P2Y1 Receptors Expressed by C1 Neurons Determine Peripheral Chemoreceptor Modulation of Breathing, Sympathetic Activity, and Blood Pressure

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Abstract—Catecholaminergic C1 cells of the rostral ventrolateral medulla (RVLM) are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Overactivation of this reflex is thought to contribute to increased sympathetic activity and hypertension; however, molecular mechanisms linking peripheral chemoreceptor drive to hypertension remain poorly understood. We have recently determined that activation of P2Y1 receptors in the RVLM mimicked effects of peripheral chemoreceptor activation. Therefore, we hypothesize that P2Y1 receptors regulate peripheral chemoreceptor drive in this region. Here, we determine whether P2Y1 receptors are expressed by C1 neurons in the RVLM and contribute to peripheral chemoreceptor control of breathing, sympathetic activity, and blood pressure. We found that injection of a specific P2Y1 receptor agonist (MRS2365) into the RVLM of anesthetized adult rats increased phrenic nerve activity (≈55%), sympathetic nerve activity (38±6%), and blood pressure (23±1 mm Hg), whereas application of a specific P2Y1 receptor antagonist (MRS2179) decreased peripheral chemoreceptor-mediated activation of phrenic nerve activity, sympathetic nerve activity, and blood pressure. To establish that P2Y1 receptors are expressed by C1 cells, we determine in the brain slice preparation using cell-attached recording techniques that cells responsive to MRS2365 are immunoreactive for tyrosine hydroxylase (a marker of C1 cells), and we determine in vivo that C1-lesioned animals do not respond to RVLM injection of MRS2365. These data identify P2Y1 receptors as key determinants of peripheral chemoreceptor regulation of breathing, sympathetic nerve activity, and blood pressure.

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Key Words: hypertension ■ medulla oblongata ■ purinergic effects ■ sleep apnea, obstructive
pre-Bözinger complex blunted the respiratory response evoked by peripheral chemoreceptor activation in awake rats.23 These results suggest that purinergic signaling contributes to the peripheral chemoreflex mechanism. However, the identity of P2 receptors regulating the peripheral chemoreflex at this level of the brain stem is unknown.

We have recently determined that P2Y1 receptors are expressed in the RVLM.31 Although these receptors do not influence cardiorespiratory responses to hypercapnia, application of a P2Y1 receptor agonist into this region mimicked effects of peripheral chemoreceptor activation by increasing breathing and blood pressure.31 Therefore, we hypothesize that P2Y1 receptors are differentially expressed by C1 neurons and function as key determinants of peripheral chemoreceptor drive through this region. Consistent with this possibility, we find that inhibition of P2Y1 receptors in the RVLM decreased breathing, sympathetic nerve activity (SNA), and blood pressure responses to cyanide-induced activation of peripheral chemoreceptors in anesthetized rats. Furthermore, NTS terminals at this level of the RVLM are immunoreactive for vesicular nucleotide transporter (VNUT). To establish that P2Y1 receptors are key determinants of peripheral chemoreceptor drive through this region, we determine in vivo that C1-lesioned animals do not respond to RVLM injection of MRS2365. We find that P2Y1 receptors are key determinants of peripheral chemoreceptor regulation of breathing, SNA, and blood pressure.

**Methods**

All procedures were performed in accordance with National Institutes of Health and the University of Connecticut and University São Paulo Animal Care and Use Guidelines. An expanded Methods section is available in the online-only Data Supplement.

**Results**

This study consists of both in vivo and in vitro experiments. First, to determine whether purinergic signaling in the RVLM contributes to peripheral chemoreceptor regulation of breathing, sympathetic activity, or blood pressure, we measured these parameters during cyanide-induced activation of peripheral chemoreceptors after bilateral RVLM injections of saline, a nonspecific P2 receptor blocker (pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid [PPADS]) or a specific P2Y1 receptor blocker (MRS2179).32 To further support the possibility that purinergic signaling contributes to peripheral chemoreceptor drive, we determine the extent to which NTS terminals in the RVLM are immunoreactive for VNUT and VGLUT2. Although our focus is on the peripheral chemoreflex, we also tested the possibility that purinergic signaling via P2Y1 receptors contributes to other reflexes mediated by C1 cells, including the somatosympathetic reflex and the baroreflex. Second, to determine which neurons express P2Y1 receptors, we used slice-patch recording techniques to measure neuronal responses to focal application of a specific P2Y1 receptor agonist (MRS2365).33 As in previous studies,10,31 we define RTN chemoreceptors as cells that respond to 15% CO2 with ≥1.5 Hz increase in firing rate. Neurons that did not exhibit this minimum firing rate response to 15% CO2 were considered nonchemosensitive. Previous evidence suggests that the majority of CO2/H+-insensitive neurons in this region are presympathetic neurons that regulate blood pressure,33 approximately two-thirds of which are C1 cells known to express TH and one third are non-C1 cells. Therefore, we use TH immunoreactivity to confirm the identity of MRS2365-responsive cells recorded in vitro, and in parallel we test MRS2365 responsiveness in C1-lesioned animals.

**Purinergic Signaling in the RVLM Contributes to Peripheral Chemoreceptor Regulation of Breathing, Sympathetic Outflow, and Blood Pressure**

To determine whether purinergic signaling contributes to peripheral chemoreceptor transduction in the RVLM, we measured breathing, sympathetic outflow, and blood pressure responses to peripheral chemoreceptor activation after bilateral RVLM injections of saline or a purinergic receptor antagonist (Figure 1A).34 The injection center was 250 µm below the facial motor nucleus and 200 µm rostral to the caudal end of this nucleus (Figure 1B).35 Bilateral injections of PPADS (100 µmol/L, 50 nL) into the RVLM did not change baseline mean arterial pressure (MAP; 119±5 mm Hg compared with saline 121±6 mm Hg), splanchnic SNA (sSNA; 101±11% of control), or phrenic nerve activity (PNA) activity (97±4% of control value). However, PPADS treatment strongly inhibited cardiorespiratory responses to peripheral chemoreceptor activation. For example, PPADS attenuated the increase in sSNA (78±10% versus saline 211±13%; P=0.015; n=7), MAP (12±3 mm Hg versus saline 25±6 mm Hg; P=0.037), and PNA amplitude (84±4% versus saline 121±14%; P=0.038) and frequency (45±7% versus saline 118±8%; P=0.026) elicited by cyanide (Figure 1A, 1C–1F).

Previous evidence showed that P2Y1 receptors are expressed in the RVLM.34 However, P2Y1 receptors do not influence CO2 responsiveness in vitro or in vivo, suggesting that these receptors relay a purinergic signal disparate from CO2-evoked ATP release, but still essential to autonomic regulation of cardiorespiratory homeostasis. To determine whether P2Y1 receptors are part of the peripheral chemoreceptor circuit, we repeated the experiments described above using the specific P2Y1 receptor antagonist (MRS2179) alone or in combination with a nonspecific ionotropic glutamate receptor antagonist (kynurenic acid; Figure 2B). As previously reported,31 bilateral RVLM injections of MRS2179 (100 µmol/L to 50 nL) did not change resting MAP (123±6 mm Hg compared with saline 122±8 mm Hg; P=0.21), sSNA (99±9% of control; P=0.77), or PNA (102±5% of control; P=0.86), suggesting that P2Y1 receptor blockade does not alter basal activity of C1 cells. Interestingly, bilateral injections of MRS2179 into this same region decreased the cyanide-induced pressor response from 22±3 to 15±2 mm Hg (P=0.036; n=6/group; Figure 2A and 2C) and decreased the sympathoexcitatory response from 218±17% to 126±8% (P=0.028; Figure 2A and 2D). Similarly, MRS2179 also decreased peripheral chemoreceptor activation of PNA amplitude and frequency by 25±6% (P=0.037; Figure 2A and 2E) and 33±7% (P=0.032; Figure 2A and 2F), respectively. Consistent with previous evidence,36 bilateral RVLM injections of kynurenic acid decreased baseline PNA amplitude.
Injections of kynurenic acid into the RVLM also blunted the peripheral chemoreceptor–mediated increase in breathing amplitude, SNA, and blood pressure (Figure 2A, 2C–2F). The effect of kynurenic acid on PNA frequency was reduced by MRS2179 (137±6 versus 169±4 breaths/min; Figure 2A and 2F). In addition, the combination of kynurenic acid and MRS2179 further decreased peripheral chemoreceptor–mediated changes in MAP (64% inhibition), sSNA (69% inhibition), and phrenic nerve discharge amplitude (68% inhibition) by more than either blocker alone (Figure 2A, 2C–2E). These results indicate that purinergic
Purinergic Signaling in the RVLM Contributes to the Excitatory Somatosympathetic Reflex But Not the Inhibitory Baroreflex

To determine whether P2Y1 receptor-dependent modulation of C1 cells is specific to the peripheral chemoreceptor, we also tested effects of MRS2179 on 2 other reflexes mediated by C1 cells: the somatosympathetic reflex which is thought to be mediated by glutamate in the RVLM and the baroreflex which is inhibitory and largely mediated by gamma-aminobutyric acid in the RVLM. The somatosympathetic reflex was represented by 2 characteristic excitatory peaks in the sSNA in response to intermittent sciatic nerve stimulation under baseline conditions (control) and after injections of saline or MRS2179 (100 µmol/L to 50 nL). As shown in Figure 3A, latencies of the peaks of sSNA (85±3 and 176±3 ms, respectively; n=4) were not significantly altered by bilateral injection of MRS2179 (sSNA: 89±4 and 179±5 ms, respectively; P=0.145; n=5). However, bilateral injection of MRS2179 decreased the area under the curve of each sSNA peak by 25±4% (P=0.042) and 22±5% (P=0.044), respectively (Figure 3A and 3B).

The baroreflex was measured by raising arterial pressure with phenylephrine (5 µg/kg, IV) and lowering arterial pressure with sodium nitroprusside (30 µg/kg, IV). A baroreflex curve relating MAP and SNA was constructed for each rat under control conditions and 10 minutes after bilateral injections of saline or MRS2179 (100 µmol/L to 50 nL; n=5). We found that the baroreflex operated around a comparable MAP50 in all groups (MRS2179: 116±9 versus saline 118±6 mmHg; P=0.46; Table). Injections of MRS2179 into the RVLM did not change the range (135±11% versus saline 138±13%; P=0.37) and the gain (5.3±0.5 versus saline 5.4±1%; P=0.71) of the sympathetic baroreflex (Table).

To confirm in vivo that MRS2179 is specific to P2Y1 receptors and does not disrupt glutamatergic signaling, we tested effects of MRS2179 on cardiorespiratory responses to RVLM injections of glutamate in urethane-anesthetized rats. Injection of MRS2179 (100 µmol/L to 50 nL; n=5) in the RVLM did not change the increase in MAP (23±4 mmHg versus saline 27±2 mmHg; P=0.64), sSNA (34±8% versus saline 33±9%; P=0.084), PNA amplitude (17±2% versus saline 18±4%; P=0.13), or PNA frequency (14±2% versus saline 16±4%; P=0.077) evoked by unilateral injection of glutamate (10 mmol/L to 50 nL) in the RVLM (Figure S1 in the online-only Data Supplement).

Together, these results suggest that application of MRS2179 into the RVLM does not antagonize glutamate receptors. These results also suggest that purinergic signaling via P2Y1 receptors in the RVLM contributes to excitatory (ie, peripheral chemoreflex and the somatosympathetic reflex) but not inhibitory baroreflex control of sympathetic activity. This study focuses on the peripheral chemoreflex because overactivation of this reflex is thought to contribute to hypertension associated with obstructive sleep apnea.

VNUT Is Expressed by NTS Neuronal Terminals in the RVLM

Our observation that P2Y1 receptors in the region of the RVLM contribute to the peripheral chemoreflex suggests that synapses activated in the RVLM during peripheral chemoreceptor stimulation release purinergic signaling molecules. To build on this possibility, we injected the anterograde tracer biotinylated dextran amine (BDA) into the cNTS (Figure 4A and 4B) and subsequently performed immunohistochemistry to determine whether cNTS terminals in the RVLM express VNUT, the protein responsible for vesicular storage and release of nucleotides. Considering that purinergic

Table. Average Parameters of Sigmoid Baroreflex Curves in Rats Treated With Saline or MRS2179

<table>
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<tr>
<th>Group</th>
<th>n</th>
<th>Baseline MAP, mmHg</th>
<th>MAP50, mmHg</th>
<th>Upper Plateau, %</th>
<th>Lower Plateau, %</th>
<th>Range, %</th>
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<tr>
<td>Saline (control)</td>
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<td>117±3</td>
<td>118±6</td>
<td>157±9</td>
<td>18±4</td>
<td>135±11</td>
<td>5.4±1</td>
</tr>
<tr>
<td>MRS2179</td>
<td>5</td>
<td>115±8</td>
<td>116±9</td>
<td>158±7</td>
<td>19±6</td>
<td>138±13</td>
<td>5.3±0.5</td>
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Baroreflex analysis after bilateral injection of saline or MRS2179 (100 µmol/L to 50 nL) injection into RVLM. Curves relating splanchnic nerve activity (sSNA) and mean arterial pressure (MAP) were generated by lowering MAP with sodium nitroprusside (SNP, 30 µg/kg, IV) and increasing MAP with phenylephrine (Phe, 5 µg/kg, IV). Baseline sSNA was set to 100%, and minimum sSNA was determined after intravenous injection of hexamethonium (30 mg/kg, IV). RVLM indicates rostral ventrolateral medulla.
nucleotides are known to be coreleased with glutamate at certain central synapses and cNTS projections to the RVLM are known to be glutamatergic, we also assayed for VGLUT2 to determine whether glutamate and nucleotides are colocalized in the same terminals.

We focused our observations on the marginal layer of the ventrolateral medulla between Bregma −11.3 and Bregma −12.2 because this region contains chemosensitive neurons and C1 cells, and it is known to receive a dense input from the cNTS. BDA-labeled varicosities were assumed to be terminals (putative synapse). This possibility is supported by our evidence that the majority of BDA labeling was immunoreactive for VNUT and VGLUT2. Figure 4C–4F shows examples of this staining where terminals from the cNTS (green) are immunoreactive for VGLUT2 (red) and VNUT (blue). A total of 126 terminals from 31 RVLM regions (n=4 rats) were counted, and 72 (57%) were positive for both VGLUT2 and VNUT, 25 (20%) were VGLUT2-positive only, 8 (6%) were VNUT-positive only, and 21 (17%) lacked any discernible immunoreactivity (Figure 4G). Although it is not surprising to find numerous BDA-labeled terminals in which no other immunoreactivity could be detected, it should be noted that in the absence of a vesicular marker, at least some BDA labeling may reflect cut axons rather than terminals.

C1 Cells But Not RTN Chemoreceptors Express P2Y1 Receptors

To determine the cellular distribution of P2Y1 receptors in the region of the RVLM, we used the brain slice preparation to make cell-attached recordings of action potential frequency in response to 15% CO2 and focal application of MRS2365 (100 µmol/L). We found that the majority of RVLM neurons (62 of 78) could be differentiated based on responsiveness to either CO2/H+ or MRS2365. Note that an increase of ≥0.5 Hz immediately after MRS2365 application was the cutoff for a neuron to be considered MRS2365 sensitive. For example, 22 of 25 chemosensitive neurons (88%; ie, ≥1.5 Hz increase in firing rate during 15% CO2) demonstrated no appreciable response to focal application of MRS2365 (Figure 5A). Conversely, 37 of 53 CO2/H+-insensitive neurons (≈70%) exhibited a robust firing rate response to focal application of MRS2365. Furthermore, responsiveness to MRS2365 was retained in synaptic block solution (Figure 5D and 5E) and blunted by bath application of MRS2179, a specific P2Y1 receptor blocker (3 µmol/L; Figure 5B and 5C). All MRS2365-insensitive but CO2/H+-sensitive neurons had baseline firing rates <1 Hz (data not shown), consistent with type 1 RTN chemosensitive neurons. However, 3 CO2/H+-sensitive neurons did respond to MRS2365, and each of these exhibited basal activity reminiscent of type II chemoreceptors (ie, ≥1 Hz), suggesting that a small subset of chemosensitive neurons may express P2Y1 receptors. Nevertheless, our results clearly show that the majority of P2Y1 receptors are expressed by CO2/H+-insensitive cells (Figure 6A). After recording, we gained whole-cell access to fill a subset of MRS2365-sensitive cells with biocytin (included in the pipette internal solution) for later determination of their immunohistochemical phenotype. We found that 9 of 18 MRS2365-sensitive cells were TH-immunoreactive, thus
confirming that at least half of MRS2365-responsive cells are C1 neurons (Figure 6B–6E). The identity of MRS2365-sensitive cells that were TH− is less clear. Together, these results suggest that P2Y1 receptors are differentially but not exclusively expressed by C1 neurons.

To further support the possibility that C1 cells preferentially express P2Y1 receptors, we tested MRS2365 responsiveness in C1-lesioned animals. To preferentially destroy C1 cells, we made bilateral RVLM injections of saporin (SAP) (an immunotoxin) that was conjugated to an antibody for dopamine β-hydroxylase (anti–DβH–SAP; 4.2 ng/100 nL) as previously described.15,41 Two weeks after anti–DβH–SAP injection, TH labeling was examined within the ventrolateral medulla to confirm specificity of the lesion to C1 cells. To confirm specificity of the C1 lesion, we also examined Phox2b immunoreactivity. Phox2b is a transcription factor strongly expressed by chemosensitive RTN neurons, but only weakly expressed by C1 cells. Therefore, we define C1 cells as TH positive and Phox2b negative (TH+/Phox2b−). Animals treated with anti–DβH–SAP showed an 86±4% reduction in the number of cells that were TH positive and Phox2b negative compared with saline-injected animals (Figure S2). The number of cells that were immunoreactive for Phox2b was unaffected by DβH–SAP treatment, suggesting that only C1 cells were affected. Furthermore, the toxin did not affect the number of choline acetyltransferase-positive facial motor neurons or TH-positive cells outside the C1 (eg, A2 and A5; Figure S2). These results demonstrate selective lesion of C1 neurons.

Two weeks after DβH–SAP treatment, vagotomized and urethane-anesthetized C1-lesioned animals with intact carotid sinus nerves (n=7) exhibit reduced sSNA (72±6% of control; P=0.032) but normal resting MAP (116±4 mm Hg versus

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**Figure 5.** P2Y1 receptors are functionally expressed by CO2-insensitive rostral ventrolateral medulla (RVLM) neurons but not retrotrapezoid nucleus (RTN) chemoreceptor neurons. A, Trace of firing rate from a chemosensitive RTN neuron shows that increasing CO2 from 5% to 15% increases firing rate by ~3 Hz. After returning to 5% CO2, focal application (arrows) of the selective P2Y1 receptor agonist (MRS2365, 100 µmol/L) had no effect on firing rate. B, Trace of firing rate from a CO2-insensitive neuron in the RVLM shows a robust and repeatable firing rate response to MRS2365. MRS2365 responsiveness was blunted by bath application of the selective P2Y1 receptor antagonist (MRS2179, 3 µmol/L) and fully recovered in wash. C, Summary data show the MRS2365 firing rate response of CO2-sensitive neurons (gray bar, n=19) and CO2-insensitive neurons (black bars, n=5) under control conditions and in the presence of the P2Y1 receptor blocker MRS2179 (blocker). D, Firing rate trace from a CO2-insensitive RVLM neuron shows that MRS2365 responsiveness was retained in the presence of synaptic block solution (high Mg2+ and low Ca2+ solution, see Methods in the online-only Data Supplement). ▼ designates DC current injection; // designates 10-minute time breaks. E, Average data (n=5) show the firing rate response to MRS2365 under control conditions and in the presence of synaptic block solution (low Ca2+). *P<0.05.

**Figure 6.** P2Y1 receptors are differentially expressed by C1 cells. A, Summary box plot shows that cells in the rostral ventrolateral medulla (RVLM) can be differentiated based on responsiveness to CO2 (15%) and MRS2365 (100 µmol/L). Chemosensitive retrotrapezoid nucleus neurons were largely MRS2365 insensitive (white bar, n=22), whereas the majority of CO2-insensitive neurons are activated by MRS2365 (red bar, n=46). After recording, cells were filled with biocytin for later immunohistochemical characterization. C1 cells were identified based on strong tyrosine hydroxylase (TH) immunoreactivity and weak phox2b labeling. Triple immunolabeling shows that a biocytin-filled MRS2365-sensitive cell (B, green) is immunoreactive for TH (C, red) and weakly immunoreactive for phox2b (D, blue); the merged image is shown in E. We found that 9 of 18 MRS2365-sensitive cells tested were TH positive. Scale bar, 20 µm.
Consistent with our hypothesis, C1-lesioned animals showed reduced sSNA (2±3% versus saline: 131±7%; P=0.0043) and MAP (1±3 mm Hg versus saline: 25±2 mm Hg; P=0.0051) responses to unilateral RVLM injection of MRS2365 (100 µmol/L to 50 nL; Figure 7A–7C, 7H–7K). The respiratory responses of C1-lesioned animals to MRS2365 were also decreased compared with control animals (Figure 7A, 7H–7K), suggesting that C1 cells project to RTN chemoreceptors possibly as a means of integrating sympathetic activity with respiratory drive. We found that C1-lesioned animals showed attenuation in the SNA and MAP, but not in PNA amplitude or frequency, responses elicited by cyanide (Figure 7A, 7D–7G), suggesting that C1 cells are key determinants of the sympatoexcitatory response to peripheral chemoreceptor activation. Although C1 cells also contribute to the peripheral chemoreceptor ventilatory reflex,36 the cyanide-induced ventilatory responses were retained in C1-lesioned animals suggesting compensation by other components of the respiratory circuit (eg, chemosensitive RTN neurons).

### Discussion

Purinergic signaling has been shown to contribute to central and peripheral chemoreflex control of cardiorespiratory function. However, molecular determinants of purinergic modulation of autonomic function remain poorly defined, and potential contribution of purinergic signaling to reflex regulation of blood pressure and sympathetic tone remains unclear. Here, we show that purinergic signaling at the level of the RVLM contributes to peripheral chemoreceptor regulation of breathing, sympathetic outflow, and blood pressure by a P2Y1 receptor–dependent mechanism. We show that P2Y1...
receptors are preferentially expressed on C1 cells, but not chemosensitive neurons, and C1-lesioned animals do not respond to RVLM injections of a P2Y1 agonist. In addition, in vivo inhibition of P2Y1 receptor signaling in the RVLM decreased peripheral chemoreceptor–mediated activation of breathing, SNA, and blood pressure, but did not change baroreflex control of sympathetic outflow or cardiorespiratory responses to RVLM injections of glutamate. We also found that pharmacological blockade of P2Y1 receptors in the RVLM caused a modest decrease in the somatosympathetic reflex. These results suggest that pharmacological blockade of P2Y1 does not alter excitability of C1 cells in a nonspecific manner and that P2Y1 receptors contribute to excitatory (glutamatergic) reflex control of C1 cells and sympathetic outflow. Consistent with this possibility, we show that inhibition of both P2Y1 receptors and glutamate receptors virtually abolished cardiorespiratory responses to peripheral chemoreceptor activation, suggesting that purinergic nucleotides are coreleased with glutamate in the RVLM by peripheral chemoreceptor inputs. In addition, our anatomic evidence shows that ≈60% of NTS terminals in the RVLM are immunoreactive for both VGLUT2 and VNUT. This finding is consistent with evidence that ATP is coreleased with glutamate at certain central synapses, and together these results suggest that peripheral chemoreceptor drive is relayed through the RVLM, in part, by a P2Y1-dependent mechanism. Considering that overactivation of peripheral chemoreceptor drive, as occurs during obstructive sleep apnea (OSA), is associated with increased SNA and hypertension, we propose that P2Y1 receptors could represent a therapeutic target for the treatment of OSA-induced hypertension.

P2Y1 Receptors Regulate the Peripheral Chemoreflex at the Level of the RVLM

It is well established that bulbospinal presynaptic neurons located in the RVLM (ie, C1 and non-C1 cells) are critical determinants of reflex control of the cardiovascular system. Present and previous evidence shows that targeted destruction of C1 cells virtually eliminated the sympathoexcitatory response to peripheral chemoreceptor activation in anesthetized rats. Furthermore, selective activation of C1 cells by channelrhodopsin-2 has been shown to increase breathing, sympathetic activity, and blood pressure, whereas inhibition of C1 cells by activation of the allostatin receptor did the opposite. However, despite the critical role of C1 cells in regulation of cardiorespiratory function, the identity of neurotransmitters and downstream effectors responsible for peripheral chemoreflex control of autonomic function at the level of the ventrolateral medulla remains incomplete.

Our results indicate that peripheral chemoreflex control of breathing, sympathetic activity, and blood pressure depends on both glutamate and purinergic signaling at the level of the RVLM. Specifically, we show in anesthetized rats that bilateral injections of kynurenic acid significantly decreased the ventilatory, sympathetic, and pressor responses to peripheral chemoreceptor activation. These results are consistent with previous studies that used kynurenic acid or APV ([2R]-amino-5-phosphonovaleric acid; specific N-methyl-D-aspartate receptor blocker) to attenuate the response of RVLM presynaptic cells to peripheral chemoreceptor drive. In addition, we found that the majority of NTS terminals in the RVLM were immunoreactive for VGLUT2 (77%). Although we do not know whether VGLUT2-positive NTS neurons that innervate the RVLM actually mediate the chemoreflex or contact C1 cells, these results are consistent with previous evidence and suggest that glutamate contributes to peripheral chemoreflex in the RVLM.

We also discovered that purinergic signaling via P2Y1 receptors expressed on C1 cells contributes to peripheral chemoreceptor regulation of breathing and blood pressure. This finding builds on the possibility that ATP is a key transmitter throughout the peripheral chemoreflex circuit. For example, as part of the first step in peripheral chemotransduction glomus cells—the chemosensory unit of the carotid bodies—release ATP to activate P2X2 and P2X3 receptors on sensory nerve endings, which then relays this excitatory drive to neurons in the cNTS. In the cNTS, application of ATP in awake rats mimicked cardiorespiratory responses (ie, bradycardia, hypertension, and tachypnea) to peripheral chemoreceptor activation. Also consistent with a role of purinergic signaling in peripheral chemoreflex, cNTS injections of glutamate receptor antagonists did not block sympathetic or bradycardic components of the chemoreceptor reflex in awake rats or in the working heart-brain stem preparation, whereas simultaneous antagonism of glutamate and P2 receptors in this region reduced pressor and sympathetic responses to chemoreflex activation. In addition, presynaptic neurons in the RVLM are activated by exogenous application of ATP analogs and application of purinergic agonists into this region increased sympathetic tone and blood pressure in anesthetized rats. Furthermore, inhibition of P2X receptors within the more caudal RVLM (at the level of the Bötzinger complex) blunted the ventilatory, but not pressure response elicited by peripheral chemoreceptor activation in conscious rats. In light of our evidence that P2Y1 receptors expressed on C1 cell mediate the peripheral chemoreceptor pressure response, we propose that differential expression of P2 receptors throughout the ventrolateral medulla could allow for parallel processing of respiratory and cardiovascular components of the peripheral chemoreflex.

It is also possible that P2Y1 receptors regulate other excitatory reflexes at the level of the RVLM. For example, we show that pharmacological blockade of P2Y1 receptors in the RVLM caused a modest reduction in the somatosympathetic reflex by reducing the SNA peak elicited by electric stimulation of the sciatic nerve in anesthetized rats. Previous evidence reported this reflex to be mediated largely by glutamate at this level of the RVLM. Our evidence suggests that purinergic signaling via P2Y1 receptors in the RVLM may also contribute to the increase in sympathetic outflow evoked by stimulation of somatic afferents. Conversely, the inhibitory baroreflex was not affected by RVLM application of a P2Y1 receptor antagonist. These results are consistent with previous evidence that P2Y1 receptor blockade in the RVLM did not affect basal cardiorespiratory parameters and suggests that P2Y1 receptors preferentially regulate excitatory reflex control of C1 cells.
P2Y1 receptors are best known for their role in paracrine signaling between astrocytes and neurons, but there is some evidence that these receptors are present postsynaptically and contribute to synaptic transmission. For example, P2Y1 receptors appear to be expressed postsynaptically in layer V pyramidal neurons, and activation of these receptors has been shown to decrease synaptic strength and plasticity, in part, by inhibiting voltage-sensitive Ca\(^{2+}\) channels. Our results are consistent with the possibility that P2Y1 receptors are situated postsynaptically on C1 cells; however, contrary to the studies noted above, we find that activation of P2Y1 on C1 cells increased excitability by activating a yet to be identified inward conductance (voltage-clamp data are not shown). It should be noted that P2Y1 receptors may also be expressed by other cell types in the RVLM, including astrocytes, and our evidence that MRS2365 responsiveness was retained in synaptic block media does not rule out a potential indirect contribution of astrocytes to the peripheral chemoreflex.

**Physiological Significance**

OSA is defined as the occurrence of repetitive episodes of upper airway obstruction during sleep. OSA is a major health problem affecting ≤20% of adults in the United States and is considered a major risk factor for cardiovascular disease (eg, heart failure, stroke, hypertension, and coronary heart disease). The link between OSA and cardiovascular disease is thought to result from repeated apneic/hypoxic-mediated activation of the sympathetic nervous system via peripheral chemoreceptors, which leads to hypertension and other cardiovascular problems. Previous evidence showed that C1 cells are key determinants of the sympathetic excitatory response to peripheral chemoreceptor activation. In addition, an animal model of OSA showed that chronic intermittent hypoxia increased sympathoexcitatory responsiveness to RVLM injections of ATP, suggesting that purinergic signaling in this region contributes to OSA-induced activation of sympathetic activity and hypertension. Here, we show that purinergic signaling contributes to the peripheral chemoreflex at the level of the RVLM by a P2Y1 receptor–dependent mechanism. Therefore, P2Y1 receptors in the RVLM may represent new avenues for the treatment of hypertension resulting from overactivation of peripheral chemoreceptors.

**Perspectives**

Catecholaminergic C1 cells in the RVLM are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Overactivation of this reflex is thought to contribute to increased sympathetic activity and hypertension; however, molecular mechanisms linking peripheral chemoreceptor drive to hypertension remain poorly understood. Here, we show that P2Y1 receptors are preferentially expressed on C1 cells but not on chemoreceptor neurons, and C1-lesioned animals do not respond to injections of a P2Y1 agonist in the ventrolateral medulla. In addition, inhibition of P2Y1 receptor signaling in the ventrolateral medulla decreased peripheral chemoreceptor-mediated activation of breathing, sympathetic outflow, and blood pressure, as well as the somatosympathetic reflex, but did not change the baroreflex activation. Furthermore, inhibition of both P2Y1 receptors and glutamate receptors virtually abolished cardiorespiratory responses to peripheral chemoreceptor activation, suggesting that purinergic nucleotides are coreleased with glutamate in the RVLM by peripheral chemoreceptor inputs. Our results incorporate the already-established notion that RVLM/C1 neurons are excited by peripheral chemoreceptors via a direct glutamatergic input from cNTS. To this notion, we have added the concept that purinergic signaling also contributes to peripheral chemoreflex control of autonomic function at the level of the RVLM/C1 neurons via a P2Y1-dependent mechanism.

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**Disclosures**

None.

**References**

Regulation of ventral surface CO2/H+ signalling.


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**Novelty and Significance**

**What Is New?**

- We show for the first time that (1) purinergic signaling via P2Y1 receptors regulates peripheral chemoreceptor control of breathing, sympathetic nerve activity, and blood pressure; (2) activation of P2Y1 receptors in the rostral ventrolateral medulla mimics effects of peripheral chemoreceptor activation in control animals, but not in C1-lesioned animals; and (3) P2Y1 receptors are preferentially expressed on blood pressure-regulating C1 cells but not in respiratory chemoreceptor neurons.

**What Is Relevant?**

- Overactivation of the peripheral chemoreflex by repeated bouts of hypoxia is thought to contribute to hypertension and cardiovascular mortality associated with obstructive sleep apnea. Our evidence that P2Y1 receptors are differentially expressed by blood pressure-regulating cells and function as key determinants of peripheral chemoreceptor regulation of blood pressure identifies P2Y1 receptors as potential therapeutic targets for the treatment of hypertension associated with conditions, such as obstructive sleep apnea. We expect that our findings will be of great interest to a broad audience in the basic, clinical, and pharmaceutical community.

**Summary**

Catecholaminergic C1 cells in the rostral ventrolateral medulla are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Overactivation of this reflex is thought to contribute to increased sympathetic activity and hypertension; however, molecular mechanisms linking peripheral chemoreceptor drive to hypertension remain poorly understood. Here, we use a combination of immunohistochemistry and in vivo and in vitro electrophysiological approaches to show that P2Y1 receptors are differentially expressed by C1 cells and function as important determinants of peripheral chemoreceptor regulation of breathing, sympathetic outflow, and blood pressure. These results suggest that P2Y1 receptors expressed on C1 cells represent a therapeutic target for the treatment of hypertension resulting from overactivation of peripheral chemoreceptors.
P2Y1 Receptors Expressed by C1 Neurons Determine Peripheral Chemoreceptor Modulation of Breathing, Sympathetic Activity, and Blood Pressure
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P2Y1-receptors expressed by C1 neurons determine peripheral chemoreceptor modulation of breathing, sympathetic activity and blood pressure

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Supplemental Methods

In vivo preparation.

Animal use was in accordance with guidelines approved by the University of São Paulo Animal Care and Use Committee. All in vivo experiments were performed in male Wistar rats weighing 250-280 g. The surgical procedures and experimental protocols were similar to those previously described. Briefly, general anesthesia was induced with 5% halothane in 100% O₂. A tracheostomy was made and the halothane concentration was reduced to 1.4-1.5% until the end of surgery. The femoral artery was cannulated (polyethylene tubing, 0.6 mm o.d., 0.3 mm i.d., Scientific Commodities, Lake Havasu City, Arizona, USA) for measurement of arterial pressure (AP). The femoral vein was cannulated for administration of fluids and drugs. The occipital plate was removed, and a micropipette was placed in the medulla oblongata via a dorsal transcerebellar approach for microinjection of drugs. A skin incision was made over the lower jaw for placement of a bipolar stimulating electrode, next to the mandibular branch of the facial nerve, as previously described. The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. To prevent any influence of artificial ventilation on phrenic nerve activity (PNA), the vagus nerve was cut bilaterally as follows.

Splanchnic sympathetic nerve activity (sSNA) was recorded as previously described. The right splanchnic nerve was isolated via a retroperitoneal approach, and the segment distal to the suprarenal ganglion was placed on a pair of teflon-coated silver wires that had been bared at the tip (250 μm bare diameter; A-M Systems, Carlsborg, WA, USA). The nerves and wires were embedded in adhesive material (Kwik-Cast Sealant, WPI, USP, Sarasota, FL USA), and the wound was closed around the exiting recording wires.

Upon completion of the surgical procedures, halothane was replaced by urethane (1.2 g/kg) administered slowly i.v. All rats were ventilated with 100% O₂ throughout the experiment. Rectal temperature was maintained at 37°C. End-tidal CO₂ was monitored throughout each experiment with a capnometer (CWE, Inc, Ardmore, PA, USA) that was calibrated twice per experiment with a calibrated CO₂/N₂ mix. This instrument provided a reading of <0.1% CO₂ during inspiration in animals breathing 100% O₂ and provided an asymptotic, nearly horizontal reading during expiration. The adequacy of anesthesia was monitored during a 20 min stabilization period by testing for the absence of withdrawal responses, pressor responses, and changes in PNA to a firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at an initial dose of 1 mg/kg i.v. and the adequacy of the anesthesia was thereafter gauged solely by the lack of increase in AP and PNA rate or amplitude to a firm toe pinch. Approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria throughout the recording period (2 hours).

C1 lesion model

The injections of the toxin anti-dopamine beta-hydroxylase-saporin (anti DβH-SAP; Advanced Targeting Systems, San Diego, CA, USA) or saline were made while the rats were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (7 mg/kg) administered intraperitoneally. Surgery was performed using standard aseptic methods. After surgery, rats were treated with the antibiotic ampicillin (100 mg/kg) and the analgesic ketorolac (0.6 mg/kg, s.c.). The anti DβH-SAP conjugate was administered into the RVLM region by pressure injection using glass pipettes with an external tip diameter of 25 μm. These glass pipettes also allowed recordings of field potentials, which were used to direct the electrode tip to the desired
The rats received bilateral injections (100 nl) placed symmetrically in the RVLM at the level of the C1, i.e., 100-200 μm below the lower edge of the field and 1.8 mm lateral to the midline and 100-200 μm caudal to the caudal end of the facial field. Animals were maintained for 2 weeks before they were used in physiological experiments. Consistent with previous evidence, the toxin produced no obvious phenotype under resting conditions. The dose of anti DβH-SAP used in the present study (4.2 ng/100 nl) was selected based on previous experiments investigating the effects of injecting anti DβH-SAP into the C1 and A5 region. In addition, we did not observe any obvious gliosis or any other readily observable cytological difference between control and anti DβH-SAP rats (data not shown).

**In vivo recordings of physiological variables**

As previously described, mean arterial pressure (MAP), phrenic nerve activity (PNA), sSNA and end-expiratory CO₂ (etCO₂) were digitized with a micro1401 (Cambridge Electronic Design), stored on a computer, and processed off-line with version 6 of Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Integrated phrenic nerve activity (∫PNA) and integrated splanchnic nerve activity (∫SNA) were obtained after rectification and smoothing (τ = 0.015 and 2s, respectively) of the original signal, which was acquired with a 30-300 Hz bandpass filter. ∫SNA was normalized within animals by assigning a value of 100 to resting SNA and a value of 0 to the minimum value recorded either during administration of a dose of phenylephrine that saturated the baroreflex (5 μg/kg, i.v.) or after ganglionic blockade (hexamethonium; 30 mg/kg, i.v.). Nerve activity was rectified and averaged over 1-s intervals and stored on hard disk for subsequent analysis. Noise was subtracted from the recordings prior to performing any calculations of evoked changes in SNA. A direct physiological comparison of the absolute level of nerve activity across nerves is not possible because of nonphysiological factors (e.g., nerve electrode contact, size of nerve bundle) and the ambiguity in interpreting how a given increase in voltage in one nerve relates to an increase in voltage in another nerve. Thus, all nerve activities were defined to be at their baseline physiological state just prior to their activation. These activities were normalized to 100%, and percent change was used to compare the magnitude of increase or decrease across nerves from these physiological baselines. PNA amplitude (PNA amp) and PNA frequency (PNA freq) were normalized in each experiment by assigning to each of the two variables a value of 100 at saturation of the chemoreflex (high CO₂) and a value of 0 to periods of apnea.

Potassium cyanide was used (KCN, 40 μg/0.1 ml, i.v.) to activate peripheral chemoreceptors because in vivo responses to KCN are robust, reversible, and blocked by carotid denervation.

**Tracer injections**

Tracer injections were made while the rats were anaesthetized with a mixture of ketamine (80 mg kg⁻¹) and xylazine (5mg kg⁻¹) administered i.p. Surgery used standard aseptic methods, and after surgery, the rats were treated with the antibiotic ampicillin (100 mg kg⁻¹) and the analgesic ketorolac (0.6 mg kg⁻¹, s.c.). A group of four rats received pressure injections of the anterograde tracer biotinylated dextran amine (BDA-lysine fixable, MW 10000; 10% w/v in 10 mm phosphate buffer, pH 7.4; Molecular Probes) into the commissural part of the nucleus of the solitary tract (cNTS) (25 μm tip diameter glass pipettes). These injections were made 0.4 mm caudal to the calamus scriptorius, in the midline and 0.3-0.5mm below the dorsal surface of the brainstem. These rats were allowed to survive 7-10 days following which they were...
anaesthetized with pentobarbital (60 mg kg\(^{-1}\), i.p.) and perfused transectadially with fixative as described below.

**Somatosympathetic reflex analysis**

The somatosympathetic reflex was activated by electrical stimulation (5–15 V; 50 sweeps; 0.2-ms pulses at 1 Hz) of the sciatic nerve and the average response of sSNA peak was analyzed off-line. The area under the curve (AUC) of the sympathoexcitatory peak, less baseline of sSNA, was determined. sSNA were rectified and smoothed at 5-ms time constant to analyze the somatosympathetic reflex.

**Baroreflex analysis**

Animals underwent a series of reflex tests that were performed in the same order separated by 5-10 minutes with drug doses established in previous studies\(^7\). The drugs were prepared in sterile isotonic saline for intravenous (i.v) injections. The baroreflex stimulation was examined by raising arterial pressure with the phenylephrine (5 μg/kg, i.v) and lowering arterial pressure with sodium nitroprusside (30 μg/kg, i.v). A baroreflex curve relating MAP and SNA as constructed for each rat. The SNA at resting MAP was set at 100%, and SNA after hexamethonium (10 mg/kg i.v) was set as the minimum (i.e., 0) value. Boltzmann sigmoidal curves, with use of the equation SNA = (A1 - A2)/{1 + exp [A3 • (MAP - A4)]} + A2, were fitted to the experimental data points by use of the software program Sigma Plot (Jandel Corporation, Point Richmond, CA), where A1 defines the upper plateau of the curve, A2 defines the lower plateau of the curve, A3 describes the distribution of the gain along the curve, and A4 (MAP50) is the midpoint of the curve. Maximum gain (Gmax) was calculated using the formula Gmax = A3 • (A1 - A2)/4\(^8\).

**Brain slice preparation**

All procedures were performed in accordance with National Institutes of Health and University of Connecticut Animal Care and Use Guidelines. Slices containing the RVLM were prepared as previously described\(^5\). Briefly, neonatal rats (7-12 days postnatal) were decapitated under ketamine/xylazine anesthesia and transverse brain stem slices (300 μm) were cut using a microslicer (DSK 1500E; Dosaka, Kyoto, Japan) in ice-cold substituted Ringer solution containing (in mM): 260 sucrose, 3 KCl, 5 MgCl\(_2\), 1 CaCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), 10 glucose, and 1 kynurenic acid. Slices were incubated for ~30 min at 37°C and subsequently at room temperature in normal Ringer solution (in mM): 130 NaCl, 3 KCl, 2 MgCl\(_2\), 2 CaCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 10 glucose. Both substituted and normal Ringer solutions were bubbled with 95% O\(_2\)-5% CO\(_2\), extracellular pH (pHo = 7.35).

**Slice-patch electrophysiology**

Individual slices were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (~2 ml min\(^{-1}\)) with a bath solution of normal Ringer solution (same as incubation Ringers above) bubbled with 95% O\(_2\)-5% CO\(_2\) (pHo = 7.35). The pH of the bicarbonate-based bath solution was decreased to 6.90 by bubbling with 15% CO\(_2\). All recordings were made with an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322A A/D converter, and recorded using pCLAMP 10.0 software (Molecular Devices). Recordings were obtained at room temperature (~22 °C) with patch electrodes pulled from borosilicate glass capillaries (Warner Instruments) on a two-stage puller (P89; Sutter
Instrument) to a DC resistance of 4–6 MΩ when filled with an internal solution containing the following (in mM): 120 KCH$_3$SO$_3$, 4 NaCl, 1 MgCl$_2$, 0.5 CaCl$_2$, 10 HEPES, 10 EGTA, 3 Mg-ATP, 0.2% biocytin, and 0.3 GTP-Tris (pH 7.2); electrode tips were coated with Sylgard 184 (Dow Corning). All recordings of neuronal firing rate were performed in the cell-attached configuration to ensure minimal alteration of the intracellular milieu. Firing rate histograms were generated by integrating action potential discharge in 10-s bins and plotted using Spike 5.0 software.

**Drugs**

All drugs were purchased from Sigma unless otherwise indicated. For *in vivo* experiments, the non-specific P2-receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), the P2Y1-receptor antagonist MRS2179 (Tocris Bioscience) and the P2Y1-receptor agonist MRS2365 (Tocris Bioscience) were diluted to 100 µM in sterile saline (pH 7.4) and injected into the RVLM using single-barrel glass pipettes (tip diameter of 25 µm) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp, Cleveland, OH).

For each injection we delivered a volume of 50 or 100 nl over a period of 5s. These glass pipettes also allowed recordings of field potential properties that were used to help direct the electrode tip to the desired site. Injections in the RVLM region were guided by recordings of the facial field potential and injected into the RVLM using single-barrel glass pipettes (tip diameter of 25 µm) connected to a pressure injector (Picospritzer III, Parker Hannifin Corp, Cleveland, OH).

Application times were 600 ms, and vehicle control experiments were performed to ensure agonist responses were not attributable to pressure artifacts.

**Histology: in vivo experiments**

At the end of each *in vivo* experiment rats were deeply anesthetized with halothane, injected with heparin (500 units, intracardially) and finally perfused through the ascending aorta with 150 ml of phosphate-buffered saline (pH 7.4) followed by formaldehyde (4% in 0.1 M phosphate buffer; pH 7.4) (Electron Microscopy Sciences, Fort Washington, PA, USA). Brains were removed and stored in the perfusion fixative for 24-48 h at 4°C. Series of coronal brain sections (40 µm) were cut using a vibrating microtome (Vibratome 1000S Plus, USA) and stored in a cryoprotectant solution at -20°C for up to 2 weeks (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) pending histological processing. All histochemical procedures were performed using free-floating sections according to previously described protocols. Tyrosine hydroxylase (TH) was detected with a mouse antibody (1:10,000,
Chemicon, Temecula), and Phox2b was detected with a rabbit antibody (1:800, gift from J.-F. Brunet, Ecole Normale Supérieure, Paris, France). These primary antibodies were detected by incubation with appropriate secondary antibodies tagged with biotinylated donkey anti-mouse (1:1000, Jackson) or (donkey anti-rabbit, Jackson, West Grove, PA, USA), followed by the ABC kit (Vector, Burlingame, CA, USA) and subsequent colourisation with 3-3-di-aminobenzidine (DAB). Choline acetyltransferase (ChAT) was detected with a goat anti-ChAT antibody (1:50, Chemicon, raised against human placental ChAT) and revealed with the DAB colourimetric method using biotinylated donkey anti-goat (1:500, Jackson) and subsequent colourisation with DAB. The specificity of the antibodies has been validated previously. TH-positive neurons with or without Phox2b were plotted and counted in 7 coronal sections per rat. Each section was 240 μm apart, and the middle section was selected to coincide with the caudal end of the facial motor nucleus.

For cell mapping, counting and imaging, a conventional Zeiss Axioskop 2 multifunction microscope (brightfield, darkfield and epifluorescence) was used for all observations. Injection sites (fluorescent microbeads), and TH-labelled neurons were plotted using a previously described computer-assisted mapping technique based on the use of a motor-driven microscope stage controlled by Neulucida software. The Neulucida files were exported to NeuroExplorer software (MicroBrightfield, Colchester, VT) to count the various types of neuronal profiles within a defined area of the pons and brainstem. When appropriate, selected Neulucida files were also exported to the Canvas 9 software drawing program (ACD Systems of America, Miami, FL, USA) for final modifications. Section alignment between brains was done relative to a reference section. To align sections around the C1 region, the most caudal section containing an identifiable cluster of facial motor neurons was identified in each brain and assigned the level of 11.6 mm caudal to Bregma (Bregma -11.6 mm). Levels rostral or caudal to this reference section were determined by adding a distance corresponding to the interval between sections multiplied by the number of intervening sections. Images were captured with a SensiCam QE 12-bit CCD camera (resolution 1376×1040 pixels, Cooke Corp., Auburn Hills, MI, USA). The neuroanatomical nomenclature is adopted from Paxinos and Watson.

**Histology: in vitro experiments**

Recorded slices were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 16 to 24 hours at 4°C and rinsed in phosphate-buffered saline (PBS). Slices were blocked and permeabilized in tris-buffered saline (TBS) containing 10% normal horse serum and 0.5% Triton X-100 for 1 h at room temperature. After three TBS washes, the slices were incubated in primary antibodies; rabbit anti-Phox2b (gift of Dr. Brunet, 1:1000) and sheep anti-tyrosine hydroxylase (Millipore, 1:1000), or VGLUT2 (Millipore, 1:1000) and VNUT (MBL, 1:400), or 48 hours at 4°C. After three TBS washes, the slices were incubated in secondary antibodies (Jackson Labs); alexa488–conjugated streptavidin (1:1,000), Cy3-conjugated goat anti-sheep (1:500) and Cy5-conjugated goat anti-rabbit (1:200), or alexa488-conjugated streptavidin, Cy3-conjugated goat anti-guinea pig (1:500) and Cy5-conjugated goat anti-rabbit for 1 h at room temperature. Slices were washed a final three times in TBS and mounted in Vectashield. Images were collected on a Leica TCS SP2 confocal microscope equipped with 488-, 543-, and 633-nm laser lines and tunable emission wavelength detection. For each slice, biocytin-positive cell, or terminal, was identified and confocal z-stacks were collected sequentially for the other two channels to detect the neuronal antigens. Neurons were scored as TH-, or Phox2b-, immunopositive and NTS terminals were scored as VGLUT2-, or VNUT-, immunopositive if the
immunoreactivity for the protein marker was detected in the biocytin-positive cell body, or terminal. Biocytin neurons and terminals were scored as immunonegative if immunoreactivity for the protein marker was not detected in the biocytin filled cell or terminal, but immunopositive cells and terminals were observed nearby. To determine background fluorescence of the tissue and nonspecific binding of secondary antibodies, we tested secondary antibodies without preincubation in primary antibody.

Statistics
Data are reported as mean ± standard error of the mean. Statistical analysis was performed using Sigma Stat version 3.0 software (Jandel Corporation, Point Richmond, CA). T-test, paired T-test or one way ANOVA followed by the Newman-Keuls multiple comparisons test were used as appropriate (p<0.05 unless otherwise stated).
Reference List

Figure S1: Purinergic blockade do not change the cardiorespiratory effects of activation of RVLM/C1 neurons. A, Recordings from one rat showing the effect of MRS2179 (100 μM - 50 nl) into the RVLM/C1 region on changes in arterial pressure (AP), splanchnic sympathetic nerve activity (sSNA) and phrenic nerve activity (PNA) induced by glutamate (10 mM - 50 nl) injection. Responses were recorded 10 min after bilateral injection of saline or MRS2179 in the RVLM/C1 region. B, Changes in mean arterial pressure (ΔMAP), C, sSNA (ΔsSNA), D, PNA amplitude (ΔPNA ampl) and E, PNA frequency (ΔPNA freq) elicited by glutamate injection in the RVLM/C1 during saline or MRS2179 injections into RVLM/C1 region. N = 5 rats/group.
Figure S2: Injection of anti DβH-SAP into the RVLM selectively destroys C1 neurons. C1 cells were identified immunohistochemically as TH-positive and Phox2b-negative cells. A-B, photomicrographs at the level of the C1 region (-11.80 mm from Bregma) from control (A) and C1 lesion (B) animals. C-D, photomicrographs show normal TH-immunolabeling in the nearby A5 (C) and A2 (D) regions. E, computer assisted plots showing the distribution of C1 cells (●) and Phox2b-positive chemosensitive neurons (□) over several coronal planes. Note that the numbers on the right side of each drawing designate caudal distance from bregma. Scale bar is 1 mm. F, average number of TH neurons per section from 7 rats. Counts were made from a 1 in 6 series of 40 μm coronal sections. G, average number of Phox2b+/TH neurons (i.e., control). Abbreviations: VII, facial motor nucleus; Amb, ambiguus nucleus; IO, inferior olivary nucleus; py, pyramidal tract, Sp5, spinal trigeminal tract. Black arrows represent the Phox2b-positive neurons and white arrows represent the TH-positive neurons.