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CICLO XXXI

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Key role of 5-HT_{1A} receptors in the modulation of the neuronal network underlying the respiratory rhythm generation in rabbits and lampreys

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“There is no royal road to science,
and only those who do not dread the fatiguing climb of its steep paths
have a chance of gaining its luminous summits.”

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Summary

1. It is well known that serotonin (5-HT) and its ionotropic and metabotropic receptors are involved in the regulation of many brain functions, including breathing. To date, at least 14 different 5-HT receptor (5-HTR) subtypes have been identified in mammals, which are grouped into seven families ($5\text{-}HT_1$-$5\text{-}HT_7$). These 5-HTRs are G-protein-coupled receptors except the ligand-gated ion channel $5\text{-}HT_3R$. In mammals and specifically in neonatal or juvenile rodents, 5-HT has been reported to play an excitatory effect on respiration by acting on the preBötzinger complex (preBötC), the neuronal aggregate responsible for inspiratory rhythm generation. However, whether its action is circumscribed to the preBötC and present also in adult preparations, particularly of other animal species, is unknown. Furthermore, no information is available on the role of 5-HT within the neighboring respiration-related regions of the ventral respiratory column.

   In the lamprey, 5-HT is importantly involved in neural circuits controlling locomotion through an action on $5\text{-}HT_{1A}Rs$, but its role within the respiratory rhythm generator network, i.e. the paratrigeminal respiratory group (pTRG) has not yet been studied**.**

   The present research was undertaken to investigate the respiratory function of 5-HT in adult rabbits and lampreys. The respiratory role of 5-HT was investigated by means of bilateral microinjections of 5-HT as well as of specific 5-HTR agonists and antagonists in α-chloralose-urethane anesthetized, vagotomized, paralyzed and artificially ventilated adult rabbits. In addition, the respiratory function of 5-HT and especially that of inhibitory $5\text{-}HT_{1A}Rs$ was studied in *in vitro* brainstem lamprey preparations making use

of appropriate agonists and antagonists applied to the bath and/or bilaterally microinjected into the pTRG. The presence of inhibitory circuits possibly involved in serotoninergic modulation was investigated in both animal species by means of the local application of strychnine and bicuculline. Finally, immunohistochemical studies were performed to corroborate the results obtained with GABA or glycine receptor antagonists.

2. In the rabbit, bilateral microinjections of 5-HT caused excitatory effects on respiratory activity only when applied to the preBötC, but not in the neighboring respiration-related regions, namely the more rostral Bötzinger complex (BötC) and the more caudal inspiratory portion of the ventral respiratory group (iVRG). These effects were mediated by 5-HT_{1A}Rs and 5-HT_3Rs as shown by microinjections of specific agonists of different 5-HTRs. Unexpectedly, the blockade of 5-HT_{1A}Rs by methysergide (the 5-$HT_{1/2}$R antagonist) or (S)-WAY 100135 (the selective 5-HT_{1A}R antagonist) induced excitatory respiratory effects. Microinjections of the 5-HT_3R antagonist ondansetron did not influence respiration, but prevented (S)-WAY 100135-induced responses. The blockade of $GABA_A$ receptors by bicuculline within the preBötC prevented the effects of the 5-HT_{1A}R agonist 8-OH-DPAT. The involvement of GABAergic inhibition and 5-HT_{1A}R-mediated disinhibition is corroborated by immunohistochemical data, showing 5-HT_{1A}R-immunoreactive structures located in close apposition to GABAergic neurons in the preBötC region.
3. In the lamprey, 5-HT either applied to the bath or bilaterally microinjected into the pTRG did not cause obvious effects on respiratory activity. However, methysergide applied to the bath as well as (S)-WAY 100135 applied to the

bath or microinjected into the pTRG induced depressant respiratory effects or even apnea, thus revealing that 5-HT exerts a 5-HT_{1A}R-mediated potent inhibitory influence on respiration and contributes to maintain baseline levels of respiratory activity. Both GABA and glycine are involved in these effects since microinjections of bicuculline or strychnine, either alone or in combination, into the pTRG prevented (S)-WAY 100135-induced apnea. In agreement with the notion that GABA-immunopositive fibres, but not GABA-immunoreactive neurons are present in the pTRG region, we found that 5-HT_{1A}Rs are widely expressed in close apposition to the soma of glycine-immunoreactive cells located within the pTRG region.

4. This study is the first to provide evidence that 5-HT plays an excitatory role in the modulation of respiratory activity in the adult rabbit by acting on the preBötC, through the activation of 5-HT_{1A}Rs and 5-HT_3Rs, without affecting the neighbouring BötC and iVRG. Interestingly, (S)-WAY 100135 is ineffective during 5-HT_3R blockade, thus suggesting a presynaptic role of 5-HT_{1A}Rs. The finding that the effects of the 5-HT_{1A}R agonist 8-OH-DPAT are prevented by bicuculline indicates an involvement of a GABAergic inhibitory circuit modulated by postsynaptic inhibitory 5-HT_{1A}Rs. Immunohistochemical data are consistent with this interpretation.

   We show for the first time that in the lamprey respiratory network 5-HT exerts a tonic influence on respiration and contributes to maintain baseline levels of respiratory activity by a potent inhibitory control on both GABAergic and glycinergic mechanisms. Consistently with immunohistochemical data, the 5-HT_{1A}R-mediated inhibition is conceivably effected on glycinergic cells and on GABAergic terminals. The finding that

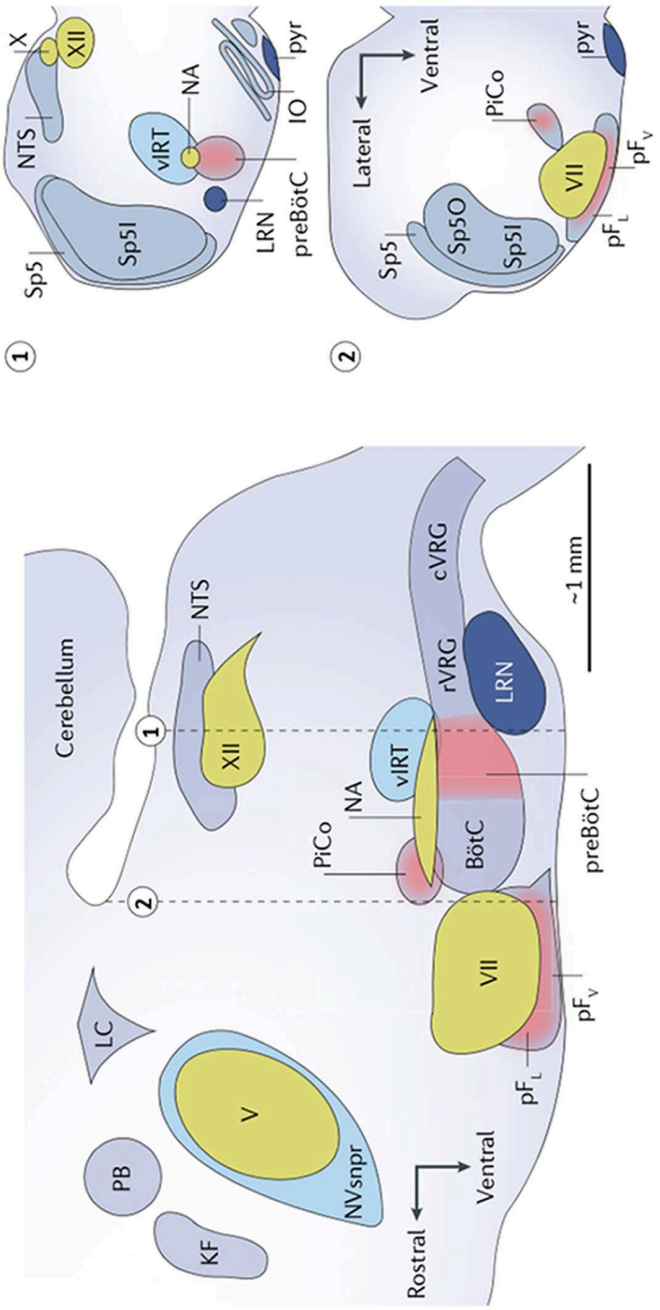
in the lamprey inhibitory neurons are inhibited through the activation of 5-HT_{1A}Rs closely recalls the neuronal organization of the mammalian respiratory network and especially that of rodents, in which the inhibitory neurons are glycinergic. The presence in the lamprey of only 5-HT_{1A}Rs supports the suggestion that they represent the ancestral archetypical 5-HTRs. Interestingly, at variance with previous results in neonatal or juvenile rodents showing varied presynaptic and postsynaptic 5-HTRs in the regulation of respiratory activity, only 5-HT_{1A}Rs and 5-HT_3Rs have been found in the rabbit. 5-HT_3R subtype has not been previously described in mammals, but in the respiratory network of the turtle, an animal species encountered much later than lampreys in the evolution. Taking together, the results support the notion that some important features of the neuronal network subserving respiratory rhythm generation are conserved throughout phylogeny.

Introduction

It is well known that serotonin (5-HT) and its ionotropic and metabotropic receptors are involved in the regulation of many brain functions, including breathing (for review, see Jacobs & Azmitia, 1992; Hodges & Richerson, 2008, 2010; Hilaire *et al.*, 2010). To date, at least 14 different 5-HT receptor (5-HTR) subtypes have been identified in mammals, which are grouped into seven families ($5\text{-}HT_1$-$5\text{-}HT_7$) (Kroeze *et al.*, 2002; Nichols & Nichols, 2008). These 5-HTRs are G-protein-coupled receptors except the ligand-gated ion channel $5\text{-}HT_3R$. The inhibitory $5\text{-}HT_{1A}R$ is a major determinant of the activity of serotoninergic cells and of 5-HT release because of its both presynaptic and postsynaptic location (Chalmers & Watson, 1991). The $5\text{-}HT_{1A}R$ is estimated to have differentiated around 650 million years ago from the $5\text{-}HT_1$ subfamily around the time during which vertebrates diverged from invertebrates (Peroutka & Howell, 1994). Most of the invertebrate 5-HTRs resemble either the mammalian $5\text{-}HT_{1A}Rs$ or the $5\text{-}HT_7Rs$ (Kroeze *et al.*, 2002), thus suggesting that the $5\text{-}HT_{1A}$ or $5\text{-}HT_7$ subclasses represent the ancestral archetypical 5-HTRs.

Breathing movements are produced by bilateral and symmetrical 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, post-inspiration or expiration phase 1, and expiration phase 2 (active expiration). 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; Del Negro *et al.*, 2018). Several brainstem structures have been found to have a respiratory function. They include pontine nuclei (comprising the pontine respiratory group), the dorsal respiratory group, corresponding to the nucleus of the solitary tract (NTS), the ventral respiratory column comprising the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), also identified as ventral parafacial respiratory group (pF_V) and lateral parafacial respiratory group (pF_L), the Bötzinger complex (BötC), the preBötzinger complex (preBötC), the inspiratory portion of the ventral respiratory group (iVRG) and the caudal expiratory component of the ventral respiratory group (cVRG). A recently discovered brainstem region, named 'Postinspiratory Complex' (PiCo), is located rostral to the preBötC, dorsal to the BötC and caudal to the facial nucleus (Anderson *et al.*, 2016). The PiCo is characterized by rhythm-generating properties and is considered necessary and sufficient for generating postinspiratory activity *in vivo*. On the basis of these results, a 'triple oscillator model' has been proposed in which inspiration, postinspiration and active expiration are generated by three distinct excitatory rhythmogenic microcircuit, i.e. preBötC, PiCo and pFRG, respectively (Anderson & Ramirez, 2017). The main components of the mammalian central network underlying respiratory pattern generation are illustrated in Fig. 1.


X
XII
NTS
vIRT
NA
pyr
IO
Sp5
Sp5I
LRN
preBötC
1
Lateral
Ventral
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Sp5O
Sp5I
VII
pF_V
pF_L
2
Cerebellum
NTS
XII
cVRG
rVRG
LRN
vIRT
NA
PiCo
BötC
preBötC
~1 mm
LC
PB
KF
V
NVsnpr
VII
pF_V
pF_L
Rostral
Ventral

Figure 1

Fig. 1. Localization of the main structures involved in the control of breathing in mammals. Parasagittal view of the brainstem containing the mammalian respiratory network. Respiratory rhythmogenic sites are shown in red: the preBötzinger complex (preBötC) for inspiration, the lateral portion of the parafacial respiratory group (pF_L) for expiration and the more medial chemosensitive ventral parafacial respiratory group (pF_V), rhythmogenic only in perinatal period, as well as the more recently described 'Postinspiratory Complex' (PiCo), hypothesized to underlie postinspiration. Other rhythmogenic sites, such as the whisking-related ventral intermediate reticular formation (vIRT) and the masticatory trigeminal principal sensory nucleus (NVsnpr), are shown in blue. Cranial motor nuclei controlling airway resistance muscles, the hypoglossal motor nucleus (XII) and the nucleus ambiguus (NA), as well as facial muscles, the facial motor nucleus (VII) and the trigeminal motor nucleus (V), are shown in green. Brainstem sites associated with breathing motor pattern or sensorimotor integration are shown in grey: the rostral ventral respiratory group (rVRG) containing inspiratory, that is, phrenic and external intercostal, premotoneurons and the caudal ventral respiratory group (cVRG) containing expiratory premotoneurons. Other sites include the pontine Kölliker-Fuse nucleus (KF) and parabrachial nucleus (PB), the nucleus of the solitary tract (NTS) and the expiratory BötC. Also shown is the locus coeruleus (LC) that receives preBötC projections, the cerebellum and the lateral reticular nucleus (LRN). Insets **1** and **2** show transverse sections at the level of the preBötC (dotted line 1) and pF (dotted line 2). Additional structures in the insets include the spinal trigeminal tract (Sp5), the spinal trigeminal sensory nucleus oralis (Sp5O) and the interpolaris (Sp5I), the inferior olive (IO) and the pyramidal tract (pyr). Modified from Del Negro *et al.*, 2018.

Despite varied 5-HT effects on the respiratory motor output have been described, the bulk of evidence seems to support the conclusion that 5-HT neurons play an excitatory role on respiratory activity in mammals (Jacobs & Azmitia, 1992; Hodges & Richerson, 2008, 2010; Hilaire *et al.*, 2010). Recent results (Ptak *et al.*, 2009) have shown that excitatory amino acid-induced augmentation of the endogenous release of 5-HT and substance P (SP) from the raphe system in *in vitro* neonatal and in *in situ* juvenile rat preparations causes increases in the respiratory motor output in consequence of the activation of the preBötC, the neuronal aggregate responsible for the inspiratory rhythm generation (Smith *et al.*, 1991; Feldman *et al.*, 2013; Del Negro *et al.*, 2018). More recently, an optogenetic study by DePuy and colleagues (2011) performed in anesthetized mice demonstrated that stimulation of raphe obscurus serotoninergic neurons activates breathing and potentiates the central respiratory chemoreflex. However, no information is available on the role of 5-HT within the neighbouring respiration-related regions and in particular within important neural substrates possibly involved in respiratory rhythm generation, i.e. the BötC and the iVRG (Smith *et al.*, 2007, 2009; Mutolo *et al.*, 2002, 2005; Bongianni *et al.*, 2010). Impairments of central 5-HT mechanisms have been reported to be implicated in the pathophysiology of some respiratory disorders, such as sleep apnoea (Kubin *et al.*, 1998; Kubin, 2016; Lipford *et al.*, 2016 also for further Refs.) and sudden infant death syndrome (Paterson *et al.*, 2006; Duncan *et al.*, 2010; Machaalani & Waters, 2014; Praveen & Praveen, 2016 also for further Refs.). Interestingly, 5-HT has also been suggested to have a key role in autoresuscitation after anoxic stresses (e.g. Erickson & Sposato, 2009; Chen *et al.*, 2013; but see also Leiter, 2009) or opioid-induced respiratory depression (e.g. Sahibzada *et al.*, 2000; Dutschmann *et al.*, 2009; Manzke *et al.*, 2009; Shevtsova *et al.*, 2011; Ren *et al.*, 2015). The 5-HTRs are

widely expressed in the VRG and 5-HT-induced excitatory effects on respiratory activity obviously depend on the combined activation of presynaptic and postsynaptic 5-HTRs within the neural structures involved in respiratory control (e.g. Hodges & Richerson, 2008, 2010; Nichols & Nichols, 2008; Dutschmann *et al.*, 2009; Hilaire *et al.*, 2010). In addition, disinhibition, i.e. 5-HT-mediated inhibition of inhibitory neurons, has been proposed to contribute to the excitatory respiratory effects (e.g. Manzke *et al.*, 2009; Shevtsova *et al.*, 2011; Corcoran *et al.*, 2014). Although GABA and glycine receptors are expressed in the preBötC of adult mammals and both GABA and glycine exert a potent inhibitory control of inspiratory activity, recent results show that the endogenous release of these inhibitory amino acids is not essential for inspiratory rhythm generation (Bongianni *et al.*, 2010; Kam *et al.*, 2013; Del Negro *et al.*, 2018 also for further Refs). In this context, it is worth noting that, in mammals, studies on the effects of 5-HT on respiration have hitherto been performed mostly in neonatal or juvenile rodent preparations (e.g. Al Zubaidy *et al.*, 1996; Pena & Ramirez, 2002; Schwarzacher *et al.*, 2002; Gunther *et al.*, 2006; Ptak *et al.*, 2009; Niebert *et al.*, 2011; Corcoran *et al.*, 2014; for review, see Hodges & Richerson, 2008, 2010; Hilaire *et al.*, 2010), thus studies in adult preparations of other animal species appear to be of interest.

The lamprey (Fig. 2) is a lower vertebrate which diverged from the main vertebrate line around 560 million years ago (Kumar & Hedges, 1998) and has proved to be highly useful to identify the neuronal circuits underlying rhythmic motor behaviours, such as locomotion (e.g. Grillner *et al.*, 1995; Grillner, 2003,2006) and respiration (e.g. Cinelli *et al.*, 2013; Bongianni *et al.*, 2016).

Fig. 2. Lamprey *(Petromyzon marinus)*

The isolated brainstem of the adult lamprey 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 (Rovainen, 1974, 1977, 1979; Guimond *et al.*, 2003), while the paratrigeminal respiratory group (pTRG) that is responsible for the respiratory rhythm generation is located in a region rostral to the trigeminal motor nucleus (Mutolo *et al.*, 2007, 2010, 2011; Cinelli *et al.*, 2013, 2014, 2017; 2016; but see also Milsom, 2018). Baseline respiratory activity is interrupted by prolonged vagal bursts followed by changes in respiration, which often consist of a pause followed by an increase in respiratory frequency. These bursts, also named 'coughs', occur at lower and irregular frequency (Rovainen, 1977, 1996; Thompson, 1985; Martel *et al.*, 2007) and probably have a different central pattern generator (Hoffman *et al.*, 2016; see also Milsom, 2018).

Endogenously released excitatory amino acids, but not GABA and glycine, have been shown to have an essential role in the respiratory rhytmogenesis (Rovainen, 1983; Martel *et al.*, 2007; Bongianni *et al.*, 1999, 2006, 2016; Cinelli *et al.*, 2013, 2014). Only GABAergic influences have a modulatory role at the pTRG level. On the other hand, GABAergic and glycinergic inputs to neurons within the vagal motoneuron region mediate changes in respiratory frequency through

ascending excitatory projections to the pTRG (Cinelli *et al.*, 2014). Furthermore, the pTRG has been anatomically and functionally characterized (Cinelli *et al.*, 2013, 2014, 2016, 2017; for review see Bongianni *et al.*, 2016; Missaghi *et al.*, 2016) and is sensitive to opioids, neurokinins, acetylcholine and ATP (Mutolo *et al.*, 2007, 2010, 2011; Cinelli *et al.*, 2017). Fig. 3 illustrates some prominent connections within the respiratory network and neurotransmitter influences on both the pTRG and the respiratory motoneuron region.

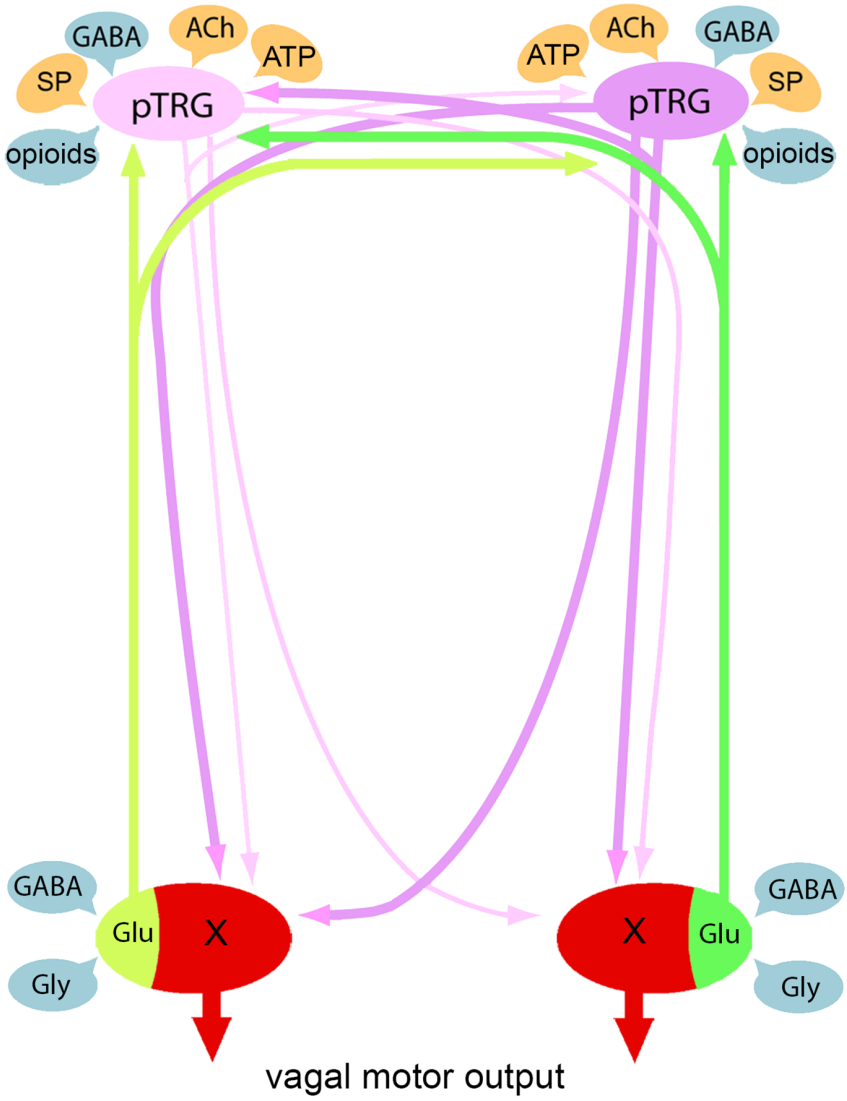

Fig. 3. Schematic drawing representing findings on the connectivity within the respiratory network of the lamprey and relevant neurotransmitter influences. The pTRG region is shown with its projections (pink) to ipsilateral and contralateral vagal motoneuron regions (red) and to the contralateral pTRG. Excitatory (orange) and inhibitory (blue) influences on the pTRG region and the vagal motoneuron region are illustrated. Glutamatergic (Glu) projections to the pTRG (green) from neurons located in the vagal area have also been reported. ACh, acetylcholine; ATP, adenosine triphosphate; GABA, γ-aminobutyric acid; Gly, glycine; pTRG, paratrigeminal respiratory group region; SP, substance P; X, vagal motoneuron region. Modified from Bongianni *et al.*, 2016.

The pTRG shows many similarities with the preBötC (Smith *et al.*, 1991; Feldman *et al.*, 2013; Bongianni *et al.*, 2016; Cinelli *et al.*, 2017). In fact, the two rhythm generators are located rostrally in the medulla, have opioid, SP and ATP sensitivity and appear to have similar underlying rhythm generating ionic currents (Mutolo *et al.*, 2007, 2010, 2011; Cinelli *et al.*, 2017). In mammals, the respiratory network receives multiple modulatory inputs which have several functions in controlling respiratory rhythmic activity (Doi & Ramirez, 2008) and differentially regulate the amplitude and frequency of respiratory activity. The study of their effects is of interest since it could also provide insights into the mechanisms generating the respiratory rhythm. One of the key neuromodulator is 5-HT (Doi & Ramirez, 2008). The general organization of the serotoninergic system in the lamprey is relatively well described and appears to share similarities with that of other vertebrates (Pierre *et al.*, 1992; Antri *et al.*, 2006; Abalo *et al.*, 2007; Cornide-Petronio *et al.*, 2013). Furthermore, it has been reported that the 5-ht1a transcript (i.e. the expression of the 5-HT_{1A}R gene) is distributed throughout the entire central nervous system (Cornide-Petronio *et al.*, 2013). Interestingly, the expression of 5-ht1a transcript was also found within a region that may correspond to the pTRG.

In the lamprey, 5-HT is importantly involved in neural circuits controlling locomotion (Grillner *et al.*, 1995). 5-HT slows the frequency of fictive locomotor activity (Harris-Warrick & Cohen, 1985; Matsushima & Grillner, 1992; Buchanan, 2001; for review see Grillner, 2006) as it does in most other motor systems in mammals (Jordan *et al.*, 2008). This network effect has been linked to a 5-HT-mediated reduction of a calcium-dependent potassium conductance underlying the slow afterhyperpolarization (sAHP) following action potentials, which in turn can influence spike frequency adaptation and increase neuronal excitability (Wallen *et*

al., 1989; Hill *et al.*, 2003). While this effect has been claimed to account for the changes in fictive and simulated network activity (e.g. Hellgren *et al.*, 1992; Grillner *et al.*, 1995, 2005), this conclusion is complicated by the wide range of cellular and synaptic effects of 5-HT. These include a hyperpolarization of the resting membrane potential, a reduction of glutamatergic synaptic transmission, and an increase or decrease in inhibitory inputs (Harris-Warrick & Cohen, 1985; Buchanan & Grillner, 1991; Parker & Grillner, 1999, 2000; Svensson *et al.*, 2001; Biro *et al.*, 2006; Parker, 2006). In the control of locomotor activity mainly postsynaptic 5-HT_{1A}Rs appear to be involved (e.g. Wikstrom *et al.*, 1995; Zhang & Grillner, 2000; Hill *et al.*, 2003; Wang *et al.*, 2014), but a presynaptic role of other 5-HTR subtypes (i.e. 5-HT_{1D}) has been reported (Schwartz *et al.*, 2005; see also Grillner, 2003, 2006). 5-HT and dopamine also modulate $Ca_v1.3$ calcium channels involved in postinhibitory rebound, a common property of rhythm generating networks in both vertebrates and invertebrates (Wang *et al.*, 2011). Despite the wide knowledge on the lamprey serotoninergic system, no information is available on the role of 5-HT and particularly of 5-HT_{1A}Rs within the respiratory rhythm generating network.

Taking into account that comparative studies in different animal species and a more deep knowledge of neuronal rhythmogenic circuits and their evolution are important targets in neuroscience, the present research was undertaken to investigate the respiratory function of 5-HT in adult rabbits and lampreys. The respiratory role of 5-HT within the rostral VRG subregions, i.e. the preBötC and the adjacent BötC and iVRG, was studied by means of microinjections of 5-HT as well as of specific 5-HTR agonists and antagonists in anesthetized, vagotomized, paralyzed and artificially ventilated adult rabbits. In addition, the effects of 5-HT and 5-HT_{1A}R agonism and antagonism on respiration were investigated in *in vitro* brainstem

lamprey preparations. 5-HT and 5-HT_{1A}R agonist 8-OH-DPAT as well as the 5-$HT_{1/2}$R antagonist methysergide and the selective 5-HT_{1A}R antagonist (S)-WAY 100135 were applied to the bath and/or bilaterally microinjected into the pTRG. The presence of inhibitory circuits possibly subserving serotoninergic modulation was investigated in both animal species by means of the local application of strychnine and bicuculline. Immunohistochemical studies were performed to corroborate the results obtained with GABA or glycine receptor antagonist.

Methods

Ethical approval

All 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 animal suffering and to reduce the number of animals used. At the end of the experiment rabbits were euthanized with an overdose of anesthetic. In lampreys, anesthesia performed to obtain brainstem preparations was terminal (see below).

Protocol 1: rabbits

Animal preparation

Experiments were carried out on 85 male New Zealand white rabbits (2.8-3.4 kg) purchased from the Pampaloni Farm and Laboratory Animal Co. (Fauglia, Pisa, Italy). Rabbits were maintained on a 12-h light/12-h dark cycle with food and water *ad libitum*. Experimental procedures and details on the methods employed have previously been fully described (Bongianni *et al.*, 1997, 2002, 2008, 2010; Mutolo *et al.*, 2002, 2005; Pantaleo *et al.*, 2011). The animals were anesthetized (ear marginal vein) with a mixture of α-chloralose (40 mg/kg i.v.; Sigma-Aldrich, St. Louis, MO, USA) and urethane (800 mg/kg i.v.; Sigma-Aldrich), supplemented (femoral vein) when necessary (4 mg/kg and 80 mg/kg, respectively). The adequacy of anesthesia was assessed by the absence of reflex withdrawal of the hindlimb in response to

noxious pinching of the hindpaw. In paralyzed animals (see below), the depth of anesthesia was assessed by ascertaining the presence of a stable and regular pattern of phrenic nerve activity as well as the absence of fluctuations in arterial blood pressure whether spontaneous or in response to somatic nociceptive stimulation. After cannulation of the trachea, polyethylene catheters were inserted into a femoral artery and vein for monitoring arterial blood pressure and for drug administration, respectively. Both C5 phrenic roots were dissected free, cut distally and prepared for recordings. Both cervical vagus nerves were separated from the sympathetic trunks for subsequent vagotomy. The animal was placed in a prone position and fixed in a stereotaxic instrument by a stereotaxic head holder and vertebral clamps (Baltimore Instrument, Baltimore, MA, USA); the head was ventroflexed to facilitate recordings from the medulla. The dorsal surface of the medulla was widely exposed by occipital craniotomy, and the dura and arachnoid membranes were removed. The posterior part of the cerebellum was removed by gentle suction to provide access to the rostral part of the medulla. All exposed tissues were covered with warm paraffin oil (~38°C). Body temperature was maintained at 38.5-39°C by a heating blanket controlled by a rectal thermistor probe. The animals were vagotomized, paralyzed (gallamine triethiodide 4 mg/kg i.v., supplemented with 2 mg/kg every 30 min; Sigma-Aldrich) and artificially ventilated with oxygen-enriched room air. The oxygen (80%) was added to the inlet of the respirator. End-tidal CO_2 partial pressure was maintained approximately at the level of spontaneous breathing (28.5-32 mmHg) by adjusting the frequency and stroke volume of the respiratory pump.

Recording procedures

Efferent phrenic nerve activity was recorded with bipolar platinum electrodes from desheathed C5 phrenic roots, amplified, full-wave rectified and passed through a leaky integrator (low-pass RC filter, time constant 100 ms) to obtain a 'moving average' of the activity, usually referred to in the literature as 'integrated' activity. Extracellular recordings from medullary neurons were made with tungsten microelectrodes (5-10 MΩ impedance as tested at 1 kHz). The most rostral extent of the area postrema on the midline was defined as the obex and used as a standard point of anatomic reference. Neuronal activity was recorded from rostral expiratory neurons of the BötC (3.0-4.5 mm rostral to the obex, 2.4-3.2 mm lateral to the midline and 3.5-4.6 mm below the dorsal medullary surface), from the iVRG (from 0.7 caudal to 2.0 mm rostral to the obex, 2.3-3.2 mm lateral to the midline and 3.0-3.5 mm below the dorsal medullary face) and from the transition zone between the BötC and the iVRG where a mix of inspiratory and expiratory neurons is present (2.1-2.9 mm rostral to the obex, 2.4-3.2 mm lateral to the midline and 3.5-4.2 mm below the dorsal medullary surface). The latter region has already been extensively investigated in the rabbit with lesion and neuropharmacological approaches (Bongianni *et al.*, 2008, 2010; Mutolo *et al.*, 2002, 2005; Pantaleo *et al.*, 2011; Stucke *et al.*, 2015) and corresponds to the preBötC described in adult cats and rats (see e.g. Connelly *et al.*, 1992; Schwarzacher *et al.*, 1995; Rekling & Feldman, 1998; Solomon *et al.*, 1999; Feldman & Del Negro, 2006; Feldman *et al.*, 2013; Del Negro *et al.*, 2018). A strain-gauge manometer was used for monitoring arterial blood pressure. End-tidal CO_2 partial pressure was monitored by an infrared CO_2 analyzer (Capnocheck Plus, Smiths Medical PM, Waukesha, WI, USA). Cardiorespiratory variables were acquired and analyzed using a personal computer equipped with an

analogue-to-digital interface (Digidata 1440, Molecular Devices, Sunnyvale, CA, USA) and appropriate software (Axoscope, Molecular Devices).

Microinjection procedures and experimental protocol

Bilateral microinjections were performed into the BötC, preBötC and iVRG regions as defined by neuronal recordings. In each experiment, recordings of neuronal activity preceded drug microinjections. The patterns of neuronal discharges recorded in the BötC and the preBötC have been already illustrated in our previous reports (e.g. Mutolo *et al.*, 2002; Bongianni *et al.*, 2008, 2010). In addition, the localization of the preBötC was confirmed by evaluating the excitatory respiratory responses induced by bilateral microinjections (30-50 nl) of 20 mM D,L-homocysteic acid (DLH; Sigma-Aldrich), a broad-spectrum excitatory amino acid agonist, into this region (e.g. Solomon *et al.*, 1999; Wang *et al.*, 2002; Monnier *et al.*, 2003; Mutolo *et al.*, 2005; Bongianni *et al.*, 2008, 2010; Stucke *et al.*, 2015).

The following drugs were used: 5 mM 5-HT (endogenous agonist at 5-HTRs; Tocris Bioscience, Bristol, UK), 1 mM (*R*)-(+)-8-Hydroxy-DPAT hydrobromide (8-OH-DPAT; full 5-HT_{1A}R agonist, more active enantiomer; Tocris Bioscience), 1 mM BP 554 maleate (selective 5-HT_{1A}R agonist; Tocris Bioscience), 1 mM CP 93129 dihydrochloride (potent and highly selective 5-HT_{1B}R agonist; Tocris Bioscience), 1 mM TCB-2 (high affinity 5-HT_{2A}R agonist; Tocris Bioscience), 1 mM BW 723C86 hydrochloride (selective 5-HT_{2B}R agonist; Tocris Bioscience), 5 mM 1-phenylbiguanide hydrochloride (PBG, selective 5-HT_3R agonist; Sigma-Aldrich), 1 mM BIMU 8 (potent 5-HT_4R full agonist; Tocris Bioscience), 1 mM LP 44 (high affinity 5-HT_7R agonist; Tocris Bioscience), 5 mM methysergide maleate (mixed 5-$HT_{1/2}$R antagonist; Tocris Bioscience), 5 mM (S)-WAY 100135 dihydrochloride

(potent and selective 5-HT_{1A}R antagonist; Tocris Bioscience), 5 mM ondansetron hydrochloride (selective 5-HT_3R antagonist; Tocris Bioscience), 5 mM SB 258719 hydrochloride (selective 5-HT_7R antagonist; Tocris Bioscience), 5 mM strychnine hydrochloride (glycine receptor antagonist; Sigma-Aldrich), 5 mM bicuculline methiodide ($GABA_A$ receptor antagonist; Sigma-Aldrich). Drugs were dissolved in 0.9% NaCl solution except for LP 44, BP 554, BW 723C86 and (S)-WAY 100135 that were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and diluted to volume with 0.9% NaCl solution. In the final solution, the concentration of DMSO was < 10%. Only one drug was tested in each preparation, unless otherwise stated. Drug concentrations were selected in preliminary trials. They were in the same range as those previously used in *in vivo* preparations (Merahi & Laguzzi, 1995; Sevoz *et al.*, 1996; Comet *et al.*, 2007; Monti *et al.*, 2008; Rauch *et al.*, 2008; Bongianni *et al.*, 2010; Valic *et al.*, 2010; Ostrowski *et al.*, 2014; Zhang *et al.*, 2014; Sardari *et al.*, 2015).

Bilateral microinjections (30-50 nl) were performed in succession using a single glass micropipette (tip diameter 10-25 μm) by applying pressure using an air-filled syringe connected to the micropipette by polyethylene tubing. 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 duration of each injection ranged from 5 to 10 s. Control injections of equal volumes of the vehicle solution at the responsive sites were also made. In a few experiments (2 with 5-HT and 1 for 8-OH-DPAT), preBötC injection sites were confirmed by injecting green fluorescent latex microspheres (LumaFluor, New City, NY, USA) added to the drug solution. In an attempt to ensure an accurate representation of the preBötC location, these microinjections were performed only

unilaterally according to the stereotaxic coordinates derived from the results of neuronal activity recordings and DLH microinjections on the contralateral side.

Histology

At the end of the experiment, the brain was perfused via a carotid artery with 0.9% NaCl solution and then with 10% formalin solution. After at least a 48-h immersion in 10% formalin solution, the brain was placed in a hypertonic sucrose solution. Frozen 20-μm coronal sections stained with Cresyl violet were used for the histological control of pipette tracks and injection sites, as already shown in our previous reports (e.g. Mutolo *et al.*, 2002, 2005; Bongianni *et al.*, 2008, 2010) , as well as of fluorescent microsphere spots. Coronal sections of the medulla in which injection sites were marked by fluorescent microspheres were examined by a light and epifluorescence microscope (Eclipse E400, Nikon, Japan) equipped with the Nikon Intensilight C-HGFI mercury-fibre illuminator. A Nikon DS-Fi1 digital camera was used to take photomicrographs. Illustration were prepared in Adobe Photoshop CS6 (Adobe Systems Incorporated). The atlas of Shek et al. (1986) was used for comparison.

Immunohistochemistry

Three rabbits were deeply anesthetized with an overdose of pentobarbitone sodium (100 mg/kg I.V.; Sigma-Aldrich). The brains were perfused via a carotid artery with ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) and subsequently with 500 ml of ice-cold 4% paraformaldehyde in PBS. The brains were postfixed for 48 h at 4°C in 4% paraformaldehyde and cryoprotected in 20% sucrose in PBS solution for at least 1 week. Then, 30 μm-thick serial coronal sections of the medulla were cut

with a cryostat, placed in an anti-freeze solution and stored at −20°C until use. Free-floating sections were rinsed (3 x 10 min) in PBS containing 0.3% Triton X-100 (PBS-TX), blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature, and washed again three times as above. Sections were then incubated for 3 days at 4°C with a rabbit polyclonal antibody anti-GABA dissolved in BSA (1:1000; NBP1-78346; Novus Biologicals, Cambridge, UK) and a mouse monoclonal antibody against 5-HT_{1A}R (clone 19A9.2) dissolved in BSA (1:500; MAB11041; Millipore Corporation, Billerica, MA, USA). After washing in PBS-TX (3 x 10 min), slices were subsequently incubated for 2 h in the dark at room temperature with a mixture of Alexa Fluor 488 goat anti-rabbit IgG (1:200; Invitrogen, Life Technologies, Carlsband, CA, USA) and Alexa Fluor 568 goat anti-mouse IgG (1:200; Invitrogen) dissolved in BSA. After washing in PBS-TX (3 x 10 min), tissue sections were mounted on gelatin-coated slides and coverslipped with glycerol containing 2.5% diazabicyclooctane (Sigma-Aldrich). Coronal sections of the medulla were examined by a epifluorescence microscope (Eclipse E400) equipped with the Nikon Intensilight C-HGFI mercury-fibre illuminator. Photomicrographs of key results were taken using a Nikon DS-Fi1 digital camera. Illustrations were prepared in Adobe Photoshop CS6 (Adobe Systems Incorporated). Images were only adjusted for brightness and contrast.

Although our rabbit polyclonal antibody anti-GABA has not been previously used in the rabbit, we are confident that it is specific for GABA. In fact, the pattern of distribution of GABAergic neurons produced by this antibody is similar to that obtained with different anti-GABA antibodies or with anti-GAD antibodies in the ventrolateral medulla of rabbits and rats (Blessing, 1990; Ellenberger, 1999), and in particular within the preBötC of the rat (Koizumi *et al.*, 2013). The specificity of the

primary antibody against GABA cannot be validated by using Western blot analysis. In fact, owing to the very small size of GABA molecule, this technique cannot be applied (Novus Biologicals and other suppliers producing antibodies against GABA). Also the mouse monoclonal antibody against 5-HT_{1A}Rs has not been previously used in the rabbit. However, the 5-HT_{1A} R expression pattern observed in the present study in the preBötC of the rabbit closely recalls that observed with a different antibody in the preBötC of the rat (Dutschmann *et al.*, 2009; Manzke *et al.*, 2009). Admittedly, Western blot validation of the specificity of the primary antibodies in the rabbit brain tissue would be appropriate. On the other hand, protein structures appear to be highly conserved throughout evolution and this antibody demonstrated cross-reaction with rats and humans (Millipore Corporation). This makes us confident that this antibody is specific also for rabbit brain tissue.

Data collection and statistical analysis

We measured the respiratory frequency (breaths/min), the inspiratory (T_I) and expiratory (T_E) times, as well as the peak amplitude of the integrated phrenic nerve activity that was normalized relative to mean control values (baseline) and expressed in arbitrary units (AU). The phrenic minute output (neural minute ventilation) was calculated as the product of phrenic tidal activity and respiratory frequency. The slope of the straight line drawn from the onset to 90% of the maximum level of the phrenic ramp was considered a reliable estimate of the inspiratory rate of rise (e.g. Bongianni *et al.*, 2002, 2008, 2010; Mutolo *et al.*, 2002, 2005). Respiratory variables were measured for an average of five consecutive breaths in the period immediately preceding each trial and at the time when the maximum response to drug microinjections occurred. In the same periods, systolic and diastolic blood pressure

were measured at 2-s intervals. Mean arterial pressure was calculated as the diastolic pressure plus one-third of the pulse pressure. Owing to the small variations in respiratory and cardiovascular variables within a measurement period, average values for each period were taken as single measurements for the purpose of statistical analysis (GraphPad Prism 7, GraphPad Software, Inc., La Jolla, CA, USA). The recovery of control values was followed for a maximum of two hours. In each preparation, we considered respiratory activity fully recovered when respiratory frequency and peak phrenic amplitude were within ± 3% of control values. Paired *t*-tests were employed to evaluate changes in cardiorespiratory variables induced by each drug or by vehicle solutions. Unpaired *t*-tests were employed to compare drug-induced cardiorespiratory changes in different preparations. One-way repeated-measures ANOVA followed by Bonferroni's multiple comparisons test was used to assess the effects of two or more treatments on cardiorespiratory variables. For simplicity, only respiratory frequency and peak phrenic amplitude were considered in this latter type of statistical analysis. Changes in respiratory variables were also expressed as percentage variations of control values. All values are presented as means ± SEM; $p < 0.05$ was considered as significant. In the legends, the level of significance is indicated as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Protocol 2: lampreys

Animal preparation

Experiments were carried out on 43 young adult (12-15 cm) sea lampreys (*Petromyzon marinus*). Animal preparation and experimental procedures were similar to those described in previous reports (Bongianni *et al.*, 1999; Mutolo *et al.*, 2007, 2010, 2011; Cinelli *et al.*, 2013, 2014, 2016, 2017). The animals were deeply anesthetized with tricaine methanesulphonate (100 mg l^{-1}; MS 222, Sigma-Aldrich, St. Louis, MO, USA). Then, the animals were immediately dissected to obtain isolated brainstem preparations, and thus the anesthesia was terminal. Lampreys were 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 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. Under these experimental conditions, already used in all previous studies on the isolated brainstem-spinal cord of the lamprey, the respiratory frequency is similar to that of spontaneous respiration of intact lampreys in the aquarium at 9-10 °C (Rovainen, 1977, 1996; Martel *et al.*, 2007; see also Cinelli *et al.*, 2014 for further Refs.). The brain was exposed and the choroid plexus removed; the brain tissue rostral to the optic tectum was cut and removed. A transection was made caudal to the obex at the level of the spinal cord. The roof of the isthmic region

was cut along the midline and the alar plates were spread laterally and pinned down.

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). Extracellular neuronal activity was recorded with fine (0.1 mm shaft diameter) tungsten microelectrodes (5 MΩ impedance at 1 kHz). Neuronal activity was recorded 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 (not shown; see e.g. Mutolo *et al.*, 2007; Cinelli *et al.*, 2013, 2014, 2017). These recordings were performed only with the purpose of ascertaining the localization of the pTRG for the subsequent execution of drug microinjections (see below). The obex was used as a reference point to evaluate coordinates of recording and microinjection sites. All the raw and integrated signals were analyzed by a personal computer supplied with an analog-to-digital interface (50 kHz sampling rate; Digidata 1440, Molecular Devices) and appropriate software (Axoscope, Molecular Devices). Clampfit software (Molecular Devices) was used for offline analysis.

Drug application and histology

Drugs were applied to the perfusing solution to reveal their respiratory effects. They could also be microinjected into the pTRG to ascertain their effects at this level. The preparation was perfused with the control solution for at least 60 min before control recordings to improve stability of respiratory motor output. The following drugs were

used: 40 μM or 1 mM 5-HT (endogenous agonist at 5-HTRs; Tocris Bioscience); 40 μM (*R*)-(+)-8-Hydroxy-DPAT hydrobromide (8-OH-DPAT; full 5-HT_{1A}R agonist, more active enantiomer; Tocris Bioscience); 100 μM methysergide maleate (mixed 5-$HT_{1/2}$R antagonist; Tocris Bioscience); 10, 100 μM or 1 mM (S)-WAY 100135 dihydrochloride (potent and selective 5-HT_{1A}R antagonist; Tocris Bioscience), 1 mM strychnine hydrochloride (glycine receptor antagonist; Sigma-Aldrich), 1 mM bicuculline methiodide ($GABA_A$ receptor antagonist; Sigma-Aldrich). Drugs were usually dissolved in distilled water, except for (S)-WAY 100135 that was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and diluted to volume with the perfusing solution. In the final solution, the concentration of DMSO was < 10%. Stock solutions were made up and stored as small aliquots in a freezer until use. Stock solutions were diluted in the perfusing solution to the final desired concentration immediately prior to bath application or to microinjections. Unless otherwise stated, only one drug was tested in each preparation. Drug concentrations, selected in preliminary trials, were similar to those employed in previous studies (see e.g. Johnson *et al.*, 2001; Antri *et al.*, 2008; Mutolo *et al.*, 2011; Cinelli *et al.*, 2014; Ostrowski *et al.*, 2014; Sardari *et al.*, 2015). Drugs applied to the bath were allowed to perfuse the brainstem for ~ 20 min. After completion of each drug challenge, either with bath application or microinjections, the preparation was allowed to recover by perfusing it with the control solution.

Bilateral microinjections (0.5-1 nl) of different drugs into the pTRG were performed by means of glass micropipettes (tip diameter 10-20 μm) and by applying pressure pulses of 50-100 ms with a Picospritzer (General Valve Corporation, Fairfield, NJ, USA) connected to the injection pipette (for the microinjection procedures see e.g. Cinelli *et al.*, 2014, 2017). 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 ranged from 20 to 30 s. The inactive dye Fast Green (0.2 %, Sigma-Aldrich) was added to the drug solution to visually assess the spread and the approximate localization of the injectate within the pTRG by judging the position of the dye spot with respect to the sulcus limitans of His and the isthmic Müller cell I_1 (Cinelli *et al.*, 2013, 2014, 2017). The depth of the injection (~ 0.3 mm below the dorsal surface) was inferred from that of rhythmic extracellular neuronal activity previously recorded in each preparation. On some occasions (n = 3) green fluorescent latex microspheres (LumaFluor) were added (dilution 1:3) to the drug solution (1 mM (S)-WAY 100135) for post hoc confirmation of injection sites (for details see Cinelli *et al.*, 2014). Control microinjections of equal volumes of the vehicle solution with 0.2 % Fast Green dye were also made. After each experiment, the brainstem was fixed (4 % formalin in 0.1 M phosphate buffer (PB), pH 7.4, overnight), cryoprotected with 30 % sucrose, frozen, and cut at 20 µm thickness on a cryostat. Coronal sections stained with cresyl violet were used for the histological control.

Immunohistochemistry

Experiments were performed for immunohistochemical detection of glycinergic neurons and 5-HT_{1A}R expression within the pTRG. The dissection, fixation and sectioning of the lamprey brains were performed as previously described (Cinelli *et al.*, 2013, 2014, 2017). The preparation was similar to that employed in the electrophysiological experiments described above. The brains were dissected out of the surrounding tissue and fixed by immersion in 2 % formalin /0.1 M PB pH 7.4 for

12-24 hours. The brains were postfixed for 24-48 h after which they were cryoprotected in 20% sucrose/PB for 3-12 hours. Transverse 20 µm-thick sections were made using a cryostat, collected on gelatin-coated slides and stored at -20°C until further processing. Primary and secondary antibodies were diluted in 1% bovine serum albumin (BSA), 0.3 % Triton X-100 in 0.1 M PB. Sections were incubated for 24 h at 4°C with a rat polyclonal anti-glycine antibody (1.5000; IG1002; ImmunoSolutions, Jesmond, New South Wales, Australia) and a mouse monoclonal antibody anti-serotonin receptor 1_A (clone 19A9.2; 1:500; MAB11041; Millipore Corporation). After a thorough rinse in 0.1 M PB pH 7.4, sections were incubated with a mixture of Alexa Fluor 488 goat anti-mouse IgG (1:200; Invitrogen) and Alexa Fluor 568 goat anti-rat IgG (1:200; Invitrogen) for 2 h at room temperature. Sections were then coverslipped with glycerol containing 2.5% diazabicyclooctane (Sigma-Aldrich). Photomicrographs of key results were taken using a Nikon DS-Fi1 digital camera and software. Illustrations were prepared in Adobe Photoshop CS6 and Adobe Illustrator CS6 (Adobe Systems Incorporated). Images were only adjusted for brightness and contrast. The specificity of glycine antibody used is known from previous studies in the lamprey (Mahmood *et al.*, 2009; Cinelli *et al.*, 2016). The mouse monoclonal antibody against 5-HT_{1A}Rs has not been previously used in the lamprey. However, given the reasons already mentioned in the Methods of *Protocol 1*, we are confident that this antibody is specific also for lamprey neuronal structures.

Data analysis

Respiratory frequency (cycles min^{-1}), vagal burst duration (ms, measured on raw activity), and peak amplitude of integrated vagal activity (taken as an index of the intensity of vagal bursts, arbitrary units) were measured and averaged for 20 s in

the period immediately preceding each trial (control values). The respiratory variables were also recorded and measured at 1-min intervals during the experimental challenge and after recovery. In two experiments where (S)-WAY 100135 was bilaterally microinjected into the pTRG, long trace recordings were also performed. As a rule, average values of respiratory variables observed in control conditions and at the time when the maximum response occurred were taken for statistical analysis (GraphPad Prism 7, GraphPad Software, Inc.). We considered respiratory activity fully recovered when the studied variables were within ± 3% of their control values. One-way repeated-measures ANOVA followed by Bonferroni's multiple comparisons tests were used to assess the effects of two different treatments. The time courses of respiratory responses to bath application of (S)-WAY 100135 were also investigated at short-time intervals by using the same statistical procedure. Student's paired *t*-tests were also employed when appropriate. Changes in respiratory variables were also expressed as percentage variations of control values. The number of preparations is indicated by *n*. An investigation on the prolonged vagal bursts, described as "coughs", was considered beyond the scope of the present research. All values are presented as means ± SEM; $p < 0.05$ was considered as significant.

Results

Protocol 1: rabbits

Excitatory respiratory effects of 5-HT

Bilateral microinjections of 5 mM 5-HT (150-250 pmol; n = 6) into the preBötC region induced excitatory effects on inspiratory activity (Fig. 4A) mainly consisting of increases in respiratory frequency from 44.1 ± 2.2 to 59.4 ± 2.0 breaths min^{-1} (35.9 ± 5.6 %; $t_{(5)}$ = 7.09, p = 0.0009) due to reductions in T_I (from 0.49 ± 0.01 to 0.41 ± 0.01 s; $t_{(5)}$ = 7.81, p = 0.0006) and T_E (from 0.89 ± 0.06 to 0.61 ± 0.03 s; $t_{(5)}$ = 5.83, p = 0.0021). These effects were associated with small, but significant reductions in peak phrenic amplitude (8.3 ± 1.7 %; $t_{(5)}$ = 43.2, p < 0.0001). Increases in both inspiratory rate of rise (11.5 ± 3.7 %; $t_{(5)}$ = 3.09, p = 0.027) and phrenic minute output (24.7 ± 5.3 %; $t_{(5)}$ = 4.88, p = 0.0045) were observed. The respiratory responses induced by 5-HT developed progressively, showing a maximum within 10 min while a complete recovery was observed within 60 min. Bilateral microinjections of 5 mM 5-HT into the BötC (n = 4) and the iVRG (n = 4) did not cause any obvious and consistent effects on respiratory activity. Histograms in Fig. 4B illustrate changes in some respiratory variables in response to 5 mM 5-HT microinjected into the preBötC, the BötC and the iVRG.

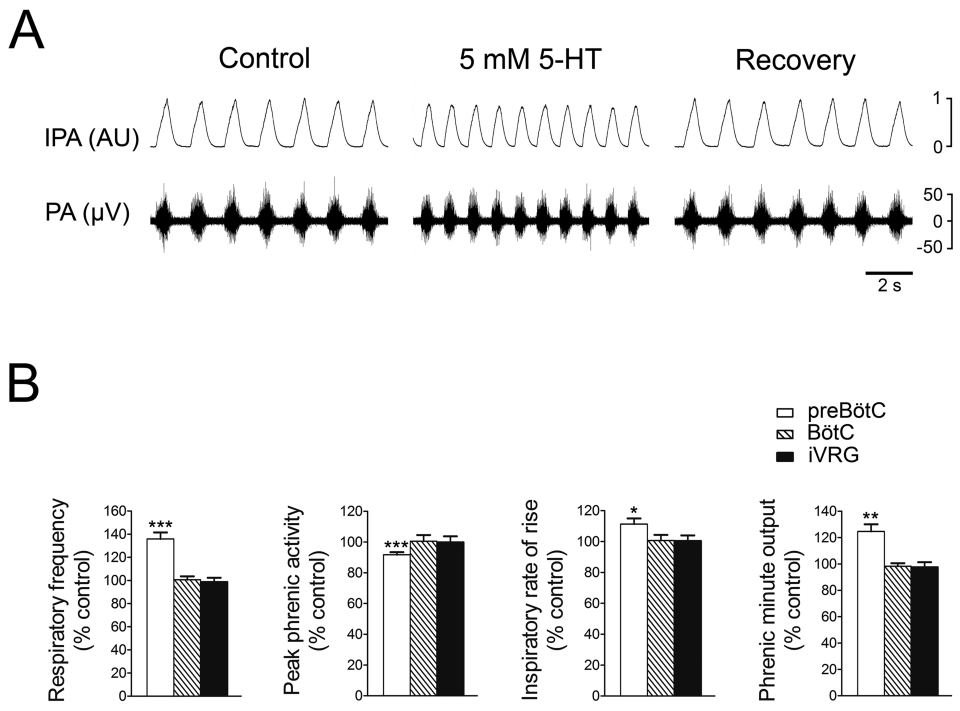

Fig. 4. Respiratory responses caused by bilateral microinjections of 5 mM 5-HT into different subregions of the ventral respiratory group in the rabbit

A. Only 5-HT applied to the preBötC region caused marked excitatory effects on the respiratory motor output. Traces are integrated phrenic nerve activity (IPA) and raw phrenic nerve activity (PA) under control conditions, ~10 and 60 min (recovery) after the completion of 5-HT microinjections. AU, arbitrary units. **B.** Histograms illustrating changes in respiratory frequency, peak phrenic activity, inspiratory rate of rise and phrenic minute output elicited by 5 mM 5-HT microinjected into the preBötC (n = 6), the BötC (n = 4) and the iVRG (n = 4). Values are means ± SEM. *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

Respiratory effects of 5-HT receptor agonists microinjected into the preBötC

To ascertain the contribution of different types of receptors possibly involved in 5-HT-induced respiratory effects, microinjections of selective receptor agonists were performed into the preBötC. Bilateral microinjections of the 5-HT_{1A}R agonist 8-OH-DPAT at 1 mM (30-50 pmol; $n = 6$) induced mean increases in respiratory frequency of 46.4 ± 6.8 % (from 43.7 ± 0.6 to 63.9 ± 2.9 breaths min^{-1}; $t_{(5)} = 6.09$, $p = 0.001$) due to reductions in both T_I (from 0.55 ± 0.01 to 0.41 ± 0.02 s; $t_{(5)} = 7.09$, $p = 0.0009$) and T_E (from 0.82 ± 0.01 to 0.54 ± 0.03 s; $t_{(5)} = 9.14$, $p = 0.0003$) accompanied by reductions in peak phrenic amplitude (12.1 ± 0.9 %; $t_{(5)} = 11.67$, $p < 0.0001$). Consistent increases in the inspiratory rate of rise (18.5 ± 5.9 %; $t_{(5)} = 3.43$, $p = 0.0187$) and the phrenic minute output (28.9 ± 6.8 %; $t_{(5)} = 5.17$, $p = 0.0035$) were also seen. Maximum effects and complete recovery occurred 10 and 90 min after the completion of the injections, respectively (Fig. 5A,C). Similar excitatory effects were induced by bilateral microinjections of 1 mM BP 554 (30-50 pmol; $n = 4$), another selective 5-HT_{1A}R agonist (Fig. 5C). Indeed, in the above mentioned experiments we used (*R*)-(+)-8-Hydroxy-DPAT hydrobromide, i.e. the enantiomer form of this agonist, that has been described as selective for 5-HT_{1A}Rs (e.g. Wieronska *et al.*, 2015). However, since it has been reported that this agonist, probably in the racemic form (±)-8-Hydroxy-DPAT (Sigma-Aldrich) can be a partial agonist at the 5-HT_7Rs (Manzke *et al.*, 2009; Corcoran *et al.*, 2014), we applied 1 mM (*R*)-(+)-8-Hydroxy-DPAT hydrobromide in the presence of the 5-HT_7R antagonist SB 258719 ($n = 4$). A repeated-measures ANOVA performed to evaluate this issue provided significant results ($F_{(2,6)} = 76.56$, $p = 0.0026$ for respiratory frequency; $F_{(2,6)} = 25.20$, $p = 0.0152$ for peak phrenic amplitude). *Post hoc* analysis showed that bilateral microinjections of 5 mM SB 258719 (150-250 pmol) did not

change respiratory activity within 10 min, while microinjections of 1 mM 8-OH-DPAT performed at the same sites after this time interval caused increases in respiratory frequency (from 44.2 ± 2.1 to 62.2 ± 2.7 breaths min^{-1}; 43.1 ± 5.1 %, p = 0.006) and reductions in peak phrenic amplitude (9.7 ± 1.6 %; p = 0.0239). All these outcomes indicate that 8-OH-DPAT effects were mediated only by 5-HT_{1A}Rs. Accordingly, local application to the preBötC of the 5-HT_7R agonist LP 44 (1 mM, 30-50 pmol) in few trials (n = 4) did not cause any obvious change in respiratory variables (Fig. 5C).

Excitatory effects on inspiratory activity were obtained using a specific agonist of the excitatory 5-HT_3Rs (Fig. 5B,C). Bilateral microinjections of 5 mM PBG (150-250 pmol; n = 6) caused increases in respiratory frequency of 15.8 ± 3.5 % (from 41.3 ± 1.3 to 47.7 ± 2.5 breaths min^{-1}; $t_{(5)}$ = 4.03, p = 0.01) due to reductions in both T_I (from 0.58 ± 0.04 to 0.47 ± 0.02 s; $t_{(5)}$ = 5.27, p = 0.0033) and T_E (from 0.89 ± 0.03 to 0.80 ± 0.04 s; $t_{(5)}$ = 3.36, p = 0.0201). Peak phrenic amplitude did not show significant changes, while the inspiratory rate of rise (18.1 ± 4.9 %; $t_{(5)}$ = 3.84, p = 0.0122) and phrenic minute output (10.5 ± 3.4 %; $t_{(5)}$ = 2.97, p = 0.0310) increased. Maximum effects were seen within 10 min, while the complete recovery occurred within 60 min after the completion of the injections. On the contrary, bilateral microinjections of the 5-HT_{1B}R agonist CP 93129 (1 mM, 30-50 pmol; n = 4), the 5-HT_{2A}R agonist TCB-2 (1 mM, 30-50 pmol; n = 4), the 5-HT_{2B}R agonist BW 723C86 (1 mM, 30-50 pmol; n = 4) and the 5-HT_4R agonist BIMU 8 (1 mM, 30-50 pmol; n = 4) did not affect the ongoing pattern of breathing (Fig. 5C).

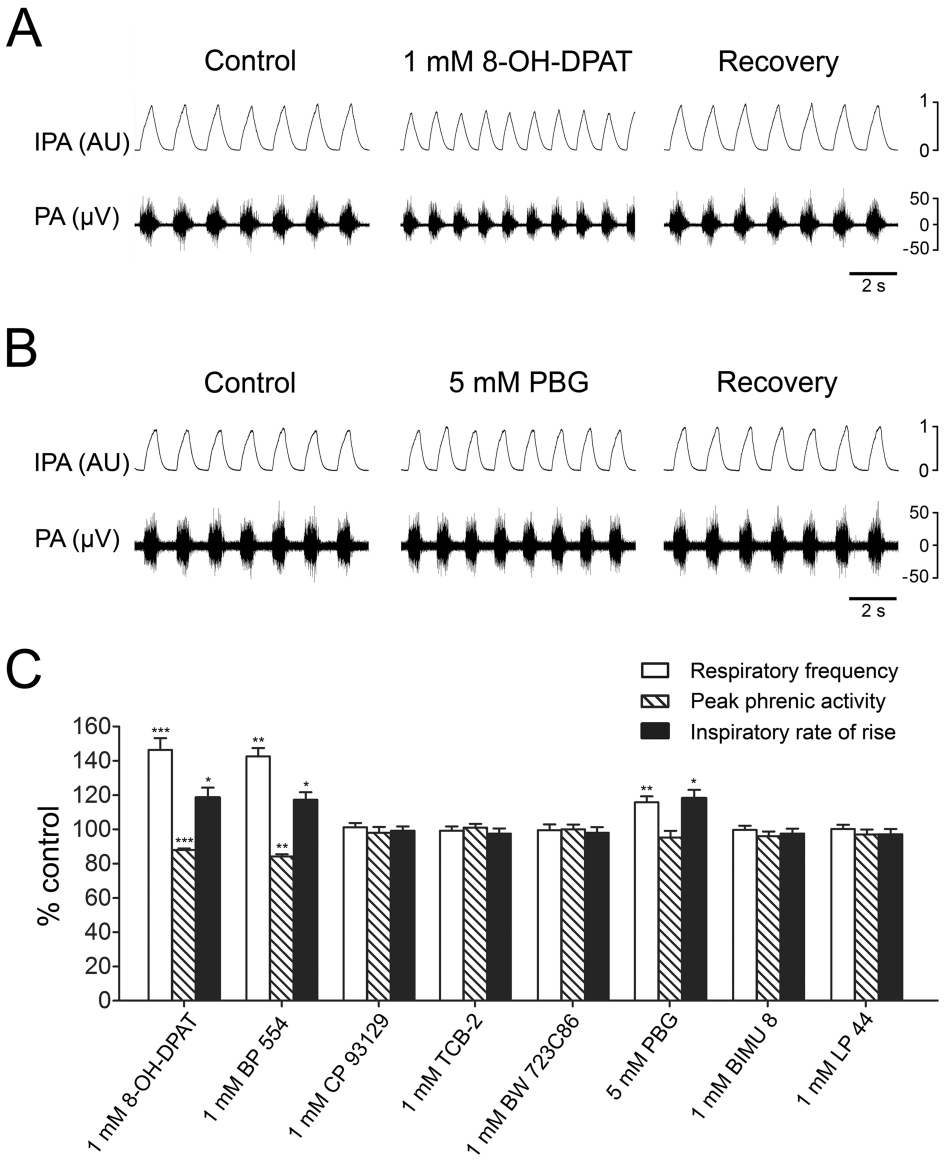

Fig. 5. Respiratory responses elicited by 5-HT receptor agonists microinjected into the preBötC of the rabbit

A. Respiratory effects ~ 10 min after bilateral microinjections of the 5-HT_{1A}R agonist 8-OH-DPAT. Recovery 90 min after 8-OH-DPAT. **B.** Respiratory effects ~ 10 min after bilateral microinjections of the 5-HT_3R agonist PBG. Recovery 60 min after PBG. AU, arbitrary units; IPA, integrated phrenic nerve activity; PA, raw phrenic nerve activity. **C.** Histograms illustrating changes in respiratory frequency, peak phrenic activity and inspiratory rate of rise elicited by 8-OH-DPAT (n = 6), BP 554 (n = 4), CP 93129 (n = 4), TCB-2 (n = 4), BW 723C86 (n = 4), PBG (n = 6), BIMU 8 (n = 4) and LP 44 (n = 4). Values are means ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Respiratory effects of 5-HT receptor antagonists microinjected into the preBötC

Somewhat unexpectedly, bilateral microinjections of the 5-$HT_{1/2}$R antagonist methysergide at 5 mM (150-250 pmol; n = 5) increased respiratory frequency from 43.9 ± 0.5 to 58.0 ± 1.2 breaths min^{-1} (31.9 ± 1.7 %; $t_{(4)}$ = 16.46, p < 0.0001) due to reductions in both T_I (from 0.62 ± 0.01 to 0.49 ± 0.01 s; $t_{(4)}$ = 9.44, p = 0.0007) and T_E (from 0.74 ± 0.02 to 0.54 ± 0.02 s; $t_{(4)}$ = 36.51, p < 0.0001). These effects were accompanied by reductions in peak phrenic amplitude (8.0 ± 1.6 %; $t_{(4)}$ = 16.06, p < 0.0001) and increases in the inspiratory rate of rise (16.2 ± 3.1 %; $t_{(4)}$ = 4.88, p = 0.0081) and phrenic minute output (21.4 ± 2.0 %; $t_{(4)}$ = 4.75, p = 0.0089). Changes in some respiratory variables have been reported in Fig. 6A. On the basis of the results obtained with 5-HTR agonists, we inferred that methysergide-induced effects were caused by the blockade of 5-HT_{1A}Rs. As illustrated in Fig. 6, bilateral microinjections of the selective 5-HT_{1A}R antagonist (S)-WAY 100135 at 5 mM (150-250 pmol; n = 6) induced marked increases in respiratory frequency (from 43.5 ± 0.3 to 68.6 ± 1.2 breaths min^{-1}; 57.5 ± 3.4 %; $t_{(5)}$ = 18.29, p < 0.0001) due to decreases in both T_I (from 0.57 ± 0.01 to 0.39 ± 0.01 s; $t_{(5)}$ = 10.60, p = 0.0001) and T_E (from 0.80 ± 0.01 to 0.48 ± 0.01 s; $t_{(5)}$ = 19.20, p < 0.0001). Reductions in peak phrenic amplitude (17.0 ± 3.3 %; $t_{(5)}$ = 4.81, p = 0.0048) and increases in both the inspiratory rate of rise (19.9 ± 2.02 %; $t_{(5)}$ = 10.56, p = 0.0001) and phrenic minute output (30.6 ± 5.4 %; $t_{(5)}$ = 6.32, p = 0.0015) were also observed (Fig. 6A,B). The excitatory effects of both methysergide and (S)-WAY 100135 developed progressively and reached a maximum within 10 min after the completion of the injections. A complete recovery was observed within 90 min. On the contrary, microinjections of the 5-HT_3R antagonist ondansetron (5 mM, 150-250 pmol; n = 4) did not alter the breathing pattern (see Fig. 6A and Fig. 7A, middle panel), thus

indicating that 5-HT_3Rs do not exert a tonic influence on baseline respiratory activity. A repeated-measures ANOVA revealed that microinjections of 5 mM ondansetron performed in the same four preparations prevented the respiratory responses induced by 5 mM PBG microinjected into the same sites after an interval of ~ 5 min ($F_{(2,6)} = 0.04$, $p = 0.95$ for respiratory frequency; $F_{(2,6)} = 0.83$, $p = 0.4455$ for peak phrenic amplitude), thus showing that 5-HT_3Rs were completely blocked (Fig. 7A, right panel).

We reasoned that the excitatory effects induced by the blockade of presynaptic 5-HT_{1A}Rs could be due to an increase in the release of 5-HT acting in turn on 5-HT_3Rs. An additional set of experiments ($n = 4$) provided results consistent with this interpretation. A repeated-measures ANOVA ($F_{(5,15)} = 41.04$, $p = 0.0044$ for respiratory frequency; $F_{(5,15)} = 5.247$, $p = 0.0478$ for peak phrenic activity) showed (*post hoc* analysis) that bilateral microinjections of 5 mM ondansetron prevented the respiratory responses caused by microinjections of 5 mM (S)-WAY 100135 into the same sites made after ~ 5 min and that microinjections of 5 mM 5-HT performed ~ 20 min after (S)-WAY 100135, i.e. during the blockade of both 5-HT_3Rs and 5-HT_{1A}Rs, failed to induce any appreciable change in the respiratory motor output (Fig. 7B1). Of note, the blockade of both 5-HT_3Rs and 5-HT_{1A}Rs did not cause any change in the control respiratory variables (Fig. 7B1, middle panel). *Post hoc* analysis also proved that the characteristic excitatory responses to 5 mM 5-HT microinjections were evoked again after > 90 min, i.e. after a time interval judged sufficient for the complete washout of (S)-WAY 100135 ($p = 0.0241$ for respiratory frequency, $p = 0.0457$ for peak phrenic activity). An example of these findings is reported in Fig. 7B2.

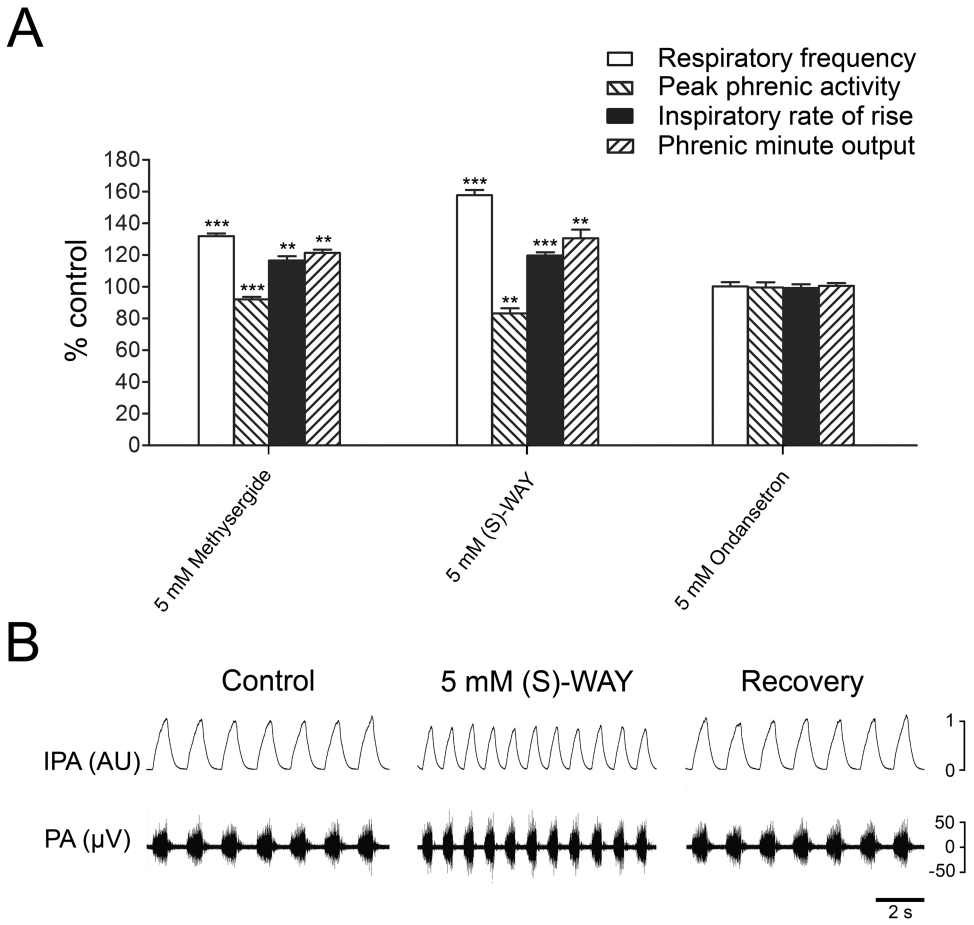

Fig. 6. Respiratory effects of some 5-HT receptor antagonists microinjected into the preBötC of the rabbit

A. Histograms illustrating changes in respiratory frequency, peak phrenic activity, inspiratory rate of rise and phrenic minute output induced by methysergide (n = 5), (S)-WAY 100135 (n = 6) and ondansetron (n = 4). Values are means ± SEM. **p < 0.01; ***p < 0.001. **B.** Respiratory effects ~ 10 min after bilateral microinjections of the 5-HT_{1A}R antagonist (S)-WAY 100135. Recovery ~ 80 min after (S)-WAY 100135. AU, arbitrary units; IPA, integrated phrenic nerve activity; PA, raw phrenic nerve activity.

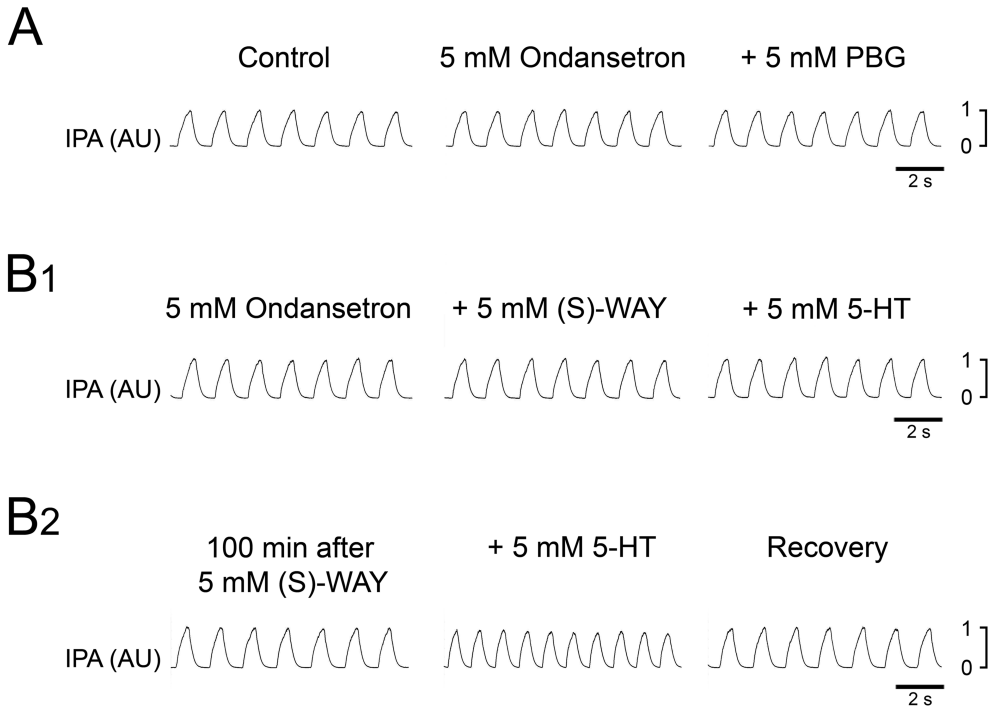

Fig. 7. Role of 5-HT$_3$ receptor antagonist in counteracting the effects mediated by 5-HT$_3$ and 5-HT$_{1A}$ receptors in the rabbit

A. Microinjections of ondansetron did not change the ongoing pattern of breathing, but prevented the excitatory responses of PBG injected into the same sites after an interval of 5 min (as observed 15 min after PBG). **B1.** During the blockade of 5-HT$_3$Rs, microinjections of (S)-WAY 100135 performed ~5 min after the completion of ondansetron microinjections did not cause obvious respiratory changes. After an interval of ~20 min (i.e. during the blockade of both 5-HT$_3$Rs and 5-HT$_{1A}$Rs), microinjections of 5-HT were ineffective (observation made at ~ 15 min after 5-HT). **B2.** After an interval > 90 min after (S)-WAY 100135 scheduled to allow a complete washout of the employed drugs, 5-HT induced again the characteristic excitatory responses (observation made ~ 10 min after the injections). The recovery ~ 60 min after 5-HT has also been reported. AU, arbitrary units; IPA, integrated phrenic nerve activity.

Role of disinhibition in the respiratory responses caused by 5-HT$_{1A}$ receptor activation

The possibility that 8-OH-DPAT effects were mediated by inhibition of inhibitory neurons located within the preBötC (Manzke *et al.*, 2009; Corcoran *et al.*, 2014) was addressed by investigating the responses induced by this agonist during the blockade of glycine or GABA$_A$ receptors. In the study of 8-OH-DPAT effects during glycine receptor blockade (n = 5), a repeated-measures ANOVA showed significant changes in the respiratory variables ($F_{(2,8)}$ = 105.2, $p < 0.0001$ for respiratory frequency; $F_{(2,8)}$ = 73.7, p = 0.0009 for peak phrenic amplitude). In more detail (*post hoc* tests), bilateral microinjections of 5 mM strychnine (150-250 pmol) induced within 5 min mild increases in respiratory frequency from 43.5 ± 0.7 to 53.5 ± 1.6 breaths min^{-1} (23.1 ± 4.1%; p = 0.0132) associated with decreases in peak phrenic amplitude (21.2 ± 3.4%; p = 0.0089) in agreement with our previous results (Bongianni *et al.*, 2010). The subsequent (~ 10 min interval) microinjections of 1 mM 8-OH-DPAT at the same sites caused within 10 min consistent further increases in respiratory frequency of 36.4 ± 5.4% (from 53.5 ± 1.6 to 72.7 ± 1.9 breaths min^{-1}; p = 0.0054) accompanied by further decreases in peak phrenic amplitude (11.2 ± 0.4%; p = 0.0003). These outcomes are illustrated in Fig. 8A.

Our observation on the effects of 8-OH-DPAT during GABA$_A$ receptor blockade provided the following results (Fig. 8B). In agreement with our previous findings (Bongianni *et al.*, 2010), bilateral microinjections of 5 mM bicuculline (150-250 pmol; n = 4) into the preBötC elicited within 3 min a pattern of breathing characterized by an overall decrease in respiratory frequency and the presence of two alternating different levels of peak phrenic activity. A repeated-measures ANOVA showed significant changes in the respiratory variables ($F_{(2,6)}$ = 35.6, p = 0.0031 for

respiratory frequency; $F_{(4,12)} = 85.3$, $p = 0.0007$ for peak phrenic amplitude). *Post hoc* tests showed that bilateral microinjections of bicuculline induced decreases in respiratory frequency from 38.3 ± 2.0 to 28.7 ± 3.4 breaths min^{-1} (26.2 ± 5.1%; $p = 0.0263$) associated with a level of peak phrenic activity higher than control (21.8 ± 1.5 %; $p = 0.0009$) and a second level of peak phrenic activity lower than control (26.7 ± 1.7%; $p = 0.0088$). The statistical analysis also showed that the subsequent (~10 min interval) bilateral microinjections of 1 mM 8-OH-DPAT at the same sites did not induce significant changes in the already disordered pattern of breathing, thus showing that bicuculline-induced disinhibition actually prevented 8-OH-DPAT excitatory effects. In more detail, paired t-tests showed that respiratory variables maintained similar values ($p > 0.05$; details of statistical analysis not shown). Respiratory activity recovered control levels within 90 min.

To corroborate these results, we sought for the presence of GABAergic neurons and 5-HT_{1A}Rs within the preBötC by double-labelling experiments ($n = 3$). GABA-immunoreactive structures were found (green signal) within the region corresponding to the preBötC (Fig. 9). The nucleus ambiguus, along with stereotaxic coordinates and data derived from the histological control of injection sites (see below), were used to identify the location of the preBötC region, that resulted to be ventral and slightly medial to the nucleus ambiguus. As illustrated in Fig. 9, photomicrographs at a higher magnification of the portion of section (white rectangle in A) including part of the preBötC show that GABAergic-immunoreactive structures (A1, green signal) are present in this region and that 5-HT_{1A}Rs (A2, red signal) are widely expressed in association with the soma of GABAergic-immunoreactive neurons (A3, merged image).

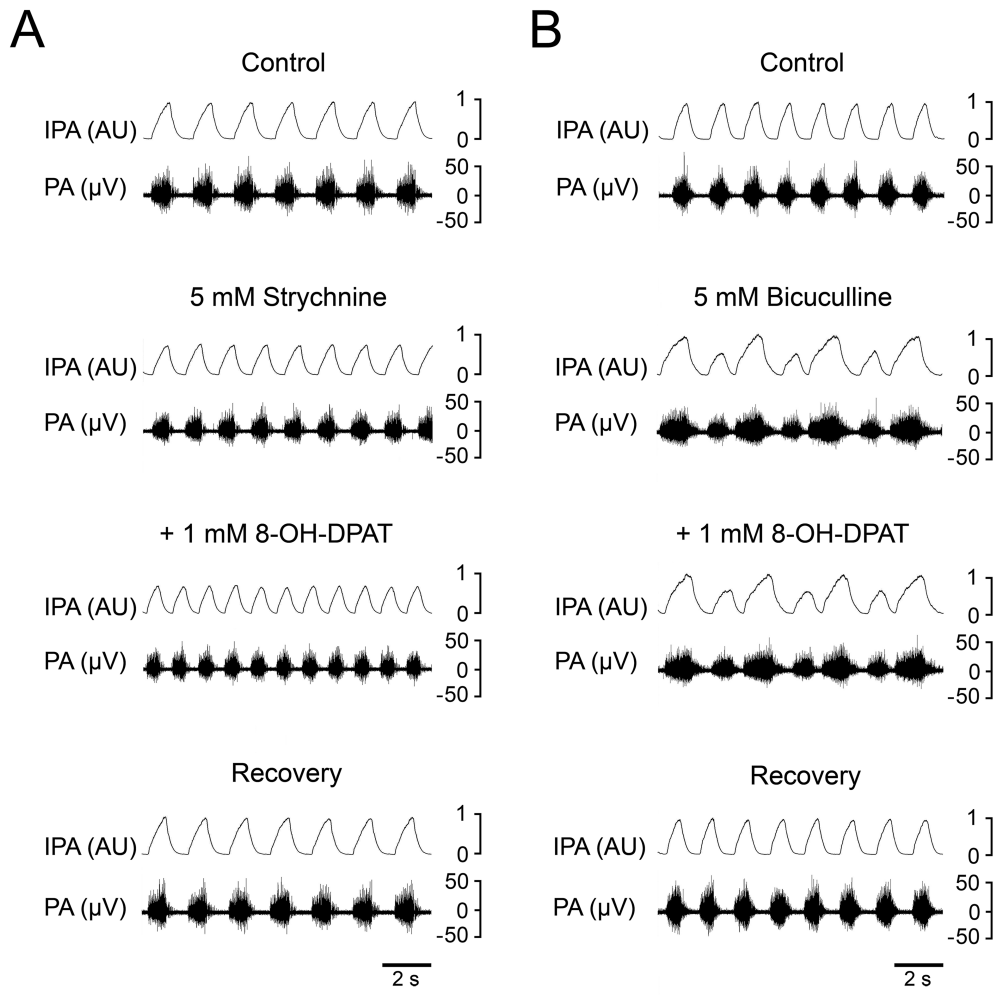

Figure 8. Glycine and $GABA_A$ receptors in the mediation of 8-OH-DPAT respiratory effects in the rabbit

A. Increases in respiratory frequency and decreases in peak phrenic amplitude observed ~ 5 min following strychnine microinjections into the preBötC. Additional changes in respiratory activity ~ 10 min after the subsequent 8-OH-DPAT microinjections performed into the same sites. Recovery ~ 90 min after 5-HT_{1A}R agonist microinjections. **B.** A breathing pattern characterized by the presence of two alternating different levels of peak phrenic activity ~ 5 min after bicuculline microinjections into the preBötC. Absence of changes in this altered pattern of breathing ~ 15 min following the subsequent 8-OH-DPAT microinjections into the same sites. Recovery taken ~ 70 min after 5-HT_{1A}R agonist microinjections. AU, arbitrary units; IPA, integrated phrenic nerve activity; PA, raw phrenic nerve activity.

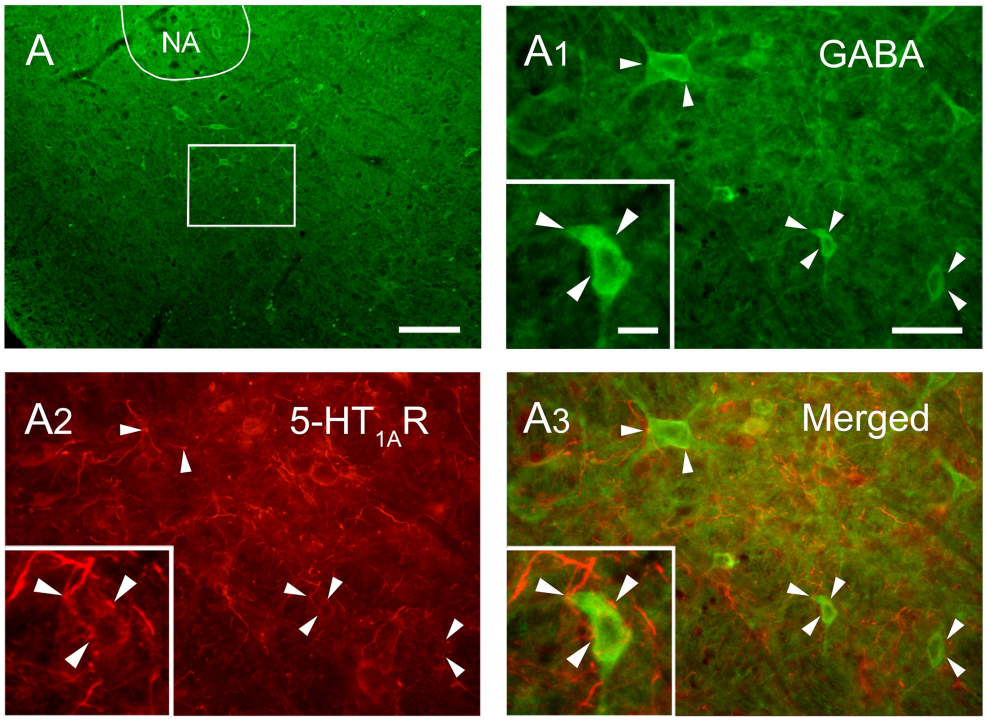

Fig. 9. Double immunostaining of GABAergic structures and 5-HT_{1A} receptors in the preBötC of the rabbit

A. Photomicrograph of a coronal section of the medulla oblongata at the level of the preBötC (~ 2.6 mm rostral to the obex) showing the distribution of GABA-immunoreactive structures (green signal). NA, nucleus ambiguus. The white box ventral and slightly medial to the NA delineates a portion of the preBötC region. (A1-A3) Photomicrographs at a higher magnification of the portion of the transverse section indicated by the white box in A showing GABAergic immunoreactive structures (A1, green signal), 5-HT_{1A}R binding sites (A2, red signal) and merged image (A3). Arrowheads point to the localization of 5-HT_{1A}R-immunoreactive structures located in close apposition to GABAergic neurons. The insets (higher magnification) indicate the location of some 5-HT_{1A}R-immunoreactive dots in close proximity to a single GABAergic neuron. Scale bars: A, 200 µm; A1-A3, 50 µm; insets, 10 µm.

Controls

The location of injection sites was confirmed by the histological control (Fig. 10). The distribution of sites within the preBötC where 5 mM 5-HT was microinjected turned out to be ventral to the nucleus ambiguus and is diagrammatically illustrated in Fig. 10B. An example of the localization of fluorescent beads is reported in Fig. 10C. The specificity of drug-induced responses was confirmed by the results obtained with control microinjections. According to previous studies (e.g. Mutolo *et al.*, 2002, 2005; Bongianni *et al.*, 2010), bilateral microinjections of 5 mM 5-HT into medullary regions sufficiently far (> 0.8 mm) from the responsive area did not induce any significant change in the ongoing respiratory activity. These control microinjections were performed both in the lateral direction (4 trials) and in the rostrocaudal direction, i.e. the microinjections performed into the BötC and iVRG. In addition, similar control bilateral microinjections (4 trials) were made into the nucleus tractus spinalis nervi trigemini or the nucleus gigantocellularis of the same preparations already employed in the study of 5-HT-induced respiratory effects. Control microinjections of equal volumes of the vehicle solutions performed in some preparations before 5-HT (3 trials) or (S)-WAY 100135 (3 trials) microinjections (0.9% NaCl solution and DMSO <10% in saline, respectively) into the preBötC did not cause appreciable effects. Noticeably, all the observed respiratory responses occurred without any significant concomitant change in mean arterial blood pressure that ranged between 90 and 103 mmHg in the different preparations. In the same preparation, small and inconsistent changes in mean arterial blood pressure were observed during each experiment (as a rule < 5%).

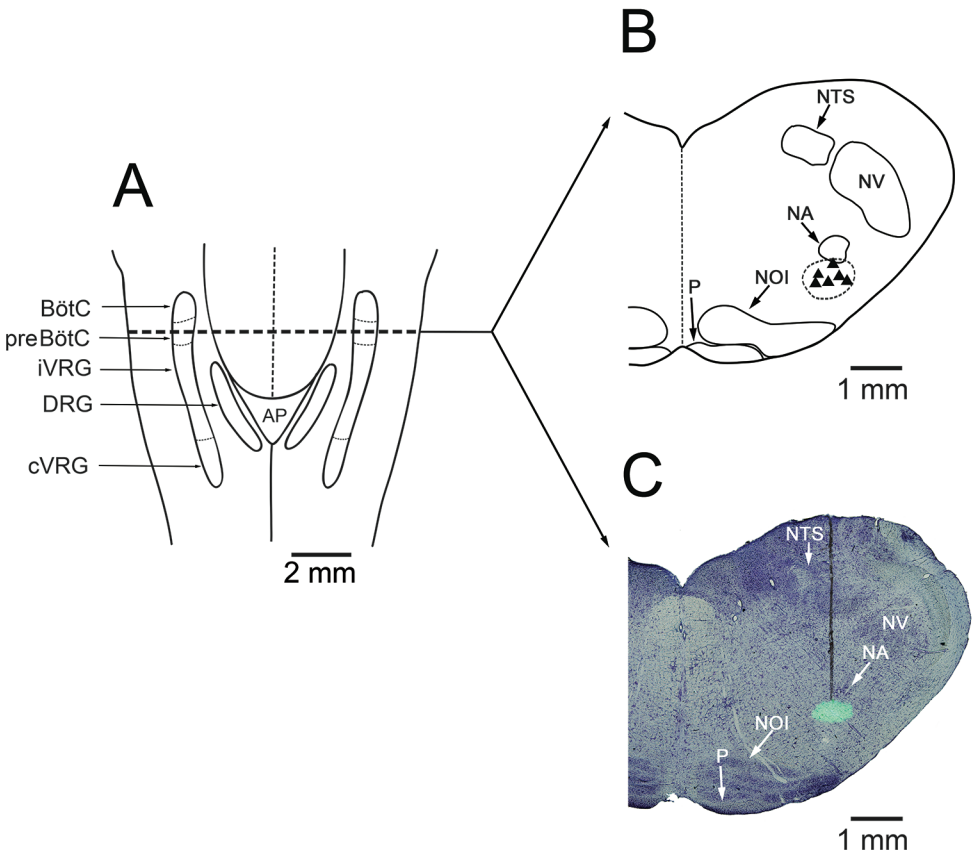

Fig. 10. Localization of injection sites in the rabbit

A. Diagrammatic representation of a dorsal view of the medulla oblongata of the rabbit illustrating some of the main components of the respiratory network. AP, area postrema; BötC, Bötzinger complex; cVRG, caudal ventral respiratory group; DRG, dorsal respiratory group; iVRG, inspiratory ventral respiratory group; preBötC, preBötzinger complex. **B.** Diagram of a coronal section of the medulla oblongata at the level (~ 2.5 mm rostral to the obex) indicated in A (dashed line) showing the distribution of sites (▲) where microinjections of 5 mM 5-HT were performed. Outlines of the map derive from a selected section of one histological preparation. The atlas of Shek et al. (1986) was used for comparison. **C.** Photomicrograph of a coronal section of the medulla oblongata at the same level as in B showing an example of the location of fluorescent beads added to the 5-HT solution microinjected into the preBötC. The histological section is counterstained with Cresyl violet. Light-field and fluorescent photomicrographs have been superimposed. The preBötC region is approximately indicated by dashed lines in **B**. NA, nucleus ambiguus; NOI, nucleus olivaris inferior; NTS,

nucleus tractus solitarii; NV, nucleus tractus spinalis nervi trigemini; P, tractus pyramidalis.

Protocol 2: lampreys

Role of 5-HT_{1A} receptors within the lamprey respiratory network

Given the pivotal role of 5-HT_{1A}Rs in the modulation of a different rhythmic motor activity in the lamprey, i.e. locomotion, we focused our attention on this receptor subtype. At variance with the results obtained in the rabbit, bath application in three lampreys of 40 μM 5-HT or 40 μM 8-OH-DPAT, the selective 5-HT_{1A}R agonist, as well as microinjections of 1 mM 5-HT or 1 mM 8-OH-DPAT into the pTRG of four lampreys did not cause obvious and consistent effects on respiratory activity (not shown). Thus, we sought to ascertain possible tonic serotoninergic influences by bath application of both the 5-$HT_{1/2}$R antagonist methysergide at 100 μM (n = 3) and the selective 5-HT_{1A}R antagonist (S)-WAY 100135 at 100 μM (n = 4). Surprisingly, both antagonists induced apnea. The apneic responses induced by (S)-WAY 100135 are illustrated in Fig. 11. Respiratory frequency that was 60.5 ± 2.7 cycles min^{-1} under control conditions started to decrease within 2 min after bath application of the antagonist and apnea ensued within 10 min. These latter effects were accompanied by progressive reductions in peak vagal activity. Respiratory activity completely recovered within 70 min washout.

The effects of S-WAY 100135 at the pTRG level, investigated by using microinjection techniques, proved to be dose-dependent. Bilateral microinjections of S-WAY 100135 at 10 μM (5-10 fmol; n = 3) were ineffective, at 100 μM (0.05-0.1 pmol; n = 3) caused depressant effects on respiratory activity and at 1 mM (0.5-1 pmol; n = 4) induced apnea. As illustrated in Fig. 12A, S-WAY 100135 at 100 μM reduced within 5 min respiratory frequency from 58.5 ± 1.9 to 36.5 ± 5 cycles min^{-1} (38.3 ± 6.9 %; $t_{(3)}$ = 6.535, p = 0.0073) and peak vagal activity (41 ± 3.5 %; $t_{(3)}$ = 14.1, p = 0.0008), without significant concomitant changes in the duration of vagal

burst. This decrease in respiration persisted for ~ 10 min. The recovery was achieved within 40 min. The apnea induced by 1 mM (S)-WAY 100135 microinjected into the pTRG ensued ~ 3 min after the completion of the injections and persisted for ~ 15 min. Respiratory activity completely recovered within ~ 60 min (Fig. 12B)

The localization of one (S)-WAY 100135 injection site into the pTRG is illustrated in Fig. 13. It is consistent with the results of our previous studies (Mutolo *et al.*, 2007, 2011; Cinelli *et al.*, 2014).

In 3 preparations, control microinjections of equal volumes of the vehicle solution containing 0.2 % Fast Green dye at the responsive sites (3 trials) as well as bilateral microinjections of 1 mM S-WAY 100135 at sites > 0.4 mm far from the responsive region (4 trials) did not induce any apparent respiratory response. These control microinjections were made into the trigeminal motor nucleus as well as at more rostral sites into the mesencephalic region (see Cinelli *et al.*, 2013).

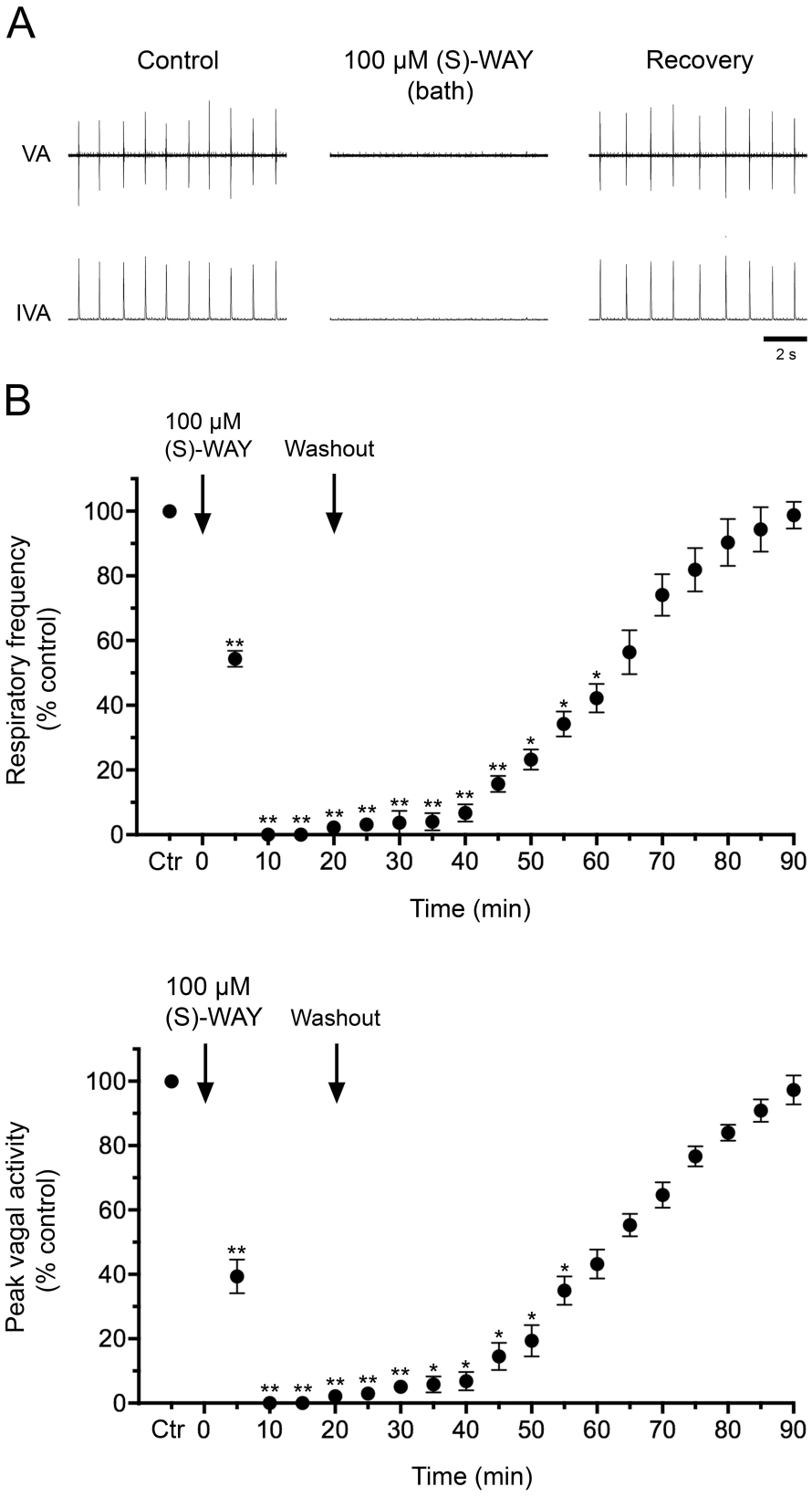
A
Control
100 µM (S)-WAY
(bath)
Recovery
VA
IVA
2 s
B
100 µM
(S)-WAY
Washout
Respiratory frequency
(% control)
Time (min)
100 µM
(S)-WAY
Washout
Peak vagal activity
(% control)
Time (min)

Figure 11

Fig. 11. Respiratory responses to (S)-WAY 100135 in the lamprey

A. Apneic response induced ~ 10 min after bath application of 100 μM (S)-WAY 100135 in one preparation. A complete recovery was observed after ~ 70 min washout. Traces are raw vagal nerve activity (VA) and integrated vagal nerve activity (IVA). **B.** Time course of respiratory responses induced by bath application of 100 μM (S)-WAY 100135 (n = 4). The antagonist caused within a few min progressive reductions in both respiratory frequency and peak vagal activity. Complete apnea ensued within 10 min. Ctr, control. The onset of (S)-WAY 100135 bath application and of washout is indicated by arrows. Values are means ± SEM. *p < 0.05, **p < 0.01 compared with control.

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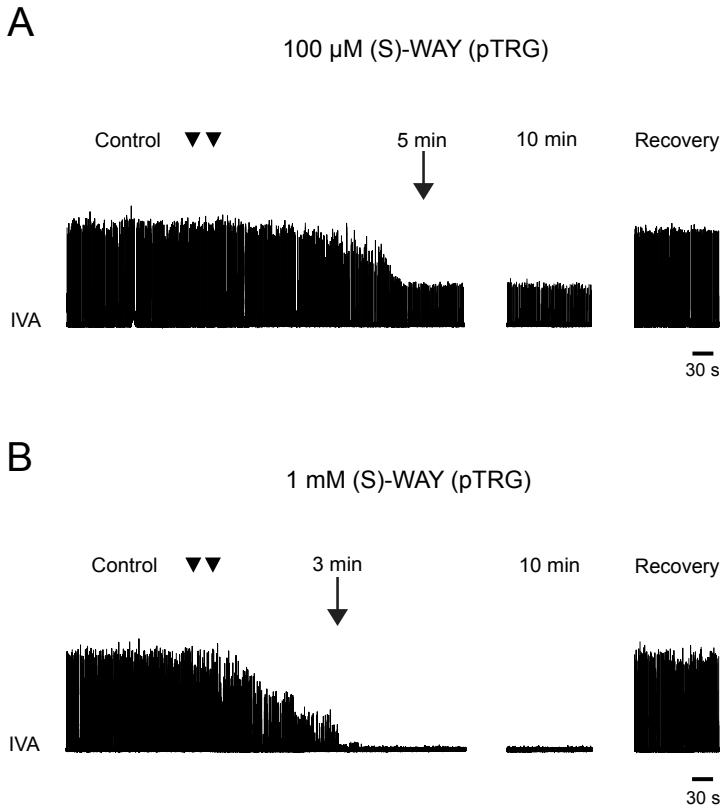

Fig. 12. Respiratory effects of (S)-WAY 100135 microinjected into the pTRG of the lamprey

Long trace recordings of integrated vagal nerve activity (IVA) in one brainstem preparation under control conditions and at different times following bilateral microinjections of 100 µM and 1 mM (S)-WAY 100135 (arrowheads). **A.** (S)-WAY 100135 at 100 µM induced depressant effects on respiratory activity that reached a maximum at ~ 5 min (arrow) and persisted for ~ 10 min. Recovery ~ 40 min after the injections. **B.** (S)-WAY 100135 at 1 mM caused depressant effects on respiratory activity within 1 min and apnea ~ 3 min (arrow) after the completion of the microinjections. Apneic effects lasted for ~ 15 min. Recovery ~ 60 min after the 5-HT_{1A}R antagonist. Pauses in the ongoing respiratory activity indicate the presence of 'coughs'.

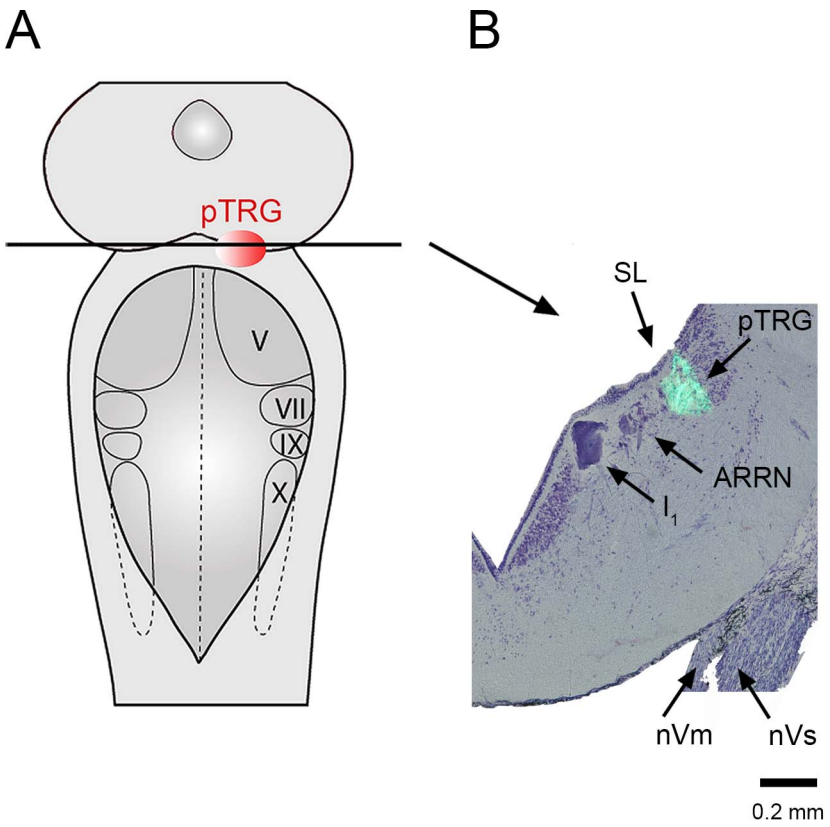

Fig. 13 Localization of the pTRG injections sites in the lamprey

A. The pTRG region (red area) where drugs were microinjected is represented on a schematic view of the dorsal mesencephalon-rhombencephalon of the lamprey. **B.** Photomicrograph of a coronal section (indicated by the arrow) at the level of the pTRG (unbroken line) showing an example of the location of fluorescent beads (green) added to the 1 mM (S)-WAY 100135 solution microinjected into the pTRG. The section is counterstained with Cresyl Violet. Light field and fluorescence photomicrographs have been superimposed. ARRN, anterior rhombencephalic reticular nucleus; I_1, isthmic Müller cell; nVm, motor root of the trigeminal nerve; nVs, sensory root of the trigeminal nerve; pTRG, paratrigeminal respiratory group; SL, sulcus limitans. V, trigeminal motor nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus. The ARRN and I_1 were used as anatomical landmarks to identify the pTRG region.

Involvement of inhibitory GABAergic and glycinergic mechanisms in the respiratory responses caused by 5-HT_{1A} receptor blockade

We hypothesized that the apneic responses induced by the blockade of 5-HT_{1A}Rs could be mediated by disinhibition of GABAergic and glycinergic mechanisms acting on pTRG neurons. To address this issue, the respiratory responses induced by the 5-HT_{1A}R antagonist were investigated during the blockade of glycine and/or $GABA_A$ receptors. Interestingly, blockade of glycine receptors by strychnine prevented (S)-WAY 100135-induced apnea. In the analysis of (S)-WAY 100135-induced effects during glycine receptor blockade (n = 4), a repeated-measures ANOVA provided these results: $F_{(2,6)} = 42.37$, $p = 0.001$ for respiratory frequency, $F_{(2,6)} = 3.58$, $p = 0.144$ for peak vagal amplitude and $F_{(2,6)} = 0.45$, $p = 0.554$ for the duration of vagal bursts. In more detail (*post hoc* tests), bilateral microinjections of 1 mM strychnine (0.5-1 pmol) did not change respiratory activity, in agreement with our previous findings (Cinelli *et al.*, 2014). The subsequent (~ 5 min interval) microinjections of 1 mM (S)-WAY 100135 at the same sites caused within 10 min only mild decreases in respiratory frequency of 16.05 ± 1.45 % (from 60.7 ± 4.2 to 51 ± 3.6 cycles min^{-1}; $p = 0.0093$) without significant changes in peak activity and duration of vagal bursts. Respiratory activity recovered control levels within 60 min after (S)-WAY 100135 microinjections. These outcomes are illustrated in Fig. 14A.

In this context, it should be recalled that we have already investigated the role of $GABA_A$ receptors within the pTRG (Cinelli *et al.*, 2014). In agreement with our previous results, bilateral microinjections of 1 mM bicuculline (0.5-1 pmol; n = 4) into the pTRG elicited a pattern of breathing characterized by irregular, prolonged vagal bursts that progressively developed and stabilized in a double-burst pattern within 5 min. As illustrated in Fig. 14B, our investigation on the effects of (S)-WAY

100135 during $GABA_A$ receptor blockade proved that also bicuculline prevented (S)-WAY 100135-induced apnea. The results of a repeated-measures ANOVA were as follows: $F_{(2,6)} = 52.53$, $p = 0.0047$ for respiratory frequency, $F_{(2,6)} = 0.0843$, $p = 0.8306$ for peak vagal amplitude, and $F_{(3,9)} = 63.79$, $p = 0.0011$ for vagal burst duration. *Post hoc* analysis showed that bilateral microinjections of bicuculline did not modify respiratory frequency and peak vagal amplitude, while the duration of the first and second vagal burst changed from 29 ± 2.3 ms (control value) to 35.6 ± 1.9 ms (23.5 ± 4.6%; $p = 0.0265$) and to 43 ± 2.2 ms (49 ± 5.8%; $p = 0.0059$), respectively. The same statistical analysis also revealed that the subsequent (~ 5 min interval) bilateral microinjections of 1 mM (S)-WAY 100135 at the same sites did not induce apnea, but restored a more regular pattern of breathing characterized by a single-burst pattern and a complete recovery of vagal burst duration (27.8 ± 1.5 ms; $p > 0.05$ vs controls). However, the respiratory frequency was lower than control (from 59 ± 3.8 to 49.9 ± 2.7 cycles min^{-1}, -15.15 ± 1.34 %; $p = 0.0199$). Respiratory activity recovered control levels within 60 min after (S)-WAY 100135 microinjections.

The concomitant blockade of both $GABA_A$ and glycine receptors completely prevented the responses induced by (S)-WAY 100135 microinjections. In more details, bilateral microinjections of 1 mM strychnine combined with 1 mM bicuculline into the pTRG ($n = 4$) induced within 5 min a double-burst pattern of breathing similar to that already observed after the injection of 1 mM bicuculline alone. The subsequent (~ 5 min interval) microinjections of 1 mM (S)-WAY 100135 did not induce any further changes in the ongoing respiratory activity (Fig. 14C). Simple comparisons by using paired *t* tests showed no differences in the respiratory frequency as well as in the other considered respiratory variables (statistical data not

shown). Respiratory activity recovered within 60 min.

To corroborate these results, we sought for the presence of 5-HT_{1A}Rs and glycinergic neurons within the pTRG by double-labelling experiments (n = 4). As illustrated in Fig. 15, glycine-immunoreactive neurons (red signal) were found in this region. In addition, photomicrographs at a higher magnification of the portion of the transverse section (white box in B) at the pTRG level display glycine-immunoreactive neuronal structures ($C_{1,}$ red signal) and 5-HT_{1A}R binding sites (C_2, green signal) widely expressed in close apposition to the soma of glycine-immunoreactive cells (C_3, merged image). Immunoreactivity for GABAergic neurons was not performed since previous studies have shown that GABA-immunopositive fibres, but not GABA-immunoreactive neurons are present within this region (Robertson *et al.*, 2007; Villar-Cervino *et al.*, 2008; Cinelli *et al.*, 2014). No attempt was made to reveal the presence of 5-HT_{1A}R on GABAergic terminals.

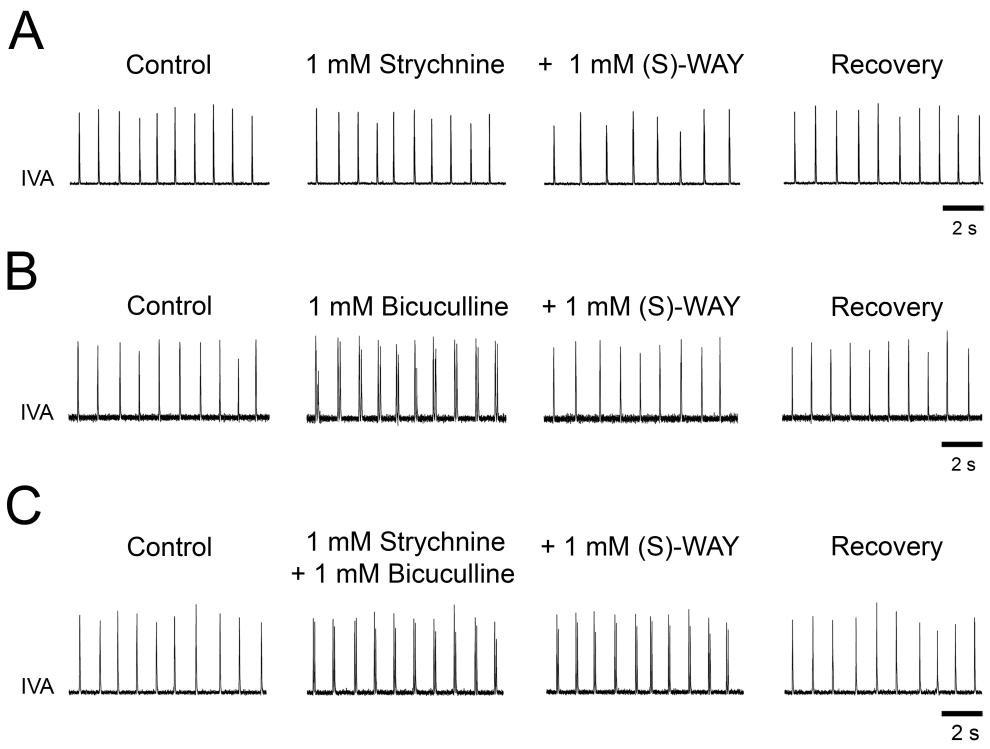

Fig. 14. Role of glycine and $GABA_A$ receptors in counteracting the respiratory effects induced by 5-HT_{1A} receptor antagonism in the lamprey pTRG

A. Bilateral microinjections of 1 mM strychnine into the pTRG did not change the ongoing pattern of breathing, but prevented the apneic response induced by 1 mM (S)-WAY 100135 injected into the same sites after an interval of ~5 min (observation made ~ 10 min after (S)-WAY 100135). However, the 5-HT_{1A}R antagonist still induced small, but consistent reductions in respiratory frequency. Recovery was taken ~ 60 min after (S)-WAY 100135 injections. **B.** Bilateral microinjections of 1 mM bicuculline into the pTRG caused within 5 min the appearance of a double-burst breathing pattern. At that time, microinjections of 1 mM (S)-WAY 100135 performed into the pTRG did not cause apnea. However, ~ 10 min after the injections the double-burst breathing pattern faded out and a regular pattern of breathing with a respiratory frequency slightly lower than control ensued. Respiratory activity recovered control levels within ~ 60 min. **C.** Microinjections of 1 mM strychine combined with 1 mM bicuculline induced within 5 min a pattern of breathing similar to that already observed during $GABA_A$ receptor blockade. At that time, bilateral microinjections of 1 mM (S)-WAY 100135 into the same sites did not change the already altered pattern of breathing (observation made ~ 10 min after (S)-WAY 100135 injections). A complete recovery was observed after ~ 60 min. Traces are integrated vagal nerve activity (IVA).

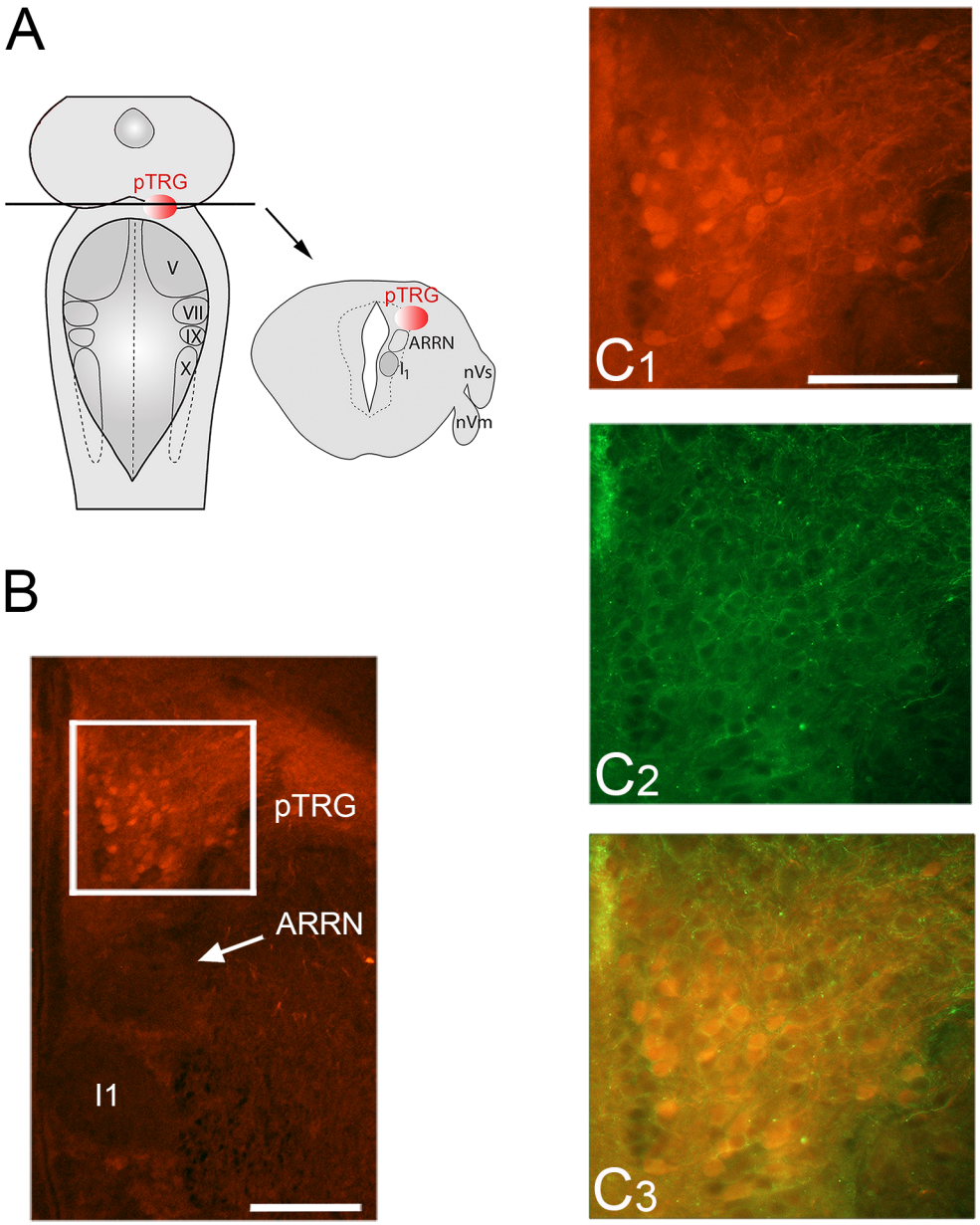

Figure 15

Fig. 15. Immunohistochemical labelling of glycinergic neurons and 5-HT_{1A} receptors in the pTRG region of the lamprey

A. Schematic illustration of a dorsal view of the lamprey mesencephalon/rhombencephalon showing the level (unbroken line) of the a coronal section (arrow) where the pTRG is located (red area). **B**. Photomicrograph of a transverse section at the pTRG level displaying glycine immunoreactivity (red signal). **C_{1-3}**, photomicrographs at a higher magnification of a portion of the transverse section (white box in B) illustrating glycine-immunoreactive neuronal structures (C_1, red signal), 5-HT_{1A}R binding sites (C_2, green signal) and merged image (C_3). Note the presence of 5-HT_{1A}R-immunoreactive structures located in close apposition to glycinergic neurons. Scale bars: B, 100 μm; C_{1-3}, 50 μm. ARRN, anterior rhombencephalic reticular nucleus; I_1, isthmic Müller cell; nVm, motor root of the trigeminal nerve; nVs, sensory root of the trigeminal nerve; pTRG, paratrigeminal respiratory group; V, trigeminal motor nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus. The ARRN and I_1 were used as anatomical landmarks to identify the pTRG region.

Discussion

This study is the first to provide evidence that 5-HT plays an excitatory role in the modulation of respiratory activity in the adult rabbit by acting on the preBötC, without affecting the neighbouring BötC and iVRG. We demonstrate that 5-HT microinjections performed into the preBötC have excitatory effects on respiration through the activation of 5-HT_{1A}Rs and 5-HT_3Rs. Unexpectedly, we also show that the 5-HT_{1A}R antagonist (S)-WAY 100135 microinjected into the preBötC causes excitatory respiratory effects, whereas the 5-HT_3R antagonist ondansetron does not influence respiration. Interestingly, (S)-WAY 100135 is ineffective during 5-HT_3R blockade, thus suggesting a presynaptic role of 5-HT_{1A}Rs. On the other hand, the effects of the 5-HT_{1A}R agonist 8-OH-DPAT are prevented by bicuculline microinjections into the preBötC. This latter finding indicates an involvement of a GABAergic inhibitory circuit and is corroborated by immunohistochemical data.

The results obtained in the adult lamprey show that 5-HT either applied to the bath or microinjected into the pTRG has no obvious effects on the medullary respiratory network. However, we show for the first time that it exerts a tonic influence on respiration as revealed by the effects induced by methysergide or (S)-WAY 100135. 5-HT_{1A}Rs contribute to maintain baseline levels of respiratory activity by mediating a potent inhibitory control of both GABAergic and glycinergic mechanisms impinging onto the pTRG rhythmogenic neurons. The 5-HT_{1A}R-mediated inhibition is conceivably effected on glycinergic cells and on GABAergic terminals, in agreement with the notion that GABAergic neurons are not present in the pTRG region (Robertson *et al.*, 2007; Villar-Cervino *et al.*, 2008; Cinelli *et al.*, 2014). Immunohistochemical data are consistent with the presence of 5-HT_{1A}Rs on

glycinergic neurons and corroborate present findings. Some important features of the 5-HT mechanisms involved in the modulation of the respiratory network appear to be conserved throughout evolution.

Methodological considerations

Microinjection procedures in mammals have been extensively discussed in previous reports (Nicholson, 1985; Lipski *et al.*, 1988; Nicholson & Sykova, 1998; see also e.g. Bongianni *et al.*, 1997, 2002, 2008, 2010; Mutolo *et al.*, 2002, 2005). In agreement with our previous findings (Bongianni *et al.*, 1997, 2002, 2008, 2010; Mutolo *et al.*, 2002, 2005), in the rabbit injection sites were selected by using stereotaxic coordinates and especially extracellular recordings of respiratory neurons from VRG subregions (see e.g. Von Euler, 1986; Bianchi *et al.*, 1995). In particular, extracellular recordings allowed us to accurately define the preBötC region, where we encountered patterns of neuronal activities similar to those described in other animal species (see e.g. Connelly *et al.*, 1992; Schwarzacher *et al.*, 1995; Rekling & Feldman, 1998). The localization of the injection sites was confirmed by DLH-induced tachypneic responses (see e.g. Solomon *et al.*, 1999; Bongianni *et al.*, 2002, 2010; Wang *et al.*, 2002; Monnier *et al.*, 2003; Mutolo *et al.*, 2005; Fong & Potts, 2006; Stucke *et al.*, 2015). On the other hand, the effects of DLH microinjections into the preBötC region of the rabbit have already been described (Bongianni *et al.*, 1997, 2008, 2010; Mutolo *et al.*, 2002, 2005; Stucke *et al.*, 2015). Excitatory respiratory effects induced by DLH microinjections into the preBötC have been compared with the responses induced in neighbouring regions and have been taken as a functional marker for the preBötC in various animal models (see e.g. Solomon *et al.*, 1999; Krolo *et al.*, 2005; Radocaj *et al.*, 2015). Furthermore, the localization of

the injection sites was ascertained by the histological control of pipette tracks and the localization of fluorescent beads microinjected into the preBötC. The specificity of the observed responses is corroborated by the absence of respiratory effects in response to control microinjections. In addition, respiratory responses were not accompanied by appreciable changes in arterial blood pressure, thus ruling out any role of baroreceptor reflexes in their development. On the other hand, in the rabbit alterations in blood pressure were usually observed following drug microinjections into the BötC, but not into the preBötC (see e.g. Bongianni *et al.*, 1997, 2008, 2010).

Present findings, along with the results of some previous studies (Mutolo *et al.*, 2002, 2005; Bongianni *et al.*, 2008, 2010), confirm that the rabbit can be a suitable animal model for studies on the neural control of breathing and/or respiratory rhythm generation. Although most of the basic knowledge on the functional organization of respiratory neurons largely derives from past experiments on adult cats and more recently on rodents, it should be recalled that rabbits have also been widely used in recent and pioneering important studies on the control of breathing and on the localization of respiration-related regions (Gromysz & Karczewski, 1981; Yamamoto & Lagercrantz, 1985; Jiang & Shen, 1991; Stucke *et al.*, 2015; for reviews, see Von Euler, 1986; Bianchi *et al.*, 1995; Hilaire & Duron, 1999).

In the lamprey, microinjections into the pTRG were performed by using a Picospritzer and a procedure validated in our previous studies (Mutolo *et al.*, 2007, 2010, 2011; Cinelli *et al.*, 2013, 2014, 2017). We are confident that the obtained respiratory effects were related to a responsive area localized within the rostrolateral trigeminal region, i.e. the pTRG. To identify this region the first step was to record respiration-related neuronal discharges and to perform in the same region the

microinjections. Only microinjections that were associated with characteristic spots of dye within this region were considered. As in our previous reports (Cinelli *et al.*, 2014, 2016), on some occasions the localization of the injection sites was verified by the presence of green florescent latex microspheres added to the injectate. Control microinjections support the specificity of the observed effects and the accuracy of the localization of pTRG injection sites. As previously discussed (Bongianni *et al.*, 2002), it should be noted that concentrations of neuroactive drugs required in the 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 rabbit preBötC as a target of 5-HT within the rostral VRG

One of the main points of interest of the present study in the rabbit is the potent 5-HT-mediated activation of the respiratory motor output through an action on the preBötC. This finding is in keeping with the vast majority of data obtained in neonatal and juvenile *in situ* rodent preparations (e.g. Al Zubaidy *et al.*, 1996; Pena & Ramirez, 2002; Schwarzacher *et al.*, 2002; Gunther *et al.*, 2006; Ptak *et al.*, 2009; Niebert *et al.*, 2011; Corcoran *et al.*, 2014; for review, see Hilaire *et al.*, 2010; Hodges & Richerson, 2008, 2010). However, our results are at variance with those obtained in the dog by Radocaj et al. (2015) who found that the local application of 8-OH-DPAT produced dose-dependent decreases in the activity of preBötC neurons, while microinjections of 5-HT and 8-OH-DPAT along the whole extent of the ventral respiratory column did not produce any effect on respiration. They suggested that the tachypneic effects of intravenous 8-OH-DPAT injections could be attributed

to other brainstem areas. However, species differences cannot be completely ruled out (see also Hilaire *et al.*, 2010).

Indeed, medullary serotoninergic neurons, mainly located in the brainstem raphe nuclei and parapyramidal regions, project extensively to the brainstem respiratory network including the preBötC and, in addition, to hypoglossal and phrenic motoneurons (Ptak *et al.*, 2009; see also Hodges & Richerson, 2008; Paterson *et al.*, 2009; Hilaire *et al.*, 2010; Ramirez *et al.*, 2013). Interestingly, these 5-HT projections are generally immunoreactive also for the neuropeptides SP and thyrotropin-releasing hormone (for review, see Hodges & Richerson, 2008). Accordingly, it has been shown that raphe magnus stimulation increases respiratory frequency in mice due to the release of 5-HT and, possibly, SP in the preBötC (Doi & Ramirez, 2010). A recent study by DePuy et al. (2011) in mice by using optogenetics demonstrated that the activation of raphe obscurus serotoninergic neurons stimulates breathing and that transfected serotoninergic neurons have multiple targets in both the lower brainstem and the spinal cord. Their observations extend previous evidence that stimulation of the raphe obscurus activates breathing and are consistent with the excitatory action of 5-HT on phrenic motoneurons and many brainstem respiratory neurons, including those of the preBötC and the retrotrapezoid nucleus.

The mechanism by which raphe 5-HT neurons affect the respiratory network (Richter *et al.*, 2003; Hodges & Richerson, 2008, 2010) and their role in the respiratory rhythmogenesis (e.g. Pena & Ramirez, 2002; Toppin *et al.*, 2007) have been debated. These issues have been recently clarified, at least to a great extent, by Ptak et al. (2009). They showed in *in vitro* neonatal and in *in situ* juvenile rat preparations that the stimulation of the raphe obscurus, that is reciprocally connected

with the preBötC, excites simultaneously both preBötC neurons and the respiratory motor output by means of the release of 5-HT and SP. In accordance with these findings, our previous studies have already shown that SP microinjections into the preBötC region, but not in the BötC and iVRG, exert intense excitatory respiratory effects (see e.g. Bongianni *et al.*, 2008 also for further Refs). In the light of the present results we can suggest that not only SP, but also 5-HT microinjections into the preBötC can be additional tools for the physiological identification of this VRG subregion. In conclusion, the preBötC proved to be an important site of action of 5-HT, although we cannot exclude that other responsive brainstem respiration-related areas may exist. Indeed, 5-HTRs have been reported to be widely expressed in the VRG at least in some animal species (Hodges & Richerson, 2008, 2010; Dutschmann *et al.*, 2009; Hilaire *et al.*, 2010), but unexpectedly we found that 5-HT microinjected into regions adjacent to the preBötC was ineffective. On the other hand, to our knowledge, no extensive investigations exist on the effects of microinjections of 5-HT into the BötC or the iVRG. We believe that this topic deserves further immunohistochemical and neurophysiological studies.

Excitatory role of 5-HT_{1A} and 5-HT_3 receptors within the rabbit preBötC

Somewhat at variance with previous studies on neonatal or juvenile rodents showing an involvement of varied 5-HTRs in the control and even in the generation or maintenance of respiratory activity (e.g. Pena & Ramirez, 2002; Schwarzacher *et al.*, 2002; Gunther *et al.*, 2006; Ptak *et al.*, 2009; Niebert *et al.*, 2011; Corcoran *et al.*, 2014; see Hodges & Richerson, 2008, 2010; Hilaire *et al.*, 2010 also for further Refs), we found that 5-HT_{1A}Rs and 5-HT_3Rs are involved in 5-HT-induced excitatory effects on respiration at the level of the preBötC in the adult rabbit. It

seems too difficult to propose an exhaustive explanation for such discrepancy. Probably it is mainly related to differences in the animal species and developmental stage. Our outcomes have been proven by several trials with 5-HTR agonists and antagonists and noticeably by the lack of 5-HT effects during the blockade of both 5-HT_{1A} Rs and 5-HT_3Rs. To our knowledge, this is the first report showing that 5-HT_3Rs have an excitatory role at the preBötC level. However, they do not seem to exert a tonic influence on respiration under basal conditions since the 5-HT_3R antagonist was ineffective. Nevertheless, the presence of 5-HT_3Rs allowed us to provide a tentative explanation of the excitatory effects of the 5-HT_{1A}R antagonist (S)-WAY 100135 by suggesting an action of this drug at the presynaptic level. Indeed, 5-HT_{1A} autoreceptors in the raphe nuclei have been extensively described only on serotoninergic neurons (Nichols & Nichols, 2008). Although presynaptic 5-HT_{1A}Rs have been found to be involved in the regulation of the release of neurotransmitters including GABA and/or glutamate (e.g. Luttgen *et al.*, 2005; Dergacheva *et al.*, 2011; Ostrowski *et al.*, 2014; Stiedl *et al.*, 2015), only suggestions have been advanced on the possible role of presynaptic 5-HT_{1A}Rs located on serotoninergic terminals involved in the release of 5-HT at sites far from serotoninergic neurons (Wang & Ramage, 2001). Here, we provide only indirect evidence of the presence of such presynaptic receptors. A specifically designed immunohistochemical analysis should be performed to ascertain our proposal as well as the presence of 5-HT_3 Rs on preBötC neurons.

Disinhibition phenomena underlying respiratory effects due to 5-HT_{1A} receptor activation within the rabbit preBötC

The interpretation of bicuculline-induced effects is very difficult, but it seems conceivable that bicuculline microinjections do not affect a segregate inhibitory pathway involved in the mediation of 5-HT-induced effects. Rather, they cause a generalized blockade of the GABAergic inhibitory control within the preBötC that may lead to a disruption of the ongoing pattern of breathing. For more details on this matter we refer to previous report (Bongianni *et al.*, 2010; see also Kam *et al.*, 2013). In any case, we found that bicuculline prevented the excitatory effects of the 5-HT_{1A}R agonist, thus the hypothesis was advanced that these effects were due to disinhibition phenomena caused by inhibition of GABAergic neurons located in the preBötC region (e.g. Kuwana *et al.*, 2006; Shevtsova *et al.*, 2011; Koizumi *et al.*, 2013) via postsynaptic inhibitory 5-HT_{1A}Rs, with a possible additional regulatory role of presynaptic 5-HT_{1A}Rs. Thus, these findings imply that GABAergic neurons exert a tonic inhibitory influence on preBötC neurons. These conclusions are strongly corroborated by immunohistochemical results showing the presence of 5-HT_{1A}R-immunoreactive dots in close apposition to the soma of GABAergic immunoreactive neurons of the preBötC, consistently with the possibility of a postsynaptic localization of 5-HT_{1A}Rs. The existence of postsynaptic and presynaptic 5-HT_{1A}Rs within the preBötC region should be ascertained in further studies. Present findings are consistent with the presence of 5-HT_{1A}Rs on GABAergic neurons in other brain structures (e.g. Luttgen *et al.*, 2005; Ostrowski *et al.*, 2014; Stiedl *et al.*, 2015). Interestingly, a prominent role of GABAergic inhibitory control at the level of the preBötC has already been described in the rabbit (Bongianni *et al.*, 2010). Furthermore, the presence of a disinhibitory mechanism is in general agreement with

the results of previous studies in rodents (e.g. Manzke *et al.*, 2009; Corcoran *et al.*, 2014) in which, however, an involvement of glycinergic neurons has been found. In conclusion, it seems that regardless of the animal species disinhibition involving inhibitory neurons either glycinergic or GABAergic can be a prominent mechanism subserving 5-HT excitatory effects on respiratory activity (Manzke *et al.*, 2009; Shevtsova *et al.*, 2011; Corcoran *et al.*, 2014).

In conclusion, the main novel findings of the present study in the rabbit are that: 1) 5-HT exerts its modulatory action only at the level of the preBötC through 5-HT_{1A}Rs and 5-HT_3Rs, without affecting the neighbouring respiration-related regions; 2) 5-HT_{1A}Rs are endogenously activated, while 5-HT_3Rs do not play any tonic role on respiratory activity; 3) excitatory respiratory effects induced by the activation of 5-HT_{1A}Rs involve preBötC GABAergic neurons. These outcomes are complex, however, considering the novelty of present findings we believe that would be of interest to advance a proposal on their functional significance.

We propose the existence of two separate serotoninergic mechanisms (Fig. 16) that, according to the available literature, can reasonably be brought into action by different stressors such as hypoxia (Erickson & Sposato, 2009; Chen *et al.*, 2013) or hypercapnia (Phillipson *et al.*, 1977; Berthon-Jones & Sullivan, 1984; Buchanan *et al.*, 2015) and by opioid-induced respiratory depression (Sahibzada *et al.*, 2000; Dutschmann *et al.*, 2009; Manzke *et al.*, 2009; Ren *et al.*, 2015). Under basal conditions, 5-HT release should be minimum, if any, because the blockade of both 5-HT_3Rs and 5-HT_{1A}Rs within the preBötC (see Fig. 7B1, middle panel) does not alter the ongoing respiratory activity. However, it strongly increases when these mechanisms are activated. The first mechanism consists of the release of 5-HT tuned by 5-HT_{1A}R-mediated presynaptic inhibition and the activation of the ionotropic

excitatory 5-HT_3Rs located on preBötC excitatory neurons that are inspiratory and rhythmogenic. The second mechanism, engaging the same or separate serotoninergic afferent pathways, involves the release of 5-HT (possibly controlled by presynaptic 5-HT_{1A}Rs) causing the activation of inhibitory 5-HT_{1A}Rs on a specific set of GABAergic cells innervating the preBötC excitatory inspiratory neurons and the consequent disinhibition phenomena leading to the activation of inspiratory rhythmic activity. These mechanisms should activate simultaneously both the respiratory motor output and hypoglossal motoneurons resulting in hyperventilation and increased airway patency (see Ptak *et al.*, 2009). Details on these mechanisms obviously require further investigations. However, in connection with present findings, it can be recalled that optogenetic stimulation of the entire population of preBötC neurons in the rat potently drives inspiratory activity *in vivo* (Alsahafi *et al.*, 2015) consistently with its rhythmogenic role and with the presence of a core of excitatory neurons. The subpopulation of excitatory and rhythmogenic preBötC neurons has been shown to belong to the family of glutamatergic $Dbx1^+$ neurons of the mouse that have preinspiratory or inspiratory firing patterns (Cui *et al.*, 2016). We can also speculate that preBötC neurons expressing 5-HT_3Rs (first mechanism) pertain to a similar neuronal subpopulation of the rabbit. Both glycinergic (Sherman *et al.*, 2015) and GABAergic (Cui *et al.*, 2016) neurons represent important components of the preBötC neuronal population, although, as already stated, not essential for inspiratory rhythm generation in intact mammals (Bongianni *et al.*, 2010; Kam *et al.*, 2013; Del Negro *et al.*, 2018 also for further Refs). Our findings show an important role of GABAergic inhibition (second mechanism) that can be hypothesized to correspond in the mouse, at least in part, to the GABAergic inhibitory mechanism activated by optogenetic stimulation of neurons expressing

both somatostatin and GABA (Cui *et al.*, 2016). For a comprehensive review on the preBötC microcircuit and its interactions with additional breathing microcircuits see Del Negro et al. (2018).

Present results in the adult rabbit may be of interest for some respiratory disorders in which, as already mentioned, an impairment of central 5-HT mechanisms has been found. Despite the fact that therapeutic approaches based on drugs affecting the 5-HT system so far have been proved to be unsuccessful (Oertel *et al.*, 2007; Dempsey *et al.*, 2010), it seems conceivable that our results could provide hints for the development of novel therapeutic strategies since they focus the attention on the preBötC, i.e. a region characterized by the presence of multiple receptors that subserve the integration of many neuromodulatory inputs to generate the breathing behaviour (Doi & Ramirez, 2008; Del Negro *et al.*, 2018).

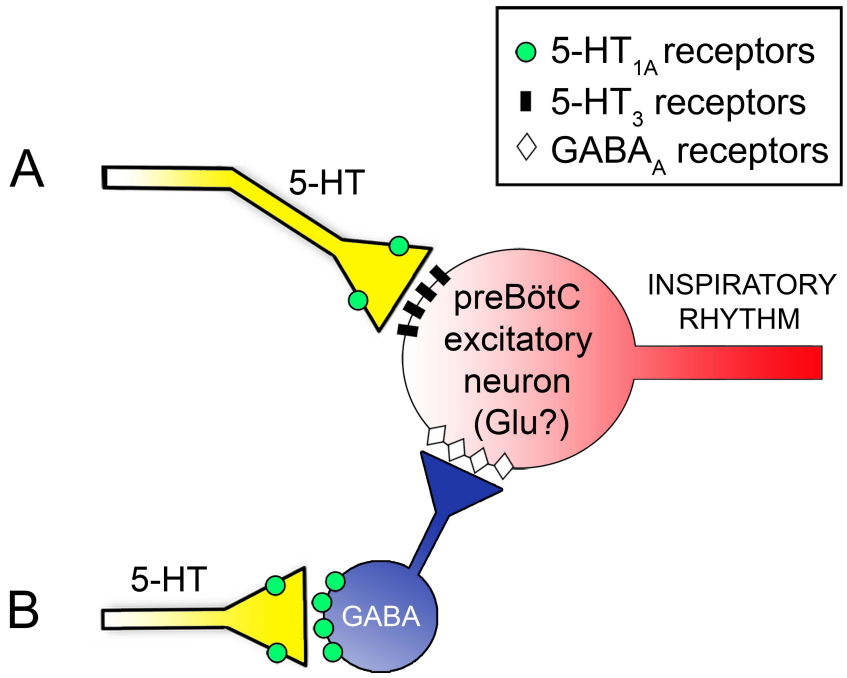

Fig. 16. Schematic drawing, partially inferred from the present results, representing two hypothetical 5-HT mechanisms in the rabbit medulla oblongata responsible for the activation of preBötC excitatory neurons

Under basal conditions only presynaptic 5-HT_{1A}Rs are endogenously activated, as suggested by the differential effects of specific 5-HT_3R and 5-HT_{1A}R antagonists, and minimize or even annihilate 5-HT release at the level of the synaptic knobs on preBötC cells. In any case, the release of 5-HT should be minimum or even absent, since the blockade of both 5-HT_{1A}Rs and 5-HT_3Rs does not alter baseline respiratory activity. Conceivably, different stressors, such as hypoxia, hypercapnia or opioid-induced respiratory depression, have been reported to activate serotoninergic mechanisms. The augmented release of 5-HT overcomes presynaptic inhibition and stimulates postsynaptic 5-HT_3Rs on preBötC cells (**A**). Simultaneously, the increased 5-HT release also activates the inhibitory 5-HT_{1A}Rs on a specific set of GABAergic neurons that exert an inhibitory control on preBötC cells through $GABA_A$ receptors. This induces disinhibition of preBötC neurons (probably glutamatergic) and the subsequent excitatory effects on respiratory activity (**B**). Presynaptic 5-HT_{1A}Rs might be present also at the level of synaptic terminals on GABAergic neurons. Present results do not allow us to advance any hypothesis on the respiratory or non-respiratory discharge pattern of these inhibitory neurons.

Tonic control of the lamprey pTRG through 5-HT_{1A} receptors

Surprisingly, 5-HT either applied to the bath or microinjected into the pTRG did not produce any change in respiratory activity. This could suggest the absence of 5-HTRs within the rhythm generating respiratory network and, therefore, an involvement of 5-HTRs in the respiratory control. However, a potent tonic influence on respiration was disclosed by employing methysergide and, especially, the 5-HT_{1A}R antagonist (S)-WAY 100135. In this study, we have focused our attention only on the 5-HT_{1A}R subtype. At present, since other antagonists have not been tested, we cannot completely exclude the influence of different 5-HTR subtypes on respiratory activity. Importantly, this tonic influence contributes to maintain basal respiratory activity and only decreases in respiration could be mediated by this receptor mechanism. Consistently with the present knowledge in mammals (Doi & Ramirez, 2008; Del Negro *et al.*, 2018), other mechanisms are known to contribute to the respiratory control in the lamprey. Endogenous opioids are not required for the generation of baseline respiratory activity, but exert a depressant modulatory role due to μ-opioid receptors and, to a minor extent, δ-opioid receptors (Mutolo *et al.*, 2007). Other neurotransmitters or neuromodulators have excitatory respiratory effects and contribute to respiratory rhythm generation, such as neurokinin receptor agonists, in particular the NK1 receptor agonist SP (Mutolo *et al.*, 2010; Cinelli *et al.*, 2013, 2017), acetylcholine (Mutolo *et al.*, 2011) and glutamate (Cinelli *et al.*, 2013). On the contrary, ATP did not prove to have a role in generating baseline respiratory activity, but produces a biphasic response consisting of an initial excitation followed by a secondary inhibition due to the breakdown of ATP into adenosine (Cinelli *et al.*, 2017). As to the role of inhibitory neurotransmitters within the pTRG, GABAergic mechanisms have a tonic modulatory role while a glycinergic

tonic influence is lacking (Cinelli *et al.*, 2014). However, both GABA and glycine have clear inhibitory effects as proved by the counteracting effects of bicuculline and strychnine on (S)-WAY 100135-induced depressant responses.

Most of 5-HT-immunoreactive neurons in the central nervous system of the adult sea lamprey have been found in the diencephalon, rhombencephalon and spinal cord. Noticeably, strong resemblances between lampreys and other vertebrates in the spatio-temporal pattern of development of brainstem serotoninergic population exist. In addition, shared patterns of ascending and descending serotoninergic pathways are present in lampreys and jawed vertebrates (Antri *et al.*, 2006; Abalo *et al.*, 2007). As already mentioned, one of the major target of the serotoninergic cell activity is the 5-HT_{1A}R and the comparison of the expression of these receptors between sea lamprey and other vertebrates reveals a conserved pattern in most of the brain regions (Cornide-Petronio *et al.*, 2013). Serotoninergic cells have been described in close vicinity to or within the pTRG. However, in our opinion, most of the projections to the pTRG probably originate from the dense serotoninergic population of neurons located in the isthmic region, which corresponds to the so-called superior raphe nuclei, located in the caudal mesencephalon and rostral rhombencephalon and present in all jawed vertebrates. (Pierre *et al.*, 1992; Antri *et al.*, 2006; Abalo *et al.*, 2007).

Disinhibition phenomena underlying respiratory responses due to 5-HT_{1A} receptor antagonism within the lamprey pTRG

No significant effects were induced by strychnine microinjections into the pTRG, while bicuculline microinjections caused a characteristic double-burst pattern with an interval between vagal duplets similar to that present under control conditions. These

findings are quite similar to those reported by us in a previous study (Cinelli *et al.*, 2014) and recall those observed in adult rabbits (Bongianni *et al.*, 2010; present results) and adult rodents (Feldman *et al.*, 2013; Janczewski *et al.*, 2013; Kam *et al.*, 2013 also for further Refs). They have been attributed to an increase in the excitability of rhythmogenic respiratory neurons present in the pTRG or in the preBötC. The results obtained by applying the 5-HT_{1A}R antagonist (S)-WAY 100135 following the blockade of $GABA_A$ and/or glycine receptors demonstrate that both GABA and glycine inhibitory mechanisms are involved in the (S)-WAY 100135-induced respiratory responses. At present, the circumstances under which 5-HT_{1A}Rs modulation are brought into action are obscure and only matter of speculation. The present study provides evidence of the existence of glycine-immunoreactive neurons within the pTRG, in agreement with previous findings on the distribution of glycine immunoreactivity in the brain of adult sea lamprey (Villar-Cervino *et al.*, 2008). Glycine-immunoreactive neurons were observed in the rostral rhombencephalon, in particular at the level of the isthmus. They could also be present in a region that may correspond, at least in part, to the pTRG. The presence of 5-HT_{1A}R-immunoreactive structures in close apposition to the soma of glycinergic-immunoreactive neurons of the pTRG corroborates our results and confirms that glycinergic cells are a target of 5-HT modulation within the pTRG.

As already mentioned, 5-HT and its receptors are also involved in the control of the lamprey locomotor rhythm by reducing segmental burst frequency and slowing the intersegmental phase coupling (for review see Grillner, 2006). Of note, the blockade of 5-HT_{1A}Rs reversibly increases the frequency of locomotor activity, thus implying that there is an endogenous release of 5-HT with an important regulatory role (Zhang & Grillner, 2000). On the whole, 5-HT has an inhibitory action on

locomotion, while at the level of the respiratory network it has an excitatory effect unmasked by the local application of the 5-HT_{1A}R antagonist during $GABA_A$ and glycine receptor blockade. The serotoninergic mechanisms subserving regulation of the respiratory network suggested by present results are schematically summarized in Fig. 17. As shown in the Figure, 5-HT_{1A}Rs are present both presynaptically on GABAergic terminals and postsynaptically on glycinergic neurons. These receptors are endogenously activated and have suppressant effects on the release of the inhibitory neurotransmitters, thus contributing to maintain basal respiratory activity. Indeed, the actual circuit underlying the modulation of the pTRG by 5-HT and GABA could be more complex than that reported in Fig. 17. The Figure also help to explain how the (S)-WAY 100135-mediated disinhibition of both inhibitory mechanisms induces apnea (prevented by their blockade) and, in addition, how the blockade of a single mechanism can still cause slight depressant effects on respiration due to a concomitant (S)-WAY 100135-induced disinhibition of the other mechanism. Any further interpretation seems, at present, highly speculative.

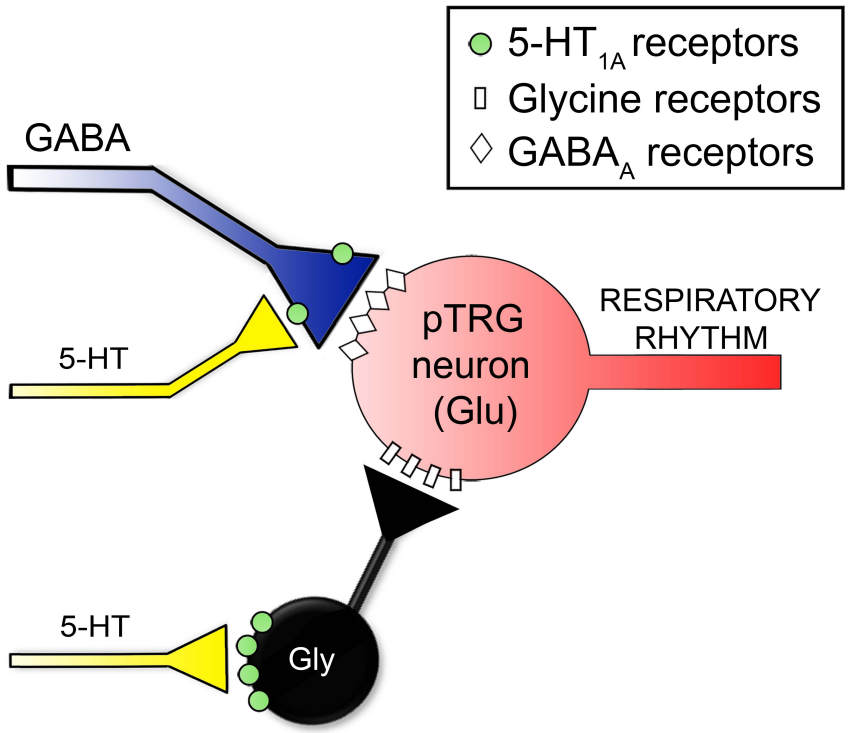

Fig. 17. Schematic drawing, partially inferred from the present results, representing two hypothetical inhibitory mechanisms mediated by 5-HT$_{1A}$ receptors within the pTRG region of the lamprey

Under basal conditions, both presynaptic 5-HT$_{1A}$Rs on GABAergic terminals and postsynaptic 5-HT$_{1A}$Rs on glycinergic neurons are endogenously activated and strongly reduce or completely suppress the release of the inhibitory neurotransmitters. 5-HTRs endogenous activation contributes to maintain the basal rhythmic respiratory activity. The blockade of 5-HT$_{1A}$Rs by the specific antagonist (S)-WAY 100135 induces apnea owing to disinhibition of both inhibitory GABAergic and glycinergic mechanisms acting on pTRG excitatory neurons. Accordingly, the blockade of both these inhibitory mechanisms, or only of a single one, completely abolishes (S)-WAY 100135-induced apneic responses. Nevertheless, since small depressant effects on respiration occurred after (S)-WAY 100135 microinjections during glycine receptor blockade, a concomitant disinhibition of the GABA release via presynaptic 5-HT$_{1A}$Rs on GABAergic terminals can be suggested. On the other hand, slight depressant effects on respiration were seen after (S)-WAY 100135 microinjections during GABA$_A$ receptor blockade, thus indicating a possible associated disinhibition of pTRG glycinergic neurons operated by 5-HT$_{1A}$Rs located on their somata.

Comparative and phylogenetic considerations

Previous studies on neonatal or juvenile rodents have shown an involvement of varied presynaptic and postsynaptic 5-HTRs in the regulation of respiratory activity (e.g. Pena & Ramirez, 2002; Schwarzacher *et al.*, 2002; Gunther *et al.*, 2006; Ptak *et al.*, 2009; Niebert *et al.*, 2011; Corcoran *et al.*, 2014; see Hodges & Richerson, 2008, 2010; Hilaire *et al.*, 2010 also for further Refs). 5-$HT_{2A/2B/2C}$, 5-HT_4 and 5-HT_{1A} are the most frequently encountered receptor subtypes. Interestingly, the presence of a disinhibitory mechanism triggered by 5-HT and mediated by 5-HT_{1A}Rs on preBötC glycinergic neurons has been reported in rodents (Manzke *et al.*, 2009; Corcoran *et al.*, 2014). At variance, present results in the rabbit suggest the existence of two separated serotoninergic mechanisms that modulate respiratory activity (Fig. 16), one mediated by excitatory 5-HT_3Rs located on preBötC rhythmogenic neurons and the other mediated by inhibitory 5-HT_{1A}Rs on GABAergic neurons of the preBötC region. The main differences are the presence in the rabbit of the 5-HT_3Rs not previously described in the respiratory network of mammals and the disinhibitory mechanism triggered by 5-HT_{1A}Rs on GABAergic neurons. In this context, it should be emphasized that present results on the lamprey respiratory network, where only 5-HT_{1A}Rs have been found until now, support the suggestion that 5-HT_{1A}Rs may represent an ancestral archetypical 5-HTR. Furthermore, the presence of inhibitory neurons inhibited through the activation of 5-HT_{1A}Rs in the lamprey pTRG recalls the neuronal organization of the mammalian respiratory network, particularly that of rodents in which the inhibitory neurons are glycinergic. Interestingly, 5-HT_3Rs are present not only in the rabbit, but also in the turtle respiratory network, consistently with the later differentiation of this animal species around 220 million years ago in the Mesozoic Era (Johnson *et al.*, 2001). In agreement with the conclusions of

several previous studies on other lamprey neural structures (Grillner, 2006), the results support the notion that important functional characteristics of the neuronal network subserving respiratory rhythm generation are conserved throughout phylogeny (see also Kinkead, 2009).

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