Figures and figure supplements

CO₂-evoked release of PGE2 modulates sighs and inspiration as demonstrated in brainstem organotypic culture

David Forsberg et al
Figure 1. PGE$_2$ and CO$_2$ increase sigh activity via EP3R signaling. Respiratory activity was recorded in vivo in a two-chamber plethysmograph (a). Sighs, defined by an increase in inspiratory volume and respiratory cycle period with a biphasic inspiration (b), increase in frequency after intracerebroventricular injection (i.c.v.) of PGE$_2$. This effect is absent in mice lacking EP3R (Ptger3$^{-/-}$, c, arrows, d). I.c.v. injection of PGE$_2$ also increases the tidal volume ($V_T$) in wild-type C57BL/6J (WT) mice (e). The sigh frequency is increased by hypercapnic (5% CO$_2$ in normoxia) conditions in wild-type and Ptger3$^{-/-}$ mice but less so in Ptger3$^{-/-}$ mice (f). In wild-type mice, the increase is abolished after i.c.v. injection of PGE$_2$ (f). Hypercapnic exposure causes an increase in respiratory frequency ($F_R$), tidal volume ($V_T$), and minute ventilation ($V_E$) (g), but the increase is attenuated in Ptger3$^{-/-}$ mice. Data are presented as means $\pm$ SD. *$p<0.05$ Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.004

The following source data is available for figure 1:

Source data 1. In vivo plethysmography data.

DOI: 10.7554/eLife.14170.005
Figure 2. Brainstem slice cultures have a preserved structure and neurons with functional potential. Brainstem slices containing the preBotC were used to create slice cultures. Anatomical landmarks, including the nucleus ambiguus (NA), nucleus tractus solitarius (NTS), and nucleus hypoglossus (XII; a), as well as the distinct expression of NK1R (b, c, g) enabled the identification of the preBotC region. The brainstem slice displayed MAP2-/Tuj1-positive neurons expressing NK1R (b, c), VGlut2 (d), and/or KCC2 (e). The abundant MAP2-/Tuj1-positive cells demonstrated a preserved neuronal network within the preBotC (g). KCC2 expression was found in the NTS, NA, and preBotC (e). DIV, days in vitro. Arrowheads: double-labeled cells. Scale bars: 100 μm in b–f, 500 μm in g. DOI: 10.7554/eLife.14170.007
Figure 2—figure supplement 1. Protein expression pattern is preserved during cultivation. The expression pattern of neuronal markers NK1R, MAP2, Tuj1 and KCC2 and the astrocyte marker GFAP did not change during cultivation for 3 weeks. DIV: days in vitro. Scale bars: 100 µm. DOI: 10.7554/eLife.14170.008
Figure 2—figure supplement 2. Slices flatten during cultivation. The gross morphology of the slices changed slightly during cultivation due to thinning and spreading. DIV: days in vitro. Scale bar: 500 μm.
DOI: 10.7554/eLife.14170.009
Figure 2—figure supplement 3. Brainstem slice cultures are viable. An individual single necrotic cell (8 ± 3%, n=257, propidium iodide-stained) were found in the brainstem slice cultures (N=12) that had been cultivated for 3 weeks, but no large clusters of necrotic cells were detected (a, d). The few necrotic cells were observed in the thickest regions, indicating that diffusion-based oxygenation is critically dependent on slice thickness. Oxygen glucose deprivation (OGD) for 1 hr produced clear positive PI-staining throughout the slice (b, N=5). Cultures also showed low apoptotic activity (2 ± 1%, n=187), as evaluated by caspase-3 staining (c, d, N=20). DIV: days in vitro. N: slices, n: cells. Scale bars: 100 μm.
DOI: 10.7554/eLife.14170.010
Neuronal electrical activity indicates preserved networks. Neurons in a preBötC slice (7 DIV), patched in the whole-cell configuration in current-clamp mode (a), exhibit regular rhythmic bursting activity (b). The neurons exhibited a hyperpolarized resting potential, action potentials, synaptic input, and spontaneous electrical activity, with epochs of action potential activity (b, c). The different measured variables indicated healthy and normally functioning neurons (d). Depicted here are two simultaneously patched neurons that also received common synaptic input (e, arrows). Spiking epochs occurred simultaneously, suggesting synchronized network oscillations. Direct connectivity between the depicted neurons showed that they were neither chemically nor electrically synaptically connected to each other. This finding indicates that the observed correlation was induced by common input from a preserved network structure. AP: action potential. DIV: days in vitro.

Source data are available in a separate source data file. DOI: 10.7554/eLife.14170.011

The following source data is available for figure 3:

Source data 1. Electrophysiology patch clamp data. DOI: 10.7554/eLife.14170.012
Figure 3—figure supplement 1. Cells of brainstem slice cultures retain neuronal electrical properties. Recorded neurons showed correlated epochs of spontaneous activity (a, b) and received synaptic input that was often synchronized (a, arrow), suggesting common presynaptic neurons. Note the similarity in neuronal properties of the recorded neighboring neurons (a, b). Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.013
Figure 4. Neural activity in the preBoTc is arranged in a functional respiratory network with respiratory-related motor output. In the preBoTc slice (a), a cross-correlation analysis of Ca²⁺ time-lapse imaging data (Figure 4—figure supplement 1) revealed small-world network-structured correlated activity in the preBoTc (b–d). The number of correlating cell pairs did not change over time (e), nor did the small-world network parameter or connectivity (f). TMR-SP-positive regions contained more correlated cell pairs than TMR-SP-negative regions (621 ± 284, N=14 and 56 ± 48, N=9, respectively, p<0.05), although there was no difference in the number of active cells (112 ± 57, N=14 and 144 ± 68, N=9, respectively, N.S., g). As in the preBoTc, the nucleus hypoglossus maintained correlated neural network activity (h). Ten percent of the cells (n=8–12/slice) in the hypoglossal nucleus exhibited a regular spiking frequency of ~50–100 mHz (i). The multicolored bar indicates the correlation coefficient in b–h; warmer colors indicate more strongly correlated activity between two cells connected by the line. DIV: days in vitro. A.U.: arbitrary units. w: week. N: number of slices, n: number of cells. Scale bars: 500 μm in a, 100 μm in b–d and g–h. Multicolored bar: color-coded correlation coefficient values. Data are presented as means ± SD. Source data are available in a separate source data file.
DOIs: 10.7554/eLife.14170.014
The following source data is available for figure 4:
Source data 1. Correlation data preBoTc.
DOI: 10.7554/eLife.14170.015
Source data 2. Frequency data with DAMGO.
DOI: 10.7554/eLife.14170.016
Figure 4—figure supplement 1. Single cell events provide information about correlated activity. Ca$^{2+}$ signals are measured in over 200 regions of interest in a single experiment. Events, here simplified as peak maxima with a minimum 20% increase above baseline, over time (lines) are identified. The locations of such events, or rather variations in the Ca$^{2+}$ signal, in both time and space are used to calculate the correlation coefficient between the cells (Smedler et al., 2014). Those correlation coefficients are then drawn as lines between their corresponding cells, providing a graphical image of the network structure. DIV: days in vitro. Scale bar: 100 μm. Multicolored bar: color-coded correlation coefficient values. DOI: 10.7554/eLife.14170.017
Figure 4—figure supplement 2. Spontaneous Ca\textsuperscript{2+} activity is preserved for 3 weeks. NK1R-expressing neurons exhibit rhythmic Ca\textsuperscript{2+} activity after 1, 2, and 3 weeks of cultivation, even during treatment with TTX, which inhibits synapse signaling. There were no significant differences in the average frequency between the control and TTX groups for all time points.
frequency or regularity among slice cultures of different ages. n=840 at 7 DIV, n=621 at 14 DIV and n=456 at 21 DIV. DIV: days in vitro. n: number of cells. *p<0.05.
DOI: 10.7554/eLife.14170.018
Figure 5. Breathing brainstem in a dish: ongoing/persistent rhythmic XII motor activity. The connected preBo¨tC neural networks generate respiratory-related motor neuronal output delivered through the 12th cranial nerve (XII). The hypoglossal nucleus/nerve discharge frequency varied among the brainstem slice cultures but did not depend on brainstem slice culture age (a, N=16 at 7 DIV, N=3 at 14 DIV, and N=6 at 21 DIV). The regularity of respiration-related motor activity, measured as CV (coefficient of variation), remained stable during 3 weeks of culture (b). The µ-opioid receptor agonist DAMGO (0.5 µM) silenced the XII nerve activity in 5/5 brainstem slice cultures, as depicted here in (c) from a 7-DIV brainstem culture (filtered trace, above, and rectified and smoothed trace, below). DAMGO lowered the Ca^{2+}. In the hypoglossal nucleus, DAMGO (0.5 µM) lowered the frequency of regularly-spiking cells (f, g). N: number of slices. Data are presented as means ± SD. *p<0.05 Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.020
The following source data is available for figure 5:

Source data 1. 12th cranial nerve electrophysiology recordings.
DOI: 10.7554/eLife.14170.021
Source data 2. Frequency data with DAMGO.
DOI: 10.7554/eLife.14170.022
Source data 3. High potassium frequency data.
DOI: 10.7554/eLife.14170.023
Source data 4. Network topology and frequency data with DAMGO.
DOI: 10.7554/eLife.14170.024
Figure 5—figure supplement 1. Rhythmic respiratory-related output is preserved. Here, the rhythmic respiratory-related output, recorded from the hypoglossal motor nucleus of a 3-week-old brainstem slice culture (N=6), is displayed. Panels show filtered (above) and rectified and smoothed (below).
traces of the extracellular recording. Rhythmic activity was maintained for 137 min (a). The frequency was regular (b) and increased after Substance P application (1 μM; N=7, c). Thirty minutes after Substance P application, the respiratory-related rhythm returned to the control frequency (d). The activity was inhibited by DAMGO (0.5 μM; N=5, e). N: number of slices.

DOI: 10.7554/eLife.14170.025
Figure 6. Gap junctions are necessary to maintain part of the correlated respiratory network. In the respiratory regions, the gap junction proteins Cx43 (a, N=9), Cx32 (b, c, N=8), and Cx26 (d, arrowheads; double-labeling with NK1R, N=5) are present. Gap junction inhibitors CBX (e) and 18-α-GA (f) reduced network synchronization in the preBötC. Notably, the Ca$^{2+}$ activity of individual NK1R-positive cells was not affected (h–j, m). Correlating cell pair numbers decreased to 21% (N=8) and 20% (N=6) of their respective controls after treatment with CBX and 18-α-GA, respectively (k). Network properties were not affected by GZA, an analog to CBX that lacks the ability to block gap junctions, (g, j–k, N=7) or aCSF (N=8). An initial increase in fluorescence intensity was noted after adding CBX and GZA but not after adding 18-α-GA, indicating an immediate excitatory effect of CBX and GZA (l). 18-α-GA reduced the number of active cells in the network at 1 min after application (53%), but CBX did not (91%, N.S.). At the same time point, an Figure 6 continued on next page
increased number of active cells were observed with GZA treatment (139%). After 10 min, a reduction of the number of active cells was found after treatment with both 18-α-GA and CBX (54% and 43%). However, the number of active cells returned to normal after GZA application (89%, N.S.; l). DIV: days in vitro. N: number of slices. Scale bars: 10 μm in a, c, and d, 100 μm in others. Multicolored bar: color-coded correlation coefficient values. Data are presented as means ± SD. *p<0.05. Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.027

The following source data is available for figure 6:

Source data 1. Gap junction inhibition data.
DOI: 10.7554/eLife.14170.028
Figure 6—figure supplement 1. A gap junction-independent network is present within the preBotC. Gap junction inhibitors did not affect the general topology of the respiratory network (a–c). Data are presented as means ± SD. Source data are available in a separate source data file. DOI: 10.7554/eLife.14170.029
PGE₂ modulates preBotC network activity. PGE₂ lowered the Ca^{2+} signaling frequency of the preBotC network in WT mice but not in Ptger3^{-/-} mice (a–b). The effect was attenuated but not abolished by Riluzole (b). PGE₂ also increased signal amplitude and length (a–b), an effect that was abolished after Riluzole application (b). Ptger3 is expressed in the preBotC (c, d), and 20% of the EP3Rs were of the α (G_{i}-protein coupled) subtype and 77% of the γ (G_{s}-protein coupled) subtype (e). Hypercapnic exposure (pCO₂ elevated from 4.6 to 6.6 kPa) did not affect the signal frequency of the preBotC (f–g). DIV: days in vitro. Scale bars: 50 μm in c and 10 μm in d. *p<0.05 Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.030

The following source data is available for figure 7:

**Source data 1.** PGE2 data preBotC.

DOI: 10.7554/eLife.14170.031

**Source data 2.** Hypercapnia data preBotC.

DOI: 10.7554/eLife.14170.032

**Source data 3.** Hypercapnia data preBotC 2.

DOI: 10.7554/eLife.14170.033
Figure 7—figure supplement 1. Hypercapnia had no effect on the preBotC. Hypercapnic exposure (pCO$_2$ elevated to 6.6 kPa) did not affect the preBotC network structure. DIV: days in vitro. DOI: 10.7554/eLife.14170.034
Figure 8. pFRG/RTN brainstem slice culture. pFRG/RTN slices were selected based on the location of the facial nucleus (VII; a). In the brainstem slice culture, pFRG/RTN expressed the neuronal markers NK1R (b), KCC2 (c), Phox2b (c), vGlut2 (d), and MAP2 (d). The pFRG/RTN neurons also retained adequate electrical properties and generated spontaneous action potentials individually or in clusters (e–f). Data are presented as box plots with minimum and maximum values. DIV: days in vitro. Scale bars: 100 μm.

DOI: 10.7554/eLife.14170.035

The following source data is available for figure 8:

Source data 1. pFRG/RTN characterization.

DOI: 10.7554/eLife.14170.036
Figure 8—figure supplement 1. Cultivation of pFRG/RTN slices. NK1R expression was preserved in the pFRG/RTN during cultivation (upper panel). Whole-cell recordings from neurons in 1-week-old pFRG/RTN brainstem slice cultures shows the existence of spontaneous synaptic input as well as excitable membrane properties (middle panel). The overall morphology of the pFRG/RTN brainstem slice cultures changed slightly during cultivation, becoming thinner (lower panel). DIV: days in vitro. Scale bars: 100 μm in upper panel, 500 μm in lower panel.

DOI: 10.7554/eLife.14170.037
The pFRG/RTN respiration-related network generates correlated neural activity and responds to CO$_2$.

The pFRG/RTN network is arranged in a small-world manner just ventral to the facial nucleus. The network structure was preserved during cultivation (a–d). The number of correlating cell pairs did not change with longer culturing times, but the number of active cells was higher at 3 weeks than at 2 weeks ($45 \pm 27\% < 76 \pm 19\%$, p<0.05; e).

The network parameters were stable during cultivation (f). The pFRG/RTN network did not respond to the $\mu$-opioid receptor agonist DAMGO (0.5 $\mu$M; n=420, N=4; a), but the average network frequency increased with higher potassium concentrations (22 ± 5 mHz and 38 ± 7 mHz, N=12; b). Both the neural network and individual NK1R/TMR-SP-labeled cells responded to increases in CO$_2$ pressure (pCO$_2$ elevated to 6.6 kPa), indicating that the chemosensitivity was preserved in the pFRG/RTN brainstem slice culture. Suramin, a P2 receptor antagonist, and TNP-APT, a P2X receptor antagonist, attenuated the CO$_2$ response but did not abolish it (g).

DIV: days in vitro. Scale bars: 100 $\mu$m. Multicolored bar: color-coded correlation coefficient values. N: number of slices, n: number of cells. Data are presented as means ± SD. *p<0.05. Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.038

The following source data is available for figure 9:

Source data 1. Correlation data pFRG/RTN.
DOI: 10.7554/eLife.14170.039

Source data 2. Hypercapnia data.
DOI: 10.7554/eLife.14170.040

Source data 3. High potassium frequency data.
DOI: 10.7554/eLife.14170.041

Source data 4. Riluzole and TTX data.
DOI: 10.7554/eLife.14170.042
Figure 9—figure supplement 1. Spontaneous Ca\(^{2+}\) activity is preserved during cultivation. NK1R-expressing neurons have spontaneous Ca\(^{2+}\) activity after 1, 2, and 3 weeks of cultivation. This activity remains during synaptic inhibition via addition of TTX. Within the culture, the frequency varied.
between cells, but there were no significant differences in average frequency or regularity among cultures of different ages. n=315 at 7 DIV, n=429 at 14
DIV, and n=192 at 21 DIV. DIV: days in vitro. n: number of cells. *p<0.05.
DOI: 10.7554/eLife.14170.043
Figure 9—figure supplement 2. Hypercapnia reduces mean path lengths in the pFRG/RTN of wild-type mice. A decrease in mean path length during hypercapnia was seen in the wild-type pFRG/RTN (N=18). Other parameters remained unchanged. No parameters were affected in the Ptger3−/− pFRG/RTN (N=7). N: number of slices. DIV: days in vitro. Scale bars: 100 μm. Multicolored bar: color-coded correlation coefficient values. Data are presented as means ± SD. *p<0.05. Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.044
Correlated pFRG/RTN network activity is not dependent on gap junctions, but hypercapnic responses are. Blocking gap junctions in the pFRG/RTN did not change the functional network structure of the respiratory center or alter its frequency (a and c, N=7). However, hypercapnic responses (CO$_2$) were abolished when gap junctions were inhibited by 18-α-GA (b, top trace; c, left graph, N=7). GZA (a structural analog of CBX without gap junction-inhibiting properties) increased the frequency, and hypercapnia increased it further (b, middle trace; c, middle graph, N=7). An initiated hypercapnic response was attenuated but not completely reversed by 18-α-GA (b, bottom trace; c, lower graph, N=5). This dynamic was not seen after application of GZA. DIV: days in vitro. Scale bars: 200 μm. N: number of slices. Multicolored bar: color-coded correlation coefficient values. Data are presented as means ± SD. *p<0.05. Source data are available in a separate source data file.
Network structure in the pFRG/RTN is not dependent on gap junctions. Blocking gap junctions in the pFRG did not change the network parameters, correlating cell pairs, or number of active cells (N=7). Data are presented as means ± SD. N: slices. Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.051
Figure 11. PGE$_2$ is released during hypercapnia. The aCSF contents exhibited an increase in microenvironmental PGE$_2$ levels during hypercapnia in 12 out of 12 slices. Here, the PGE$_2$ concentration of a brainstem slice culture is displayed during control and hypercapnic periods (a). When gap junctions were inhibited (18-α-GA, blue line), the PGE$_2$ levels remained unaltered during hypercapnia (N=4). The average PGE$_2$ level throughout the whole experiment was not affected by hypercapnia, but the peak value was higher during hypercapnia than under control conditions (b). N: number of slices.

Data are presented as means ± SD. *p<0.05. Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.052

The following source data is available for figure 11:

**Source data 1.** Hypercapnia PGE$_2$ ELISA data.

DOI: 10.7554/eLife.14170.053
Figure 11—figure supplement 1. mPGEs-1 is expressed in astrocytes in the proximity of the ventral border of the pFRG. Expression of mPGEs-1, critical for PGE$_2$ production, was found in GFAP-expressing astrocytes (arrowheads) proximal to the ventral medullary border in acute frozen brainstem tissue. This was evident in both wild-type mice and transgenic mice with GFAP-driven expression of GFP (N=11/11 and 6/6 respectively). N: number of slices. Scale bars: 100 μm. * indicates the ventrolateral edge of the brainstem.
DOI: 10.7554/eLife.14170.054
Figure 12. PGE$_2$ alters respiratory network activity. In the pFRG/RTN, PGE$_2$ increased the frequency of respiratory (NK1R-expressing) neurons. This PGE$_2$ effect was absent in brainstem slice cultures lacking EP3R (Ptger3$^{-/-}$; a–b). EP3Rs were present in NK1R-expressing neurons in the pFRG/RTN (c, arrowheads, f) and co-localized with Phox2b (d, arrowheads). EP3Rs were also found on S100B-expressing astrocytes (e, arrowheads). Staining was performed on acutely fixed tissue (c–e) and brainstem slice cultures (f). qRT-PCR showed an abundance of the EP3R (G$_s$-protein coupled) in the pFRG/RTN (N=7; g). N: number of slices. DIV: days in vitro. Scale bars: 100 μm. Data are presented as means ± SD. *p<0.05 Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.055

The following source data is available for figure 12:

**Source data 1.** PGE$_2$ frequency data pFRG/RTN.

DOI: 10.7554/eLife.14170.056
Figure 13. PGE$_2$, acting through EP3R, is crucial for the hypercapnic response. Pharmacological inhibition of EP3R by the EP receptor antagonist AH6809 inhibited the response to hypercapnia (increased pCO$_2$) in the

Figure 13 continued on next page
Figure 13 continued

pFRG/RTN (N=6, n=472, N.S.; a–b). The hypercapnic response was also absent in pFRG/RTN slices lacking EP3R (Ptger3<sup>−/−</sup>; N=5, n=348, N.S.; c–d). Layout of the lentivirus containing Halo57 (ER2) and eGFP genes under the control of the EP3R promoter (Ptger3) used for optogenetics (WPRE=gene enhancing element; e). During optogenetic silencing of Ptger3-expressing cells, no frequency changes were observed in response to hypercapnia (f, top trace; g, left graph). The hypercapnic response was also reversed by activating Ptger3-Halo57 (f, middle and bottom trace; g, middle and right graph). Red line: Halo57 activation in response to 625 nm light. N: slices, n: cells. Data are presented as means ± SD. *p<0.05. Source data are available in a separate source data file.

DOI: 10.7554/eLife.14170.058

The following source data is available for figure 13:

**Source data 1.** EP antagonist data.

DOI: 10.7554/eLife.14170.059

**Source data 2.** Hypercapnia EP3R data.

DOI: 10.7554/eLife.14170.060

**Source data 3.** Optogenetics data.

DOI: 10.7554/eLife.14170.061
Figure 13—figure supplement 1. Optogenetic silencing of Ptger3-expressing cells decreases respiration-related activity. Transduced slices show the expression of EGFP in the pFRG/RTN, localized through Phox2b staining (a, left). Phox2b-positive cells express EGFP after transduction (a, right, arrowheads). Silencing Ptger3 cells with Halo57 stimulation decreased the frequency of the entire pFRG/RTN (b). General depolarization caused by an increase in the potassium concentration to 9 mM increased the average network frequency in both the preBotC and pFRG/RTN during optogenetic inhibition (N=6; c). Red line: Halo57 activation in response to 625 nm light. N: number of slices. DIV: days in vitro. Scale bars: 100 μm (a, left), 10 μm (a, right). Data are presented as means ± SD. *p<0.05. Source data are available in a separate source data file. DOI: 10.7554/eLife.14170.062
Figure 14. Model of how PGE$_2$ modulates respiration and sighs in the preBötC and pFRG/RTN. Systemic inflammation, through the proinflammatory cytokine IL-1β and hypoxia, induces the production of PGE$_2$ in blood brain barrier (BBB) endothelial cells (Hofstetter et al., 2007). PGE$_2$ subsequently induces respiratory depression and increases sigh activity via the inhibitory G-protein coupled receptor EP3R$_\alpha$ in the preBötC. In the pFRG/RTN, PGE$_2$ plays a role in the response to elevated pCO$_2$. CO$_2$ directly modulates connexin 26 (Cx26) hemichannels, leading to ATP release. The results in this study suggest that Cx26 also releases PGE$_2$, possibly from mPGEs-1$^+$ astrocytes. PGE$_2$ increases respiratory activity via the stimulatory G-protein coupled receptor EP3R$_\gamma$ on pFRG/RTN neurons. Thus, inflammation, hypoxia, and hypercapnia alter respiratory neural network and motor output and breathing activity through distinct effects of PGE$_2$ in the pFRG/RTN and the preBötC, respectively. Chronically elevated PGE$_2$ levels, as observed during ongoing inflammation, may decrease the central pattern generators’ ability to respond to hypoxic and hypercapnic events. In extreme cases, this decrease may have fatal consequences.

DOI: 10.7554/eLife.14170.064