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


Institute for Experimental and Clinical Pharmacology and Toxicology, University of Lübeck, 23562 Lübeck, Germany; DZHK (German Research Centre for Cardiovascular Research), partner site Hamburg/Lübeck/Kiel, 23562 Lübeck, Germany; Institute of Physiology, University of Zürich, CH-8057 Zürich, Switzerland; Central Institute of Mental Health, Medical Faculty of Mannheim/University of Heidelberg, 68159 Mannheim, Germany; Institute of Physiology, University of Zürich, 23526 Lübeck, Germany; Cardiovascular and Metabolic Sciences, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, 13125 Berlin, Germany; DZHK (German Research Centre for Cardiovascular Research), partner site Berlin, 13125 Berlin, Germany; Department of Diagnostic and Interventional Neuroradiology, University Medical Center Hamburg-Eppendorf, 20251 Hamburg, Germany; Department of Neurology, University Medical Center Hamburg-Eppendorf, 20251 Hamburg, Germany; Priority Area Asthma and Allergy, Research Center Borstel, 23045 Borstel, Germany; Institute for Pharmaceutical Biology, University of Bonn, 53115 Bonn, Germany; Charité Universitätsmedizin Berlin, 10117 Berlin; and Department of Pharmacology, Max Planck Institute for Heart and Lung Research, 61231 Bad Nauheim, Germany

Carbon dioxide (CO₂), the major product of metabolism, has a strong impact on cerebral blood vessels, a phenomenon known as cerebrovascular reactivity. Several vascular risk factors such as hypertension or diabetes dampen this response, making cerebrovascular reactivity a useful diagnostic marker for various vascular pathologies, but its functional relevance, if any, is still unclear. Here, we found that GPR4, an endothelial H+ receptor, and endothelial Gαq/11 proteins mediate the CO₂/H+ effect on cerebrovascular reactivity in mice. CO₂/H+ leads to constriction of vessels in the brainstem area that controls respiration. The consequential washout of CO₂, if cerebrovascular reactivity is impaired, reduces respiration. In contrast, CO₂ dilates vessels in other brain areas such as the amygdala. Hence, an impaired cerebrovascular reactivity amplifies the CO₂ effect on anxiety. Even at atmospheric CO₂ concentrations, impaired cerebrovascular reactivity caused longer apneic episodes and more anxiety, indicating that cerebrovascular reactivity is essential for normal brain function. The site-specific reactivity of vessels to CO₂ is reflected by regional differences in their gene expression and the release of vasoactive factors from endothelial cells. Our data suggest the central nervous system (CNS) endothelium as a target to treat anxiety and affective disorders associated with vascular diseases.

Endothelial dysfunction | Brain endothelial cells | Hypercapnia | Respiration | Anxiety

Cerebral blood flow (CBF) supplies energy substrates to the brain and removes metabolic products. Therefore, CBF is tightly controlled (1, 2). Since the 19th century, it has been known that carbon dioxide (CO₂)H+ is one of the strongest stimuli for increasing brain perfusion (3–5). While it may seem plausible that as the major product of metabolism CO₂ increases CBF to enhance its removal and replenish nutrients, a physiological function of cerebrovascular reactivity to CO₂ has never been proven experimentally. Also, the molecular mechanisms underlying cerebrovascular reactivity are still debated. This lack of knowledge is surprising because many patients suffering from neurological, cardiovascular, and metabolic diseases show major alterations in cerebrovascular reactivity. In fact, cerebrovascular reactivity is routinely monitored as a diagnostic marker to detect early stages of vascular pathology (6). In the context of the multifaceted conditions associated with vascular risk factors, it has been difficult to delineate the functional consequences of impaired cerebrovascular reactivity. Therefore, we have investigated the mechanisms underlying CO₂-induced perfusion changes in the brain. Based on the obtained knowledge, we were able to selectively interfere with cerebrovascular reactivity and unexpectedly found that its integrity is required for the regulation of respiration and emotional behavior.

Results

CO₂-Induced CBF Response Depends Partially on GPR4. As an important mediator of the neuronal response to CO₂H+ ATP is released by erythrocytes (7) and parenchymal cells (8). ATP regulates the diameter of cerebral arterioles by acting on purinergic P₂Y receptors, including P₂Y₂, in endothelial and smooth muscle cells of the brain (9–11). Therefore, we examined the effect of CO₂ to cerebrovascular reactivity. Thus, we have investigated the mechanisms underlying CO₂-induced perfusion changes in the brain. Based on the obtained knowledge, we were able to selectively interfere with cerebrovascular reactivity and unexpectedly found that its integrity is required for the regulation of respiration and emotional behavior.

Significance

The ability of blood vessels to respond to endogenous and exogenous stimuli is of high importance. Several diseases lead to an impairment of vascular reactivity, especially in the brain. Here, we show that the functional consequences of impaired cerebrovascular reactivity differ between brain areas and depend on whether vessels constrict or dilate as a response to CO₂. A loss of vascular reactivity to carbon dioxide induces anxiety and changes respiration, even at a basal state. Area-specific vascular responses can be explained by characteristic gene expression patterns and release of vasoactive mediators.


The authors declare no competing interest.

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Data deposition: Microarray data have been deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress) under accession no. E-MTAB-8523.

1 To whom correspondence may be addressed. Email: jan.wenzel@uni-luebeck.de or markus.schwaninger@uni-luebeck.de.

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deleting brain endothelial P₂Y₂ on CO₂-induced cerebral perfusion. We generated a mouse line that carries the brain endothelial-specific cre driver Slco1c1-CreERT² (12) combined with a loxP-flanked P₂Y₂ gene (13) to delete this receptor selectively in the brain endothelium (P₂y2⁻/⁻KO mice) and confirmed the knockout by using mRNA quantification and calcium imaging in primary forebrain endothelial cells (PFBECs) (14). To investigate the effect of CO₂ on CBF, mice were artificially ventilated with normal air or a gas mix containing increased CO₂ concentrations without changing oxygen levels. CBF was measured by laser speckle imaging. CO₂ induced a similar increase in cortical perfusion of control and P₂y2⁻/⁻ mice (Fig. 1 A and B). Thus, the endothelial P₂Y₂ receptor is not involved in increasing cortical blood flow upon CO₂ exposure. Therefore, we tested the alternative hypothesis that CO₂/H⁺ could be directly sensed by specific receptor proteins in the brain vasculature. In buffered biological systems, CO₂ is rapidly converted into protons and bicarbonate. Protons mediate most of the physiological effects of CO₂, such as respiratory stimulation (15) or fear responses (16). In addition, cerebral perfusion reacts to acidosis with strong vasodilation. Recently, several groups reported that some orphan G protein-coupled receptors (GPCRs) were activated by H⁺ in a narrow physiological range (17, 18). Among them, GPR4 and GPR68 are expressed in vessels (18, 19). To examine the role of H⁺ sensing by GPCRs during a CO₂-induced CBF increase, we investigated knockout mice for each of the receptors (15). GPR4 and GPR68 are increased CO₂ concentrations without changing oxygen levels. CBF was measured by laser speckle imaging. CO₂ induced a similar increase in cortical perfusion of control and P₂y2⁻/⁻ mice (Fig. 1 A and B). Thus, the endothelial P₂Y₂ receptor is not involved in increasing cortical blood flow upon CO₂ exposure. Therefore, we tested the alternative hypothesis that CO₂/H⁺ could be directly sensed by specific receptor proteins in the brain vasculature. In buffered biological systems, CO₂ is rapidly converted into protons and bicarbonate. Protons mediate most of the physiological effects of CO₂, such as respiratory stimulation (15) or fear responses (16). In addition, cerebral perfusion reacts to acidosis with strong vasodilation. Recently, several groups reported that some orphan G protein-coupled receptors (GPCRs) were activated by H⁺ in a narrow physiological range (17, 18). Among them, GPR4 and GPR68 are expressed in vessels (18, 19). To examine the role of H⁺ sensing by GPCRs during a CO₂-induced CBF increase, we investigated knockout mice for each of the receptors (15). GPR4 and GPR68 are expressed in vessels (18, 19). To examine the role of H⁺ sensing by GPCRs during a CO₂-induced CBF increase, we investigated knockout mice for each of the receptors (15). GPR4 and GPR68 are expressed in vessels (18, 19). To examine the role of H⁺ sensing by GPCRs during a CO₂-induced CBF increase, we investigated knockout mice for each of the receptors (15). GPR4 and GPR68 are expressed in vessels (18, 19). To examine the role of H⁺ sensing by GPCRs during a CO₂-induced CBF increase, we investigated knockout mice for each of the receptors (15).
knockout decreased CO2-induced blood flow stimulation (Fig. 1 C and D), whereas we found no effect of the deletion of GPR68 on this response (Fig. 1 E and F). In the periphery, GPR68 is mainly expressed in smooth muscle cells while GPR4 has been mostly detected in endothelial cells, also in the brain (18–20).

In line with the described localization, we detected GPR68 expression in brain vessel fragments containing smooth muscle cells, pericytes, and endothelial cells, but not in PFBECs (SI Appendix, Fig. S1C), whereas GPR4 was enriched in both vessel fragments and PFBECs compared to whole-brain lysate (SI Appendix, Fig. S1D). In situ hybridization confirmed that Gpr68 mRNA is colocalized with smooth muscle cells in vessels of the brain (SI Appendix, Fig. S1F), whereas Gpr4 mRNA is colocalized mainly with endothelial cells in brain tissue (Fig. 1G and SI Appendix, Fig. S1E). In contrast to previous reports on another GPR4 knockout mouse line (21), we did not observe any morphological changes of the brain microvasculature (SI Appendix, Fig. S1 G and H).

The involvement of GPR4 implies that increased CO2/H+ concentrations are sensed by endothelial rather than by smooth muscle cells. To characterize the endothelial signaling pathway, we stimulated PFBECs with increased CO2 in a physiological, bicarbonate-buffered solution, lowering the pH to ~7.0. We measured PGF2α and nitrate as stable products of the endothelial-derived vasodilating mediators prostacyclin and nitric oxide (NO) and found both to be increased upon CO2 stimulation (Fig. 1 H and I). Prostacyclin release but not NO release was dependent on GPR4, indicating more than one mechanism by which CO2 induces vasodilation via endothelial cells. GPR4 is able to induce GO− and cAMP, as well as Gq/11-mediated signaling pathways (18, 22, 23). Unexpectedly, CO2 did not increase, but rather decreased cAMP production in PFBECs (SI Appendix, Fig. S2A), indicating another mechanism in the brain endothelium, such as coupling to Gq/11 pathways (23). Indeed, we observed a similar decrease in prostacyclin release when we treated PFBECs with a specific and effective inhibitor of Gq/11 signaling (SI Appendix, Fig. S2 B and C). Additionally, by blocking this pathway the NO release was reduced in PFBECs after stimulation with CO2 (24) (SI Appendix, Fig. S2D).

To verify the involvement of Gq/11 proteins, we used a strategy that was applied successfully in previous studies (13) and is based on the parallel deletion of Gna11 and Gnaq as the respective gene products are able to compensate for each other. We combined a Gna11 knockout, loxP-flanked Gnaq alleles (13), and the brain endothelial-specific Slo/1-CreER12 driver (12), leading to a tamoxifen-inducible knockout of Gq/11 in brain endothelial cells (Gq/11 beKO mice). We confirmed the deletion by quantifying Gnaq mRNA levels (SI Appendix, Fig. S3A) and intracellular Ca2+ concentrations in PFBECs in response to ATP (SI Appendix, Fig. S3B). As seen with the Gq/11 inhibitor, the increase of prostacyclin and NO release upon CO2 stimulation was clearly reduced in PFBECs of Gq/11 beKO mice compared to controls (SI Appendix, Fig. S3 C and D). Supporting the involvement of NO, the endothelial NO synthase (eNOS) was phosphorylated by CO2 and this activation was impaired in PFBECs of Gq/11 beKO animals (SI Appendix, Fig. S3E). Overall, the data suggest that CO2/H+ increase cortical CBF via the endothelial H+−sensitive receptor GPR4, intracellular Gq/11 proteins, and the release of prostacyclin and NO.

CO2-Induced CBF Response Depends on Endothelial Gq/11 Signaling.

To investigate the role of endothelial Gq/11 signaling in the CO2/H+−induced cerebrovascular response in vivo, we used a gas mix containing 10% or 20% CO2 to ventilate Gq/11 beKO mice. These stimuli profoundly increased arterial Pco2 and reduced arterial pH with no difference between genotypes (SI Appendix, Fig. S4 A–C). At a basal state, we did not find any changes in venous blood gases in Gq/11 beKO mice (SI Appendix, Fig. S4 D–F). The brain endothelial-specific Gq/11 deletion interfered with the CO2-induced CBF increase to an even greater extent than Gpr4 knockout (Fig. 2A–E). After a shorter stimulus of 10% CO2, there was almost no effect of CO2 on cortical perfusion in Gq/11 beKO mice (SI Appendix, Fig. S4 G and H). In addition, endogenously induced CO2/H+ elevation via hyperventilation increased CBF in control mice but less so in Gq/11 beKO animals (SI Appendix, Fig. S4 I and J). Even short apneic periods (3 s) markedly increased CBF in control mice but less so in Gq/11 beKO animals (Fig. 2 F–I). In contrast, the whole-genome knockout of only Gna11 did not affect cortical CO2-induced perfusion (SI Appendix, Fig. S4 K and L). As deleting Gq/11 in all endothelial cells of the body, using another cre mouse line, induced arterial hypertension (13)—which has been shown to be a confounding factor in CBF studies—we examined the blood pressure in Gq/11 beKO mice using telemetry. In contrast to the global endothelial deletion, the brain endothelial-specific deletion of Gq/11 in Gq/11 beKO mice did not affect blood pressure or heart rate (SI Appendix, Fig. S4 M and N).

Laser speckle imaging is not suitable for measuring absolute perfusion in tissues. Therefore, we used arterial spin labeling MRI to quantify brain perfusion. We did not detect a difference in brain perfusion between Gq/11 beKO and control mice (Fig. 2 J and K), which suggests that endothelial Gq/11 signaling may not play a role in the unstimulated cerebrovascular tone, at least during anesthesia. However, CO2 exposure again led to a prominent increase in cerebral perfusion in control mice (Fig. 2 L), whereas this effect was diminished in Gq/11 beKO animals (Fig. 2 J and L). The impaired vascular reactivity in Gq/11 beKO mice is stimulus-specific because the CBF response to the vasodilatory anesthetic isoflurane did not differ between Gq/11 beKO and control mice, in contrast to the response to CO2 (SI Appendix, Fig. S4O). An unchanged vessel density and normal coverage of cortical vessels by pericytes, smooth muscle cells, and basement membrane proteins (SI Appendix, Fig. S5 A–F) support the finding that endothelial Gq/11 signaling is not necessary for baseline perfusion or normal vessel morphology in the brain, but is essential for the reactivity to CO2/H+

Cerebral arterioles have been reported to dilate upon CO2/H+−induced diameter changes in arterioles by using a method described recently (26). As expected from the above data, the deletion of endothelial Gq/11 signaling led to a loss of CO2-induced arteriolar vessel dilation in cortical brain slices (Fig. 2 M–O), whereas normal reactivity was observed upon calcium withdrawal and exposure to a high concentration of potassium (SI Appendix, Fig. S5 G and H). Demonstrating that the reduced response to CO2/H+ was not due to a general morphological or functional impairment of vessel reactivity. In support of this, diameters of unstimulated arterioles were not altered in cortical slices of Gq/11 beKO mice (Fig. 2P). Taken together, our data indicate that endothelial cells play a critical role during CO2−induced blood flow responses in the brain as they are able to sense H+ changes by GPR4 and Gq/11 signaling and mediate the subsequent vascular reaction.

Impaired Vascular Reactivity to CO2 Aggravates CO2-Evoked Fear Response. Having established a mouse model of impaired cerebrovascular reactivity, we were able to investigate the physiological functions of CO2/H+−induced perfusion changes. Elevated CO2 concentrations elicit several behavioral and respiratory responses that help lower CO2 in the body. As a prominent effect, CO2 leads to what has been interpreted as a suffocation alarm (27), consisting of fear and panic reactions in mice and humans by activating chemosensitive brain areas, including the basolateral amygdala (16). To investigate cerebrovascular reactivity in the amygdala, we prepared acute brain slices of this area and measured arteriolar diameter changes after a CO2 stimulus. Again, as seen in cortical slices before, elevated CO2 concentrations increased vessel diameters, a response that was almost absent in slices of Gq/11 beKO mice.

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Fig. 2. Endothelial Gαq/11 signaling mediates CO₂-induced perfusion increase in the cortex. (A) Representative images of laser speckle recordings of Gαq/11−/− and control mice ventilated with different CO₂ concentrations taken before (air) and during CO₂ stimulation. Color scale indicates arbitrary units. An exemplary region of analysis is indicated by the white dotted box. (B and D) Quantification of laser speckle imaging measuring cortical perfusion of Gαq/11−/− and control mice during artificial ventilation with 10% CO₂ (B) or 20% CO₂ (D). (C and E) Areas under the curves shown in B or D, respectively. Mann–Whitney U test, *P < 0.05; n = 11 to 13 mice per group. (F and H) Quantification of laser speckle imaging measuring cortical perfusion of Gαq/11−/− and control mice during and after short apneic periods of 2 (F) or 3 (H) seconds. *P < 0.05 (RM-ANOVA with Bonferroni posttest); n = 6 to 7 mice per group. (G and I) Areas under the curves shown in F or H, respectively. Mann–Whitney U test, *P < 0.05; n = 6 to 7 mice per group. (J) Representative images of arterial spin labeling (ASL-MRI) of Gαq/11−/− and control mice before (air) and during stimulation with 10% CO₂. (K) Quantification of ASL-MRI perfusion measurements in the cortex of unstimulated Gαq/11−/− and control mice; n = 14 to 15 mice per group. (L) Difference in ASL-MRI perfusion measurements in the cortex of CO₂-exposed and unexposed Gαq/11−/− and control mice. Student’s t test, **P < 0.01; n = 14 to 15 mice per group. (M) Representative images of arterial spin labeling (ASL-MRI) of Gαq/11−/− and control mice before and during stimulation with CO₂. (N) Representative traces of diameter measurements in acute cortical brain slices of Gαq/11−/− and control mice before and during stimulation with CO₂. (O) Change in arteriolar diameters after stimulation with CO₂ in acute cortical brain slices of Gαq/11−/− and control mice (1 arteriole per animal, mean of 3 different sites of each vessel; n = 5 to 8 mice per group). Mann–Whitney U test, *P < 0.05. (P) Baseline diameters of the measured arterioles in acute cortical brain slices of Gαq/11−/− and control mice; n = 5 to 8 mice per group. Data are means ± SEM.

(Fig. 3 A–C). However, no changes were seen in the effects of control stimuli (SI Appendix, Fig. S6 A and B) or baseline diameter (Fig. 3D). As CO₂-induced blood flow response was impaired in the amygdala, we exposed freely moving mice to 10% CO₂ for 10 min to determine freezing behavior as a measure of the fear response. Confirming its known effect, 10% CO₂ increased freezing in control mice (Fig. 3 E and F) (16). Even more pronounced was the response of Gαq/11−/− mice, which
showed significantly more freezing behavior upon CO₂ exposure than control mice (Fig. 3 E and F). These findings point to an important influence of vessel reactivity on the chemosensitivity response. During everyday activities, such as speaking in humans or sniffing in mice, breathing is irregular, resulting in small alterations in blood CO₂/pH and corresponding changes in cerebral coordination, grip strength, and memory, as well as explorative activity and hedonic behavior did not differ between genotypes (Supplementary Appendix, Fig. S6 C–G), indicating that cerebrovascular reactivity is specifically required to regulate the chemosensitive fear and anxiety-like behavior, probably because it compensates for small fluctuations of blood CO₂/pH levels. This mechanism may contribute to the increased anxiety level that is observed in obese humans and animals in which cerebrovascular reactivity is impaired (29, 30).

**Fig. 3.** Impaired vascular reactivity to CO₂ in the amygdala leads to increased fear responses. (A) Representative images of stained arterioles in acute amygdala slices of Gαq/11 beKO and control mice before and during stimulation with CO₂. (B) Representative traces of diameter measurements in acute amygdala slices of Gαq/11 beKO and control mice during stimulation with CO₂. (C) Change in arteriolar diameters after stimulation with CO₂ in acute amygdala slices of Gαq/11 beKO and control mice (1 arteriole per animal, mean of 3 different sites of each vessel, n = 17 mice per group). Student’s t test, *P < 0.05. (D) Baseline diameters of the measured arterioles in acute amygdala slices of Gαq/11 beKO and control mice; n = 17 mice per group. (E) Representative track reports of Gαq/11 beKO and control mice exposed to normal air or CO₂ exposure in Gαq/11 beKO and control mice. *P < 0.05, ***P < 0.001 (2-way ANOVA with Bonferroni posttest); n = 16 to 17 mice per group. (F) Representative track reports of Gαq/11 beKO and control mice during a 10-min open field test and quantification of the time mice spent in the inner zone of the open field arena. Student's t test, *P < 0.05; n = 13 to 16 mice per group. (G) Representative track reports of Gαq/11 beKO and control mice during a 5-min elevated plus maze test and quantification of the time mice spent in the closed arm of the maze. Student’s t test, *P < 0.05; n = 13 to 16 mice per group. Data are means ± SEM.

**Impaired Cerebrovascular Reactivity Decreases CO₂-Evoked Respiration and Prolongs Apneic Episodes.** Importantly, CO₂ regulates respiration. CO₂ increases breathing frequency and tidal volume by acting on different central areas, most of which are located in the brainstem (31). Direct sensor proteins for increased CO₂/H⁺, including GPR4 and TASK2, were identified in neurons of the retrotrapezoid nucleus (RTN) (15). Brainstem neurons that are involved in sensing CO₂ are closely associated with vessels (32, 33), placing them in an ideal position to rapidly sense CO₂ changes. To examine vascular reactivity in the RTN we exposed acute brainstem slices to CO₂, measuring the arteriolar response in the RTN. In contrast to the findings in the cortex and amygdala, RTN arterioles responded to CO₂ with constriction (Fig. 4 A–C), revealing opposite vascular reactivity in different brain areas as previously reported in rats (26). Notably, RTN vessels of Gαq/11 beKO mice did not constrict upon CO₂ exposure. As shown already for cortex and amygdala, arterioles of the RTN responded to Ca²⁺ withdrawal and K⁺ exposure like control vessels (Supplementary Appendix, Fig. S7 A and B) and had the same baseline diameter (Fig. 4D). Thus, CO₂-induced cerebrovascular reactivity depends on endothelial Gαq/11 signaling in all territories.
although the effects on vessel diameters differ. To evaluate whether the opposing reactivity of RTN and cortical arterioles is specific for CO2 stimulation, we employed several other vasoactive factors. Sodium nitroprusside, endothelin-1, the thromboxane receptor agonist U46619, and ATP (SI Appendix, Fig. S7 C–F) had similar effects on the diameter of arterioles in cortex and brainstem slices.

To determine whether the peculiar, CO2-induced vasoconstriction in the RTN impacts respiration, we measured breathing parameters during CO2 exposure in awake Gαq\textsubscript{11}\textsuperscript{beKO} and control mice. First, we used a head-out plethysmography setup and exposed control and Gαq\textsubscript{11}\textsuperscript{beKO} mice to different CO2 concentrations, as recorded by whole-body plethysmography during the inactive period and quantification of the number of apneic phases; n = 9 to 10 mice per group. (G) Representative apnea phases of Gαq\textsubscript{11}\textsuperscript{beKO} and control mice recorded by whole-body plethysmography during the inactive period and quantification of the number of apneic phases; n = 9 to 10 mice per group. (H) Mean duration of all recorded apnea within 1 h during the inactive period of the day for each mouse in Gαq\textsubscript{11}\textsuperscript{beKO} and control mice. Student’s t test, *P < 0.05; n = 9 to 10 mice per group. Data are means ± SEM.

Fig. 4. Impaired vascular reactivity to CO2 leads to respiratory changes. (A) Representative images of stained arterioles in acute RTN slices of Gαq\textsubscript{11}\textsuperscript{beKO} and control mice before and during stimulation with CO2. (B) Representative traces of diameter measurements in acute RTN slices of Gαq\textsubscript{11}\textsuperscript{beKO} and control mice during stimulation with CO2. (C) Change in arteriolar diameters after stimulation with CO2 in acute RTN slices of Gαq\textsubscript{11}\textsuperscript{beKO} and control mice (1 arteriole per animal, mean of 3 different sites of each vessel). Student’s t test, **P < 0.01; n = 14 to 16 mice per group. (D) Baseline diameters of the measured arterioles in acute RTN slices of Gαq\textsubscript{11}\textsuperscript{beKO} and control mice; n = 14 to 16 mice per group. (E) Representative respiratory flow traces of Gαq\textsubscript{11}\textsuperscript{beKO} and control mice exposed to different concentrations of CO2 and the quantification thereof, recorded by head-out plethysmography. *P < 0.05 (RM-ANOVA with Bonferroni posttest). (F) Respiration flow of Gαq\textsubscript{11}\textsuperscript{beKO} and control mice exposed to different CO2 concentrations, as recorded by whole-body plethysmography. *P < 0.05 (RM-ANOVA with Bonferroni posttest); n = 7 to 9 mice per group. (G) Representative apnea phases of Gαq\textsubscript{11}\textsuperscript{beKO} and control mice recorded by whole-body plethysmography during the inactive period and quantification of the number of apneic phases; n = 9 to 10 mice per group. (H) Mean duration of all recorded apnea within 1 h during the inactive period of the day for each mouse in Gαq\textsubscript{11}\textsuperscript{beKO} and control mice. Student’s t test, *P < 0.05; n = 9 to 10 mice per group. Data are means ± SEM.
Gαq/11−/− mice and performed microarrays to determine mRNA expression. The preparations from brainstem and cortex were similar in terms of endothelial marker genes, but we found the mRNA expression of Nos3 (eNOS) and of genes involved in prostanooid synthesis and sensing to differ significantly between vascular fragments of the cortex and the brainstem (SI Appendix, Table S1). None of these genes differed between control and Gαq/11−/− mice (SI Appendix, Table S2).

To characterize the differences between brain areas further, we prepared primary brain endothelial cells from the brainstem and from the subcortical telencephalon (SCT) containing the amygdala (Fig. 5A). Cultured cells were almost pure endothelial cells (SI Appendix, Fig. S9 A and B) as described previously for the whole forebrain (14). Interestingly, we found again some changes in the expression of prostanooid-related genes as well as lower expression of the Nos1 and Nos3 mRNA in brainstem endothelial cells compared to SCT endothelial cells (SI Appendix, Fig. S10A). When we stimulated the cells with CO2, endothelial cells from the brainstem released less NO than the cells from the cortex (Fig. 5B), indicating a different reactivity to CO2. Differences in the release of the prostacyclin derivative PGF2α and PGE2 were even larger. Brainstem endothelial cells released less of these vasodilatatory prostanooids in response to CO2 than SCT endothelial cells (Fig. 5 C and D). In contrast, the CO2-induced release of thromboxane A2, as assessed by TXB2 concentrations, and the release of PGF2α did not differ between endothelial cells of the SCT and the brainstem (Fig. 5 E and F). Interestingly, vasodilation of RTN arterioles in response to the prostacyclin analog iloprost was diminished in comparison to arterioles of the cortex (Fig. 5G), supporting the role of prostanooids as possibly different between brain areas. All in all, the gene expression data as well as the different release of vasoactive compounds indicate a highly specialized vasculature that supports the functions of the surrounding brain region, such as breathing regulation in the brainstem. In addition, these findings confirm that NO and prostacyclin mediate CO2-induced vasodilation in the cortex.

Discussion

In this study, we demonstrate a hitherto unknown role of brain endothelial cells in CO2-induced hyperemia and show that a loss of this cerebrovascular reactivity affects several effects of CO2 on the central nervous system (CNS). Interestingly, the impaired CO2 reactivity is associated with dysfunctions in fear and breathing already with atmospheric CO2 concentrations. The response of the brain vasculature to CO2 is thought to be mediated by changes in pH rather than in CO2 or HCO3− concentrations (4). In keeping with the role of H+ GPR4, an endothelial H+−sensing GPCR, partially mediates the CO2-induced hyperperfusion in the cortex. GPR4 can activate Gαq/11 signaling pathways (23) and endothelial Gαq/11 signaling, as well as GPR4, are instrumental for the CO2-induced vascular response. These findings also suggest that endothelial cells form the first line of chemosensors, which convert metabolic blood changes rapidly into vascular diameter responses. Endothelial cells in the brain play a crucial role in blood flow reactivity, either by conducted hyperpolarization (36) or the release of vasoactive mediators (37). In line with reports that endothelial-derived vasoactive NO is involved in CO2-induced CBF increase (38, 39), we found that CO2 increased NO release in a Gαq/11-dependent manner. The NO-dependent component of the hypercapnia-induced hyperemia in the brain is strongest at low concentrations of CO2 (40, 41), which fits our finding that a loss of brain endothelial Gαq/11 signaling abrogates the CBF response at lower CO2 concentrations but only partially reduces the response at higher CO2 concentrations. Whether the released NO is due to eNOS activation is unclear at this stage. Alternatively, the neuronal NO synthase is expressed in endothelial cells and involved in the CO2-induced perfusion response in the brain (42, 43). In addition to endothelial cells, pericytes, astrocytes, and neurons may contribute to the residual reactivity that still occurred.

Fig. 5. Release of vasoactive substances differs between subcortical-telencephalic and brainstem endothelial cells. (A) Scheme of the brain areas that were used for the preparation of area-specific primary endothelial cells. (B) Assessment of NO release by measuring nitrate concentrations in the supernatant of SCT or brainstem endothelial cells after 20-min stimulation with 5% (control) or 15% CO2. **P < 0.01 (2-way ANOVA with Bonferroni posttest); n = 4 to 6 per group, 2 independent experiments. (C) PGF2α as a surrogate for prostacyclin release from SCT or brainstem endothelial cells after 20-min stimulation with 5% (control) or 15% CO2. ***P < 0.001 (2-way ANOVA with Bonferroni posttest); n = 5 to 6 per group, 2 independent experiments. (D) PGE2 release from SCT or brainstem endothelial cells after 20-min stimulation with 5% (control) or 15% CO2. ***P < 0.001 (2-way ANOVA with Bonferroni posttest); n = 5 to 6 per group, 2 independent experiments. (E) TXB2 as a surrogate for TXA2 release from SCT or brainstem endothelial cells after 20-min stimulation with 5% (control) or 15% CO2. **P < 0.01 (2-way ANOVA with Bonferroni posttest); n = 5 to 6 per group, 2 independent experiments. (F) PGF2α release from SCT or brainstem endothelial cells after 20-min stimulation with 5% (control) or 15% CO2. n = 3 to 5 per group, 2 independent experiments. (F) PGF2α release from SCT or brainstem endothelial cells after 20-min stimulation with 5% (control) or 15% CO2. n = 3 to 5 per group, 2 independent experiments. Absolute values of prostanooids released by endothelial cells are shown in SI Appendix, Fig. S10B. (G) Representative traces of diameter measurements in acute cortical and RTN brain slices of C57BL/6 mice during stimulation with 1 μM iloprost and quantification thereof (1 arteriole per animal, mean of 3 different sites of each vessel, n = 3 mice per group). Student’s t test, *P < 0.05. Data are means ± SEM.
at high CO₂ concentrations in Gαq⁹/¹¹ beKO mice (44–46). Neurons sense pH changes and modulate cerebrovascular reactivity, mediated most likely by the neuronal NO synthase (46). Gαq⁹/¹¹ signaling in brain endothelial cells not only controls the release of vasoactive molecules, but also the activity of ion channels in capillaries (47, 48) that are involved in the regulation of vascular reactivity (36, 49). The activation of endothelial Gαq⁹/¹¹ signaling in the brain leads to an arteriolar dilation that depends on NOS activity (50). At the membrane, other, still unidentified endothelial Gαq⁹/¹¹-coupled receptors might contribute to CO₂-induced cerebrovascular reactivity, but neither GPR68 nor P.Y₂, both of which are involved in endothelial shear stress responses (13, 20), affected the CO₂-induced perfusion increase.

Currently, the coupling between vessels and neurons is mostly studied in the neuro-to-vascular direction. Conversely, the vascular tone has also a direct impact on neuronal activity in the cortex (51). In support of this idea, our data suggest that impaired CO₂/H⁺-induced cerebrovascular reactivity modulates the behavioral and respiratory effects of CO₂. Normal cerebrovascular reactivity apparently attenuates the behavioral effects of CO₂, probably by facilitating its washout from most parts of the brain (SI Appendix, Fig. S11). If cerebrovascular reactivity fails to maintain CO₂/H⁺ homeostasis in the brain, CO₂-induced fear is unleashed and helps to avoid exogenous CO₂ sources. In contrast, cerebrovascular reactivity seems to retain CO₂ in the RTN, thereby stimulating the CO₂ effect on respiration and enhancing CO₂ elimination from the body. This concept is in line with the recent finding that constriction of local vessels at the ventral medullary surface of the brainstem increases CO₂-induced breathing activity, whereas a decreased respiratory response was observed after local vasodilation (26). The unique features of cerebrovascular reactivity in the RTN could be related to lower production of vasodilatory or an increased release of vasoconstrictive mediators upon CO₂/H⁺ stimulation (52). Supporting this idea, we found highly specialized gene expression in vessels of cortex and brainstem. Gene expression favors the synthesis of the vasodilating NO in the cortex or SCT. To assess the endothelial release of vasoactive mediators, we established the primary culture of endothelial cells originating from different brain areas. In these brain area-specific cell populations, CO₂ induced the release of vasodilative factors from endothelial cells of the SCT but not the brainstem. In contrast, CO₂-stimulated the release of thromboxane similarly in both endothelial populations. It was shown before that the synthesis of prostanoids plays a role during hypercapnia-induced perfusion increase (53, 54) and that prostanoids, including the constrictive thromboxane, are released during hypercapnia (54, 55). We conclude that upon CO₂/H⁺ stimulation the release of NO and prostanoids differs in the vessels of different parts of the brain but the initial endothelial Gαq⁹/¹¹-mediated mechanism is the same. It is well described that brain areas respond differently to a hypercapnic stimulus, including negative responses that lead to hypoperfusion (56). In the brainstem, nuclei that are located very close to each other have been described to respond to CO₂ in different ways (26, 56–58). Collectively, all effects seem to serve the goal of removing CO₂ from the brain, with the notable exception of the brainstem (SI Appendix, Fig. S11). Importantly, our findings show that specialization of vessels does not only appear along the vascular tree in the brain (10) but also depends on the surrounding brain area.

Impaired cerebrovascular reactivity has an impact on the behavioral and respiratory functions of mice already when breathing normal air, which may be explained by small fluctuations in blood CO₂ concentration during everyday activities, such as sniffig.

Similar effects occur during speaking or sighing in humans. These small changes are sufficient to affect both the CBF (28) and the pH in brain extracellular fluids (59), and we have shown that short apnic periods increase cortical perfusion in a Gαq⁹/¹¹-dependent manner. Thus, short and rapid vascular responses to even small changes in blood CO₂ levels control normal brain function, at least in CO₂-sensitive areas. Impaired cerebrovascular reactivity to CO₂ is a key diagnostic feature of endothelial dysfunction (59) that develops in metabolic syndrome and in several vascular diseases (60). Our data suggest that endothelial dysfunction in the brain contributes to the pathogenesis of sleep apnea and anxiety disorders, and maybe other diseases that are often associated with metabolic syndrome (29, 30, 34). Thus, endothelial dysfunction in the brain and altered cerebrovascular reactivity should be considered as a therapeutic target in several diseases, including metabolic syndrome.

Materials and Methods

Mice. Brain endothelial-specific knockout (beKO) animals were generated by crossing the bacterial artificial chromosome (BAC)-transgenic SloC1-CreER² strain (12), which expresses the tamoxifen-inducible CreER² recombinase under control of the mouse SloC1 regulatory sequences in brain endothelial cells, with mice carrying lox-flanked alleles. GPR4 and GPR68 whole-genome knockout mice have been described previously (15). All animal experiments were approved by the local animal ethics committee (Regierungspräsidium Karlsruhe; Ministerium für Landwirtschaft, Umwelt und ländliche Räume, Kiel, Germany). For details see SI Appendix.

Laser Speckle Imaging. Mice were anesthetized and 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 CO₂ concentration of 35 to 45 mmHg that was continuously controlled during the experiments with a capnometer. Laser speckle imaging was performed and regions of interest were set over big cortical vessels. Flux intensities were recorded throughout CO₂ stimulation (10 or 20%, combined with 21% O₂, rest N₂) and normalized to baseline values for each region of interest. For details see SI Appendix.

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 (26) with slight changes. For details see SI Appendix. Single arterioles were identified in brain slices by typical ring-like labeling (Figs. 2M, 3A, and 4C) and a diameter of 3–10 μm. RTN slices were taken from the ventral surface below the caudal end of the facial nucleus; amygdala slices were taken 1 to 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. For further method descriptions see SI Appendix.

Data Availability. Microarray data have been deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-8521. All other original data files are available from the authors upon request.

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