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Supplementary Information for

Impaired endothelium-mediated cerebrovascular reactivity promotes anxiety and respiration disorders in mice

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Supplementary Information

Supplementary Methods

Mice. All mouse lines were established on a C57BL/6 background. We used littermate mice that were sex- and age-matched between experimental groups. In most experiments, mice were between 8 and 18 weeks old. All animal experiments were approved by the local animal ethics committee (Regierungspräsidium Karlsruhe; Ministerium für Landwirtschaft, Umwelt und ländliche Räume, Kiel, Germany). Mice were kept at constant temperature (22°C) on a 12-hour light/ dark cycle and were provided with standard laboratory chow (2.98 kcal g^{-1}; Altromin, Hannover, Germany) and water ad libitum.
Brain endothelial-specific knockout (beKO) animals were generated by crossing the BAC-transgenic *Slco1c1-CreER^{T2}* strain (1), which expresses the tamoxifen-inducible CreER^{T2} recombinase under control of the mouse *Slco1c1* regulatory sequences in brain endothelial cells, with mice carrying loxP-flanked alleles. Mice that carried a floxed Gα_q allele (*Gnaq$^{fl/fl}$*) and were Gα_{11}-deficient (*Gna11$^{-/-}$*) (2) (*Gnaq$^{fl/fl}$*::*Gna11$^{-/-}$*) were used to knock out the G$\alpha_{q/11}$ pathway in brain endothelial cells. Littermates lacking the Cre transgene and heterozygous for *G*α_{11} (*Gnaq$^{fl/fl}$*::*Gna11$^{-/+}$*) were used as controls and received tamoxifen in parallel to knockout animals. *P2yr2^{Fl}* mice (3) were crossed with the *Slco1c1-CreER^{T2}* strain to generate a brain endothelial-specific deletion of P_2Y_2 (*P2yr2^{beKO}*). GPR4 and GPR68 whole-genome knockout mice have been described previously (4).
All animals used to examine the brain endothelial deletion of the G$\alpha_{q/11}$-pathway or the P_2Y_2 receptor were injected with 1 mg tamoxifen (Sigma-Aldrich) per 20 g body weight dissolved in 90% miglyol® 812 /10% ethanol i.p. every 12 h for 5 consecutive days. Investigators were blinded to the treatment or genotype of mice or both in all experiments and analyses.

CO_2-induced fear response. For baseline measurements, mice were placed in a small box (26.5 cm x 20.5 cm x14.0 cm) that was prefilled with normal air (21% O_2/79% N_2) and exposed to constant flow-through of air (1 l/min). The box was closed by a lid and mice were video-monitored for 10 min. CO_2 stimulation was performed in the same way but the gas mixture contained 10 % CO_2, 21 % O_2, and 69 % N_2. Videos were analyzed using special software (ANY-maze, Stoelting Co.) regarding freezing behavior. Freezing was expressed as total duration during the 10-min stimulation.

Head-out plethysmography. Respiratory activity was assessed using a head-out plethysmograph that has been described previously (5). Briefly, the system contains a glass made head-out plethysmograph attached to a chamber that was filled and continuously ventilated (1 l/min) with specific gas mixtures. Mice were positioned in the plethysmograph with the head protruding into the ventilated chamber. After positioning and baseline recording, the CO_2 concentration was increased in a stepwise manner (0.5 %, 3 %, 8 %, balance O_2, each step for 8 min). Hypercapnic exposure was performed in hyperoxia to minimize contributions of peripheral chemoreceptors to the hypercapnic ventilatory reflex. However, we performed also experiments with the indicated CO_2 concentrations, 21 % O_2 and rest N_2 to measure effects under normal air conditions. For airflow measurement, a calibrated pneumotachograph (PTM 378/1.2; Hugo Sachs Elektronic) and a differential pressure transducer (8T-2; Gaeltec), coupled to an amplifier (HSE-IA; Hugo Sachs Elektronic) were attached to the top port of each plethysmograph. For each animal the amplified analog signal from the pressure transducer was digitized via an A/D converter (DT301 PCI; Data Translation) at a sampling rate of 2,000/sec. Notocord hem 3.5 (Notocord) was used for data calculation. Mean values were calculated using the last 2 min of each stimulation episode. The minute volume was calculated as the product of breathing frequency and tidal volume.

Whole-body plethysmography and determination of apneic episodes. In addition to head-out plethysmography, ventilatory responses were measured in awake, freely moving mice by whole-body plethysmography (EMKA Technologies). A mass flow regulator provided a quiet, constant, and smooth flow through the animal chamber (0.5 l/min). The typical protocol entailed four sequential, incremental CO_2 challenges (10-min exposures to 0.5 %, 3 %, 8 %, and 10 % CO_2, balance O_2) after a 30-min acclimatization period. Again, hypercapnic exposure was performed in hyperoxia. Ventilatory flow signals were recorded, amplified, digitized, and analyzed using Iox 2.7 (EMKA Technologies). To exclude periods of sniffing that occurred mainly during baseline periods and low CO_2 concentrations we removed signals that were below 40 µl tidal volume and above 400 bpm breathing frequency. Mean values for minute volume were calculated as described above.
To determine apneic episodes, plethysmographic recordings were performed during the inactive phase of mice without any stimulus for at least 1.5 h. After a 30-min acclimatization period, apneic episodes were determined within a 1-h period and defined by at least 100 % longer duration of one breath than the mean breath length within the 6 seconds before using the software described above.

Laser speckle imaging. Mice were anesthetized with an induction dose of 3-4% isoflurane and, throughout the surgery, with 2 % isoflurane. During the measurements, anesthesia was switched to ketamine (70 µg/g body weight, bela-pharm) / xylazine (16 µg/g body weight, Bayer AG). Body temperature was maintained at 37 ± 0.2°C throughout the study using a feedback-regulated warming system (TCAT-2LV, Physitemp Instruments). A small ventilatory tube was inserted into the trachea after tracheotomy and connected to a small animal ventilation device (MiniVent, Harvard Apparatus). Ventilation volume was constant and ventilation frequency was adapted to a physiological expiratory CO_2 concentration of 35-45 mmHg that was continuously controlled during the experiments with a capnometer (microCapstar, CWE Inc.). The skin over the head was removed but the skull remained intact for the whole experiment. Laser speckle imaging (FLPI, Moor instruments) was performed using a recording rate of 0.25 Hz or 25 Hz (during apnea recordings). Regions of interest were set over big cortical vessels. Flux intensities were recorded throughout CO_2 stimulation (10 or 20 %, combined with 21 % O_2, rest N_2) and were normalized to baseline values for each region of interest. Due to gas diffusion along the tubing, the mix containing 10 % CO_2 resulted in a CO_2 concentration of 7.8 % and the 20 % CO_2 mix in a concentration of 11.3 % at the outlet of the ventilation tube.
Hypoventilation was performed by decreasing the ventilation frequency down to half of the initially identified optimal frequency for each mouse for 120 sec, leading to a robust increase in expiratory CO_2. Apneic periods were induced by stopping the artificial ventilation for 2 or 3 seconds as indicated.

Arteriolar reactivity in acute brain slices. Vascular reactivity of small arterioles in slices of different brain areas was assessed using a protocol that was described previously (6) with slight changes. In detail, mice were decapitated under deep isoflurane anesthesia and brains were removed immediately. Transverse sections (150 µm) were prepared using a vibratome (VT1200, Leica) in carbogen-gassed (5% CO_2, 95 % O_2), cold, artificial cerebrospinal fluid (ACSF) containing: NaCl 130 mM, KCl 3 mM, $NaHCO_3$ 26.5 mM, $MgCl_2$ 2 mM, NaH_2PO_4 1.25 mM, ascorbic acid 0.4 mM, $CaCl_2$ 2 mM, and D-glucose 10 mM. After slicing, tissue was stained for vessels with 2 µg/ml isolectin GS-IB_4 conjugated to Alexa Fluor 568 (Thermo Fischer Scientific) at 37°C in ACSF gassed with carbogen for 30 min.
Imaging was performed using a high-speed calcium imaging setup (FEI GmbH) mounted on the Axio Examiner D1 upright fluorescent microscope (Zeiss).

Slices were placed in a flow chamber that was perfused with ACSF containing U46619 (125 nM, Tocris) with a flow rate of 2 ml/min and a bath temperature of 35°C. The perfusate was continuously gassed with 5 % CO_2 / 21 % O_2 / 74 % N_2 to maintain a pH of 7.4. CO_2 stimulation was performed for 5 min by switching the solution to ACSF equilibrated with 15 % CO_2 / 21 % O_2 / rest N_2, leading to a pH of 7.0.
Substances were applied to the perfusate for 5 min leading to the following final concentrations: endothelin-1: 100 nM, sodium nitroprusside: 1 µM, U46619: 1 µM, iloprost: 1µM, ATP: 1mM. Single arterioles were identified in brain slices by typical ring-like labeling (see Fig. 2M, 3A, 4A) and a diameter of >10 µm. RTN slices were taken from the ventral surface below the caudal end of the facial nucleus; amygdala slices were taken 1 – 1.5 mm above the ventral surface of the forebrain and the area between the cortical and thalamic/hypothalamic structures was imaged; cortical vessels were identified in slices taken from the somatosensory cortex.
After CO_2 stimulation, slices were exposed first to a Ca^{2+}-free ACSF containing 5 mM EGTA and 200 µM papaverine (Sigma-Aldrich) to dilate arterioles, and afterward to a 60 mM K^+ solution to induce a constriction. Only vessels that responded to Ca^{2+} removal and K^+ exposure were considered as functional and included in the analysis.
Vascular reactivity to the aforementioned stimuli was analyzed using FIJI (www.fiji.sc/Fiji). If needed, image sequences (0.25 Hz) were registered before applying a linear region of interest perpendicular to the vessel direction. The distance between the peaks of the fluorescence intensity profile was plotted throughout the whole imaging sequence using a macro (https://github.com/omsai/blood-vessel-diameter) and was used as a measure to determine the vessel diameter. At least three regions per arteriole were measured and the mean value of the last 2 minutes of stimulation was taken to calculate diameter changes. Calculated diameters were verified by measuring manually at single time points. All analyses were done blinded for the brain region and genotype.

Blood pressure recordings. Mice were anesthetized using isoflurane for implantation of the telemetric pressure transducer (Data Sciences International). The catheter was introduced into the left carotid artery and the transmitter positioned subcutaneously at the back (7). Blood pressure and heart rate were measured continuously for 24 h. Values were stored every minute and analyzed using the DSI software.

Elevated plus maze. Anxiety-related exploration was observed and video recorded in an elevated plus maze for 5 min. The arena consisted of a plus-shaped maze with two arms (5 cm wide) closed by side walls and two arms without walls; intertrial cleaning was performed with 70% ethanol.

Open field test. Animals were observed and video recorded for 10 min during their active period in a 50 x 50 cm^2 arena, illuminated with 25 lux; intertrial cleaning was performed with 70% ethanol.

Rotarod. Animals were set on a rotating rod, with a starting speed of 2.5 rpm, increasing to 25 rpm in 4.5 min, and the latency until they fell was scored (maximal time: 5 min); intertrial cleaning was performed with 70% ethanol.

Grip strength test. Animals were held at the tail and lowered until they could hold the grid, first two times with only the forepaws and then two times with all four paws. After few seconds, animals were pulled horizontally until they lost the grid. The grip strength meter displayed the power of the grip in g.

Novel cage exploration. Animals were observed under red light conditions for 5 min during their active period in a clean, unused, standard macrolon cage (type II) with a little sawdust; intertrial cleaning was performed with 70% ethanol

Novel object recognition. The novel object recognition experiment consisted of 7 trials with 6 min each in a round grey open field arena illuminated with 25 lux. In trial 1, the mice were habituated to the open field. In the trials 2-6, the mice could explore 4 objects, which were different in surface structure, color, and design. For every mouse, the location of every object was always fixed, whereas between mice, object combination and location of the objects were balanced. In trial 7, the second object was replaced by a new, unfamiliar object. Number of approaches and time at the familiar objects versus the new object were measured. Between the trials, mice were gently removed and kept in their homecage for maximally 4 min while all objects and the open field were cleaned with 70% ethanol.

Conditioned taste aversion. Mice were trained to drink water from 2 bottles in two sessions per day (30 min each, 10:00 and 16:00) and deprived of water during the rest of the day; the amount of water consumed was measured. On the 3rd day, the mice got one bottle with saccharin solution (0.5 %) in their morning session. 45 min after this session the mice got a single i.p. injection with lithium (0.14 M at a dose of 2% of body weight). 48h and 2 weeks after the conditioning, animals were given the possibility to choose in a session between saccharin and water. The preference for saccharin was analyzed.

Blood gas measurements. Venous blood from the facial vein was collected from awake mice into heparinized capillary tubes and immediately analyzed with a blood gas analyzer (Combiline, Eschweiler) for pH, pCO_2, and pO_2. For arterial blood gases during artificial ventilation mice were anesthetized with isoflurane and ketamine/xylazine according to the regime described in the laser speckle imaging part. A catheter (ID 0.28 mm, OD 0.61 mm, Smiths Medical ASD Inc., USA) filled with 0.9% NaCl solution containing heparin (100 IU/ml) was inserted into the right common carotid artery for arterial blood sampling. After tracheotomy mice were mechanically ventilated (150 strokes/min-1, stroke volume of 220 µl, MiniVent Model 845, Hugo Sachs Elektronik, Germany) using a gas mixture containing 21% O_2 and 79% N_2. At least 15 min of recovery was allowed before blood was collected at the basal condition. Subsequently, inspiratory gas was supplemented with increasing CO_2 concentrations (10 and 20%) at constant O_2. Each CO_2 concentration was applied for 10 min before blood sampling. Arterial blood samples were analysed for pH, pO_2, and pCO_2. An additional blood sampling was performed after a 3-seconds lasting apneic period that led to subsequent changes in blood gases compared to baseline ($paCO_2$: 43.23±1.75 vs. 38.38±1.97, $p=0.08$; paO_2: 86.96±4.95 vs. 119.8±5.75, $p<0.001$; pH: 7.312±0.013 vs. 7.358±0.014, $p<0.05$; mean±SEM, n=15). However, blood sampling itself took several seconds and thus, these samples contained partly blood from the re-ventilation period.

Arterial spin labeling MRI. Anesthesia was induced by 4% isoflurane. All MRI studies were performed under ~0.8-1% isoflurane in a gas mixture containing 21% O_2 and rest nitrogen. Respiration rate and body temperature were continuously monitored using a MRI-compatible system and temperature was maintained at 37 ± 0.2^{o}C throughout the study using a feedback-regulated warming system (Small Animal Instruments Inc.). Mice were scanned using a 7 Tesla small animal MRI scanner (ClinScan, Bruker) with a ^{1}H receive-only 2x2 mouse brain surface array coil. The array coil was placed closely to the neck and back of the head. Anatomical image data were acquired using a T2-weighted turbo spin-echo sequence with the following sequence parameters: echo time – TE = 65 ms, repetition time – TR = 3000 ms, field of view – FoV = 16×16 mm^2, image matrix = 192×192, turbo factor = 9, number of slices = 15, slice thickness = 0.5 mm, readout bandwidth = 100 Hz/pixel, 50% phase oversampling, and total acquisition

time = 3:15 min. CBF was measured by arterial spin labeling (ASL) with Q2TIPS (quantitative imaging of perfusion using a single subtraction with interleaved thin-slice TI1 periodic saturation) (8) with TI1/TI2/SS = 900/1400/1375 ms and with 45 control-label pairs. Further ASL scanning parameters were: Echo planar imaging technique with TE =7.3 ms, TR =3000 ms, FoV =16×16 mm^2, image matrix = 64×64, number of slices = 3, slice thickness = 0.5 mm, partial Fourier factor =6/8, readout bandwidth = 4340 Hz/pixel, and total acquisition time = 4:38 min. ASL imaging was performed before and after applying 10 % CO_2 (21 % O_2, balance N_2).

Primary endothelial cells. Primary forebrain endothelial cells (PFBECs) of mice were cultured as described in detail previously (9). Brain area-specific primary endothelial cells of the subcortical telencephalon (SCT) or the brainstem were cultured under the same conditions as PFBECs but beforehand the tissue was dissected (see Fig. 5A). As the amount of vessels is far less than with PFBECs we pooled 5 instead of 1-2 brains per 6 wells of a 24-well plate. PFBECs and SCT endothelial cells reached confluency usually after 5-6 days, whereas brainstem endothelial cells after approximately 7-10 days in culture. Experiments were performed on confluent cells.
For CO_2 stimulation experiments, cells were starved for 20 h before the experiment started. Starving medium contained 1 % PDS (plasma-derived bovine serum) instead of 20 % as in normal culture medium. After starving, cells were covered with a Krebs-Ringer solution (NaCl 130 mM, NaH_2PO_4 1.25 mM, ascorbic acid 0.4 mM, KCl 3 mM, $NaHCO_3$ 26.5 mM, $MgCl_2$ 2 mM, $CaCl_2$ 2 mM, D-glucose 10 mM) that was pregassed with either 5 % CO_2 or 15 % CO_2 at 37°C (resulting in a pH of 7.4 or 7.0, respectively). After 20 min of incubation with CO_2 at 37°C, supernatant was collected and cells frozen in liquid nitrogen for further analyses. For blocking the $G\alpha_{q/11}$ signaling pathway, PFBECs were incubated with 10 µM of the specific inhibitor FR900359[25] or DMSO as control for 20 min at 37°C.

Cyclic AMP assay. The amount of cAMP generated in PFBECs during a stimulation period of 30 min as described above (PFBECs) was determined after lysis of the cells according to the manufacturer´s protocol using the Direct cAMP ELISA kit (ENZO Life Sciences).

Nitric oxide (NO) release assay. The amount of NO released by PFBECs after a stimulation period of 20 min was determined in the supernatant according to the manufacturer´s protocol using the nitrate/nitrite fluorometric assay kit (Cayman). Before starting the assay, we stabilized the pH of the supernatant by mixing it with a HEPES solution, resulting in a final concentration of 10 mM HEPES and a pH of 7.4. Afterward, we followed the manufacturer´s protocol to measure the amount of NO-derived nitrate in the supernatant.

Prostanoid release assays. We measured prostaglandin E_2, thromboxane B_2 as a surrogate for thromboxane A_2 synthesis, prostaglandin $F_{2\alpha}$, or 6-keto $PGF_{1\alpha}$ as a surrogate for prostacyclin synthesis in the supernatant of primary endothelial cells. The amount of prostanoids released by the cells after a stimulation period of 20 min was determined in the supernatant according to the manufacturer´s protocol using the PGE_2, $TBXB_2$, $PGF_{2\alpha}$, or PGF-1α assay kit (all Cayman). Before starting the assay, we stabilized the pH of the supernatant by mixing it with a HEPES solution, resulting in a final concentration of 10 mM HEPES and a pH of 7.4. Afterward, we followed the manufacturer´s protocol to measure the amount of prostanoids in the supernatant.

Ca^{2+} imaging. To detect changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), PFBECs were cultured on cover glasses until reaching confluency. PFBECs were incubated with Fura-2AM (2 µM with 0.05% pluronic 127, and 10 µM probenecid, all Life technologies) in Krebs-Ringer solution (see PFBECs) for 30 min at 37 °C. Then, they were incubated in Krebs-Ringer solution containing only probenecid (10 µM, 30 min 37 °C) before measurement. PFBECs were placed in

a flow chamber that was perfused with Krebs-Ringer solution (flow rate: 2 ml/min) that was continuously gassed with 5 % CO_2 / 95 % O_2. $[Ca^{2+}]_i$ was measured using a high-speed calcium imaging setup (FEI GmbH). For data acquisition and quantification we used live acquisition and offline analysis software (FEI GmbH). Changes in the fluorescence ratio (F_{340}/F_{380}) were normalized to baseline mean values during the 60 sec before stimulating each region (F_0) of interest (ROI) and were shown as $\Delta F/F_0$. At least 20 cells per sample were randomly chosen and analyzed. Stimulation with ATP (10 - 30 µM) for 1 min was used to determine $[Ca^{2+}]_i$ responses of the P_2Y-$G\alpha_{q/11}$ signaling cascade in PFBECs. A threshold of 10 % increase above baseline was defined and number of responding cells was counted.

Western Blotting. PFBECs were cultured on 6-well plates and lysed in 4x SDS buffer, incubated at 95 °C for 10 min, and then loaded on SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes, which were then incubated with the indicated primary antibodies (phospho-eNOS (Ser1177): Cell Signaling Technology, 9571S; eNOS: BD Transduction, 610296) overnight at 4 °C and, subsequently, with HRP-conjugated secondary antibodies for 1-2 h at room temperature (Rabbit IgG-HRP: Santa Cruz, sc2301; Mouse IgG-HRP: Santa Cruz, sc-2005). For detection we applied enhanced chemiluminescence (SuperSignal West Femto Substrate, Thermo Scientific) and a digital detection system (FUSIO SOLO S, Vilber).

Immunohistochemistry. We perfused mice with PBS containing heparin (10 IU/ml). To characterize vascular morphology we stained methanol-fixed cryosections with antibodies specific for the following antigens: anti-CD31 1:500 (BD Pharmingen, #557355); anti-CD13 1:400 (AbD Serotec, #MCA2183GA); anti-SMA 1:200 (Acris, #DM00105); and anti-collagen IV 1:1000 (Abcam, #ab6586 or #ab19808). Two to four pictures of cortical stainings per mouse were taken and analyzed. Stainings were quantified in a partially automated manner using a custom macro implemented into the image analysis software Fiji (www.fiji.sc/Fiji), as described previously (10).

RNAscope *in situ* hybridization. Brain tissue of adult C57BL7N mice was sectioned and postfixed in 4% PFA for 30 min. After dehydration, sections were used for fluorescence *in situ* hybridization using RNAscope multiplex fluorescent assay (Advanced Cell Diagnostics) according to the manufacturer's instructions. Afterward, sections were immunostained with anti-collagen IV antibodies (1:2000, EMD Millipore, #AB769) or anti-smooth muscle actin antibodies (1:400, Millipore, #C6198) and cover slipped in Invitrogen ProlongTM Gold antifade reagent (Thermo Fischer Scientific). *Gpr4*, *Gpr68*, and *Pecam* transcript localization was assessed using a confocal microscope (Leica SP5).

Quantitative RT-PCR. RNA was isolated from whole forebrain, vessel fragments, or brain endothelial cells by Nucleo Spin RNA Kit (Macherey-Nagel) according to the manufacturer´s instructions. RNA (similar amount of each sample) was transcribed with Moloney Murine Leukemia Virus Reverse Transcriptase and random hexamer primers (Promega). The following primers were used for quantitative real-time PCR: *Ppia* forward, 5´-AGG TCC TGG CAT CTT GTC CAT-3´, *Ppia* reverse, 5´-GAA CCG TTT GTG TTT GGT CCA-3´; *Gpr4* forward 5´-AGT CGG GAC CAA GTC AGA GA-3´, *Gpr4* reverse, 5´-GGT GGA GAC TGA GGA GTG GA-3´; *Gpr68* forward, 5´-TGA CCT ACC TGT GGG ACA GAA-3´, *Gpr68* reverse, 5´-ATC ACT GGT GGT AGG CGG A-3´; *P2yr2* forward, 5'-CCT CAC CAC CTC AAG AGC AG-3', *P2yr2* reverse,5'-TAT CCC AGT TCG TCC CCC TC-3`; *Gnaq* forward,5'-TTA TCA CCT ACC CCT GGT TCC-3', *Gnaq* reverse, 5'-TCG ACT AGG TGG GAA TAC ATG A-3'; *Nos1* forward, 5´-AGAGGAGAGGAAGAGCTACAAGG-3', *Nos1* reverse, 5´-CCAGGCCGAAGACTGAGAAC-3'; *Nos3* forward, 5´-TGTGACCCTCACCGCTACAA-3', *Nos3* reverse, 5´-GCACAATCCAGGCCCAATC-3'; *Ptgs1* forward, 5´-TAGGCCACGGGGTAGACCTT-3',

Ptgs1 reverse, 5´-GTAGCGCATCAACACGGACG-3'; *Ptgs2* forward, 5´-AGGCCTCCATTGACCAGAG-3', *Ptgs2* reverse, 5´-GCCATTTCCTTCTCTCCTGTAA-3'; *Ptgds* forward, 5´-CAACCGGATAAGTGCATTCA-3', *Ptgds* reverse, 5´-TTGAGAGTGACAGAGCAAAGG-3'; *Ptgis* forward, 5´-CAGGATGAAGCCGACGCTCA-3', *Ptgis* reverse, 5´-CTGAGCAGGGCGTTGTAGGA-3'; *Ptges* forward, 5´-CACACTGCTGGTCATCAAGAT-3', *Ptges* reverse, 5´-GGTTGGCAAAAGCCTTCTT-3'; *Ptges2* forward, 5´-TGCAGGATGATGTACGGGTA-3', *Ptges2* reverse, 5´-CACACCATACACTGCCAGGT-3'; *Ptges3* forward, 5´-TTTGACCGTTTCTCTGAGATGA-3', *Ptges3* reverse, 5´-GCGATGACAACAGCCCTTAC-3'; *Prxl2b* forward, 5´-ACCCCAGTGTGATGAAGAGGT-3', *Prxl2b* reverse, 5´-ACAGGGATTCTTTGTGTTCCA-3'. Quantitative RT-PCR was performed according to the following protocol: after preincubation for 10 min at 95 °C the amplification was performed for 30 sec at 95 °C, 30 sec at 60 °C, 30 sec at 72 °C, and 1 min at 60 °C (38 cycles). Amplification was quantified using Platinum SYBR Green qPCR SuperMix (Invitrogen). Quantified results were normalized to *Ppia* using the ΔΔCt method.

Microarray. RNA was isolated from vessel fragments from cortex and brainstem of single mice using the RNAeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The RNA was processed and hybridized on the Affymetrix Mouse Gene 2.0 Array (Thermo Fisher Scientific, Germany) as previously described (11).
For the choice of prostaglandin-related genes, purinergic receptors, and acid-base metabolism-genes we checked in a database whether genes are expressed in cells of the brain vasculature first (http://betsholtzlab.org/VascularSingleCells/database.html). Only genes that were described to be expressed in endothelial cells, pericytes, or smooth muscle cells underwent a detailed analysis.

Statistical analysis. GraphPad Prism (v. 5.0) was used for statistical analysis. All data are presented as means ± standard error of the mean (SEM). For statistical comparison of two groups we used two-tailed Student`s *t*-test after confirming normal distribution of data with the Shapiro-Wilk normality test. In case of unequal variances, we applied Welch´s correction. If data values did not pass a normality test, we applied the two-tailed nonparametric Mann-Whitney test to compare two groups. For more than two groups the analysis was performed by one-way ANOVA with subsequent Bonferroni post-hoc test. Time-dependent data or data including more than one variable were analyzed with two-way ANOVA followed by Bonferroni post-hoc test. A p value < 0.05 was considered statistically significant.

Supplemental Figures

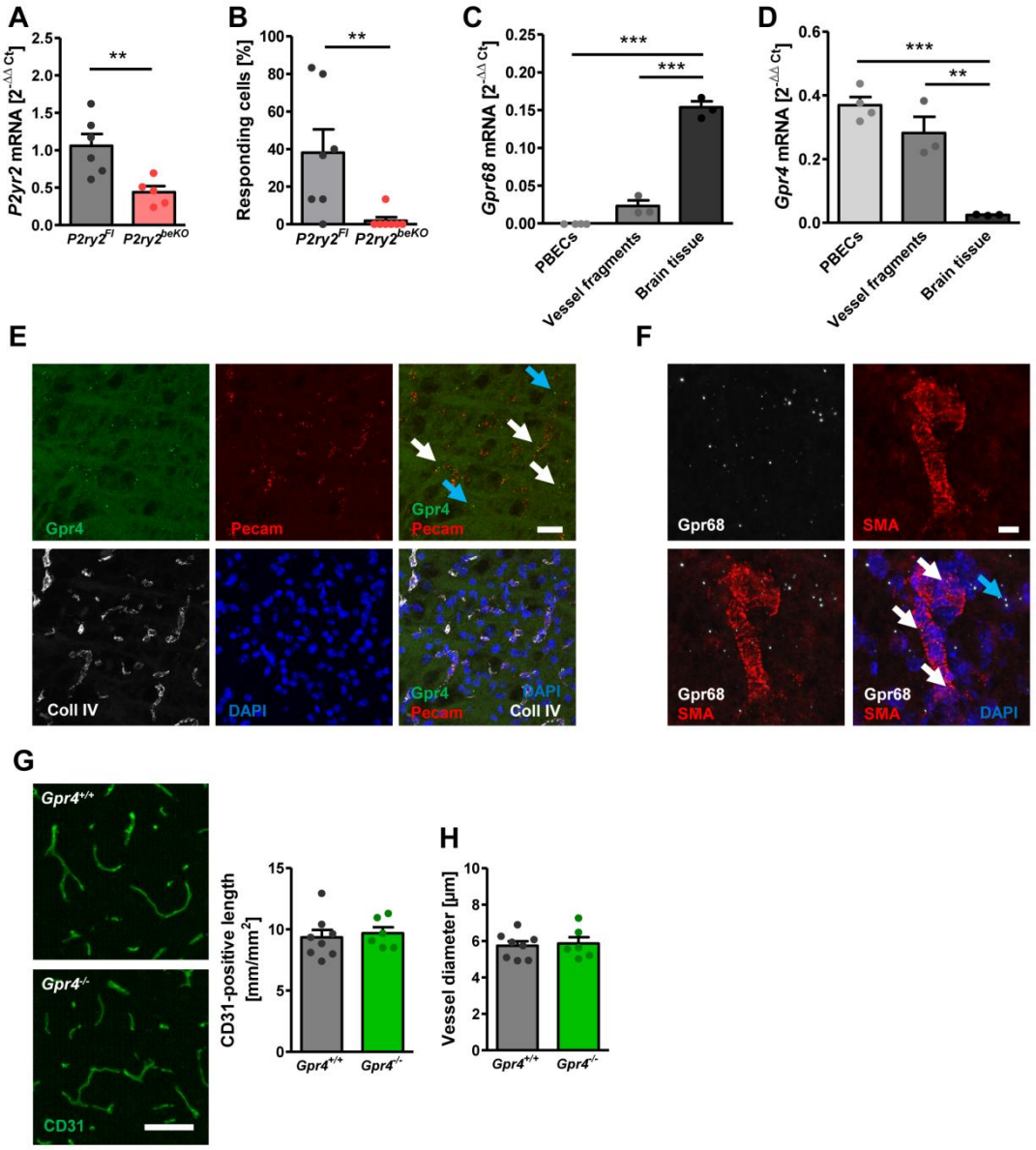

Figure S1. (**A**) mRNA levels of *P2ry2* in PFBECs derived from *P2ry2*^{beKO} and control mice as measured by quantitative RT-PCR. Mann-Whitney test, **P < 0.01. n = 5-6 per group. (**B**) Effect of ATP (10 µM) on intracellular calcium ($[Ca^{2+}]_i$) in PFBECs derived from *P2ry2*^{beKO} and control mice. To measure $[Ca^{2+}]_i$, cells were stained with Fura-2. The percentage of cells responding to the stimulus is shown. Mann-Whitney test, **P < 0.01, n = 7 per group. (**C**) mRNA levels of *Gpr68* in PFBECs, vessel fragments, and whole-brain lysates of wild-type mice. 1-way-ANOVA: F(2,7) = 223.5, P < 0.0001. ***P < 0.001 between indicated groups (Bonferroni post-test). n = 3-4 per group. (**D**) mRNA levels of *Gpr4* in PFBECs, vessel fragments, and whole-brain lysates of wild-type mice. 1-way-ANOVA: F(2,7) = 31.71, P < 0.0003; **P < 0.01, ***P < 0.001 between indicated groups (Bonferroni post-test). n = 3-4 per group. (**E**) *In situ* hybridization of *Gpr4* mRNA shows coexpression with the endothelial cell marker *Pecam* and colocalization with the basement membrane protein collagen IV in most cases. Exemplary vascular *Gpr4* expression is indicated by white arrows. Blue arrows indicate *Gpr4*-positive cells that are not colocalized with

vessels. Scale bar, 50 µm. (**F**) *In situ* hybridization of *Gpr68* mRNA shows colocalization with the smooth muscle cell marker SMA (smooth muscle actin). Exemplary vascular *Gpr68* expression is indicated by white arrows. The blue arrow indicates *Gpr68*-positive cells that are not colocalized with vessels. Scale bar, 20 µm. (**G**) Representative images and quantification of CD31 staining to visualize vessel length in the cortex of $Gpr4^{+/+}$ and $Gpr4^{-/-}$ mice. n = 6-8 animals per group. (**H**) Quantification of vessel diameter in the cortex of $Gpr4^{+/+}$ and $Gpr4^{-/-}$ mice. n = 6-8 animals per group. Data are means ± SEM.

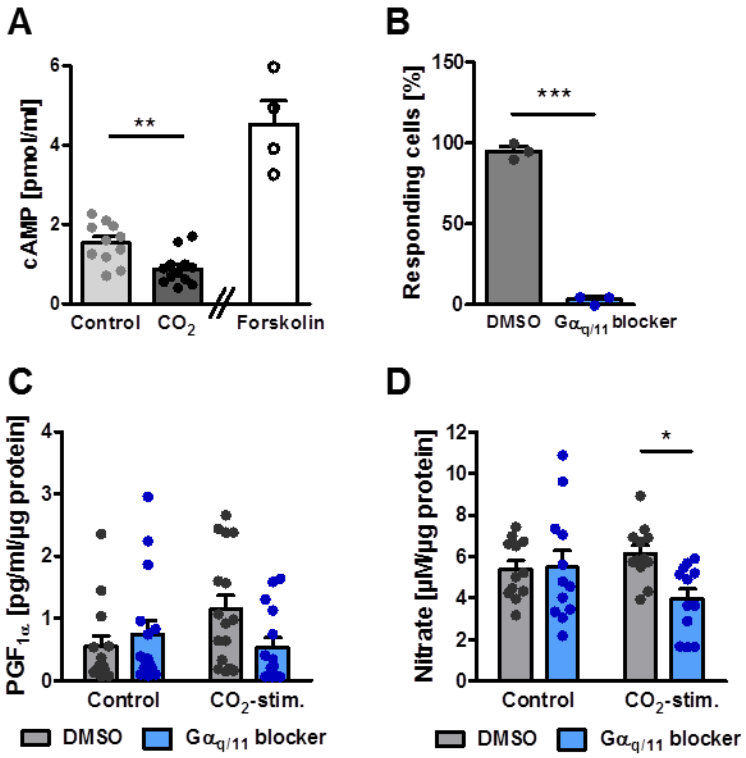

Figure S2. (**A**) Cyclic AMP concentrations in PFBECs after 30-min stimulation with 15 % CO_2 or 1 µM forskolin. Student's *t*-test, **P < 0.01, n = 11-12 per group. (**B**) Effect of ATP (30 µM) on $[Ca^{2+}]_i$ in PFBECs either pretreated with DMSO or 10 µM of the specific $G\alpha_{q/11}$ inhibitor FR900359. To measure $[Ca^{2+}]_i$, cells were stained with Fura-2. The percentage of cells responding to the stimulus is shown. Student's *t*-test, ***P < 0.001, n = 3 per group. (**C**) $PGF_{1\alpha}$ as a surrogate for prostacyclin release of PFBECs either pretreated with vehicle (DMSO) or the specific $G\alpha_{q/11}$ inhibitor FR900359 (10 µM) before exposure to 5 % (control) or 15 % CO_2, 2-way-ANOVA: CO_2 concentration: $F(1,57) = 1.104$, $P = 0.298$; treatment: $F(1,57) = 0.949$, $P = 0.334$; interaction: $F(1,57) = 4.148$, $P = 0.046$; n = 14-16 per group. (**D**) Assessment of NO release by measurement of nitrate concentrations in the supernatant of PBECs after exposure to 15 % CO_2, either pretreated with vehicle (DMSO) or the specific $G\alpha_{q/11}$ inhibitor FR900359 (10 µM). 2-way-ANOVA: CO_2 concentration: $F(1,44) = 0.541$, $P = 0.466$; treatment: $F(1,44) = 3.779$, $P = 0.058$; interaction: $F(1,44) = 4.566$, $P = 0.038$; *P < 0.05 between indicated groups (Bonferroni post-test). n = 12 per group. Data are means ± SEM.

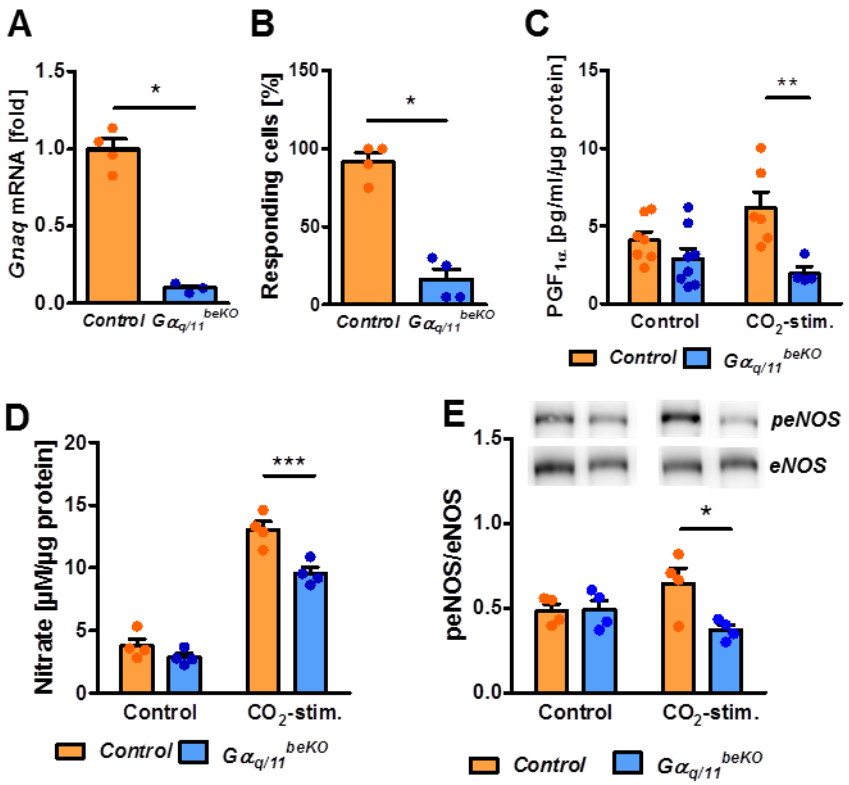

Figure S3. (**A**) mRNA levels of *Gnaq* in PFBECs derived from $G\alpha_{q/11}^{beKO}$ and control mice. Mann-Whitney test, *P < 0.05. n = 3-4 per group. (**B**) Effect of ATP (30 µM) on $[Ca^{2+}]_i$ in PFBECs derived from $G\alpha_{q/11}^{beKO}$ and control mice. To measure $[Ca^{2+}]_i$, cells were stained with Fura-2. The percentage of cells responding to the stimulus is shown. Mann-Whitney test, *P < 0.05, n = 4 per group. (**C**) $PGF_{1\alpha}$ as a surrogate for prostacyclin release of PFBECs of $G\alpha_{q/11}^{beKO}$ and control mice after 30-min stimulation with 5 % (control) or 15 % CO_2, 2-way-ANOVA: CO_2 concentration: F(1,21) = 0.592, P = 0.450; genotype: F(1,21) = 12.87, P = 0.002; interaction: F(1,21) = 4.003, P = 0.059; **P < 0.01 between indicated groups (Bonferroni post-test), n = 4-8 per group. (**D**) Assessment of NO release by measurement of nitrate concentrations in the supernatant of PFBECs of $G\alpha_{q/11}^{beKO}$ and control mice after 30-min stimulation with 5 % (control) or 15 % CO_2, 2-way-ANOVA: CO_2 concentration: F(1,12) = 243.7, P < 0.0001; genotype: F(1,12) = 18.68, P = 0.001; interaction: F(1,12) = 6.120, P = 0.029; ***P < 0.001 between indicated groups (Bonferroni post-test), n = 4 per group. (**E**) Analysis of western blots against phosphorylated and total eNOS protein amount of PFBECs of $G\alpha_{q/11}^{beKO}$ and control mice after 20-min stimulation with 5 % (control) or 15 % CO_2, 2-way-ANOVA: CO_2 concentration: F(1,12) = 0.137, P = 0.718; genotype: F(1,12) = 5.167, P = 0.042; interaction: F(1,12) = 5.694, P = 0.034; *P < 0.05 between indicated groups (Bonferroni post-test), n = 4 per group.

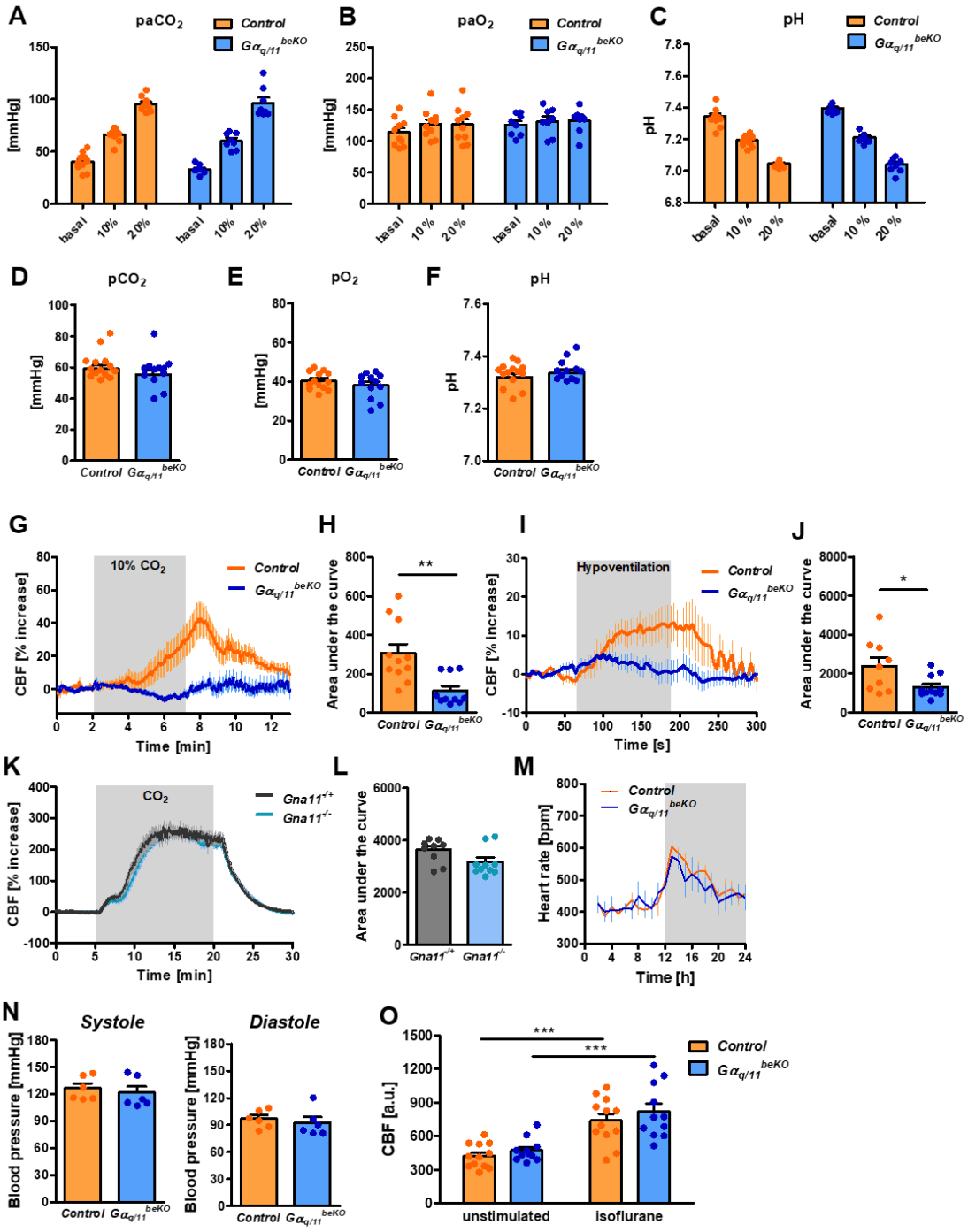

Figure S4. (**A-C**) Arterial blood gases determined under laser speckle imaging conditions after treating mice with different CO_2 concentrations. n = 8-10 mice per group. (**D-F**) Venous blood gases in awake $G\alpha_{q/11}^{beKO}$ and control mice. n = 12-13 animals per group. (**G**) Quantification of laser speckle imaging measuring cortical perfusion of $G\alpha_{q/11}^{beKO}$ and control mice during artificial ventilation with 10 % CO_2 for 5 min. (**H**) Areas under the curves shown in (G). Mann-Whitney test, **P < 0.05, n = 10-11 mice per group. (**I**) Quantification of laser speckle imaging of $G\alpha_{q/11}^{beKO}$ and control mice that were artificially hypoventilated for 120 seconds at half of their

optimal breathing frequency. (**J**) Areas under the curves shown in (I). Mann-Whitney test, *P < 0.05, n = 9-11 mice per group. (**K**) Quantification of laser speckle imaging of $G\alpha_{11}$ knockout and control mice during artificial ventilation with 20 % CO_2. (**L**) Areas under the curves shown in (K). n = 9-10 mice per group. (**M**) Circadian time course of heart rate determined by telemetry of $G\alpha_{q/11}^{beKO}$ and control mice. n = 6 animals per group. bpm, beats per min. (**N**) Systolic and diastolic blood pressure in $G\alpha_{q/11}^{beKO}$ and control mice determined by telemetry. n = 6 animals per group. (**O**) Cortical CBF of $G\alpha_{q/11}^{beKO}$ and control mice under unstimulated conditions and during stimulation with 2 % isoflurane. CBF was measured by laser speckle imaging and is expressed in arbitrary units. RM-ANOVA: genotype: $F(1,21) = 1.06$, $P = 0.314$; treatment: $F(1,21) = 64.78$, $P < 0.0001$; interaction: $F(1,21) = 0.115$, $P = 0.738$; ***P < 0.001 between indicated groups (Bonferroni post-test). n = 11-12 mice per group. Data are means ± SEM.

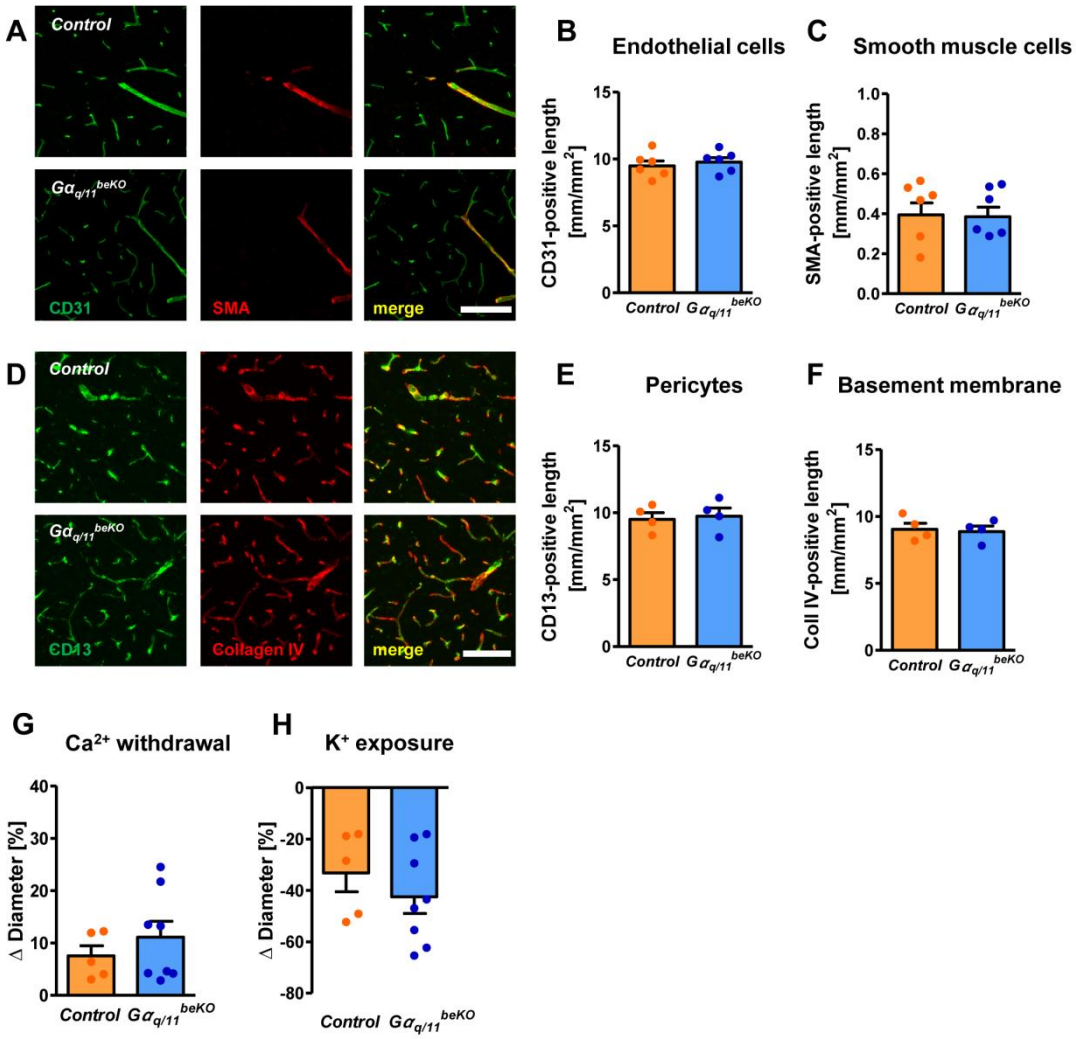

Figure S5. (**A**) Representative stainings of the endothelial cell marker CD31 and the smooth muscle cell marker SMA in cortical brain slices of $G\alpha_{q/11}^{beKO}$ and control mice. (**B**, **C**) Quantification of the images shown in (A). n = 6 mice per group. (**D**) Representative images of cortical brain slices of $G\alpha_{q/11}^{beKO}$ and control mice that were double-stained for the pericyte marker CD13 and the basement membrane protein collagen IV. (**E**, **F**) Quantification of the images shown in (D). n = 4 mice per group. (**G**) Diameter changes in arterioles in acute cortical brain slices of $G\alpha_{q/11}^{beKO}$ and control mice after Ca^{2+} withdrawal by applying a Ca^{2+}-free perfusion buffer containing the Ca^{2+} chelator EGTA (5 mM) and papaverine (200 µM). n = 5-8 animals per group. (**H**) Diameter changes in arterioles in acute cortical brain slices of $G\alpha_{q/11}^{beKO}$ and control mice after adding 60 mM K^+ to the perfusate. n = 5-8 animals per group. Data are means ± SEM.

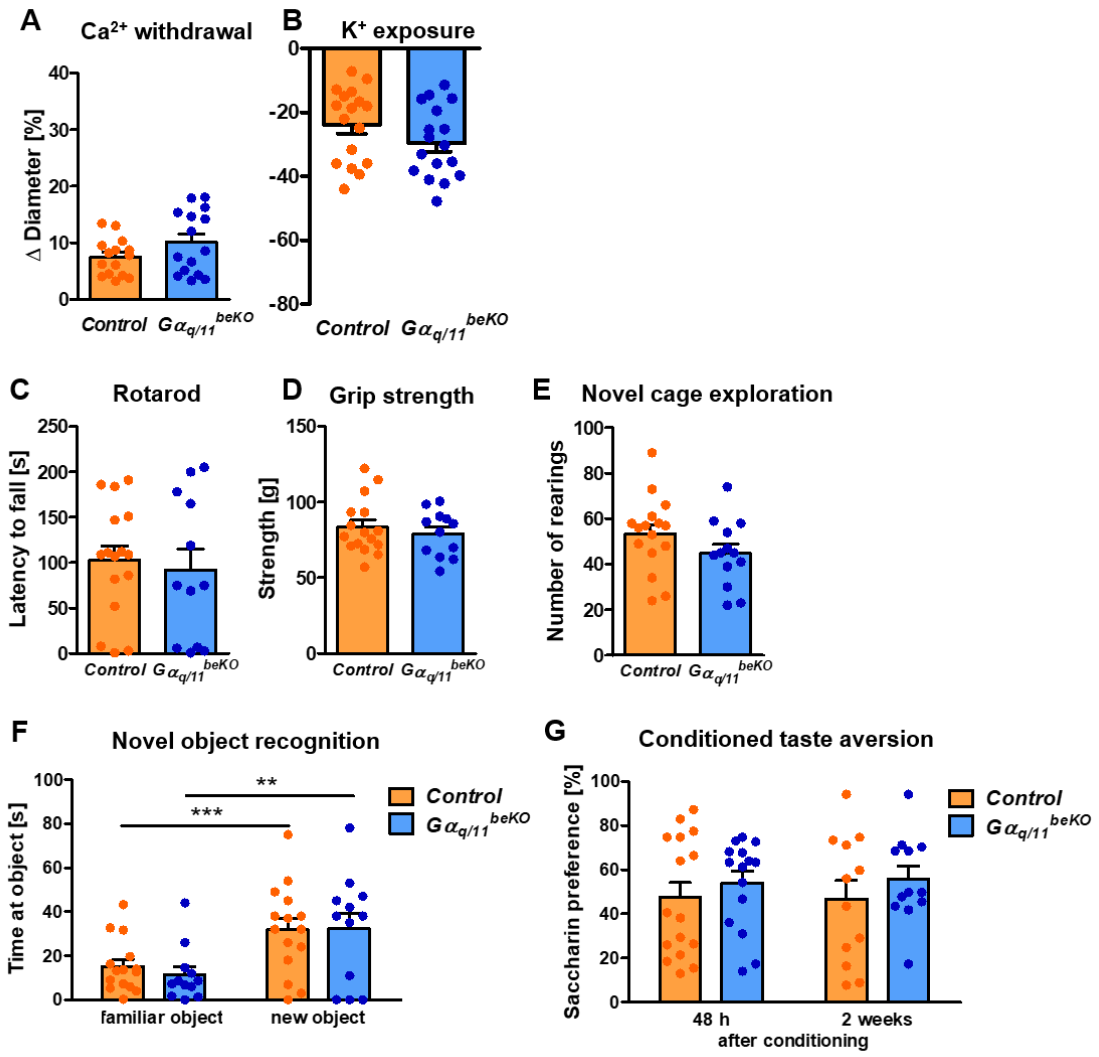

Figure S6. (**A**) Diameter changes in arterioles in acute amygdala slices of $G\alpha_{q/11}{}^{beKO}$ and control mice after Ca^{2+} withdrawal by applying a Ca^{2+}-free perfusion buffer containing the Ca^{2+} chelator EGTA (5 mM) and papaverine (200 µM). n = 17 animals per group. (**B**) Diameter changes in arterioles in acute amygdala slices of $G\alpha_{q/11}{}^{beKO}$ and control mice after adding 60 mM K^+ to the perfusate. n = 17 animals per group. (**C**) Latency to fall from a rotarod of $G\alpha_{q/11}{}^{beKO}$ and control mice. n = 12-16 animals per group. (**D**) Grip strength of $G\alpha_{q/11}{}^{beKO}$ and control mice. n = 12-16 animals per group. (**E**) Number of rearings in a novel cage environment of $G\alpha_{q/11}{}^{beKO}$ and control mice. n = 13-16 animals per group. (**F**) Time spent by $G\alpha_{q/11}{}^{beKO}$ and control mice at familiar objects and a new object. RM-ANOVA: object: $F(1,25) = 41.92$, $P < 0.0001$; genotype: $F(1,25) = 0.076$, $P = 0.785$; interaction: $F(1,25) = 0.540$, $P = 0.469$. n = 12-15 mice per group. (**G**) Saccharin preference assessed at two time points after inducing taste aversion by injecting LiCl in $G\alpha_{q/11}{}^{beKO}$ and control mice. 2-way-ANOVA: time: $F(1,51) = 0.072$, $P = 0.933$; genotype: $F(1,51) = 1.389$, $P = 0.244$; interaction: $F(1,51) = 0.035$, $P = 0.852$. **$P < 0.01$, ***$P < 0.001$ between indicated groups (Bonferroni post-test). n = 12-16 animals per group. Data are means ± SEM.

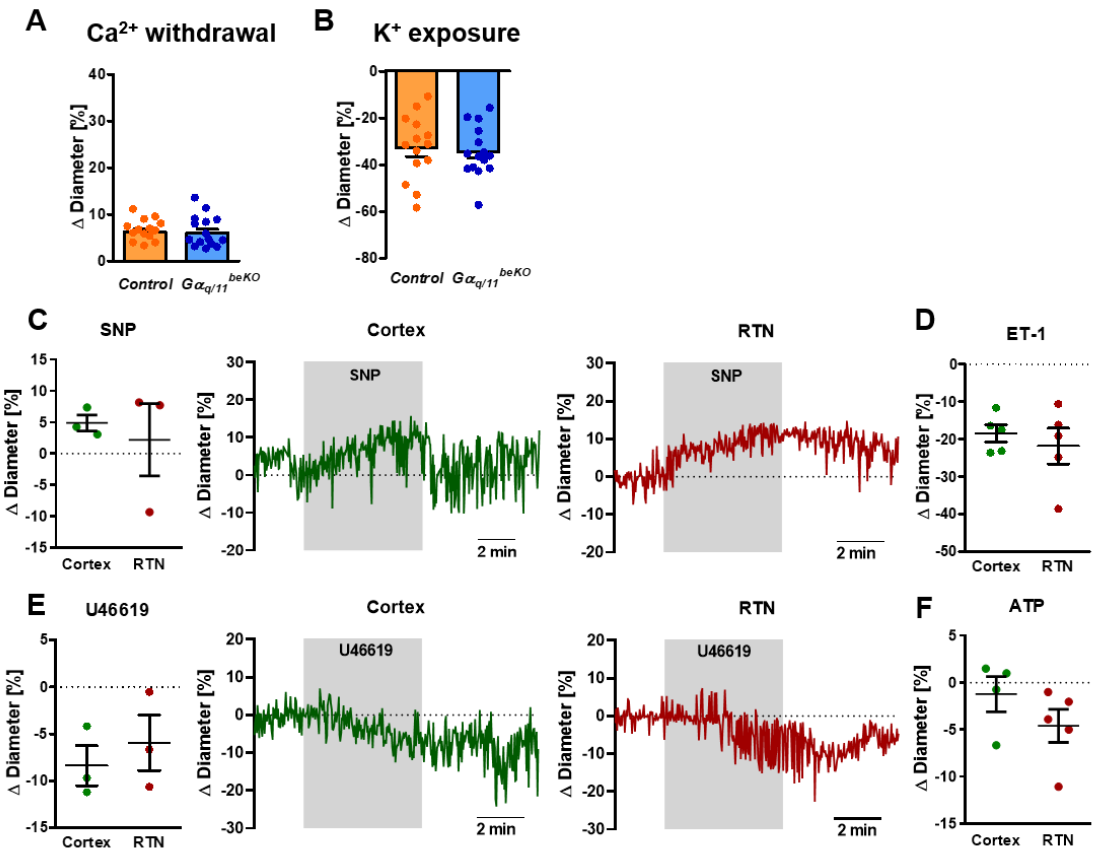

Figure S7. (**A**) Diameter changes in arterioles in acute RTN slices of $G\alpha_{q/11}{}^{beKO}$ and control mice after Ca^{2+} withdrawal by applying a Ca^{2+}-free perfusion buffer containing the Ca^{2+} chelator EGTA (5 mM) and papaverine (200 µM). n = 14-16 animals per group. (**B**) Diameter changes in arterioles in acute RTN slices of $G\alpha_{q/11}{}^{beKO}$ and control mice after adding 60 mM K^+. n = 14-16 animals per group. (**C**) Representative traces of diameter measurements in acute cortical and RTN brain slices of C57BL/6 mice during stimulation with 1 µM sodium nitroprusside (SNP) and quantification thereof (1 arteriole per animal, mean of 3 different sites of each vessel, n = 3 mice per group). (**D**) Quantification of diameter measurements in acute cortical and RTN brain slices of C57BL/6 mice during stimulation with 100 nM endothelin-1 (ET-1; 1 arteriole per animal, mean of 3 different sites of each vessel, n = 3 mice per group). (**E**) Representative traces of diameter measurements in acute cortical and RTN brain slices of C57BL/6 mice during stimulation with 1 µM U46619 and quantification thereof (1 arteriole per animal, mean of 3 different sites of each vessel, n = 3 mice per group). (**F**) Quantification of diameter measurements in acute cortical and RTN brain slices of C57BL/6 mice during stimulation with 1 mM ATP (1 arteriole per animal, mean of 3 different sites of each vessel, n = 3 mice per group). Data are means ± SEM.

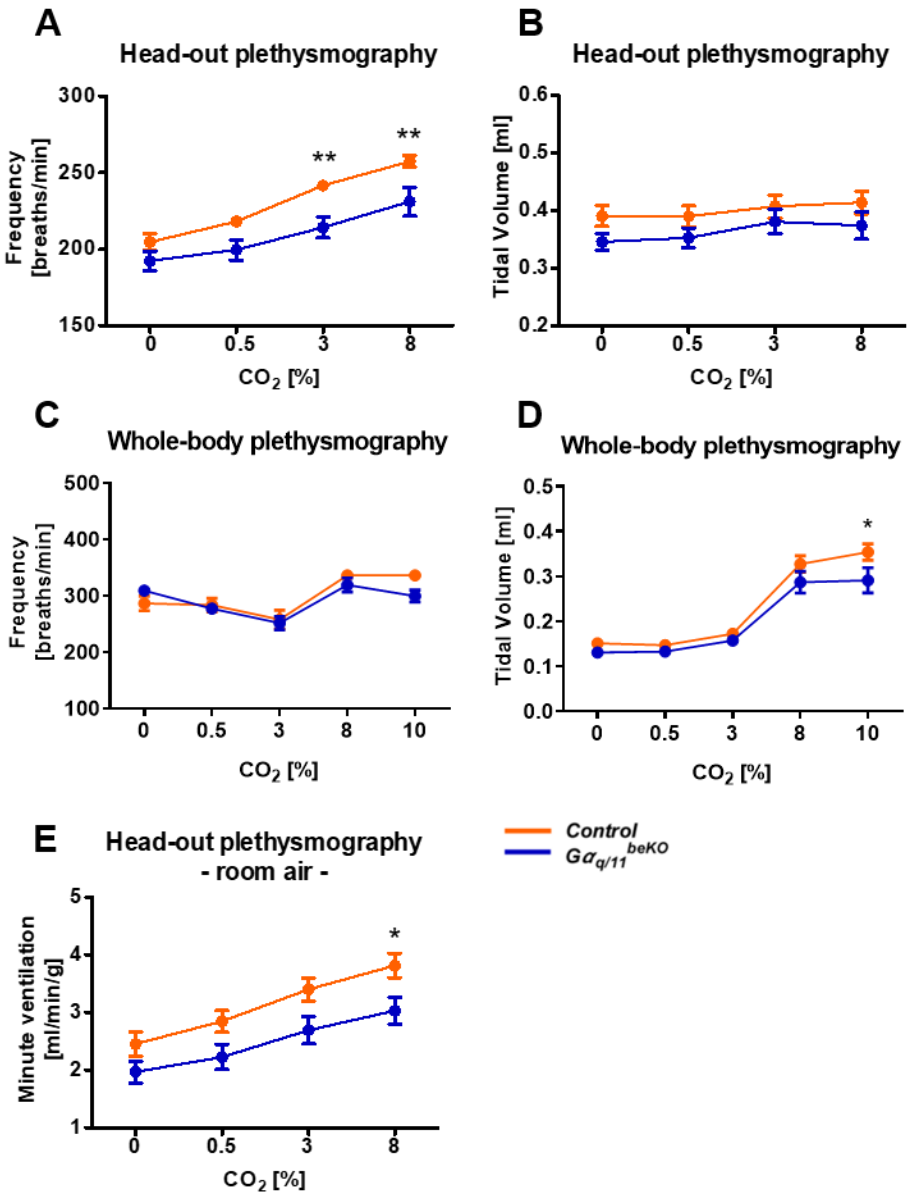

Figure S8. (**A**) Respiratory frequency of $G\alpha_{q/11}^{beKO}$ and control mice exposed to different CO_2 concentrations, as recorded by head-out plethysmography. **P < 0.01 (RM-ANOVA with Bonferroni post-test). n = 16 mice per group. (**B**) Respiratory tidal volume of $G\alpha_{q/11}^{beKO}$ and control mice exposed to different CO_2 concentrations, as recorded by head-out plethysmography. n = 16 mice per group. (**C**) Respiratory frequency of $G\alpha_{q/11}^{beKO}$ and control mice exposed to different CO_2 concentrations, as recorded by whole-body plethysmography. n = 7-9 mice per group. (**D**) Respiratory tidal volume of $G\alpha_{q/11}^{beKO}$ and control mice exposed to different CO_2 concentrations, as recorded by whole-body plethysmography. n = 7-9 mice per group. *P < 0.05 (RM-ANOVA with Bonferroni post-test). (**E**) Minute ventilation of $G\alpha_{q/11}^{beKO}$ and control mice exposed to different CO_2 concentrations, as recorded by head-out plethysmography in room air. *P < 0.05 (RM-ANOVA with Bonferroni post-test). n = 16 mice per group. Data are means ± SEM.

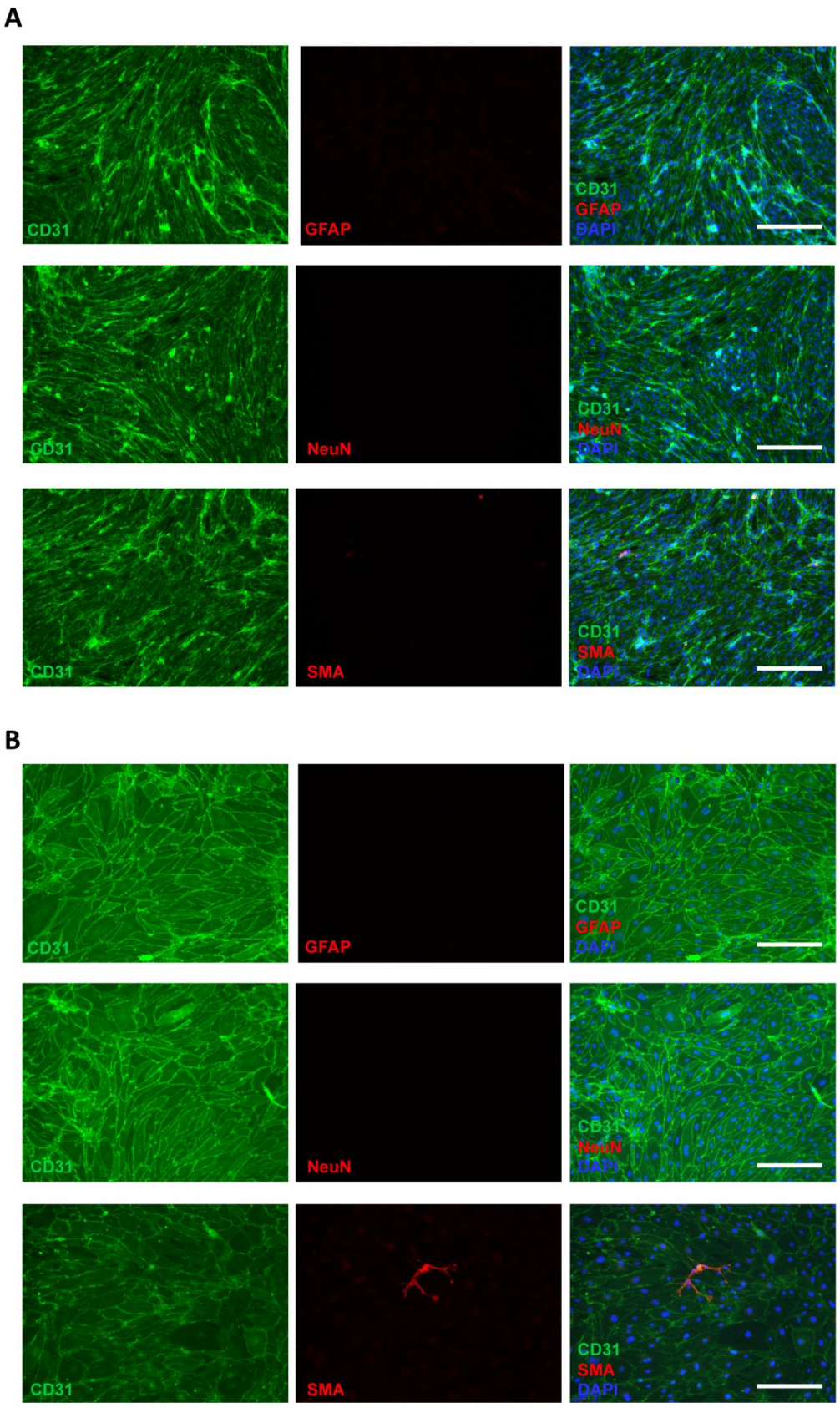
A
CD31
GFAP
CD31
GFAP
DAPI
CD31
NeuN
CD31
NeuN
DAPI
CD31
SMA
CD31
SMA
DAPI
B
CD31
GFAP
CD31
GFAP
DAPI
CD31
NeuN
CD31
NeuN
DAPI
CD31
SMA
CD31
SMA
DAPI

Figure S9. (**A**-**B**) Immunohistochemical stainings against the endothelial cell marker CD31, the astrocyte marker GFAP, the neuronal marker NeuN, and the pericyte/smooth muscle cell marker SMA showed an almost pure culture of endothelial cells after isolation from the subcortical telencephalon (**A**) or the brainstem (**B**).

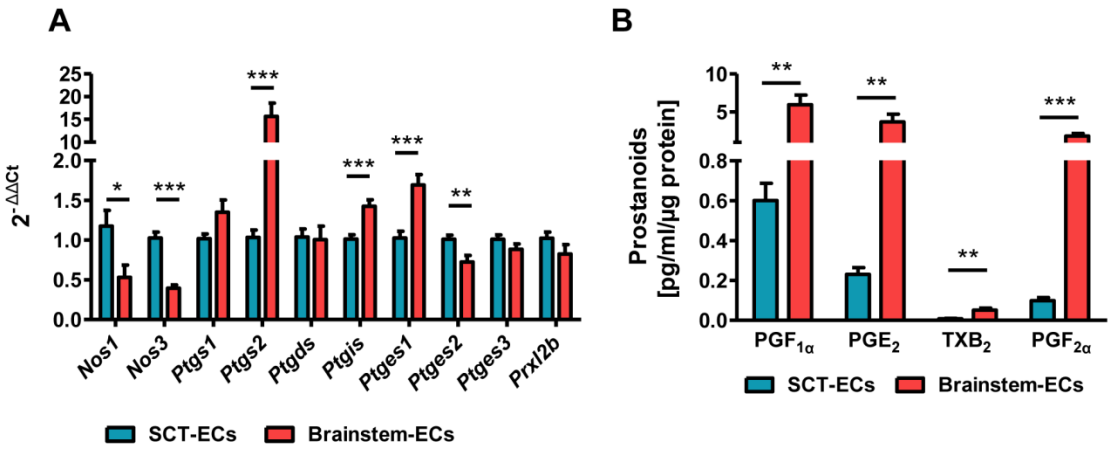

Figure S10. (**A**) Quantitative PCR measurements of mRNA expression related to nitric oxide or prostanoids measured in primary endothelial cells of the subcortical telencephalon (SCT-ECs) or the brainstem (Brainstem-ECs). *P < 0.05, **P < 0.01, ***P < 0.001, Student´s t-test. n = 10 per group, two independent experiments. (**B**) Assessment of protein amount-normalized release of different prostanoids by untreated SCT-ECs and Brainstem-ECs. These values correspond to the control values of Fig. 5C-5F. **P < 0.01, ***P < 0.001, Student´s t-test. n = 5-6 per group, two independent experiments.

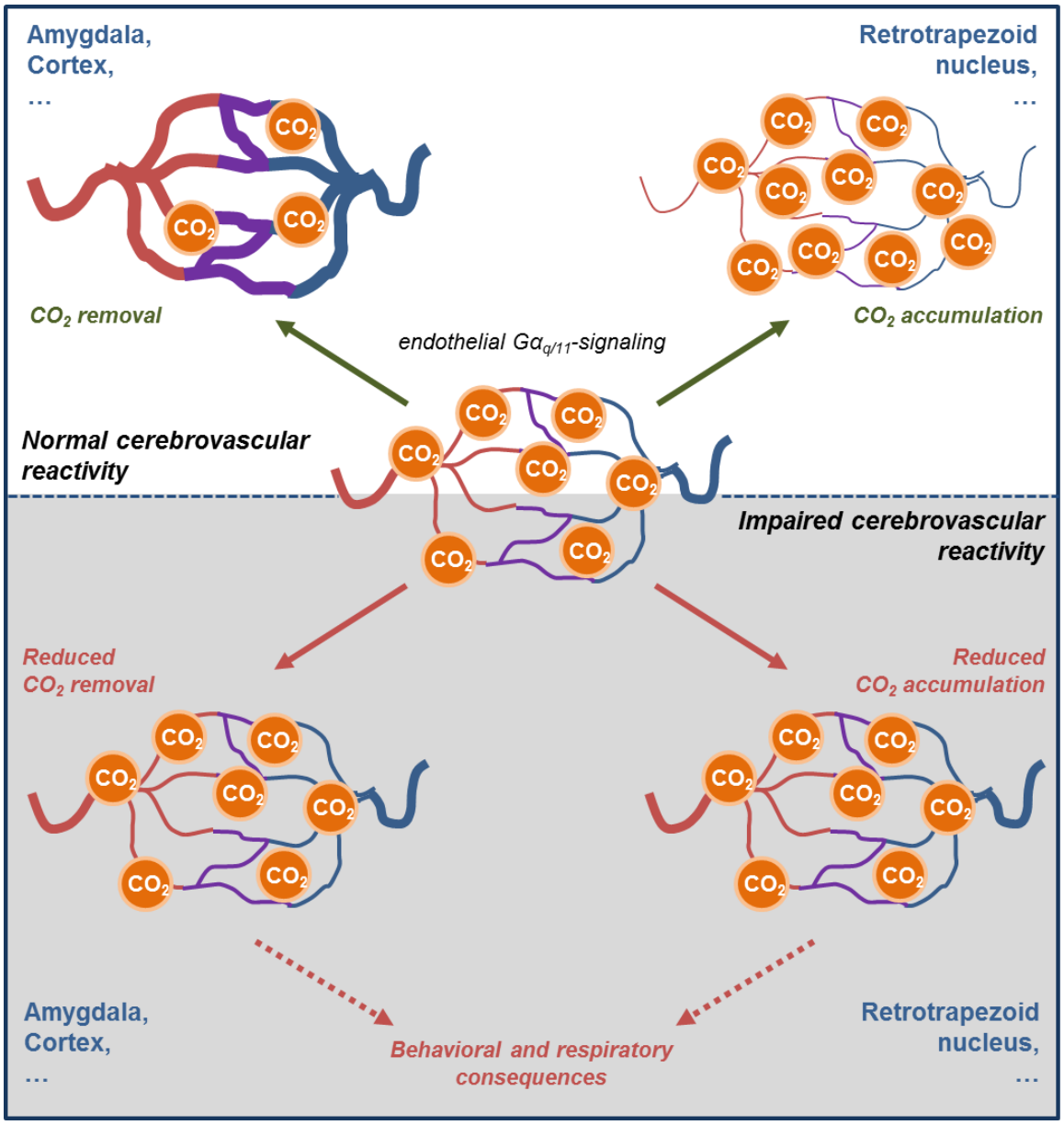
Amygdala,
Cortex,
…
Retrotrapezoid
nucleus,
…
CO2 removal
CO2 accumulation
endothelial Gαq/11-signaling
Normal cerebrovascular
reactivity
Impaired cerebrovascular
reactivity
Reduced
CO2 removal
Reduced
CO2 accumulation
Amygdala,
Cortex,
…
Behavioral and respiratory
consequences
Retrotrapezoid
nucleus,
…

Figure S11. Mechanisms of how an impaired cerebrovascular reactivity influences behavior and respiration. In most brain areas, including cortex and amygdala, healthy vessels dilate in response to an increased CO_2 concentration (upper part, left). This effect is dependent on endothelial $G\alpha_{q/11}$ signaling and leads to a washout of the increased CO_2 from the respective brain area. In contrast, vessels of the RTN region of the brainstem constrict upon CO_2 exposure leading to an increase in local CO_2 concentrations (upper part, right) and thereby to the activation of chemosensitive respiratory centers. With an impaired cerebrovascular reactivity, as shown for $G\alpha_{q/11}^{beKO}$ mice, CO_2 does not dilate vessels in the amygdala or cortex. Therefore, CO_2 is not removed properly from those areas (lower part, left). Also, vessels of the RTN do not respond to CO_2 in case of an impaired cerebrovascular reactivity preventing the accumulation of CO_2 in the RTN that is expected with normal vascular reactivity (lower part, right). According to our concept, endothelial dysfunction and the associated loss of vascular CO_2 reactivity induce changes in CO_2-sensitive brain areas that have respiratory and behavioral functions.

Table S1

Expression profile of vessel fragments of different brain regions

	Gene	Cortex [Signal log_2]	Brainstem [Signal log_2]	p-value
Endothelial cell marker	*Abcb1a*	11.43±0.09	11.12±0.13	p > 0.05
	Cldn5	13.04±0.07	12.86±0.13	p > 0.05
	Flt1	12.33±0.08	12.07±0.17	p > 0.05
	Ocln	10.49±0.04	10.31±0.07	p > 0.05
	Pecam1	11.70±0.08	11.45±0.09	p > 0.05
	Slco1c1	12.60±0.04	12.40±0.12	p > 0.05
	Tek	11.16±0.11	10.70±0.21	p > 0.05
	Tie1	11.22±0.10	10.93±0.16	p > 0.05
Pericyte/SMC marker	*Acta2*	10.34±0.11	9.45±0.21	**p < 0.05**
	Anpep	9.36±0.11	9.00±0.11	p > 0.05
	Cspg4	8.34±0.06	8.09±0.17	p > 0.05
	Des	8.79±0.03	8.19±0.11	**p < 0.05**
	Kcnj8	9.08±0.11	9.51±0.12	p > 0.05
	Mcam	9.98±0.09	9.59±0.15	p > 0.05
	Pdgfrb	11.28±0.09	11.05±0.20	p > 0.05
Astrocyte marker	*Aldh1l1*	7.38±0.09	7.79±0.10	p > 0.05
	Aqp4	7.30±0.10	8.13±0.18	**p < 0.05**
	Gfap	8.06±0.14	9.12±0.11	**p < 0.05**
	Gjb6	7.21±0.10	7.08±0.07	p > 0.05
	S100b	8.03±0.21	9.06±0.13	**p < 0.05**
	Slc1a2	8.61±0.09	7.99±0.09	**p < 0.05**
	Slc1a3	9.67±0.10	8.82±0.11	**p < 0.05**
Nitric oxide	*Nos1*	7.79±0.07	7.39±0.04	p > 0.05
	Nos3	10.15±0.10	9.60±0.24	**p < 0.05**
	Gucy1a2	8.20±0.07	8.01±0.08	p > 0.05
	Gucy1a3	9.02±0.07	8.83±0.09	p > 0.05
	Gucy1b3	8.98±0.07	8.70±0.10	p > 0.05
Prostanoids	*Ptgs1*	7.33±0.06	6.81±0.07	**p < 0.05**
	Ptgs2	6.02±0.09	5.87±0.07	p > 0.05
	Ptgds	12.53±0.05	12.20±0.05	**p < 0.05**
	Ptgis	9.31±0.08	8.95±0.11	**p < 0.05**
	Ptges	7.51±0.09	7.43±0.02	p > 0.05
	Ptges2	6.84±0.12	7.13±0.07	p > 0.05
	Ptges3	10.05±0.03	10.14±0.08	p > 0.05
	Ptgir	6.32±0.07	6.23±0.06	p > 0.05
	Ptgfr	6.93±0.07	6.62±0.04	**p < 0.05**
	Tbxa2r	8.28±0.08	8.09±0.09	p > 0.05
	Ptgdr2	5.86±0.05	6.02±0.05	p > 0.05
	Ptger1	7.50±0.10	7.25±0.09	p > 0.05
	Ptger3	6.07±0.06	5.80±0.06	p > 0.05
	Ptger4	5.98±0.07	5.97±0.05	p > 0.05
Purinergic	*Entpd1*	9.64±0.08	9.17±0.12	**p < 0.05**
	Nt5e	6.47±0.06	6.83±0.13	p > 0.05
	Adora1	8.33±0.06	8.36±0.12	p > 0.05

	Adora2a	8.65±0.11	8.49±0.17	p > 0.05
	Adora2b	7.57±0.06	7.99±0.09	**p < 0.05**
	P2ry1	6.87±0.07	7.03±0.11	p > 0.05
	P2ry2	6.79±0.09	6.45±0.08	p > 0.05
	P2ry6	7.18±0.06	6.91±0.09	p > 0.05
	P2ry14	9.45±0.14	9.78±0.22	p > 0.05
	P2rx1	6.08±0.07	5.84±0.06	p > 0.05
	P2rx4	8.87±0.15	8.63±0.18	p > 0.05
	P2rx7	8.19±0.30	7.91±0.29	p > 0.05
Acid-base metabolism	*Car2*	8.64±0.06	9.37±0.08	**p < 0.05**
	Car4	12.21±0.03	12.15±0.06	p > 0.05
	Car5a	7.18±0.09	7.06±0.07	p > 0.05
	Car5b	7.53±0.07	7.70±0.07	p > 0.05
	Car7	7.49±0.04	7.15±0.05	p > 0.05
	Car8	6.41±0.11	6.43±0.16	p > 0.05
	Car10	8.00±0.15	8.18±0.04	p > 0.05
	Car11	9.80±0.05	9.43±0.12	p > 0.05
	Car14	8.74±0.12	9.51±0.12	**p < 0.05**
	Slc9a1	8.84±0.10	8.74±0.14	p > 0.05
	Slc9a2	7.72±0.08	7.59±0.12	p > 0.05
	Slc9a3	5.27±0.06	5.36±0.08	p > 0.05
	Slc9a4	5.65±0.07	5.72±0.07	p > 0.05
	Slc9a5	7.32±0.05	7.27±0.06	p > 0.05
	Slc4a3	7.97±0.10	7.70±0.14	p > 0.05
	Slc4a4	9.41±0.10	9.89±0.08	**p < 0.05**
	Slc4a8	7.36±0.06	7.45±0.11	p > 0.05

Table S1. Microarray expression values of vessel fragments derived from cortex and brainstem. Shown is the mRNA expression of typical marker genes for endothelial cells, pericytes/smooth muscle cells (SMCs), or astrocytes. The latter recapitulate known regional variations in astrocytic gene expression. Additionally, gene expression of nitric oxide (NO)-related, prostanoid-related, purinergic system-related, or acid-base metabolism-related genes is shown. The main extracellular ectonucleotidase *Entpd1* that converts extracellular ATP into adenosine was lower in the brainstem than in the cortex. The two carbonic anhydrases (*Car2* and *Car14*) as well as the bicarbonate transporter *Slc4a4* are higher expressed in the brainstem than in the cortex indicating a more efficient handling of CO_2 and its derivatives in the brainstem that is presumably exposed to high CO_2. levels.Student's *t*-test, $p < 0.05$ is considered significant and highlighted in bold. n = 7 mice per group. Data are means ± SEM.

Table S2

Vascular expression of control and $G\alpha_{q/11}^{beKO}$ mice in different brain regions

	Gene	Control [Signal log_2]	$G\alpha_{q/11}^{beKO}$ [Signal log_2]	p-value
Cortex	*Nos3*	10.15±0.10	10.25±0.09	p > 0.05
	Ptgs1	7.33±0.06	7.12±0.09	p > 0.05
	Ptgds	12.53±0.05	12.62±0.06	p > 0.05
	Ptgis	9.31±0.08	9.23±0.13	p > 0.05
	Ptgfr	6.93±0.07	6.81±0.07	p > 0.05
	Entpd1	9.64±0.08	9.69±0.06	p > 0.05
	Adora2b	7.57±0.06	7.45±0.06	p > 0.05
	Car2	8.64±0.06	8.47±0.07	p > 0.05
	Car14	8.74±0.12	8.99±0.04	p > 0.05
	Slc4a4	9.41±0.10	9.20±0.05	p > 0.05
Brainstem	*Nos3*	9.60±0.24	9.62±0.23	p > 0.05
	Ptgs1	6.81±0.07	6.87±0.13	p > 0.05
	Ptgds	12.20±0.05	12.11±0.09	p > 0.05
	Ptgis	8.95±0.11	9.11±0.11	p > 0.05
	Ptgfr	6.62±0.04	6.30±0.05	p > 0.05
	Entpd1	9.17±0.12	9.10±0.10	p > 0.05
	Adora2b	7.99±0.09	7.73±0.05	p > 0.05
	Car2	9.37±0.08	9.14±0.11	p > 0.05
	Car14	9.51±0.12	9.45±0.13	p > 0.05
	Slc4a4	9.89±0.08	9.97±0.06	p > 0.05

Table S2. Microarray expression values of vessel fragments derived from control and $G\alpha_{q/11}^{beKO}$ mice of cortex and brainstem. Shown is the mRNA expression of genes that differed in their expression between brainstem and cortical vessel fragments (see Table S1). n = 7 mice per group. Data are means ± SEM.

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