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SYMPOSIUM REVIEW

# Independent purinergic mechanisms of central and peripheral chemoreception in the rostral ventrolateral medulla

Thiago S. Moreira[1], Ian C. Wenker[2], Cleyton R. Sobrinho[1], Barbara F. Barna[1], Ana C. Takakura[3] and Daniel K. Mulkey[4]

[1]*Department of Physiology and Biophysics, University of São Paulo, São Paulo, SP 05508, Brazil*
[2]*Department of Pharmacology, University of Virginia, Charlottesville, VA 22908, USA*
[3]*Department of Pharmacology, University of São Paulo, São Paulo, SP 05508, Brazil*
[4]*Department of Physiology and Neurobiology, University of Connecticut, Storrs, CT 06269, USA*

**Abstract** The rostral ventrolateral medulla oblongata (RVLM) contains two functionally distinct types of neurons that control and orchestrate cardiovascular and respiratory responses to hypoxia and hypercapnia. One group is composed of the central chemoreceptor neurons of the retrotrapezoid nucleus, which provides a $CO_2/H^+$-dependent drive to breathe and serves as an integration centre and a point of convergence of chemosensory information from other central and peripheral sites, including the carotid bodies. The second cluster of RVLM cells forms a population of neurons belonging to the C1 catecholaminergic group that controls sympathetic vasomotor tone in resting conditions and in conditions of hypoxia and hypercapnia. Recent evidence suggests that ATP-mediated purinergic signalling at the level of the RVLM co-ordinates cardiovascular and respiratory responses triggered by hypoxia and hypercapnia by activating retrotrapezoid nucleus and C1 neurons, respectively. The role of ATP-mediated signalling in the RVLM mechanisms of cardiovascular and respiratory activities is the main subject of this short review.

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**Corresponding author** T. S. Moreira: Department of Physiology and Biophysics, Institute of Biomedical Science, University of São Paulo, Av. Prof. Lineu Prestes 1524, 05508-000, São Paulo, SP, Brazil. Email: tmoreira@icb.usp.br
I. C. Wenker: Department of Pharmacology, University of Virginia, 1300 Jefferson Park Avenue, PO Box 800735, Charlottesville, VA 22908-0735, USA. Email: icw7f@virginia.edu

**Abbreviations** Cx26, connexin 26; NTS, nucleus of the solitary tract; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral medulla oblongata.

**Thiago S. Moreira** received a PhD in Physiology from the Federal University of Sao Paulo (UNIFESP), Brazil. In 2009, he joined the Institute of Biomedical Science at the University of Sao Paulo, Brazil as an Assistant Professor and in 2014 became Associate Professor. During recent years, his research has focused on the brainstem network that co-ordinates respiration and circulation. **Ian C. Wenker** holds an MS from Wright State University and a PhD from the University of Connecticut. For his PhD thesis, he studied intrinsic properties of brainstem cardiorespiratory neurons and astrocytes with brain slice patch-clamp recording techniques and earned a predoctoral fellowship from the American Heart Institute. He is now a postdoctoral fellow at the University of Virginia, exploring the brainstem circuitry involved in autonomic reflex control of the cardiorespiratory system.

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## Introduction

Respiratory chemoreception is the ability of an organism to sense changes in blood gases (i.e. $O_2/CO_2/H^+$) to provide for the homeostatic control of respiratory and cardiovascular systems and can be divided into central and peripheral components. Central chemoreception relies on specialized cells within the brainstem that sense $CO_2/H^+$ and output to increase breathing and sympathetic nerve activity (Guyenet *et al.* 2010; Moreira *et al.* 2011). Peripheral chemoreceptors of the carotid body sense changes in $O_2/CO_2/H^+$ and also regulate breathing and sympathetic outflow, via synapses through the central nervous system (Kumar & Prabhakar, 2012). It is well established that the rostral ventrolateral medulla (RVLM) contains two important subsets of neurons involved in cardiorespiratory control during the chemoreflexes, namely the $CO_2/H^+$-sensitive neurons of the retrotrapezoid nucleus (RTN) that function as central respiratory chemoreceptors (Mulkey *et al.* 2004; Guyenet *et al.* 2010) and presympathetic catecholaminergic C1 neurons that control sympathetic vasomotor tone in response to a number of reflexes, including the peripheral chemoreflex (Guyenet, 2006; Guyenet *et al.* 2013).

The role of ATP as a neurotransmitter was first described in the enteric nervous system several decades ago (Burnstock *et al.* 1970). Since then, purinergic signalling has been found to contribute at all levels of the nervous system, including enteric, autonomic and central (Burnstock, 2007). The mechanisms of ATP signalling are equally diverse. They include many ionotropic (P2X) and metabotropic (P2Y) receptor subtypes (Fredholm *et al.* 1994), as well as varying methods of transmission, including vesicular, volume-regulated anion channel and gap junction hemichannel release of ATP from neuronal and non-neuronal cells (Burnstock, 2007).

This short review addresses the role of purinergic signalling in the RTN chemoreceptor and C1 presympathetic neuronal control of the central and peripheral chemoreflexes. To learn more about purinergic signalling in respiratory control, the reader is referred to reviews by Erlichman and colleagues (2010) and Funk (2013). In addition, this review focuses on central purinergic mechanisms; however, purinergic signalling is also critical peripherally in the carotid bodies (Piskuric & Nurse, 2013).

## Purinergic signalling in the RVLM: the RTN and central chemoreception

The defining properties of central respiratory chemoreceptors include the following: (i) intrinsic $CO_2/H^+$ sensitivity *in vivo* and *in vitro*; (ii) an excitatory neurochemical phenotype; and (iii) projection to the respiratory pattern generator. While there are a number of chemosensitive respiratory neurons that are likely to contribute to central chemoreception (Nattie & Li, 2012), the chemosensitive neurons of the RTN fulfil all three of these criteria and are the focus of this review. They are highly activated by increasing arterial $P_{CO_2}$ *in vivo*, independently of respiratory activity (Mulkey *et al.* 2004; Takakura *et al.* 2006). Retrotrapezoid nucleus neurons are directly activated by $CO_2/H^+$, as demonstrated in neuronal recordings from brain slices (Mulkey *et al.* 2004; Onimaru *et al.* 2012) and acutely dissociated preparations (Wang *et al.* 2013). Retrotrapezoid nucleus neurons are glutamatergic, and their selective stimulation *in vivo* results in rapid and robust breathing activity (Abbott *et al.* 2009; Kanbar *et al.* 2010), while selective inhibition blunts whole-animal breathing responses to hypercapnia (Marina *et al.* 2010; Takakura *et al.* 2014), thus indicating that RTN chemoreceptors provide an excitatory drive to breathe.

As denoted above, at least some of the $CO_2/H^+$ sensitivity of RTN neurons is intrinsic, and this appears to be mediated partly by TWIK-related acid-sensitive K-2 channels (TASK-2; Wang *et al.* 2013). However, adult RTN neurons receive numerous excitatory and inhibitory inputs, including polysynaptic inputs from the carotid body, pulmonary receptors, hypothalamus, nucleus of the solitary tract (NTS), periaqueductal grey matter, spinal cord, dorsolateral pons and raphe nuclei (Rosin *et al.* 2006; Takakura *et al.* 2006; Moreira *et al.* 2007; Barna *et al.* 2014). If some of these inputs are chemosensitive themselves (e.g. carotid body inputs surely are and some NTS inputs could be), then part of the *in vivo* chemosensitivity of RTN neurons could be synaptically driven. However, pharmacological blockade of excitatory inputs has little to no effect on their $CO_2/H^+$ sensitivity *in vivo*, at least in an anaesthetized, hyperoxic state (Mulkey *et al.* 2004), underscoring their intrinsic chemosensitivity.

In the past decade, a role for paracrine release of ATP (i.e. purinergic signalling) in the RVLM has been found to be crucial for proper central chemoreception (Thomas & Spyer, 1999, 2000; Spyer *et al.* 2004; Gourine *et al.* 2005). Work from our group confirmed and extended some of these earlier studies (Fig. 1). We found that blocking P2 receptors in the RTN produces a reduction in the amplitude and frequency of phrenic nerve activity and in the pressor responses elicited by hypercapnia in anesthetized and conscious rats (Wenker *et al.* 2012; Sobrinho *et al.* 2014; B. F. Barna, A. C. Takakura, D. K. Mulkey and T. S. Moreira, unpublished results). At the cellular level, bath application of P2-receptor antagonists blunted the $CO_2/H^+$-evoked firing rate response of RTN neurons in brain slice recordings (Gourine *et al.* 2010; Wenker *et al.* 2010, 2012). The contribution of purinergic signalling to chemosensitive RTN neuronal activity was found to be independent of temperature and stimulus strength and was wholly retained when synaptic activity was blocked using high-$Mg^{2+}$, low-$Ca^{2+}$ solution (Wenker *et al.* 2012).

Our group also found that connexin hemichannel blockers were effective at blunting the purinergic component of RTN neuronal $CO_2/H^+$ sensitivity (Wenker *et al.* 2012). This observation is congruent with a series of experiments from the laboratory of Nicholas Dale, where they demonstrated that $CO_2$-evoked ATP release at the ventral surface is likely to be mediated by $CO_2$-sensitive connexin 26 (Cx26) hemichannels (Huckstepp *et al.* 2010*a*, 2010*b*; Meigh *et al.* 2013). Using ATP-sensing microelectrodes, they found that $CO_2$, and not $H^+$, was the stimulus for ATP release in brain slices containing the ventral surface, and this process is dependent on functional connexin hemichannels (Huckstepp *et al.* 2010*b*). In cultured HeLa cells, transfection with Cx26 and preloading with ATP was enough to recapitulate the $CO_2$-dependent ATP release observed in brain slices (Huckstepp *et al.* 2010*a*). Furthermore, in a series of elegant molecular studies they were able to demonstrate that $CO_2$ binds directly to Cx26 channels, resulting in their opening (Meigh *et al.* 2013).

Although the above experiments provide evidence for a purinergic role in central chemoreception and the ability of Cx26 to sense $CO_2$ and release ATP, major questions remain. For instance, which cells are releasing ATP? Based on experiments using synaptic blockade (Mulkey *et al.* 2004; Wenker *et al.* 2012), it is clear that fast chemical synapses do not provide for the purinergic drive in the RTN region. The common interpretation has been that

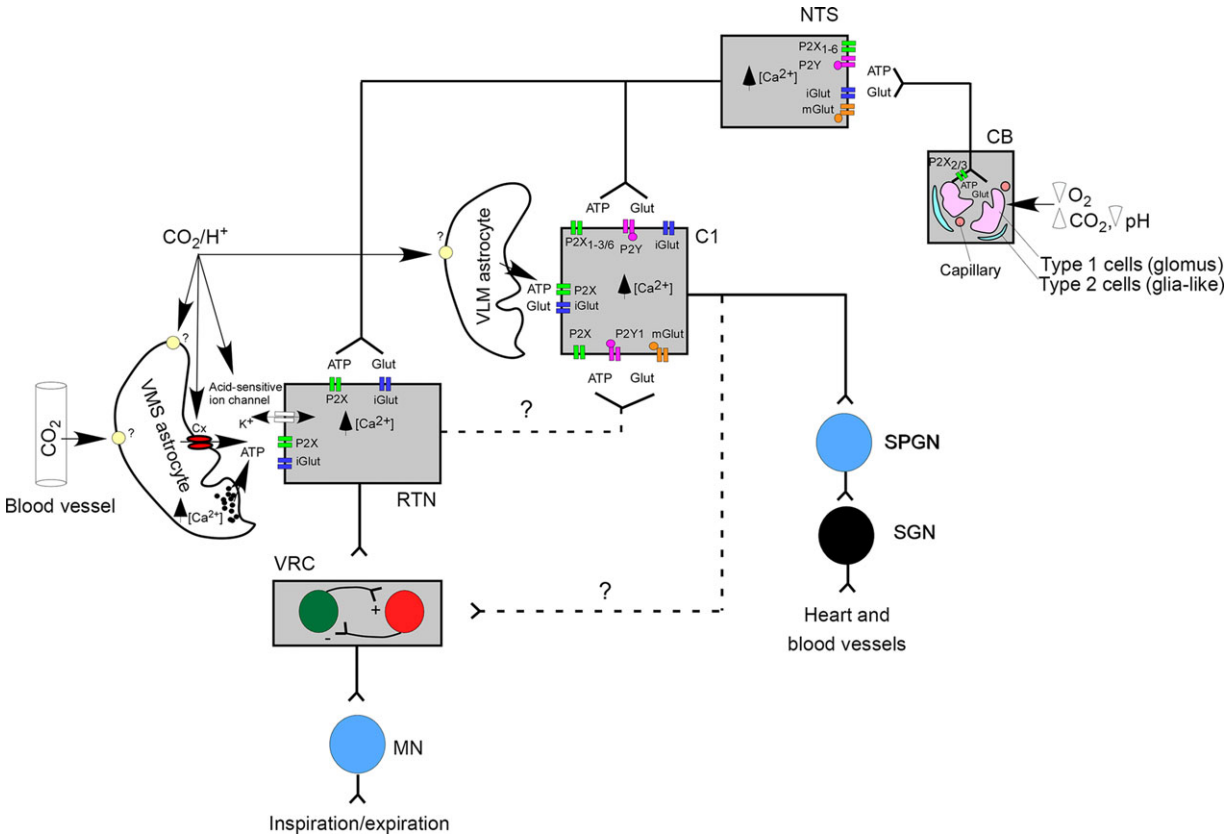


**Figure 1. Schematic model of the possible medullary mechanisms involved in the control of cardio-respiratory responses caused by raising cerebral arterial $P_{CO_2}$ and lowering arterial $P_{O_2}$**
Signals from central or peripheral chemoreceptors may directly or indirectly affect the activity of several medullary areas, including the NTS, C1 region, RTN and VRC, which affect sympathetic discharge to the heart and blood vessels and motorneurons to the respiratory muscles (Pankratov *et al.* 2006; Braga *et al.* 2007; Moraes *et al.* 2011; Wenker *et al.* 2013). An essential step for hypercapnia-induced increase in breathing is activation of RTN neurons by $CO_2/H^+$, directly or indirectly from VMS astrocytes, which in turn send excitatory signals to activate the VRC, either directly or through activation of metabotropic and ionotropic glutamate/purinergic receptors in the C1 region (Takakura & Moreira, 2011; Wenker *et al.* 2013). Release of ATP by astrocytes may be a calcium-dependent exocytotic process triggered by intracellular acidification and/or a leak through connexin channels (primarily connexin 26) opened by molecular $CO_2$ via carbamylation (Huckstepp *et al.* 2010*a*, 2010*b*). Signals from the RTN that activate metabotropic receptors in the C1 region may also increase sympathetic activity to the cardiovascular system. Abbreviations: ATP, adenosine triphosphate; C1, C1 adrenergic region; CB, carotid body; Glut, glutamate; iGlut, ionotropic glutamatergic receptors; mGlut, metabotropic glutamatergic receptors; MN, motor neuron; NTS, nucleus of the solitary tract; P2X, ionotropic purinergic receptors; P2Y, metabotropic purinergic receptors; RTN, retrotrapezoid nucleus; SGN, sympathetic ganglionic neurons; SPGN, sympathetic preganglionic neurons; VLM, ventrolateral medulla; VMS, ventral medullary surface; and VRC, ventral respiratory columm.

ATP is released by astrocytes, because astrocytes have been found to release ATP in response to a number of different physiological stressors (Butt, 2011; Ota *et al.* 2013). Work by Gourine and colleagues (2010) demonstrated that optogenetic stimulation of astrocytes produced ATP release and respiratory effects when the light was directed at the ventral surface. The investigators also found that ATP was released in the RVLM in response to $CO_2/H^+$ stimulation, via $Ca^{2+}$-dependent vesicular release. The pharmacology used to block vesicular release could affect any cell type, and astrocyte-specific loss-of-function experiments was not done. By itself, this leaves open the possibility that other cell types could be responsible for the $CO_2/H^+$-evoked ATP release. However, the ATP release was unaffected by tetrodotoxin, a blocker of neuronal action potentials, and genetically identified astrocytes were found to elevate intracellular $Ca^{2+}$ in response to $CO_2/H^+$. In addition, cultured brainstem astrocytes have demonstrated $H^+$-mediated ATP release (Kasymov *et al.* 2013). Thus, although it remains possible that ATP could be released by other cell types, astrocytes appear to be the likely candidates. In separate experiments, Nicholas Dale's laboratory also produced data supporting astrocytes as the ATP-releasing cells (Huckstepp *et al.* 2010*a*, 2010*b*). Looking at fluorescent dye uptake into cells (dyes that can traverse Cx26 channels) during elevated $CO_2$, they found that it mostly co-localized with glial fibrillary acid protein, a marker for astrocytes (Huckstepp *et al.* 2010*b*). However compelling the apparent $CO_2$-dependence of these data, it is of course only correlative, and future studies will require cell-specific loss of function, as has been done in the cortex (Lalo *et al.* 2014), to confirm that astrocytes are indeed responsible for the purinergic drive to breath.

Another open question is, by what mechanism(s) does purinergic signalling alter the function of RTN chemosensitive neurons? For example, purinergic receptor subtypes and downstream cellular mechanisms of membrane depolarization (e.g. ion channels) are incompletely understood. Based on the purinergic agonist profile described by Mulkey and colleagues (2006), RTN neurons are excited by direct activation of P2Y receptors and inhibited by indirect activation (i.e. via interneurons) of P2X receptors. However, based on the purinergic antagonist profile of the $CO_2/H^+$ responses of neurons in the RVLM, Gourine and colleagues (2010) suggested that the receptors might be of the P2X variety. The former case may be open to some contention because newer, more subtype-selective pharmacological agents have since been developed for purinergic receptors (Fredholm *et al.* 1994). Our group's only results using these agents show that P2Y1 receptors (for a review of purinergic receptor subtypes see Fredholm *et al.* 1994) do not contribute to $CO_2/H^+$ sensitivity of the RTN (Wenker *et al.* 2012), although, serendipitously, they do regulate the activity of local catecholamine neurons in the RVLM (see next section; Fig. 1). The use of the ever-improving purinergic pharmacology and cell specific loss-of-function genetics will no doubt improve our understanding of purinergic mechanisms in central chemoreception.

## Purinergic signalling in the RVLM: the C1 neurons and the peripheral chemoreflex

The increased sympathetic outflow elicited by peripheral chemoreceptors is mediated primarily by activation of the presympathetic neurons of the RVLM, the majority of which are C1 neurons (Fig. 1; Guyenet *et al.* 2013). In support of this idea, selective lesion of the C1 neurons with dopamine-$\beta$-hydoxylase-conjugated saporin toxin virtually abolishes the sympathoexcitatory response to peripheral chemoreflex activation (Schreihofer & Guyenet, 2000). The cardiorespiratory effects of peripheral chemoreceptors are mediated in part by direct glutamatergic inputs from the NTS to C1 neurons. Indirect pathways also exist, and the best documentation is a di-synaptic input that relays via the chemosensitive neurons of the RTN (Koshiya *et al.* 1993; Sun & Reis, 1995; Paton *et al.* 2001; Moreira *et al.* 2006; Takakura *et al.* 2006; Takakura & Moreira, 2011).

In addition to glutamatergic neurotransmission, the C1 neuronal activity can be modulated by purinergic signalling, both by exogenous agonists and endogenously, during autonomic reflex control. In studies from the last two decades, spinally projecting RVLM neurons were found to express P2Y and P2X receptors functionally (Sun *et al.* 1992; Ralevic *et al.* 1999), and activation of these receptors in the RVLM also increases cardiorespiratory parameters (i.e. fictive breathing and blood pressure) in anaesthetized rats (Ralevic *et al.* 1999). Later, it was purported that these P2X receptors were important for RVLM reflex control of cardiorespiratory function. For example, inhibition of P2X receptors within the ventrolateral medulla blunted the ventilatory but not the pressure response elicited by peripheral chemoreceptor activation in conscious rats (Moraes *et al.* 2011). The interpretation that P2X receptors are responsible is based on the relatively low pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate concentration used, which *in vivo* is dubiously selective over P2Y receptors. Nevertheless, if it is true, this could be explained by P2X receptor expression on nearby RTN chemoreceptor neurons (Gourine *et al.* 2010) or by differential expression of P2X and P2Y receptors amongst RVLM neurons (as in Ralevic *et al.* 1999).

More recently, a role for P2Y signalling in C1 neuronal control of the peripheral chemoreflex has been put forward by our group. Specifically, we found that P2Y1 receptors are robustly expressed by C1 neurons but not by

nearby RTN chemoreceptors *in vitro* (Wenker *et al.* 2013). This was determined by the fact that action potential firing of $CO_2/H^+$-sensitive (i.e. RTN chemosensitive) neurons in this region were unaffected by application of a P2Y1-specific agonist, whereas $CO_2/H^+$-insensitive neuronal firing was greatly increased. In addition, many of these $CO_2/H^+$-insensitive, P2Y1-expressing neurons were immunoreactive for tyrosine hydroxylase, a marker for C1 neurons in this region. The expression of P2Y1 receptors by C1 neurons was confirmed *in vivo* by showing that the cardiorespiratory responses induced by P2Y1 agonist injection in the RVLM were blunted in C1-lesioned animals (Wenker *et al.* 2013). Additionally, selective inhibition of P2Y1 receptors in the RVLM decreased peripheral chemoreceptor-mediated activation of breathing and sympathetic outflow. Importantly, this did not change cardiorespiratory outflow during baroreflex or RVLM stimulation, indicating that pharmacological blockade of P2Y1 receptors does not directly alter excitability of C1 cells and that ATP is released during the chemoreflex to stimulate P2Y1 receptors (Wenker *et al.* 2013). Corroborating this idea, we found that approximately 60% of caudal NTS neuron varicosities in the RVLM are immunoreactive for both vesicular glutamate and nucleotide transporters (VGLUT2 and VNUT; Wenker *et al.* 2013), which at other central synapses are sufficient machinery to allow for ATP and glutamate co-release (Gordon *et al.* 2009).

Together, these results suggest that peripheral chemoreceptor drive is relayed, in part, by ATP and glutamate co-release from NTS neuron terminals acting on P2Y1 and ionotropic glutamatergic receptors expressed on C1 neurons (Fig. 1). Interestingly, this purinergic mechanism appears to be distinct from those involved in RTN chemoreception. By this, we mean that although ATP is released in the RVLM during the central $CO_2$ chemoreflex, P2Y1 receptors do not appear to influence the cardiorespiratory effects of this reflex (Wenker *et al.* 2013), Of course, this has been tested only in anaesthetized hyperoxic conditions. It is known that while central and peripheral chemoreflexes operate via separate sensors, they do influence the activity of one another (Blain *et al.* 2010). Thus, in different conditions (i.e. $CO_2/O_2$ levels or conscious state) it is possible that the P2 receptors in the RVLM could contribute to central–peripheral chemoreflex interactions.

Finally, it is important to point out that astrocytes in the RVLM are capable of releasing ATP to affect C1 neurons. Optogenetic stimulation of astrocytes within the ventrolateral medulla excites presympathetic C1 neurons via an ATP-dependent mechanism (Marina *et al.* 2013). This is particularly interesting because evidence exists that glial cells release ATP in response to various stimuli, including hypoxia (Aley *et al.* 2006), and hypoxia produces ATP release in the RVLM (Gourine *et al.* 2005). Thus, depending on the conditions, purinergic signalling of a number of varieties could co-ordinate the output of RVLM neurons.

## Conclusions and clinical perspectives

In this review, we have discussed a number of independent purinergic mechanisms of RTN and C1 neurons that influence breathing and autonomic control of the chemoreflexes. It is not surprising that purinergic signalling is so important in this region, because it contributes to autonomic control via varying mechanisms at several levels of the nervous system, both peripherally and centrally (Gourine *et al.* 2009).

The central and peripheral chemical drive to breathe is associated with several widespread autonomic disorders. Deficits in central chemical drive are associated with central sleep apnoea, a debilitating disease with few therapies besides constant positive airway pressure (Dempsey *et al.* 2014). In addition, disruption of the drive to breath is thought to contribute to mortality of certain pathologies, including sudden infant death syndrome, stroke and epilepsy (Kinney *et al.* 2009; Davis *et al.* 2013; Massey *et al.* 2014). Finally, in obstructive sleep apnoea, certain forms of hypertension and heart failure, the sensitization of peripheral chemoreceptor drive, particularly the sympathetic component, is observed, and this overactivity is thought contribute to the pathology (Narkiewicz *et al.* 1999; Schultz *et al.* 2007).

In recent years, purinergic signalling has been proposed to be an excellent system to target for therapies of numerous pathologies (Jacobson & Boeynaems, 2010; Burnstock, 2014), mainly due to novel pharmacological agents being developed. As more detailed understanding of the purinergic mechanisms involved in the chemical drive to breath is uncovered, it may be possible to treat the aforementioned pathologies with the newly developed purinergic agents. Recent work by Marina and colleagues (2013) demonstrates the utility of targeting purinergic mechanisms by transducing an ectonucleotidase, which breaks down ATP, in the RVLM to treat a rat model of heart failure (Marina *et al.* 2013). Based on our recent data, we have proposed that P2Y1 receptors could represent a therapeutic target for the treatment of cardiorespiratory diseases in which the peripheral chemoreflex is sensitized (Wenker *et al.* 2013). Most of the new purinergic pharmacological agents are ATP analogues and do not cross the blood–brain barrier, making them less practical for use in the central nervous system. It is therefore imperative that brain-permeable agents are developed (Burnstock, 2008). Better pharmacology, combined with further understanding of the specific purinergic receptor subtypes and signalling pathways involved in chemoreflex

control by RTN and C1 neurons, may allow for novel therapeutic strategies for cardiorespiratory diseases.

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## Additional information

### Competing interests

None declared.

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