The Journal of Physiology

# ATP and astrocytes play a prominent role in the control of the respiratory pattern generator in the lamprey

Elenia Cinelli, Ludovica Iovino and Donatella Mutolo

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

**Key points**

- The paratrigeminal respiratory group (pTRG) is responsible for the respiratory pattern generation in the lamprey.
- The role of ATP and astrocytes, known to control respiratory activity in mammals, was investigated in the lamprey respiratory network.
- ATP microinjected into the pTRG induces a biphasic response consisting of marked increases in respiratory frequency mediated by P2X receptors followed by a decrease in the respiratory motor output due to the ATP metabolite adenosine.
- We provide evidence that astrocytes are involved in the genesis of the normal respiratory pattern, ATP-induced responses and acidification-induced increases of the respiratory activity.
- The function of astrocytes in rhythmic networks appears to be phylogenetically conserved.

**Abstract** The role of ATP and astrocytes in respiratory rhythm modulation has been recently investigated in neonatal rodents. However, no information on the role of ATP and astrocytes within the respiratory network of the lamprey is available, particularly within the paratrigeminal respiratory group (pTRG), the proposed respiratory central pattern generator. To address these issues, the present study was carried out on isolated brainstems of the adult lamprey. Bath application of ATP caused marked increases in respiratory frequency followed by decreases in the respiratory motor output, mediated by the ATP metabolite adenosine at the level of the pTRG. Bath applications and microinjections of agonists and antagonists of purinergic receptors showed that ATP increased respiratory activity through an action on pTRG P2X receptors. To disclose the respiratory role of astrocytes, we used bath application of the gliotoxin aminoadipic acid, which dramatically depressed the respiratory motor output that, however, promptly recovered following glutamine application. Furthermore, the excitatory responses to ATP-$\gamma$-S (a non-hydrolysable ATP analogue), but not to substance P, microinjected into the pTRG, were abolished. Finally, we also demonstrated that acidification-induced increases in respiratory activity were ATP-independent, but mediated by the astrocytes' glutamate–glutamine cycle. The results show for the first time that ATP and especially astrocytes strongly contribute to the modulation of the lamprey respiratory pattern. Their role in the modulation or maintenance of rhythmic neuronal activities appears to be phylogenetically conserved.

(Resubmitted 9 June 2017; accepted after revision 21 July 2017; first published online 22 July 2017)
**Corresponding author** D. Mutolo: Dipartimento di Medicina Sperimentale e Clinica, Sezione Scienze Fisiologiche, Università degli Studi di Firenze, Viale G.B. Morgagni 63, 50134 Firenze, Italy. Email: donatella.mutolo@unifi.it

E. Cinelli and L. Iovino contributed equally to this work.


DOI: 10.1113/JP274749

**Abbreviations** AAA, L-2-aminoadipic acid; ADO, adenosine; $\alpha\beta$meATP, $\alpha,\beta$-methyleneadenosine 5′-triphosphate trisodium salt; ARRN, anterior rhombencephalic reticular nucleus; ATP-$\gamma$-S, adenosine 5′-[$\gamma$-thio]triphosphate tetralithium salt; CPG, central pattern generator; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GFAP, glial fibrillary acidic protein; Gln, glutamine; MRS 2179, 2′-deoxy-$N^6$-methyladenosine 3′,5′-bisphosphate tetrasodium salt; MRS 2365, [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo [3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt; P2XR, P2X receptor; P2YR, P2Y receptor; PB, phosphate buffer; PBS, phosphate-buffered saline; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium; preBötC, preBötzinger complex; pTRG, paratrigeminal respiratory group; SP, substance P; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP.

## Introduction

Astrocytes are the most abundant glial cells in the brain and are an important element of several functions in physiological and pathophysiological conditions (for review see Fellin, 2009; Perea *et al.* 2014; Harada *et al.* 2015). Several lines of evidence indicate that interactions between neurons at pre- and postsynaptic level and astrocytes (the so called 'tripartite synapses') are of extreme importance in the modulation of synaptic and network activities (see e.g. Araque *et al.* 1999; Halassa *et al.* 2007; Fellin, 2009). Astrocytes can respond to different neurotransmitters, such as glutamate, GABA, acetylcholine and ATP, and can control the activity of neurons through the uptake of neurotransmitters or the release of gliotransmitters, such as glutamate, D-serine, GABA and ATP, thus contributing to neuronal network function (Abbracchio *et al.* 2009; Fellin, 2009; Perea *et al.* 2014). Interestingly, some studies indicate that astrocytes regulate neuronal activities involved in the generation of rhythmic behaviours, such as respiration, locomotion and mastication (Hülsmann *et al.* 2000; Baudoux & Parker, 2008; Huxtable *et al.* 2010; Okada *et al.* 2012; Morquette *et al.* 2015; Oku *et al.* 2016).

It is well known that ATP plays a role in the control of the preBötzinger complex (preBötC), the proposed mammalian respiratory central pattern generator (CPG; for review see Feldman & Del Negro, 2006), and that astrocytes contribute to this purinergic modulation (Erlichman *et al.* 2010; Huxtable *et al.* 2010; Funk *et al.* 2015). ATP, which can act on different types of ionotropic P2X receptors (P2XRs) and metabotropic P2Y receptors (P2YRs) in the nervous system (Abbracchio *et al.* 2009; Burnstock, 2014), appears to exert potent excitatory effects on respiratory frequency mainly via $P2Y_1$Rs within the preBötC (Lorier *et al.* 2007; Huxtable *et al.* 2009; Zwicker *et al.* 2011; Funk, 2013). Furthermore, ATP is rapidly degraded to adenosine (Zimmermann, 2000), which has been reported to cause post-ATP decrease in the respiratory motor output (Huxtable *et al.* 2009; Zwicker *et al.* 2011; Funk, 2013). Interestingly, it is also widely accepted that in the mammalian respiratory network ATP-mediated mechanisms and astrocytes are involved in peripheral and central chemoreception (Gourine *et al.* 2005, 2010; Huckstepp *et al.* 2010; Wenker *et al.* 2010; Sobrinho *et al.* 2014; Murali & Nurse, 2016; Turovsky *et al.* 2016; for review see Erlichman *et al.* 2010; Funk, 2013; Funk *et al.* 2015; Moreira *et al.* 2015; Guyenet *et al.* 2016).

The isolated brainstem of the adult lamprey spontaneously generates rhythmic respiratory activity *in vitro* and the putative respiratory CPG is located in the paratrigeminal respiratory group (pTRG), a region rostral to the trigeminal motor nucleus (Mutolo *et al.* 2007, 2010, 2011; Cinelli *et al.* 2013, 2014; for review see Bongianni *et al.* 2016). Baseline (fast) respiratory activity is interrupted by prolonged bursts of vagal activity followed by changes in respiration, which often consist of a pause followed by an increase of respiratory frequency. These bursts, also called 'coughs', occur at lower and irregular frequency (Rovainen, 1977, 1996; Thompson, 1985; Martel *et al.* 2007) and probably have a different CPG (see also Hoffman *et al.* 2016).

No information is available on the role played by the purinergic mechanisms in breathing control of the lamprey, a lower vertebrate that diverged from the main vertebrate line around 560 million years ago (Kumar & Hedges, 1998) and has proved to be highly useful for demonstrating that the basic features of rhythmogenic networks are conserved throughout evolution (for review see Bongianni *et al.* 2016; Grillner & Robertson, 2016; Missaghi *et al.* 2016; Ramirez *et al.* 2016). Furthermore, knowledge of the functional role of astrocytes in lamprey rhythmic activities is limited to only one report (Baudoux & Parker, 2008) on the involvement of glial cells in the spinal cord locomotor activity.

At present, no specific studies on peripheral and central respiratory chemoreceptors in the lamprey are available. Results by Rovainen (1977, 1979) seem to suggest a minimal central respiratory $CO_2$/pH sensitivity in adult lampreys. In addition, recently Hoffman *et al.* (2016) found in the larval lamprey that the periodic prolonged bursts, described as 'coughs', are modulated by central sensitivity to $CO_2$. Interestingly, a novel central mechanism specifically sensitive to pH changes has also been described in the spinal cord of the adult lamprey (Jalalvand *et al.* 2016*a*, *b*).

The present study was undertaken to investigate the function of the purinergic signalling and astrocytes

within the lamprey respiratory network. Experiments were carried out on isolated brainstems of adult lampreys by using bath application or microinjections of drugs into the pTRG to address the following issues: (1) the presence and type of purinergic receptors at the pTRG level by investigating the effects of ATP and those of specific agonists and antagonists on respiratory activity; (2) the function of astrocytes in the control of the respiratory pattern generator by the application of the gliotoxin L-2-aminoadipic acid (AAA; Baudoux & Parker, 2008; Sherpa *et al.* 2014); (3) the involvement of astrocytes and ATP in the increase in respiratory activity induced by the acidification of the perfusing solution at constant $P_{CO_2}$; (4) the immunohistochemical detection of astrocytes at the level of the pTRG under control conditions and after AAA application.

## Methods

### Ethical approval

Experiments were carried out on 65 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 h light–12 h dark cycle. All animal care and experimental procedures were conducted in accordance with Italian legislation and the official regulations of the European Communities 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. The experiments conform to the principles and regulations of *The Journal of Physiology*, as described in the editorial by Grundy (2015). During the investigation, all efforts were made to minimize animal suffering and to reduce the number of animals used.

### Animal preparation

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). The animals were deeply anaesthetized by immersion in a solution containing 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 anaesthesia was terminal. Lampreys were transected below the gills, muscles and connective tissues 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 bath pH at 7.4. Under these experimental conditions, the respiratory frequency is similar to that of spontaneous respiration of lampreys in the aquarium at 9–10°C (Rovainen, 1977, 1996; Martel *et al.* 2007; see also Cinelli *et al.* 2014 for further references). In addition, we would like to note that similar conditions of temperature and pH have been used in all studies on the isolated brainstem–spinal cord of the lamprey. 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. 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). As in previous studies (e.g. Mutolo *et al.* 2007; Cinelli *et al.* 2014; see also Cinelli *et al.* 2013), 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). 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 analysed by a personal computer supplied with an analog-to-digital interface (50 kHz sampling rate; Digidata 1440, Molecular Devices, Sunnyvale, CA, USA) 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. Effective drugs could also be microinjected into the pTRG when we aimed at ascertaining whether their effects were mediated at this level. As soon as the brainstem was prepared, its respiratory motor output displayed some variability at the beginning, but

became very stable within ∼60 min. For this reason, the preparation was left to stabilize in the control solution for at least 60 min before control recordings. The following drugs were used: 10 $\mu$M or 1 mM adenosine 5′-triphosphate disodium salt hydrate (ATP; a P2R agonist, Sigma-Aldrich), 1 mM adenosine 5′-[$\gamma$-thio]triphosphate tetralithium salt (ATP-$\gamma$-S; a non-hydrolysable ATP analogue and P2R agonist, Sigma-Aldrich), 0.5 mM adenosine (an $A_{1,2,3}$R agonist, Sigma-Aldrich), 10 $\mu$M 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; an $A_1$R antagonist, Sigma-Aldrich), 100 $\mu$M pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium (PPADS; a P2R antagonist, Tocris Bioscience, Bristol, UK), 1 mM [[(1*R*,2*R*,3*S*,4R,5*S*)-4-[6-amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS 2365; a selective $P2Y_1$R agonist, Tocris Bioscience), 100 $\mu$M 2′-deoxy-$N^6$-methyladenosine 3′,5′-bisphosphate tetrasodium salt (MRS 2179; a selective $P2Y_1$R antagonist, Tocris Bioscience), 1 mM $\alpha,\beta$-methyleneadenosine 5′-triphosphate trisodium salt ($\alpha\beta$meATP; a $P2X_{1,3}$R agonist, Tocris Bioscience), 10 $\mu$M 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP (TNP-ATP; a $P2X_{1,3}$R antagonist, Tocris Bioscience), 5 mM L-glutamine (Gln; Sigma-Aldrich), 1 mM AAA (a potent and specific gliotoxin, Sigma-Aldrich), 1 $\mu$M substance P (SP; Tocris Bioscience). Drugs were dissolved in distilled water except for DPCPX and AAA, which were dissolved in 0.1 M NaOH and phosphate-buffered saline (PBS; pH 7.4), respectively. Stock solutions were made up, aliquoted and stored as small aliquots in a freezer until use. Only ATP and adenosine were prepared fresh on the day of the experiment. 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 and application times were similar to those employed in previous studies (Lorier *et al.* 2007, 2008; Baudoux & Parker, 2008; Huxtable *et al.* 2009, 2010; Mutolo *et al.* 2010, 2011; Zwicker *et al.* 2011; Cinelli *et al.* 2013; Sherpa *et al.* 2014). As a rule, each drug was allowed to perfuse the brainstem for 30 min, with the exception of AAA, which was applied to the bath for ∼60 min or more (see Results) owing to its relatively slowly developing action (Baudoux & Parker, 2008; Sherpa *et al.* 2014). After completion of each drug challenge, the preparation was allowed to recover by perfusing it with the control solution. In eight experiments, the respiratory effects induced by reducing the pH of the perfusing solution from 7.4 to 7.0 (see Rovainen, 1977) for 10 min were studied. The pH was adjusted to 7.0 by decreasing the $NaHCO_3$ concentration to 6 mM and adjusting the NaCl concentration at 103 mM (see also Harada *et al.* 1985; Dubreuil *et al.* 2009).

Bilateral microinjections (0.5–1 nl) of different drugs were performed by means of glass micropipettes (tip diameter 10–20 $\mu$m) and by applying pressure pulses of 50–100 ms with a Picospritzer (General Valve Corp., Fairfield, NJ, USA) connected to the injection pipette (for the microinjection procedures see e.g. Cinelli *et al.* 2014). 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 injection. The localization of the pTRG was judged by 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). 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, New City, NY, USA) were added (dilution 1:3) to the injectate (1 mM ATP) 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 $\mu$m thickness on a cryostat. Coronal sections stained with cresyl violet were used for the histological control.

### Immunohistochemistry

Experiments were performed for immunohistochemical detection of astrocytes under control conditions ($n = 2$) or ∼60 min after bath application of 1 mM AAA ($n = 2$). The dissection, fixation and sectioning of the lamprey brains were performed as previously described (Cinelli *et al.* 2013, 2014). 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 4% formalin in 0.1 M PB pH 7.4 for 4 h, after which they were cryoprotected in 20% sucrose in PB for 3–12 h. Transverse 20 $\mu$m-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 0.3% Triton X-100 in 0.1 M PB. Sections were incubated for 24 h at 4°C with a rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:400; Z0334; DakoCytomation, Glostrup, Denmark). The specificity of the antibody used for the detection of astrocytes is known from a previous study in the lamprey (Wasowicz *et al.* 1994). After a thorough rinse in PBS containing 0.3% Triton X-100, sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:400; Invitrogen, Life Technologies, Carlsbad, CA, USA) for 2 h at room temperature. Sections were then coverslipped with a proper mounting medium to obtain staining of nuclei with

4′,6-diamidino-2-phenylindole (DAPI) (Vectashield, hard set mounting medium with DAPI, Vector Laboratories, Burlingame, CA, USA). Photomicrographs of key results were taken using a Nikon DS-Fi1 (Nikon, Japan) digital camera and software. Illustrations were prepared in Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA). Images were only adjusted for brightness and contrast.

### Data analysis

We performed sample size calculation and power analysis for the main experimental conditions (see e.g. Fisher & Van Belle, 1996). We considered the direction of effects one-tail according to expected results (only one population is considered since the analysis for paired samples was employed). Furthermore, we considered the probability $\alpha$ of a Type I error equal to 0.05 (95%) and the probability $\beta$ of a Type II error equal to 0.20 (80%). The minimum difference in respiratory frequency to be detected was set at 10 with a standard deviation ranging from 5 to 7. The calculated sample sizes were very low and consistent with those reported in the study. Actually, the effects of the different treatments were relatively intense, while their variability was relatively low. 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 one experiment with 10 $\mu$M ATP applied to the bath and in four experiments with low-pH solutions applied following AAA and Gln (see Results), long trace recordings were performed. One-way repeated-measures ANOVA followed by the Student–Newman–Keuls test was used to assess the effects of each drug as well as the effects caused by reducing the pH of the perfusing solution. As a rule, average values of respiratory variables observed in control conditions, at the time when the maximum response occurred, and after recovery were considered for statistical analysis (Sigma Stat; Systat Software, San Jose, CA, USA). However, this procedure was not followed when we applied AAA (see data in the text and Fig. 4). Student's paired *t* test was also employed when appropriate. Vagal burst duration has not been reported in the Result since it did not show significant changes in any of the considered experimental conditions. Changes in respiratory variables were also expressed as percentage variations of control values. The number of preparations is indicated by *n*. The same preparations could be used for different types of trials. 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

### ATP-induced biphasic respiratory responses

Bath application of 10 $\mu$M ATP ($n = 6$) caused marked increases in respiratory frequency, from 61.3 ± 1.8 to 86.0 ± 1.5 cycles min$^{-1}$ (+40.3 ± 3.5%; $P < 0.001$), without significant changes in peak amplitude of vagal bursts (Fig. 1*A*). Increases in respiratory frequency started within 2 min after bath application and reached a maximum within 5 min. Maximum effects persisted for ∼10 min. Then, respiratory frequency decreased gradually, reaching a minimum within 20 min (51.5 ± 1.9 cycles min$^{-1}$, −19.5 ± 2.7%; $P < 0.01$). These latter effects were accompanied by reductions in peak vagal activity (−16.7 ± 3.4%; $P < 0.05$; Fig. 1*A*). Respiratory activity recovered within a 60 min washout. Bilateral microinjections of 1 mM ATP (0.5–1 pmol) into the pTRG (in 5 of these preparations) increased respiratory frequency from 60.5 ± 2.4 to 87.7 ± 1.3 cycles min$^{-1}$ (+45.7 ± 5.2%; $P < 0.001$) without significant changes in peak vagal activity (Fig. 1*B*). The respiratory responses started within 1 min after the injections and reached a maximum within 5 min. Respiratory frequency remained elevated for ∼15 min after the injections. Thereafter, it declined below control levels, reaching a minimum within 20 min (47.1 ± 3.5 cycles min$^{-1}$, −23.0 ± 4.1%; $P < 0.001$). Concomitant decreases in peak vagal activity (−22.8 ± 2.7%; $P < 0.05$) were observed (Fig. 1*B*). Respiratory activity recovered within 60 min after the injections. 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 ATP at sites 0.4 mm or more from the responsive region either before or after ATP microinjections into the pTRG (4 trials in all) did not induce any apparent respiratory response (data not shown).

### Role of adenosine in ATP-induced respiratory depression

Bilateral microinjections ($n = 5$) of 1 mM ATP-$\gamma$-S (0.5–1 pmol), a non-hydrolysable ATP analogue, were performed into the pTRG. They caused marked increases in respiratory frequency, from 60.8 ± 1.8 to 89.5 ± 3.0 cycles min$^{-1}$ (+47.6 ± 5.7%; $P < 0.001$), without significant changes in peak vagal activity (Fig. 2*A*). The respiratory responses started within 1 min after the injections and reached a maximum within 5 min. Respiratory frequency remained elevated for ∼20 min and recovered gradually within 60 min after the injections. Noticeably, ATP-induced decreases in respiratory frequency were not observed. Furthermore, as illustrated in Fig. 2*B*, microinjections ($n = 5$) of

0.5 mM adenosine (0.25–0.5 pmol) into the pTRG reduced both the respiratory frequency, from 62.0 ± 2.6 to 45.4 ± 1.3 cycles min$^{-1}$ (−26.5 ± 2.2%; $P < 0.001$), and peak vagal activity (−23.8 ± 3.6%; $P < 0.001$). The respiratory response appeared within 2 min after the injections and reached a minimum within 5 min. Respiratory activity remained depressed for 10 min and then gradually recovered within 60 min. Bath application ($n = 3$) of 10 $\mu$M DPCPX, an $A_1$R antagonist, did not alter the respiratory activity, but prevented the decreases in respiratory activity caused by adenosine microinjected into the pTRG (Fig. 2*C*). In the presence of DPCPX (same preparations), 1 mM ATP microinjected into the pTRG (Fig. 2*D*) elicited only increases in respiratory frequency, from 62.7 ± 1.3 to 84.3 ± 4.7 cycles min$^{-1}$ (+40.4 ± 5.7%; $P < 0.01$). Finally, to determine whether endogenous ATP is involved in the control of baseline respiratory activity, the effects of the P2R antagonist PPADS were investigated. Bath application ($n = 3$) of 100 $\mu$M PPADS did not alter respiratory activity, but prevented the increases in respiratory frequency in response to microinjections of 1 mM ATP-$\gamma$-S into the pTRG (not shown).

## Involvement of P2R subtypes in ATP-induced respiratory responses

First we investigated whether $P2Y_1$Rs have a role since their mediation of ATP-evoked increases in respiratory frequency at the level of the mammalian preBötC is well known (Lorier *et al.* 2007). Bilateral microinjections ($n = 4$) of the $P2Y_1$R agonist MRS 2365 (1 mM; 0.5–1 pmol) into the pTRG did not change respiratory activity (Fig. 3*A*). In addition, bath application ($n = 4$) of 100 $\mu$M MRS 2179, a $P2Y_1$R antagonist, did not affect respiration, nor prevent the increases in respiratory frequency (from 62.0 ± 3.0 to 88.1 ± 2.8 cycles min$^{-1}$, +42.6 ± 5.7%; $P < 0.001$) in response to ATP-$\gamma$-S microinjected into the pTRG (Fig. 3*A*). On the other hand, bilateral microinjections ($n = 5$) of the $P2X_{1,3}$R agonist $\alpha\beta$meATP (1 mM; 0.5–1 pmol) caused marked increases in respiratory frequency, from 61.4 ± 3.1 to 84.1 ± 3.4 cycles min$^{-1}$ (+37.4 ± 4.1%, $P < 0.001$; Fig. 3*B*), without changes in peak vagal activity. The respiratory responses started within 1 min after the injections and attained a maximum within 5 min. Respiratory frequency remained elevated for 15 min and recovered within 45 min after the injections. Furthermore, bath application ($n = 4$) of the $P2X_{1,3}$R

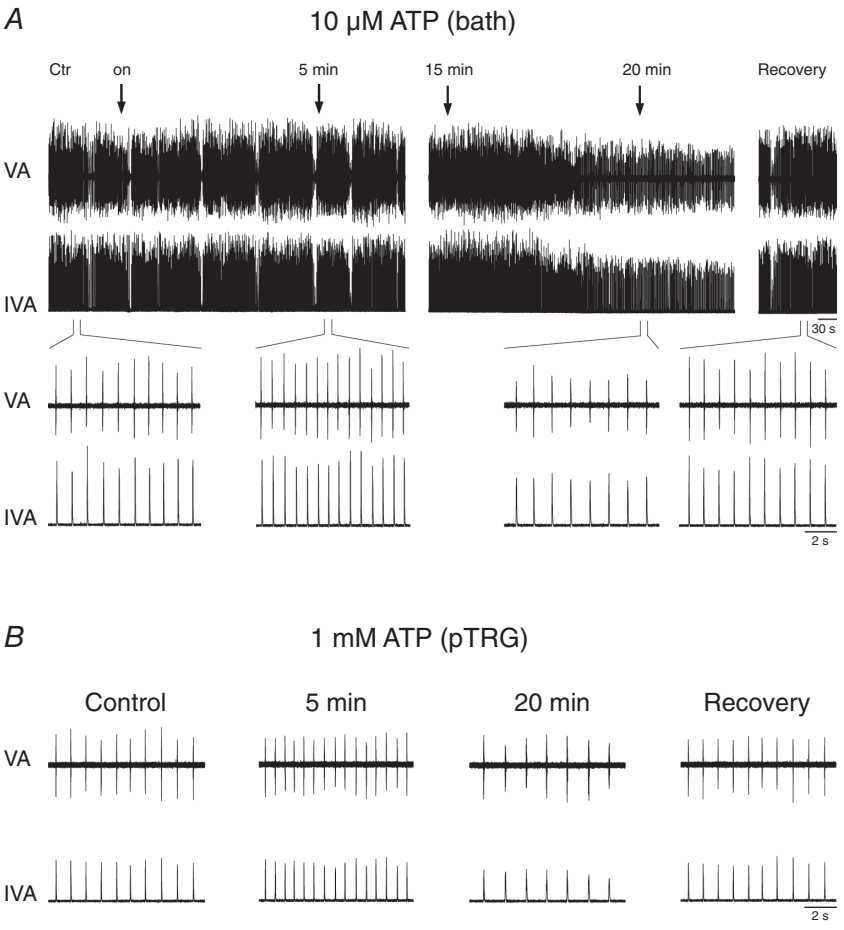


**Figure 1. Respiratory responses to ATP**
*A*, biphasic responses induced by bath application of 10 $\mu$M ATP in one preparation. Long trace recordings of raw vagal activity (VA) and integrated vagal activity (IVA) under control conditions (Ctr), at different times following bath application of 10 $\mu$M ATP (on) and after 60 min washout (Recovery). Expanded traces show control baseline respiratory activity, maximum increases in respiratory frequency (5 min), maximum decreases in respiratory frequency and peak vagal activity (20 min) and recovery. Pauses in the ongoing (fast) respiratory activity indicate the presence of 'coughs'. *B*, similar biphasic responses induced by bilateral microinjections of 1 mM ATP into the pTRG of a different preparation.

antagonist TNP-ATP at 10 $\mu$M did not modify baseline respiratory activity, but prevented the excitatory effects caused by 1 mM $\alpha\beta$meATP microinjected into the pTRG (Fig. 3*B*). In four additional preparations, bath application of 10 $\mu$M TNP-ATP prevented the excitatory responses of 1 mM ATP-$\gamma$-S microinjected into the pTRG (Fig. 3*B*).

## Mediation of ATP-induced respiratory responses by astrocytes

The contribution of astrocytes to the modulation of the respiratory activity was investigated by using the gliotoxin AAA. The time course of drug-induced responses is reported in Fig. 4. Bath application ($n = 4$) of 1 mM AAA progressively increased respiratory frequency (from 57.6 ± 3.3 to 74.6 ± 3.3 cycles min$^{-1}$, +30.8 ± 10.4%; $P < 0.05$) and peak vagal activity (+47.5 ± 5.2%; $P < 0.05$), showing the maximum effect after 30 min. Then, the frequency and amplitude of vagal bursts progressively decreased and after 60 min they turned out to be reduced ($P$ always $< 0.05$) by 34.1 ± 3.4% (38.2 ± 3.7 cycles min$^{-1}$) and 32.0 ± 8.8%, respectively. In the absence of washout, this level of respiratory depression persisted for relatively long periods (up to 120 min in two preparations). Surprisingly, if after 60 min the preparation was perfused with the control solution to allow recovery, respiratory activity continued to decrease and after 30 min washout displayed further marked reductions ($P$ always $< 0.001$) in respiratory frequency (4.5 ± 4.5 cycles min$^{-1}$; −91.8 ± 8.2%) and peak vagal activity (−88.8 ± 11.2%). However, bath application of 5 mM Gln in the same preparation caused a rapid recovery of respiratory variables (Fig. 4), consistent with the glial function to provide neurons with Gln for glutamate synthesis (for review see Broer & Brookes, 2001). AAA-induced respiratory depression was long-lasting as ascertained in two additional preparations after 3 h washout. Gln application restored respiration, even under these circumstances. It is worth noting that bath application of 5 mM Gln under control conditions (3 trials before AAA application) did not appreciably change respiratory motor output.

To evaluate the possible involvement of astrocytes in the ATP-induced effects, bilateral microinjections ($n = 4$) of 1 mM ATP-$\gamma$-S (0.5–1 pmol) into the pTRG ~50 min after bath application of 1 mM AAA was performed. No changes in respiratory activity were seen. By contrast, microinjections of 1 $\mu$M SP (0.5–1 fmol; 4 trials), which has an excitatory role within the pTRG (Mutolo *et al.* 2010; Cinelli *et al.* 2013), executed either after (2 trials) or before (2 trials) ATP-$\gamma$-S microinjections, markedly increased respiratory frequency and peak vagal activity within 2 min after the injections (see Fig. 5). The presence of astrocytes under control conditions and their alterations ~60 min following bath application of 1 mM AAA were observed by immunohistochemical analysis. AAA altered the morphology of GFAP-reactive structures: astrocytes exhibited shorter principal branches that appeared twisted and bent as compared with controls. GFAP immunostaining of astrocytes at the level of the pTRG under control conditions as well as after bath application of 1 mM AAA is shown in Fig. 6.

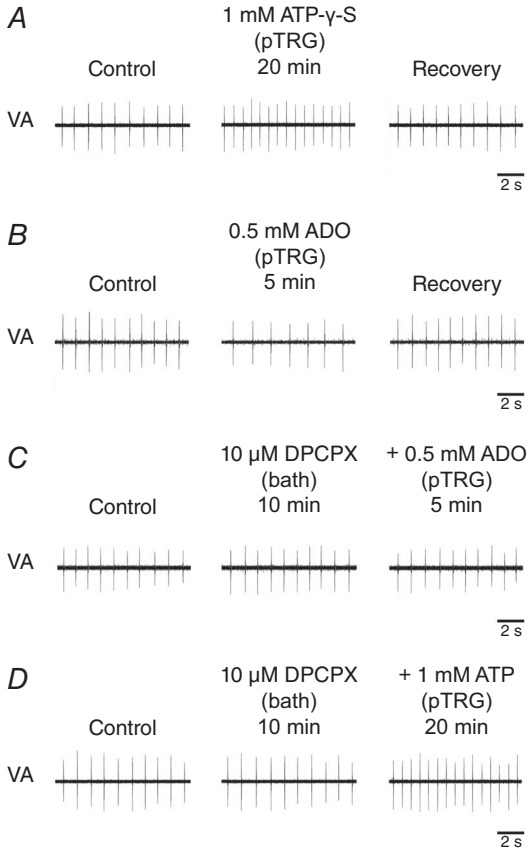


**Figure 2. Respiratory effects caused by ATP hydrolysis**
*A*, excitatory respiratory effects 20 min after bilateral microinjections of 1 mM ATP-$\gamma$-S into the pTRG. *B*, depressant respiratory effects 5 min after bilateral microinjections of 0.5 mM adenosine (ADO) into the pTRG. *C*, absence of appreciable respiratory effects 10 min after bath application of the $A_1$R antagonist DPCPX (10 $\mu$M) as well as 5 min following 0.5 mM ADO microinjected into the pTRG in the presence of this antagonist in the bath. *D*, in the presence of 10 $\mu$M DPCPX, only increases in respiratory frequency were observed 20 min after microinjections of 1 mM ATP into the pTRG. Traces are raw vagal activity (VA).

## Role of astrocytes and ATP in the acidification-induced increases in respiration

Because acidification has been reported to increase respiratory activity in the lamprey (Rovainen, 1977), the involvement of ATP and astrocytes was investigated. The pH of the perfusing solution was reduced from 7.4 to 7.0 at constant $P_{CO_2}$ in four preparations. As shown in

Fig. 7, this manoeuvre increased respiratory frequency (from 58.7 ± 3.8 to 78.1 ± 3.8 cycles min$^{-1}$, +33.5 ± 3.6%; $P < 0.001$) and peak vagal activity (+45.3 ± 5.9%; $P < 0.001$). These effects became obvious within 2 min after bath application of the low-pH perfusing solution and reached a maximum within 5 min. Perfusion of these preparations with the control solution led to a complete recovery within 5 min. After recovery, bath application of 100 $\mu$M PPADS did not affect baseline respiratory activity (see above) nor excitatory respiratory responses to the low-pH perfusing solution applied in close succession (Fig. 7). After 1 h washout, we investigated the contribution of astrocytes to the excitatory effects caused by acidification. AAA (1 mM) applied for ∼50 min decreased both respiratory frequency (from 59.5 ± 2.7 to 42.9 ± 3.1 cycles min$^{-1}$, −27.3 ± 3.2%; $P < 0.01$) and peak vagal activity (−21.0 ± 6.1%; $P < 0.01$). The subsequent immediate perfusion of the preparations with the low-pH solution did not evoke any increase in the respiratory motor output (Fig. 7). In four additional preparations, the same experimental procedure, already adopted to reveal the effects of low pH as well as those of AAA and Gln, gave similar results (Fig. 8*A* and *B*). Of note, after Gln-induced resumption of rhythmic respiratory activity, the response to the low-pH perfusing solution was completely restored (Fig. 8*C*) and displayed similar increases in both respiratory frequency (from 59.5 ± 3.1 to 78.5 ± 4.3 cycles min$^{-1}$, +32.5 ± 3.2%; $P < 0.001$) and peak vagal activity (+43.1 ± 6.1%; $P < 0.001$).

## Discussion

This study is the first to provide evidence that ATP and astrocytes play important roles in the lamprey respiratory network through an action at the level of the respiratory CPG. In more detail, ATP microinjected into the pTRG induces a biphasic response consisting of marked increases in respiratory frequency mediated by P2XRs followed by decreases in the respiratory motor output due to the degradation of ATP to adenosine. Bath application of the gliotoxin AAA causes pronounced increases followed by depression of the respiratory motor output that is restored by Gln application, thus suggesting that the glia glutamate–glutamine cycle is crucial for the maintenance of the respiratory rhythmic activity. We demonstrate that ATP-induced increases in respiratory frequency are mediated by astrocytes located within the pTRG since they are no longer evoked by ATP-$\gamma$-S microinjected into the pTRG after bath application of AAA. The results also reveal for the first time the existence of a central, ATP-independent, pH sensitivity that requires astrocyte metabolic support. The immunohistochemical demonstration of AAA-induced alterations of pTRG astrocytes strongly corroborates present findings.

### Purinergic modulation of the pTRG

Our results provide evidence that the purinergic signalling system is present within the pTRG and that ATP

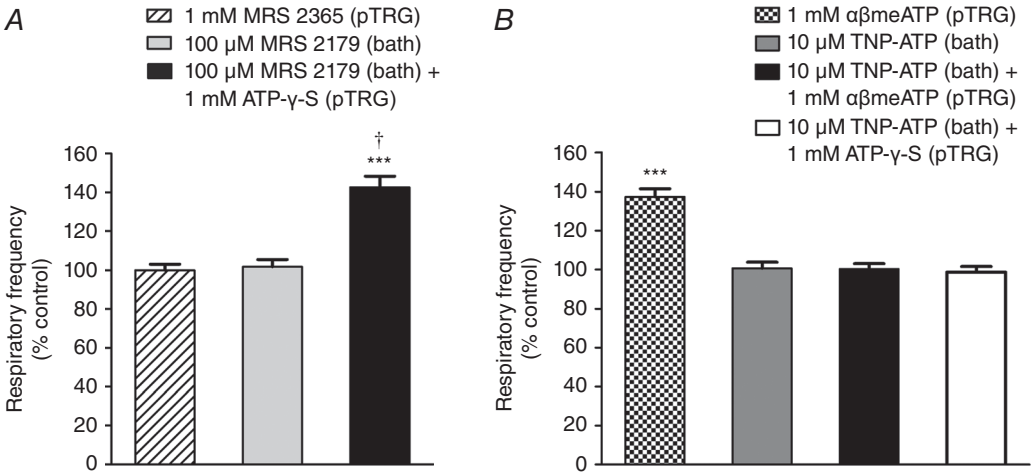


**Figure 3. Role of P2 receptors in ATP-induced respiratory responses**
*A*, bar graphs illustrating that neither microinjections ($n = 4$) of the $P2Y_1R$ agonist MRS 2365 (1 mM) into the pTRG nor bath application ($n = 4$) of the $P2Y_1R$ antagonist MRS 2179 (100 $\mu$M) changed the respiratory frequency. Noticeably, in the presence of MRS 2179, 1 mM ATP-$\gamma$-S microinjected into the pTRG increased the respiratory frequency. *B*, bar graphs illustrating that microinjections ($n = 5$) of the $P2X_{1,3}R$ agonist $\alpha\beta$meATP (1 mM) caused marked increases in respiratory frequency. Bath application ($n = 4$) of the $P2X_{1,3}R$ antagonist TNP-ATP (10 $\mu$M) did not modify the respiratory activity, but prevented the excitatory effects induced by 1 mM $\alpha\beta$meATP microinjected into the pTRG. More importantly, the $P2X_{1,3}R$ antagonist prevented respiratory responses induced by 1 mM ATP-$\gamma$-S microinjected into the pTRG ($n = 4$). Values are means ± SEM. ***$P < 0.001$, compared with control; †$P < 0.001$, compared with MRS 2179.

causes biphasic respiratory responses that are related to ATP hydrolysis. In fact, only increases in respiratory frequency are evoked by the non-hydrolysable ATP agonist ATP-$\gamma$-S, while observed decreases in respiratory frequency are mimicked by the ATP metabolite adenosine (Zimmermann, 2000), thus suggesting that the balance between ATP- and adenosine-induced effects could ultimately determine the actual respiratory frequency. Similar ATP-induced biphasic respiratory responses have been reported in previous studies on the preBötC of neonatal rats (Lorier *et al.* 2007; Huxtable *et al.* 2009; Funk, 2013). In contrast, the preBötC appears to be insensitive to ATP in mice and only after blockade of $A_1$Rs with DPCPX can the ATP-induced increases in respiratory frequency be obtained (Zwicker *et al.* 2011). In this animal species adenosine-mediated inhibition counteracts ATP-mediated excitation on account of the predominant expression of an ectonucleotidase isoform which rapidly degrades ATP to adenosine. Furthermore, it could be expected that also ADP, derived from ATP hydrolysis (Zimmermann, 2000) and known to increase respiratory frequency by acting at the preBötC level (Huxtable *et al.* 2009), plays a role within the lamprey respiratory network. However, since ADP is an agonist of P2YRs (e.g. Burnstock, 2014), which are not involved in the ATP-induced respiratory responses (present results), we can reasonably exclude any ADP contribution to the control of breathing in the lamprey. The finding that adenosine causes depressant effects on respiratory activity is consistent with the results of several previous studies in different animal species (e.g. Herlenius & Lagercrantz, 1999; Mironov *et al.* 1999; Huxtable *et al.*

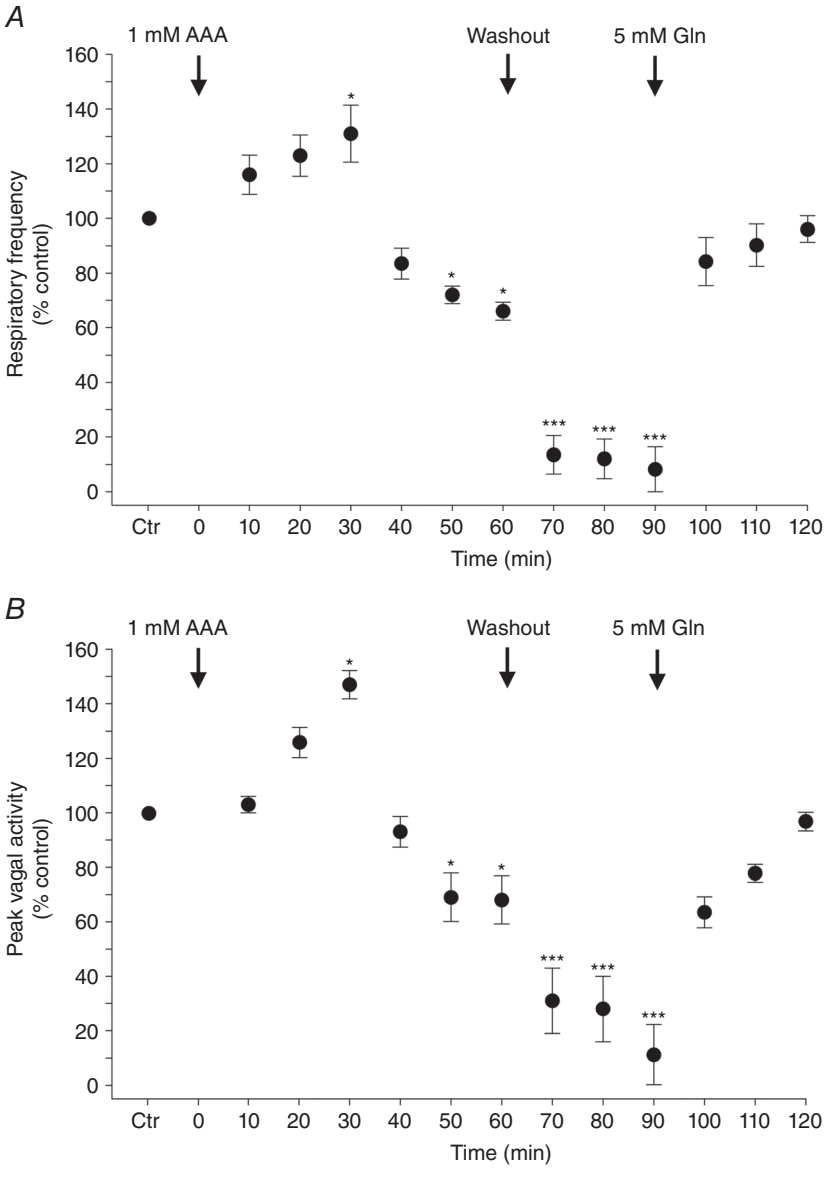


**Figure 4. Time course of respiratory responses following bath application of the gliotoxin aminoadipic acid and glutamine**
Bath application ($n = 4$) of 1 mM AAA caused increases in respiratory frequency (*A*) and peak vagal activity (*B*) followed by progressive decreases in both these respiratory variables that continued to decrease even after washout. Ctr, control. Arrows indicate the onset of bath application of 1 mM AAA, control perfusing solution (washout) and 5 mM Gln. Note that bath application of Gln caused the rapid recovery of respiratory activity. Values are means ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with control.

2009; Zwicker *et al.* 2011; Funk, 2013). In this context, it seems appropriate to recall that purinergic neurotransmitters were found for the first time to be involved in the regulation of rhythmic movements in frog embryos through an ATP–adenosine biphasic effect (Dale & Gilday, 1996).

The lack of respiratory effects induced by $A_1R$ and P2R antagonists indicates that endogenous adenosine and ATP do not exert a tonic influence on baseline respiratory activity. This finding does not completely exclude the possibility of a tonic purinergic modulation of respiration since the isolated brainstem may lack potential sources of ATP. The possibility remains that ATP does not modulate ongoing respiratory activity at all (see also Lorier *et al.* 2007). However, the presence of specific purinergic receptors within the pTRG and the respiratory effects of exogenous ATP strongly suggest that ATP could affect respiration when released in response to specific stimuli whose nature is at present unknown. We believe that the antagonists at the employed concentrations block totally their specific receptors. In fact, the respiratory effects induced by microinjections of adenosine and ATP-$\gamma$-S into the pTRG are prevented by the presence of DPCPX and PPADS, respectively. In agreement with our interpretation, ATP increases only the respiratory frequency during $A_1R$ blockade. Finally, our results on the purinergic antagonists are in keeping with previous observations in *in vitro* preparations from postnatal animals (Lorier *et al.* 2007; Huxtable *et al.* 2009; Zwicker *et al.* 2011), although it has been reported that adenosine can exert a tonic inhibitory influence on the respiratory frequency in fetal or newborn mammals (Herlenius & Lagercrantz, 1999; Huxtable *et al.* 2009; but see Mironov *et al.* 1999). These latter findings suggest that the potency of adenosine-related respiratory modulation decreases during the first days after birth.

In contrast with previous findings showing that $P2Y_1Rs$ are primarily involved in the ATP-evoked excitatory respiratory effects within the preBötC (Lorier *et al.* 2007), here we demonstrate that responses induced by ATP microinjected into the pTRG are not due to the activation of $P2Y_1Rs$. Our results clearly indicate that $P2X_{1,3}Rs$ are responsible for ATP-induced responses. In this context, it is interesting to recall that the purinergic signalling system appeared very early during phylogenesis and is present in all types of cells and tissues (Verkhratsky & Burnstock, 2014). In particular, P2XRs were the earliest purinergic receptors to appear and their structure remains almost unchanged during evolution. The metabotropic P2YRs appeared much later during evolution, more specifically in sharks and rays (Verkhratsky & Burnstock, 2014, also for further references). Thus, although no information is available, to our knowledge, on the presence and cellular localization of purinergic receptors in the lamprey, it seems plausible that $P2Y_1Rs$ are not expressed at least within the pTRG or even in the entire lamprey brain.

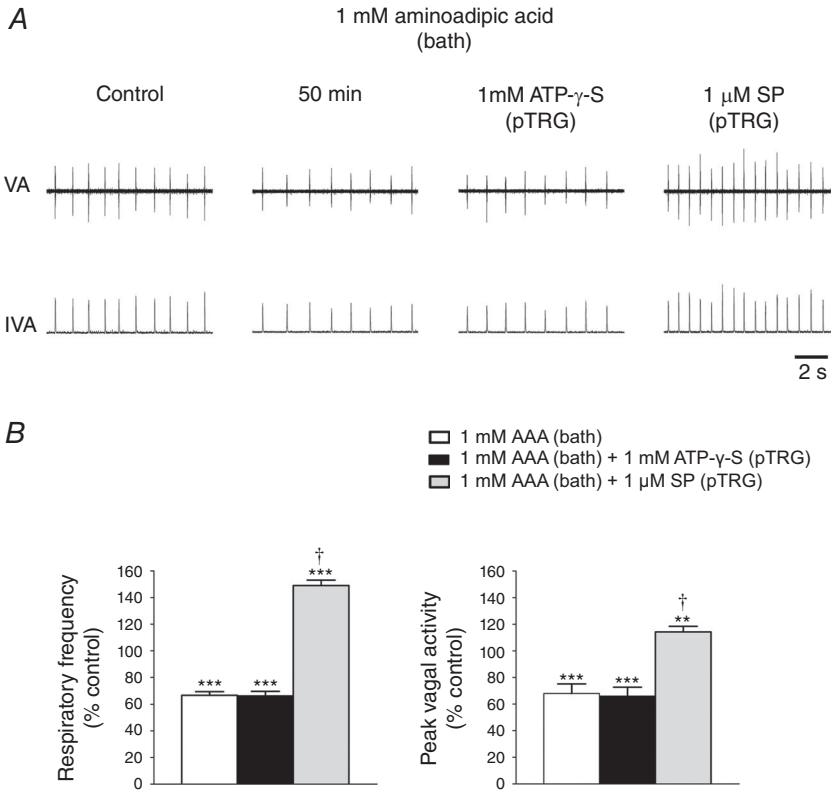


**Figure 5. Astrocyte mediation of the increases in respiratory activity evoked by ATP-$\gamma$-S and SP**
*A*, after bath application of 1 mM AAA for 50 min, bilateral microinjections of 1 mM ATP-$\gamma$-S into the pTRG did not change respiratory activity, while similar microinjections of 1 $\mu$M SP markedly increased respiratory activity. Traces are raw vagal activity (VA) and integrated vagal activity (IVA). *B*, bar graphs illustrating the changes in respiratory frequency and peak vagal activity induced by microinjections of ATP-$\gamma$-S and SP into the pTRG 50 min after bath application of 1 mM AAA ($n = 4$). Values are means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control; †$P < 0.001$ compared with AAA.

Despite P2YRs being primarily involved in the respiratory control within the preBötC (Lorier *et al.* 2007; Zwicker *et al.* 2011), also P2XRs have an important respiratory role in mammals since they are engaged in central chemosensitivity (Gourine *et al.* 2005, 2010; Mulkey *et al.* 2006; Moreira *et al.* 2015).

### Contribution of astrocytes to the generation of respiratory pattern and ATP-mediated responses

Our results show for the first time that astrocytes strongly contribute to the modulation of the lamprey respiratory CPG. They are consistent with previous findings showing that respiratory activity in medullary slices of neonatal mice and rats was markedly reduced or even blocked by using fluoroacetate or methionine sulfoximine, two different glial toxins that block glial metabolism (Hülsmann *et al.* 2000; Huxtable *et al.* 2010). As also reported in these previous studies, Gln restores respiratory activity indicating that glutamatergic neurotransmission, crucial for the lamprey respiratory rhythm generation (Bongianni *et al.* 1999; Martel *et al.* 2007; Cinelli *et al.* 2013), is controlled by the glia glutamate–glutamine cycle. Glutamate released from synaptic terminals is converted by astrocytes to Gln, which is then released, taken up by synaptic terminals and

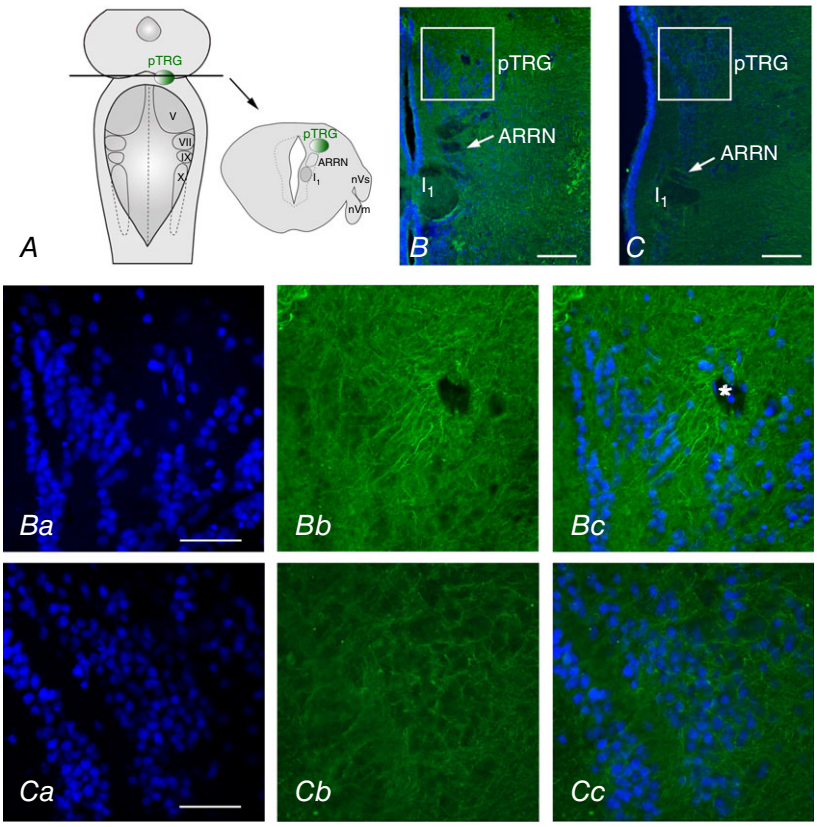


**Figure 6. Immunohistochemical labelling of astrocytes in the pTRG region and aminoadipic acid-induced alterations**
*A*, schematic illustration of a dorsal view of the lamprey mesencephalon/rhombencephalon showing the level (unbroken line) of the coronal section (arrow) where the pTRG is located (green area). *B* and *C*, photomicrographs of transverse sections at the pTRG level displaying GFAP immunostaining of astrocytes (green) and DAPI staining of nuclei (blue) under control conditions (*B*) and ∼60 min after application of 1 mM AAA (*C*). *Ba–c*, photomicrographs at a higher magnification of a portion of the transverse section (white box in *B*) illustrating DAPI staining of nuclei (*Ba*), many GFAP-reactive prolongations (*Bb*) and merged image (*Bc*). Note the extensive filling of the neuropil and the dense supply of blood vessels (*) by astrocytic processes as well as that astrocytes are conceivably associated with cell nuclei, including those of neurons. *Ca–c*, photomicrographs at a higher magnification of a portion of the transverse section (white box in *C*) illustrating DAPI staining of nuclei (*Ca*), altered morphology of GFAP-reactive structures (*Cb*) and merged image (*Cc*). Scale bars in *B* and *C*, 100 $\mu$m; in *Ba–c* and *Ca–c*, 50 $\mu$m. 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. [Colour figure can be viewed at wileyonlinelibrary.com]

here converted into glutamate (for review see Broer & Brookes, 2001). Interestingly, the possibility that astrocytes modulate and maintain the respiratory rhythm is coherent with previous findings (Hülsmann *et al.* 2000; Huxtable *et al.* 2010). More interestingly, it has been recently reported that a subset of astrocytes in the preBötC exhibited rhythmic $Ca^{2+}$ increases preceding inspiratory neuronal activity (Okada *et al.* 2012; Oku *et al.* 2016) and that their activation triggered bursting of inspiratory neurons (Okada *et al.* 2012; but see also Schnell *et al.* 2011; Phillips *et al.* 2016), thus raising the possibility that astrocytes have an active role in respiratory rhythm generation.

In the present study, we employed the gliotoxin AAA since it has already been used in the lamprey by Baudoux & Parker (2008) to ascertain the role of astrocytes in the spinal cord locomotor network. This gliotoxin is a blocker of the $Na^{+}$-dependent glutamate transporter which depolarizes astrocytes leading to increases in intracellular $Ca^{2+}$ levels and to swelling and death of astrocytes (McBean, 1994; Pannicke *et al.* 1994; Alvarez-Maubecin *et al.* 2000; Sherpa *et al.* 2014). The application time and the latency of the effects were similar to those described in previous reports using not only AAA, but also other gliotoxins in different preparations, thus implying that a relatively long latency is necessary to impair astrocytes (Hülsmann *et al.* 2000; Baudoux & Parker, 2008; Huxtable *et al.* 2010; Sherpa *et al.* 2014). Our results show that AAA caused increases in the frequency and amplitude of vagal bursts followed by a progressive decrease in both these respiratory variables that continued even after washout. The mechanisms underlying these outcomes are at present obscure and only some hypotheses can be advanced. It can be suggested that the initial increase in the respiratory motor output could be due to increased levels of extracellular glutamate within the respiratory network caused by the blockade of glial glutamate uptake and subsequent neuronal depolarization (Baudoux & Parker, 2008). In addition, it has been shown (Alvarez-Maubecin *et al.* 2000) that AAA has a direct depolarizing action on rat locus coeruleus neurons and possibly an indirect excitatory effect on the same neurons via an electrotonic coupling between neurons and glia. The following reduction in the respiratory motor output could be reasonably ascribed to the diffusion of glutamate. Any further comment on this topic would be highly speculative. The above mentioned depolarizing action implies that AAA acts also on neurons. No information is at present available that this gliotoxin affects these cells, causing swelling and death or functional impairments (e.g. Takada & Hattori, 1986; McBean, 1994; Brown & Kretzschmar, 1998; Alvarez-Maubecin *et al.* 2000; Baudoux & Parker, 2008; Sherpa *et al.* 2014).

The finding that exogenous Gln restores respiration clearly indicates that the glia glutamate–glutamine cycle within the lamprey respiratory network is crucial for the generation and/or maintenance of baseline respiratory activity. Thus, the hypothesis can be advanced that the glia glutamate–glutamine cycle (Hülsmann *et al.* 2000; Huxtable *et al.* 2010) could be a common feature of the respiratory network of different animal species.

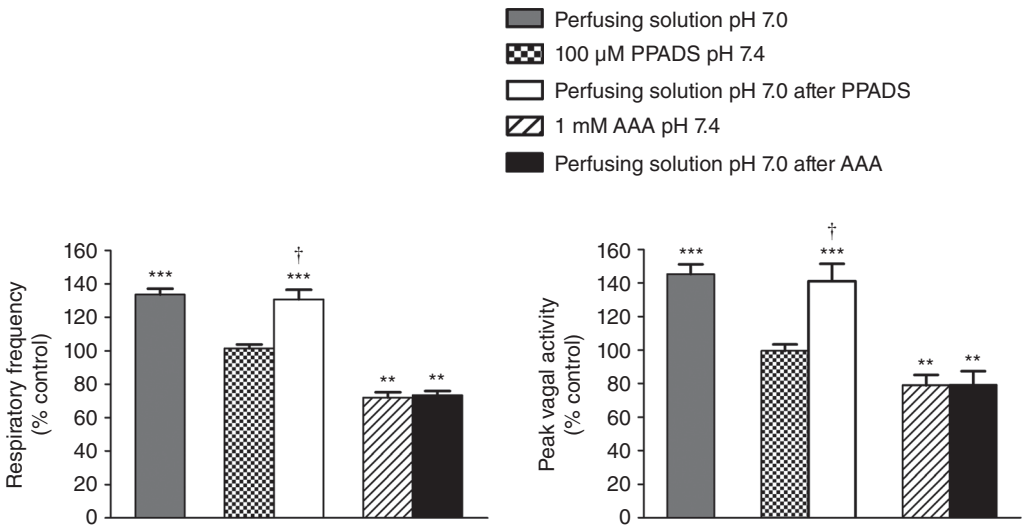


**Figure 7. Crucial role of astrocytes in the low pH-induced respiratory responses**
Bar graphs illustrating changes in respiratory frequency and peak vagal activity induced by reducing the pH of the perfusing solution from 7.4 to 7.0 under control conditions, after bath application of 100 $\mu$M PPADS or 1 mM AAA ($n = 4$). Note that the increases in the respiratory motor output due to the reduction of the pH are prevented only by AAA. Values are means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control; †$P < 0.01$ compared with PPADS.

An important result of the present study is that ATP-induced increases in respiratory frequency are mediated by astrocytes located within the pTRG since they are no longer evoked by ATP-$\gamma$-S microinjected into the pTRG after AAA application. On the other hand, the fact that the excitatory effects in response to SP microinjections into the pTRG (Mutolo *et al.* 2010; Cinelli *et al.* 2013; for review see Bongianni *et al.* 2016)

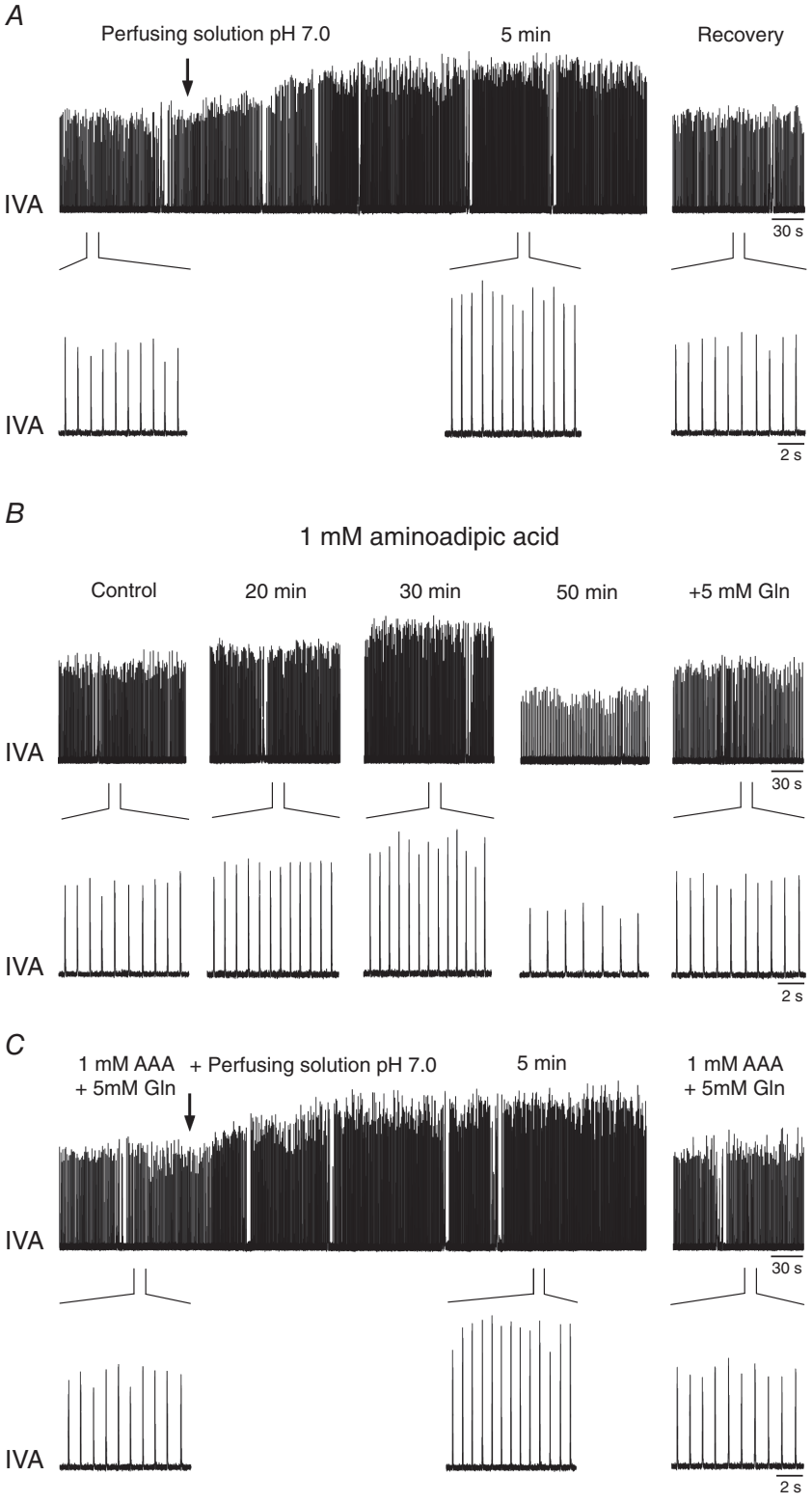


**Figure 8. Glutamine restores respiratory activity and low pH-induced respiratory responses in one preparation in the presence of aminoadipic acid**
Long trace recordings of integrated vagal activity (IVA) and expanded traces at selected times are shown. The presence of 'coughs' is revealed by pauses in the baseline (fast) respiratory activity. *A*, increases in respiratory frequency and peak vagal activity caused by reducing the pH of the perfusing solution from 7.4 to 7.0 (arrow). Maximum effects taken 5 min after low-pH application and recovery taken 5 min after washout. *B*, respiratory responses at different times following bath application of 1 mM AAA and effects observed 5 min after the subsequent bath application of 5 mM Gln. Note that Gln application restored respiratory activity. *C*, similar increases in respiratory frequency and peak vagal activity induced by a low-pH perfusing solution (arrow) after Gln-induced resumption of respiratory activity. Recovery taken after 5 min washout (pH 7.4) is also shown.

are obtained in the same conditions strongly suggests that ATP acts mainly on astrocytes to modulate the respiratory CPG. Although a direct ATP action on pTRG neurons cannot be completely excluded, the hypothesis can be advanced that ATP induces glutamate release from astrocytes, thus activating pTRG neurons involved in the respiratory rhythm generation and increasing respiratory frequency (see e.g. Funk, 2013; Funk *et al.* 2015). However, the results do not rule out a possible contribution of other gliotransmitters. In conclusion, all these findings strongly suggest that AAA selectively impairs function of astrocytes, but not that of the neuronal circuit subserving respiratory rhythm generation. Alterations in neuronal activity appear to be secondary to astrocyte impairment. Actually, exogenous Gln restored respiratory activity and SP still induced its characteristic excitatory responses. The immunohistochemical demonstration that astrocyte GFAP immunoreactivity within the pTRG is closely associated with neurons (see also Okada *et al.* 2012) and that astrocytes display evident altered anatomical characteristics (see e.g. Sherpa *et al.* 2014, also for further references) following 60 min perfusion with AAA strongly support our interpretation.

### Mediation of low pH-induced respiratory responses by astrocytes

To tentatively disclose one condition under which ATP could be released, we investigated whether increases in respiratory activity following acidification of the perfusing solution at constant $P_{CO_2}$ were due to ATP. Our results show that an ATP-independent mechanism is brought into action since the P2R antagonist PPADS does not prevent low pH-induced respiratory responses. We provide the first clear demonstration that pH sensory mechanisms are present in the lamprey respiratory network and that astrocytes play a key role in the mediation of these responses by providing metabolic support. Of note, a novel pH-sensing system in the lamprey spinal cord that acts to restore pH to physiological levels by reducing motor activity has been suggested (Jalalvand *et al.* 2016*a*, *b*; but see Santin *et al.* 2016 for critique). Thus, it is not surprising that a similar system could exist at the level of the lamprey respiratory network. Several lines of evidence indicate that astrocytes located within the mammalian brainstem detect changes in the $CO_2/H^+$ levels, thus contributing to central respiratory chemosensitivity (for review see Funk *et al.* 2015; Moreira *et al.* 2015; Guyenet *et al.* 2016); astrocytes can respond through several different mechanisms leading to increases in intracellular $Ca^{2+}$ concentration and subsequent release of gliotransmitters (Gourine *et al.* 2010; Wenker *et al.* 2010; Huckstepp *et al.* 2010; Sobrinho *et al.* 2014; Turovsky *et al.* 2016). It is widely accepted that ATP is an important mediator of chemoreception, at least at the level of the retrotrapezoid nucleus, a region located close to the ventral surface of the medulla where both neurons and astrocytes have been shown to be involved in the central chemoreception (e.g. Gourine *et al.* 2005, 2010; Mulkey *et al.* 2006; Wenker *et al.* 2010; Huckstepp *et al.* 2010; Turovsky *et al.* 2016; for review see Erlichman *et al.* 2010; Funk, 2013; Funk *et al.* 2015; Moreira *et al.* 2015; Guyenet *et al.* 2016). However, it has been shown that purinergic signalling is not required for $CO_2$/pH sensing within the nucleus tractus solitarii and medullary raphe (Sobrinho *et al.* 2014). Our results are consistent with these observations in mammals. In this connection, it seems interesting to mention that Huda *et al.* (2013) have provided evidence that extracellular acidification depolarizes astrocytes, compromises their glutamate uptake and contributes to the modulation of excitatory synaptic transmission within the nucleus tractus solitarii of the rat.

Admittedly, very scanty knowledge is available on central respiratory chemoreceptors in non-air-breathing fishes (Hoffman *et al.* 2016, also for further references). At present, we have no evidence that the pH-sensitive mechanism located in the lamprey brainstem is also responsive to changes in $P_{CO_2}$. This subject would deserve further investigation (see e.g. Harada *et al.* 1985; Kawai *et al.* 2006). However, this is far beyond the scope of the present research. Indeed, in aquatic animals, hypoxia rather than hypercapnia appears to play a major role in driving compensatory responses to maintain adequate oxygen supply to the brain, which is highly vulnerable to oxygen deprivation. The existence of specialized oxygen sensors in the central nervous system has been suggested, but only recently demonstrated at the level of astrocytes both in rats and mice (Angelova *et al.* 2015, also for further references). This topic is very interesting and could be addressed in further studies on the brainstem respiratory control in lampreys and other animal species.

## Conclusions

Growing evidence indicates that astrocytes regulate neuronal excitability and synaptic transmission and that neuron–glia interactions affect neural network activity in physiological and pathological conditions (for review see Fellin, 2009; Perea *et al.* 2014; Harada *et al.* 2015). Although the exact mechanism underlying astrocyte modulation of respiratory activity is unclear, present results show for the first time that they operate in the adult lamprey respiratory network and, in particular, at the level of the CPG, i.e. the pTRG. The results strongly suggest that the contribution of ATP and astrocytes to the modulation and/or maintenance of rhythmic neuronal activities is highly conserved throughout evolution (see Hülsmann *et al.* 2000; Baudoux & Parker, 2008; Huxtable *et al.* 2010; Okada *et al.* 2012; Morquette *et al.* 2015; Oku *et al.* 2016). The notion that some neural mechanisms

are phylogenetically conserved probably ascribes to them a great functional significance. Our findings may help understand the mechanisms underlying rhythmic firing and may have broad implications for other neuronal rhythmogenic networks. They could also provide hints for further studies on the astrocyte function in both lower vertebrates and mammals. Indeed, understanding circuit function and evolution has always been a central goal of neuroscience (e.g. Clandinin & Marder, 2016). On the other hand, it should be noted that the application of ATP or glial toxins to the CPGs of rhythmic activities making use of microinjection techniques has been performed, to our knowledge, mainly in neonatal rodents. A more extensive use of this experimental approach in adult mammals could be desirable (see e.g. Holleran *et al.* 2001; Costa *et al.* 2013). An interesting outcome of the present study is the demonstration that a $CO_2$-independent pH sensory mechanism is present in the neuronal respiratory network of the lamprey. The existence of a similar system in the mammalian brainstem respiratory regions remains to be ascertained.

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## Additional information

### Competing interest

The authors declare no conflict of interest.

### Author contributions

E.C. and L.I. conceived and designed the experiments. E.C., L.I. and D.M. collected, assembled, analysed and interpretated data for the work and developed the experimental design. E.C., L.I. and D.M. drafted the article or revised it critically for important intellectual content. D.M. wrote the manuscript in interactions with all authors. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

### Acknowledgements

This study was supported by grants from the Ministry of Education, University, and Research of Italy. E.C. is supported by a Postdoctoral Fellowship from the Fondazione Internazionale Menarini. We gratefully acknowledge Prof. Fulvia Bongianni and Prof. Tito Pantaleo for their helpful discussions, their valuable insights and review of this manuscript. We also thank Prof. Maria Grazia Giovannini for the generous gift of the anti-glial fibrillary acidic protein (GFAP) antibody.