstem of adult lampreys spontaneously generates respiratory neuronal activity *in vitro.* The vast majority of respiratory motoneurons are located in the facial, glossopharyngeal and, especially, in the vagal nuclei, while the neural aggregate responsible for respiratory rhythm generation appears to be located bilaterally in a region rostrolateral to the trigeminal motor nucleus, named the paratrigeminal respiratory group (pTRG). This region has been suggested to correspond to the pre-Bötzinger complex, the hypothesized kernel of the inspiratory rhythm-generating network in mammals.

3. Although acetylcholine (ACh) has long been known to play an important role in the neural control of breathing, no information is available on the role played by ACh in the regulation of respiratory activity in the lamprey. The present study was performed to investigate whether ACh affects respiratory activity and to identify the muscarinic (mAChRs) or nicotinic receptors (nAChRs) involved. Since, at variance with mammals, respiratory rhythmic activity persisted during blockade of ionotropic glutamate, GABA and glycine receptors, an attempt was also made to test the hypothesis that other neurotransmitters including ACh were implicated in maintaining the respiratory rhythm.

4. Experiments were carried out on *in vitro* brainstem preparations of the adult lamprey. The vagal motor output was used to monitor respiratory activity. Drugs were applied either through the perfusing solution or via bilateral microinjections (0.5-1 nl) into the pTRG or into the region of vagal motoneurons.

5. Bath application of 100 μM physostigmine (an acetylcholinesterase inhibitor) as well as microinjections of 1 mM physostigmine into the pTRG induced marked increases in respiratory frequency without significant changes in peak amplitude and duration of vagal bursts. Similar microinjections into the region of vagal motoneurons did not cause any obvious effect. Bath application of 1 μM nicotine (a nAChR agonist) increased respiratory frequency, whilst bath application of 10-50 μM pilocarpine (a non-selective mAChR agonist) and 10 μM atropine (a non-selective mAChR antagonist) did not alter respiratory activity.

6. To test whether nAChRs are involved in the control of baseline respiratory activity, the effects of various nAChR antagonists were investigated. Bath application of 100 μM D-tubocurarine (a non-selective nAChR antagonist) or 0.25 μM α-bungarotoxin (an α7 selective nAChR antagonist) caused reductions in respiratory frequency and increases in the duration of vagal bursts. No respiratory effects were observed following bath application of 10 μM dihydro-β-erythroidine (an α4β2 selective nAChR antagonist) or 10 μM hexamethonium (a preferentially blocker of nAChRs at autonomic ganglia). Microinjections of 1 mM nicotine into the pTRG increased respiratory frequency without changes in the duration and amplitude of vagal bursts. Microinjections of 1 mM D-tubocurarine and 2.5 μM α-bungarotoxin into the pTRG decreased respiratory frequency and increased vagal burst duration without changes in their peak.

7. A cocktail of antagonists for NMDA (100 μM D-AP5), non-NMDA (20 μM CNQX), $GABA_A$ (10 μM bicuculline) and glycine (10 μM strychnine) receptors was added to the perfusing solution. This cocktail solution did not abolish respiratory activity, but decreased respiratory frequency and increased the duration of vagal bursts, without concomitant changes in their peak amplitude. Bath application of 10 μM methysergide (a non-selective 5-HT receptor antagonist), 500 μM MCPG (a non-selective group I/group II metabotropic glutamate receptor antagonist), 100 μM suramin or 100 μM PPADS (broad spectrum antagonists at P2X and P2Y purinergic receptors), 10 μM CP-99,994 (a NK1 receptor antagonist), 10 μM MEN 10376 (a NK2 receptor antagonist) and 10 μM SB 222200 (a NK3 receptor antagonist) did not change the respiratory motor output. On the contrary, bath application of 100 μM D-tubocurarine or 0.25 μM

α-bungarotoxin as well as microinjections of 1 mM D-tubocurarine into the pTRG abolished respiratory activity.

8. This study is the first to demonstrate that ACh plays an important excitatory role within the lamprey respiratory network under basal conditions and possibly under still undefined physiological conditions which imply increased respiratory drive to vagal motoneurons such as locomotion or increased sensory inputs. This excitatory role appears to be mediated by pTRG α7 nAChRs. Furthermore, the results indicate that ACh also contributes to maintaining the respiratory rhythm when fast synaptic transmission due to glutamatergic, GABAergic and glycinergic mechanisms is abolished. The excitatory function of ACh is mediated exclusively by α7 nAChRs located in the pTRG, that is confirmed as the possible respiratory central pattern generator in the lamprey. Present findings encourage studies to further characterize cholinergic mechanisms and their possible function under physiological and pathophysiological conditions.

INTRODUCTION

Central pattern generators for respiration

Vital circuits in the mammalian brainstem control respiratory movements and maintain homeostasis of the internal physiological state of the brain and body. Breathing is a primal homeostatic neural process regulating levels of oxygen and carbon dioxide in blood and tissues which are crucial for life. Rhythmic respiratory movements must occur continuously throughout life and originate from neural activity generated by specially organized circuits in the brainstem. The automatic and seemingly simple nature of the act of inspiration and expiration masks the complexity of the neural machinery involved. Indeed, although neuroscientists have been conducting intensive investigations of neural circuits involved in respiratory rhythm and pattern generation for more than a century, the underlying neural mechanisms are still not fully understood, and many key issues remain unresolved. Rhythmic movements such as breathing are thought to be produced by central pattern generators (CPGs). These are specialized neuronal circuits in the central nervous system that are intrinsically capable of generating rhythmic activity and motor output without rhythmic inputs from other central circuits or sensorimotor feedback signals (Marder & Calabrese, 1996; Grillner, 2006; Grillner & Jessell, 2009). The rhythmic activities generated by CPGs emerge from a combination of synaptic interactions among spatially distributed populations of neurons and intrinsic cellular properties of these neurons. In addition, the CPGs are incorporated into larger neural systems and operate under the control of various central and peripheral sensory inputs that modify the CPG-generated motor pattern, adjusting it to the internal and/or external environment.

Such inputs regulate not only the frequency and amplitude of the output rhythmic motor activity, but can also reconfigure the CPGs by transforming the operational rhythmogenic and neural pattern formation mechanisms. Thus, CPGs not only serve as generators of spatio-temporal patterns of neural activity, but also function as neural substrates for sensorimotor integration. Understanding the complex neural processes involved in the operation of different CPGs, and in particular the respiratory CPG, including the mechanisms underlying the circuit dynamic reconfiguration under different conditions, represents a central and challenging problem in neuroscience.

Respiratory network in mammals

Breathing movements are produced by a spatially distributed pontine–medullary respiratory network generating rhythmic patterns of alternating inspiratory and expiratory activities that drive and coordinate the activity of spinal and cranial motoneurons (Von Euler, 1986; Bianchi *et al.*, 1995). The motor pattern during normal breathing is considered to consist of three phases: inspiration (I), post-inspiration (post-I) or expiration phase 1 (E1) and expiration phase 2 (E2). Respiratory rhythm in adult mammals probably results from synaptic interactions between respiratory neurons located in the lower brainstem, and particularly in the medulla oblongata (Von Euler, 1986; Bianchi *et al.*, 1995; Haji *et al.*, 2000; Feldman & Del Negro, 2006; Alheid & McCrimmon, 2008; Doi & Ramirez, 2008). **Figure 1** illustrates the localization of the main structures involved in the control of breathing.

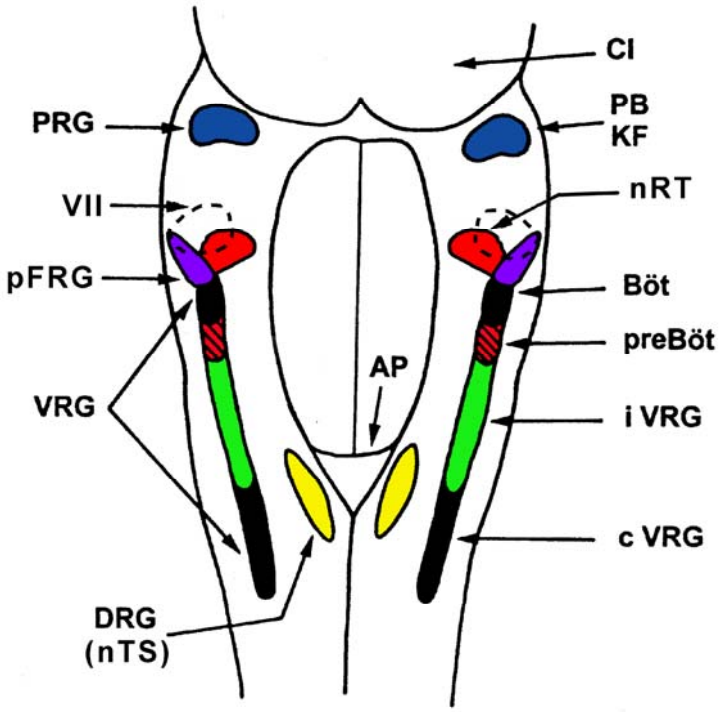

Fig. 1. Dorsal view of the brainstem of the rabbit illustrating the main structures involved in the control of breathing. AP, area postrema; Böt, Bötzinger complex; CI, colliculus inferior; cVRG, caudal ventral respiratory group; DRG, dorsal respiratory group; iVRG, intermediate or inspiratory ventral respiratory group; KF, Kölliker-Fuse nucleus; nRT, retrotrapezoid nucleus; NTS, nucleus tractus solitarii; PB, parabrachial nuclei; preBötC, pre-Bötzinger complex; pFRG, parafacial respiratory group; PRG, pontine respiratory group; VRG, ventral respiratory group; VII, nucleus facialis.

Respiratory neurons have been reported to be present in the rostral pons at the level of the parabrachial and Kölliker-Fuse nuclei, *i.e.* in a region corresponding, to a larger extent, to the 'pneumotaxic centre' and now designated as the pontine respiratory group (PRG). Respiratory medullary neurons appear to be concentrated in two main aggregates, the dorsal respiratory group (DRG) and the ventral respiratory group (VRG).

Respiratory neurons in these compartments are typically classified on the basis of their firing patterns (*e.g.* decrementing and augmenting) and their phases of activity relative to the breathing cycle, such as: early-inspiratory (early-I) neurons with a decrementing firing pattern during inspiration, ramp-inspiratory (ramp-I) neurons with an augmenting firing pattern during inspiration, post-I neurons with a decrementing firing pattern during expiration, augmenting expiratory (aug-E) neurons with an augmenting firing pattern during expiration; and a heterogeneous population of pre-inspiratory (pre-I) neurons that start firing before the onset of inspiration and continue throughout the inspiratory phase. These neurons could be more properly called phase-spanning E-I neurons. Phase spanning I-E neurons have also been described. Although the functional role of the PRG in the generation and control of the respiratory activity has not been fully established, the above-mentioned pontine regions have been shown to interact with multiple medullary compartments. These interactions provide strong modulation of medullary respiratory network activity and control respiratory phase transitions (Cohen, 1979; St John, 1998; Mutolo *et al.*, 1998; Dutschmann & Herbert, 2006; Ezure & Tanaka, 2006; Alheid & McCrimmon, 2008; Morschel & Dutschmann, 2009).

The VRG is located in the ventrolateral medulla and corresponds to a longitudinal column of neurons extending from the cervical spinal cord to the facial nucleus: it is associated with the nucleus retrofacialis, nucleus ambiguus and nucleus retroambigualis. The VRG includes several rostro-caudally arranged compartments: the Bötzinger complex (BötC), the pre-Bötzinger complex (preBötC), the intermediate or inspiratory VRG (iVRG) and the caudal part of the VRG (cVRG).

The BötC and the preBötC have been implicated in respiratory rhythm generation, while the DRG, the iVRG and the cVRG are considered mainly output systems. The DRG is closely associated with the nuclear complex of the solitary tract (nTS) and contains mostly bulbospinal inspiratory premotor neurons. The iVRG compartment contains bulbospinal inspiratory (ramp-I) excitatory neurons that project to spinal phrenic and intercostal inspiratory motoneurons and shape the inspiratory motor output. Briefly, these neurons receive excitatory inputs from the preBötC excitatory neurons and are inhibited during expiration by the BötC inhibitory neurons; both of these inputs along with other modulatory drives shape and control the characteristic ramp-like pattern of inspiratory activity (Smith *et al.*, 2007; Alheid & McCrimmon, 2008).

Expiratory neurons are mainly concentrated in the cVRG and the BötC. Most of cVRG expiratory neurons are bulbospinal neurons that project to spinal thoracic and lumbar expiratory motoneurons and that receive their excitatory input from more rostral regions of the medulla (Von Euler, 1986; Bianchi *et al.*, 1995; Iscoe, 1998). This compartment is presumed to be the expiratory counterpart to the iVRG. Convergent inputs, including those from the BötC that are synaptically integrated in the cVRG, shape the pattern of discharge of expiratory cVRG

neurons, and drive the expiratory motor output. Recent lines of evidence have indicated that neurons located in the cVRG have a crucial role in determining not only the expiratory, but also the inspiratory components of the cough motor pattern, thus supporting the view that the cVRG population of neurons does not represent merely an output system (Bongianni *et al.*, 1994; 2005; Pantaleo *et al.*, 2002; Mutolo *et al.*, 2009; 2010a). The BötC, with predominantly aug-E neurons, is considered to be a major source of expiratory activity during normal breathing and exerts extensive inhibition on medullary respiratory neurons (Von Euler, 1986; Bianchi *et al.*, 1995). The function of the BötC and its interactions with other VRG compartments have been extensively investigated (Fedorko & Merrill, 1984; Ezure & Manabe, 1988; Jiang & Lipski, 1990; Tian *et al.*, 1998; Ezure *et al.*, 2003; Shen *et al.*, 2003). BötC neurons have been reported to have an inhibitory function on all medullary respiratory neurons and even on phrenic motoneurons. However, evidence has been provided for an excitatory role of these neurons on at least part of the cVRG expiratory neurons (Mutolo *et al.*, 2002; 2005; Smith *et al.*, 2007; 2009; Bongianni *et al.*, 2008; 2010).

Adjacent and caudal to the BötC, the preBötC has been of intense interest because it is thought to function as a kernel structure for the genesis of the respiratory rhythm (Feldman *et al.*, 1991; Feldman & Del Negro, 2006). In adult animals, it has been defined in the rat (St Jacques & St John, 1999; Monnier *et al.*, 2003), the cat (Connelly *et al.*, 1992; Schwarzacher *et al.*, 1995) and the rabbit (Mutolo *et al.*, 2002; 2005; Bongianni *et al.*, 2008; 2010) and contains several types of inspiratory and expiratory propriobulbar neurons. Interestingly, the preBötC has been suggested to play a crucial role in respiratory rhythm generation both in neonatal and adult animals (Smith *et al.*, 1991; Rekling & Feldman, 1998; Smith

et al., 2000; Feldman & Del Negro, 2006). This region has been mainly investigated in medullary slices from neonatal rodents since it can express autorhythmic or pacemaker-like neuronal activity that generates a simple pattern of inspiratory activity (Smith *et al.*, 1991; Feldman & Del Negro, 2006). This activity is proposed to be based on excitatory synaptic interactions within the preBötC and intrinsic cellular mechanisms involving the persistent sodium current (I_{NaP}) and the calcium-activated non-selective cationic current (I_{CAN}). Collectively, these (and other) neuronal currents and excitatory synaptic interactions provide mechanisms for regenerative initiation, maintenance and termination of inspiratory activity in the isolated preBötC *in vitro*. An important and controversial issue is the role of preBötC neurons that show bursting-pacemaker activity in the absence of fast glutamatergic, GABAergic and glycinergic transmission in respiratory rhythm generation (Thoby-Brisson & Ramirez, 2001; Del Negro *et al.*, 2002a; 2005; Peña *et al.*, 2004; Feldman & Del Negro, 2006). One ionic mechanism for pacemaker activity is proposed to generate bursts via the I_{CAN} and its bursting properties are selectively blocked by flufenamic acid (FFA). The other ionic mechanism generates bursting-pacemaker activity through the I_{NaP} and its bursting properties are blocked by riluzole (RIL). Co-application of RIL and FFA eliminates the respiratory rhythm in rodent medullary slices *in vitro*, while respiratory activity persists upon blockade of either I_{NaP} or I_{CAN} alone (Del Negro *et al.*, 2005). The two drugs probably induce a lowering of the excitability of all neurons which express I_{NaP} or I_{CAN}, regardless of their effects on pacemaker neurons (Thoby-Brisson & Ramirez, 2001; Del Negro *et al.*, 2002a; 2005; Peña *et al.*, 2004; Feldman & Del Negro, 2006). Accordingly, the rhythmogenic role of pacemaker neurons does not seem to be obligatory since rhythmic activity could be restored by increasing

network excitability with substance P application in the presence of RIL and FFA (Del Negro *et al.*, 2005). Therefore, rhythm generation appears to be an emergent property of non-pacemaker neurons that are interconnected by excitatory synaptic interactions. All these neuronal features have been incorporated in the so-called "group-pacemaker" hypothesis (see Feldman & Del Negro, 2006). Mechanisms underlying rhythmic inspiratory pattern generation in the preBötC under more physiological conditions (*i.e.* when the preBötC is embedded in the intact brainstem) are more complex (Pierrefiche *et al.*, 1998; Smith *et al.*, 2000; 2007; Bongianni *et al.*, 2002b; 2008; 2010; Mutolo *et al.*, 2005).

Recently, two partially overlapping respiration-related regions (**Fig. 1**), *i.e.* the retrotrapezoid nucleus and the parafacial respiratory group (pFRG), have been described rostral to the VRG in close vicinity of the BötC (Alheid *et al.*, 2002; Feldman & Del Negro, 2006; Smith *et al.*, 2009). Consequently, some Authors have introduced the term "ventral respiratory column" (VRC; Alheid *et al.*, 2002) that is intended to be inclusive of the VRG. In particular, neurons that fire before and after the inspiratory phase and are silent during inspiration have been described in the pFRG. Also these neurons have been called pre-I neurons like those encountered in the preBötC (Mellen *et al.*, 2003; Onimaru & Homma, 2003; Feldman & Del Negro, 2006; Janczewski & Feldman, 2006). Furthermore, it has been suggested that there may be two distinct oscillators for respiratory rhythm generation: the first drives the inspiratory activity and is located in the preBötC, the second drives expiratory activity and is located within the pFRG. Experiments performed on *in vitro* preparations lead to the suggestion that a subpopulation of preBötC neurons expressing both neurokinin-1 and μ-opioid receptors plays an essential role in the genesis of rhythmic inspiratory activity (Gray *et al.*, 2001; Mellen *et al.*, 2003;

Feldman & Del Negro, 2006). Alternatively, the pFRG is considered a semi-independent oscillator that initiates inspiration and is coupled to the preBötC, although the preBötC is still considered the main inspiratory rhythm generator (Mellen *et al.*, 2003; Onimaru & Homma, 2003). In adult animals the retrotrapezoid nucleus have been shown to be one of the main brainstem structure involved in the chemosensitivity (Nattie & Li, 1995; Nattie, 2000; Feldman *et al.*, 2003). Although pFRG neurons of newborn rats distinguish themselves from preBötC neurons by their poor responsiveness to opioids both *in vitro* and *in situ* (Takeda *et al.*, 2001; Janczewski & Feldman, 2006), the fact that neurons comprising the pFRG are still unidentified anatomically and have not been demonstrated in the adult animal (Fortuna *et al.*, 2008) remains an important limitation in our understanding of their precise role in respiratory control.

Neurons located in the pFRG have been also suggested to constitute the precursor of chemosensitive neurons of the retrotrapezoid nucleus. Nevertheless, it has been reported that in the *in situ* rat preparation some neurons located in the retrotrapezoid nucleus/ pFRG region that are silent during eupneic breathing display a rhythmic activity during hypercapnia coincident with abdominal late expiratory discharge. This suggests that critical metabolic conditions, such as hypercapnia or hypoxia, provide an additional excitatory drive to these late expiratory neurons that can switch on their bursting state (Abdala *et al.*, 2009). According to the suggestions of Abdala *et al.* (2009), it seems reasonable to hypothesize that these hypercapnia-activated expiratory neurons may excite not only the pre-motor circuits that generate the expiratory motor output, but also preBötC inspiratory neurons to promote the onset of inspiration and restore

rhythmic phrenic bursts (see Feldman & Del Negro, 2006; Abdala *et al.*, 2009 also for further references).

Respiratory network in the lamprey

The lamprey (**Fig. 2**) is a lower vertebrate which diverged from the main vertebrate line around 450 million years ago (Hotton, 1976) and has proved to be highly useful to identify the cellular mechanisms underlying neural control of rhythmic motor behaviour, such as locomotion *i.e.* the CPGs for locomotion (Grillner, 2006; Grillner & Jessell, 2009).

Fig. 2. Lamprey (*Petromyzon marinus*)

The general organization of lamprey nervous system is highly similar to that of other vertebrates, including mammals. A major benefit of this animal model is the comparative simplicity of its nervous system relative to that of mammals. Since several properties of the respiratory network are highly retained throughout evolution (see *e.g.* Mutolo *et al.*, 2007; 2010b; Kinkead, 2009), a comparative

approach could be important to derive broader biological principles and a more comprehensive view of the basic neural mechanisms responsible for respiratory rhythm generation. In fact, they are still not fully elucidated and remain highly debated (see *e.g*. Feldman & Del Negro, 2006; Smith *et al.*, 2009). The possibility of isolating and maintaining the entire central nervous system in physiological conditions *in vitro* for many hours up to a few days is a significant additional advantage of the lamprey preparation. The neural mechanisms underlying respiratory rhythmogenesis and the role of neurotransmitters and neuromodulators within the respiratory network of the lamprey have been investigated (Rovainen, 1983; 1985; Thompson, 1985; Russell, 1986; Bongianni *et al.*, 1999; 2002a; 2006; Martel *et al.*, 2007; Mutolo *et al.*, 2007; 2010b).

In lampreys the respiratory pump consists of seven gills pores located on each side of the buccal cavity (Rovainen, 1979). The gills are surrounded by "spring-like" cartilaginous baskets also covered by a layer of striated muscle fibers. These branchial muscles receive innervation from the Vth, IXth and Xth cranial nerves. Breathing is produced by synchronous contractions of the branchial muscles that force water out of the gill openings; the inhalation phase is passive and is produced by the elastic recoil of cartilaginous baskets surrounding the gill sacs (Rovainen, 1977; 1979).

The isolated brain spontaneously produces respiratory neuronal activity *in vitro*; this activity closely resembles that underlying the respiratory behaviour of intact animals and persists after transections of the brain at the obex and isthmus levels (Rovainen, 1977; 1983; Thompson, 1985; Bongianni *et al.*, 1999; 2006; Mutolo *et al.*, 2007; 2010b). Thus, both the neural network responsible for respiratory rhythm generation and respiratory motoneurons are located within the

brainstem (**Fig. 3a**). Studies performed using electrical stimulations, lesions and neuronal recordings have suggested that bilateral CPGs for respiration are located within the lateral trigeminal region (Rovainen, 1985; Thompson, 1985; Russell, 1986; Martel *et al.*, 2007). This region has been recently named the paratrigeminal respiratory group (pTRG; Mutolo *et al.*, 2007). It has been suggested that the pTRG plays a pivotal role in respiratory rhythm generation (Mutolo *et al.*, 2007; 2010b) and that its function could correspond to that of the preBötC in mammals. The vast majority of respiratory motoneurons are located in the facial, glossopharyngeal and, especially, in the vagal nuclei (Rovainen, 1974; 1977; 1979; Guimond *et al.*, 2003), while respiration-related neurons possibly responsible for respiratory rhythm generation have been encountered within the pTRG (Mutolo *et al.*, 2007; 2010b).

The majority of extracellular recordings from the pTRG neurons (Mutolo *et al.*, 2007) reveals discharges starting before the onset of the activity in vagal motoneurons and continuing after the end of vagal bursts. Other recordings show either late discharge patterns, *i.e.* neuronal firing starting at the end of vagal bursts, or in same instances, action potentials synchronous with vagal activity. The discharge patterns described above may suggest different functions for the neurons from which they were derived. However, the neuronal mechanisms responsible for respiratory rhythmogenesis have not yet been identified and any attempt to ascribe a specific role to each type of encountered respiration-related neuron would be highly speculative. Inspiratory and expiratory interneurons with different discharge patterns have also been encountered in medullary regions involved in the respiratory rhythmogenesis of mammals, such as the preBötC and adjacent regions of the ventral respiratory column (see “Respiratory network in mammals”). Although

pacemaker neurons may be crucial for respiratory rhythm generation, other types of neurons that have different discharge patterns may also play an important role not only in rhythm generation, but also in pattern formation, *i.e.* in determining the amplitude and duration of vagal bursts (see *e.g.* Bianchi *et al.*, 1995; Smith *et al.*, 2000; Mutolo *et al.*, 2002; 2005; Bongianni *et al.*, 2006). More recently, intracellular recordings, that are the only suitable to ascertain the presence of respiratory neurons in this region and to discriminate cells from by-passing fibers, have provided evidence that respiratory neurons with different discharge patterns are present within the pTRG (Mutolo *et al.*, 2010b). In fact some neurons are tonically active (**Fig. 3b**), while others display different types of respiration-related activity. The majority of intracellular recordings reveals the presence of neurons firing synchronously with the vagal activity (**Fig. 3c**). Other recordings show either late discharge patterns, *i.e.* neurons with action potentials starting at the end of vagal bursts (**Fig. 3d**), or, in some instances, early and late discharge patterns, *i.e.* neurons with action potentials firing before the onset and after the end of vagal nerve activity (**Fig. 3e**). Finally, some recordings show transient hyperpolarization of membrane potential during the vagal burst (**Fig. 3f**). Unfortunately, neurons in the pTRG are small and so far it has proved impossible to maintain recordings for periods long enough to conduct detailed electrophysiological and/or pharmacological studies.

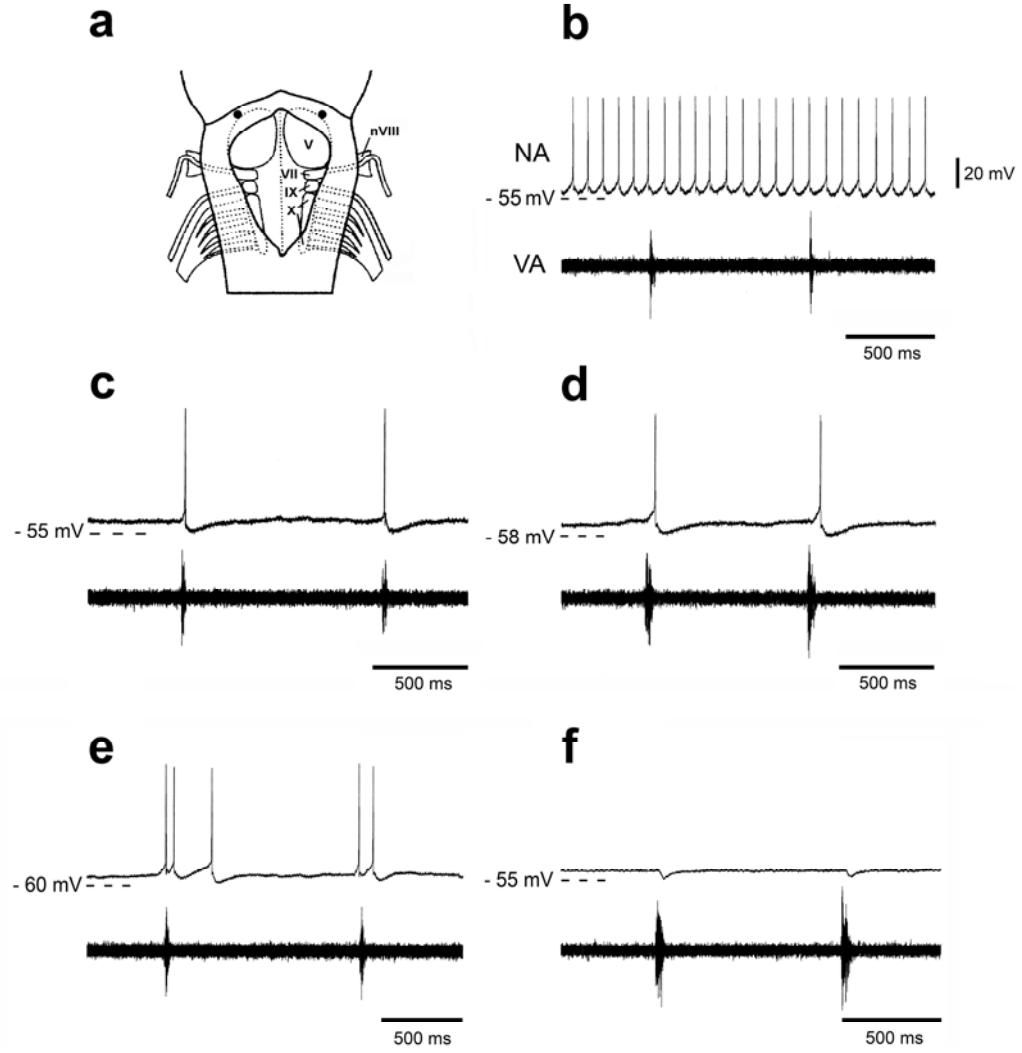

Fig. 3. Discharge patterns of respiration-related neurons encountered in the pTRG. (**a**) Diagrammatic illustration of a dorsal view of the lamprey rhombencephalon showing the small area (●) located within the rostrolateral trigeminal region and termed the pTRG where intracellular recordings of respiration-related neuronal activities were performed. V, trigeminal motor nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus; nVIII, vestibular nerve. (**b**) Recordings from a neuron tonically active. (**c**) Recordings from a neuron firing synchronously with vagal bursts. (**d**) Recordings from a neuron firing at the end of vagal bursts. (**e**) Recordings from a neuron firing before and after the onset of vagal bursts. (**f**) Recordings from a neuron which displayed hyperpolarization during the vagal bursts. Traces are neuronal activity (NA) and raw vagal nerve activity (VA). From Mutolo *et al.* (2010b).

Furthermore, it has been shown (Bongianni *et al.*, 1999) that endogenously released excitatory amino acids are involved in the control of the timing and intensity components of the breathing pattern through an action on ionotropic and metabotropic glutamate receptors (Bongianni *et al.*, 1999; 2002a).

Evidence has been provided as regards the fact that the respiratory activity is crucially dependent upon the activation of non-*N*-methyl-D-aspartate (non-NMDA) receptors, given that the blockade of these receptors completely suppresses the respiratory motor output (Bongianni *et al.*, 1999). Inhibitory synaptic mechanisms mediated by GABA and glycine are not essential for respiratory rhythm generation, although they appear to contribute to maintaining a stable and regular pattern of breathing (Rovainen, 1983; Bongianni *et al.*, 2006). In addition, recent findings indicate that pTRG neurons behave like those of the preBötC (Gray *et al.*, 1999) since microinjections into the pTRG of the μ-opioid receptor agonist DAMGO abolish the respiratory rhythm (Mutolo *et al.*, 2007), whereas those of substance P cause marked increases in respiratory frequency (Mutolo *et al.*, 2010b). Furthermore, the presence of the inspiratory burst-promoting currents described in mammals (Thoby-Brisson & Ramirez, 2001; Del Negro *et al.*, 2002a; 2005; Peña *et al.*, 2004; Feldman & Del Negro, 2006), *i.e.* the I_{CAN} and the I_{NaP}, has been ascertained also in the lamprey respiratory network. Interestingly, respiratory activity is abolished by blocking both these currents, but is restored by substance P microinjections into the pTRG (Mutolo *et al.*, 2010b), thus suggesting that the rhythmogenic role of pacemaker neurons is not obligatory not only in rodents (Feldman & Del Negro, 2006), but also in the lamprey and that rhythm generation is an emergent property of non-pacemaker neurons that are interconnected by excitatory synaptic interactions ("group-pacemaker" hypothesis).

Role of acetylcholine in the respiratory control

In vertebrates the spontaneous respiratory pattern is generated within the brainstem and involves several neural mechanisms as well as diverse neurotransmitter and neuromodulator regulation (Von Euler, 1986; Bianchi *et al.*, 1995; Haji *et al.*, 2000; Feldman *et al.*, 2003; Doi & Ramirez, 2008). Acetylcholine (ACh) has long been known to play an important role in the neural control of breathing (Dikshit, 1934; Gesell *et al.*, 1943; Metz, 1962; Weinstock *et al.*, 1981; Murakoshi *et al.*, 1985; Gillis *et al.*, 1988; Nattie & Li, 1990; Burton *et al.*, 1994; 1995; 1997; Haji *et al.*, 1996; Bellingham & Funk, 2000; Boudinot *et al.*, 2008), including central chemosensitivity (Dev & Loeschcke, 1979; Fukuda & Loeschcke, 1979; Haxhiu *et al.*, 1984; Nattie *et al.*, 1989; Monteau *et al.*, 1990; Burton *et al.*, 1997; Boudinot *et al.*, 2004). Impairments in central cholinergic mechanisms have been implicated in the pathophysiology of some important neurological disorders affecting the respiratory control, such as sudden infant death syndrome and sleep apnea (Kinney *et al.*, 1995; Bellingham & Funk, 2000; Benarroch *et al.*, 2001; Lydic *et al.*, 2002; Gilman *et al.*, 2003; Duncan *et al.*, 2008).

Alterations of ACh synthesis, release, degradation, or activation of ACh receptors in the medulla result in perturbations of the respiratory pattern (Shao & Feldman, 2009). The most consistent central effect of ACh on respiratory activity has been reported to be excitatory both *in vivo* (Gesell *et al.*, 1943; Weinstock *et al.*, 1981; Haxhiu *et al.*, 1984; Gillis *et al.*, 1988; Nattie *et al.*, 1989; Nattie & Li, 1990; Burton *et al.*, 1997) and *in vitro* (Murakoshi *et al.*, 1985; Monteau *et al.*, 1990; Burton *et al.*, 1994; 1995; Shao & Feldman, 2000; 2001; 2005; Hatori *et al.*, 2006). However, it is controversial whether activation of ACh receptors located on neurons

of the medullary respiratory network is excitatory or inhibitory (Salmoiraghi & Steiner, 1963; Jordan & Spyer, 1981; Bradley & Lucy, 1983; Bohmer *et al.*, 1987; 1989) probably due to variability or lack of precise information concerning the location of recording sites and the states of anesthesia (Foutz *et al.*, 1987).

Recently, experiments performed on *in vitro* medullary slices from neonatal rodents have shown that ACh can modulate respiratory activity by acting on both nicotinic and muscarinic receptors (nAChRs and mAChRs, respectively) within the preBötC (Smith *et al.*, 1991; Feldman & Del Negro, 2006; Shao & Feldman, 2009). Activation of nAChRs in the preBötC affects the excitability of preBötC inspiratory neurons and produces an increase in respiratory frequency (Shao & Feldman, 2001). It is well known that nAChRs are ligand-gated ion channels formed as a pentameric assemblies of subunits (Albuquerque *et al.*, 2009). Interestingly, in the ventrolateral medulla the nAChRs subunits α4, α7 and β2 are present and native functional nAChRs other than α4, α7 and β2 are found in a limited number of brain regions (Albuquerque *et al.*, 2009; cfr. Shao & Feldman, 2002 for further references). The predominant subtype of nAChRs involved in this respiratory effect is an α4β2 subunit combination (Shao & Feldman, 2002; Shao *et al.*, 2008). On the other hand, also activation of the mAChRs in the preBötC has been reported to induce increases in respiratory frequency (Shao & Feldman, 2000). The involvement of ACh and related receptors within the preBötC in respiratory regulation has been confirmed in experiments in which the acetylcholinesterase inhibitor physostigmine was employed (Shao & Feldman, 2005). Evidence has also been provided that nicotine modulates glutamatergic transmission at the level of preBötC inspiratory neurons in rat medullary slices (Shao & Feldman, 2001), in agreement with the general notion that nAChRs can presynaptically affect both glutamatergic and GABAergic

neurotransmission (Fregosi & Pilarski, 2008; Albuquerque *et al.*, 2009). Interestingly, despite the large body of literature dealing with the ACh role in the control of breathing, experiments performed by application of AChR antagonists have not yet ascertained whether the release of endogenous ACh is needed to generate baseline respiratory activity (Murakoshi *et al.*, 1985; Monteau *et al.*, 1990; Burton *et al.*, 1994; 1995; Shao & Feldman, 2000; 2001; 2002; 2005; Hatori *et al.*, 2006).

Nicotinic receptors have attracted wide research interests not only for their role in cholinergic regulation of respiratory pattern, but also for their mediation of the effects of nicotine from tobacco smoke, a matter of considerable significance for public health. In addition, recent studies have disclosed the effects of perinatal exposure to nicotine on neonatal control of breathing (Fregosi & Pilarski, 2008). The involvement of ACh in the control of breathing has also been shown in acetylcholinesterase knockout mice (Boudinot *et al.*, 2004) and in particular the role of α4β2 nAChRs has been confirmed in knockin animals (Shao *et al.*, 2008).

No information is available on the role played by ACh in the regulation of respiratory activity in the lamprey respiratory network and, especially, at the level of the pTRG (Mutolo *et al.*, 2007; 2010b).

The presence of neurons with pacemaker properties within the respiratory rhythm-generating network is an important and controversial issue. As a rule, the bursting-pacemaker activity of preBötC neurons has been ascertained and analyzed in the absence of fast synaptic transmission due to blockade of glutamate, $GABA_A$ and glycine receptors (Del Negro *et al.*, 2002b; 2005; Tryba *et al.*, 2003; Peña *et al.*, 2004). Unexpectedly, preliminary experiments in the *in vitro* brainstem preparation of the lamprey in an attempt to ascertain the presence of neurons with pacemaker properties showed that respiratory rhythmic activity, although at reduced frequency,

persisted under these conditions. Thus, this intriguing issue was further investigated in the present study. Conceivably, other neurotransmitters, with an excitatory function on respiratory activity, including ACh, (Bianchi *et al.*, 1995; Haji *et al.*, 2000; Doi & Ramirez, 2008) could be involved in maintaining the respiratory rhythm.

Aims of the present research

The present study was carried out on *in vitro* brainstem preparations of adult lampreys to investigate whether ACh affects respiratory activity possibly through an action on pTRG neurons and to identify the ACh receptors involved. Since one of the main outcome of this study was that endogenous ACh exerts excitatory influences on respiratory activity by acting on α7 nAChRs located within the pTRG, an attempt was also made to test the hypothesis that ACh as well as other neurotransmitters maintain the respiratory rhythm during blockade of glutamate, $GABA_A$ and glycine receptors, thus possibly providing further insights into the basic mechanisms generating the respiratory rhythm.

EXPERIMENTAL PROCEDURES

Lamprey brainstem preparation

Experiments were carried out on 85 young adult lampreys (*Petromyzon marinus*). All animal care and experimental procedures were conducted in accordance with the Italian legislation and the official regulations of the European Communities Council on the use of laboratory animals (directive 86/609/EEC). The study was approved by the Animal Care and Use Committee of the University of Florence. All efforts were made to minimize the number of animals used. Animal preparation and experimental procedures were similar to those described in previous reports (Bongianni *et al.*, 1999; 2002a; 2006; Mutolo *et al.*, 2007; 2010b). The animals were anesthetized with tricaine methanesulphonate (100 mg l^{-1}; MS 222, Sigma-Aldrich, St. Louis, MO, USA) and transected below the gills. Muscles and connective tissues were removed and the isolated brain-spinal cord was mounted dorsal side up in a Sylgard-lined recording chamber continuously perfused with a cold solution using a peristaltic pump. The chamber volume was 3.0 ml and the perfusion rate was set at 2.5 ml min^{-1}. Bath temperature was maintained at 9-10 °C. The solution (control solution) flowed from a reservoir and had the following composition (in mM): 91 NaCl, 2.1 KCl, 2.6, $CaCl_2$, 1.8 $MgCl_2$, 4 glucose, 23 $NaHCO_3$. The solution was continuously bubbled with 95% O_2-5% CO_2 to oxygenate and maintain the pH in the bath at 7.4. The brain was exposed dorsally and the choroid plexus removed; the brain tissue rostral to the optic tectum was cut and removed. Caudally, a transection was made below the obex, maintaining a minimum length of spinal cord to hold the preparation. Recordings and microinjections within the pTRG as well as neighboring sites were performed

under microscope control (Stemi 2000, Zeiss, Göttingen, Germany). These maneuvers were facilitated by cutting the roof of the isthmic region along the midline, spreading the alar plates laterally and pinning them down. In agreement with previous reports (Thompson, 1985; Russell, 1986; Mutolo *et al.*, 2007; 2010b), these procedures had no significant effect on respiratory activity.

Recording procedures

Efferent respiratory activity was recorded bilaterally from the vagal nerves by means of suction electrodes. The signals were amplified, full-wave rectified and integrated (low-pass filter, time constant 10 ms). In control trials, brainstem preparations spontaneously produced a stable and regular fictive respiratory rhythm for at least 12 h (Rovainen, 1985; Thompson, 1985; Russell, 1986; Bongianni *et al.*, 1999; 2002a; 2006; Mutolo *et al.*, 2007; 2010b). In the present experiments, under control conditions respiratory frequency ranged from 54 to 72 cycles min^{-1}, while the duration of vagal bursts ranged from 21 to 36 ms. Extracellular recordings of neuronal activity were made with fine (shaft diameter 0.1 mm) tungsten microelectrodes (5 MΩ impedance at 1 kHz). The obex was used as a standard point of anatomical reference to evaluate coordinates of recording and microinjection sites. Neuronal activity was recorded from vagal motoneurons (0.4-0.6 mm rostral to the obex, 0.3-0.4 mm lateral to the midline and 0.15-0.25 mm below the dorsal surface of the rhombencephalon) as well as from respiration-related neurons of the pTRG (1.8-2.0 mm rostral to the obex, 0.8-1.0 mm lateral to the midline and 0.25-0.3 mm below the dorsal surface of the rhombencephalon). For data acquisition and analysis, a personal computer equipped with an analog-to-digital interface (sampling rate 50 kHz; Digidata 1200,

Axon Instruments, Union City, CA, USA) and appropriate software (Axoscope, Axon Instruments) was used. Off-line analysis was performed using Clampfit software (Axon Instruments).

Drug application

Drugs were either applied through the perfusing solution or microinjected at different sites of the rostrolateral trigeminal region as well as into the region of vagal motoneurons. In each experiment, the preparation was perfused with the control solution for at least 60 min before control recordings. Unless otherwise stated, only one drug was tested in each preparation.

The following drugs were dissolved in the control solution and applied to the bath: 100 μM physostigmine (an acetylcholinesterase inhibitor, Sigma-Aldrich), 10-50 μM pilocarpine hydrochloride (a mAChR agonist, Tocris Bioscience, Bristol, UK), 10 μM atropine sulfate salt (a non-selective mAChR antagonist, Sigma-Aldrich), 1 μM nicotine hydrogen tartrate salt (a nAChR agonist, Sigma-Aldrich), 100 μM D-tubocurarine chloride hydrate (a non-selective nAChR antagonist, Sigma-Aldrich), 0.25 μM α-bungarotoxin (an α7 selective nAChR antagonist, Sigma-Aldrich), 10 μM dihydro-β-erythroidine hydrobromide (an α4β2 selective nAChR antagonist, Sigma-Aldrich), 10 μM hexamethonium chloride (a preferentially blocker of nAChRs at autonomic ganglia, Sigma-Aldrich), 100 μM pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS) and 100 μM suramin sodium salt (broad spectrum antagonists at P2X and P2Y purinergic receptors, Sigma-Aldrich), 500 μM (RS)-α-Methyl-4-carboxyphenylglycine disodium salt (MCPG, a non-selective group I/group II metabotropic glutamate receptor antagonist, Tocris), 10 μM

methysergide maleate (a non-selective 5HT receptor antagonist, Tocris), 10 μM CP-99,994 (a NK1 receptor antagonist that was a gift from Pfizer Inc. Groton, CT, USA), 10 μM MEN 10376 (a NK2 receptor antagonist, Tocris), 10 μM SB 222200 (a NK3 receptor antagonist, Tocris). All these drugs, except for pilocarpine, were dissolved in distilled water made up to a stock solution and stored as small aliquots in a freezer until use. Pilocarpine was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to produce stock solutions. Stock solutions were diluted to final desired concentration in control solution immediately prior to application. Control experiments with equivalent amounts of DMSO in the perfusing solution were also scheduled.

The drug concentrations and the application time were similar to those employed in previous studies (Bongianni *et al.*, 2002a; Shao & Feldman, 2002; Le Ray *et al.*, 2003; 2004; Lorier *et al.*, 2007; St John & Leiter, 2008; Shao *et al.*, 2008; Mutolo *et al.*, 2010b). The brainstem was perfused with each drug for 30 min. After completion of each trial, the preparation was allowed to recover by perfusing it with the control solution. To avoid possible confounding effects of desensitization (Albuquerque *et al.*, 2009), only one concentration of physostigmine and nicotine was used in each preparation. In some experiments, we also ascertained the effectiveness of receptor blockade induced by 100 μM D-tubocurarine or 0.25 μM α-bungarotoxin. First, the antagonist was applied alone for 30 min and subsequently 1 μM nicotine was added for 20 min.

Bilateral microinjections (0.5-1 nl) of physostigmine (1 mM), nicotine (1 mM), D-tubocurarine (1 mM) and α-bungarotoxin (2.5 μM) were performed by means of glass micropipettes (tip diameter 10-15 μm) and by applying pressure pulses of 50-100 ms with a Picospritzer (General Valve Corporation, Fairfield, NJ,

USA) connected to the injection pipette. The injected volume was estimated by measuring the diameter of a droplet ejected from the tip of the pipette. Bilateral microinjections were performed using a single micropipette that was withdrawn after the first microinjection and then introduced contralaterally for the second injection. The interval between the two microinjections was ≤ 20 s. Bilateral microinjections were performed into the pTRG and the region of vagal motoneurons on the basis of both extracellular recordings and coordinates. The concentrations of microinjected drugs were selected in preliminary trials and were in the same range as those employed in previous microinjection studies (e.g. Aberger *et al.*, 2001; Le Ray *et al.*, 2003). For further considerations on the concentrations of neuroactive drugs see Mutolo *et al.* (2007). The inactive dye Fast Green (0.2 %, Sigma-Aldrich) was added to the drug solution to visually assess the spread of the injection. The dye spots had as a rule a diameter of about 0.2 mm (see also Mutolo *et al.*, 2007). Control microinjections of the perfusing solution with Fast Green were also made.

In 27 experiments, a cocktail of antagonists for NMDA receptors (100 μM D-(–)-2-amino-5-phosphonopentanoic acid, D-AP5, Tocris), non-NMDA receptors (20 μM 6-cyano-7-nitroquinoxaline-2,3-dione, CNQX, Tocris), $GABA_A$ receptors (10 μM bicuculline methiodide, Sigma-Aldrich) and glycine receptors (10 μM strychnine, Tocris) was applied through the perfusing solution. In 3 of these experiments D,L-homocysteic acid (DLH), a broad-spectrum excitatory amino acid agonist (Sigma-Aldrich), was added to the bath to test the effectiveness of glutamate receptor blockade. In 2 experiments, CNQX and D-AP5 were applied first to abolish the respiratory rhythm (Bongianni *et al.*, 1999) and the other components of the cocktail solution were added subsequently. In 2 experiments the cocktail

solution contained higher concentrations of CNQX up to 100 μM. The drug concentrations and the application time were similar to those employed in previous studies in lampreys (Bongianni *et al.*, 1999; 2006) or mammals (Del Negro *et al.*, 2002b; 2005; Tryba *et al.*, 2003; Peña *et al.*, 2004). In each experiment, the preparation was perfused with the cocktail solution for 60 min and thereafter was allowed to recover by perfusing it with the control solution. After blockade of glutamate, $GABA_A$ and glycine receptors, some drugs were either added to the cocktail perfusing solution or microinjected into the pTRG. After completion of each trial, the preparation was allowed to recover by perfusing it with the cocktail solution.

Histology

The fixed brains (4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 overnight) were cryoprotected with 30 % sucrose, frozen, and cut at 25-μm thickness on a cryostat. Coronal sections stained with cresyl violet were used for the histological analysis.

Data analysis

Respiratory frequency (cycles min^{-1}), vagal burst duration (ms, measured from raw activity), and peak amplitude of integrated vagal activity (taken as a reliable index of the intensity of vagal bursts, arbitrary units) were measured and averaged for 20 s both in the period immediately preceding each trial (control values) and at 2-min intervals during drug application. Average values of respiratory variables observed in control conditions and at the time when the maximum response occurred were considered for statistical analysis (Sigma Stat, Jandel Scientific Software, San

Rafael, CA, USA). Paired *t*-tests were employed to evaluate changes in respiratory variables induced by each drug added to the bath. The same statistical analysis was used to evaluate changes in respiratory variables induced by each agonist in the presence of the specific antagonist and to assess the effects of drug microinjections. In microinjection trials, respiratory variables were measured and averaged for 20 s immediately before the microinjections, as well as at the time when the maximum response occurred. Changes in respiratory variables were also expressed as percentage variations of control values. The number of preparations employed in each set of drug challenges is indicated by *n*. The number of trials reported on some occasions implies that some of them were executed in a single preparation. All values are presented as means ± s.e.m. $P < 0.05$ was considered as significant.

RESULTS

Respiratory role of ACh

A total number of 6 preparations was employed to investigate the respiratory responses to physostigmine. Bath application of 100 μM physostigmine induced marked increases in respiratory frequency from 65.1 ± 4.7 to 89.3 ± 6.5 cycles min^{-1} (37.2 ± 2.2 %; n = 6; P < 0.01) without significant changes in peak amplitude and duration of vagal bursts (**Fig. 4a**). Increases in respiratory frequency started within ~ 5 min after bath application, developed progressively and reached a maximum within ~ 15 min. Respiratory activity recovered within 60 min washout. In the same preparations, we performed bilateral microinjections into the pTRG or the vagal motoneuron region on the basis of both extracellular recordings and coordinates. Bilateral microinjections of physostigmine (1 mM; 0.5-1 pmol) into the pTRG increased respiratory frequency from 61.1 ± 2.5 to 82.8 ± 3.4 cycles min^{-1} (36.1 ± 7.3 %; n = 4; P < 0.01) without significant changes in peak amplitude and duration of vagal bursts (**Fig. 4b**). The respiratory responses started within 1 min after the injections and reached a maximum within ~ 5 min. Respiratory activity recovered gradually within 25 min after the injections. Bilateral microinjections of physostigmine (1 mM; 0.5-1 pmol) into the region of vagal motoneurons (n = 3) did not cause any obvious effect (**Fig. 4c**).

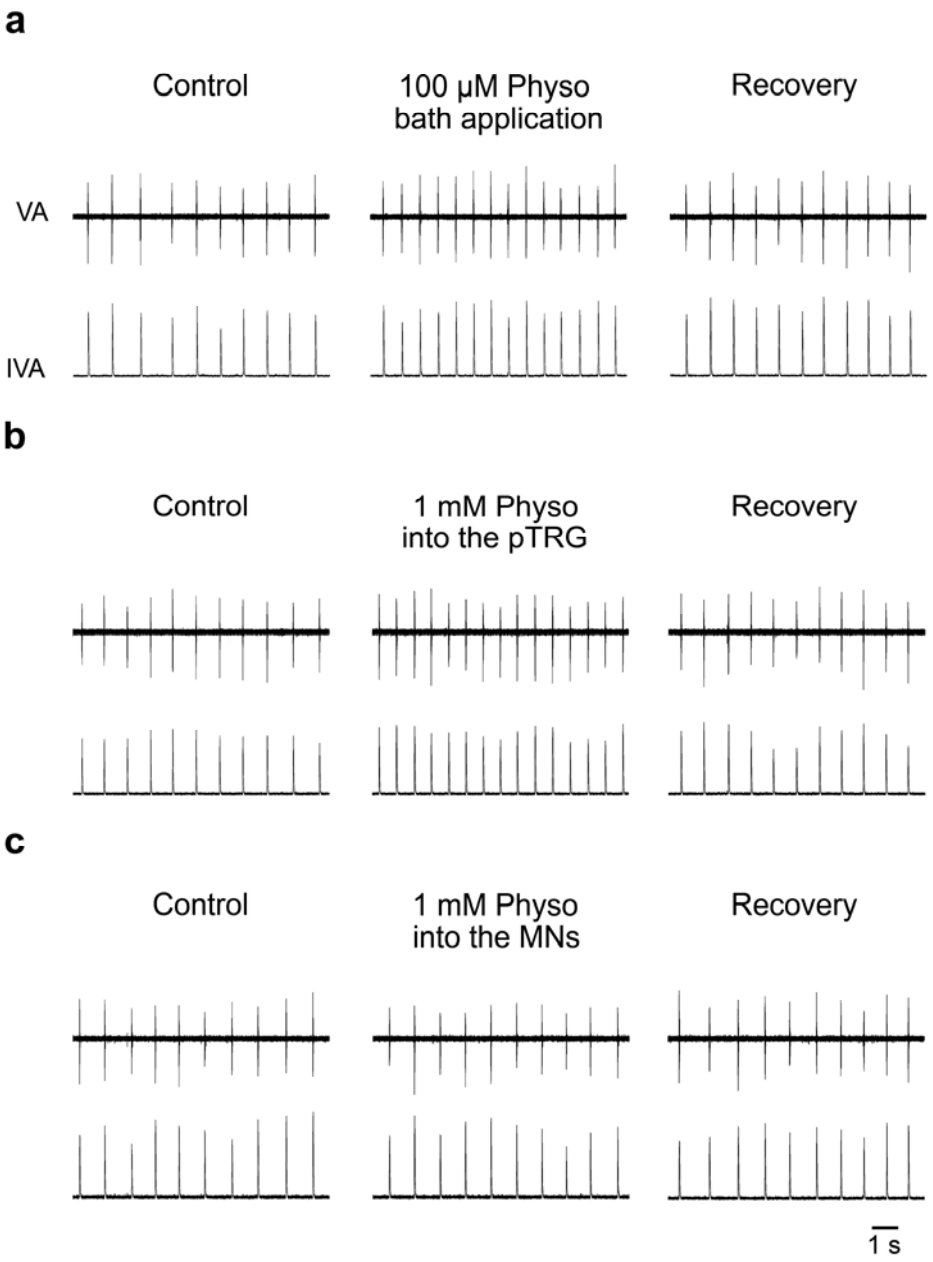

Fig. 4. **Respiratory responses caused by the acetylcholinesterase inhibitor physostigmine (Physo).** Increases in respiratory frequency ~ 15 min after bath application of 100 µM physostigmine (**a**) and ~ 5 min after bilateral microinjections of 1 mM physostigmine into the pTRG (**b**). (**c**) No changes in respiratory activity ~ 5 min following bilateral microinjections of 1 mM physostigmine into the vagal motoneurons (MNs). VA, raw vagal nerve activity; IVA, integrated vagal nerve activity.

The localization of injections sites is illustrated in **Fig. 5**. Since the injectate contained 0.2 % Fast Green dye, the microinjections were specifically associated with the appearance of spots of dye confined within these respiration-related regions. Equivalent volumes of the perfusing solution containing Fast Green dye microinjected at the same locations (3 trials) as well as bilateral microinjections of 1 mM physostigmine at neighbouring sites 0.4 mm or more away from the responsive region (4 trials) did not cause any respiratory response.

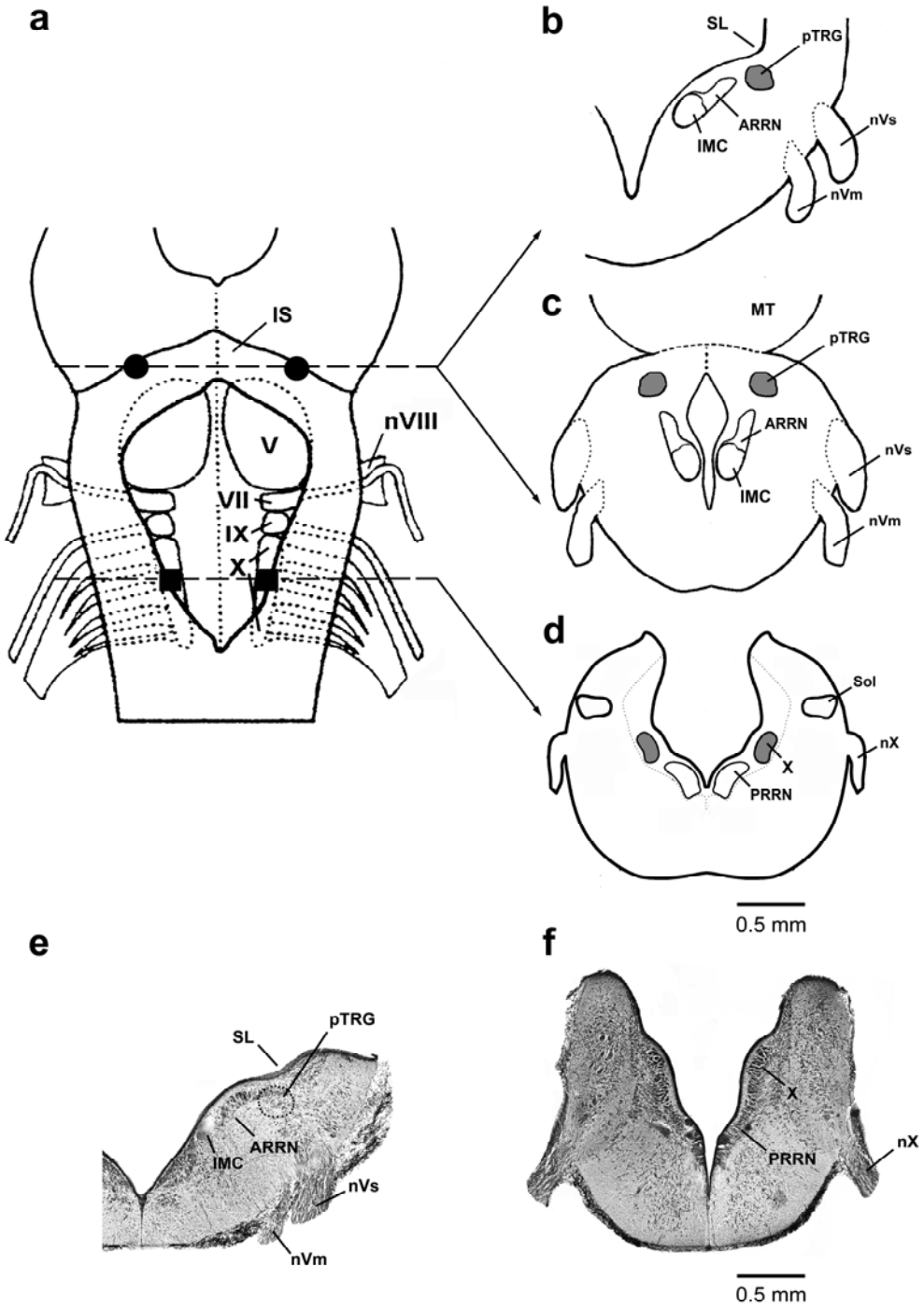

Fig. 5. **Localization of recording and injection sites.** (**a**) Diagrammatic illustration of a dorsal view of the lamprey rhombencephalon showing the area located within the rostrolateral trigeminal region termed the pTRG (●) and the vagal motoneuron region (■) where respiration-related neuronal activity was recorded and microinjections were performed. IS, isthmus; V, trigeminal motor nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus; nVIII, vestibular nerve. (**b, c**) Schematic drawings of transverse

sections of the rostral rhombencephalon at the same level as indicated in **a** (dashed line) showing the location of the pTRG (shaded areas). The outlines of the map reported in **b** derive from a selected section of one histological preparation (camera lucida redrawing) of the lamprey brainstem. In this case, unlike the transverse section in **c**, the roof of the isthmic region was cut along the midline, the alar plates were spread laterally and pinned down to perform recordings and microinjections within the pTRG (see Methods). Some relevant structures are indicated: ARRN, anterior rhombencephalic reticular nucleus; IMC, isthmic Müller cell; MT, mesencephalic tectum; nVm, motor root of the trigeminal nerve; nVs, sensory root of the trigeminal nerve; SL, sulcus limitans of His. (**d**) Diagram of a transverse section of the caudal rhombencephalon at the level indicated in **a** (dashed line) showing the location of the vagal motor nucleus (shaded areas). nX, vagal nerve; PRRN, posterior rhombencephalic reticular nucleus; Sol, nucleus of the solitary tract. The schematic drawings in **c** and **d** are derived and modified from Auclair *et al*. (2004). (**e, f**). Photomicrographs of transverse sections already schematically illustrated in **b** and **d** showing the location of the pTRG region (outlined by the dotted line) and the vagal motor nucleus, respectively.

To identify the receptors involved in ACh-mediated effects, specific agonists and antagonists acting on nAChRs and mAChRs were employed. Bath application of 1 μM nicotine (n = 5) increased respiratory frequency from 65.7 ± 4.2 to 91.1 ± 3.4 cycles min^{-1} (39.7 ± 5.2 %; $P < 0.001$). Peak amplitude and duration of vagal bursts did not vary (**Fig. 6**). These responses started within 2 min after bath application, developed progressively and reached a maximum within ~ 10 min. Respiratory activity recovered within 60 min washout. In 4 preparations, bath application of pilocarpine at 10 μM, or even at 50 μM in 2 cases, did not alter respiratory activity (**Fig. 6**). Bath application of 10 μM atropine (n = 4) did not affect the respiratory motor output (not shown).

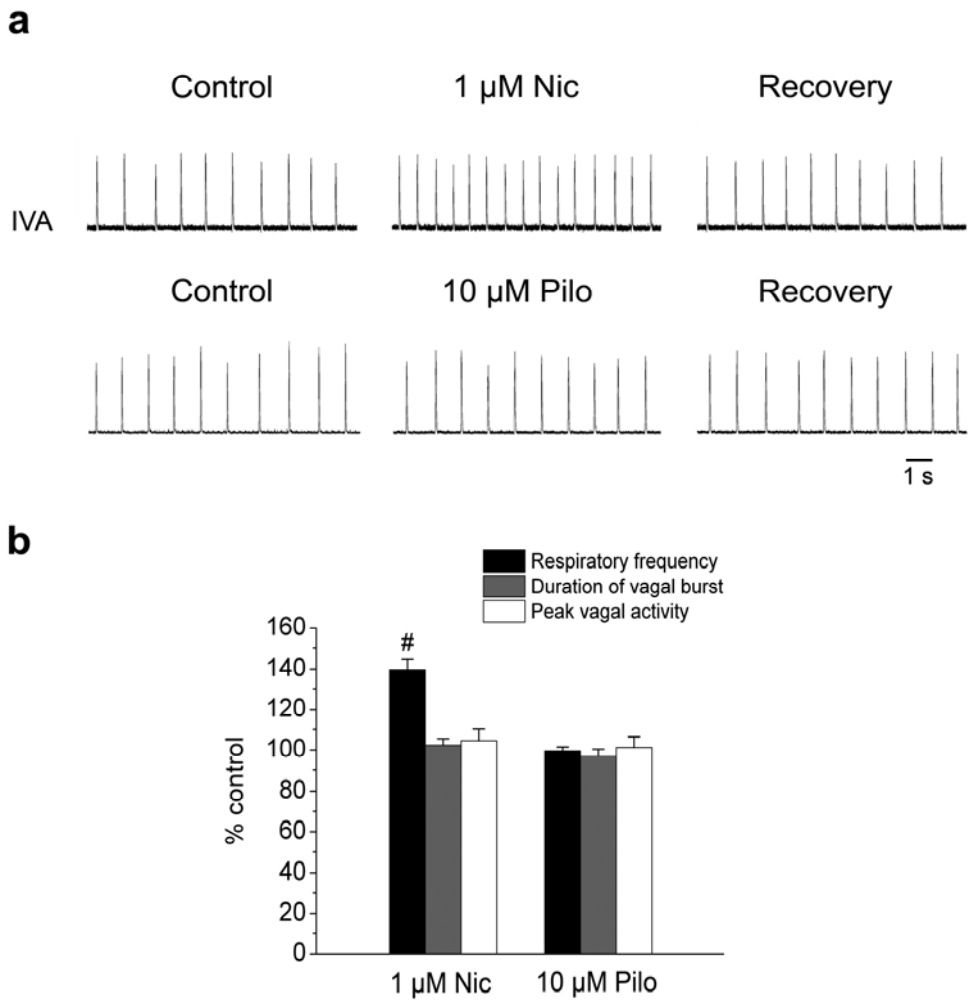

Fig. 6. **Respiratory responses to nicotinic and muscarinic receptor agonists.** (**a**) Increases in respiratory frequency ~10 min after bath application of 1 μM nicotine (Nic). No changes in respiratory activity ~10 min after bath application of 10 μM pilocarpine (Pilo). IVA, integrated vagal nerve activity. (**b**) Histograms illustrating maximum changes in respiratory frequency, vagal burst duration and peak vagal activity induced by bath application of 1 μM nicotine (n = 5) or 10 μM pilocarpine (n = 4).Values are means ± s.e.m. $^{\#}P < 0.001$.

To test whether nAChRs are involved in the control of baseline respiratory activity, we investigated the effects of various nAChR antagonists. Bath application of 100 μM D-tubocurarine (n = 6) caused reductions in respiratory frequency from 63.5 ± 3.6 to 47.8 ± 3.3 cycles min^{-1} (25.8 ± 6.0 %; $P < 0.05$) and increases in the duration of vagal bursts from 25.6 ± 1.3 to 38.0 ± 3.5 ms (49.1 ±15.0 %; $P < 0.05$) without significant changes in their peak amplitude (**Fig. 7a**). Bath application of 0.25 μM α-bungarotoxin (n = 6) provoked decreases in respiratory frequency (from 63.2 ± 2.4 to 47.5 ± 4.2 cycles min^{-1}; 25.2 ± 5.0 %; $P < 0.05$) and increases in the duration of vagal bursts (from 27.9 ± 1.7 to 38.8 ± 3.5 ms; 39.8 ± 10.6 %; $P < 0.05$) without variations in their peak amplitude. In some of these preparations, the presence of 100 μM D-tubocurarine (n = 2) or 0.25 μM α-bungarotoxin (n = 2) in the bath proved to prevent the excitatory effects on respiration induced by the application of 1 μM nicotine (not shown). On the contrary, no respiratory effects were observed following bath application of 10 μM dihydro-β-erythroidine (n = 4) and 10 μM hexamethonium (n = 3; not shown). Histograms in **Fig. 7b** show changes in respiratory frequency, duration and peak amplitude of vagal bursts induced by D-tubocurarine and by other nAChR antagonists acting on different subtypes of nAChRs. The respiratory responses started within ~ 5 min after bath application of D-tubocurarine or α-bungarotoxin, developed gradually and reached a maximum within ~ 15 min. Respiratory activity recovered within 60 min washout.

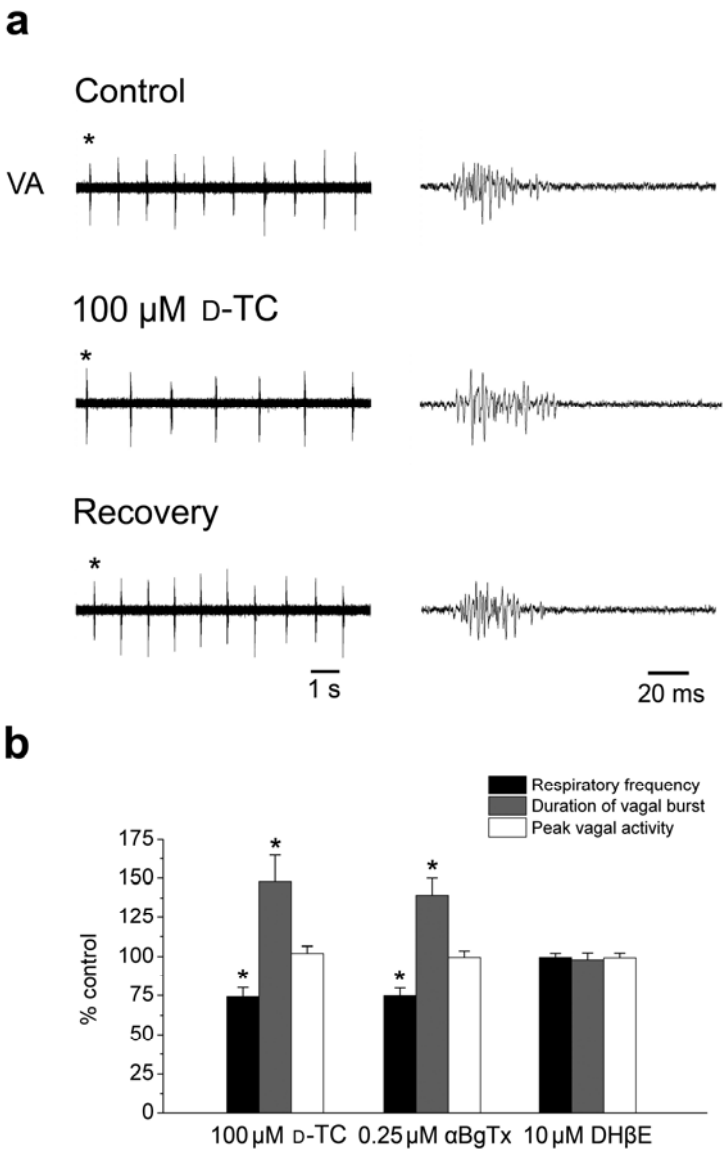

Fig. 7. Respiratory effects induced by nicotinic receptor antagonists. (**a**) Decreases in respiratory frequency ~15 min after bath application of 100 μM D-tubocurarine (D-TC). Traces are raw vagal nerve activity (VA). The first vagal bursts (marked with an asterisk) are shown with an expanded time scale on the right of each trace to illustrate changes in their duration. (**b**) Histograms showing maximum changes in respiratory frequency, vagal burst duration and peak vagal activity induced by 100 μM D-TC (n = 6), 0.25 μM α-bungarotoxin (αBgTx, n = 6) or 10 μM dihydro-β-erythroidine (DHβE, n = 4). Values are means ± s.e.m. *P < 0.05.

Nicotinic receptors within the pTRG

The respiratory responses induced by bilateral microinjections of nicotine, D-tubocurarine and α-bungarotoxin into the pTRG are illustrated in **Fig. 8**. Microinjections of 1 mM nicotine (0.5-1 pmol; $n = 6$) caused significant increases in respiratory frequency from 64.2 ± 3.4 to 88.5 ± 5.6 cycles min^{-1} (37.3 ± 2.6 %; $P < 0.001$) without significant changes in the duration and amplitude of vagal bursts. Microinjections of 1 mM D-tubocurarine (0.5-1 pmol; n = 6) caused decreases in respiratory frequency from 67.1 ± 3.4 to 49.7 ± 4.5 cycles min^{-1} (25.7 ± 5.8 %; $P < 0.01$) and increases in vagal burst duration from 26.2 ± 1.4 to 35.7 ± 2.0 ms (36.7 ± 5.6 %; $P < 0.01$) without changes in peak vagal activity. Similarly, microinjections of 2.5 μM α-bungarotoxin (1.25-2.5 fmol; $n = 6$) decreased respiratory frequency (from 62.5 ± 3.1 to 48.6 ± 3.4 cycles min^{-1}; 22.3 ± 5.0 %; $P < 0.01$) and increased the duration of vagal bursts (from 28.9 ± 1.7 to 35.0 ± 2.3 ms; 22.9 ± 2.5 %; $P < 0.01$) without changes in their peak amplitude. All these respiratory responses started within 1 min and reached a maximum within ~ 5 min. Respiratory activity recovered gradually within 60 min after the injections. Bilateral microinjections at neighbouring sites 0.4 mm or more away from the responsive region (nicotine, 4 trials; D-tubocurarine, 6 trials; α-bungarotoxin, 4 trials) did not cause any respiratory change. Bilateral microinjections of nicotine ($n = 2$), D-tubocurarine ($n = 3$) and α-bungarotoxin ($n = 2$) performed in the same preparations into the region where vagal motoneurons are located did not alter respiratory activity (not shown).

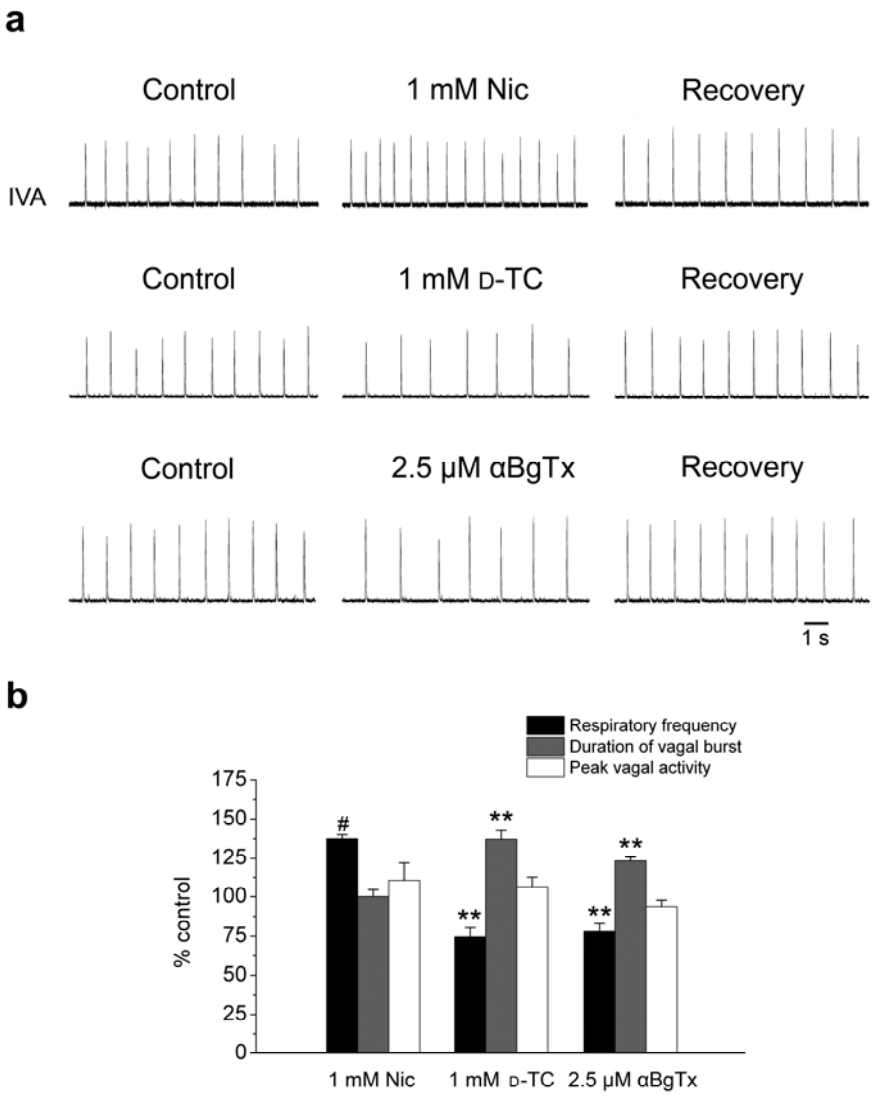

Fig. 8. Respiratory role of nicotinic receptors within the pTRG. (**a**) Respiratory responses to bilateral microinjections of 1 mM nicotine (Nic), 1 mM D-tubocurarine (D-TC) or 2.5 μM α-bungarotoxin (αBgTx) into the pTRG. Changes in integrated vagal nerve activity (IVA) ~5 min after the injections. (**b**) Histograms showing maximum changes in respiratory frequency, vagal burst duration and peak vagal activity induced by 1 mM Nic (n = 6), 1 mM D-TC (n = 6) or 2.5 μM αBgTx (n = 6) microinjected into the pTRG. Values are means ± s.e.m. $^{**}P < 0.01$; $^{\#}P < 0.001$.

Blockade of ionotropic glutamate, GABA and glycine receptors

A cocktail of antagonists for NMDA (100 μM D-AP5), non-NMDA (20 μM CNQX), $GABA_A$ (10 μM bicuculline) and glycine (10 μM strychnine) receptors was applied through the perfusing solution (Del Negro *et al.*, 2002b; 2005; Tryba *et al.*, 2003; Peña *et al.*, 2004) Respiratory activity was not abolished. The analysis of cumulative data from preparations in which blockade of these receptors was performed (n = 27) showed that respiratory frequency decreased from 66.1 ± 2.1 to 36.1 ± 1.3 cycles min^{-1} (44.3 ± 2.6 %; $P < 0.001$), the duration of vagal bursts increased from 28.6 ± 0.5 to 59.9 ± 2.3 ms (109.5 ± 6.3 %; $P < 0.001$), without concomitant changes in their peak amplitude (**Fig. 9**). The respiratory responses developed progressively and reached a maximum within ~ 30 min. Respiratory activity recovered within 60 min washout. In 3 of these experiments, 50 μM DLH added to the bath 30 min after the application of the cocktail solution did not affect respiratory activity. In 2 experiments, 20 μM CNQX and 100 μM D-AP5 were applied first to abolish the respiratory rhythm (Bongianni *et al.*, 1999), a result that was achieved within ~ 10 min. Thereafter, the other components of the cocktail were added to the bath. Respiration was restored and displayed within ~ 10 min characteristics similar to those described above following cocktail application. In addition, bath application of cocktail solutions containing higher concentrations of CNQX (up to 100 μM) provided similar results (n =2).

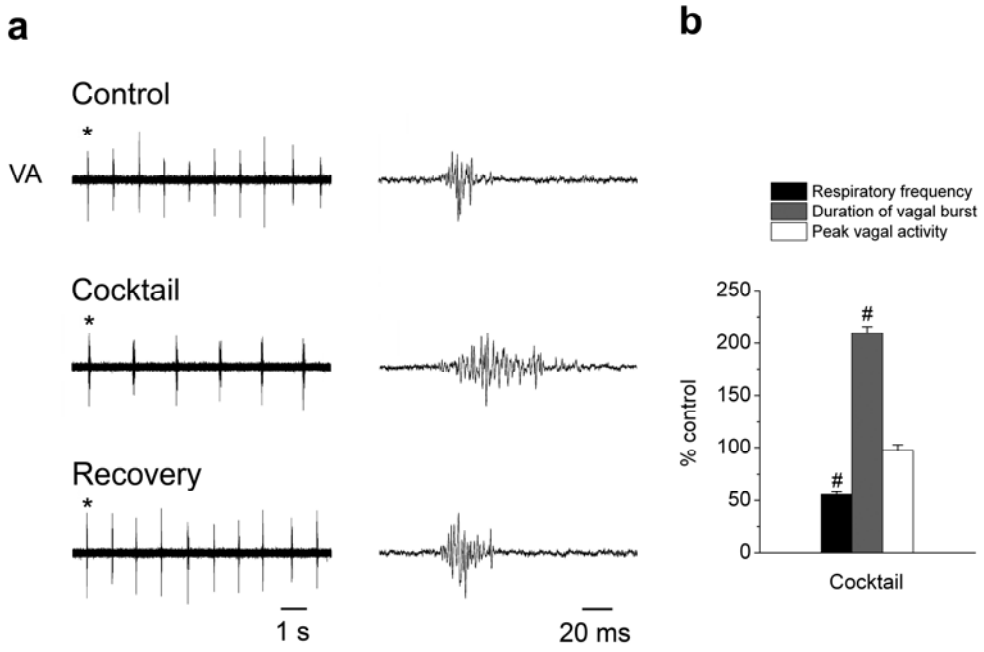

Fig. 9. Persistence of respiratory activity after blockade of glutamate, $GABA_A$ and glycine receptors (cocktail). (**a**) Respiratory rhythm characterized by reduced frequency and increased duration of raw vagal nerve activity (VA) ~ 30 min following the application of a cocktail of antagonists for NMDA (100 μM D-AP5), non-NMDA (20 μM CNQX), $GABA_A$ (10 μM bicuculline) and glycine (10 μM strychnine) receptors. The first vagal bursts (marked with an asterisk) are shown with an expanded time scale on the right of each trace to illustrate changes in their duration. (**b**) Histograms showing maximum changes in respiratory frequency, vagal burst duration and peak vagal activity induced by bath application of the cocktail solution ($n = 27$). Values are means ± s.e.m. $^{\#}P < 0.001$.

Experiments were carried out to verify whether respiratory activity could be affected during cocktail perfusion (>30 min) by the application of receptor antagonists of different neurotransmitters. Bath application of 10 μM methysergide (*n* = 4), 500 μM MCPG (*n* = 3) and 100 μM suramin or 100 μM PPADS (*n* = 3) did not change the respiratory motor output (**Fig. 10a**). In the same preparations, after appropriate (> 60 min) washout with the cocktail solution, bath application of 10 μM CP-99,994 (*n* = 4), 10 μM MEN 10376 (*n* =3) and 10 μM SB 222200 (*n* = 3) did not cause any respiratory effects (not shown). On the contrary, in 7 additional preparations bath application of 100 μM D-tubocurarine (*n* = 5) or 0.25 μM α-bungarotoxin (*n* = 2) abolished respiratory activity within ~ 10 min (**Fig. 10b**). Respiratory activity recovered within 60 min washout with the cocktail solution. After recovery, in 3 out of these preparations, bilateral microinjections of 1 mM D-tubocurarine (0.5-1 pmol) into the pTRG caused depressant effects on respiratory activity within 1 min and the suppression of vagal bursts within ~ 5 min (**Fig. 10b**). Rhythmic respiratory activity recovered gradually within 60 min after the injections. Bilateral microinjections performed 0.4 mm or more away from the responsive region (4 trials) did not affect respiratory activity.

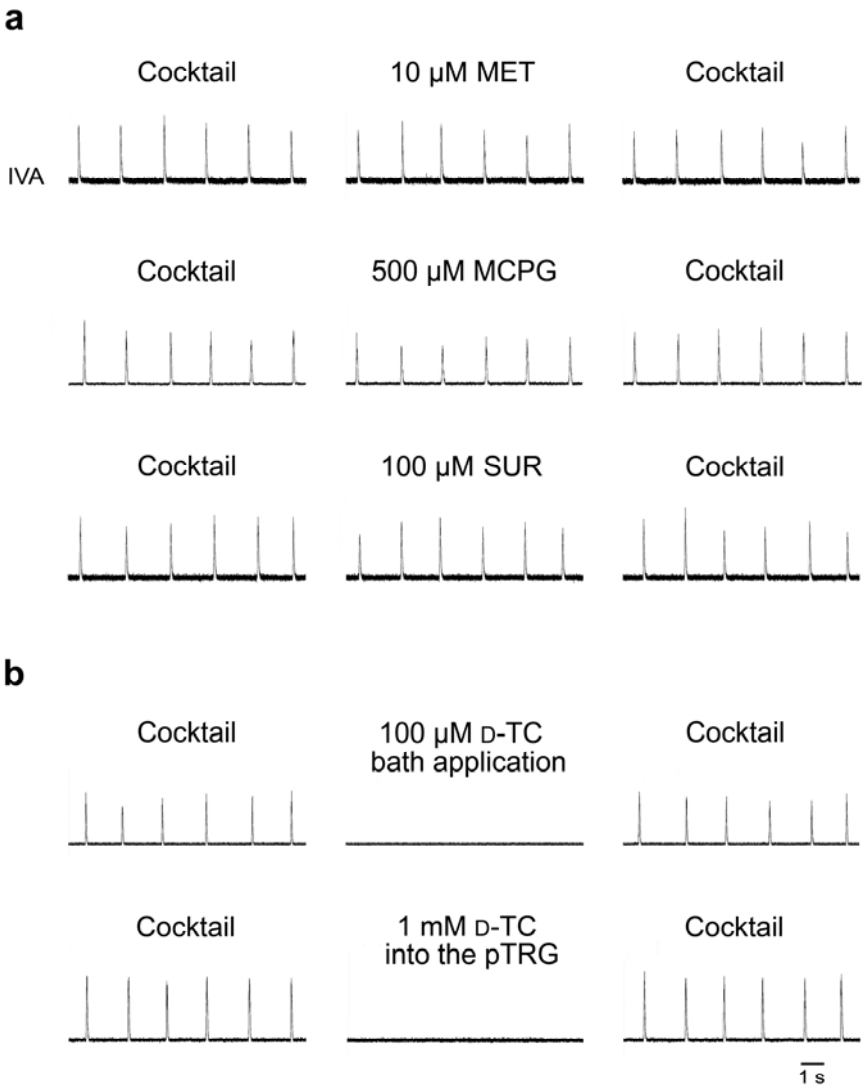

Fig. 10. Role of nicotinic receptors in maintaining the respiratory rhythm during blockade of glutamate, $GABA_A$ and glycine receptors (cocktail). (**a**) No change in integrated vagal nerve activity (IVA) ~10 min after bath application of 10 µM methysergide (MET), 500 µM MCPG or 100 µM suramin (SUR). (**b**) Bath application of 100 µM D-tubocurarine (D-TC) and microinjections of 1 mM D-TC into the pTRG abolished respiratory rhythmic activity within ~ 10 and 5 min, respectively.

In 5 additional preparations, neurons with respiration-related activity were recorded within the pTRG both before and after cocktail application (**Fig. 11**). These neurons maintained rhythmic activity synchronous with vagal bursts at decreased frequency after cocktail application. Under these conditions, rhythmic neuronal and vagal activities displayed an increase in frequency that reached a maximum within ~ 10 min in response to bath application of 1 μM nicotine (n = 2). On the contrary, they were abolished within the same time interval following bath application of 100 μM D-tubocurarine (n = 3).

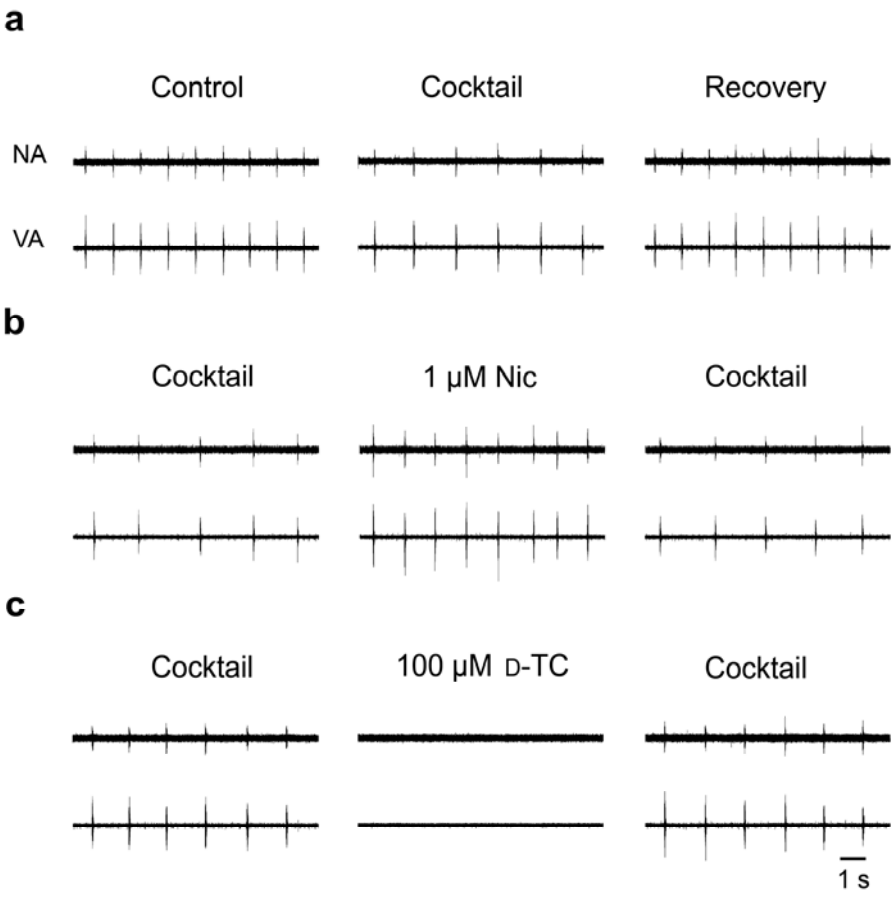

Fig. 11. Role of nicotinic mechanisms within the pTRG in maintaining the respiratory rhythm during blockade of glutamate, $GABA_A$ and glycine receptors (cocktail). (**a**) Depressant response on respiration-related neuronal activity (NA) of the pTRG and on raw vagal nerve activity (VA) ~ 35 min after cocktail application. (**b**) Excitatory effects on vagal and pTRG neuronal respiratory activity in response to 1 µM nicotine (Nic) ~ 10 min after bath application of the drug during cocktail perfusion. (**c**) Suppression of both vagal and pTRG neuronal activity ~10 min after bath application of 100 µM D-tubocurarine (D-TC) during cocktail.

DISCUSSION

We have shown that ACh plays an important excitatory role within the lamprey respiratory network under basal conditions and possibly under still undefined physiological conditions which imply increased respiratory drive to vagal motoneurons, such as locomotion or increased sensory inputs (Gravel *et al.*, 2007). This excitatory role is mediated by pTRG α7 nAChRs. We have found that respiratory rhythmic motor output, although at reduced frequency, persisted during blockade of glutamate, $GABA_A$ and glycine receptors. This respiratory activity was not affected by blockade of 5HT, purinergic, NK and metabotropic glutamate receptors, but was suppressed by blockade of pTRG α7 nAChRs, thus suggesting that under these conditions ACh *per se* is capable of maintaining the respiratory rhythm. The pTRG is confirmed to be a region displaying functional characteristics appropriate for a CPG.

The finding that ACh has an excitatory effect on respiration is in agreement with previous results obtained in mammals both *in vivo* (Gesell *et al.*, 1943; Weinstock *et al.*, 1981; Haxhiu *et al.*, 1984; Gillis *et al.*, 1988; Nattie *et al.*, 1989; Nattie & Li, 1990; Burton *et al.*, 1997) and *in vitro* (Murakoshi *et al.*, 1985; Monteau *et al.*, 1990; Burton *et al.*, 1994; 1995; Shao & Feldman, 2000; 2001; 2005; Hatori *et al.*, 2006). However, the effects of locally applied ACh on neurons of the mammalian medullary respiratory network are not consistent among different types of experiments probably due to differences in the animal species, the states of anesthesia, the localization of recording sites and the distribution and density of

AChRs (Salmoiraghi & Steiner 1963; Jordan & Spyer, 1981; Bradley & Lucy, 1983; Bohmer *et al.*, 1987; 1989; Bianchi *et al.*, 1995; Haji *et al.*, 2000).

Noticeably, present findings show that ACh affects respiratory activity through a specific action on the pTRG. Cholinergic inputs to pTRG may arise from several regions of the lamprey brain where cholinergic neurons have been mapped (Pombal *et al.*, 2001). In the brainstem, choline acetyltransferase-immunoreactive neurons included various groups of cranial motoneurons and two distinct groups of neurons in the tegmentum at the mesopontine border, in an area encompassing the isthmus and the caudal mesencephalon. In particular, immunoreactive cells were encountered close to the isthmic Müller cells located in the anterior rhombencephalic reticular nucleus. The latter appears to be in close proximity with the pTRG (see **Fig. 2**). Cholinergic inputs from the mesencephalic locomotor region to reticulospinal neurons are excitatory since ACh induces a nAChR-mediated depolarization of reticolospinal cells involved in the initiation and control of locomotion (Le Ray *et al.*, 2003). Nicotinic activation of lamprey reticulospinal neurons involved in the control of locomotion is due to α-bungarotoxin-sensitive receptors, *i.e.* α7 nAChRs. Furthermore, the activity of reticulospinal neurons is amplified by an excitatory glutamatergic drive arising from a group of brainstem neurons which receive mAChR-mediated inputs from the mesencephalic locomotor region (Smetana *et al.*, 2010). The results of these studies have suggested that ACh acting on both nAChRs and mAChRs has an important role in initiating, maintaining and enhancing the locomotor output. It seems reasonable to hypothesize that endogenous ACh could affect respiration when released in response to stimuli that induce locomotion, *i.e.* under conditions

that also require an enhanced respiratory drive to vagal motoneurons (Gravel *et al.*, 2007).

Interestingly, the depressant effects on respiratory frequency and the increases in the duration of vagal bursts evoked by α7 nAChR blockade indicate that during normal breathing α7 nAChRs are activated by endogenously released ACh and have a role in the modulation of the intensity of baseline respiratory activity. Thus, nAChR-expressing neurons within the pTRG may affect the glutamatergic rhythm-generating network (Bongianni *et al.*, 1999) by exerting a tonic influence that can be removed by D-tubocurarine and α-bungarotoxin. Few studies have provided evidence that ACh can play such a role in the mammalian respiratory network acting on nAChRs (Hatori *et al.*, 2006) or mAChRs (Burton *et al.*, 1994; 1995). At variance with previous studies in *in vitro* preparations from neonatal rodents demonstrating that the activation of both nAChRs and mAChRs (Shao & Feldman, 2000; 2001; 2005; Shao *et al.*, 2008) or exclusively of mAChRs (Monteau *et al.*, 1990; Burton *et al.*, 1995) mediates ACh excitatory effects on respiration, our results clearly show that ACh affects respiration in the lamprey by acting solely on nAChRs. The lack of respiratory effects in response to bath application of pilocarpine and atropine indicates that mAChRs do not contribute to respiratory regulation, although these receptors have been demonstrated to be present in the lamprey brainstem and involved in the control of locomotion (Le Ray *et al.*, 2004; Smetana *et al.*, 2010). At variance with previous findings showing that the predominant subtype of nAChRs in the preBötC of neonatal rats involved in the nicotine-induced respiratory effects is an α4β2 subunit combination (Shao & Feldman, 2001; 2002; Shao *et al.*, 2008), it has been found that only α7 nAChRs are expressed within the pTRG. These

discrepancies may depend upon differences in the animal species employed possibly due to differences in the subunit composition of nAChR subtypes (Albuquerque *et al.*, 2009). However, in this context it should be also mentioned that recent neuroanatomical studies have shown that α7 nAChRs are more common in the preBötC region than electrophysiological studies would suggest (Dehkordi *et al.*, 2004), although the way in which these receptors modulate the excitatory drive to preBötC neurons has not yet been elucidated. In agreement with the general notion that nAChRs can presynaptically modulate both glutamatergic and GABAergic neurotransmission (Fregosi & Pilarski, 2008; Albuquerque *et al.*, 2009), evidence has been provided that nicotine acts on α4β2 nAChRs of preBötC inspiratory neurons by modulating glutamatergic neurotransmission (Shao & Feldman, 2001). Although information is lacking on the cellular localization of nAChRs in the lamprey respiratory network, the finding that nicotine-induced activation or D-tubocurarine- and α-bungarotoxin-induced blockade of α7 nAChRs still produces respiratory effects during cocktail application provides evidence against a presynaptic localization of α7 nAChRs on glutamatergic neurons. Our results indicate that activation or blockade of α7 nAChRs causes changes in respiratory frequency without concomitant variations in the amplitude of vagal bursts, thus suggesting that separate mechanisms are involved in the timing and intensity control of respiratory activity. In agreement with the view that changes in respiratory frequency are due to an action on the central mechanisms generating the respiratory rhythm (Feldman & Del Negro, 2006; Mutolo *et al.*, 2010b), respiratory responses induced by bath application of nAChR agonists or antagonists were mimicked by microinjections of the same drugs into the pTRG, but not by similar microinjections within the region where vagal motoneurons are located.

Results obtained with microinjections are consistent with those observed in perfusion experiments. We are confident that drug microinjections targeted the pTRG. In agreement with previous findings (Mutolo *et al.*, 2007; 2010b), injection sites were selected by using coordinates and, especially, by recordings from respiration-related neurons. Only microinjections that were associated with characteristic spots of dye within the pTRG evoked respiratory responses. The localization of injection sites was confirmed by the histological control. Microinjections into neighbouring sites 0.4 mm or more away from the responsive area as well as into the region where motoneurons are located failed to induce significant changes in respiratory activity. It is also unlikely that respiratory responses resulted from non-specific effects of volume or pressure, since control injections of equal volumes of the vehicle solution performed at the same locations did not provoke any obvious respiratory response. The drug concentrations were in the same range as those employed in previous studies (Aberger *et al.*, 2001; Bongianni *et al.*, 2002a; Shao & Feldman, 2002; Le Ray *et al.*, 2003; 2004; Lorier *et al.*, 2007; St John & Leiter, 2008; Shao *et al.*, 2008; Mutolo *et al.*, 2010b; 2007). As previously discussed (Mutolo *et al.*, 2007; 2010b), it should be noted that concentrations of neuroactive drugs required in microinjection studies are higher than those used in perfusion studies. This may be accounted for by several complex factors that drastically reduce the actual concentration at the level of the injected area (Nicholson, 1985; Nicholson & Sykova, 1998).

The finding that respiratory rhythm persists following cocktail application is surprising and at variance with previous results obtained in *in vitro* medullary slices from neonatal rodents showing that the respiratory output was abolished, despite preBötC pacemaker neurons continued to burst (Del Negro *et al.*, 2002b; 2005;

Tryba *et al.*, 2003; Peña *et al.*, 2004). Interestingly, after blockade of both NMDA and non-NMDA receptors most pacemaker neurons ceased to rhythmically burst, but resumed endogenous bursting after subsequent blockade of GABAergic and glycinergic inhibition, in the absence of a rhythmic respiratory output (Tryba *et al.*, 2003). Present results confirm that blockade of both non-NMDA and NMDA receptors (Bongianni *et al.*, 1999) abolishes the respiratory motor output and provide evidence that respiratory activity is restored after subsequent blockade of $GABA_A$ and glycine receptors (Bongianni *et al.*, 2006). We are confident that non-NMDA receptors were completely blocked since similar respiratory responses were obtained by bath application of cocktail solutions containing higher concentrations of CNQX. In addition, DLH did not alter respiratory activity of preparations perfused with the cocktail solution. In the lamprey, rhythmic respiratory discharges persisted after bath application of the cocktail solution both in the pTRG and vagal motoneurons. Some neurotransmitters known to have an excitatory function on respiratory activity (Bianchi *et al.*, 1995; Haji *et al.*, 2000; Doi & Ramirez, 2008) did not affect respiratory rhythm during cocktail application. On the contrary, respiratory activity of pTRG neurons and vagal motoneurons increased in frequency in response to nicotine and was silenced by D-tubocurarine and α-bungarotoxin. However, only neurons of the pTRG proved to be responsive to the activation or blockade of α7 nAChRs, thus indicating that nAChR-expressing neurons of the pTRG contribute to respiratory rhythm generation and drive transmission to vagal motoneurons after cocktail-induced blockade of glutamate, $GABA_A$ and glycine receptors. We cannot completely exclude that other excitatory neurotransmitters contribute to the maintenance of the respiratory rhythm. It should also be mentioned that α7 nAChR blockade as well as bath application of the cocktail solution caused

increases in the duration of vagal bursts. The reasons of this outcome are obscure. The absence of the inhibitory control *per se* does not affect burst duration (Bongianni *et al.*, 2006). Thus, the hypothesis can be advanced that reductions in the excitatory input to pTRG neurons could decrease the excitatory coupling between pTRG neurons resulting in an increase in vagal burst duration (see e.g. Shao & Feldman, 2001).

The results obtained by cocktail application imply that inhibition can be exerted not only on glutamatergic neurons, but also on cholinergic neurons of the lamprey respiratory network. The absence of the inhibitory control cannot obviously affect the glutamatergic rhythm-generating network (Bongianni *et al.*, 1999; 2006) since glutamate receptors are blocked. However, it can still cause the disinhibition of cholinergic neurons and the resumption and maintenance of the respiratory rhythm through a different neural circuit involving cholinergic neurons. We cannot exclude that in the lamprey brainstem neurons partially or completely deprived of glutamatergic transmission rely on parallel cholinergic transmitter signaling due to the corelease of glutamate and ACh. This latter has been proved to occur at the spinal level in other animal species (Grillner & Jessell, 2009).

How the respiratory drive is conveyed to vagal motoneurons during cocktail application is not clear, owing to the blockade of glutamatergic transmission that, as a rule, mediates the excitatory input to motoneurons in the lamprey as well as to the absence of a proved involvement of other neurotransmitters. In this context, it should be mentioned that a combined electrical and chemical transmission has been described in the lamprey (Rovainen, 1979; Batueva, 1987; Grillner & Jessell, 2009). Further investigations are needed to ascertain the presence of electrical coupling or

the existence of still unidentified neurotransmitters implicated in the mediation of the excitatory drive to vagal motoneurons.

Studies in invertebrate or vertebrate motor systems have led to the conclusion that rhythmic motor patterns depend on a combination of synaptic and intrinsic membrane properties (Marder & Bucher, 2001; Peña *et al.*, 2004). Removal of a single rhythm-generating mechanism may thus change the configuration of a network, but will not necessarily abolish motor output. The presence of different rhythm-generating mechanisms could represent not only a safety factor, but may allow the respiratory network to adjust to changes in the behavioral, environmental and metabolic states. The relative contribution of different rhythm-generating mechanisms can be altered in a state-dependent manner by neuroactive agents which can activate, modify or terminate CPG (Marder & Bucher, 2001). The present study indicates that ACh acting on α7 nAChRs of pTRG neurons not only modulates respiratory rhythm under basal conditions, but is also capable *per se* of maintaining the respiratory rhythm when other more usual fast synaptic mechanisms are impaired. In agreement with a view expressed several years ago by Von Euler (1997) and recently taken again into consideration (Mellen, 2010), our results demonstrate that even in a very simple lower vertebrate most probably the respiratory CPG is a neural system characterized by degeneracy and a great deal of redundancy.

In several disorders related to central control of breathing, including sudden infant death syndrome and sleep apnea (Kinney *et al.*, 1995; Bellingham & Funk, 2000; Benarroch *et al.*, 2001; Lydic *et al.*, 2002; Gilman *et al.*, 2003; Duncan *et al.*, 2008), ACh seems to play a critical role. Noteworthy, it has been reported that prenatal nicotine exposure may predispose newborns to sudden infant death

syndrome (Fregosi & Pilarski, 2008; Shao & Feldman, 2009). Our study shows that ACh-mediated neural mechanisms play an important role in the basal control of breathing as well as under conditions of impaired glutamatergic, GABAergic and glycinerigic transmission. These results encourage future studies to further characterize cholinergic mechanisms involved in the control of breathing both in mammalian preparations and in lower vertebrates that appear to retain throughout evolution some basic functional characteristics of the respiratory CPG.

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