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P2Y1-receptors expressed by C1 neurons determine peripheral chemoreceptor modulation of breathing, sympathetic activity and blood pressure

Ian C. Wenker[1,*], **Cleyton R. Sobrinho**[2,*], **Ana C. Takakura**[3], **Daniel K. Mulkey**[1,+], and **Thiago S. Moreira**[2,+]

[1]Dept. of Physiology and Neurobiology, University of Connecticut, Storrs, CT 06269, USA

[2]Dept. of Physiology and Biophysics, University of São Paulo, São Paulo, SP, 05508, Brazil

[3]Dept. of Pharmacology, University of São Paulo, São Paulo, SP, 05508, Brazil

Abstract

Catecholaminergic C1 cells of the rostral ventrolateral medulla (RVLM) are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Over-activation 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 if 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 (PNA) (~55%), sympathetic nerve activity (SNA) (38 ± 6%), and blood pressure (23 ± 1 mmHg), whereas application of a specific P2Y1-receptor antagonist (MRS2179) decreased peripheral chemoreceptor-mediated activation of PNA, SNA 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 (TH, a marker of C1 cells), and we determine *in vivo* that C1 lesion animals do not respond to RVLM injection of MRS2365. These data identify P2Y1-receptors as key determinants of peripheral chemoreceptor regulation of breathing, SNA and blood pressure.

[+]**Address correspondence to:** Thiago S. Moreira, Ph.D., Department of Physiology and Biophysics, Institute of Biomedical Science, University of São Paulo, Av. Prof. Lineu Prestes, 1524. 05508-000, São Paulo, SP, Brazil, Phone: +55 (11) 3091-7764, Fax : +55 (11) 3091-7285, tmoreira@icb.usp.br, Daniel K. Mulkey, Ph.D, Department of Physiology and Neurobiology, Pharmacy Building, Rm216, University of Connecicut, 75 North Eagleville Road, Unit 3156, 06269-3156, Storrs, CT, USA, Phone: (860) 486-5572, Fax: (860) 486-3303, daniel.mulkey@uconn.com.
[*]Both authors contribute equally to the study

Disclosures
None

Keywords

peripheral chemoreceptor; RVLM; purinergic signaling; blood pressure; hypertension; ventrolateral medulla; obstructive sleep apnea

Introduction

Peripheral chemoreceptors located in the carotid sinus sense changes in arterial CO_2 and O_2 and communicate this information to cardiorespiratory centers to regulate breathing and sympathetic outflow to ensure adequate ventilation-perfusion matching in tissues. There is strong evidence that over-activation of the peripheral chemoreflex by repeated bouts of hypoxia contributes to hypertension and cardiovascular mortality associated with obstructive sleep apnea[1–4]. However, despite intense investigation molecular mechanisms linking peripheral chemoreceptor drive to hypertension remain poorly understood.

It is well known that peripheral chemoreceptor afferents first synapse in the caudal portion of the nucleus tractus solitarii (cNTS)[5,6] before relaying this information primarily to the rostral ventrolateral medulla (RVLM) including at the level of the retrotrapezoid nucleus (RTN)[7–9]. This brainstem region contains at least two functionally discrete but anatomically overlapping populations of neurons; CO_2/H^+-sensitive RTN neurons that appear to function as respiratory chemoreceptors[10], and C1 and non-C1 cells that control sympathetic vasomotor tone and arterial pressure[11–14]. Evidence suggests that both RTN chemoreceptors[9] and C1 cells[15] receive peripheral chemoreceptor drive. The transmitter basis for this drive is thought to depend largely on glutamate since NTS terminals in this region are immunoreactive for vesicular glutamate transporter 2 (VGLUT2, a marker of glutamatergic cells;[16,17]) and bilateral injections of kynurenic acid (glutamate receptor blocker) blunted peripheral chemoreceptor-mediated activation of breathing and blood pressure[9,18]. However, purinergic signaling has also been implicated in the peripheral chemoreflex[19–22] including at the level of the RVLM[23]. For example, chemosensitive RTN neurons[24,25] and presympathetic RVLM neurons[26] are activated by purinergic agonists, application of purinergic agonists into the RVLM increased breathing[27,28] and blood pressure[29,30] in anesthetized rats, and inhibition of P2 receptors in the nearby Bötzinger and 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 brainstem 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 expressed by C1 cells, we determine in the brain slice preparation that cells responsive to specific P2Y1 receptor agonist (MRS2365) are immunoreactive for tyrosine hydroxylase (TH, a marker of C1 cells), and we determine *in vivo* that C1 lesion 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 if 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 non-specific P2-receptor blocker (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/or 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% CO_2 with ≥ 1.5 Hz increase in firing rate. Neurons that did not exhibit this minimum firing rate response to 15% CO_2 were considered non-chemosensitive. Previous evidence suggests that the majority of CO_2/H^+-insensitive neurons in this region are presympathetic neurons that regulate blood pressure[10], 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 if 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 (Fig. 1A). The injection center was 250 μm below the facial motor nucleus and 200 μm rostral to the caudal end of this nucleus (Fig. 1B)[10,34]. Bilateral injections of PPADS (100 μM, 50 nl) into the RVLM did not change baseline MAP (119 ± 5 mmHg compared to saline 121 ± 6 mmHg), sSNA (101 ± 11% of control) or 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%, vs. saline: 211 ± 13%, $p = 0.015$, $N = 7$), in MAP (12 ± 3 mmHg, vs. saline: 25 ± 6 mmHg, $p = 0.037$) and in PNA amplitude (84 ± 4% vs. saline: 121 ± 14%, $p = 0.038$) and frequency (45 ± 7% vs. saline: 118 ± 8%, $p = 0.026$) elicited by cyanide (Fig. 1A; 1C–F).

Previous evidence showed that P2Y1-receptors are expressed in the RVLM[31]. However, P2Y1-receptors do not influence CO_2-responsivness *in vitro* or *in vivo*, suggesting these receptors relay a purinergic signal disparate from CO_2-evoked ATP release, but still essential to autonomic regulation of cardiorespiratory homeostasis. To determine if 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 non-specific ionotropic glutamate receptor antagonist (kynurenic acid)

(Fig. 2B). As previously reported[31], bilateral RVLM injections of MRS2179 (100 μM - 50 nl) did not change resting MAP (123 ± 6 mmHg compared to saline 122 ± 8 mmHg, 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 mmHg (p = 0.036, N = 6/group) (Figs. 2A & C) and decreased the sympathoexcitatory response from 218 ± 17% to 126 ± 8% (p = 0.028) (Figs. 2A & D). Likewise, MRS2179 also decreased peripheral chemoreceptor activation of PNA amplitude and frequency by 25 ± 6% (p = 0.037) (Fig. 2A & E) and 33 ± 7% (p = 0.032) (Fig. 2A & F), respectively. Consistent with previous evidence[35], bilateral RVLM injections of kynurenic acid decreased baseline PNA amplitude (59 ± 5%) and increased PNA frequency (46 ± 4%), but did not affect sSNA or MAP. Injections of kynurenic acid into the RVLM also blunted the peripheral chemoreceptor-mediated increase in breathing amplitude, SNA and blood pressure (Figs. 2A, 2C–F). The effect of kynurenic acid on PNA frequency was reduced by MRS2179 (137 ± 6 vs. 169 ± 4 breaths/min; Figs. 2A & 2F). In addition, the combination kynurenic acid and MRS2179 further decreased peripheral chemoreceptor-mediated changes in MAP (64% inhibition), sSNA (69% inhibition), PND amplitude (68% inhibition) by more than either blocker alone (Figs. 2A, 2C–E). These results indicate that purinergic signaling via P2Y1-receptors is an important component of peripheral chemoreceptor drive through the RVLM.

Injections located outside the RVLM region often reached the facial motor nucleus or dorsal to it (3 out of 4) and one injection (1 out of 4) was located in the parapyramidal region (data not shown). Bilateral injections of PPADS, MRS2179, or kynurenic acid into the facial motor nucleus or parapyramidal region did not change the cyanide-induced respiratory or pressor responses in these animals (data not shown).

Purinergic signaling in the RVLM contributes to the excitatory somatosympathetic reflex but not the inhibitory baroreflex

To determine if P2Y1-receptor dependent modulation of C1 cells is specific to the peripheral chemoreflex, we also tested effects of MRS2179 on two other reflexes mediated by C1 cells; the somatosympathetic reflex which is thought to be mediated by glutamate in the RVLM[36] and the baroreflex which is inhibitory and largely mediated by GABA in the RVLM[37]. The somatosympathetic reflex was represented by two 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 μm - 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 injection (sSNA: 89 ± 4 and 179 ± 5 ms, respectively, p = 0.145, N = 5). However, bilateral RVLM injections of MRS2179 decreased the area under the curve (AUC) of each sSNA peak by 25 ± 4% (p = 0.042) and 22 ± 5% (p = 0.044), respectively (Figs. 3A–B).

The baroreflex was measured by raising arterial pressure with 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 under control conditions and 10 min after bilateral injections of saline or MRS2179 (100 μm - 50 nl, N = 5). We found that the baroreflex operated around a comparable MAP50 in all groups (MRS2179: 116 ± 9 vs. saline: 118 ± 6 mmHg, p = 0.46) (Table 1). Injections of MRS2179 into the RVLM did not change the range (135 ± 11% vs. saline: 138 ± 13%, p = 0.37) and the gain (5.3 ± 0.5, vs. saline: 5.4 ± 1%, p = 0.71) of the sympathetic baroreflex (Table 1).

To confirm *in vivo* that MRS2179 is specific to P2Y1-receptors and does not disrupt glutamatergic signaling, we test effects of MRS2179 on cardiorespiratory responses to

RVLM injections of glutamate in urethane-anesthetized rats. Injection of MRS2179 (100 μm - 50 nl, N = 5) in the RVLM did not change the increase in MAP (23 ± 4 mmHg, vs. saline: 27 ± 2 mmHg; p = 0.064), sSNA (34 ± 8%, vs saline: 33 ± 9%; p = 0.084), PNA amplitude (17 ± 2%, vs. saline 18 ± 4%, p = 0.13) or PNA frequency (14 ± 2%, vs. saline 16 ± 4%, p = 0.077) evoked by unilateral injection of glutamate (10 mM - 50 nl) in the RVLM (Fig. S1).

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 (i.e., peripheral chemoreflex and the somatosympathetic reflex) but not inhibitory baroreflex control of sympathetic activity. This study focuses on the peripheral chemoreflex because over activation 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 possibly, we injected the anterograde tracer BDA into the caudal NTS (cNTS) (Figs. 4A–B) and subsequently performed immunohistochemistry to determine if cNTS terminals in the RVLM express VNUT, the protein responsible for vesicular storage and release of nucleotides[38]. Considering that purinergic nucleotides are known to be co-released with glutamate at certain central synapses[38] and cNTS projections to the RVLM are known to be glutamatergic[9], we also assayed for VGLUT2 to determine if glutamate and nucleotides are co-localized 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[9,10,25,31] neurons and C1[15,17] cells and it is known to receive a dense input from the cNTS[9]. 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/or VGLUT2. Figure 4C–F show 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 (Fig. 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% CO_2 and focal application of MRS2365 (100 μM). We found that the majority of RVLM neurons (62 out of 78) could be differentiated based on responsiveness to either of CO_2/H^+ or MRS2365. Note that an increase of ≥ 0.5 Hz immediately following MRS2365 application was the cutoff for a neuron to be considered MRS2365-sensitive. For example, 22 of 25 chemosensitive neurons (88%) (i.e. ≥ 1.5 Hz increase in firing rate during 15% CO_2) demonstrated no appreciable response to focal application of MRS2365 (Fig. 5A). Conversely, 37 of 53 CO_2/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 (Figs 5D and 5E) and blunted by bath application of MRS2179, a specific P2Y1-receptor blocker[32] (3

μM, Fig. 5B, 5C). All MRS2365-insensitive but CO_2/H^+-sensitive neurons had baseline firing rates less than 1 Hz (data not shown), consistent with Type 1 RTN chemosensitive neurons[39]. However, three CO_2/H^+-sensitive neurons did respond to MRS2365 and each of these exhibited basal activity reminiscent of Type II chemoreceptors (i.e. ≥ 1 Hz)[39], 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 CO_2/H^+-insensitive cells (Fig. 6A). After recording we gained whole-cell access to fill a subset of MRS2365-senstive 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 (Figs 6B–E). 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 are preferentially express P2Y1-receptors, we tested MRS2365 responsiveness in C1 lesioned animals. To preferentially destroy C1 cells, we made bilateral RVLM injections of saporin (an immunotoxin) that was conjugated to an antibody for dopamine-β-hydroxylase (anti DβH-SAP; 4.2 ng/100 nl) as previously described[15,40]. 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 weekly 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 to saline injected animals (Suppl. Fig 2). The number of cell 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 ChAT-positive facial motor neurons or TH-positive cells outside the C1 (e.g., A2 and A5) (Fig. 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 mmHg, vs. saline: 119 ± 8 mmHg, p = 0.11). Consistent with our hypothesis, C1 lesion animals showed reduced sSNA (2 ± 3%, vs. saline: 131 ± 7%, p = 0.0043) and MAP (1 ± 3 mmHg, vs. saline: 25 ± 2 mmHg, p = 0.0051) responses to unilateral RVLM injection of MRS2365 (100 μM - 50 nl) (Figs. 7A–C; 7H–K). The respiratory responses of C1 lesion animals to MRS2365 were also decreased compared to control animals (Figs. 7A and 7H–K), suggesting that C1 cells project to RTN chemoreceptors possibly as a means of integrating sympathetic activity with respiratory drive. We found that C1 lesion animals showed attenuation in the SNA and MAP, but not in PNA amplitude or frequency, responses elicited by cyanide (Figs. 7A, 7D–G), suggesting that C1 cells are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Although C1 cells also contribute to the peripheral chemoreceptor ventilatory reflex[35], the cyanide induced ventilatory responses was retained in C1 lesion animals suggesting compensation by other components of the respiratory circuit (e.g., 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 remain 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, sympathetic nerve activity 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 non-specific 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 co-released with glutamate in the RVLM by peripheral chemoreceptor inputs. In addition, our anatomical 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 co-released with glutamate at certain central synapses[32] and together these results suggest that peripheral chemoreceptor drive is relayed through the RVLM, in part, by a P2Y1-dependent mechanism. Considering that over-activation of peripheral chemoreceptor drive, as occurs during OSA, is associated with increased sympathetic nerve activity and hypertension[2,3,41], 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 presympathetic neurons located in the RVLM (i.e., C1 and non-C1 cells) are critical determinants of reflex control of the cardiovascular system[14,42]. Present and previous[15] evidence shows that targeted destruction of C1 cells virtually eliminated the sympathoexcitatory response to peripheral chemoreceptor activation in anesthetized rats. Moreover, selective activation of C1 cells by channelrhodopsin-2 has been shown to increase breathing, sympathetic activity and blood pressure[43–45], whereas inhibition of C1 cells by activation of the allostatin receptor did the opposite[46]. 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[35] or APV (specific NMDA receptor blocker)[47] to attenuate the response of RVLM presympathetic 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 if VGLUT2-positive NTS neurons that innervate the RVLM actually mediate the chemoreflex or contact C1 cells, these results are consistent with previous evidence[9] 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 chemoreceptor 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 P2×2 and P2×3 receptors on sensory nerve endings, which then relays this excitatory drive to neurons in the cNTS[22]. In the cNTS, application of ATP in awake rats mimicked cardiorespiratory responses (i.e., bradycardia, hypertension and tachypneia) to peripheral chemoreceptor activation[48,49]. 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[50] or in the working heart-brainstem preparation[51], whereas simultaneous antagonism of glutamate and P2 receptors in this region reduced pressor and sympathetic responses to chemoreflex activation[19]. In addition, presympathetic neurons in the RVLM are activated by exogenous application of ATP analogues[26,29,30] and application of purinergic agonists into this region increased sympathetic tone[29] and blood pressure [29,30] 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[23]. 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-recpetors 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 electrical 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[36]. 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-recpetor blockade in the RVLM did not affect basal cardiorespiratory parameters[31] 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[52] but there is some evidence that these receptors are present postsynaptically and contribute to synaptic transmission[53–55]. 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[53–55] in part by inhibiting voltage-sensitive Ca^{2+} channels[54]. Our results are consistent with the possibly 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 is not shown). It should be noted that P2Y1-receptors may also be expressed by other cells types in the RVLM including astrocytes, and our evidence that MRS2365-responsivness was retained in synaptic block media does not rule out a potential indirect contribution of astrocytes to the peripheral chemoreflex.

Physiological Significance

Obstructive sleep apnea (OSA) is defined as the occurrence of repetitive episodes of upper airway obstruction during sleep. OSA is a major health problem affecting up to 20% of adults in the United States and is considered a major risk factor for cardiovascular disease[3,4,56,57], e.g., 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[56]. Previous evidence showed that C1 cells are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation[15]. In addition, an animal model of OSA showed that chronic intermittent hypoxia increased sympathoexcitatory responsiveness to RVLM injections of ATP[28], 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 over-activation of peripheral chemoreceptors.

Perspectives

Catecholaminergic C1 cells in the RVLM are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Over-activation 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 chemoreceptors neurons and C1 lesioned-animals do not respond to injections of a P2Y1 agonist in the ventrolateral medulla. Additionally, 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 co-released 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[9]. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

MAP	mean arterial pressure
NTS	nucleus tractus solitarii
PPADS	pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PNA	phrenic nerve activity

RVLM	rostral ventrolateral medulla oblongata
RTN	retrotrapezoid nucleus
SNA	sympathetic nerve activity
TH	tyrosine hydroxylase
VGLUT2	vesicular glutamate transporter 2
VNUT	vesicular nucleotide transporter

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Novelty and Significance

1) What is new?

We show for the first time that i) purinergic signaling via P2Y1-receptors regulates peripheral chemoreceptor control of breathing, sympathetic nerve activity and blood pressure; ii) activation of P2Y1-receptors in the RVLM mimics effects of peripheral chemoreceptor activation in control animals, but not in C1 lesion animals; and iii) P2Y1-receptors are preferentially expressed on blood pressure regulating C1 cells, but not in respiratory chemoreceptors neurons.

2) What is relevant?

Over-activation of the peripheral chemoreflex by repeated bouts of hypoxia is thought to contribute to hypertension and cardiovascular mortality associated with obstructive sleep apnea (OSA). Our evidence that P2Y1-recepors 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 like OSA. We expect that our findings will be of great interest to a broad audience in the basic, clinical, and pharmaceutical community.

3) Summary

Catecholaminergic C1 cells in the RVLM are key determinants of the sympathoexcitatory response to peripheral chemoreceptor activation. Over-activation 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 over-activation of peripheral chemoreceptors.

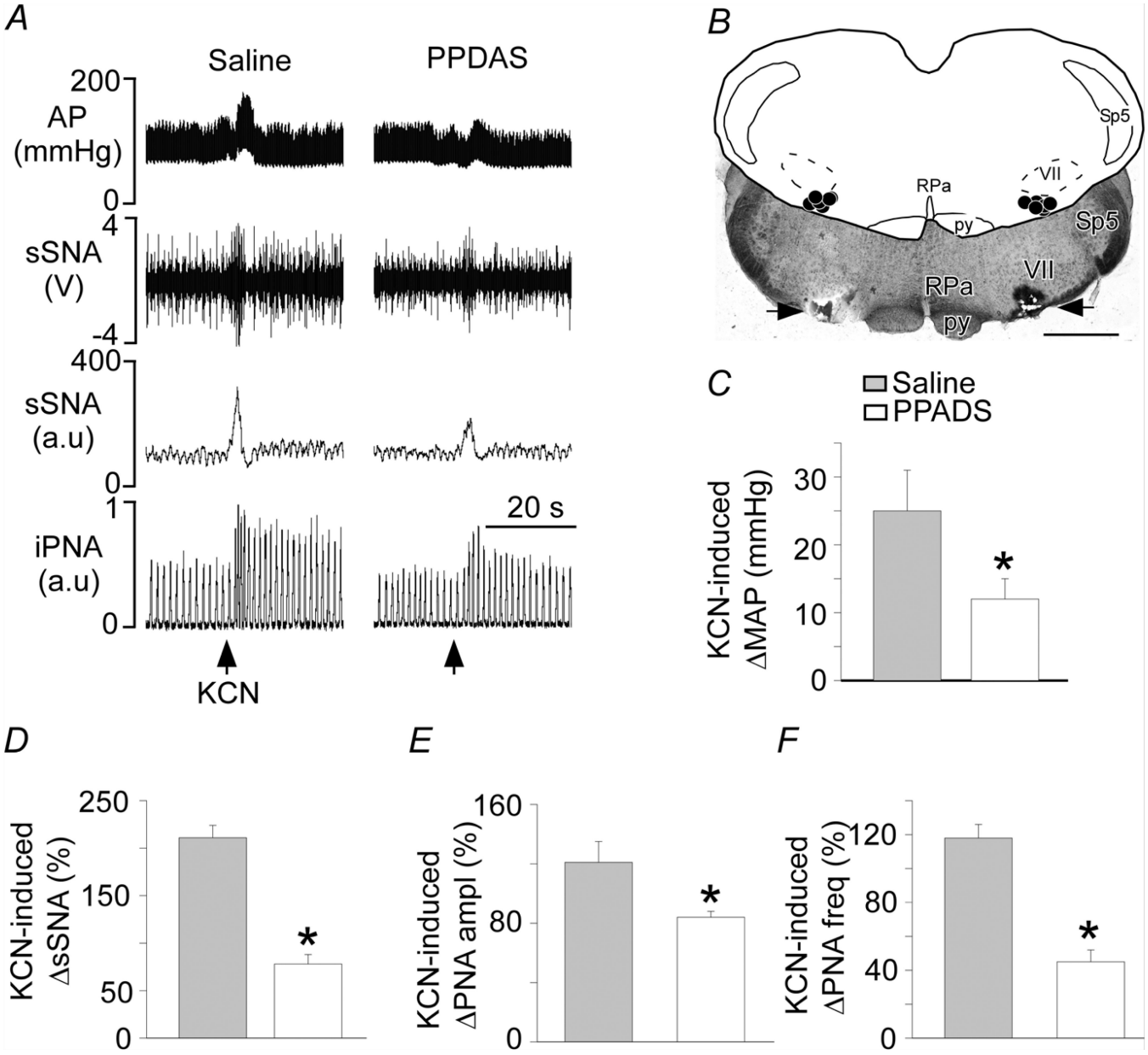

Figure 1. Purinergic signaling in the RVLM is necessary for peripheral chemoreceptor regulation of breathing, blood pressure and sympathetic activity

Traces of arterial pressure (AP), raw and integrated (∫) splanchnic sympathetic nerve activity (sSNA) and integrated phrenic nerve activity (∫PNA) show the response of a urethane-anaesthetized rat to activation of peripheral chemoreceptors with cyanide (KCN: 40 μg/0.1 ml i.v.) 5 min after bilateral RVLM injections of saline and PPADS (100 μM, 50 nl). **B**, Computer-assisted plot and representative photomicrograph show PPADS injection sites in the RVLM (Bregma −11.6)[58]. Scale bar = 1 mm. **C–F**, Summary data (N = 6 rats/group) shows that RVLM injections of PPADS decreased effects of peripheral chemoreceptor activation on MAP (C), sSNA (D), PNA amplitude (E) and PNA frequency (F). Abbreviations: VII, facial motor nucleus, py, pyramidal tract; RPa, raphe pallidus; Sp5, spinal tract of trigeminal nerve. *, designates $P < 0.05$.

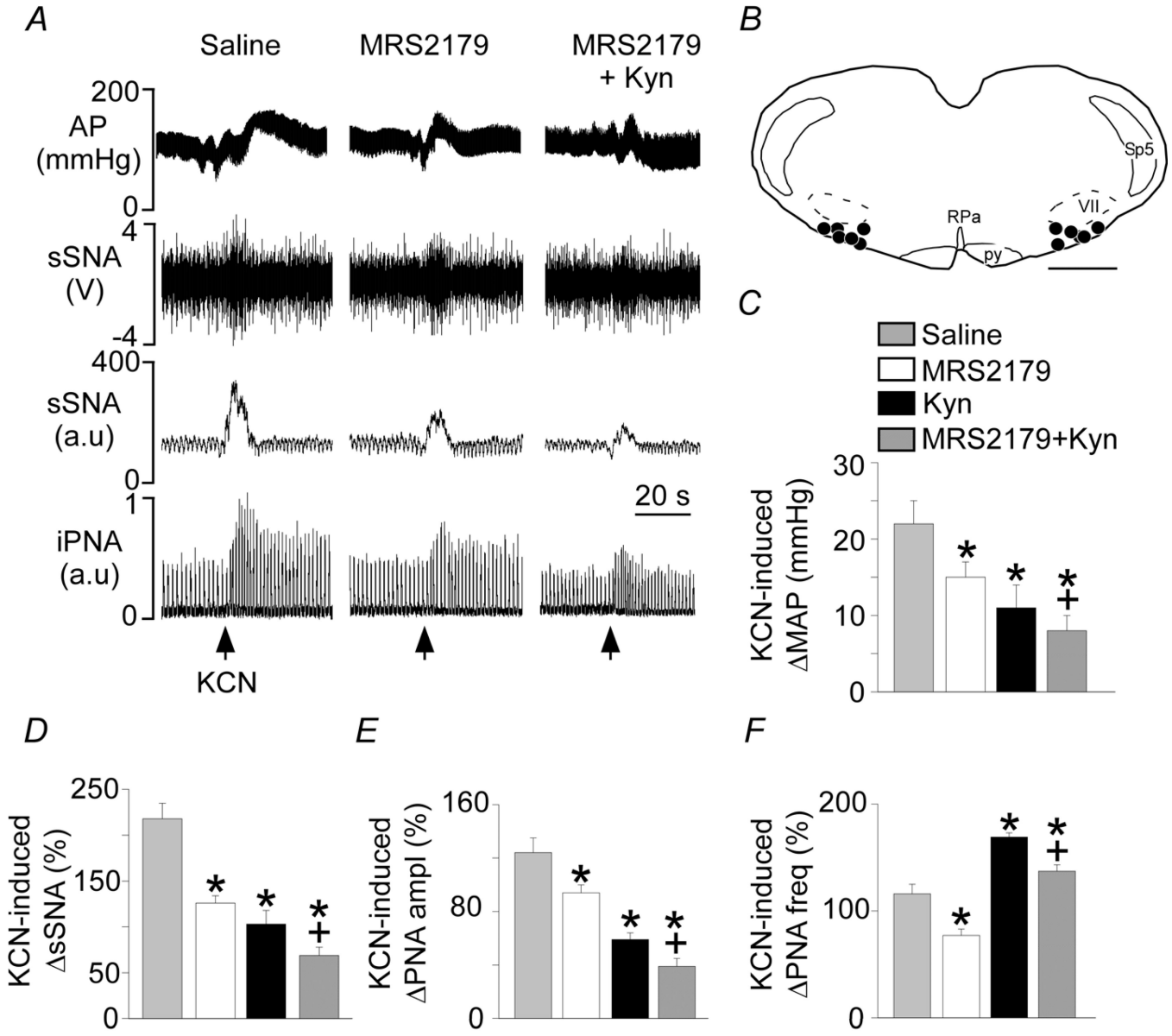

Figure 2. P2Y1 and ionotropic glutamate receptors regulate peripheral chemoreflex in the RVLM

A, traces of arterial pressure (AP), raw and integrated (∫) splanchnic sympathetic nerve activity (sSNA) and integrated phrenic nerve activity (∫PNA) show the response of a urethane-anaesthetized rat to KCN (40 μg/0.1 ml i.v.)-induced activation of the peripheral chemoreflex 5 min after bilateral RVLM injections of saline (left), MRS2179 (100 μM, 50 nl) alone (middle), and MRS2179 and Kynurenic acid (100 mM, 50 nl) (right). **B**, computer-assisted plot of injection sites in the RVLM (Bregma −11.6)[58]. Scale bar = 1 mm. **C–F**, Summary data (N = 6 rats/group) shows that RVLM injections of MRS2179 and kynurenic acid alone and in combination decreased effects of peripheral chemoreceptor activation on MAP (C), sSNA (D), PNA amplitude (E) and PNA frequency (F). Abbreviations: VII, facial motor nucleus, py, pyramidal tract; RPa, raphe pallidus; Sp5, spinal tract of trigeminal nerve. *, designates $P < 0.05$.

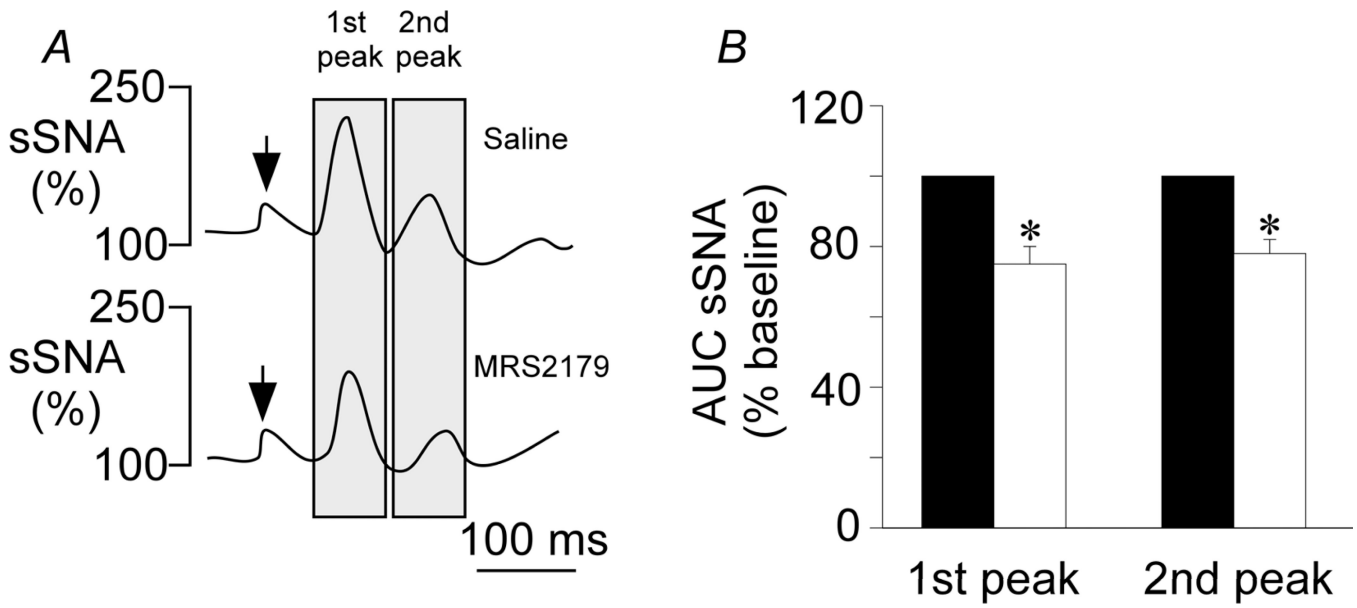

Figure 3. Purinergic signaling via P2Y1 receptors in the RVLM contributes to the somatosympathetic reflex

A, grouped effect of sciatic nerve-evoked stimulation of sSNA at control period (saline injection) and after bilateral injection of MRS2179 (100 μM - 50 nl, N = 5) into the RVLM. Arrows indicate the time of stimulation. **B**, grouped data illustrating the effects of saline or MRS2179 on the area under the curve (AUC) of the first and second sympathoexcitatory peaks for sSNA (A). *, designates $P < 0.05$ compared with control (saline group).

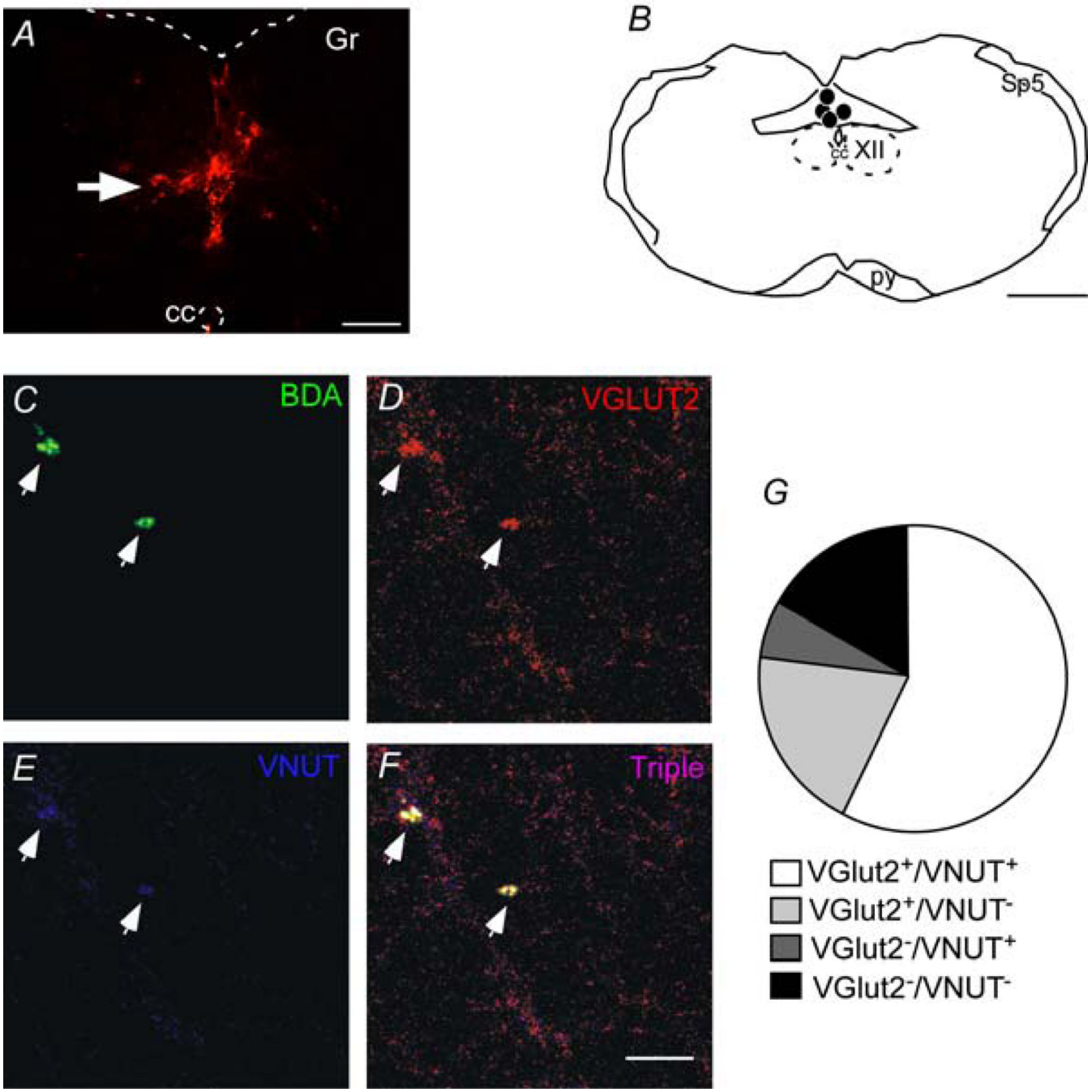

Figure 4. Projections from the caudal NTS to the RVLM at the level of the RTN are immunoreactive for VGLUT2 and VNUT

Fluorescent microbeads were injected with BDA to confirm the location of injection sites. **A–B**, computer-assisted plot (A) and representative photomicrograph (B) of the boxed region in panel A shows BDA injection sites in the caudal NTS (cNTS). All injection sites were within 200 μm of the caudal edge of the area postrema (Bregma = −13.8 mm)[58]. Scale bar = 200 μm. **C–E**, digital images show that the majority of BDA labeled cNTS terminals in the RVLM (green, C) are immunoreactive for VGLUT2 (red, D) and VNUT (blue, E). **F**, merged image. The double arrow designates a cNTS terminal in the RVLM that was VNUT-positive but VGLUT2-negative. Scale bar = 25 μm. **G**, summary data (N = 4 rats) shows the

proportion of cNTS terminals in the RVLM that were immunopositive for VGLUT2 and/or VNUT. Abbreviations: XII, hypoglossal nucleus cc, central canal; Gr, gracile nucleus; py, pyramidal tract; Sp5, spinal tract of trigeminal nerve.

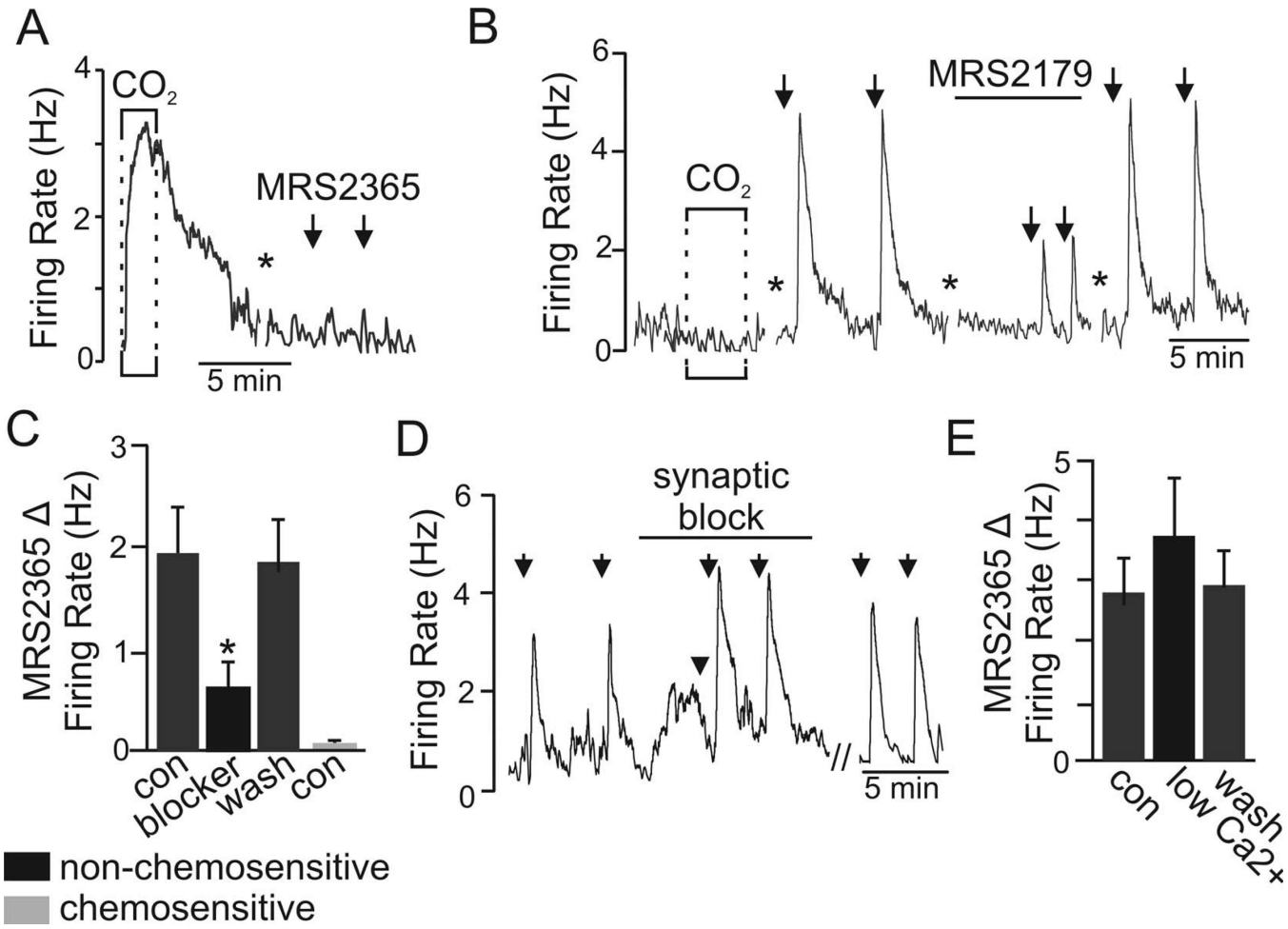

Figure 5. P2Y1-receptors are functionally expressed by CO_2-insensitive RVLM neurons but not RTN chemoreceptor neurons

A, trace of firing rate from a chemosensitive RTN neuron shows that increasing CO_2 from 5 to 15% increases firing rate by ~3 Hz. After returning to 5% CO_2, focal application (arrows) of the selective P2Y1- receptor agonist (MRS2365, 100 μM) had no effect on firing rate. **B**, trace of firing rate from a CO_2-insensitive neuron in the RVLM shows a robust and repeatable firing rate response to MRS2365. MRS2365-responsivness was blunted by bath application of the selective P2Y1-receptor antagonist (MRS2179, 3μM) and fully recovered in wash. **C**, summary data shows the MRS2365 firing rate response of CO_2-sensitive neurons (grey bar, N=19) and CO_2-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 CO_2-insensitive RVLM neuron shows that MRS2365-responsivness was retained in the presence of synaptic block solution (high Mg^{2+} and low Ca^{2+} solution, see supplemental methods). ▼, designates DC current injection; //, designates 10 min time breaks. **E**, average data (N = 5) shows the firing rate response to MRS2365 under control conditions and in the presence of synaptic block solution (low Ca^{2+}). *, designates $P < 0.05$.

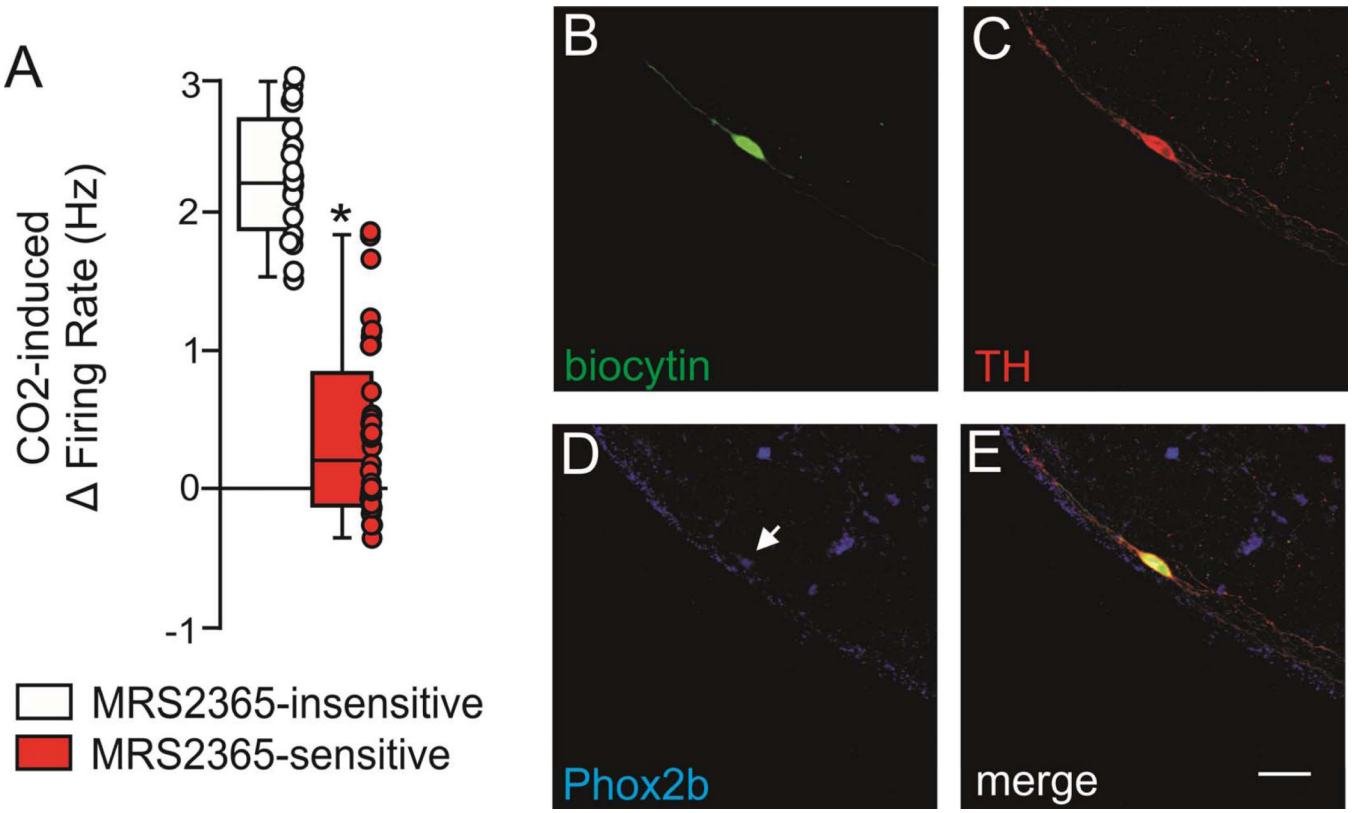

Figure 6. P2Y1-receptors are differentially expressed by C1 cells

A, summary box plot shows that cells in the RVLM can be differentiated based on responsiveness to CO_2 (15%) and MRS2365 (100 μM). Chemosensitive RTN neurons were largely MRS2365-insensitive (white bar, N = 22), whereas the majority of CO_2-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. **B-E**, 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.

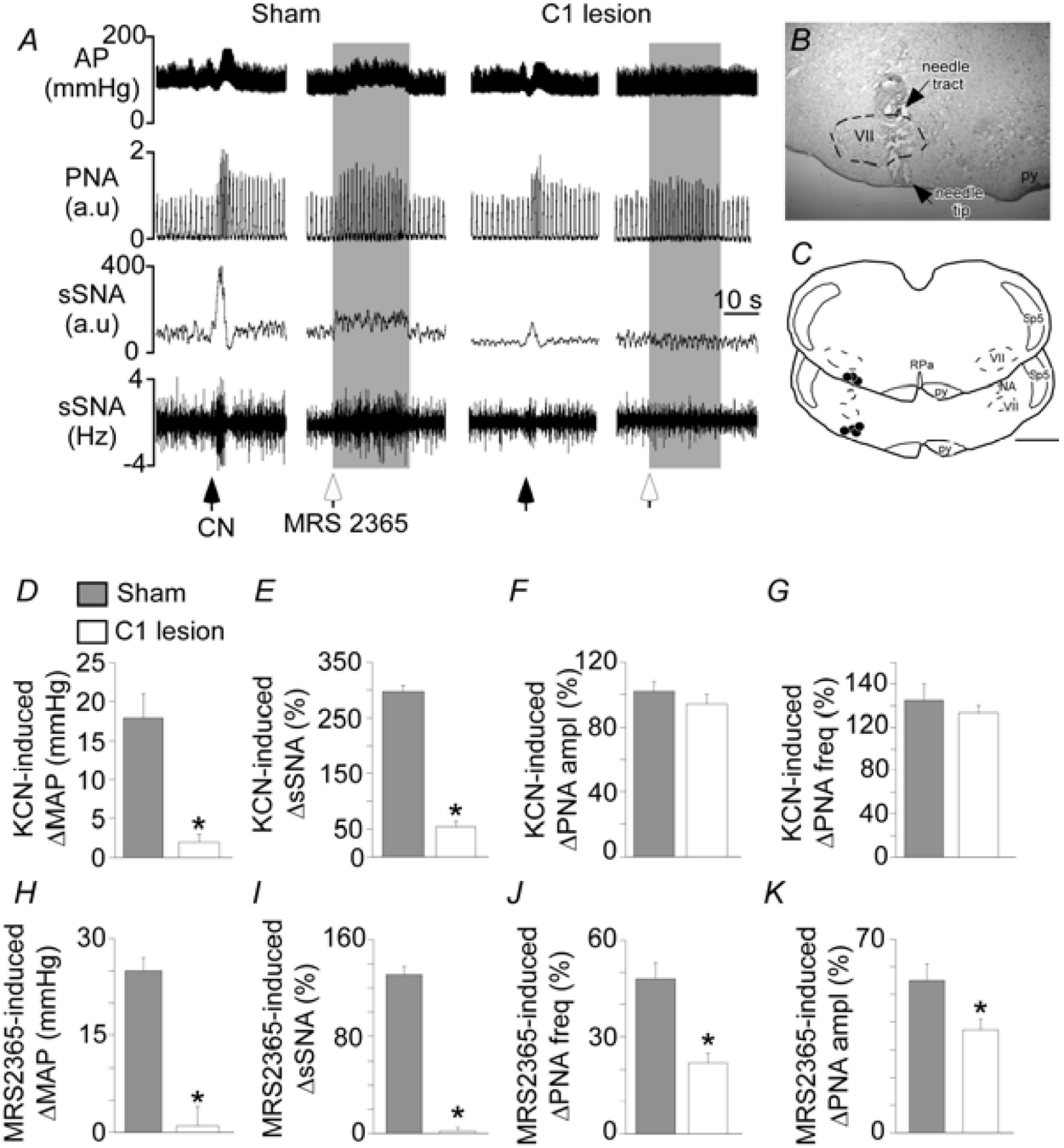

Figure 7. Depletion of C1 neurons decreased pressor and sympathoexcitatory responses to activation of peripheral chemoreceptors and decreased cardiorespiratory responses to activation of P2Y1-receptors in the RVLM

We made bilateral RVLM injections of anti DβH-SAP (4.2 ng/100 nl) to selectively lesion C1 cells (see supplemental figure S1). **A**, traces of arterial pressure (AP), raw and integrated (∫) splanchnic sympathetic nerve activity (sSNA) and integrated phrenic nerve activity (∫PNA) show the response of a urethane-anaesthetized sham rat (left) and C1 lesion rat (right) to activation of peripheral chemoreceptors with cyanide (KCN: 40 μg/0.1 ml i.v.) and unilateral RVLM injection of MRS2365 (100 μM, 50 nl). **B**, representative photomicrograph shows that MRS2365 was injected into the RVLM at the level of the RTN. **C**, plot of

MRS2365 injection sites (Bregma −11.6 and −11.8)[58]. Scale bar = 1 mm. **D–G**, summary data (N = 7 rats/group) shows MAP (D), sSNA (E), PNA amplitude (F) and PNA frequency (G) responses of sham and C1 lesion animals to activation of peripheral chemoreceptors. Note that C1 lesion animal's exhibit reduced pressor and sympathoexcitatory responses but otherwise normal respiratory response to peripheral chemoreceptor activation. **H–K**, summary data (N = 7 rats/group) shows MAP (H), sSNA (I), PNA amplitude (J) and PNA frequency (K) responses of sham and C1 lesion animals to unilateral RVLM injection of MRS2365. These results confirm that P2Y1-receptors expressed on C1 cells are required for peripheral chemoreflex in the RVLM. Abbreviations: VII, facial motor nucleus, py, pyramidal tract; RPa, raphe pallidus; Sp5, spinal tract of trigeminal nerve. *, designates $P < 0.05$.

Table 1

Average parameters of sigmoid baroreflex curves in rats treated with saline or MRS2179

Group	n	Baseline MAP (mmHg)	MAP50 (mmHg)	Upper plateau (%)	Lower plateau (%)	Range (%)	Gain max (%/mmHg)
Saline (control)	5	117±3	118±6	157±9	18±4	135±11	5.4±1
MRS2179	5	115±8	116±9	158±7	19±6	138±13	5.3±0.5

Baroreflex analysis after bilateral injection of saline or MRS2179 (100 μM - 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, i.v) and increasing MAP with phenylephrine (Phe - 5 μg/kg, i.v). Baseline sSNA was set to 100%, and minimum sSNA was determined after intravenous injection of hexamethonium (30 mg/kg, i.v).