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RESEARCH REPORT

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Key role of 5-HT$_{1A}$ receptors in the modulation of the neuronal network underlying the respiratory rhythm generation in lampreys

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Abstract

In mammals, 5-HTexcitatory respiratory effects imply 5-HT$_{1A}$ receptor-mediated disinhibition of pre-Bötzinger complex neurons. In the lamprey, 5-HT$_{1A}$ receptors are involved in the neural control of locomotion, but their role in the respiratory regulation, particularly at the level of the putative respiratory rhythm generator, the paratrigeminal respiratory group (pTRG), is not known. We here investigate the respiratory function of inhibitory 5-HT$_{1A}$ receptors within the pTRG of the isolated brainstem of the adult lamprey. The 5-HT$_{1A}$ receptor agonists either bath applied or microinjected into the pTRG did not cause significant effects. However, the selective 5-HT$_{1A}$ receptor antagonist (S)-WAY 100135 bath applied or microinjected into the pTRG induced depressing respiratory effects or even apnoea, thus revealing that 5-HT exerts a 5-HT$_{1A}$ receptor-mediated potent tonic influence on respiration and contributes to maintain baseline levels of respiratory activity. Microinjections of strychnine or bicuculline, either alone or in combination, into the pTRG prevented (S)-WAY 100135-induced apnoea. In addition, immunohistochemical studies corroborate the present findings suggesting that 5-HT$_{1A}$ receptors are widely expressed in close apposition to the soma of glycine-immunoreactive cells located within the pTRG region. The results show that in the lamprey respiratory network, 5-HT exerts a tonic influence on respiration by a potent inhibitory control on both GABAergic and glycinergic mechanisms. The observed disinhibitory effects resemble the excitatory respiratory modulation exerted by 5-HT$_{1A}$ receptor-mediated inhibition of glycinergic and/or GABAergic neurons present in mammals, supporting the notion

Abbreviations: 5-HT, serotonin; 5-HTR, 5-HT receptor; 5-HT$_{1A}$Rs, 5-HT$_{1A}$ receptors; ARRN, anterior rhombencephalic reticular nucleus; DMSO, dimethyl sulfoxide; Glu, glutamate; Gly, glycine; I$_1$, isthmic Müller cell; IVA, integrated vagal nerve activity; IX, glossopharyngeal motor nucleus; nVm, motor root of the trigeminal nerve; nVs, sensory root of the trigeminal nerve; PB, phosphate buffer; PBS, phosphate-buffered saline; pre-BötC, pre-Bötzinger complex; pTRG, paratrigeminal respiratory group; SL, sulcus limitans of His; SP, substance P; V, trigeminal motor nucleus; VA, raw vagal activity; VII, facial motor nucleus; X, vagal motor nucleus.

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that some features of the neuronal network subserving respiratory rhythm generation are highly conserved throughout phylogeny.

KEYWORDS

central pattern generator, control of breathing, disinhibition, inhibitory amino acids, serotonin

1 | INTRODUCTION

Serotonin (5-HT) with its ionotropic and metabotropic receptors contributes to regulate many brain functions, including breathing (for review see Hilaire et al., 2010; Hodges & Richerson, 2008, 2010; Jacobs & Azmitia, 1992). To date, at least 14 different 5-HT receptor (5-HTR) subtypes have been identified in mammals, which are grouped into seven families (5-HT_1-5-HT_7; Kroeze, Kristiansen, & Roth, 2002; Nichols & Nichols, 2008). These 5-HTRs are G protein-coupled receptors except the ligand-gated ion channel 5-HT_3R. The inhibitory 5-HT_{1A}R is a major determinant of serotoninergic cell activity and of 5-HT release because of both its presynaptic and postsynaptic location (Chalmers & Watson, 1991). The 5-HT_{1A}R is estimated to have differentiated around 650 million years ago from the 5-HT_1R subfamily around the time during which vertebrates diverged from invertebrates (Peroutka & Howell, 1994). Most of the invertebrate 5-HTRs resemble either the mammalian 5-HT_{1A}Rs or the 5-HT_7Rs (Kroeze et al., 2002), thus suggesting that the 5-HT_{1A} or 5-HT_7 subclasses represent the ancestral archetypical 5-HTRs.

Varied effects of 5-HT on the respiratory motor output have been described in mammals mainly related to the complexity of the mode of action of this neurotransmitter. However, the bulk of evidence seems to support the conclusion that 5-HT neurons play an excitatory neuromodulation of respiratory activity in mammals (Depuy, Kanbar, Coates, Stornetta, & Guyenet, 2011; Hilaire et al., 2010; Hodges & Richerson, 2008, 2010; Iovino et al., 2019; Ptak et al., 2009). 5-HT-induced excitatory effects on respiration obviously depend on the combined activation of presynaptic and postsynaptic 5-HTRs within the neural structures involved in the respiratory control (e.g. Dutschmann et al., 2009; Hilaire et al., 2010; Hodges & Richerson, 2008, 2010; Nichols & Nichols, 2008). According to previous observations in rodents (Corcoran et al., 2014; Manzke et al., 2009), recently we have provided evidence in adult rabbits (Iovino et al., 2019) that 5-HT-induced excitatory respiratory effects are exerted mainly through a 5-HT_{1A}R-mediated inhibition of inhibitory neurons at the level of the pre-Bötzinger complex (pre-BötC), the neuronal aggregate widely accepted to be responsible for the inspiratory rhythm generation (Del Negro, Funk, & Feldman, 2018; Feldman, Del Negro, & Gray, 2013; Janczewski, Tashima, Hsu, Cui, & Feldman, 2013; Smith, Ellenberger, Ballanyi, Richter, & Feldman, 1991). In more detail, a 5-HT_{1A}R-mediated inhibition of glycinergic neurons has been found in rodents and cats (Corcoran et al., 2014; Manzke et al., 2009) and of GABAergic neurons in rabbits (Iovino et al., 2019).

The lamprey, a lower vertebrate which diverged from the main vertebrate line around 560 million years ago (Kumar & Hedges, 1998), has proved to be highly useful to identify the neuronal circuits underlying rhythmic motor behaviours, such as locomotion (e.g. Grillner, 2006; Grillner et al., 1995; Grillner & El Manira, 2019) and respiration (e.g. Bongianni, Mutolo, Cinelli, & Pantaleo, 2016; Cinelli et al., 2013). The isolated brainstem of the adult lamprey spontaneously generates respiratory neuronal activity in vitro. The putative respiratory central pattern generator is located in the paratrigeminal respiratory group (pTRG), a region rostral to the trigeminal motor nucleus (Mutolo, Bongianni, Einum, Dubuc, & Pantaleo, 2007; Mutolo, Bongianni, Cinelli, & Pantaleo, 2010; Mutolo, Cinelli, Bongianni, & Pantaleo, 2011; Cinelli, Iovino, & Mutolo, 2013; Cinelli, Mutolo, Contini, Pantaleo, & Bongianni, 2014; Cinelli et al., 2016; Cinelli et al., 2017; for review see Bongianni et al., 2016; Milsom, 2018). The pTRG shows many similarities with the mammalian pre-BötC (see e.g. Bongianni et al., 2016; Cinelli et al., 2017; Del Negro et al., 2018; Feldman et al., 2013; Smith et al., 1991). In fact, the two rhythm generators are under an extensive neuromodulatory control (Doi & Ramirez, 2008; Ramirez, Dashevskiy, Marlin, & Baertsch, 2016) and display similar sensitivity to opioid, substance P (SP), acetylcholine and ATP (Cinelli et al., 2013, 2017; Mutolo et al., 2007, 2010, 2011).

The general organization of the serotoninergic system in the lamprey is relatively well described and appears to share similarities with that of other vertebrates (Abalo et al., 2007; Antri, Cyr, Auclair, & Dubuc, 2006; Cornide-Petronio, Anadon, Barreiro-Iglesias, & Rodicio, 2013; Pierre et al., 1992). 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 corresponds to the pTRG. 5-HT is importantly involved in neural circuits controlling locomotion (Grillner et al., 1995) by slowing the frequency of fictive locomotor activity (Harris-Warrick & Cohen, 1985; Matsushima & Grillner, 1992; Buchanan, 2001;

for review see Grillner, 2006; Grillner & El Manira, 2019). In this control, mainly postsynaptic 5-HT_{1A}Rs appear to be involved (e.g. Hill, Svensson, Dewael, & Grillner, 2003; Wang, Grillner, & Wallen, 2014; Wikstrom, Hill, Hellgren, & Grillner, 1995; Zhang & Grillner, 2000). A presynaptic role of 5-HT_{1D}Rs has also been reported (Schwartz, Gerachshenko, & Alford, 2005). The role of 5-HT and 5-HTR subtypes, especially 5-HT_{1A}Rs, in modulating the respiratory rhythm generating network of the lamprey is unknown.

Comparative studies may offer a deeper understanding of neuronal rhythmogenic circuits and their evolution. Thus, the goals of this study were to investigate serotoninergic signalling in the lamprey respiratory network, in particular to explore whether the5-HT_{1A}R-mediated excitation of mammalian respiratory network that occurs via disinhibition of glycinergic and/or GABAergic neurons (Corcoran et al., 2014; Iovino et al., 2019; Manzke et al., 2009), also occurs in the lamprey. To this end, we examined the effects on the respiratory activity generated by adult lamprey brainstems in vitro induced by bath application and microinjections into the pTRG of selective 5-HT_{1A}R agonists and of a selective 5-HT_{1A}R antagonist. The effects of the local application of $GABA_A$ and/or glycinergic receptor antagonists were also studied. Immunohistochemical studies were performed to corroborate the results obtained with the glycine receptor antagonist strychnine.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

Experiments were carried out on 44 young adult (12–15 cm) sea lampreys (*Petromyzon marinus*) of either sex. Animals were captured in small rivers in Maine and Massachusetts and delivered by ACME Lamprey Company (Harrison, ME, USA). Lampreys (fasted) were kept in an aerated freshwater aquarium at 5°C, with a 12-hr light–12-hr dark cycle. Animal care and experimental procedures were conducted in accordance with Italian legislation and the official regulations of the European Community Council on the use of laboratory animals (DecretoLegislativo 4/3/2014 no. 26 and Directive 2010/63/UE). The study was approved by the Animal Care and Use Committee of the University of Florence. All efforts were made to minimize both the number of animals used and their suffering.

2.2 | Animal preparation

Animal preparation and experimental procedures were similar to those described in previous reports (Bongianni, Deliagina, & Grillner, 1999; Cinelli et al., 2013, 2014, 2016, 2017; Mutolo et al., 2007, 2010, 2011). The animals were deeply anaesthetized with tricaine methanesulphonate (100 mg/L; MS 222, Sigma-Aldrich, St. Louis, MO, USA). To obtain the isolated brainstem preparations, the lamprey was immediately transected below the gills. Therefore, the anaesthesia was terminal. 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. The dead space was 7.5 ml. Thus, a drug added to the reservoir took ~3 min to reach the chamber. This time was considered in the evaluation of the beginning of drug effects. Bath temperature was maintained at 9–10°C. The bath solution had the following composition (in mM): 91 NaCl, 2.1 KCl, 2.6, $CaCl_2$, 1.8 $MgCl_2$,4 glucose and 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 at 9–10°C throughout the experiment. Under these experimental conditions, 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 references). The brain rostral to the optic tectum was cut and removed. A transection was made caudal to the obex (cervical region) maintaining a minimum length of spinal cord for holding the preparation. The roof of the isthmic region was cut along the midline, and the alar plates were spread laterally and pinned down.

2.3 | 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 (see e.g. Cinelli et al., 2017; Cinelli et al., 2013, 2014; Mutolo et al., 2007, 2011). 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 recorded signals were analysed by a personal computer supplied with an analog-to-digital interface (50 kHz sampling rate; Digidata 1440, Molecular Devices, Sunnyvale, CA) and appropriate software (Axoscope, Molecular Devices).

Clampfit software (Molecular Devices) was used for offline analysis.

2.4 | Drug application and histology

Drugs were applied to the bath solution to reveal their respiratory effects. They were also 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: (*R*)-(+)-8-Hydroxy-DPAT hydrobromide (8-OH-DPAT; full 5-HT_{1A}R agonist, more active enantiomer; Cat. # 1080, Tocris Bioscience, Bristol, UK) either bath applied (10, 50 and 100 μM) or microinjected (1 mM); BP 554 maleate (selective 5-HT_{1A}R agonist; Cat. # 0556, Tocris Bioscience) microinjected (1 mM); (S)-WAY 100135 dihydrochloride (potent and selective 5-HT_{1A}R antagonist; Cat. # 1253, Tocris Bioscience) either bath applied (100 μM) or microinjected (10, 100 μM and 1 mM); strychnine hydrochloride (glycine receptor antagonist; Cat. # S8753, Sigma-Aldrich) and bicuculline methiodide ($GABA_A$ receptor antagonist; Cat. # 14343, Sigma-Aldrich) microinjected both at 1 mM. Drugs were usually dissolved in distilled water, except for (S)-WAY 100135 and BP 554 that were dissolved in 100% dimethyl sulfoxide (DMSO; Cat. # D8418, Sigma-Aldrich) and diluted to volume with the perfusing solution. In the final solution for bath application experiments, DMSO concentration was 0.1%, while in the final solution for microinjection experiments DMSO concentration was always ≤1%. The pH of drug solutions was ~7.4. 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 were selected in preliminary trials. They were similar to those employed in previous studies (see e.g. Cinelli et al., 2014; Johnson, Wilkerson, Henderson, Wenninger, & Mitchell, 2001; Ostrowski, Ostrowski, Hasser, & Kline, 2014; Sardari, Rezayof, & Zarrindast, 2015). In these trials, as in a previous study (Mutolo et al., 2010), control experiments with equivalent amounts of DMSO in the perfusing solution were performed. They showed that the solvent had no effect on respiratory activity. 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 with microinjections, the preparation was allowed to recover by perfusing it with the control solution for a maximum of 2 hr. Higher concentrations of neuroactive drugs are required with local application due to the exponential decay of drug concentration with the distance from the pipette tip as well as to other factors including the large dilution volume constituted by the extracellular space and the continuous washout in the recording chamber. Microinjection procedures have been extensively discussed in previous reports (Lipski, Bellingham, West, & Pilowsky, 1988; Nicholson, 1985; Nicholson & Sykova, 1998; see e.g. Bongianni, Mutolo, Carfi, & Pantaleo, 2002; Mutolo, Bongianni, Nardone, & Pantaleo, 2005).

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) connected to the injection pipette. The volume was estimated by measuring the diameter of a droplet ejected from the tip of the pipette with an ocular micrometre in a dissecting microscope. 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., 2017, 2013, 2014). 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. Control microinjections of equal volumes of the vehicle solution with 0.2% Fast Green dye were also made prior to drug injections. On some occasions (n = 3) green fluorescent latex microspheres (LumaFluor, New City, NY) were added (dilution 1:3) to the drug solution (1 mM (S)-WAY 100135) for post hoc confirmation of injection sites (see also Cinelli et al., 2014). 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.

2.5 | Immunohistochemistry

Experiments were performed for immunohistochemical detection of glycinergic neurons and 5-HT_{1A}R expression within the pTRG (n = 4). The dissection, fixation and sectioning of the lamprey brains were performed as previously described (Cinelli et al., 2017, 2013, 2014). The preparation was similar to that employed in the electrophysiological experiments described above. The brains were dissected out of the surrounding tissue, fixed by immersion in 2% formalin/0.1 M PB pH 7.4 for 12 hr and cryoprotected in 20% sucrose/PB for 3–12 hr. 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) and 0.3% Triton X-100 in 0.1 M PB. Sections were incubated for 24 hr at 4°C with a rat polyclonal anti-glycine antibody (1:5,000; Cat. # IG1002; Lot # 1131; ImmunoSolutions, Jesmond, New South Wales; RRID: AB_10013222) and a mouse monoclonal anti-serotonin receptor 1_Aantibody (clone 19A9.2; 1:500; Cat. # MAB11041; Lot # 2424802; Millipore Corporation, Billerica, MA; RRID: AB_10806903). After a thorough rinse in 0.01 M phosphate-buffered saline (PBS), sections were incubated with a mixture of Alexa Fluor 488 goat anti-mouse IgG (1:200; Cat. # A-11077; Invitrogen, Life Technologies, Carlsbad, CA; RRID: AB_141874) and Alexa Fluor 568 goat anti-rat IgG (1:200; Cat. # A-11029; Invitrogen; RRID: AB_138404) for 2 hr at room temperature. Sections were then coverslipped with glycerol containing 2.5% diazabicyclooctane (Sigma-Aldrich). The specificity of glycine antibody used is known from previous studies in the lamprey (Cinelli et al., 2016; Mahmood, Restrepo, & El Manira, 2009) and is consistent with the finding of glycine immunonegative areas, such as that corresponding to the anterior rhombencephalic reticular nucleus (see Figure 5b). The mouse monoclonal antibody against 5-HT_{1A}Rs has been used in the mammalian central nervous system (Azizi et al., 2017; Iovino et al., 2019; Li et al., 2018; Millon et al., 2016). It has not been previously used in the lamprey. The specificity of the 5-HT_{1A}R antibody can be hypothesized since 5-HT_{1A}Rs are highly conserved in the central nervous system throughout the vertebrate evolution (Cornide-Petronio et al., 2013) and, in addition, 5-HT_{1A}R labelling with the employed antibody is virtually absent in some regions adjacent to the pTRG (see Figure 5b). However, we have not provided any evidence that this antibody is really specific also in the lamprey brain. Therefore, we have to admit that its cross-reactivity with other antigens is possible. For these reasons, we will refer to 5-HT_{1A}R-like immunoreactivity in the text. Furthermore, no immunoreactivity was detected when primary antibody was omitted from the immunohistochemical processing.

Whenever antibody expression levels were compared, tissues were processed simultaneously. Photomicrographs of key results were taken using a Nikon DS-Fi1 (Nikon, Japan) digital camera and software. Images were captured and processed in Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA; RRID: SCR_014199) using identical settings. Images were only adjusted for brightness and contrast. Illustrations were prepared in Adobe Photoshop CS6 and Adobe Illustrator CS6 (Adobe Systems Inc., San Jose, CA; RRID: SCR_010279).

2.6 | Data collection and analysis

Data collection and analysis were performed according to procedures already described in our previous reports (see e.g. Cinelli et al., 2017). We measured respiratory frequency (cycles/min), 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) that were normalized by expressing it as a fraction (or percentage) of mean control value (relative unit, RU). All these variables were measured and averaged for 20 s in the period immediately preceding each trial (control values) as well as 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, at the time when the drug-induced responses occurred and in the recovery period were taken for statistical analysis (Prism 7, GraphPad Software Inc., La Jolla, CA; RRID: SCR_002798). 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 differences in the effects of a series of experimental conditions on the same group of animals. In particular, this analysis was used to investigate the time course of respiratory responses to bath application or microinjections of (S)-WAY 100135 and related recovery (5-min intervals). 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 analysis of the prolonged vagal bursts, described as "coughs" (see Cinelli et al., 2017 also for further references), was considered beyond the scope of the present research. All values are presented as means ± *SEM*; $p < .05$ was considered as significant.

3 | RESULTS

3.1 | Role of 5-HT_{1A}Rs 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, that is locomotion, we focused on this receptor subtype. Bath application ($n = 3$) of 100 μM 8-OH-DPAT, a selective 5-HT_{1A}R agonist, did not within 10 min cause any obvious or consistent effect on the respiratory frequency (from 61.4 ± 2.1 to 60.8 ± 1.8 cycles/min; $p > .05$, Student's paired *t* test). Amplitude and duration of vagal bursts were also unaffected by 100 μM 8-OH-DPAT (p always $> .05$, Student's paired *t* tests). Lower concentrations of 8-OH-DPAT (10 or 50 μM) were also without effect. Similarly, bilateral microinjections of 1 mM 8-OH-DPAT into the

pTRG (0.5–1 pmol; $n = 4$) did not produced any significant effect on respiratory frequency (from 59.8 ± 1.6 to 61.1 ± 1.5 cycles/min; $p > .05$, Student's paired t test). The other respiratory variables did not display significant changes (p always > 0.05; Student's paired t tests). The lack of 8-OH-DPAT effects was somewhat surprising. However, this finding was corroborated by some experiments performed making use of another 5-HT_{1A}R agonist. Bilateral microinjections into the pTRG of 1 mM BP 554 (0.5–1 pmol; $n = 3$) did not induce within 10 min any significant change in the respiratory frequency (from 60.3 ± 2.0 to 60.7 ± 1.7 cycles/min; $p > .05$, Student's paired t test). Also, the amplitude and duration of vagal bursts were not affected (p always > 0.05; Student's paired t tests). Thus, we examined possible tonic serotoninergic influences on respiration by bath application of the selective 5-HT_{1A}R antagonist (S)-WAY 100135 at 100 μM ($n = 4$). Unexpectedly, this drug induced apnoea (Figure 1a). The time course of respiratory responses and their recovery are illustrated (Figure 1b). Respiratory frequency was 60.5 ± 2.7 cycles/min under control conditions and started to decrease within 2 min after bath application of the antagonist. Apnoea ensued within 10 min. These latter effects were accompanied by progressive reductions in the amplitude of vagal activity. Respiratory activity slowly, but completely recovered after 60–70 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 different doses of S-WAY 100135 were performed. While 10 μM S-WAY 100135 (5–10 fmol; $n = 3$) was ineffective, 100 μM (0.05–0.1 pmol; $n = 4$) caused depressing effects on the respiratory activity and 1 mM (0.5–1 pmol; $n = 4$) apnoea. These respiratory responses were analysed by a one-way repeated-measures ANOVA. The analysis of the effects induced by 100 μM S-WAY 100135 provided the following results: $F_{(8,24)} = 20.33$, $p = .0064$ for respiratory frequency, $F_{(8,24)} = 33.83$, $p = .0001$ for peak vagal amplitude and $F_{(8,24)} = 1.438$, $p = .3078$ for the duration of vagal bursts. *Post hoc* analysis showed that S-WAY 100135 at 100 μM reduced within 5 min the respiratory frequency (from 58.5 ± 1.9 to 36.5 ± 5cycles/min; −38.3 ± 6.9%; $p = .0315$) and the amplitude of vagal activity (−41 ± 3.5%; $p = .0062$), without significant concomitant changes in the duration of vagal bursts. The decrease in respiration persisted for ~10 min. The recovery was achieved within 40 min (Figure 2a). The same statistical analysis of 1 mM (S)-WAY 100135 effects provided the following results: $F_{(12,36)} = 154.6$, $p < .0001$ for respiratory frequency, $F_{(12,36)} = 117.2$, $p < .0001$ for peak vagal amplitude and $F_{(12,36)} = 292.6$, $p = .0002$ for the duration of vagal bursts. The apnoea ensued within 5 min after the completion of the injections and persisted for ~15 min.

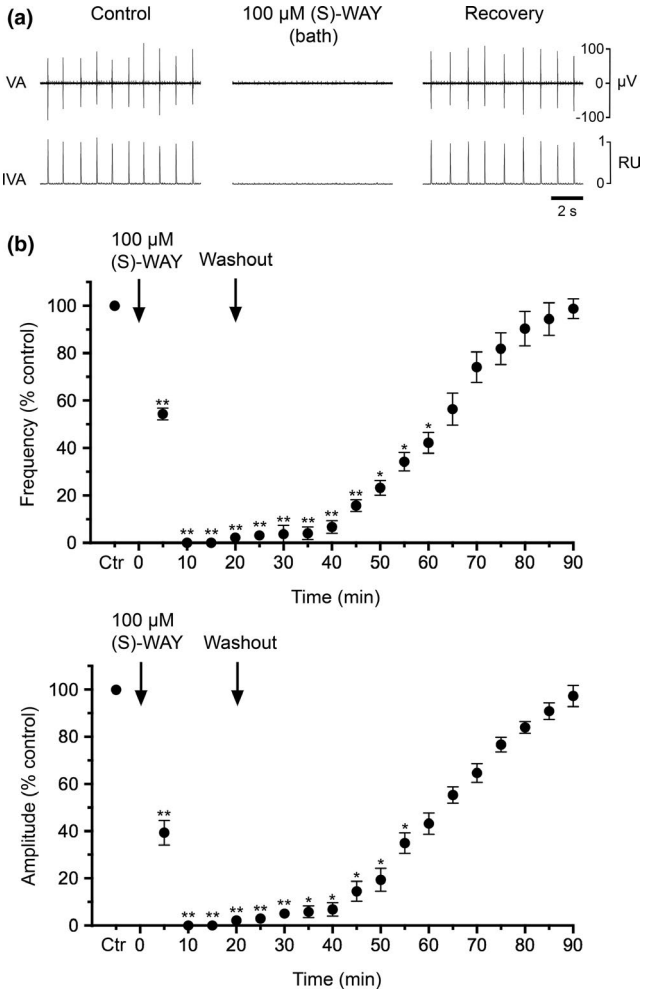

FIGURE 1 Respiratory responses to the 5-HT_{1A}R antagonist, (S)-WAY 100135. (a) Apnoeic response induced ~10 min after bath application of 100 μM (S)-WAY 100135 in one preparation. A complete recovery was observed after ~70 min washout. Traces are raw vagal nerve activity (VA) and integrated vagal nerve 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 amplitude of vagal activity. Complete apnoea ensued within 10 min. Ctr, control. The onsets of bath application of (S)-WAY 100135 and washout are indicated by arrows. Values are means ± *SEM*. *$p < .05$, **$p < .01$ compared with control

Respiratory activity completely recovered within ~60 min (Figure 2b). The time course of respiratory responses induced by 100 μM and 1 mM S-WAY 100135 and their recovery are illustrated (Figure 2c). The localization of one (S)-WAY 100135 injection site into the pTRG, ascertained with green fluorescent latex microspheres added to the injectate, is illustrated in Figure 3. This finding is consistent with that reported in our previous studies (Cinelli et al., 2014; Mutolo et al., 2007, 2011). 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

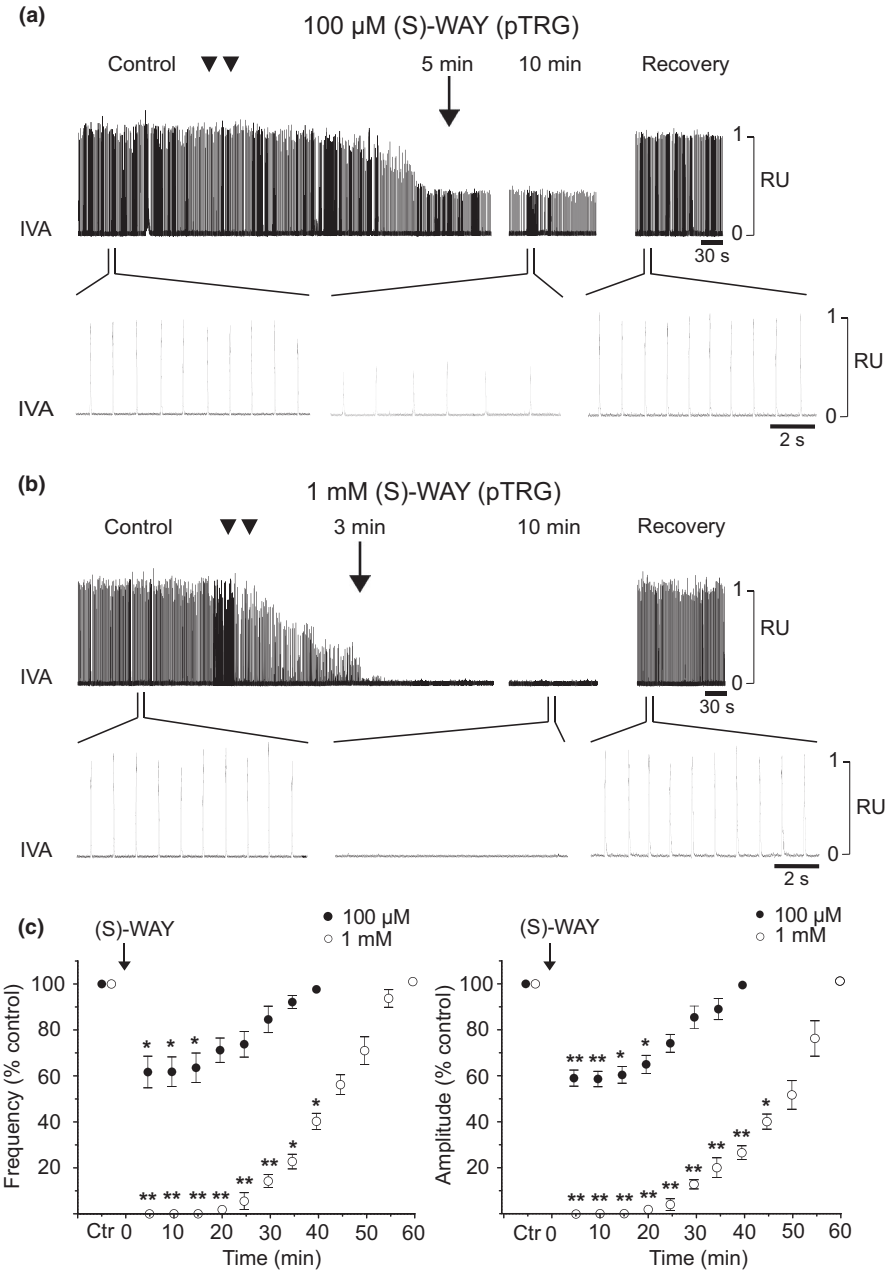

FIGURE 2 Respiratory effects of (S)-WAY 100135 microinjected into the pTRG. Long trace recordings of integrated vagal nerve activity (IVA) in one brainstem preparation under control conditions and at different times following bilateral microinjections of 100 µM and 1 mM (S)-WAY 100135 (arrowheads). (a) 100 µM (S)-WAY 100135 induced depressing effects on respiratory activity that reached a maximum at ~5 min (arrow) and persisted for ~10 min. Recovery ~40 min after the injections. Expanded traces show control baseline respiratory activity, decrease in respiratory frequency and amplitude of vagal activity 10 min after the completion of the injections and recovery of respiratory activity. (b) 1 mM (S)-WAY 100135 caused depressing effects on respiratory activity within 1 min and apnoea ~3 min (arrow) after the completion of the microinjections. Apnoeic effects lasted for ~15 min. Recovery ~60 min after the injections. Expanded traces show control baseline respiratory activity, apnoea 10 min after the completion of the injections, and recovery of respiratory activity. Pauses in the ongoing respiratory activity indicate the presence of "coughs." (c) Time courses of respiratory responses caused by microinjections of 100 µM and 1 mM (S)-WAY 100135. Ctr, control. (S)-WAY 100135 microinjections are indicated by arrows. Values are means ± *SEM*. **p* < .05, ***p* < .01 compared with control

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 also Cinelli et al., 2013).

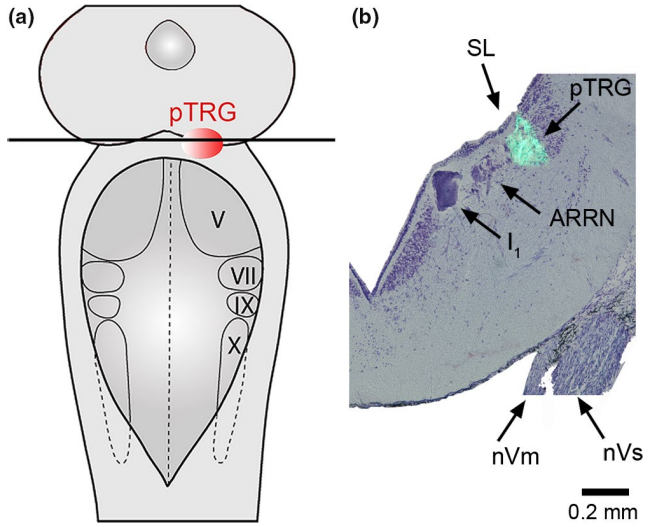

FIGURE 3 Localization of the pTRG injection site. (a) The pTRG region (red area) where drugs were microinjected is represented on a dorsal view of the mesencephalon–rhombencephalon of the lamprey. (b) Photomicrograph of a coronal section at the level of the pTRG (unbroken line in a) showing an example 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 of His; 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

3.2 | Inhibitory mechanisms involved in the mediation of respiratory responses caused by 5-HT_{1A}R blockade

Taking into account disinhibition phenomena induced by 5-HT_{1A}Rs in mammals (Corcoran et al., 2014; Iovino et al., 2019; Manzke et al., 2009), we hypothesized that the apnoeic responses could be mediated by disinhibition of glycinergic and/or GABAergic mechanisms acting on neurons located in the pTRG region. 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 bilateral microinjections of 1 mM strychnine (0.5–1 pmol; $n = 4$) into the pTRG prevented (S)-WAY 100135-induced apnoea. In the analysis of (S)-WAY 100135-induced effects during glycine receptor blockade, a one-way repeated-measures ANOVA provided these results: $F_{(3,9)} = 26.78$, $p = .0105$ for respiratory frequency, $F_{(3,9)} = 3.205$, $p = .1379$ for peak vagal amplitude and $F_{(3,9)} = 0.3474$, $p = .7179$ for the duration of vagal bursts. In more detail, *post hoc* tests revealed that bilateral microinjections of strychnine did not change respiratory activity (Figure 4a), 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 small decreases in respiratory frequency (from 60.7 ± 4.2 to 51 ± 3.6 cycles/min, −16.05 ± 1.45%; $p = .0064$) without significant changes in the amplitude and duration of vagal bursts (third panel in Figure 4a). Respiratory activity recovered control levels within 60 min after (S)-WAY 100135 microinjections.

As already described in a previous report (Cinelli et al., 2014), bilateral microinjections of 1 mM bicuculline (0.5–1 pmol; $n = 4$) into the pTRG elicited a breathing pattern characterized by irregular, prolonged vagal bursts that progressively developed and stabilized within 5 min in a double-burst pattern. Figure 4b shows that also bicuculline prevented (S)-WAY 100135-induced apnoea. In the analysis of (S)-WAY 100135-induced effects during $GABA_A$ receptor blockade, the results of a one-way repeated-measures ANOVA were as follows: $F_{(3,9)} = 42.49$, $p = .0018$ for respiratory frequency, $F_{(3,9)} = 0.3654$, $p = .6708$ for peak vagal amplitude and $F_{(4,12)} = 50.88$, $p = .0001$ for vagal burst duration (see also Cinelli et al., 2014). *Post hoc* analysis showed that bilateral microinjections of bicuculline did not modify the frequency (interval between duplets) nor modify the amplitude (mean values of each duplet) of vagal activity, while the duration of the first and second vagal burst changed from 29.0 ± 2.3 ms (control value) to 35.6 ± 1.9 ms (23.5 ± 4.6%; $p = .0176$) and to 43.0 ± 2.2 ms (49.0 ± 5.8%; $p = .004$), respectively. In particular, *post hoc* analysis revealed that the subsequent (~5 min interval) microinjections of 1 mM (S)-WAY 100135 at the same sites did not induce apnoea, but restored within ~10 min more regular pattern of breathing characterized by a single-burst pattern similar to that observed under control conditions (vagal burst duration 27.8 ± 1.5 ms; $p > .05$). However, the respiratory frequency (third panel in Figure 4b) was slightly lower than control (from 59 ± 3.8 to 49.9 ± 2.7 cycles/min, −15.15 ± 1.34%; $p = .0133$). 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 (Figure 4c). 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 (third panel in Figure 4c).

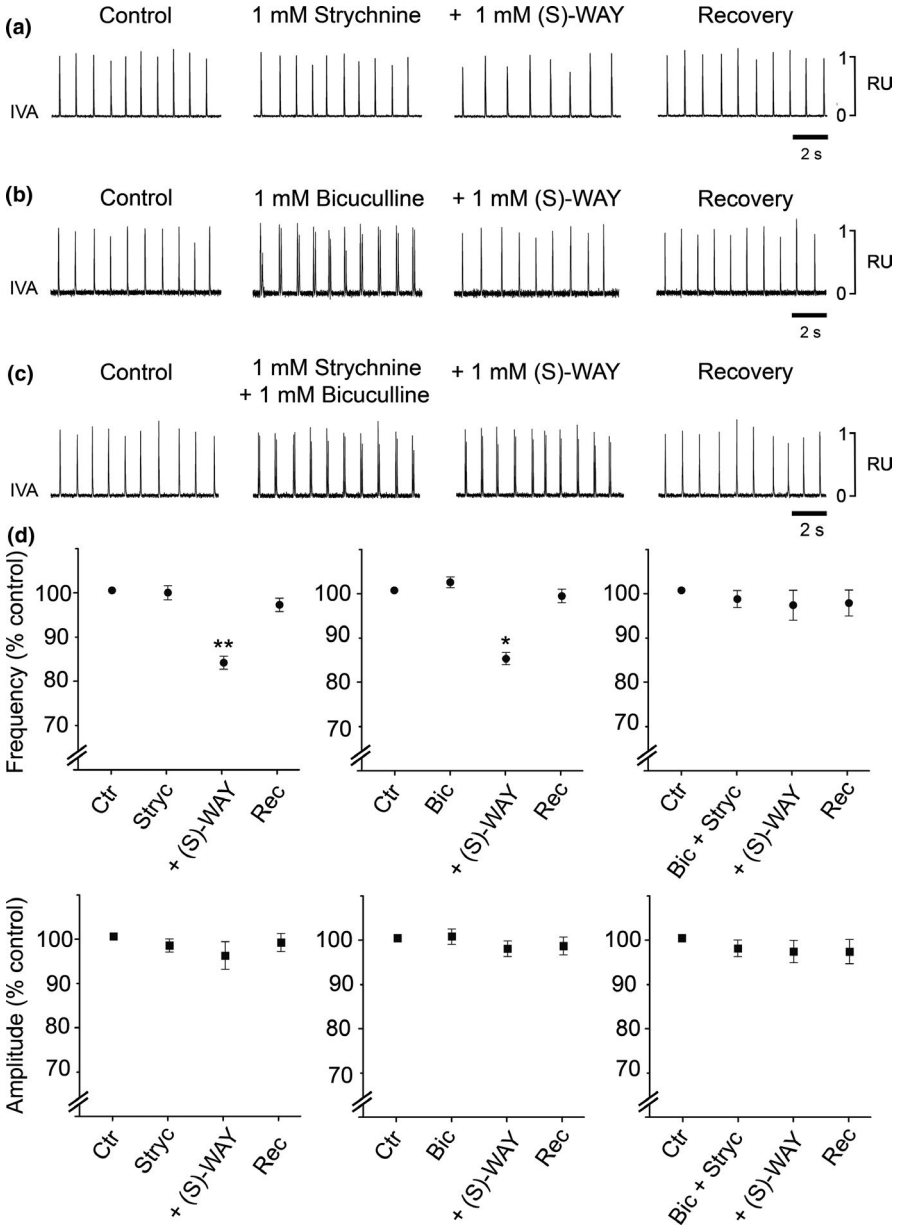

FIGURE 4 Role of glycine and $GABA_A$ receptors in counteracting the respiratory effects induced by 5-HT_{1A}R antagonism in the pTRG. (a) Bilateral microinjections of 1 mM strychnine into the pTRG did not change the ongoing pattern of breathing, but prevented the apnoeic response induced by 1 mM (S)-WAY 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 occurred ~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. The subsequent microinjections of 1 mM (S)-WAY 100135 performed into the pTRG did not cause apnoea. However, within ~10 min the double-burst breathing pattern faded out and was followed by a regular pattern of breathing characterized by a respiratory frequency slightly lower than control. Respiratory activity returned to control levels within ~60 min. (c) Microinjections of 1 mM strychnine combined with 1 mM bicuculline induced within 5 min a pattern of breathing similar to that already observed during $GABA_A$ receptor blockade. Subsequent 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). (d) Group data showing respiratory responses during blockade of glycine and/or $GABA_A$ receptors as well as during the subsequent local application of (S)-WAY 100135. Recovery ~60 min after the last injections. For simplicity, only frequency (●) and amplitude (■) of vagal bursts have been reported. Values are means ± *SEM*. **p* < .05, ***p* < .01 compared with control

Respiratory activity recovered within 60 min. These results were examined with the same statistical analysis (details on this analysis not reported). Group data showing respiratory responses during blockade of glycine and/or $GABA_A$ receptors as well as during the subsequent local application of (S)-WAY 100135 are illustrated in Figure 4d.

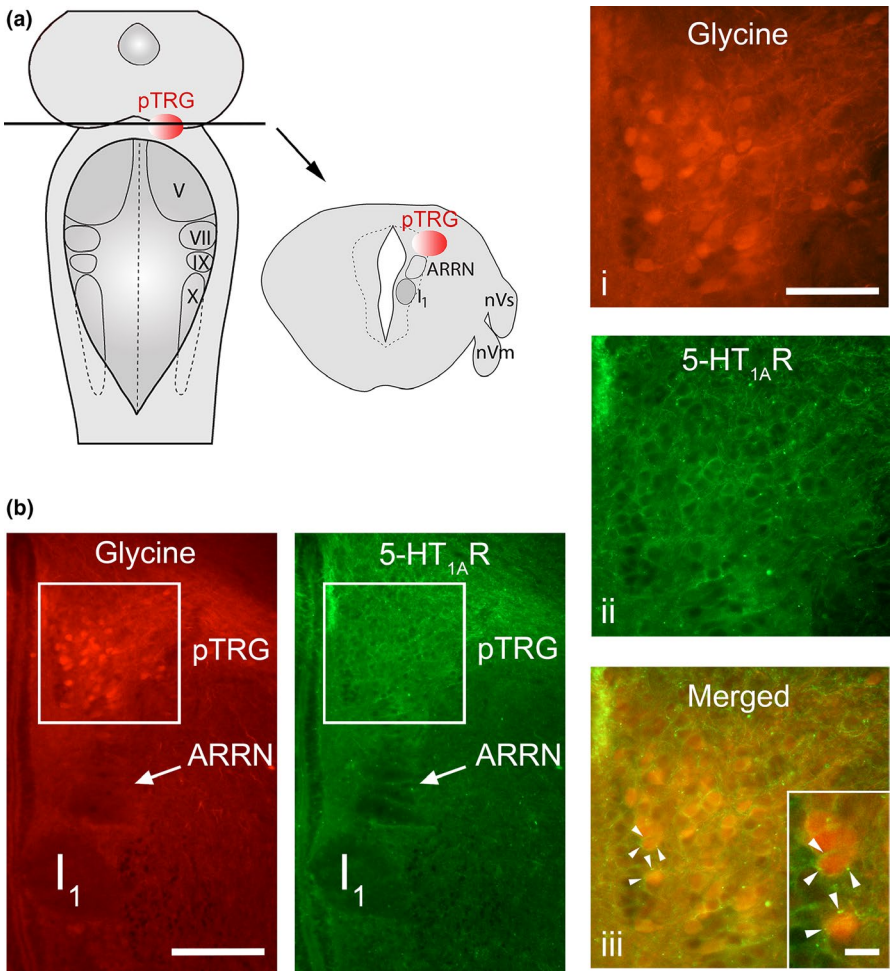

FIGURE 5 Immunohistochemical labelling of glycinergic neurons and 5-HT_{1A}Rs in the pTRG region. (a) Schematic illustration of a dorsal view of the lamprey mesencephalon/rhombencephalon showing the level (unbroken line) of a coronal section (arrow) where the pTRG is located (red area). (b) Photomicrographs of transverse sections at the pTRG level displaying glycine immunoreactivity (red signal) and 5-HT_{1A}R-like immunoreactivity (green signal). (b, panel i-iii) Photomicrographs at a higher magnification of a portion of the transverse section (white box in b) illustrating glycine-immunoreactive neuronal structures (panel i, red signal), 5-HT_{1A}R binding sites (panel ii, green signal) and merged image (panel iii). Note the presence of 5-HT_{1A}R-likeimmunoreactive structures located in close apposition to glycinergic neurons. Scale bars: (b), 100 μm; (b, panel i-iii), 50 μm; inset in panel iii, 10 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

3.3 | Immunohistochemical analysis of 5-HT_{1A}Rs and glycinergic neurons in the pTRG region

To corroborate these results, we assessed whether 5-HT_{1A}Rs are located in close proximity to glycinergic neurons within the pTRG by double-labelling experiments ($n = 4$). As illustrated in Figure 5, 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 (panel i, red signal) and 5-HT_{1A}R-like immunoreactive sites (panel ii, green signal) widely expressed in close apposition to the soma of glycine-immunoreactive cells (panel iii, 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 (Cinelli et al., 2014; Robertson, Auclair, Menard, Grillner, & Dubuc, 2007; Villar-Cervino, Barreiro-Iglesias, Anadon, & Rodicio, 2008).

4 | DISCUSSION

The results show for the first time in the adult lamprey that 5-HT exerts a tonic influence on respiration as revealed by the effects induced by the selective 5-HT_{1A}R antagonist (S)-WAY 100135. In particular, the results imply that

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 acting on glycinergic cells and on GABAergic terminals, in agreement with the notion that GABAergic neurons are not present in the pTRG region (Cinelli et al., 2014; Robertson et al., 2007; Villar-Cervino et al., 2008). Immunohistochemical data are consistent with the presence of 5-HT_{1A}Rs on glycinergic neurons and corroborate the present findings. We suggest that certain features of the 5-HT mechanisms involved in the modulation of the respiratory network are conserved throughout evolution. Admittedly, the sample size for each kind of trial was low. However, the effects of the different treatments were very clear, while their variability was relatively low. On the other hand, as already mentioned, we tried to minimize the number of animals employed.

4.1 | Tonic control of the lamprey pTRG through 5-HT_{1A}Rs

In the present study, we focused on the 5-HT_{1A}R subtype. A potent 5-HT-related tonic influence on respiration was shown by application of the 5-HT_{1A}R antagonist (S)-WAY 100135 (either applied to the bath or microinjected into the pTRG). Importantly, this tonic influence contributes to maintain basal respiratory activity. Changes in respiration could be operated by the regulation of this receptor mechanism via adjustments in the release of 5-HT. The results suggest that under in vitro conditions, this mechanism of inhibition–disinhibition mediated by 5-HT_{1A}Rs is a major determinant of basal respiratory activity since, when it is lacking, apnoea occurs, and no other mechanisms, either related to 5-HTRs or not, are brought into action to maintain respiration. However, the involvement of other 5-HTR subtypes could contribute to explain our results. In this context, it should be mentioned that at the concentrations used, the 5-HT_{1A}R antagonist (S)-WAY 100135 may also act at the level of 5-HT_{1B}Rs and 5-HT_{1D}Rs as a partial agonist (Davidson, Ho, Price, Jones, & Stamford, 1997). Interestingly, the activation of 5-HT_{1D}Rs appears to have a role in the control of the lamprey locomotor activity. Schwartz et al. (2005) found that the 5-HT_{1D}R agonist, L694-247, prolongs the frequency of ventral root bursting. They also showed that this agonist presynaptically inhibits synaptic transmission without altering postsynaptic Ca^{2+}-activated K^+ currents.

Consistently with the present knowledge in mammals (Del Negro et al., 2018; Doi & Ramirez, 2008), other mechanisms are known to contribute to the respiratory control in the lamprey (see Bongianni et al., 2016 and Cinelli et al., 2017 also for further references). Some neurotransmitters or neuromodulators have excitatory respiratory effects and contribute to respiratory rhythm generation, such as neurokinin receptor agonists, in particular the neurokinin-1 receptor agonist SP (Cinelli et al., 2013, 2017; Mutolo et al., 2010) as well as acetylcholine (Mutolo et al., 2011) and glutamate (Cinelli et al., 2013). On the contrary, ATP is not involved in the respiratory rhythmogenesis, but it can contribute to respiratory modulation (Cinelli et al., 2017), while endogenous opioids exert a depressing role by activating μ-opioid receptors and, to a minor extent, δ-opioid receptors (Mutolo et al., 2007). In the lamprey pTRG, the endogenous tonic release of excitatory amino acids, but not that of GABA and glycine, has a crucial role in the respiratory rhythmogenesis (Bongianni et al., 1999, 2016; Bongianni, Mutolo, Nardone, & Pantaleo, 2006; Cinelli et al., 2013, 2014; Martel et al., 2007; Rovainen, 1983). This occurs also in the mammalian pre-BötC (Bongianni, Mutolo, Cinelli, & Pantaleo, 2010; Del Negro et al., 2018; Kam, Worrell, Janczewski, Cui, & Feldman, 2013 also for further references). However, in the lamprey GABAergic mechanisms have a tonic modulatory role on respiration while a glycinergic tonic influence is lacking, as demonstrated by the absence of strychnine-induced respiratory effects (see also Cinelli et al., 2014). This latter finding is probably related to the tonic activation of inhibitory 5-HT_{1A}Rs on glycinergic neurons (present results). Nevertheless, both GABA and glycine have clear potent inhibitory effects as proved by the counteracting effects of bicuculline and strychnine on (S)-WAY 100135-induced depressing 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 (e.g. Abalo et al., 2007; Antri et al., 2006; Pierre et al., 1992). Noticeably, strong resemblances between lampreys and other vertebrates in the distribution and in the spatio-temporal pattern of development of brainstem serotoninergic neural populations exist (Abalo et al., 2007; Antri et al., 2006). In addition, the development of early ascending and descending serotoninergic pathways in the lamprey is similar to that of other vertebrates (Abalo et al., 2007). The comparison of the expression of 5-HT_{1A}Rs between sea lamprey and other vertebrates reveals a conserved pattern in most brain regions (Cornide-Petronio et al., 2013). Serotoninergic cells have been described in close vicinity to or within a region encompassing the pTRG. However, it seems plausible to suggest that projections to the pTRG may 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 (Abalo et al., 2007; Antri et al., 2006; Pierre et al., 1992).

4.2 | Disinhibition phenomena underlying respiratory responses due to 5-HT$_{1A}$R antagonism within the lamprey pTRG

While no significant effects were induced by strychnine microinjections into the pTRG, bicuculline microinjections caused a characteristic double-burst pattern. The lack of effects induced by bilateral microinjections of 1 mM strychnine performed into the pTRG, already observed by Cinelli et al. (2014), could probably be related to the complete inhibition of glycinergic cells via serotoninergic terminals, that is the absence of a glycinergic activity under basal conditions. On the other hand, bicuculline and gabazine microinjections were effective providing evidence of a tonic GABA$_A$ receptor-mediated modulation of the pTRG. Bicuculline-induced effects were quite similar to those reported by us in a previous study (Cinelli et al., 2014) as well as those observed in adult rabbits (Bongianni et al., 2010; Iovino et al., 2019) and adult rodents (Feldman et al., 2013; Janczewski et al., 2013; Kam et al., 2013 also for further references). These effects have been attributed to an increase in the excitability of rhythmogenic respiratory neurons in the pTRG or in the pre-BötC. 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 changes in 5-HT$_{1A}$R modulation of baseline respiration are brought into action are obscure and only a matter of speculation. In agreement with previous findings on the distribution of glycine immunoreactivity in the brain of adult sea lamprey (Villar-Cervino et al., 2008), the present study provides evidence of the existence of glycinergic-immunoreactive neurons within the pTRG. The finding of 5-HT$_{1A}$R-like 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.

The present results are consistent with previous findings in rodents. Manzke et al. (2009) demonstrated in the perfused rodent brainstem–spinal cord preparation the existence of a respiratory network modulation through 5-HT$_{1A}$R-mediated inhibition primarily affecting glycinergic neurons. Interestingly, in the same report they found in the cat complex 5-HT$_{1A}$R-induced disinhibitory mechanisms, probably acting on glycinergic postinspiratory neurons leading to excitatory respiratory modulation. On the other hand, Corcoran et al. (2014) showed in a transgenic mouse model that the excitatory effects on respiratory network were due to the 5-HT$_{1A}$R-mediated inhibition of inhibitory interneurons, that could be presumed glycinergic. Recently, we have provided evidence in the adult rabbit (Iovino et al., 2019) that 5-HT has a prominent role in the modulation of pre-BötC activity. On the basis of the novel findings presented in that study, we have suggested the existence of both pre- and postsynaptic serotoninergic mechanisms in the pre-BötC (see Fig. 8 in Iovino et al., 2019). In particular, a 5-HT$_{1A}$-mediated inhibition of pre-BötC GABAergic interneurons was demonstrated. The putative serotoninergic mechanisms subserving regulation of the respiratory network suggested by present results in the lamprey are schematically summarized in Figure 6. As shown in this Figure, 5-HT$_{1A}$Rs could be present both presynaptically on GABAergic terminals and postsynaptically on glycinergic neurons. To establish

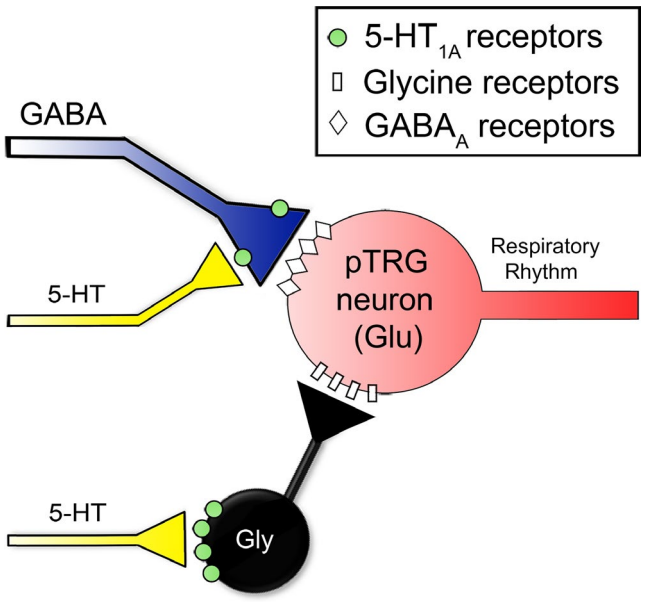

FIGURE 6 Schematic drawing, partially inferred from the present results, representing two hypothetical inhibitory mechanisms mediated by 5-HT$_{1A}$Rs within the pTRG region. Under basal conditions, both presynaptic 5-HT$_{1A}$Rs on GABAergic terminals and postsynaptic 5-HT$_{1A}$Rs on glycinergic neurons are endogenously activated and strongly reduce or completely suppress the release of the inhibitory neurotransmitters. Endogenous activation of 5-HT$_{1A}$Rs contributes to maintain the basal rhythmic respiratory activity. The blockade of 5-HT$_{1A}$Rs by the specific antagonist (S)-WAY 100135 induces apnoea owing to disinhibition of both inhibitory GABAergic and glycinergic mechanisms acting on pTRG excitatory neurons. The blockade of both these inhibitory mechanisms, completely abolishes (S)-WAY 100135-induced apnoeic responses. Present results show that also the blockade of only a single inhibitory mechanism is sufficient to prevent (S)-WAY 100135-induced apnoea, thus suggesting a synergistic action of the two inhibitory neurotransmitters. Since small depressing 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 depressing 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 postsynaptic 5-HT$_{1A}$Rs located on their somata

the prevailing pre- or postsynaptic location of 5-HT_{1A}Rs was beyond the scope of the present study. 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 Figure 6. The figure also help to explain how the (S)-WAY 100135-mediated disinhibition of both inhibitory mechanisms induces apnoea (prevented by their blockade) and, in addition, how the blockade of a single mechanism can still cause slight depressing effects on respiration probably due to a concomitant (S)-WAY 100135-induced disinhibition of the other mechanism. The absence of significant effects of the 5-HT_{1A}R agonists is difficult to interpret. Nevertheless, the circuit represented in Figure 6 could also help to provide a tentative explanation of this finding. The potent tonic inhibitory influence on both GABAergic and glycinergic mechanisms exerted by endogenously released 5-HT under basal conditions may activate maximally pre- and postsynaptic 5-HT_{1A}Rs, thus producing a sort of occlusion effect with the exogenously administered agonist. However, we must recognize that the actual explanation of our results can be more complex and other 5-HTR subtypes could have played a role. Interestingly, recent results (Ptak et al., 2009) have shown that excitatory amino acid-induced augmentation of the endogenous release of 5-HT and 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 other 5-HTR subtypes within the pre-BötC region. More recently, an optogenetic study by Depuy et al. (2011) performed in anaesthetized mice have demonstrated that more selective stimulation of raphe obscurus serotoninergic neurons activates breathing and potentiates the central respiratory chemoreflex. The presence and the role of other 5-HTR subtypes in the lamprey respiratory network would deserve further investigations.

The primary conclusion to be drawn from present results is that 5-HT_{1A}R modulation affects inhibition of pTRG neurons through glycinergic and GABAergic mechanisms. This neural organization, reminiscent of the mammalian respiratory network, along with the strong similarities between lampreys and other vertebrates in the distribution and development of brainstem 5-HT neurons, strongly supports the view that some characteristics of the neural network subserving respiration are highly conserved throughout evolution (see also Grillner, 2006; Grillner & El Manira, 2019).

COMPETING INTEREST

The authors declare no conflict of interests.

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AUTHORS' CONTRIBUTION

EC designed the study, performed the experiments, collected, analysed and interpreted data and wrote the manuscript. DM designed the study, performed and supervised the experiments, collected, analysed and interpreted data and wrote the manuscript. LI performed the experiments, collected, analysed and interpreted data. TP designed the study and wrote the manuscript. FB designed the study, analysed data and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data of the present study can be requested to the corresponding author.

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