Figures and figure supplements

Vascular control of the CO$_2$/H$^+$-dependent drive to breathe

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Figure 1. CO₂/H⁺ differentially regulates arteriole tone in the RTN by a P2Y₂ receptor dependent mechanism. (A-B) Tissue sections containing the cNTS, ROb, and RTN were prepared from an endothelial cell (Tekcre::TdTomato) and smooth muscle cell reporter mice (Myh11Cre/eGFP). Individual cells

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were dissociated and sorted to isolate enriched cell populations from each region (100–300 cells/region). Control cells for each region were prepared from slices with experimental regions removed. Fold change of each P2 receptor was determined for each region by normalizing to within group Gapdh expression as well as to control group receptor expression and plotted as log₂[fold change]; 0 on the y-axis indicates control group expression for each receptor and negative values reflect less than the control group and positive values reflect greater than control group. Of all P2 receptors detected in each region, only P2ry2 showed an expression pattern consistent with a role in vasoconstriction in the RTN; low expression in endothelial cells (A) and above baseline expression in smooth muscle cells (B) from this region. (C-F) Diameter of arterioles in the RTN, cNTS, ROb and somatosensory cortex was monitored in brain slices from adult mice over time by fluorescent video microscopy. (C-F) Diameter traces of individual arterioles in each region and corresponding summary bar graphs show that exposure to 15% CO₂ (Ci-ii), activation of P2Y₂ receptors by bath application of PSB1114 (100 μM) (Ei-ii), or activation of astrocytes by bath application of t-ACPD (50 μM) (Fi-ii) caused vasoconstriction in the RTN and dilation in all other regions of interest. (Di-Dii) The CO₂/H⁺ response of RTN vessels was blocked by a selective P2Y₂ receptor antagonist (AR-C118925; 10 μM) #, difference from baseline (one sample t-test). *, differences in each condition (one-way ANOVA with Tukey’s multiple comparison test). One symbol = p < 0.05, two symbols = p < 0.01, three symbols = p < 0.001, four symbols = p < 0.0001.
Figure 1—figure supplement 1. Gating Strategy for FACS sorting of smooth muscle cell and endothelial cell populations from a single-cell suspension.  (A) Scatter graph gating out debris from the sample. (B) Side scatter graph to gate for complexity/doublets. (C) Forward scatter graph to gate for cell size. (D) Scatter graph for DAPI and GFP (or TdTomato). Cells were gated for positive GFP (or TdTomato) and low DAPI signal.
Figure 1—figure supplement 2. CO$_2$/H$^+$-induced constriction of RTN arterioles in vitro is not dependent on neural activity, prostaglandin EP$_3$ receptors or adenosine signaling. (A) Traces of arteriole diameter shows that exposure to 15% CO$_2$ alone and in the presence of POM 1 (100 µM; an ectonucleotidase inhibitor), L-798,106 (0.5 µM; a selective EP3 receptor blocker), 8-PT (10 µM; an adenosine receptor antagonist), or TTX (0.5 µM) to block action potentials had similar effects on arteriole diameter. (B) Summary data show CO$_2$-induced change in diameter under control conditions (N = 7 vessels) and in the presence of POM 1 (N = 7 vessels), L-798,106 (N = 5 vessels), 8-PT (N = 8 vessels), and TTX (N = 7 vessels) (F$_{2,19}$ = 1.063, p>0.05, one-way ANOVA).
Figure 2. CO₂ constricts pial vessels in the RTN region by a P2Y₂ receptor-dependent mechanism to increase respiratory behavior in anesthetized mice. (A) Images of RTN pial vessels and corresponding traces of vessel diameter (N = 6 mice/condition) show that exposure to 9–10% CO₂ decreased vessel diameter under control conditions (saline) but not when P2Y₂ receptors were blocked with AR-C118925 (1 mM). (B) Images of RTN pial vessels and corresponding traces vessel diameter show that application of a P2Y₂ receptor agonist (PSB1114; 100 μM) caused a reversible constriction. (C-D) Traces of external intercostal EMG (IntEMG) and end expiratory CO₂ (etCO₂) show that blocking CO₂/H⁺-induced vasoconstriction by ventral surface application of AR-C118925 minimally affected respiratory activity at low etCO₂ levels but blunted the ventilatory response to 9–10% CO₂ (C). Conversely, at a constant etCO₂ of 5% the application of PSB1114 to mimic CO₂/H⁺ constriction increased respiratory output (D). (E-J) Summary data show (N = 6 mice/condition) effects RTN application of saline, AR-C118925 or PSB1114 on intercostal EMG amplitude (E, H), frequency (F, I) and mean arterial pressure (MAP; G, J). *, Different (RM-ANOVA followed by Bonferroni multiple-comparison test; *, p<0.05). scale bar = 200 μm.
Figure 3. Disruption of \( \text{CO}_2/\text{H}^+ \) dilation in the cNTS and ROB causes unstable breathing and apnea. (A) Trace of external intercostal muscle EMG (\( \text{IntEMG} \)) activity shows respiratory activity of an anesthetized wild type mouse breathing 2.5% \( \text{CO}_2 \) following injections of saline or U46619 (1 \( \mu \text{M}; 30 \text{nL/region} \)) into the cNTS and ROB. (B) Location of injections in the cNTS and ROB. (C) Representative Poincaré plot (50 breaths) shows breath-to-breath (\( \text{T}_{\text{TOT}} \)) interval variability following injections saline (black) or U46619 (red) conditions. (D-E) Summary data (N = 6 animals/group) shows effects of U46619 injections into the cNTS and ROB alone and in combination on the coefficient of variation of Int\( \text{EMG} \) frequency (C) and Int\( \text{EMG} \) frequency (E). (F) Summary data show that injections of U46619 injections into the cNTS and ROB lowered the \( \text{CO}_2 \) apneic threshold from 3.2 ± 0.3% to 2.1 ± 0.1% (N = 7 mice). asterisk, difference in Int\( \text{EMG} \) activity under control conditions (saline) vs. during U46619 into the NTS and/or ROB (RM-ANOVA followed by Bonferroni multiple-comparison test, \( p<0.05 \)). scale bar = 200 \( \mu \text{m} \).
Figure 4. Smooth muscle P2Y2 cKO mice show a blunted CO2 chemoreflex that can be rescued by re-expression of P2Y2 only in RTN smooth muscle cells. (A) P2Y2 transcript was detected in RTN, brainstem and cortical smooth muscle cells isolated from control mice (Taglncre::TdTomato), P2Y2 cKO mice (Taglncre::P2ry2f/f::TdTomato), and P2Y2 rescue mice (P2Y2 cKO animals that received bilateral RTN injections of AAV2-Myh11p-eGFP-2A-mP2ry2). P2ry2 transcript was not detected (n.d.) in smooth muscle cells from P2Y2 cKO mice (N = 3 runs/9 animals). P2ry2 was also not detected in cortical smooth muscle cells from either genotype. Conversely, RTN (p = 0.0073) and brainstem (p = 0.0073) but not cortical (p > 0.05) smooth muscle cells from P2Y2 rescue mice show increased P2ry2 transcript compared to P2Y2 cKO but not to the same level as cells from control mice (p = 0.0219) (ANOVA on ranks followed by Dunn multiple comparison test). (B) Left, computer-assisted plots show the center of all bilateral AAV2-Myh11p-eGFP-2A-mP2ry2 injections; each matching color pair of dots corresponds to one animal (N = 13 animals). Approximate millimeters behind bregma (Paxinos and Franklin, 2013) is indicated by numbers next to each section. Right, 2 weeks after injections we confirmed that ~80% of RTN smooth muscle Acta2-immunoreactive cells were also GFP+ (inset). (C-F) Representative traces of respiratory activity (C) and summary data show that smooth muscle-specific P2Y2 KO mice (Taglncre::P2ry2f/f) breathe normally under room air conditions but fail to increase respiratory frequency (C) or tidal volume (D) during exposure to CO2, thus resulting in diminished minute ventilation at 5–7% CO2 (E). Re-expression of P2Y2 in only RTN smooth muscle cells rescued the ventilatory response to CO2. Note that Taglncre only and P2ry2f/f only control mice show similar baseline breathing and responses to CO2. Figure 4 continued on next page.
CO₂ and so were pooled. *, Different from 0% CO₂ in condition as assessed by Tukey’s post-hoc multiple comparison test. ####, Different between genotypes (two-way ANOVA with Tukey’s multiple comparison test, p<0.0001).
Figure 4—figure supplement 1. Cardiovascular, metabolic and blood gas parameters in control and smooth muscle P2Y2 cKO mice. (A) Traces of blood pressure and ECG activity from control and P2Y2 cKO mice. (B) Summary data (N = 4/genotype) shows that control and P2Y2 cKO mice have
similar mean arterial pressures (MAP) during a 12 hr light cycle ($T_2 = 0.1152, p>0.05$) and during a 12 hr dark cycle ($T_2 = 0.1349, p>0.05$). (C) Summary (N = 4/genotype) heart rate (beats/min) during light and dark cycles show similar means for controls and P2Y$_2$cKO ($F_{1,3} = 2.896, p>0.05$). (D-E), summary (N = 4/genotype) blood pressure (D) and heart rate (E) under room air conditions and during exposure to graded increases in CO$_2$ (values were obtained during the last minute of each condition). Control and P2Y$_2$cKO animals show similar blood pressure ($F_{1,3} = 2.051, p>0.05$) and heart rate ($F_{2,3} = 2.896, p>0.05$) responses across all experimental conditions. * indicates significant change from 0% CO2. (F-H) Oxygen consumption (VO$_2$) (F), CO$_2$ production (VCO$_2$) and the respiratory exchange ratio (H) were similar between control (N = 11 animals) and P2Y$_2$cKO (N = 10 animals) mice. (I-K) Under room air conditions control and P2Y$_2$cKO mice (N = 7 animals/genotype) showed similar arteriole PO$_2$ ($T_{12} = 0.3080, p>0.05$), PCO$_2$ ($T_{12} = 0.7548, p>0.05$) and pH ($T_{12} = 0.4437, p>0.05$). Blood pressure and heart rate values were compared using a two-way ANOVA with Tukey’s post-hoc multiple comparison test. Baseline metabolic activity and blood gas values were compared using an unpaired two-way t-test.
Figure 4—figure supplement 2. Respiratory activity of Myh11Cre/eGFP::P2ry2f/f and control viral injected P2Y2 cKO mice. (A–C) We crossed Myh11Cre/eGFP::P2ry2f/f and control viral injected P2Y2 cKO mice. (A–C) We crossed Myh11Cre/eGFP::P2ry2f/f and control viral injected P2Y2 cKO mice. Control (Myh11Cre only and P2ry2f/f only) and Myh11Cre/eGFP::P2ry2f/f cKO mice show a similar respiratory frequency (A), tidal volume (B) and minute ventilation (c) under room air conditions. However, Myh11Cre/eGFP::P2ry2f/f mice exhibit a blunted ventilatory response to CO2. This respiratory phenotype is nearly identical to the respiratory phenotype of TaglnCre::P2ry2f/f mice (defined as P2Y2 cKO) described in the main text. (D–F) Bilateral RTN injections of control virus (AAV2-Myh11p-eGFP) minimally affected respiratory frequency (D), tidal volume (E) or minute ventilation (F) in TaglnCre::P2ry2f/f (P2Y2 cKO) mice. *, Difference from 0% CO2; #, differences between genotypes (two-way ANOVA with Tukey’s multiple comparison test). One symbol = p < 0.05; two symbols = p < 0.01; three symbols = p < 0.001; four symbols = p < 0.0001. Linear regressions were compared by two-tailed analysis of covariance.
Figure 4—figure supplement 3. P2Y$_2$ cKO mice show a normal ventilatory response to acute hypoxia. (A) Traces of respiratory activity from control and P2Y$_2$ cKO mice in room air (21% O$_2$) and during exposure to hypoxia (10% O$_2$). (B) Summary data (N = 8 animals/genotype) plotted as minute ventilation shows that control and P2Y$_2$ cKO exhibit similar respiratory activity under baseline conditions and during hypoxia. ***, Different from control at p<0.001 (two-way RM-ANOVA with Tukey’s multiple comparison test).
Figure 4—figure supplement 4. Functional characterization of RTN and cortical arterioles in slices from P2Y2 cKO mice. (A-B) Diameter traces of individual arterioles and summary data (bottom) show that vessels from the RTN (A) and cortex (B) in slices from P2Y2 cKO mice respond similarly to 15% CO2 and t-ACPD (50 μM). As expected, vessels from each region in slices from P2Y2 cKO mice failed to respond to bath application of the P2Y2 agonist PSB 1114 (200 nM). (C) RTN arterioles in slices from P2Y2 cKO mice 2 weeks after AAV2-Myh11p-eGFP-2A-mP2ry2 was injected bilaterally into the RTN (P2Y2 cKO rescue) constrict in response to 15% CO2, t-ACPD and PSB 1114 in a manner similar to vessels from control mice (Figure 1C). ##, different from baseline (paired two-tailed t-test; p<0.0001).
Figure 4—figure supplement 5. Cell specificity of AAV2-Myh11p-eGFP-2A-mP2ry2 transduction. Histological analysis was performed on brainstem sections collected from P2Y2 cKO mice two weeks after bilateral RTN injections of AAV2-Myh11p-eGFP-2A-mP2ry2. (Ai) Example images of virally transduced (eGFP driven by Myh11 promoter) smooth muscle cells counterstained with alpha smooth muscle actin 2 (Acta2; cyan). Overlapping Acta2 and eGFP localization represents positive smooth muscle cell transduction. 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; white) was used to visualize non-transfected negative cells. (Aii) Example images of a transduced smooth muscle cell. Some small eGFP green puncta are also observed in a DAPI-positive but Acta2-negative cell (red arrow). Inset, shows eGFP puncta not co-localized with DAPI (yellow arrows). (B) Sagittal view of an arteriole in the RTN with four eGFP labeled smooth muscle cells and some eGFP puncta not co-localized with DAPI (yellow arrows). Scale bars 20 μm.