Geoff Burnstock, purinergic signalling, and chemosensory control of breathing

Alexander V. Gourine and K. Michael Spyer

Centre for Cardiovascular and Metabolic Neuroscience, Department of Neuroscience, Physiology & Pharmacology, University College London, Gower Street, London WC1E 6BT, UK

Abstract

This article is the authors' contribution to the tribute issue in honour of Geoffrey Burnstock, the founder of this journal and the field of purinergic signalling. We give a brief account of the results of experimental studies which at the beginning received valuable input from Geoff, who both directly and indirectly influenced our research undertaken over the last two decades. Research into the mechanisms controlling breathing identified ATP as the common mediator of the central and peripheral chemosensory transduction. Studies of the sources and mechanisms of chemosensory ATP release in the CNS suggested that this signalling pathway is universally engaged in conditions of increased metabolic demand by brain glial cells - astrocytes. Astrocytes appear to function as versatile CNS metabolic sensors that detect changes in brain tissue pH, CO_2, oxygen, and cerebral perfusion pressure. Experimental studies on various aspects of astrocyte biology generated data indicating that the function of these omnipresent glial cells and communication between astrocytes and neurons are governed by purinergic signalling, - first discovered by Geoff Burnstock in the 70's and researched through his entire scientific career.

Introduction

It is a real privilege to have this opportunity to present a review of some of our research in a volume dedicated to the memory of Geoff Burnstock. He was a colleague, collaborator, and friend who both directly and indirectly influenced our research undertaken over the last two decades.

One of us (KMS) first met Geoff in 1974 at an international meeting in Tokyo which led to several informal meetings over the years in London. We were together in New York in 1978 attending a meeting organised by Chandler Brooks and spent a great deal of extramural time sampling the dining delights of the city. Geoff then tried to recruit KMS to his Department of Anatomy and Developmental Biology at the University College London (UCL). The Department had an extraordinary mixture of truly excellent scientists and had a strong emphasis on Neuroscience. Geoff offered a wonderful suite of laboratories, but for many reasons it was not the right time for KMS to move from Birmingham and the offer was declined at that time. However, two years later KMS moved to London to head the Department of Physiology at the Royal Free Hospital School of Medicine.

It took until the mid-90's for a collaboration to develop between the Burnstock and Spyer laboratories, coinciding with KMS becoming Head of UCL's Department of Physiology. This collaboration centred on the role of ATP and adenosine in the CNS control of circulation. Geoff was forceful in persuading us that the effects of adenosine are "not that important" and that we should focus on ATP-mediated signalling. In a series of pilot studies with Theresa Thomas it slowly emerged that signalling via P2 receptors might indeed play an important role in modulation of the activity of brainstem circuits that control breathing.

Our collaboration intensified in 1997 when Geoff retired from the headship of the Anatomy Department and moved to the Royal Free Hospital School of Medicine to occupy laboratory space adjacent to the Spyer's laboratories. This led to the creation of the Autonomic Neuroscience Institute, with Burnstock and Spyer as co-directors, aimed at developing a unique centre for research on the autonomic nervous system. This venture was very successful in regard of allowing Geoff to continue to be fully research active and motivate the line enquiry described in detail below. The Autonomic Neuroscience

Institute was closed on Geoff's return to the University of Melbourne in 2017 and succeeded by UCL Centre for Cardiovascular and Metabolic Neuroscience.

In 1975 Geoff together with Marcello Costa published a book "Adrenergic Neurons: Their Organization, Function and Development in the Peripheral Nervous System". AVG was familiar with Geoff's name from an early age, as the Russian edition of the book edited by AVG's father Valery Gourine and published in 1979 was always in a prominent place in the home library. AVG joined the Department of Physiology in 2000 to work in collaboration with KMS on the mechanisms of chemosensory control of breathing and had the first opportunity to meet Geoff in person. The subsequent sections of this article give a general summary of the outcomes of our research on the role of ATP in the chemosensory control of breathing which in its early years received valuable input from Geoff, which is evident from several joint publications.

Early work on the role of adenosine in the brainstem mechanisms of cardiovascular control

Early studies on purinergic signalling in the brainstem mechanisms of cardiovascular control focused on the role played by adenosine (Spyer & Thomas, 2000;Spyer *et al.*, 1997). In experiments using experimental animals (rats) it was shown that the cardiovascular changes that accompany the defence reaction are associated with the release of adenosine in the nucleus of the solitary tract and the rostral ventrolateral medulla (RVLM). Blockade of adenosine receptors was found to modify the pressor response, indicating that adaptive cardiovascular changes during fight-or-flight reactions are modulated by adenosine. The pharmacological data suggested that this adenosine is likely to be produced extracellularly following the breakdown of the released ATP (St Lambert *et al.*, 1997). This hypothesis was subsequently tested and supported by experimental data (Dale *et al.*, 2002) obtained in collaboration with Nick Dale (University of Warwick) who developed enzymatic microelectrode biosensors for real-time detection of adenosine release in the brain (Llaudet *et al.*, 2003).

First studies of the ATP effects in the brainstem

Subsequent work undertaken in close collaboration with Burnstock's laboratory aimed at understanding the role of ATP in modulation of the neuronal activity in the RVLM. First it

was found that ATP acting via P2X and P2Y receptors has a strong excitatory effect on the majority of RVLM neurons, including cells with monosynaptic projections to the spinal cord – pre-sympathetic (or sympathoexcitatory) neurons (Ralevic *et al.*, 1999). Strong cardiovascular and respiratory responses were recorded following microinjections of ATP or P2X receptor agonists into the RVLM (Thomas *et al.*, 2001). It was also observed that in anaesthetized rats microinjections of the broad spectrum P2 receptor blocker suramin or P2X receptor agonist αβ-metATP (to desensitize the receptors) into the RVLM region reduced the respiratory responses to CO_2 (Thomas *et al.*, 1999). CO_2-induced increases in the activity of the medullary inspiratory neurons were found to be blocked by P2 receptor antagonists (Thomas & Spyer, 2000).

Studies of the role of P2X receptors in the chemosensory control of breathing

Reduction of the respiratory response to CO_2 in conditions of P2 receptor blockade localised to the ventral regions of the brainstem led to a hypothesis that central respiratory chemosensitivity to CO_2 is mediated via a proxy of pH changes detected by $P2X_2$ receptors, known to be highly sensitive to changes in pH within the physiological range (King *et al.*, 1997). In the absence of selective $P2X_2$ receptor ligands, this hypothesis was tested in $P2X_2$ and $P2X_2/P2X_3$ receptor knockout mice, which became available for this project from a collaboration between Geoff and the research group led by Anthony Ford in Roche Pharmaceutical (Palo Alto). In conscious mice, the respiratory responses to the increases in the level of inspired CO_2 were found to be unaffected by genetic deletion of $P2X_2$, $P2X_3$ or both receptor subunits (Rong *et al.*, 2003). Moreover, analysis of the P2 receptor expression demonstrated that only a small proportion of inspiratory neurons identified within the ventral respiratory column express $P2X_2$ (Gourine *et al.*, 2003), - a result supported by a recent report showing low level of $P2X_2$ expression in the ventral regions of the brainstem (Kim *et al.*, 2020).

Demonstration of the pivotal role of ATP as a key mediator of chemosensory signalling in the carotid body

While the respiratory sensitivity to CO_2 was unaffected in $P2X_2$ knockout mice, the responses to hypoxia were found to be dramatically reduced in conditions of $P2X_2$ receptor deficiency (Rong *et al.*, 2003), pointing to a critical role of ATP-mediated signalling in the carotid body function. In an *ex vivo* preparation of the carotid body,

Weifang Rong observed that the increases in the carotid sinus nerve discharge evoked by hypoxia are dramatically reduced by pharmacological P2X receptor blockade, or in conditions of $P2X_2$ receptor deficiency (Rong *et al.*, 2003). Further reductions of the carotid body response to hypoxia were observed in $P2X_2/P2X_3$ double knockout mice, suggesting that the ventilatory responses in low oxygen conditions are mediated by ATP acting at heteromeric $P2X_{2/3}$ receptors expressed by the afferent terminals of the carotid sinus nerve (Gourine, 2005). The resulting publication (Rong *et al.*, 2003) was the last in a series of articles published from the collaboration between Burnstock and Spyer laboratories and has led to ATP being generally regarded as the key signalling molecule of chemosensory transduction in the carotid body. Moreover, the carotid body's P2X receptors are now recognized as a potential target for the treatment of circulatory system disease (Pijacka *et al.*, 2016).

Studies of the chemosensory ATP release in the brainstem

The data discussed above did not support the hypothesis that pH sensitivity of P2X receptors expressed by the neurons of the medullary respiratory network underlie central respiratory sensitivity to CO_2. This led to the development of a modified hypothesis that the central respiratory chemosensitivity is mediated not by pH-sensitivity of P2X receptors expressed by the respiratory neurons, but by the CO_2/acidification-induced release and actions of ATP. We suggested that chemosensory stimuli induce the release of ATP in the brain and this ATP activates neurons of the medullary respiratory networks to trigger adaptive changes in breathing (Gourine, 2005). This hypothesis was tested in collaboration with Nick Dale and Enrique Llaudet (University of Warwick) who at that time were working on the development of enzymatic amperometric biosensors for real time detection of ATP release (Llaudet *et al.*, 2005). The very first experiments with the new biosensor showed that systemic chemosensory stimuli, such as increases in inspired CO_2 (hypercapnia) or decreases in inspired oxygen (hypoxia), trigger ATP release from the chemosensitive regions of the ventral medulla oblongata (Gourine *et al.*, 2005b;Gourine *et al.*, 2005a;Huckstepp *et al.*, 2016). Blockade of ATP receptors was found to reduce the respiratory responses to both hypercapnia and hypoxia (Gourine *et al.*, 2005b;Gourine *et al.*, 2005a)), suggesting that ATP mediates (at least in part) the effects of chemosensory stimuli on the activity of the brainstem respiratory network (but these actions of ATP are mediated by P2 receptors other than $P2X_2$ or $P2X_3$, considering the phenotype of $P2X_2/P2X_3$ receptor knockout mice, as discussed above).

Identifying the source(s) and mechanisms of ATP release in response to CO_2

Electrically non-excitable cells communicate via the release of ATP, therefore, we next tested the hypothesis that in response to chemosensory stimuli (CO_2 or hypoxia) ATP is released by glial cells. Earlier investigators had noted dense glial layer covering the ventral surface of the brainstem at the locations corresponding to the classical chemosensory areas (Loeschcke, 1982) and the sites of chemosensory ATP release (Gourine *et al.*, 2005a) (for a detailed histological analysis of brainstem astrocytes see (Sheikhbahaei *et al.*, 2018a)). Subsequent studies conducted in London by AVG in collaboration with Sergey Kasparov (University of Bristol) and in Warwick by Nick Dale, revealed distinct and parallel mechanisms underlying the sensitivity of brainstem astrocytes to changes in pH and CO_2, leading to the release of ATP as a common mediator of chemosensory signalling (Huckstepp *et al.*, 2010b;Gourine *et al.*, 2010) (Figure 1a). It was found that in the brainstem astrocytes, acidification activates Na^+/HCO_3^- cotransport, leading to increases in $[Na^+]_i$, activation of the Na^+/Ca^{2+} exchanger to operate in a reverse mode, Ca^{2+} entry and exocytosis of ATP-containing vesicular compartments (Kasymov *et al.*, 2013;Turovsky *et al.*, 2016). CO_2 is sensed directly by connexin 26 hemichannels which increase their open probability (allowing egress of ATP) proportionally to the concentration of CO_2 which forms carbamate bridges between subunits (Huckstepp *et al.*, 2010a;Meigh *et al.*, 2013;Dospinescu *et al.*, 2019).

Follow up studies using *in vivo* animal models provided further evidence that ATP released by brainstem astrocytes contributes to the development of the ventilatory responses to CO_2 and hypoxia (Huckstepp *et al.*, 2010b;Gourine *et al.*, 2010;Angelova *et al.*, 2015;Sheikhbahaei *et al.*, 2018b;van de Wiel *et al.*, 2020). An important role played by astrocytes and purinergic signalling in mediating the effects of chemosensory stimuli on the activities of the brainstem cardiovascular and respiratory control networks is supported by the results of experimental studies conducted by other research groups (Lorier *et al.*, 2007;Lorier *et al.*, 2008;Huxtable *et al.*, 2009;Huxtable *et al.*, 2010;Wenker *et al.*, 2010;Zwicker *et al.*, 2011;Wenker *et al.*, 2012;Funk, 2013;Sobrinho *et al.*, 2014;Barna *et al.*, 2016;Rajani *et al.*, 2016;Cinelli *et al.*, 2017;Sobrinho *et al.*, 2017;Hawkins *et al.*, 2017;Rajani *et al.*, 2018;Reklow *et al.*, 2019;Patterson *et al.*, 2021). The notion that ATP release by astrocytes contributes to the hypoxic ventilatory response centrally was met with some scepticism and the readers are invited to evaluate

the arguments in favour and against this hypothesis presented in a series of opinion articles (Gourine and Funk, 2017; Funk and Gourine, 2018a, 2018b; Teppema, 2018).

Understanding the role of purinergic signalling in the neuronal-activity dependent control of cerebral blood flow and local brain tissue pH

There is significant evidence that neuronal activation leads to the release of purines (Pankratov *et al.*, 1998;Pascual *et al.*, 2005;Pankratov *et al.*, 2006;Wall & Dale, 2013;Sims & Dale, 2014;Badimon *et al.*, 2020;Peng *et al.*, 2020). What is the functional significance of ATP and adenosine release that parallels the increases in the neuronal activity? Using biosensor recordings in anaesthetised rats, we demonstrated the release of ATP from the central terminals of visceral afferents (Gourine *et al.*, 2008) and in the forepaw region of the cerebral cortex in response to activation of somatosensory pathways (Wells *et al.*, 2015). We also found that ATP-mediated signalling plays an important role in the mechanisms of neurovascular coupling in the cerebral cortex (Wells *et al.*, 2015). In a parallel study involving our laboratory, ATP was shown to mediate neuronal activity-dependent cerebrovascular responses at the capillary level (Mishra *et al.*, 2016). More recent data suggest that ATP triggers bicarbonate secretion by astrocytes and this release of bicarbonate helps to maintain local brain extracellular pH homeostasis in conditions of enhanced acid loads associated with increases in neuronal activity (Theparambil *et al.*, 2020) (Figure 1b).

Studies of the role of purinergic signalling in the control of circulation and breathing in pathological conditions

Astroglial dysfunction had been shown to contribute to neuropathology and disordered breathing pattern in Rett's syndrome, - an autism spectrum disorder caused by loss of function of the transcription factor MeCP2 (Lioy *et al.*, 2011). Mouse models of the disease showed that MeCP2 deficiency impairs the ability of brainstem astrocytes to detect changes in CO_2/H^+ (Turovsky *et al.*, 2015), while MeCP2 deletion specifically from astrocytes markedly reduces the ventilatory sensitivity to CO_2 (Garg *et al.*, 2015). These data provided further evidence supporting the hypothesis of an important role played by astrocytes and purinergic signalling in chemosensory control of breathing.

There is also evidence that upregulated chemosensory glial responses leading to the release and high 'ambient' concentrations of purines may play an important role in the mechanisms underlying sympathetic activation which accompanies and contributes to the progression of the circulatory system diseases, such as hypertension and heart failure. Experiments in animal models showed that blockade of ATP-mediated signalling in the RVLM slows the remodelling process in heart failure induced by myocardial infarction (Marina *et al.*, 2013), and reduces systemic arterial blood pressure in hypertension (Marina *et al.*, 2015).

Studies of the mechanosensory properties of astrocytes

In the late 90's Geoff proposed a concept of purinergic mechanosensory transduction, involving ATP as one of the signalling molecules released by cells in response to mechanical stress (Burnstock, 1999). Studies in mice with genetic deletion of $P2X_2/P2X_3$ receptors provided strong experimental evidence that this mechanism operates in the peripheral organs, such as the bladder (Cockayne *et al.*, 2000;Vlaskovska *et al.*, 2001). Recent results suggest that Geoff's concept also applies to our understanding of mechanosensory signalling in the brain. Experimental evidence was obtained suggesting that astrocytes function as intracranial baroreceptors that detect decreases in brain perfusion and trigger compensatory increases in arterial blood pressure and heart rate to preserve cerebral blood flow and oxygen delivery (Marina *et al.*, 2020). TRPV4-dependent opening of connexin 43 hemichannels leading to the release of ATP appears to be the key central event underlying mechanosensory signalling in astrocytes (Turovsky *et al.*, 2020).

Conclusion

This article gives a brief account of the research which at the beginning was motivated by the results of exploratory studies undertaken in collaboration with Geoff's laboratory. When in the year 2000 we started to explore in detail the potential role of ATP in the mechanisms underlying chemosensory control of breathing, all our research methods were tuned to study neurons and the first series of the experiments focused on studies of the neuronal mechanisms and neuronal responses. The first breakthrough came in late 2002 with the development of purine biosensors by Nick Dale and our joint first recordings of chemosensory release of ATP in the living brain. Release of ATP pointed to

the potential involvement of astrocytes, which at that time were generally considered to merely provide neurons with structural and metabolic support, while the research methods to study astrocytes *in vivo* were at early stages of development. The next significant advance came ~2008 as a result of collaboration with Sergey Kasparov and Anja Teschemacher (University of Bristol) who pioneered the use of viral vectors to target brainstem astrocytes; first to express genetically-encoded Ca^{2+} sensors and then light-sensitive proteins, allowing monitoring and control of astroglial activity *in vivo*. All the subsequent studies on various aspects of astrocyte biology generated data, indicating that the function of these omnipresent glial cells and communication between astrocytes and neurons are governed by purinergic signalling, - first discovered by Geoff Burnstock in the 70's and researched through his entire scientific career. Our collaboration with Geoff was hugely valuable to us. It allowed us to take initial steps that would otherwise have been difficult in a timely manner. He did not always understand the issues that were bedevilling us, but he knew what the end point might be and was happy to trust our judgement on the way to proceed. We believe he would take pride in what our research has achieved and particularly in the body of evidence suggesting that purinergic mechanisms may have a true potential in providing novel therapeutic targets for the treatments of some common respiratory and cardiovascular diseases.

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Author notes: Address for correspondence: A. V. Gourine, Centre for Cardiovascular and Metabolic Neuroscience, Department of Neuroscience, Physiology & Pharmacology, University College London, London WC1E 6BT, United Kingdom. a.gourine@ucl.ac.uk

Figure legend

Figure 1| Purinergic signalling mediates communication between astrocytes and neurons in conditions of increased metabolic demand. **a**, Astrocytes function as versatile metabolic sensors of the brain milieu, exquisitely sensitive to changes in brain tissue pH, partial pressures of oxygen and carbon dioxide, as well as cerebral perfusion pressure. In the brainstem, astrocytes are adjacent to, and intermingled with, the networks of neurons that generate and modulate the central respiratory and sympathetic drives. Brainstem astrocytes respond to the potential metabolic threats and via the release of ATP stimulate breathing and increase sympathetic activity (Marina *et al.*, 2018). **b**, In the cerebral cortex, astrocytes contribute to the protection of the brain milieu from acidification locally. At least one third of all astrocytes release bicarbonate to buffer extracellular H^+ loads associated with increases in neuronal activity. The underlying signalling mechanism involves neuronal activity-dependent release of ATP triggering bicarbonate secretion by astrocytes via activation of $P2Y_1$ receptors (Theparambil *et al.*, 2020).

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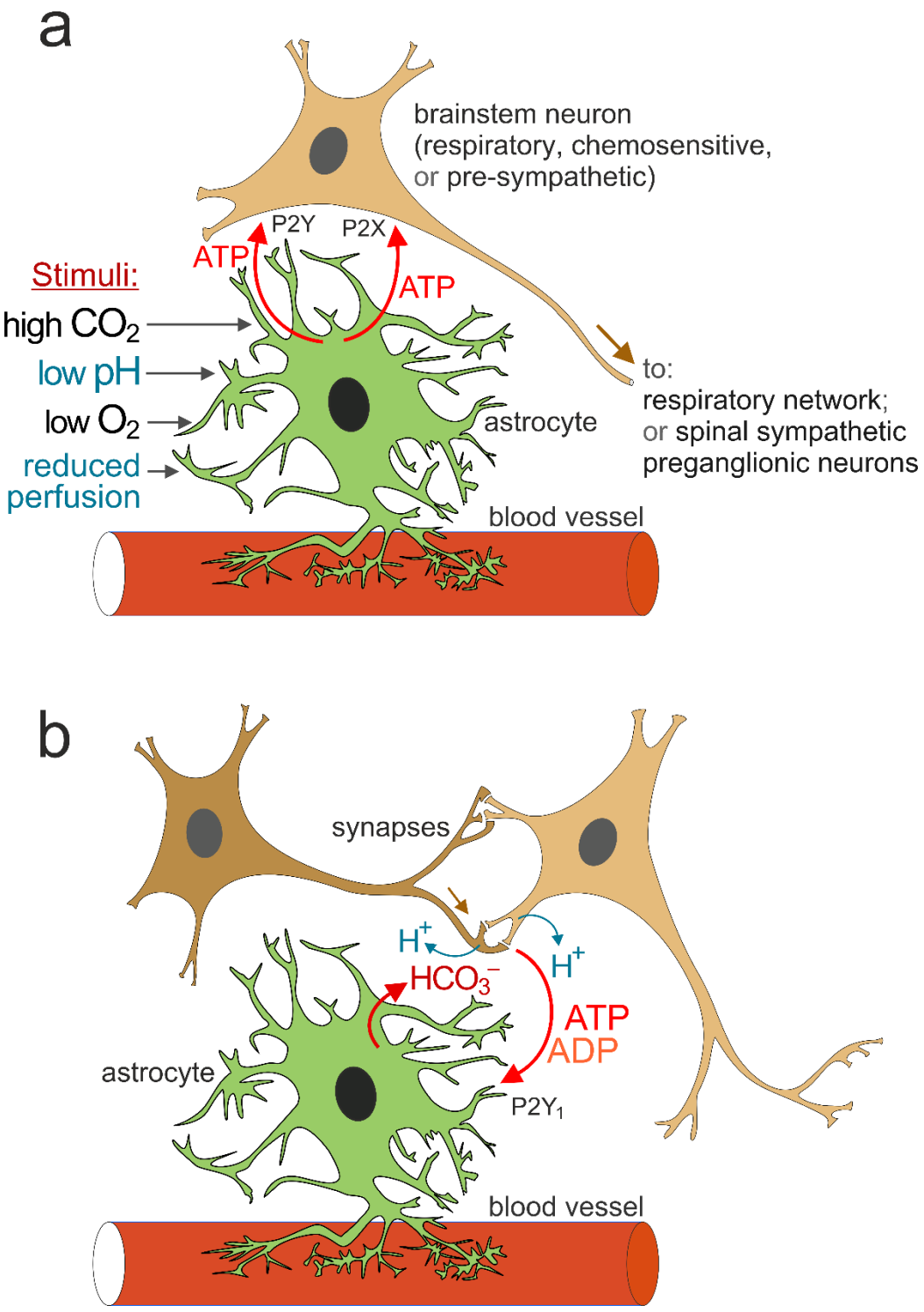
a
brainstem neuron
(respiratory, chemosensitive,
or pre-sympathetic)
P2Y
P2X
ATP
ATP
Stimuli:
high CO_2
low pH
low O_2
reduced
perfusion
astrocyte
to:
respiratory network;
or spinal sympathetic
preganglionic neurons
blood vessel
b
synapses
H^+
H^+
HCO_3^-
ATP
ADP
astrocyte
$P2Y_1$
blood vessel