Astrocytes modulate brainstem respiratory rhythm-generating circuits and determine exercise capacity

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Astrocytes are implicated in modulation of neuronal excitability and synaptic function, but it remains unknown if these glial cells can directly control activities of motor circuits to influence complex behaviors in vivo. This study focused on the vital respiratory rhythm-generating circuits of the preBötzinger complex (preBötC) and determined how compromised function of local astrocytes affects breathing in conscious experimental animals (rats). Vesicular release mechanisms in astrocytes were disrupted by virally driven expression of either the dominant-negative SNARE protein or light chain of tetanus toxin. We show that blockade of vesicular release in preBötC astrocytes reduces the resting breathing rate and frequency of periodic sighs, decreases rhythm variability, impairs respiratory responses to hypoxia and hypercapnia, and dramatically reduces the exercise capacity. These findings indicate that astrocytes modulate the activity of CNS circuits generating the respiratory rhythm, critically contribute to adaptive respiratory responses in conditions of increased metabolic demand and determine the exercise capacity.

DOI: 10.1038/s41467-017-02723-6

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Astrocytes have been proposed to modulate neuronal excitability, synaptic transmission, and plasticity. Physiology of these electrically non-excitable cells of the brain is governed by intracellular Ca\(^{2+}\), with increases in [Ca\(^{2+}\)]\(_i\), triggering release of signaling molecules or "gliotransmitters" (such as ATP/adenosine, \(\beta\)-serine, and others). Recent studies have suggested that via release of gliotransmitters astrocytes may influence activities of neural circuits controlling sleep, feeding, and chemosensing, yet it remains unknown whether astrocytes can directly modulate motor circuits and have an impact on complex behaviors. In vitro experiments with rodent brainstem slices have suggested that astroglial mechanisms may play a certain role in regulating the activities of neuronal networks producing motor rhythms, including those within the preBötC complex (preBötC)\(^{10}\) in the ventrolateral medulla that generates the rhythm of breathing\(^{11}\). However, whether such modulation is functionally important for rhythmic motor behavior has not been determined. In this study, we accordingly focused on the preBötC that produces a fundamental, clearly defined motor output, and where local astrocytic modulation of neuronal excitability and/or synaptic transmission would directly affect respiratory motor behavior. We determined the effects of compromised preBötC astroglial vesicular release mechanisms on breathing in conscious adult rats at rest and in conditions of increased metabolic demand requiring regulatory adjustments of respiratory motor activity, including during exercise. We show that blockade of vesicular release in preBötC astrocytes reduces the resting breathing rate and frequency of periodic sighs, decreases rhythm variability, impairs respiratory responses to hypoxia and hypercapnia, and dramatically reduces the exercise capacity.

Results
Vesicular release mechanisms in preBötC astrocytes in adult Sprague-Dawley male rats were disrupted by virally driven expression of either the light chain of tetanus toxin (TeLC)\(^{12}\), or the dominant-negative SNARE (dnSNARE) protein\(^{13}\) (Supplementary Table 1) to block SNARE-dependent vesicular exocytosis. Astrocyte-specific expression of TeLC or dnSNARE was controlled by an enhanced GFAP promoter (Fig. 1a). The high efficacy of TeLC expression in blocking vesicular release in brainstem astrocytes has been demonstrated previously\(^{12}\). To determine efficacy of our novel dnSNARE construct, we used total internal reflection fluorescence microscopy (TIRF) to monitor vesicular fusion events in cultured brainstem astrocytes transduced to express dnSNARE or a control transgene (CatCh-EGFP). In dnSNARE-expressing astrocytes, the number of juxtamembrane vesicles labeled with quinacrine was reduced by 67% (\(p < 0.001\); Fig. 1b). Facilitated vesicular fusion induced by the \(\text{Ca}^{2+}\) ionophore ionomycin, or the oxygen scavenger sodium dithionite, was effectively abolished in astrocytes expressing dnSNARE (Fig. 1c-e; Supplementary Fig. 1).

In conscious rats, bilateral expression of dnSNARE or TeLC in preBötC astrocytes (Fig. 1f; Supplementary Figs. 2 and 3) resulted in a significant reduction in resting breathing frequency (\(f_R\)) by 11% (94±2 vs. 106±5 min\(^{-1}\) in controls; \(n = 5\), \(p = 0.016\)) and by 11% (92±2 vs. 103±3 min\(^{-1}\) in controls; \(n = 12\), \(p = 0.011\)), respectively (Fig. 1g, h). Since dnSNARE or TeLC expression in astrocytes is likely to block exocytosis of several putative gliotransmitters, we determined the possible contribution of ATP by blocking ATP-mediated signaling within the preBötC by virally driven expression of a potent ectonucleotidase — transmembrane prostatic acid phosphatase (TMAP). TMAP expression is highly effective in preventing ATP accumulation in astroglial vesicular compartments and blocking extracellular ATP actions\(^{14-18}\). We found that bilateral expression of TMAP in the preBötC (Supplementary Figs. 2 and 3) reduced resting \(f_R\) by 12% (98±3 vs. 111±4 min\(^{-1}\) in controls, \(n = 7\), \(p = 0.017\); Fig. 1i). These results suggested that at rest, vesicular release of gliotransmitters by preBötC astrocytes provides tonic excitatory drive to the inspiratory rhythm-generating circuits.

We next assessed whether activation of preBötC astrocytes influences breathing behavior. Release of gliotransmitters by astrocytes may occur following activation of phospholipase C (PLC)\(^{12}\). To facilitate PLC-mediated release of gliotransmitters, we transduced preBötC astrocytes to express a \(G_q\)-coupled Designer Receptor Exclusively Activated by Designer Drug (DREADD)\(^{15}\) (see vector layout, Fig. 2a, f-h; Supplementary Figs. 2 and 3). As expected, the DREADD ligand clozapine-N-oxide (CNO) triggered robust increases in [Ca\(^{2+}\)]\(_i\), in brainstem astrocytes expressing DREADD\(_{Cq}\) (Fig. 2b; Supplementary Fig. 4). These responses were blocked by the PLC inhibitor U73122 (Fig. 2c). However, a PLC activity assay revealed higher resting (i.e., in the absence of CNO) levels of inositol phosphates in cultured astrocytes expressing DREADD\(_{Cq}\) (Fig. 2d). Moreover, DREADD\(_{Cq}\) expression was also found to be associated with a higher rate of spontaneous fusion of quinacrine-labeled vesicles in cultured astrocytes (Fig. 2e), and facilitated release of ATP in conditions when preBötC astrocytes were transduced to express the transgene (in experiments on acute brainstem slices, Fig. 2i, j), indicating that in the absence of an agonist, DREADD\(_{Cq}\) is constitutively active at the level of expression achieved by the viral vector used. We exploited this property of DREADD\(_{Cq}\) in order to determine whether sustained activation of PLC in preBötC astrocytes, associated with facilitated vesicular release of ATP, has an impact on the inspiratory rhythm-generating circuits. Bilateral expression of DREADD\(_{Cq}\) (\(n = 8\)) in preBötC astrocytes resulted in 26% higher baseline \(f_R\) (123±5 vs. 98±2 min\(^{-1}\) in controls, \(n = 14\), \(p < 0.001\); Fig. 2k). This effect was effectively abolished by the ectonucleotidase activity of TMAP. Co-expression of DREADD\(_{Cq}\) and TMAP in the preBötC was associated with a significant reduction of the respiratory rate below the baseline (88±3 min\(^{-1}\), \(n = 5\), \(p = 0.030\); Fig. 2k), an effect similar to that observed in conditions of TMAP expression alone (Fig. 1i).

Altered function of preBötC astrocytes also had a significant impact on other features of resting inspiratory activity. Bilateral expression of dnSNARE or TeLC in preBötC astrocytes was associated with a significant reduction in the variability of the respiratory rhythm (Fig. 3a). DREADD\(_{Cq}\) expression had an opposite effect and increased respiratory variability (Fig. 3a).

The frequency of sighs, breaths with augmented inspiration, generated periodically by the preBötC circuits, was reduced by 27% (\(p < 0.001\)) in rats expressing dnSNARE, by 25% (\(p < 0.001\)) in rats expressing TeLC, and by 26% (\(p < 0.001\)) in rats expressing TMAP in the preBötC (Fig. 3b). Sigh frequency was found to be significantly higher in rats transduced to express DREADD\(_{Cq}\) in preBötC astrocytes (by 31%, \(n = 8\), \(p < 0.001\); Fig. 3b). There is recent evidence that generation of sighs is facilitated by the actions of bombesin-like peptides\(^{18}\) and inhibited when astroglial function is compromised\(^{20}\), suggesting that sigh generation may be modulated by signaling molecules released by preBötC astrocytes in response to various stimuli, including locally released bombesin-like peptides. Indeed, we found that bombesin triggers robust [Ca\(^{2+}\)]\(_i\) responses in cultured brainstem astrocytes (Supplementary Fig. 5). Blockade of vesicular release mechanisms in preBötC astrocytes (dnSNARE expression) significantly reduced the effect of bombesin on sigh frequency in vivo (Supplementary Fig. 6), suggesting that the actions of bombesin-like peptides on preBötC circuits are potentially mediated by astrocytes. Together these results suggest that vesicular release of gliotransmitter(s) by preBötC astrocytes...
**Fig. 1** PreBötC astrocytes modulate the activity of the respiratory rhythm-generating circuits. 

**a** Schematic of AVV-sGFAP-dnSNARE-EGFP vector layout. 

**b** Summary data illustrating a reduction in the number of juxtamembrane quinacrine-labeled vesicular compartments in cultured brainstem astrocytes expressing dnSNARE. 

**c** Plots of TIRF intensity changes showing loss of quinacrine fluorescence from a proportion of labeled organelles in response to application of the Ca2\(^{2+}\) ionophore ionomycin (1 \(\mu\)M) in two individual cultured astrocytes transduced to express control transgene (black traces) or dnSNARE (red traces). In cultures of astrocytes expressing dnSNARE, digitonin was applied at the end of the recording to permeabilize the membranes, resulting in a rapid loss of quinacrine fluorescence. 

**d** Averaged temporal profile of ionomycin-induced vesicular fusion events detected in cultured astrocytes expressing control transgene or dnSNARE. 

**e** Total number of ionomycin- and sodium dithionite-induced vesicular fusion events detected in individual cultured astrocytes expressing control transgene or dnSNARE. In **b**, **d**, and **e**, numbers of individual tests performed in three different cultures prepared from different animals are indicated. 

**f** Schematic drawings of the rat brain in parasagittal and coronal projections illustrating the location of the preBötC. NAsc, semi-compact division of the nucleus ambiguus; XII, hypoglossal motor nucleus. Representative confocal image of dnSNARE-EGFP expression in preBötC astrocytes is shown on the right (scale bar: 200 \(\mu\)m). High-magnification inset shows expression of dnSNARE-EGFP in GFAP-positive preBötC astrocytes (inset scale bar: 50 \(\mu\)m). NAsc neurons are identified by choline acetyltransferase (ChAT) immunoreactivity. VS, ventral surface of the brainstem. 

**g**, **h**, and **i** Group data showing the effects of dnSNARE or TeLC expression in preBötC astrocytes on frequency distribution of all respiratory-related events detected in 30-min assay (top) and resting respiratory frequency recorded during periods of calm wakefulness and/or quiet sleep (\(f_R\), bottom) in conscious adult rats. In control animals preBötC astrocytes were transduced to express CatCh-EGFP. Group data showing the effect of TMPAP expression in preBötC region on frequency distribution of the respiratory-related events and resting \(f_R\) in conscious rats. In **g**, **h**, and **i**, number of animals in each experimental group is indicated in parentheses. \(p\) values—Mann–Whitney U rank test
modulates the variability of the respiratory rhythm and the generation of sighs.

Since hypoxia induces release of ATP by astrocytes and increases sigh frequency, we next evaluated the effects of dnSNARE or TeLC expression in preBötC astrocytes on respiratory responses to systemic hypoxia (10% O2 in the inspired air) as well as the effects of dnSNARE, TeLC, TMPAP, and DREADDGq expression on sigh generation during hypoxia. Expression of dnSNARE attenuated hypoxia-induced increases in fR by 27% (159±10 vs. 217±7 min⁻¹ in controls; Fig. 4a) and in

![Diagram of ATP sensor and expression of DREADDGq](image)

- **a** Schematic illustration of ATP sensor and expression of DREADDGq.
- **b** Fura-2 fluorescence (F/F₀) in naive and DREADDGq expressing astrocytes under control conditions and with CNO.
- **c** Fura-2 fluorescence (F/F₀) in naive and DREADDGq expressing astrocytes with U73122.
- **d** % [3H]-InsP production under control conditions and with CNO in naive and DREADDGq expressing astrocytes.
- **e** Fusion events (min⁻¹) under control conditions and with CNO in naive and DREADDGq expressing astrocytes.
- **f** Immunofluorescence images of DREADD-EGFP in ChAT and NAnc.
- **g** Immunofluorescence images of DREADD-EGFP in NeuN and GFAP.
- **h** Schematic representation of ATP release with ATP sensor on or off DREADDGq expressing side.
- **j** ATP release (µM) with ATP sensor on or off DREADDGq expressing side.
- **k** Number of events and fR (min⁻¹) with DREADDGq and TMPAP.

(Additional graphs and tables for statistical analysis are included in the article.)
Expression of TeLC or dnSNARE protein in preBötC astrocytes reduced the resting breathing rate and frequency of periodic sighs, decreased rhythm variability, impaired respiratory responses to hypoxia and hypercapnia, and dramatically reduced the exercise capacity. TeLC is a proteolytic enzyme that cleaves SNARE proteins required for vesicular fusion. In cultured astrocytes, TeLC inhibits ATP and glutamate release, blocks Ca²⁺-dependent vesicular fusion, and prevents the spread of Ca²⁺ waves triggered by mechanical stimulation—the effects consistent with the inhibition of vesicular ATP release. TIRF imaging confirmed that dnSNARE expressed in astrocytes reduces the number of juxtamembrane vesicles and effectively blocks Ca²⁺-dependent vesicular fusion. These effects are in line with the proposed mechanisms underlying the effect of dnSNARE on exocytosis in astrocytes.

Our initial design of the gain-of-function experiment with DREADDGq involved targeting preBötC astrocytes to express this receptor followed by documenting changes in respiratory activity induced by administration of CNO. However, validation experiments of our viral vector construct revealed that in the absence of a ligand, astrocytes expressing DREADDGq exhibit a higher level of PLC activity, higher rate of spontaneous vesicular fusion, and facilitated tonic release of ATP. These results suggested that DREADDGq is constitutively active when expressed in astrocytes, an observation consistent with the properties of many hM3 receptor mutants originally described. Since CNO appears to have low affinity for DREADDs and its effects are largely attributed to its conversion to clozapine, which may interact with astroglial serotonin receptors, we focused on determining the effects of the constitutive DREADDGq activity. In rats, sustained activation of PLC-mediated signaling pathways in preBötC astrocytes expressing DREADDGq was associated with higher resting breathing rate, higher frequency of periodic sighs, and increased rhythm variability. That this effect was blocked by co-expression of the potent ectonucleotidase TMPAP suggested that the stimulatory effect of DREADDGq expression in preBötC astrocytes on breathing could be mediated by direct actions of ATP and/or related purines on preBötC circuits or, alternatively, autocrine effects of ATP on Ca²⁺ in preBötC astrocytes leading to the release of other gliotransmitters, neither possibility of which we rule out here.

The role of preBötC astrocytes in the control of breathing becomes especially important during physiological metabolic challenges, such as systemic hypoxia and hypercapnia, where enhanced respiratory effort is critical to maintain homeostasis. Although expression of dnSNARE or TeLC in preBötC astrocytes reduced resting respiratory rate, minute ventilation at normoxia/eucapnia was similar to that in animals expressing control minute ventilation by 34% (Fig. 4a). TeLC expression in preBötC astrocytes had a similar effect (Supplementary Fig. 6). Disruption of either astroglial vesicular release (dnSNARE or TeLC expression) or ATP-mediated signaling (TMPAP expression) reduced the frequency of sighs during the hypoxic challenge by 34% (n = 5, p < 0.001), 36% (n = 12, p < 0.001), and 44% (n = 7, p < 0.001), respectively (Fig. 3b). DREADDGq expression in preBötC astrocytes had an opposite effect and increased frequency of sigh generation during hypoxia by 50% (n = 8, p = 0.003; Fig. 3b).

Brainstem astrocytes are sensitive to changes in PCO₂/pH and we next found that preBötC astrocytes play an important role in the development of respiratory response to elevated systemic CO₂ (hypercapnia). In conscious rats, bilateral expression of dnSNARE or TeLC in preBötC astrocytes reduced the fR responses to hypercapnia (6% inspired CO₂) by 23% (141 ± 5 vs. 182 ± 3 min⁻¹ in controls; n = 5, p = 0.005) and 20% (151 ± 6 vs. 190 ± 8 min⁻¹ in controls; n = 9, p = 0.005), respectively (Fig. 4b), concomitantly reducing minute ventilation (Fig. 4b).

We next hypothesized that astroglial control of breathing at the level of the preBötC may become particularly important during physical activity and exercise when increased oxygen demand must be supported by an enhanced respiratory effort. Accordingly, we determined whether blockade of astroglial vesicular release mechanisms impairs the exercise capacity. Bilateral expression of dnSNARE or TeLC in preBötC astrocytes resulted in a marked reduction of exercise capacity by 57% (0.5 vs. 1.2 µJ in controls; p = 0.016, n = 5) and 42% (0.7 ± 0.1 vs. 1.2 ± 0.1 µJ in controls; p < 0.001, n = 9), respectively (Fig. 5a, b). Cardi-vascular responses to exercise (increases in heart rate and systemic arterial blood pressure) were not affected (Fig. 5c), suggesting that the impaired exercise capacity is due to the respiratory, not a cardiovascular, deficit.
transgene (due to small compensatory increases in tidal volume). More marked differences in ventilation between the experimental and control groups were observed during the hypoxic challenge, indicating that preBötC astrocytes are critically important for the development of the full-scale hypoxic ventilatory response. These data are consistent with the proposed role of astrocytes as CNS oxygen sensors.

Respiratory rhythm-generating circuits are silent in the absence of CO$_2$ and require a certain level of CO$_2$ to operate. The preBötC has a neuronal H$^+$/CO$_2$-sensing mechanism, however, our results suggest that preBötC astrocytes contribute in a significant manner to the development of the respiratory response to hypercapnia. Our data support the “distributed central chemosensitivity” hypothesis, which proposes that central respiratory sensitivity to CO$_2$ (the mechanism that adjusts breathing in accordance with changes in brain parenchymal PCO$_2$/pH) is mediated by multiple central chemoreceptor sites (one being the preBötC), with each site providing tonic excitation in eucapnia and a fraction of the total response to systemic hypercapnia. Previous experimental studies suggested that the contribution of the preBötC mechanism(s) to the overall respiratory response to CO$_2$ is ~20–25%. Our experiments showed that ventilation during eucapnia and hypercapnia is similarly reduced by ~20% in conditions when vesicular release mechanisms in preBötC astrocytes are blocked and hyperoxia is applied to reduce the drive from the peripheral chemoreceptors. While current models of central respiratory CO$_2$ chemosensitivity are focused on groups of pH-sensitive neurons residing elsewhere in the brainstem, our data suggest that CO$_2$/pH chemosensitivity of the preBötC is mediated by astrocytes.

In conclusion, the data obtained in the present study indicate that astrocytes are able to modulate the activities of vital rhythmic motor circuits with a significant impact on motor behavior in vivo. We targeted astrocytes intermingled with the preBötC...
respiratory rhythm-generating circuits to express proteins that block or facilitate vesicular release mechanisms. Our results suggest that astroglial signaling involving exocytotic vesicular release of gliotransmitters provides tonic excitation of preBötC circuits that generate the inspiratory rhythm. The role of preBötC astrocytes becomes especially important in conditions such as systemic hypoxia, hypercapnia, and exercise, when homeostatic adjustments of breathing are critical to support our physiological and behavioral demands.

**Methods**

**Animals.** All animal experiments were performed in Sprague-Dawley rats (adult males 230–270 g or neonates P2–P3 of either sex) in accordance with the European Commission Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes), the UK Home Office (Scientific Procedures) Act (1986), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with project approval from the respective Institutional Animal Care and Use Committees. Animals were housed in a temperature-controlled facility with a normal light-dark cycle (12 h:12 h, lights on at 0700 hours). Tap water and laboratory rodent chow were provided ad libitum.

**Molecular approaches to block astroglial signaling.** To block vesicular release mechanisms in preBötC astrocytes, we developed a novel adeno viral vector (AVV) to drive the expression of dnSNARE protein under the control of an enhanced GFAP promoter (Fig. 1a; Supplementary Table 1). Description of validation of the transgene efficacy in blocking vesicular release mechanisms in astrocytes is
provided above. PreBotC astrocytes were also targeted to express TeLC, which blocks vesicular exocytosis via proteolytic degradation of SNARE proteins. Generation of the AVV-sGFAP-skip-TeLC (Supplementary Table 1; AVV-sGFAP-EGFP-skip-TeLC) in astrocytes and validation of the transgene efficacy in preventing vesicular ATP accumulation in astrocytes and blocking ATP-mediated signaling between astrocytes was characterized in detail previously1,12,13.

**Molecular approach to activate astrocytes.** To stimulate Gq-coupled signaling pathways we generated a AVV to express CatCh-EGFP fused with enhanced green fluorescent protein (EGFP) in astrocytes (Fig. 2a; Supplementary Table 1). DREADDcat expressed in astrocytes was found to be constitutively active. This constitutive activity of DREADDcat was lower compared to that triggered by the application of the DREADD ligand CNO (Fig. 2d), and did not appear to be detrimental to the cells expressing the transgene. Brainstem astrocytes transduced to express DREADDcat looked normal upon histological examination (Fig. 2f–i) and were able to mount unaltered [Ca2+]i responses to activation of purinoreceptors following application of ATP (Supplementary Fig. 4).

**Control transgenes.** Two control vectors were used: (1) an AVV to express calmodulin translocating channelrhodopsin variant (CatCh) fused with EGFP (CatCh-EGFP) under the control of the GFAP promoter (AVV-sGFAP-CatCh-EGFP); and (2) a LVV to express EGFP under the control of EF1α promoter (despite the use of the generic promoter, EGFP expression driven by LVVs is almost exclusively confined to astrogliosis14) (Supplementary Table 1). The choice of CatCh-EGFP was dictated by the need of having a transduced membrane-bound protein as an appropriate control in experiments where brainstem astrocytes were transduced to express DREADDcat which is also a membrane protein. Parts of both CatCh and DREADDs are facing the extracellular space and (as foreign proteins) may potentially trigger an immune response. Thus, expression of CatCh-EGFP is a much harsher control than cytoplasmic expression of EGFP, which has hardly ever been reported to cause any adverse cellular effects. Each experimental (dnSNARE, TeLC, DREADDcat or TMPAP) and control (CatCh-EGFP or EGFP) animal groups were injected with the appropriate viral vector at the same time and the experimental groups were compared to their own control groups (Figs. 1g–i and 2k). These experiments were conducted over the course of 24 months and some variations in the baseline respiratory frequency were observed (Supplementary Fig. S3). However, these differences in resting respiratory activity between animals from different control groups expressing CatCh-EGFP or EGFP in the preBotC and naive (non-transduced) rats were not statistically significant (Supplementary Fig. S3). Since no significant variations across all the control groups were observed when the frequency of respiratory sighs was analyzed, the sigh frequency data obtained in eight representative control animals transduced to express DREADDcat, looked normal upon histological examination (Fig. 2f–i) and were able to mount unaltered [Ca2+]i responses to activation of purinoreceptors following application of ATP (Supplementary Fig. 4).

**In vivo viral gene transfer.** Adult male rats (250–270 g) were anesthetized with ketamine (60 mg kg−1, intramuscular i.m.) and medetomidine (250 µg kg−1, i.m.), in a stereotaxic frame. For both bar was aimed at the bregma that was positioned 5 mm below lambda. PreBotC areas were targeted bilaterally by advancing a pipette from the dorsal surface of the brainstem. Viral vectors (see Supplementary Table 1 for viral titers) were delivered via a single microinjection (0.20–0.25 µl per side using the following coordinates: 0.9 mm rostral; 2 mm lateral; and 2.7 mm ventral from the calamus scriptorium. After the microinjections, the wound was sutured and anesthesia was reversed with atipamezole (1 mg kg−1, i.m.). For postoperative analgesia, the animals received buprenorphine (0.05 mg kg−1, i.m.) and flunixin meglumine (1 mg kg−1, s.c.) for 3 days. No complications were observed after the surgery and the animals gained weight normally.

**Cell culture.** Primary astroglial cell cultures were prepared from the brainstem tissue of rat pups (P2-P3) as described12,44. The cultures were cultured in isoflurane overdose, the brains were removed, and the ventral regions of the medulla oblongata were dissected out. Ventral brainstem tissue cuts from two to three animals were used for each cell culture preparation. After isolation, the cells were plated on poly-d-lysine-coated glass coverslips and maintained at 37 °C in a humidified atmosphere (5% CO2, 95% O2). For the application of ATP, 3.6% CO2, 95% O2, and 95% air, to the cellular vectors to 95% air, 3.6% CO2, 95% O2. For the application of ATP, the medium was replaced with Hanks’ balanced salt solution buffer. Lithium chloride was then added to reach a final concentration of 10 mM and cultures were incubated at 37 °C for an additional 30 min. To activate DREADDcat, CNO (5 µM, Tocris Bioscience) was added for 20 min. Reactions were terminated by removal of the medium and the addition of 500 µl of ice-cold methanol. [3H]-inositol phosphate-5'-monophosphate ([3H]-InsP5) production was determined by adding the samples to 2 ml Dowex columns pre-washed with a mixture of ammonium formate (2 M) and 0.1 M formic acid. Double-distilled water and a mixture of sodium tetraborate (5 mM) and sodium formate (60 mM) were used to elute unbound [3H]-inositol and glycosylphosphatidylinositol, respectively. Then, a mixture of ammonium formate (1 M) and formic acid (0.1 M) was added to the column to elute total [3H]-InsP into scintillation vials. The 500 µl aliquots of the eluted samples were then transferred in duplicates to liquid scintillation vials. Concentrations of [3H] in [3H]-InsP and total [3H]-inositol lipids were detected using a Beckman LS 5801 scintillation counter (4 min, [3H] DPM program). The results are presented as percentages of radioactive InsP ([3H]-InsP) in the total inositol lipid pool (Fig. 2d).

**PLC activity assay.** Cultured naïve astrocytes and astrocytes transduced to express DREADDcat or CatCh were incubated for 18 h in M199 medium containing 10% dextran-coated fetal calf serum and 1 µg ml−1 of [3H]-inositol (specific activity 18.5 Ci mmol−1) to label the inositol lipid pool. Immediately prior to the assay, the incubation medium was replaced with Hanks’ balanced salt solution buffer. Lithium chloride was then added to reach a final concentration of 10 mM and cultures were incubated at 37 °C for an additional 30 min. To activate DREADDcat, CNO (5 µM, Tocris Bioscience) was added for 20 min. Reactions were terminated by removal of the medium and the addition of 500 µl of ice-cold methanol. [3H]-inositol phosphate-5'-monophosphate ([3H]-InsP5) production was determined by adding the samples to 2 ml Dowex columns pre-washed with a mixture of ammonium formate (2 M) and 0.1 M formic acid. Double-distilled water and a mixture of sodium tetraborate (5 mM) and sodium formate (60 mM) were used to elute unbound [3H]-inositol and glycosylphosphatidylinositol, respectively. Then, a mixture of ammonium formate (1 M) and formic acid (0.1 M) was added to the column to elute total [3H]-InsP into scintillation vials. The 500 µl aliquots of the eluted samples were then transferred in duplicates to liquid scintillation vials. Concentrations of [3H] in [3H]-InsP and total [3H]-inositol lipids were detected using a Beckman LS 5801 scintillation counter (4 min, [3H] DPM program). The results are presented as percentages of radioactive InsP ([3H]-InsP) in the total inositol lipid pool (Fig. 2d).

**Measurements of ATP release in acute brainstem slices.** Adult rats were transduced to express DREADDcat and CatCh in astrocytes of the left and right preBotC regions. After 7 days following microinjections of viral vectors, the animals were humanely killed by isoflurane overdose and the brainstem was quickly removed and placed in chilled (4–6°C) artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 3 mM KCl, 2 mM CaCl2, 26 mM NaHCO3, 1.25 mM Na2HPO4, 1 mM MgSO4, 10 mM d-glucose saturated with 95% O2/5% CO2, pH 7.4) at 37 °C. The medulla was isolated and a horizontal 400 µm-thick slice was cut parallel to the ventral medullary surface14,15. Recordings were made in the medium (3 ml min−1) at 35 °C from the slices placed on an elevated grid to permit access of aCSF from both sides of the slice.

The design and operation of the ATP biosensors (Sarissa Biomedical) were described in detail previously11. To control for the release of nonspecific electroactive interferents, a dual recording configuration of the ATP biosensor and control (null) biosensor was used, as described13,15. A “null” biosensor (lacking enzymes but otherwise identical) current was subtracted from the current recorded by the ATP biosensor to give “net-ATP” readings, reporting release of ATP (Fig. 2i). Both sensors were initially placed in the recording chamber having no contact with the brainstem slice. Once a steady-state recording was achieved, the sensors were laid flat bilaterally (ATP sensor was placed randomly on either left or right side of the slice) in direct contact with the ventral surface of the slice in equivalent positions overlaying the preBotC. The sensors were left in place to achieve stable recordings of the ATP tone and then carefully lifted from the surface of the slice to allow measurement of tonic ATP release (Fig. 2i). Without removing the sensor from the recording chamber, their positions on the left (expressing DREADDcat) and right (expressing CatCh) sides of the brainstem slice were swapped to determine tonic ATP release from the opposite site (Fig. 2i). Sensors were calibrated before and after every recording by application of ATP (10 µM) (Fig. 2i). To convert changes in the biosensor current to changes in ATP concentration, an average of sensor calibrations before and after the recording was used.

**Measurements of respiratory activity in conscious rats.** Whole-body plethysmography was used to record respiratory activity in unrestrained conscious adult rats16–18. Briefly, 5–7 days after the injections of viral vectors the rats were placed in a Plexiglas recording chamber (1) that was flushed continuously with humidified air (21% O2, 79% N2, temperature 22–24°C, 12 h; 24°C, 12 h). In order to take into the account circadian variations of the physiological parameters, respiratory activity in all the animals was assessed at the same time of the day.
between 1100 and 1500 hours). The distance covered by the animal was recorded and exercise capacity was
maintained with the physiological ranges. Core body temperature was main-
tered (500 μs) in the 30-min assay period was analyzed, plotted, and
fitted to modify respiratory network activity.

Data analysis. The respiratory cycle duration (TTOT) was measured for each respiratory cycle after the animals had habituated to the plethysmography chamber environment for at least 60 min. The average TTOT calculated for the periods of calm wakefulness and/or quiet sleep recorded in a 30-min period following accl-
imation to the chamber environment was used to determine the resting respiratory frequency (number of breaths per minute, fR). The frequency dis-
tribution of the instantaneous rate of all respiratory-related events (including sighs and sighing) in the 30-min assay period was analyzed, plotted, and
reported as averages for each of the experimental groups. Poincaré plots of TTTOT for the nth cycle vs. TTOT for the nth+1 cycle were used to evaluate the temporal
dispersion of TTTOT. Variability of TTTOT was determined as described previously.48 Tidal volume (VT, normalized to the body weight) was determined by measuring the
pressure changes in the chamber. Calculated values of minute ventilation (V\text{E} = fR \times VT) were averaged and reported in arbitrary units. In addition to quantifying fR, we also determined the frequency of sighs—augmented breaths that occur on top of normal inspirations.49,50 A sigh was defined as a high-amplitude, biphasic increased inspiratory breath (Supplementary Fig. 3) that started near the peak of a normal inspiration and lasted for a period that exceeded the duration of the previous inspiration.49,50 Sighs were also recognizable by the lengthening of the respiratory cycle (i.e., increase in TTOT) immediately after the sigh (Supplementary Fig. 3). Sigh frequency was calculated (and verified manually) offline using Spike2 software. The frequency of augmented breaths with VT\text{TOT} that was at least 50% higher than the average eupneic breath, followed by a brief period of apnea.

In box and whisker plots, the central black dot illustrates the mean, the central line shows the median, the edges of the box define the upper and lower quartile values, and whiskers show the minimum-maximum range of the data.

The data were compared using nonparametric Mann–Whitney U by ranks test, Wilcoxon matched-pairs signed-rank test, Kruskal–Wallis analysis of variance by ranks followed by Dunn’s post hoc test, as appropriate. Differences with p < 0.05 were considered to be significant.

Data availability. The data that support the findings of this study are available from the corresponding authors upon request.

Received: 19 June 2017 Accepted: 19 December 2017

Published online: 25 January 2018

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Acknowledgements

This work was supported by The Wellcome Trust (A.V.G.), British Heart Foundation (A. V.G., Ref. RG/14/14/3076), BBSRC (S.K., Refs. BB/L019396/1 and BB/K009192/1), the Medical Research Council (S.K., Ref. MR/L020661), and in part by the Intramural Research Program of the NIH, NINDS. A.V.G is a Wellcome Trust Senior Research Fellow (Refs. 095064 and 200893), S.S. is an NIH UCL GPP Fellow. We thank Professor Shamshad Cockcroft for her help with PLC activity assay and Professor Philip Haydon for providing dnSNARE construct. We are grateful to Professor David Attwell and Dr. Richard D. Fields for their comments on an earlier version of the manuscript.

Author contributions

A.V.G. and J.C.S. designed research; S.S., E.A.T., P.S.H., A.H., and S.M.T. analyzed data; A.V.G. and S.S. wrote the paper.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-017-02723-6.

Competing interests: The authors declare no competing financial interests.

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