Vascular control of the CO₂/H⁺ dependent drive to breathe

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Abstract
Respiratory chemoreceptors regulate breathing in response to changes in tissue CO$_2$/H$^+$. Blood flow is a fundamental determinant of tissue CO$_2$/H$^+$, yet little is known regarding how regulation of vascular tone in chemoreceptor regions contributes to respiratory behavior. Previously, we showed in rat that CO$_2$/H$^+$-vasoconstriction in the retrotrapezoid nucleus (RTN) supports chemoreception by a purinergic-dependent mechanism (Hawkins et al. 2017). Here, we show in mice that CO$_2$/H$^+$ dilates arterioles in other chemoreceptor regions, thus demonstrating CO$_2$/H$^+$ vascular reactivity in the RTN is unique. We also identify P2Y$_2$ receptors in RTN smooth muscle cells as the substrate responsible for this response. Specifically, pharmacological blockade or genetic deletion of P2Y$_2$ from smooth muscle cells blunted the ventilatory response to CO$_2$, and re-expression of P2Y$_2$ receptors only in RTN smooth muscle cells fully rescued the CO$_2$/H$^+$ chemoreflex. These results identify P2Y$_2$ receptors in RTN smooth muscle cells as requisite determinants of respiratory chemoreception.
Significance Statement:
Disruption of vascular control as occurs in cardiovascular disease leads to compromised chemoreceptor function and unstable breathing. Despite this, virtually nothing is known regarding how regulation of vascular tone in chemoreceptor regions contributes to respiratory behavior. Here, we identify P2Y$_2$ receptors in RTN vascular smooth muscle cells as a novel vascular element of respiratory chemoreception. Identification of this mechanism may facilitate development of treatments for breathing problems including those associated with cardiovascular disease.
Introduction

Blood flow in the brain is tightly coupled to neural activity. Typically, an increase in neural activity triggers vasodilation to increase delivery of nutrients and clear metabolic waste (6, 21). Blood flow may also directly regulate neural activity and contribute to behavior; perhaps the most compelling evidence supporting this involves vasoregulation by CO$_2$/H$^+$ (6, 21). In most cases, CO$_2$/H$^+$ function as potent vasodilators to provide support for metabolic activity and to maintain brain pH within the narrow range that is conducive to life (1). However, CO$_2$/H$^+$ has the opposite effect on vascular tone in a brainstem region known as the retrotrapezoid nucleus (RTN) (18, 40), where CO$_2$/H$^+$ is not just metabolic waste to be removed but also functions as a primary stimulus for breathing (17). Neurons (17, 22) and astrocytes (14) in this region are specialized to regulate breathing in response to changes in tissue CO$_2$/H$^+$ (i.e., function as respiratory chemoreceptors) and so vasoconstriction may augment this function by preventing CO$_2$/H$^+$ washout and maintaining the stimulus to respiratory chemoreceptors. However, it is not known whether CO$_2$/H$^+$ induced vasoconstriction is unique to the RTN or also contributes to chemotransduction in other chemosensitive brain regions. Also, mechanisms underlying this specialized CO$_2$/H$^+$ regulation of vascular tone in the RTN are largely unknown. Previous work showed that RTN chemoreception involves CO$_2$/H$^+$-evoked ATP-purinergic signaling most likely from astrocytes (14, 39); however, downstream cellular and molecular targets of this signal are not known.

Here, we show that CO$_2$/H$^+$ vascular reactivity in the RTN is unique compared to other chemoreceptor regions. Our evidence also suggests that CO$_2$/H$^+$ constriction in the RTN enhances chemoreceptor activity and respiratory output, while simultaneous dilation in other
chemoreceptor regions may serve to limit chemoreceptor activity and stabilize breathing. We also show that endothelial cells and vascular smooth muscle cells in the RTN have a unique purinergic (P2) receptor expression profile compared to other levels of the respiratory circuit that is consistent with P2Y₂ dependent vasoconstriction. Further, at the functional level, we show pharmacologically and genetically using P2Y₂ conditional knockout (P2Y₂ cKO) mice and RTN smooth muscle-specific P2Y₂ re-expression that P2Y₂ receptors on vascular smooth muscle cells in the RTN are required for CO₂/H⁺ dependent modulation of breathing.

Results

P2Y₂ receptors are differentially expressed by vascular smooth muscle cells in the RTN

Purinergic signaling is required for CO₂/H⁺-induced constriction of RTN arterioles(18). Therefore, as a first step towards identifying the cellular and molecular basis of this response, we determined the repertoire of P2 receptors expressed by endothelial and smooth muscle cells associated with arterioles in the RTN. We also characterize the P2 receptor expression profile of vascular cells in the caudal aspect of the nucleus of the solitary tract (cNTS) and raphe obscurus (ROb), two putative chemoreceptor regions where P2 receptor signaling does not contribute to respiratory output (35). For this experiment, we used Cre-dependent smooth muscle (smMHC<sup>Cre/eGFP</sup>) and endothelial cell (Tie₂<sup>Cre-::TdTomato</sup>) reporter lines and performed fluorescence activated cell sorting to obtain enriched populations of endothelial or smooth muscle cells. Control tissue was obtained at the same level of brainstem but outside the region of interest. Each population of cells was pooled from 3-8 adult mice per region for subsequent quantitative RT-PCR for each of the fourteen murine P2 receptor subtypes. The type and relative abundance of P2 receptors expressed by each cell type was similar to the cortex (38) and
relatively consistent between brainstem regions. For example, endothelial cells from each region express ionotropic P2X4 and P2X7 and metabotropic P2Y1,2,6,12,14 receptors, albeit at varying levels relative to control (Fig. 1A). Also, P2X1,4 and P2Y2,6, and 14 receptor subtypes were detected in smooth muscle cells from each region and most were under-expressed relative to control (Fig. 1B). The P2 transcript profile of vascular cells in the RTN was fairly similar to the ROb (Figs. 1A-B) but differed from the cNTS in several ways including lower expression of P2Y6 in endothelial cells (Fig. 1A) and higher expression of P2X1, P2X4 and P2Y14 in smooth muscle cells (Fig. 1B). However, only P2Y2 in the RTN showed a distinct expression profile that was consistent across cell types with a role in vasoconstriction; P2Y2 transcript was detected at lower (-9.2 ± 0.3 log2 fold change, p=0.0013) and higher (6.8 ± 1.5 log2 fold change, p=0.0462) levels compared to control in RTN endothelial and vascular smooth muscle cells, respectively (Figs. 1A-B). This is interesting because in other brain regions activation of endothelial P2Y2 receptors favors nitric oxide-induced vasodilation (27), whereas activation of this same receptor in vascular smooth muscle cells mediates vasoconstriction (3, 23). These results identify P2Y2 receptors in vascular smooth muscle cells as potential substrates for CO2/H+ -induced ATP-dependent vasoconstriction in the RTN.

CO2/H+ differentially effects vascular tone in the RTN by a P2Y2 dependent mechanism

To further test this possibility, we used the brain slice preparation, optimized for detecting increases or decreases in vascular tone, to characterize CO2/H+ vascular responses in each chemoreceptor region of interest as well in a non-respiratory region of the somatosensory cortex. For these experiments, we target arterioles based on previously described criteria (9, 29). We verified that exposure to CO2/H+ (15% CO2; pH 6.9) under control conditions decreases the
diameter of RTN arterioles by -11.2 ± 4.5% (N=9 vessels), whereas this same stimulus consistently dilates arterioles in the cNTS (Δ +6.0 ± 2.2%; N=9 vessels), ROb (Δ +6.5 ± 2.6%; N=11 vessels) and somatosensory cortex (Δ +3.7 ± 0.9%; N=9 vessels) (F3,34=13.49; p <0.0001) (Figs. 1Ci-Cii). Consistent with a requisite role of P2Y2 receptors in this response, we found that CO2/H+ induced constriction of RTN arterioles was eliminated by blockade of P2Y2 receptors with AR-C118925 (10 μM) (Δ +0.2 ± 0.3%; N=6 vessels) (T5=1.223; p >0.05) (Figs. 1Di-Dii) and mimicked by application of a selective P2Y2 receptor agonist (PSB 1114; 200 nM) (Δ -2.3 ± 0.7%; N=11 vessels) (T10=3.460; p<0.010) (Figs. 1Ei-Eii). As a negative control, we confirmed that PSB 1114 (200 nM) minimally affects vascular tone in the cNTS (Δ -0.05 ± 0.09%; N=6 vessels; T5=0.3898; p>0.05) and ROb (Δ -0.07 ± 0.19%; N=7 vessels; T6=0.6076; p>0.05) (Figs. 1Ei-Eii), where P2Y2 receptors were detected at low levels in both endothelial and smooth muscle cells (Figs. 1A-B). We also confirmed that the response of RTN arterioles to CO2/H+ was retained when neural action potentials were blocked by bath application of TTX (0.5 μM) (Supplemental Figs. 1A-B), indicating that CO2/H+ constriction of RTN arterioles differs from canonical neurovascular coupling both in terms of polarity and dependence (or lack thereof) on neuronal activity (21). Also, despite evidence that extracellular ATP can be rapidly metabolized to adenosine (a potent vasodilator including in the RTN (18)) the response of RTN arterioles to CO2/H+ was unaffected by 8-phenyltheophylline (8 PT; 10 μM) to block adenosine A1 receptors or sodium metatungstate (POM 1; 100 µM) to inhibit ectonucleotidase activity (Supplemental Figs. 1A-B). Furthermore, exposure to CO2/H+ has been shown to trigger prostaglandin E2 (PGE2) release that may contribute to RTN chemoreception by a mechanism involving EP3 receptors (10); therefore, we also tested for a role of PGE2/EP3 signaling in CO2/H+ dependent regulation of RTN arteriole tone. We found bath application of PGE2 (1 μM)
decreased RTN arteriole tone by -2.7 ± 0.4% (T9 = 2.787, p = 0.0212). However, CO2/H+ constriction of RTN vessels was retained in the presence of L-798,106 (0.5 µM) to block EP3 receptors (-9.6 ± 4.0%, T7 = 2.675, p = 0.0318) (Supplemental Fig. 1A-B), suggesting PGE2/EP3 signaling is dispensable for CO2/H+ regulation of arteriole tone in the RTN.

Astrocytes are thought to contribute to CO2/H+ -induced vasodilation in the cortex (20) and vasoconstriction in the RTN (18). Consistent with this, we found that exposure to t-ACPD (50 µM), an mGluR agonist widely used to elicit Ca2+ elevations in astrocytes (20), caused constriction of RTN arterioles (Δ -5.9 ± 1.7%; N=8 vessels) and dilation of arterioles in the cNTS (Δ +7.6 ± 2.5%; N=8 vessels), ROb (Δ +6.8 ± 1.9%; N=8 vessels) and somatosensory cortex (Δ +2.9 ± 0.8%; N=5 vessels) (F3,25=10.18; p <0.0001) (Fig. 1Fi-Fii). These results show that CO2/H+-dependent regulation of vascular tone and roles of astrocytes in this process are fundamentally different in the RTN compared to other brain regions, and point to differential purinergic signaling mechanisms downstream of astrocyte activation.

To determine whether P2Y2 receptors in the RTN regulate CO2/H+ vascular reactivity in vivo, we measured the diameter of pial vessels on the ventral medullary surface (VMS) in the region of the RTN during exposure to high CO2 under control conditions (saline) and when P2Y2 receptor are blocked with AR-C118925. Consistent with our slice data, we found under saline control conditions that increasing end-expiratory CO2 to 9-10%, which corresponds with an arterial pH of 7.2 pH units (16), constricted VMS vessels by -7.8 ± 0.7 % (p=0.01, N = 7 animals) (Fig. 2A).

However, when P2Y2-receptors are blocked by application of AR-C118925 (10 µM) to the VMS, increasing inspired CO2 resulted in a vasodilation of 5.2 ± 0.9% (Fig. 2A) (p=0.05; N = 7
animals). Thus, in the absence of functional P2Y2 receptors, RTN vessels respond to CO2/H+ in a manner similar to other brain regions. Also consistent with our slice data, we found that activation of P2Y2 receptors by application of PSB1114 (100 µM) to the VMS constricted vessels in the region of the RTN by -6.3 ± 0.9 % (p=0.05, N = 7 animals) (Fig. 2B).

To correlate P2Y2-dependent vasoconstriction in the RTN region with respiratory behavior, we simultaneously measured systemic blood pressure and external intercostal electromyogram (IntEMG) activity (as a measure of respiratory activity) in urethane-anesthetized mice during exposure to low and high CO2 following VMS application of saline or AR-C118925. We found that VMS application of AR-C118925 (1 mM) minimally affected the CO2/H+ apneic threshold (3.3 ± 0.4% vs. saline: 3.1 ± 0.5% (p > 0.05; two-way RM ANOVA; N = 7). However at high levels of CO2 (9-10% etCO2) AR-C118925 decreased intercostal EMG amplitude by 23 ± 5% (F2,74 = 69.83; p=0.021; N = 7 animals) (Figs. 2C, E), but with no change in frequency (F2,74 = 1.09; p>0.05; N = 7 animals) (Figs. 2C, F). Conversely, VMS application of PSB1114 (100 µM) while holding etCO2 constant at 5% increased intercostal EMG amplitude by 20 ± 3% (F2,74 = 96.14; p=0.02; N = 7 animals) (Figs. 2D, H), again with no change in frequency (F2,74 = 0.79; p>0.05; N = 7 animals) (Figs. 2D, I). These treatments had negligible effects on systemic mean arterial pressure (MAP) (AR-C118925: 113 ± 5; PSB1114: 112 ± 5; saline: 114 ± 4 mmHg; F2,74 = 1.29; p>0.05; N = 7 animals) (Figs. 2G, J). These results indicate that P2Y2 dependent vasoconstriction in the RTN contributes to the drive to breathe.

If the mechanism underlying vascular control of RTN chemoreception involves maintenance of tissue CO2/H+ levels, then by this same logic, vasodilation in the cNTS and ROb should buffer...
tissue CO$_2$/H$^+$ and limit contributions of these regions to respiratory output. Such a braking mechanism may be important for stabilizing breathing since over-activation of the CO$_2$/H$^+$ chemoreflex is thought to cause unstable periodic breathing (5). To test this, we disrupted CO$_2$/H$^+$ dilation by injecting the vasoconstrictor U46619 (a thromboxane A2 receptor agonist) into the cNTS and ROb while measuring cardiorespiratory activity in anesthetized mice breathing a level of CO$_2$ required to maintain respiratory activity (2-3% CO$_2$). We found that injections of U46619 (1 μM) into the cNTS alone or together with the ROb resulted in unstable breathing as evidenced by frequent bouts of hyperventilation followed by apnea (Fig. 3A) (1.2 ± 0.6, vs. saline: 0.4 ± 0.2 apneas/min; $p < 0.001$) and increased breath to breath variability (Figs. 3C-E). Consistent with increased chemoreceptor drive, we found that application of U46619 into these regions lowered the CO$_2$ apneic threshold from 3.2 ± 0.3% to 2.1 ± 0.1% ($p < 0.05$; two-way RM ANOVA; $N = 7$) (Fig. 3F). Also, consistent with previous work (41), we found that injection of U46619 (1 μM) into the cNTS also increased systemic blood pressure (127 ± 11, vs. saline: 98 ± 4 mmHg, ($F_{3,65} = 77.62$; $p<0.01$, data not shown), whereas, application of this drug into the ROb alone had negligible effects on cardiorespiratory output. Together, these results show that CO$_2$/H$^+$ induced vasoconstriction in the RTN serves to enhance chemoreceptor drive, while simultaneous CO$_2$/H$^+$ dependent dilation in the cNTS and possibly the ROb limits chemoreceptor activity to stabilize CO$_2$/H$^+$ stimulated respiratory drive.

Smooth muscle specific P2Y$_2$ cKO mice have a blunted chemoreflex that can be rescued by targeted re-expression of P2Y$_2$ in RTN smooth muscle cells

To definitively test contributions of P2Y$_2$ receptors in vascular smooth muscle cells to respiratory activity, we created a smooth muscle cell specific P2Y$_2$ knockout mouse (P2Y$_2$ cKO
maintained on a C57BL6/J background) by crossing αSm22$^{cre}$ (JAX #: 017491) with P2Y$_2^{f/f}$ mice provided by Dr. Cheike Seye (Indiana Univ.). We confirmed that cre recombinase expression was restricted to vascular smooth muscle cells (Fig. 4A) and that P2Y$_2$ receptor transcript was not detectable in RTN smooth muscle cells from P2Y$_2$ cKO mice (Fig. 4A). Each genotype (αSm22$^{cre}$ only, P2Y$_2^{f/f}$ only and P2Y$_2$ cKO mice) was obtained at the expected ratios and gross motor activity, metabolic activity, blood gases, heart rate and blood pressure were all similar between genotypes and sexes (Table 1, Supplemental Fig. 2). Therefore, results from male and female mice of each genotype were pooled. To determine whether P2Y$_2$ cKO mice exhibit respiratory problems, we used whole-body plethysmography to measure baseline breathing and the ventilatory response to CO$_2$ in conscious unrestrained adult mice (mixed sex). Consistent with the possibly that smooth muscle P2Y$_2$ receptor-mediated vasoconstriction in the RTN augments the ventilatory response to CO$_2$, we found that P2Y$_2$ cKO mice exhibit normal respiratory activity under room air conditions (Table 1) but pronounced hypoventilation during exposure to graded increases in CO$_2$ (balance O$_2$ to minimize input from peripheral chemoreceptors). For example, minute ventilation – the product of frequency and tidal volume – of P2Y$_2$ cKO mice at 5% and 7% CO$_2$ was 28% and 35% smaller than control counterparts (Figs. 4C-F, Table 2). This chemoreceptor deficit involves a diminished capacity to increase both respiratory frequency (Fig. 4D) and tidal volume (Fig. 4E) during exposure to CO$_2$ (Table 2). Similar results were obtained in P2Y$_2$ cKO mice generated using a different smooth muscle cre line (smMHC$^{cre/eGFP}$) (Supplemental Fig. 3). Also, smooth muscle P2Y$_2$ cKO mice showed a normal ventilatory response to hypoxia (10% O$_2$; balance N$_2$, T$_7$=0.1089, p>0.05) (Supplemental Fig. 4). We confirmed in vitro that RTN arterioles in slices from P2Y$_2$ cKO do not respond to PBS 1114 (0.4 ± 0.1%, T$_7$ = 1661, p > 0.05, N=8 vessels). Interestingly, we also
found that RTN arterioles in slices from P2Y2 cKO mice fail to constrict in response to CO2/H+ or tACPD, but rather respond in a manner similar to arterioles in other brain regions. For example, both CO2/H+ and tACPD dilated RTN (CO2/H+: 4.7 ±0.2%, T9 = 3.312, p = 0.009, N=10 vessels; tACPD: 3.2 ± 0.7%, T7 = 2.864, p = 0.035, N = 8 vessels) and cortical (CO2/H+: 7.3 ± 1.5%, T0=4.307, p=0.0051, N=7 vessels; tACPD: 6.7 ± 0.9%, T0=2.739, p=0.0338, N=7 vessels) arterioles in slices from P2Y2 cKO mice (Supplemental Fig. 5A-B). This suggests CO2/H+ induced vasodilation is a general phenomenon mediated by undetermined mechanisms, but in the RTN is response is countered by smooth muscle P2Y2 receptor-mediated constriction.

We next tested whether targeted re-expression of P2Y2 in RTN smooth muscle cells would rescue the blunted chemoreflex in P2Y2 cKO mice. An adeno-associated virus (AAV2-Myh11p-eGFP-2A-mP2ry2, Vector Biolabs) was injected bilaterally into the RTN of adult P2Y2 cKO (αSm22cre/P2Y2f/f) to drive P2Y2 expression in smooth muscle cells. Two weeks after virus injection into the RTN region, we confirmed that ~80% of smooth muscle α2 actin (Acta2)-immunoreactive cells in the RTN are also GFP+ (Fig. 4B, Supplemental Fig. 6). Some background eGFP labeling was observed, but not associated with DAPI labeling (Supplemental Fig. 6aii). We also confirmed RTN smooth muscle cells from P2Y2 rescue mice show increased P2Y2 transcript levels compared to P2Y2 cKO (p=0.0073) but not to the same level as cells from control mice (p = 0.0219) (Fig. 4A). Importantly, re-expression of P2Y2 receptors in RTN smooth muscle cells resulted in a full rescue of the minute ventilatory response to CO2 (Fig. 4C-F, Table 2) (F1,7=17.75, p=0.004, N= 13 animals). Conversely, bilateral RTN injections of control virus (AAV2-Myh11p-eGFP, Vector Biolabs) minimally affected respiratory parameters of interest in P2Y2 cKO mice (Supplemental Fig. 3). Furthermore, re-expression of P2Y2
receptors in RTN smooth muscle cells also rescues CO$_2$/H$^+$ vascular reactivity. Specifically, we found in slices from P2Y$_2$ cKO mice, that RTN arterioles transfected with AAV showed a robust constriction in response to CO$_2$ (-19.9 ± 6.9%, p=0.0421), tACPD (-6.2 ± 1.9%, p=0.0092) and PSB1114 (-9.3 ± 4.9%, p=0.0154) (Supplemental Fig. 5). These results identify the first vascular element of respiratory control by showing that P2Y$_2$ receptors in RTN vascular smooth muscle cells are required for the normal ventilatory response to CO$_2$.

Discussion

These results identify P2Y$_2$ receptors in RTN smooth muscle cells as a novel vascular element of respiratory chemoreception. We show that CO$_2$/H$^+$ constriction by activation of smooth muscle P2Y$_2$ receptors is unique to the RTN and is required for the normal ventilatory response to CO$_2$, whereas simultaneous CO$_2$/H$^+$ vasodilation in other chemoreceptor regions like the cNTS and ROb may serve to stabilizes breathing. Considering these regions sense changes in CO$_2$/H$^+$ to regulate breathing, and since vasoconstriction and dilation are expected to increase and decrease tissue CO$_2$/H$^+$ levels respectively, we speculate that differential CO$_2$/H$^+$ vascular reactivity across multiple chemoreceptor regions serves to fine tune respiratory drive and stability during exposure to high CO$_2$. Understanding this novel mechanism may lead to new treatment options for breathing problems, particularly those associated with cardiovascular disease.

Experimental Limitations

There are several limitations of this work that should be recognized. First, our in vitro experiments utilized the slice preparation because it allows for easy visualization of vessels in each region of interest and pharmacological manipulation of candidate mechanisms independent
of potential confounding effects of blood pressure on myogenic tone. However, because blood vessels in brain slice have limited myogenic tone these experiments were performed in the presence of U-46619 to pre-constrict vessels ~30% (2). We also used a large stimulus (15% CO$_2$) to characterize CO$_2$/H$^+$ vascular reactivity *in vitro*. For these reasons, we also confirmed *in vivo* in the absence of U-46691 that more physiological levels of CO$_2$ (9-10%) constricted arterioles in the RTN by a P2Y$_2$-dependent mechanism. Second, we and others (41) showed that administration of U46619 into the NTS increased blood pressure. Although baroreceptor activation has a fairly mild effect on respiratory activity (28), it remains possible that the baroreflex contributed to respiratory instability observed during this experimental manipulation. Furthermore, neurons and astrocytes may express thromboxane A2 receptors (12, 30) so it is possible respiratory instability caused by injections of U46619 into the cNTS and ROB may involve direct neural or astrocyte activation. Third, CO$_2$/H$^+$ typically increases minute ventilation by increasing both rate and depth of breathing (36, 37), yet ventral surface application of drugs to manipulate P2Y$_2$ receptors only affected tidal volume (*Figs. 2E-J*). Therefore, it is possible other nearby respiratory centers were affected in this experiment. However, this issue is mitigated by more targeted genetic approaches showing that deletion of P2Y$_2$ from smooth muscle cells blunted the rate and depth of respiratory responses to CO$_2$, and re-expression of P2Y$_2$ receptors only in RTN smooth muscle cells fully rescued the CO$_2$/H$^+$ chemoreflex.

**Mechanisms contributing to vascular control of breathing**

Blood flow in the brain is controlled by several mechanisms including neural activity, which leads to vasodilation and increased blood flow by a process termed neurovascular coupling (21). Autoregulation is a mechanism that maintains cerebral blood flow in response to changes in
perfusion pressure (32). For example, myogenic tone increases with increased perfusion pressure and relaxes with decreased pressure. CO$_2$/H$^+$ also functions as a potent vasodilator in most brain regions (19), and is perhaps the most potent regulator of cerebrovascular tone since high CO$_2$/H$^+$ can impair both autoregulation (31, 34) and neurovascular coupling (25, 26). Since CO$_2$/H$^+$ is a waste product of metabolism, CO$_2$/H$^+$ vascular reactivity provides a means of matching local blood flow with tissue metabolic needs. However, at the level of the RTN this need appears secondary to chemoreceptor regulation of respiratory activity. For example, we (18) and others (40) showed that exposure to high CO$_2$/H$^+$ constricted RTN arterioles, and disruption of this response blunted the ventilatory response to CO$_2$.

The cellular and transmitter basis for CO$_2$/H$^+$ induced constriction of RTN arterioles appears to involve CO$_2$/H$^+$-evoked ATP release from local astrocytes. For example, previous work showed that i) CO$_2$/H$^+$ caused discrete release of ATP near the RTN region (15); ii) astrocytes in the RTN show Ca$^{2+}$ responses to mild acidification (14); and iii) activation of astrocytes with tACPD, an mGluR agonist widely used to elicit Ca$^{2+}$ elevations in astrocytes (20), constricted RTN arterioles by a purinergic-dependent mechanism (18) (Fig. 2B), whereas application of an ATP receptor blocker to the ventral surface blunted CO$_2$/H$^+$ induced vasoconstriction and the ventilatory response to CO$_2$ (18). Here, we extend this work by showing that CO$_2$/H$^+$ vascular responses in the RTN are opposite to other chemoreceptor regions, and we identify P2Y$_2$ receptors in vascular smooth muscle cells as requisite determinants of RTN CO$_2$/H$^+$ vascular reactivity and chemoreception. For example, P2Y$_2$ is a metabotropic receptor that couples with Gq, Go and G12 proteins (8); activation of this receptor in smooth muscle is associated with vasoconstriction (3, 23), whereas its activation in endothelial cells favors nitric oxide-mediated
vasodilation (27). Consistent with a role in vasoconstriction, we found that P2Y2 transcript was expressed at higher than control levels in RTN smooth muscle cells and at lower than control levels in RTN endothelial cells (Figs. 1A-B). Furthermore, pharmacological experiments show *in vitro* (Figs. 1C-E) and in anesthetized mice (Figs. 2A-B) that CO2/H+ -induced constriction of RTN arterioles was eliminated by blocking P2Y2 receptors with AR-C118925 and mimicked by a selective P2Y2 receptor agonist (PSB 1114). We also show in awake mice that genetic deletion of P2Y2 from smooth muscle cells blunted the ventilatory response to CO2, and re-expression of P2Y2 receptors only in RTN smooth muscle cells fully rescued the CO2/H+ chemoreflex (Figs. 4A-F). These results establish P2Y2 receptors in RTN smooth muscle cells as requisite determinants of respiratory chemoreception.

It should be noted that other cell types and signaling pathways may also contribute to RTN vascular reactivity. For example, endothelial cells including those in the RTN, express H+ activated GPR4 receptors that when activated facilitates release of vasoactive effectors including thromboxane to mediate vasoconstriction (40). Consistent with this, disruption of GPR4 signaling blunted CO2/H+ vascular reactivity in the RTN and the ventilatory response to CO2 (40). Although untested, it is also possible RTN endothelial cells release other vasoactive signals including ATP, and so may contribute to CO2/H+ -evoked purinergic-dependent constriction of arterioles in this region. Prostaglandin E2 (PGE2) is also thought to contribute to CO2/H+ vascular reactivity. For example, in the cortex CO2/H+ facilitates Ca2+ oscillations in astrocytes (14, 20) leading to enhanced arachidonic acid metabolism and release of PGE2, which triggered arteriole dilation by activation of prostaglandin (EP1) receptors (20). In the RTN, CO2/H+ also causes PGE2 release most likely from astrocytes (11) and contributes to RTN chemoreception by
a mechanism involving EP3 receptors (10). However, selective blockade of EP3 receptors minimally affected CO$_2$/H$^+$ constriction of RTN arterioles (Supplemental Fig. 1B), suggesting PGE$_2$/EP3 signaling contributes to RTN chemoreception directly by modulation of chemosensitive neurons.

In contrast to the RTN, we also found that CO$_2$/H$^+$ (Fig. 1C) and astrocyte activation by bath application of t-ACPD (Fig. 1F) dilated arterioles in other chemoreceptor regions including the cNTS and ROb. This suggests that CO$_2$/H$^+$-dependent regulation of vascular tone and roles of astrocytes in this process are fundamentally different in the RTN compared to other chemoreceptor regions. Considering vasoconstriction and dilation are expected to increase and decrease tissue CO$_2$/H$^+$ levels respectively, we propose that CO$_2$/H$^+$ constriction in the RTN supports the drive to breathe by ensuring tissue acidification is maintained to stimulate chemoreceptors in this region, whereas CO$_2$/H$^+$ dilation in the cNTS and ROb provides a means of buffering tissue H$^+$ and chemoreceptor activity to maintain respiratory stability. Conversely, if CO$_2$/H$^+$ were to cause vasoconstriction across multiple chemoreceptor regions simultaneously, this may exaggerate the ventilatory response to CO$_2$ to the point of causing unstable periodic breathing (5). Indeed, this is thought to be the cause of unstable breathing in patients with global deficits in CO$_2$/H$^+$ vascular reactivity due to congestive heart failure or cerebrovascular disease (4, 5). Consistent with this, we show that disruption of CO$_2$/H$^+$ dilatation in the cNTS and ROb by application of the vasoconstrictor U46619 while leaving RTN CO$_2$/H$^+$ constriction unperturbed, increased chemoreceptor drive as evidenced by a decrease in the CO$_2$/H$^+$ apneic threshold and caused unstable periodic breathing during exposure to higher levels of CO$_2$/H$^+$ (Fig. 3). However, further work is needed to understand whether and how differential CO$_2$/H$^+$
vascular reactivity in these chemoreceptor regions contributes to breathing problems in disease states.

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AUTHOR CONTRIBUTIONS

CMC: experimental design; collection and analysis of data; revising the manuscript; final approval of the manuscript.

TSM: experimental design; collection and analysis of data; revising the manuscript; final approval of the manuscript.

ACT: collection and analysis of data; revising the manuscript; final approval of the manuscript.

MTN: experimental design; final approval of the manuscript.

TAL: experimental design; collection and analysis of data; revising the manuscript; final approval of the manuscript.

DKM: experimental design; data analysis; drafting the manuscript; revising the manuscript, final approval of the manuscript.

DECLARATION OF INTERESTS

Nothing to declare
Figure legends

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 (Tie2Cre::TdTomato) and smooth muscle cell reporter mice (smMHCCre::eGFP). Individual cells 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 (Supplemental Table 1) 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 P2Y2 showed an expression pattern that was 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), activation of P2Y2 receptors by bath application of PSB1114 (100 µM) (Ei), or activation of astrocytes by bath application of t-ACPD (50 µM) (Fi) 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 (paired 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 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 CO₂/H⁺ dilation in the cNTS and ROb causes unstable breathing and apnea. A, trace of external intercostal muscle EMG (IntEMG) activity shows respiratory activity of an anesthetized wild type mouse breathing 2.5% CO₂ following injections of saline or U46619 (1 µM; 30 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 (TTOT) 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 IntEMG frequency (C) and IntEMG frequency (E). F, Summary data show that injections of U46619 injections into the cNTS and ROb lowered the CO2 apneic threshold from 3.2 ± 0.3% to 2.1 ± 0.1% (N = 7 mice). *, difference in IntEMG activity under control conditions (saline) vs. during U46119 into the NTS and/or ROb (RM-ANOVA followed by Bonferroni multiple-comparison test, p < 0.05). scale bar = 200 μm.

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 (αSm22Cre::TdTomato), P2Y2 cKO mice (αSm22Cre::P2Y2fl/fl::TdTomato), and P2Y2 rescue mice (P2Y2 cKO animals that received bilateral RTN injections of AAV2-Myh11p-eGFP-2A-mP2ry2). P2Y2 transcript was not detected (n.d.) in smooth muscle cells from P2Y2 cKO mice (N=3 runs/9 animals). P2Y2 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 P2Y2 transcript levels 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 (33) is indicated by numbers next to each section. Right, two weeks after injections we confirmed that ~80% of RTN smooth muscle α2 actin (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 (αSm22^{Cre::P2Y2^{f/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 αSm22^{Cre} only and P2Y2^{f/f} only control mice showed similar baseline breathing and responses to 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).

Table 1. Respiratory parameters in control (αSm22^{Cre} only and P2Y2^{f/f} only), P2Y2 cKO (αSm22^{Cre::P2Y2^{f/f}}) and P2Y2 rescue animals two weeks after bilateral RTN injections of AAV2-Myh11p-eGFP-2A-mP2ry2. No significant differences were observed ( p> 0.05).

Table 2. Cardiorespiratory and metabolic parameters under room air conditions in control and P2Y2 cKO mice. *, difference from 0% CO2; #, differences between genotypes under an experimental condition (two-way ANOVA with Tukey’s multiple comparison test). Two symbols = p < 0.01, three symbols = p < 0.001, four symbols = p < 0.0001.
# Key Resources Table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Strain, strain background (M. musculus, Tie2-Cre, C57BL6/J background) | B6.Cg-Tg(Tek-cre)1Ywa/J | Jackson Laboratories | RRID:IMSR_JAX:008863 |  |
| Strain, strain background (M. musculus, smMHC-Cre/eGFP, C57BL6/J background) | B6.Cg-Tg(Myh11-cre,-EGFP)2Mik/J | Mus Musculus | Jackson Laboratories | RRID:IMSR_JAX:007742 |
| Strain, strain background (aSM22-Cre, C57BL6/J background) | B6.Cg-Tg(Tagln-cre)1Her/J | Mus musculus | Jackson Laboratories | RRID:IMSR_JAX:017491 |
| Strain, strain background (TdT tomato reporter Ai14, C57BL6/J background) | B6.Cg-Gt(ROSA)26Sorim9(CAG-tdTomato)Hze/J | Mus musculus | Jackson Laboratories | RRID:IMSR_JAX:007909 |
| Strain, strain background (P2Y2 floxed, C57Bl6/J background) | P2Y2ff | PMID: 27856454 | Gifted by Dr. Cheike Seye (Indiana Univ.) |  |
| Transfected construct (M. musculus) | AAV2-Myh11p-eGFP-2A-mP2ry2 | This paper |  |  |
| Transfected construct \((M. \textit{musculus})\) | AAV2-\textit{Myh11p}-\textit{eGFP} | This paper | |
|---|---|---|---|
| Genetic reagent (TaKaRa TaqTM DNA Polymerase) | Taq polymerase | Promega | R001A |
| Antibody | mouse anti- α smooth muscle actin | Sigma | A5228 | 1:100 dilution |
| Sequence-based reagent | Taqman probe \textit{Gapdh} | ThermoFisher | Mm99999915_g1 |
| Sequence-based reagent | Taqman probe \textit{Rhox3} | ThermoFisher | Mm01248771_m1 |
| Sequence-based reagent | Taqman probe \textit{Aldh111} | ThermoFisher | Mm03048957_m1 |
| Sequence-based reagent | Taqman probe \textit{Acta2} | ThermoFisher | Mm00725412_s1 |
| Sequence-based reagent | Taqman probe \textit{Flt1} | ThermoFisher | Mm00438980_m1 |
| Sequence-based reagent | Taqman probe \textit{P2rx1} | ThermoFisher | Mm00435460_m1 |
| Sequence-based reagent | Taqman probe \textit{P2rx2} | ThermoFisher | Mm00462952_m1 |
| Sequence-based reagent | Taqman probe \textit{P2rx3} | ThermoFisher | Mm00523699_m1 |
| Sequence-based reagent | Taqman probe \textit{P2rx4} | ThermoFisher | Mm00501787_m1 |
| Sequence-based reagent | Taqman probe $P_{2rx5}$ | ThermoFisher | Mm00473677_m1 |
|------------------------|-------------------------|--------------|----------------|
| Sequence-based reagent | Taqman probe $P_{2rx6}$ | ThermoFisher | Mm00440591_m1 |
| Sequence-based reagent | Taqman probe $P_{2rx7}$ | ThermoFisher | Mm01199500_m1 |
| Sequence-based reagent | Taqman probe $P_{2ry1}$ | ThermoFisher | Mm02619947_s1 |
| Sequence-based reagent | Taqman probe $P_{2ry2}$ | ThermoFisher | Mm02619978_s1 |
| Sequence-based reagent | Taqman probe $P_{2ry4}$ | ThermoFisher | Mm00445136_s1 |
| Sequence-based reagent | Taqman probe $P_{2ry6}$ | ThermoFisher | Mm02620937_s1 |
| Sequence-based reagent | Taqman probe $P_{2ry12}$ | ThermoFisher | Mm01950543_s1 |
| Sequence-based reagent | Taqman probe $P_{2ry13}$ | ThermoFisher | Mm01950543_s1 |
| Sequence-based reagent | Taqman probe $P_{2ry14}$ | ThermoFisher | Mm01952477_s1 |
| Chemical compound, drug | Papain | Sigma | P4762 |
| Chemical compound, drug | 1,4-Dithioerythritol | Sigma | D8255 |
| Chemical compound, drug | Collagenase IV | ThermoFisher | 1714019 |
|-------------------------|----------------|--------------|---------|
| Chemical compound, drug | Bovine Serum Albumin | Sigma | A2153 |
| Chemical compound, drug | Trypsin Inhibitor | Sigma | T9253 |
| Chemical compound, drug | U46619 | Tocris | 1932 |
| Chemical compound, drug | Trans-ACPD | Tocris | 0187 |
| Chemical compound, drug | AR-C 118925XX | Tocris | 4890 |
| Chemical compound, drug | PSB 1114 | Tocris | 4333 |
| Chemical compound, drug | POM 1 | Tocris | 2689 |
| Chemical compound, drug | 1,3-dimethyl-8-phenyl-xantine (8-PT) | Sigma | P2278 |
| Chemical compound, drug | Prostaglandin E2 | Cayman Chemical | 14010 |
| Chemical compound, drug | L-798, 106 | Cayman Chemical | 11129 |
| Chemical compound, drug | Papaverine | Sigma | P3510 |
| Chemical compound, drug | Diltiazem | Tocris | 0685 |
### Commercial assay, kit
- Taqman Gene Expression Cells-to-CT kit
  - ThermoFisher
  - AM1728

### Commercial assay, kit
- Taqman Fast Advanced Master Mix
  - ThermoFisher
  - 4444557

### Software, algorithm
- ImageJ
  - NIH
  - RRID:SCR_003070
  - Version 2.0.0

### Software, algorithm
- Macro (for vessel analysis)
  - PMID: 28387198

### Software, algorithm
- Spike
  - Cambridge Electronic Design
  - RRID:SCR_000903
  - Version 2.0

### Software, algorithm
- Ponemah
  - DSI
  - RRID:SCR_017107
  - Version 5.23

### Software, algorithm
- QuantStudio Design and Analysis
  - ThermoFisher
  - RRID:SCR_018712
  - Version 1.5.1

### Software, algorithm
- Prism 7
  - GraphPad
  - RRID:SCR_011323
  - Version 7.03

### Other
- Griffonia Simplicifolia Lectin I isolectin B4, Dylight 594
  - Vector Laboratories
  - SL-1207-.5

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**Animals**

All procedures were performed in accordance with National Institutes of Health and University of Connecticut Animal Care and Use Guidelines. All experiments used mixed sex C57BL6/J animals at least 3 weeks of age that housed in a 12:12 light dark cycle with normal chow ad
The following transgenic cre mouse lines were used to perform single cell isolation, FACS, and pooled qPCR: smMHC\textsuperscript{Cre/eGFP} (Jax Stock #: 007742) and Tie2-Cre/TdTomato (Jax Stock #: 008863, 007909), and αSm22-Cre/TdTomato (Jax Stock #: 017491, 007909). αSm22-Cre and smMHC\textsuperscript{Cre/eGFP} mice were crossed with P2Y\textsubscript{2} floxed mice (gifted by Dr. Cheike Seye at Indiana University) to generate double floxed smooth muscle specific P2Y\textsubscript{2} cKO mice for \textit{in vitro} and \textit{in vivo} experimentation.

\textit{In vitro} arteriole slice recordings

\textit{Brainstem and Cortical Slice Preparation}

Animals (P21 and older) were decapitated under isoflurane anesthesia and transverse brainstem slices (150 μm) were prepared using a vibratome in ice-cold substituted artificial cerebrospinal fluid (aCSF) solution containing (in mM): 130 NaCl, 3 KCl, 2 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, 10 glucose. 0.4 mM L-ascorbic acid was added into aCSF while slicing. Slices were incubated for 30 minutes at 37°C and subsequently at room temperature in aCSF, equilibrated with a 5% CO\textsubscript{2}-95% O\textsubscript{2} gas mixture. Prior to imaging, each slice was incubated for thirty-five minutes with 6 mg/mL DyLight 594 Isolectin B4 conjugate (Vector Labs) to label vascular endothelium as previously described\textsuperscript{(18)}.

\textit{Imaging arterioles in vitro}

Individual brain slices containing either the caudal NTS (cNTS), raphe obscurus (ROb), RTN, or somatosensory cortex were transferred to a Plexiglas recording chamber mounted on a fixed-stage upright fluorescent microscope (Zeiss Axioskop FS) and perfused with 37°C aCSF bubbled with a 5% CO\textsubscript{2}-21% O\textsubscript{2} (balance N\textsubscript{2}). Hypercapnic solutions were made by allowing aCSF to
equilibrate with a 15% CO\textsubscript{2}-21% O\textsubscript{2} (balance N\textsubscript{2}). Arterioles were identified based on clear evidence of vasomotion under IR-DIC microscopy and bulky fluorescent labeling that indicates a thick layer of tightly wrapped smooth muscle surrounding the vessel lumen. Precapillary arterioles (as indicated by more sporadic, less uniform smooth muscle cell layer and thinner luminal diameter) were excluded from experimentation. All arterioles selected for experimentation had a luminal diameter between 8-50 µm. It should be noted that most, if not all, of the cNTS arterioles were small (average diameter was 9 µm) and ROb vasculature did not vary more than 10 µm from the midline of the slice. RTN vessels were located within 200 µm of the ventral surface and below the caudal end of the facial motor nucleus. Neocortical vessels were located in layers 1-5.

For an experiment, fluorescent images were acquired at a rate of 1 frame every 20 seconds using a 40X water objective lens, a Clara CCD Andor camera, and the NIS Advanced Research software suite (Nikon). To induce a partially constricted state in arterioles, we continuously bath applied 125 nM of a thromboxane A2 receptor agonist (U46619). U46619 has been previously shown to induce a 20-30% vasoconstriction at a concentration of 125 nM, enabling the vessel to either dilate or constrict further under experimental pharmacological studies (13). As previously described (18), we assessed arteriole viability at the end of each experiment by inducing a large vasoconstriction with a 60 mM K\textsuperscript{+} solution and then a large vasodilation with a Ca\textsuperscript{2+} free solution containing EGTA (5 mM), a phosphodiesterase inhibitor (papaverine, 200 mM), and an L-type Ca\textsuperscript{2+} channel blocker (diltiazem, 50 mM). Only one vessel was recorded per experiment and slice. Any vessel that did not respond to these solutions were excluded in data analysis. The
list of all drugs and concentrations used for in vitro and in vivo experimentation are detailed in
578 **Key Resource Table** and text where appropriate.

579

580

**Image Analysis**

581 Vessel diameter was determined using ImageJ. All images were calibrated, and pixel distance
582 was converted to millimeters. Data files underwent StackReg (Biomedical Imaging Group) to
583 stack each image over time and then three linear region of interests (ROI) were drawn orthogonal
584 to the vessel. A macro (available at https://github.com/omsai/blood-vessel-diameter [Nanda,
585 2017]) was used to determine peak to peak distance using fluorescence intensity profile plots for
586 all slices of the data file.

587

588

**Single cell isolation and qRT-PCR**

589 At least three and no greater than eight, animals (postnatal days P21-P40) were used of either of
590 the following genotypes: smMHC\textsuperscript{Cre/\textsc{eGFP}}, Tie2-Cre/TdTomato, \(\alpha\text{Sm22-Cre/TdTomato, or}\)
591 \(\alpha\text{Sm22-Cre/TdTomato/P2Y}^2_{\text{fl/fl}}\). Animals were euthanized under isoflurane anesthesia and
592 brainstem slices were prepared using a vibratome in ice cold, high sucrose slicing solution
593 containing (in mM): 87 NaCl, 75 sucrose, 25 glucose, 25 NaHCO\textsubscript{3}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 2.5 KCl, 7.5
594 MgCl\textsubscript{2}, 0.5 mM CaCl\textsubscript{2} and 5 L-ascorbic acid. Slicing solution was equilibrated with a 5% CO\textsubscript{2}-
595 95% O\textsubscript{2} gas mixture before use. Transverse brainstem slices (400 \(\mu\)m thick) were prepared and
596 then immediately enzymatically treated at 34\degree C with an initial incubation in a papain (6 mg/mL,
597 Sigma) and dithioerythritol (10 mg/mL, ThermoFisher) mixture for 30 minutes in sucrose
598 dissociation solution containing (in mM): 185 sucrose, 10 glucose, 30 Na\textsubscript{2}SO\textsubscript{4}, 2 K\textsubscript{2}SO\textsubscript{4}, 10
599 HEPES, 0.5 CaCl\textsubscript{2}, 6 MgCl\textsubscript{2}, 5 L-ascorbic acid, pH 7.4, 320 mOsm, followed by a collagenase
IV (10 mg/mL, ThermoFisher) enzyme treatment for 6 minutes. After enzyme incubation, slices were washed three times in cold dissociation solution and then transferred to an enzyme inhibitor mix containing trypsin inhibitor (10 mg/mL, Sigma), bovine serum albumin (BSA, 10 mg/mL, Sigma), and sodium nitroprusside (SNP, 2 mg/mL, ThermoFisher) in cold sucrose dissociation solution. Shortly thereafter, slices were transferred to a glass Petri dish on ice. Using a plastic transfer pipette and a 15 blade scalpel, each region of interest was microdissected out of the slices and manually separated into sterile microcentrifuge tubes. The control samples were made up of two slices: one slice with the NTS removed and the other with the ROB and RTN removed. Once microdissection is completed, the tissue chunks were warmed to 34°C for 10 minutes before trituration. A single cell suspension was achieved by trituration using a 25G and 30G needle sequentially, attached to a 3 mL syringe. Samples were triturated for an average of 5 minutes. Immediately after, the samples were placed back on ice and filtered through a 30-micron filter (Miltenyi Biotech) into round bottom polystyrene tube for fluorescence-activated cell sorting (FACS).

*Florescence-activated cell sorting (FACS)*

All cell types of interest were sorted on a BD FACSria II Cell Sorter (UConn COR²E Facility, Storrs, CT) equipped with 407 nm, 488 nm, and 607 nm excitation lasers. Five minutes before sorting, 5 µL of 100 ng/mL DAPI was added to each sample. Cells were gated based on scatter (forward and side), for singlets, and for absence of DAPI. Finally, cells were gated either to TdTomato or GFP fluorescence and sorted by 4-way purity into a sterile 96-well plate containing 5 µL of sterile PBS per sample. Between 100 and 500 cells were sorted per sample in any experiment and were processed immediately following FACS. To control for RNA
contamination in FACS droplets, allophycocyanin (APC) beads were sorted based on green fluorescence and were treated alongside experimental samples (data not shown). See Supplemental Figure 5 for FACS gating parameters.

Pooled cell qRT-PCR

A lysis reaction followed by reverse transcription was performed using the kit Taqman Gene Expression Cells-to-CT Kit (ThermoFisher) with ‘Lysis Solution’ followed by the ‘Stop Solution’ at room temperature, and then a reverse transcription with the ‘RT Buffer’, ‘RT Enzyme Mix’, and lysed RNA at 37°C for an hour. Following reverse transcription, cDNA was pre-amplified by adding 2 uL of cDNA from each sample to 8 uL of preamp master mix [5 uL TaKaRa premix Taq polymerase (Clontech), 2.5 uL 0.2X Taqman pooled probe, 0.5 uL H₂O] and thermocycled at 95°C for 3 minutes, 55°C for 2 minutes, 72°C for 2 minutes, then 95°C for 15 seconds, 60°C for 2 minutes, 72°C for 2 minutes for 16-20 cycles, and then a final 10°C hold. Amplified cDNA was then diluted 2:100 in RNase free H₂O. Each qPCR assay contained the following reagents: 0.5 uL 20X Taqman probe, 2.5 uL RNase free H₂O, 5 uL Gene Expression Master Mix or Fast Advanced Master Mix (ThermoFisher), and 2 uL diluted pre-amplified cDNA. qPCR reactions were performed in triplicate for each Taqman assay of interest on a QuantStudio 3 Real Time PCR Machine (ThermoFisher).

qRT-PCR Data Analysis

All three technical replicates were averaged to create one raw Ct values per Taqman assay. As a control, all samples were subject to cell markers of various cell types to confirm specificity: Rbfox3 for neurons, Aldh1l1 for astrocytes, Acta2 for smooth muscle cells, and Flt1 for
endothelial cells (Supplemental Table 1). Any assay that did not give a discrete Ct value was given a Ct value of 40 for analysis. Fold change was determined by using the equation $2^{(\Delta\Delta Ct)}$. 

*Gapdh* was used as a sample dependent internal control. Fold changes for all runs of each cell type were averaged; the log$_2$ of the fold change was calculated, analyzed, and plotted on bar graphs.

In vivo anesthetized preparation

Animal use was in accordance with guidelines approved by the University of São Paulo Animal Care and Use Committee. A total of 26 adult male C57BL6 (25-28 g) were used for in vivo experiments. General anesthesia was induced with 5% isoflurane in 100% O2. A tracheostomy was made, and the isoflurane concentration was reduced to 1.4-1.5% until the end of surgery. The carotid artery was cannulated (polyethylene tubing, 0.6 mm o.d., 0.3 mm i.d., Scientific Commodities) for measurement of arterial pressure (AP). The jugular vein was cannulated for administration of fluids and drugs. Rats were placed supine onto a stereotaxic apparatus (Type 1760; Harvard Apparatus) on a heating pad and core body temperature was maintained at a minimum of 36.5°C via a thermocouple. The trachea was cannulated. Respiratory flow was monitored via a flow head connected to a transducer (GM Instruments) and CO$_2$ via a capnograph (CWE, Inc.) connected to the tracheal tube. Paired EMG wire electrodes (AM-System) were inserted into the external intercostal muscle to record respiratory-related activity. After the anterior neck muscles were removed, a basio-occipital craniotomy exposed the ventral medullary surface, and the dura was resected. After bilateral vagotomy, the exposed tissue around the neck and the mylohyoid muscle was covered with mineral oil to prevent drying. Baseline parameters were allowed to stabilize for 30 min prior to recording.
In vivo recordings of physiological variables

Mean arterial pressure (MAP), external intercostal muscle activity (IntEMG) and end-expiratory CO₂ (etCO₂) were digitized with a micro1401 (Cambridge Electronic Design), stored on a computer, and processed off-line with version 7 of Spike 2 software (Cambridge Electronic Design). Integrated intercostal activity (∫IntEMG) was collected after rectifying and smoothing (τ = 0.03) the original signal, which was acquired with a 300-3000 Hz bandpass filter. Noise was subtracted from the recordings prior to performing any calculations of evoked changes in EMG. A direct physiological comparison of the absolute level of EMG activity across nerves is not possible because of non-physiological factors (e.g., muscle electrode contact, size of muscle bundle) and the ambiguity in interpreting how a given increase in voltage in one EMG relates to an increase in voltage in another EMG. Thus, muscle activity was defined according to its baseline physiological state, just prior to their activation. The baseline activity was normalized to 100%, and the percent change was used to compare the magnitude of increases or decreases across muscle from those physiological baselines.

In vivo experimental protocol

Each in vivo experiment began by testing responses to hypercapnia by adding CO₂ to the breathing air supplied by artificial ventilation. The addition of CO₂ was monitored to reach a maximum end-expiratory CO₂ between 9 and 10%, which corresponds with an estimated arterial pH of 7.2 based on the following equation: pHa = 7.955 - 0.7215 \times \log_{10} (EtCO₂). This maximum end-expiratory CO₂ was maintained for 5 min and then replaced by 100% O₂.
To determine whether local regulation of vascular tone in the region of the RTN contributes to the \( \text{CO}_2/\text{H}^+ \)-dependent drive to breathe, we made injections of saline, PSB1114 (100 \( \mu \)M), AR-C118925 (1 mM) or U46619 (1 \( \mu \)M) while monitoring IntEMG amplitude and frequency. These drugs were diluted with sterile saline (pH 7.4) and applied using single-barrel glass pipettes (tip diameter of 25 \( \mu \)m) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp, Cleveland, OH). For each injection, we delivered a volume of 30 nL over a period of 5s.

Injections in the VMS region were placed 1.9 mm lateral from the basilar artery, 0.9 mm rostral from the most rostral hypoglossal nerve rootlet, and at the VMS. The second injection was made 1-2 min later at the same level on the contralateral side. For injections located at the cNTS or ROb, we used the following coordinates: a) cNTS: 0.2-0.3 mm rostral to the calamus scriptorius, 0.3 mm lateral to midline, and 0.3 mm below the dorsal surface of the brainstem and ROb: 1.2-1.3 mm caudal to the parietal-occipital suture, in the midline, and 5-5.3 mm below the cerebelar surface.

A cranial optical window was prepared using standard protocols. For the VMS, the anterior neck muscles were removed, a basio-occipital craniotomy exposed the ventral medullary surface, and the dura was resected. Pial vessels in the VMS were located 1.9 mm lateral from the basilar artery and 0.9 mm rostral to the most rostral portion of the hypoglossal nerve rootlet. The surface of the VMS was cleaned with buffer containing (in mmol/L) the following: 135 NaCl, 5.4 KCl, 1 \( \text{MgCl}_2 \), 1.8 CaCl\(_2\), and 5 HEPES, pH 7.3., and a chamber (home-made 1.1-cm-diameter plastic ring was glued with dental acrylic cement attached to a baseplate). The chamber was sealed with a circular glass coverslip (#1943-00005, Bellco). The baseplate was affixed to the Digital
Camera (Sony, DCR-DVD3-5) and a light microscope was used for vessel imaging (x40 magnification).

**In vivo pial vessel imaging**

*Animal Preparation*

Mixed sex adult mice (>6 weeks of age) were briefly anesthetized with isoflurane (1-3%) followed with an IP injection of 300-500mg urethane to ensure a deep anesthetic state. The animal was then fixed to stereotaxic ear bars with the ventral side of the animal facing up. The trachea of the animal was then cannulated with a 18G needle to provide 1.5 mL ventilations at a rate of 150 breaths per minute of 5% CO$_2$-21% O$_2$ (balance N$_2$) via artificial ventilator (Kent Scientific). After cannulation, deep neck muscles were resected, and the cranial-pharyngeal canal and dura mater of the animal was removed. A digital camera was placed over the ventral surface and focused on the pial vasculature. Animals that did not have well perfused pial vessels were not included in experimentation. Animals that were excessively bleeding from the surgical site were sacrificed and not included in any analyses.

*In vivo Image Analysis*

Three linear ROIs were drawn over the pial vasculature we recognize as supplying blood flow to the RTN, similar to the *in vitro* methods described above. Bright field imaging was analyzed using a macro in ImageJ. Briefly, the macro measured the drop and rise of the local maxima and minima, correlating to vessel boundaries. These boundaries were then used to make a linear measurement between the two points which was vessel diameter.
**RTN Viral Injections**

Adult mice (>20 grams) were anesthetized with 3% isoflurane. The right cheek of the animal was shaved, and an incision was made to expose the right marginal mandibular branch of the facial nerve. The animals were then placed in a stereotaxic frame and a bipolar stimulating electrode was placed directly adjacent to the nerve. Animals were maintained on 1.5% isoflurane for the remainder of the surgery. An incision was made to expose the skull and two 1.5 mm holes were drilled left and right of the posterior fontanelle, caudal of the lambdoidal suture. The facial nerve was stimulated using a bipolar stimulating electrode to evoke antidromic field potentials within the facial motor nucleus. In this way, the facial nucleus on the right side of the animal was mapped in the X, Y, and Z direction using a quartz recording electrode.

The viral vector was loaded into a 1.2 mm internal diameter borosilicate glass pipette on a Nanoject III system (Drummond Scientific). Virus was injected at least -0.02 mm ventral to the Z coordinates of the facial nucleus, to ensure injection into the RTN. These same coordinates were used for the left side of the animal. In all mice, incisions were closed with nylon sutures and surgical cyanoacrylate adhesive. Mice were placed on a heated pad until consciousness was regained. Meloxicam was administered 24 and 48 h postoperatively.

**Telemetry Transmitter Placement**

Adult mice (>30 grams) were anesthetized with an induction dose of 3% isoflurane and placed into a sterile field. HD-X11 transmitters (Data Sciences International; DSI) were placed and fixed intraperitoneally with non-absorbable nylon sutures, followed by ECG lead placement on the left and right pectoralis on top of the rib cage. Finally, a pressure catheter was placed into the
left carotid artery and threaded through to the aortic arch for continuous blood pressure measurements. Animals were closed with nylon sutures and placed on a heated pad unit conscious. Mice were continuously watched for the next three days for any post-operative pain complications while meloxicam was administered 24 and 48 h postoperatively. Mice were not used for any experimentation until post-operative day 7.

Unrestrained whole-body plethysmography

Respiratory activity was measured using a whole-body plethysmograph system (Data Scientific International; DSI), utilizing animal chamber maintained at room temperature and ventilated with air (1.3 L/min) using a small animal bias flow generator. Mice were individually placed into a chamber and allowed 1 hr to acclimate prior to the start of an experiment. Respiratory activity was recorded using Ponemah 5.32 software (DSI) for a period of 15 min in room air followed by exposure to graded increases in CO₂ from 0% to 7% CO₂ (balance O₂). In separate experiments, we characterized the ventilatory response to 10% O₂ (balance N₂). Parameters of interests include respiratory frequency (F<sub>R</sub>, breaths per minute), tidal volume (V<sub>T</sub>, measured in mL; normalized to body weight and corrected to account for chamber and animal temperature, humidity, and atmospheric pressure), and minute ventilation (V<sub>E</sub>, mL/min/g). A 20 second period of relative quiescence after 5 min of exposure to each condition was selected for analysis. All experiments were performed between 9 a.m. and 6 p.m. to minimize potential circadian effects.

Comprehensive Lab Animal Monitoring (CLAMS)

Metabolic monitoring (VCO₂, VO₂) was performed using comprehensive lab animal monitoring systems (CLAMS, Columbus Instruments). In short, mice were individually housed on a 12:12
light dark cycle in plastic cages with a running wheel, regular bedding, and regular chow for one week before experimentation. Three days before the metabolic experiment, each animal was placed in the CLAMS housing cage with metered water and waste collection. Mice had two days to acclimate to the metabolic chamber; on the third day, all results were recorded for a continuous 24-hour period. After data collection, all raw results were exported and averaged out per hour. Then, light and dark periods were determined and averaged per animal for statistical analysis. Both sexes were equally represented in the data set.

**Blood Gas Analysis**

Arterial blood gasses were collected from adult mice 6 weeks of age and older (>30 grams). A RAPIDLab® 348 blood gas analyzer (Siemens) was used for all blood gas analysis; all calibrations, QC, and use were performed as indicated by the manufacturer. Animals were anesthetized with an induction dose of 3% isoflurane and then quickly switched to 1% isoflurane for the remainder of arterial blood collection. This level of isoflurane minimally affects blood gases (7, 24). The left carotid artery was exposed and quickly cannulated to allow for arterial blood to be collected and analyzed by the blood gas analyzer; no more than 5 seconds was spent between blood collection and analysis on the blood gas analyzer.

**Immunohistochemistry**

Adult mice were transcardially perfused with 20 mL of room temperature phosphate buffered saline (PBS, pH 7.4) followed by 20mL of chilled 4% paraformaldehyde (pH 7.4). The brainstem was then removed from the animal and 150 um slices were made using a Zeiss VT100S vibratome. Slices were then incubated in a 0.5% Triton-X/PBS solution for 45 minutes to
permeabilize the tissue. The slices remained in a 0.1% Triton-X/10% Fetal Bovine Serum (FBS, ThermoFisher)/PBS solution for a 12-hour primary antibody incubation of anti-mouse alpha smooth muscle actin (Sigma). The tissue was then washed three times in 0.1% Triton-X/10% FBS/PBS solution; the secondary antibody was incubated with the tissue after the third wash for 2 hours (donkey anti-mouse Alexa Fluor 647, ThermoFisher). The tissue was then washed three times in PBS before mounting on precleaned cover slides with Prolong Diamond with DAPI (ThermoFisher). Imaging of brain slices was achieved with a Leica SP8 confocal microscope.

Statistics
Data are reported as mean ± SE. Power analysis was used to determine sample size, all data sets were tested for normality using Shapiro-Wilk test, and comparisons were made using t-test, one-way or two-way ANOVA (parametric or non-parametric) followed by multiple comparison tests as appropriate. The specific test used for each comparison is reported in the figure legend and all relevant values used for statistical analysis are included in the results section.

Data Availability
All original data files are available from authors upon request.
Supplementary Figure 1. CO$_2$/H$^+$-induced constriction of RTN arterioles in vitro is not dependent on neural activity, prostaglandin EP3 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).

Supplementary Figure 2. Cardiovascular, metabolic and blood gas parameters in control and smooth muscle P2Y$_2$ cKO mice. A, Traces of blood pressure and ECG activity from control and P2Y$_2$ cKO mice. B, Summary data (N=4/genotype) shows that control and P2Y$_2$ cKO mice have similar mean arterial pressures (MAP) during a 12 hour light cycle (T$_3$=0.1152, p > 0.05) and during a 12 hour dark cycle (T$_3$=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. 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.

**Supplementary Figure 3.** Respiratory activity of smMHC$^{Cre/eGFP::P2Y_2^+/+}$ and control viral injected P2Y$_2$ cKO mice. A-C, We crossed smMHC$^{Cre/eGFP}$ with P2Y$^{+/+}$ as an alternative means of generating smooth muscle specific P2Y$_2$ cKO (smMHC$^{Cre/eGFP::P2Y_2^+/+}$) mice. Control (smMHC$^{Cre}$ only and P2Y$_2^{+/+}$ only) and smMHC$^{Cre/eGFP::P2Y_2^+/+}$ cKO mice show a similar respiratory frequency (A), tidal volume (B) and minute ventilation (c) under room air conditions. However, smMHC$^{Cre/eGFP::P2Y_2^+/+}$ mice exhibit a blunted ventilatory response to CO$_2$. This respiratory phenotype is nearly identical to the respiratory phenotype of αSm22$^{Cre::P2Y_2^+/+}$ mice (defined as P2Y$_2$ 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 αSm22$^{Cre::P2Y_2^+/+}$ (P2Y$_2$ cKO) mice. *, difference from 0% CO$_2$; #, 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.

**Supplementary Figure 4.** 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 P2Y2 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).

Supplementary Figure 5. 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 two 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 (Fig.
1C). ##, different from baseline (paired two-tailed t-test; p<0.0001).

Supplementary Figure 6. 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 um.

**Supplementary Figure 7. 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.

**Supplementary Table 1. Raw Ct values of housekeeping and cell type specific control genes.**  
Housekeeping gene *Gapdh* and cell type specific markers including *Rbfox3* (neurons), *Aldh1l1* (astrocytes), *Acta2* (smooth muscle cells), and *Flt1* (endothelial cells) were run in each qRT-PCR panel. Raw Ct values for GAPDH were used for inclusion (cutoff Ct was between 18-22) as well as internal validation.
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Figure 1

A  Endothelial Cells

- RTN
- cNTS
- ROb
- cortex

$\Delta$ diameter (%)

- CO$_2$

2 µm

5 min

B  Smooth Muscle Cells

- RTN
- cNTS
- ROb
- cortex

$\Delta$ diameter (%)

- CO$_2$

2 µm

5 min

Di  P2Y2 blocker

- AR-C 118925

5 min

Ei  PSB 1114

5 min

Fi  tACPD

5 min
Figure 2
Figure 4
|                          | αSm22^cre only | P2Y\textsubscript{2}^ff only | P2Y\textsubscript{2} cKO |
|--------------------------|----------------|-------------------------------|-------------------------|
| **Frequency** (breaths/min) | 163.4 ± 7.1    | 149.8 ± 7.2                   |                         |
| **Tidal Volume** (μL x 10^-3) | 6.8 ± 0.4      | 6.7 ± 0.5                     |                         |
| **Minute Ventilation** (mL/min/kg) | 1.1 ± 0.1     | 1.0 ± 0.1                     |                         |
| **Systolic blood pressure** (mm Hg) | 124.2 ± 0.9  | 123.0 ± 1.2                   |                         |
| **Diastolic blood pressure** (mm Hg) | 85.5 ± 0.8   | 86.7 ± 1.4                    |                         |
| **Heart rate** (beats/min)     | 473.8 ± 3.0   | 465.0 ± 5.0                   |                         |
| **VO\textsubscript{2}** (mL/kg/hr) | 4886.5 ± 127.1| 5067.0 ± 230.4                |                         |
| **VCO\textsubscript{2}** (mL/kg/hr) | 4392.8 ± 101.9| 4653.8 ± 204.5               |                         |
| **respiratory exchange ratio (RER)** | 0.89 ± 0.01 | 0.91 ± 0.01                   |                         |
| **pH**                      | 7.410 ± 0.005 | 7.406 ± 0.008                 |                         |
| **pCO\textsubscript{2}** (mmHg)     | 40.1 ± 0.4    | 40.7 ± 0.6                    |                         |
| **pO\textsubscript{2}** (mmHg)      | 88.3 ± 0.9    | 88.0 ± 0.8                    |                         |

Table 1
|                | αSm22<sup>cre</sup> only or P2Y<sub>2</sub><sup>tif</sup> only | P2Y<sub>2</sub> cKO | P2Y<sub>2</sub> cKO Rescue |
|----------------|-------------------------------------------------|----------------|--------------------------|
|                | Frequency (breaths per minute) | Tidal Volume (ml/g x10-3) | Minute Ventilation (mL/min/kg) | Frequency (breaths per minute) | Tidal Volume (ml/g x10-3) | Minute Ventilation (mL/min/kg) | Frequency (breaths per minute) | Tidal Volume (ml/g x10-3) | Minute Ventilation (mL/min/kg) |
| room air       | 163.4 ± 7.1               | 6.8 ± 0.4                       | 1.1 ± 0.1          | 149.8 ± 7.2               | 6.7 ± 0.5                       | 1.0 ± 0.1                      | 156.3 ± 9.0                       | 5.6 ± 0.1                       | 0.9 ± 0.2                      |
| 0% CO<sub>2</sub>/100% O<sub>2</sub> | 149.1 ± 5.7               | 6.7 ± 0.5                       | 1.0 ± 0.1          | 130.1 ± 7.4               | 7.1 ± 0.3                       | 0.9 ± 0.1                      | 158.3 ± 12.8                      | 6.8 ± 0.1                       | 1.1 ± 0.3                      |
| 3% CO<sub>2</sub>/97% O<sub>2</sub> | 236.6 ± 8.5 ****          | 8.7 ± 0.4 ****                  | 2.1 ± 0.1 ****     | 213.7 ± 6.5 ****          | 8.0 ± 0.3                       | 1.7 ± 0.1 **                   | 254.0 ± 6.2 ****                  | 9.5 ± 0.1 ***                   | 2.4 ± 0.3 ****                  |
| 5% CO<sub>2</sub>/95% O<sub>2</sub> | 306.0 ± 9.4 ****          | 12.9 ± 0.6 ****                 | 4.0 ± 0.3 ****     | 256.2 ± 9.6 ### ****      | 10.4 ± 0.4 #### ****           | 2.7 ± 0.2 #### ****             | 305.1 ± 9.8 #### ****           | 14.0 ± 0.1 ## ****             | 4.3 ± 0.4 #### ****             |
| 7% CO<sub>2</sub>/93% O<sub>2</sub> | 351.6 ± 9.8 ****          | 17.1 ± 1.4 ****                 | 6.0 ± 0.3 ****     | 291.3 ± 7.5 #### ****     | 13.3 ± 0.5 #### ****           | 3.9 ± 0.1 #### ****            | 335.6 ± 4.1 #### ****           | 17.0 ± 0.2 #### ****           | 5.7 ± 0.6 #### ****            |

Table 2