Purinergic and Glial Signalling in the Hypoxic Ventilatory Response

by

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Abstract

The hypoxic ventilatory response comprises of an initial increase in ventilation, followed by a secondary depression that can be life threatening in premature infants. During hypoxia, ATP is released in the ventrolateral medulla, where it is suggested to attenuate the secondary hypoxic respiratory depression. The objective of my thesis is to explore the contribution of ATP to the hypoxic ventilatory response, specifically exploring the (i) cell-types, (ii) site, (iii) receptor subtype and (iv) signalling pathways that underlie the actions of ATP. I first tested the contribution of glia. I disrupted vesicular release proteins that are necessary for gliotransmission via bilateral injection of an adenovirus controlling glial-specific expression of tetanus light chain protein (TeLC) into the ventral respiratory column (VRC). TeLC caused a decrease in ventilation during the initial phase I component of hypoxic ventilatory response when ventilation increases quickly, and also during the phase II component after ventilation has plateaued after the secondary depression. I next tested which receptor subtype is responsible for attenuating the hypoxic ventilatory depression and where in the VRC this effect was mediated. In adult rats, in vivo unilateral injection of $P2Y_1$ receptor agonist into the preBötzinger Complex (preBötC, central site of inspiratory rhythm generation) caused an increase in respiratory frequency, while in vivo injection of $P2Y_1$ receptor antagonist into the same site increased in the secondary hypoxic respiratory depression. In vivo injection of adenosine, the end product of ATP breakdown, into the preBötC had no effect on ventilation. To explore signaling cascades underlying the excitatory effects of $P2Y_1$ receptor activation in the preBötC, I tested the effect of selective pharmacological blockers on the $P2Y_1$ receptor mediated frequency increase in the neonatal rhythmic slice preparation. Experiments revealed that the $P2Y_1$ receptor mediated excitation is sensitive to calcium chelation, sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibition, antagonism of IP_3 receptors,

and protein kinase C (PKC) inhibition. Similar to the $P2Y_1$ network response, I show that the ATP activation of cultured preBötC glia via intracellular Ca^{2+} increases are also sensitive to SERCA inhibition. These findings suggest that ATP, through a process that involves gliotransmission, acts via $P2Y_1$ receptors in the preBötC to attenuate the secondary hypoxic respiratory depression in vivo, via signaling cascade that involves release of calcium from intracellular stores and PKC activation. This work provides in vivo verification of multiple hypotheses previously developed from in vitro preparations, and lays a foundation for future research in understanding the underlying mechanism of purinergic signalling and the contribution of glia to respiratory network modulation.

Preface

Ethics approval:

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board. The approved projects are entitled:

1) "Neuromodulation of inspiratory network activity in vitro", No. AUP00000255, 2007 (renewed through to 2016).
2) "In vivo approaches to deciphering the modulation of respiratory rhythm generation by ATP", No. AUP00000256, 2011 (renewed through to 2016).

Collaborations:

Some of the research conducted for this thesis forms part of an international research collaboration involving the labs of Dr. Gregory D. Funk (University of Alberta), Alexander V. Gourine (University College London) and Sergey Kasparov (University of Bristol). Part of the viral experiments featured in Chapter 2 (section 2.3.1) were conducted, by myself at the University College London in the laboratory of Alexander V. Gourine, while the remaining were completed at the University of Alberta. All viral constructs were synthesized and supplied by Sergey Kasparov (University of Bristol). Experiments (2.3.1), data analysis and figures (2.1, 2.2), and concluding discussion of these experiments (2.4, 4) are my original work.

Components of the literature review have been published as Rajani V, Zhang Y, Revill AL & Funk GD (2015), "The role of $P2Y_1$ receptor signalling in central respiratory control." *Respir Physiol Neurobiol*. In press". As lead author, I was responsible for overall manuscript organization and composition, the majority of the text and figures. YZ contributed sections of the literature review and revision. AR contributed sections of literature review and revision. GDF was the supervisory author and was involved in concept formation, manuscript organization, composition and revision. Sections from this review that I drafted have been adapted slightly to fit the scope and flow of this thesis. Aside from small changes to accommodate flow, these sections in my thesis are exactly the same as in published manuscript. This was the intent of drafting the published review. These sections are found in sections 1.5, 1.6, 3.1, 4.3.2 and Fig 4.2 of my thesis.

Experimental components of this thesis were contributed by Jennifer D. Zwicker and Venkatesh Jalubula. JDZ conducted in vivo experiments in sections 2.3.2, 2.3.3 and Fig 2.4, 2.5, which have been previously published: Zwicker, JD (2015). *ATP and central respiratory control: a three-part signalling system* (Doctoral dissertation). UNIV OF ALBERTA LIBRARIES' Institutional Repository, EBSCOhost. JDZ also conducted culture experiments that have not been published (3.2.1, 3.3.3 and Fig 3.5). I completed the synthesis of these data for their inclusion in this document. The in vitro thapsigargin experiments were my design but carried out with the assistance of VJ (3.3.1 and Fig 3.2). All data interpretation and discussion is my original work.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Definition
$[Ca^{2+}]_i$	intracellular calcium
aCSF	artifical cerebral spinal fluid
ADO	adenosine
ADP	adenosine diphosphate
ALS	amyotrophic lateral sclerosis
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AVV	adenoviral vector
BK	large conductance calcium activated potassium currents
BME	basal medium eagle
BötC	Bötzinger Complex
BP	blood pressure
BPM	breaths per minute
cAMP	cyclic adenosine monophosphate
CCHS	congenital central hypoventilation syndrome
ChR2	channel rhodopsin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
DAG	diacyl glycerol
DIA	diaphragm
DLH	DL-homocysteic acid
DMSO	dimethyl sulfoxide
dn-SNARE	dominant-negative soluble N-ethylmaleimide factor attachment protein receptor
DRG	dorsal respiratory group
E-NPP	ectonucleotide pyrophosphatase
E-NTPDase	diphosphohydrolase
EMG	electromyogram
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein (s-enhanced shortened)
GFP	green flourescent protein (e-enhanced)
GG	genioglossus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i.p.	interperitoneal
i.v.	intravenous
I_{CAN}	calcium activated non-specific cation current
Inst Freq	instantaneous frequency
IP_3	inositol triphosphate
∫PNA	integrated phrenic nerve amplitude
LC	Locus Coeruleus

Abbreviation	Definition
MRS 2179/2279	selective $P2Y_1$ receptor antagonist
MRS 2365	selective $P2Y_1$ receptor agonist
NK1	N-methyl-D-aspartate
NTS	nucleus of the solitary tract (c-caudal)
P1	adenosine receptor
P2X	purinergic receptor
P2Y	purinergic receptor
PaCO2	partial pressure arterial carbon dioxide
PaO2	partial pressure arterial oxygen
pFRG	parafacial respiratory group
PIP2	phosphatidylinositol 4,5-biphosphate
PKC	protein kinase C
PLC	phospholipase C
preBötC	preBötzinger Complex
PRG	pontine respiratory group
Rel Freq	relative frequency
ROI	region of interest
ROS	reactive oxygen species
RTN	retrotrapeziod nucleus
SD	sprague dawley
SK	small conductance calcium activated potassium currents
SP	substance P
SP-SAP	substance P conjugated to saporin toxin
SST	somatostatin
TASK	TWIK-related aid sensitive K^+ channel
TeLC	tetanus light chain protein
TIRF	total internal reflection fluorescence
TMPAP	transmembrane prostatic acid phosphatase
TNAP	tissue-nonspecific alkaline Phosphatase
TNF	tumor necrosis factor
TRH	thyrotropin-releasing hormone
TRP	transient receptor potential cation channel
UTP	Uridine triphosphate
$\dot{V}_E$	minute ventilation
VGCC	voltage gated calcium chanels
VLM	ventrolateral medulla
VRC	ventral respiratory column
VRG	ventral respiratory group (r-rostral, c-caudal)
V_T	tidal volume

Chapter 1: Introduction

"It appears that the central mechanisms responsible for rhythmic respiration are relatively simple in nature." – DB Burns, 1963

1.1 Overview

The act of breathing is the simple movement of air in and out of the lungs, yet, it is a process that requires the integration and synchronization of multiple physiological components, while remaining robust and highly adaptable to changes in both internal and external environments. A single breath requires the coordination of a number of upper airway, accessory and pump muscles that facilitate airflow, each controlled by dedicated motoneuron pools in the central nervous system (CNS). Moreover, activation patterns are all orchestrated by specialized areas of the medulla that include integration sites for multiple mechanosensory and chemosensory inputs, and the ventral respiratory column that extends throughout the medulla and pons and includes the essential kernel for inspiratory rhythm generation in the preBötzinger Complex (preBötC).

The primary physiological role of breathing and the networks controlling this motor behavior is the homeostatic control of blood gases. Changes in arterial PaO_2, $PaCO_2$ and pH are sensed by chemosensitive areas of the central and peripheral nervous systems that activate neural pathways exciting central respiratory networks to evoke homeostatic changes in ventilation. These ventilatory chemoreflexes are vital to human life. Accordingly, the failure of central areas involved in the detection and responses to low O_2 or high CO_2 contribute to a variety of neurorespiratory and developmental disorders, including apnea of prematurity, congenital central hypoventilation syndrome (CCHS), sudden infant death syndrome (SIDS) and are relevant in neurodegenerative diseases such as Parkinson's disease, amyotrophic lateral sclerosis (ALS) and multiple system atrophy.

The overarching goal of this thesis is to explore the rhythm generating network of the ventral respiratory column and the contribution of adenosine triphosphate (ATP) signalling to the biphasic hypoxic ventilatory response. I will provide insight into the:

(i) contribution of gliotransmission in the ventrolateral medulla (VLM) to the hypoxic ventilatory response in vivo (Chapter 2);

(ii) P2 receptor subtype(s) that mediate the excitatory actions of ATP during hypoxia in vivo (Chapter 2);

(iii) the brainstem site(s) at which ATP acts to increase ventilation during hypoxia (Chapter 2), and;

(iv) cell types (neurons vs glia) and signalling cascades via which ATP acts in the central respiratory network to increase ventilation (Chapter 3).

Before describing my actual research, I review in this chapter background information necessary to understand the rationale and significance of the proposed work. I review the anatomy of central respiratory networks, outlining regions that contribute to the modulation and generation of respiratory rhythm. I then discuss models of rhythm generation and the evolving views of the role played by the preBötC in this vital process, as understanding rhythm generation is important in developing hypotheses about how ATP might be acting to modulate rhythm. Following this, due to their prominence in respiratory network modulation, I discuss types and roles of glia in the CNS, before providing an overview of purinergic signalling in central chemosensitivity and the hypoxic ventilatory response.

1.2 Anatomy of respiratory networks

Respiratory neurons (i.e,, neurons that discharge in phase with some portion of the respiratory cycle) are located in three main areas of the medulla; the pontine respiratory group (PRG), the dorsal respiratory group (DRG) and the ventral respiratory column (VRC). Each is discussed briefly below, but in section 1.3, I focus further attention on the organization and function of the preBötC because it is a critical site of inspiratory rhythm generation and because my thesis focuses on the potential involvement of the preBötC, ATP and astrocytes in the hypoxic ventilatory response.

The PRG, located in the dorsolateral pons, consists of the parabrachial and the Kölliker-Fuse nuclei, the latter of which plays an important role in transition between inspiratory and expiratory phases of breathing and maintaining upper airway resistance

(Dutschmann & Herbert, 2006). The DRG, is a group of primarily inspiratory neurons located in the caudal nucleus of the solitary tract (cNTS), an integration site for sensory respiratory information. Inputs to the DRG include connections from slowly and rapidly adapting stretch receptors in the lungs and peripheral chemoreceptors (Kubin *et al.*, 2006).

The VRC consists of several compartments of respiratory neurons that are distinguished based on different respiratory-related firing and projection patterns. Rostrally to caudally, these compartments are the parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN), Bötzinger Complex (BötC), preBötC, and the rostral and caudal ventral respiratory groups (rVRG and cVRG) A schematic detailing the relative location of these components is shown in Fig 1.1.

Parafacial respiratory group/retrotrapezoid nucleus. The pFRG/RTN is a rostral group of rhythmically active neurons that contribute to the generation of active expiration. Optical imaging with voltage-sensitive dyes revealed that oscillatory activity from the pFRG/RTN immediately preceded that of inspiratory bursts, and partial bilateral electrical lesioning of the pFRG reduced respiratory frequency, suggesting its importance in rhythm generation (Onimaru & Homma, 2003). The significance of this oscillator to rhythm generation was also suggested by the opioid-induced quantal slowing of preBötC rhythm. Quantal slowing was explained by the transmission failure of pre-inspiratory drive (presumably from the pFRG/RTN) to preBötC inspiratory networks (Mellen *et al.*, 2003), suggesting a coupling of the two oscillators to maintain normal respiratory rhythm. This dual oscillator hypothesis is strengthened by the evidence of dual breathing patterns in amphibians; a buccal pattern for swallowing air and an opiate-sensitive lung pattern that suggest an evolutionary origin of coupled dual oscillators in mammalian respiratory rhythm generation (Wilson *et al.*, 2002).

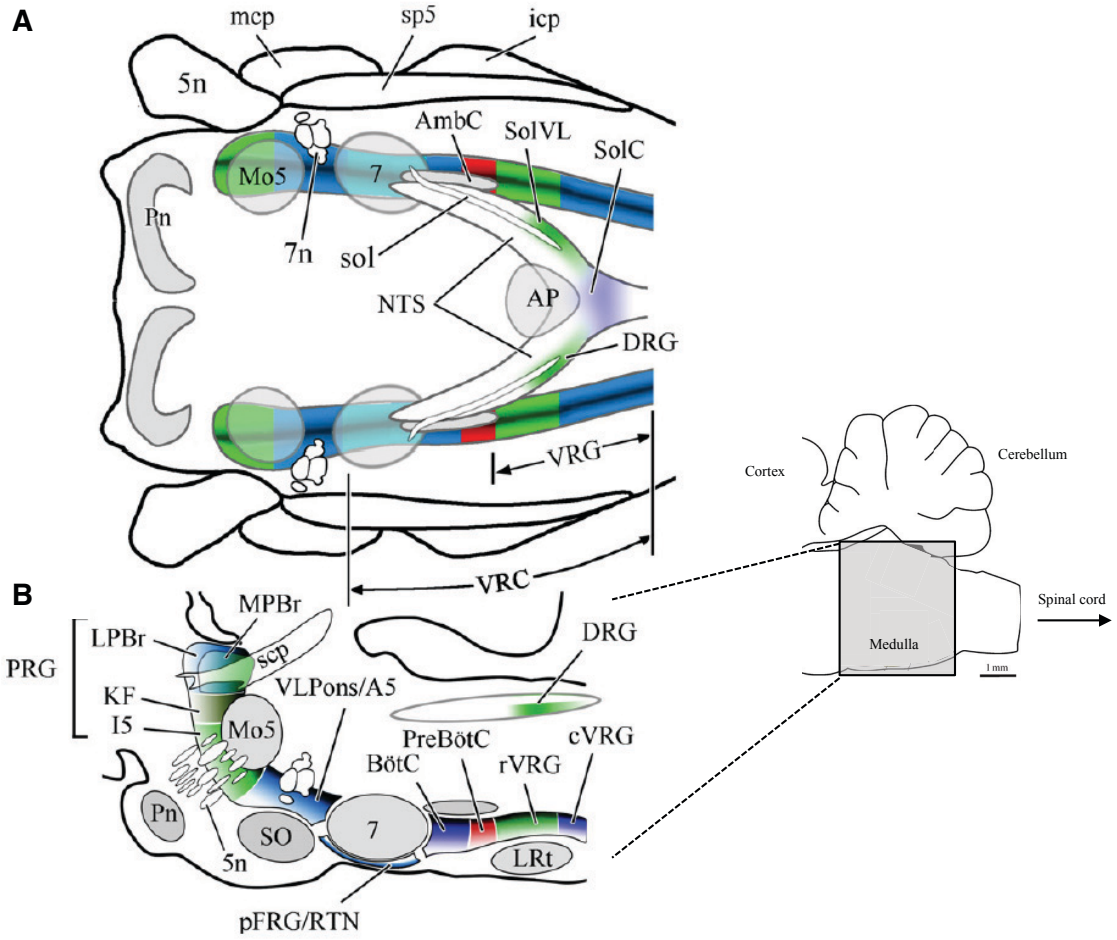

Figure 1.1 Anatomy of respiratory-related regions of the rat brainstem shown in horizontal (A) and sagittal (B) views.

Respiratory-related regions comprise a nearly continuous column in the lateral brainstem. Relative location of the medulla in reference to cerebellum, cortex, and spinal cord is shown on the right, adapted from Spyer and Gourine (2009) *Phil. Trans. R. Soc. B* vol 364 no. 1529 2603-2610I. The boundaries depicted between the various brainstem compartments reflect functional distinctions between adjacent regions relative to their impact on breathing. *Abbreviations*: 5n, trigeminal nerve; 7, facial nucleus; 7n, facial nerve; A5, A5 noradrenergic neuronal group; AmbC, compact part of nucleus ambiguus; AP, area postrema; BötC, Bötzinger complex; cVRG, caudal division of ventral respiratory group; DRG, dorsal respiratory group; I5, intertrigeminal area; icp, inferior cerebellar peduncle; KF, Kölliker-Fuse nucleus; LPBr, lateral parabrachial region; LRt, lateral reticular nucleus; mcp, medial cerebellar peduncle; Mo5, motor nucleus of the trigeminal nerve; MPBr, medial parabrachial region; NTS, nucleus of the solitary tract; pFRG, parafacial respiratory group; Pn, basilar pontine nuclei; preBötC, preBötzinger complex; PRG, pontine respiratory group; RTN, retrotrapezoid nucleus; rVRG, rostral division of ventral respiratory group; scp, superior cerebellar peduncle; SO, superior olive; sol, solitary tract; SolC, commissural subdivision of the nucleus of the solitary tract; SolVL, ventrolateral subdivision of the nucleus of the solitary tract; sp5, spinal trigeminal tract; vlPons, ventrolateral pontine region; VRC, ventral respiratory column of the medulla; VRG, ventral respiratory group. Adapted from Alheid and McCrimmon (2008) Respir Physiol Neurobiol. 64:3-11

Bötzinger Complex. First delineated as a group of predominantly expiratory neurons caudal to the facial nucleus in the cat (Lipski & Merrill, 1980), BötC neurons provide inhibitory projections throughout the VRC via glycinergic and GABAergic signalling (Alheid & McCrimmon, 2008). Though the BötC is suggested as important in phase transition between expiration and inspiration (Smith *et al.*, 2012), the necessity of its involvement in rhythm generation and normal breathing patterns is still under debate. A more thorough discussion of its proposed role in rhythm generation is provided below in Section 1.3

Rostral and caudal ventral respiratory groups. The rVRG is mainly populated by inspiratory bulbospinal premotor neurons that project to inspiratory motoneurons in the phrenic nucleus and external intercostal motoneurons (Dobbins & Feldman, 1994). As the expiratory counterpart to the rVRG, the bulbospinal neurons making up the cVRG project to abdominal and internal intercostal motoneurons (Iscoe, 1998). These regions receive input from the preBötC, BötC and pFRG/RTN regions and transmit this drive via descending tracts to activate and inhibit respective pump muscles (Alheid & McCrimmon, 2008).

preBötzinger Complex. First described in the neonatal rat brainstem spinal cord preparation (Smith *et al.*, 1991), and more recently in humans (Schwarzacher *et al.*, 2010), the preBötC is an essential site of basic inspiratory rhythm generation. The preBötC receives a variety of inputs from centres of chemosensory and mechanosensory feedback, and provides inspiratory drive to multiple respiratory nuclei that are responsible for modulation of muscle activity and changes in breathing pattern. A major focus of this thesis is on the role of the preBötC and its modulation by ATP during the hypoxic ventilatory response. I therefore describe its organization and review the main models of rhythm generation in the following section.

1.3 Models of inspiratory rhythm generation

A centuries old question has been the site and organization of the network responsible for respiratory rhythm generation. Earlier models of rhythm generation in the 20th century were influenced heavily by the simplistic, yet attractive, half-centre model for the locomotor central pattern generator (CPG), which consisted of a bistable system relying on reciprocal inhibition between contralateral and ipsilateral flexor and extensor nodes (Brown, 1914). The resulting model for the respiratory CPG, based on *the half-centre model*, consisted of a rhythm generated by two antagonistic, mutually inhibitory groups of neurons, or half-centres, driving the two observable phases of breathing, inspiration and expiration (Burns, 1963). However, closer inspection of phrenic nerve discharge in anesthetized, paralyzed cats, revealed a third, intermediate stage of respiration, the post-inspiratory phase, bridging the end of the inspiratory phase with the start of the active expiratory phase (Richter, 1982). Neurons were classified based on how their firing patterns correlated with the three phases, revealing multiple classes of respiratory neurons: post-inspiratory, early-inspiratory, ramp inspiratory and late-inspiratory along with inspiratory and expiratory premotor neurons. These observations gave rise to a model of the respiratory network that consisted of a ramp generating excitatory network of interneurons that were initially inhibited by inhibitory late inspiratory neurons, and then finally turned off by post-inspiratory (early-inspiratory) neurons (Richter *et al.*, 1986). By correlating patterns of synaptic inhibition recorded from individual neurons with patterns of respiratory neuron discharge, a connectivity map was generated in which two key groups of neurons, early inspiratory and post-inspiratory neurons were hypothesized as key, reciprocally-connected, inhibitory elements governing respiratory rhythm generation (Richter *et al.*, 1986). Further advancements in the field, however, slowed due to the lack of approaches to directly test this model.

In 1984, a novel observation by Suzue provided a new route to explore the organization of the respiratory network. It was proposed that the rhythmic behaviour recorded from the hypoglossal and phrenic nerves of the in vitro brainstem spinal cord

preparation of the neonatal rat corresponded to "breathing", which provided the opportunity to test key properties of Richter's model (Suzue, 1984). The in vitro rhythm was carefully characterized, providing solid evidence that it was indeed respiratory-related (Feldman & Smith, 1989). Moreover, the essential role of synaptic inhibition in the three-phase model proposed by Richter, came under scrutiny upon the observation that blocking synaptic Cl^--dependent inhibition in the en bloc preparation (Feldman *et al.*, 1990) (later verified with whole-cell recordings (Shao & Feldman, 1997)) affected burst duration, but did not affect cycle timing, suggesting that synaptic inhibition is important for pattern formation, but not necessary for rhythm generation.

The advantages presented by the in vitro preparation propelled a direction in research in understanding the cellular mechanisms underlying the generation and modulation of respiratory activity. A key question could now be answered: what is the source of rhythm? Rostral to caudal serial sectioning of the preparation revealed a ~350μm thick region, ranging from the caudal end of the retrofacial nucleus to a level rostral to obex, that was necessary and sufficient for respiratory rhythm generation (Smith *et al.*, 1991). This region, named the preBötC, responded to increases in extracellular potassium with increases in frequency. In addition, block of rhythm by the non-N-methyl-D-aspartate (NMDA) glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) suggested that glutamatergic signalling is critical in respiratory rhythm generation (Smith *et al.*, 1991).

In vitro analysis of the preBötC greatly increased understanding of the microcircuit in rhythm generation in vitro. In vivo experiments established the existence of a preBötC in vivo and that it contained a unique group of interneurons. The key question though, was the relevance of the in vitro data, and the preBötC itself, to rhythm generation in vivo. The challenge was to develop methods to selectively target key preBötC neurons in vivo to assess their role in the intact nervous system. So began attempts to molecularly characterize the neurons of the preBötC in order to identify unique molecular markers to enable their selective manipulation. A high density of neurokinin-1 (NK1) neurons and μ-opioid receptors

were found concentrated in the preBötC, particularly on putative rhythm generating neurons (Gray *et al.*, 1999). The next step was to try and selectively ablate these neurons, to test the hypothesis that normal breathing rhythm is generated specifically by NK1-expressing neurons. Neurons were bilaterally and selectively ablated in the preBötC using substance P (SP) conjugated to the plant toxin, saporin (SP-SAP). Ablation caused severely ataxic breathing patterns and abnormal responses to hypoxia and hypercapnia in vivo to develop over the week as the neurons died (Gray *et al.*, 2001). In addition, rats with unilateral ablation of preBötC NK1 expressing neurons exhibited disrupted breathing first during sleep (McKay & Feldman, 2008) and during wakefulness as neuronal death progressed, all of which suggest the necessity of these neurons for generating normal breathing rhythm. However, the expression of NK1 receptors rostral and caudal to the preBötC (Guyenet *et al.*, 2002) motivated a search for more specific markers of the preBötC. The identification of a more confined, subset of NK1 expressing neurons that coexpressed the peptide somatostatin (SST) suggested a more refined group of neurons that might characterize the essential components of rhythm generation in the preBötC (Stornetta *et al.*, 2003). Using novel methods of viral transfection, Feldman and colleagues tested whether SST-expressing neurons were essential for rhythm generation in the preBötC of the rat. Using an adeno-associated virus (AAV) 2, the *Drosophila* allatostatin receptor was expressed in SST-expressing cells of the rat preBötC. The addition of allatostatin caused a persistent (but reversible) apnea suggesting the importance of SST-expressing neurons in rhythm generation (Tan *et al.*, 2008). As a result, preBötC neurons necessary for rhythm generation could be defined as glutamatergic neurons possessing NK1 receptor and SST expression. Tracing these glutamatergic, NK1 and SST expressing neurons to their embryonic origins revealed that they are a subset of Dbx1-expressing progenitor cells (Gray *et al.*, 2010), which are expressed throughout the VLM. Those in the preBötC are essential for inspiratory rhythm generation in vitro and in vivo, based on the respiratory deficits that appear in Dbx1 mutant mice (Gray *et al.*, 2010). Furthermore, the novel and elegant technique of laser ablation to

selectively lesion Dbx1 neurons in mouse slices in vitro showed that rhythm generation stopped after removal of 15% of the Dbx1 preBötC population. This provided insight into the minimum number of neurons necessary for generating rhythm in the preBötC, and also revealed that some of these neurons serve a premotor role driving XII motoneurons (Wang *et al.*, 2014)..

The discovery of the preBötC and endogenously rhythmic bursting neurons, or "pacemakers" in the preBötC led to the *hybrid pacemaker network model* of inspiratory rhythm generation. It suggested that inspiratory rhythm is generated by a network of coupled pacemaker cells that posess unique membrane conductances and receive key conditional inputs to maintain and recruit additional pacemakers within a voltage range conducive to bursting. Synaptic inhibition in this model was important for shaping burst, but not rhythm generation. Two types of endogenously bursting pacemaker neurons were identified in the mouse preBötC that relied on different membrane properties to produce bursting. One type was sensitive to Cd^{2+} and the other to riluzole (blocker of the persistent sodium current) (Thoby-Brisson & Ramirez, 2001; Pena *et al.*, 2004; Tryba & Ramirez, 2004). One type of pacemaker neuron relies on a voltage-dependent persistent Na^{+} current (I_{NaP}) while the other on a Ca^{2+}-activated nonspecific cationic current (I_{CAN}), the latter of which is only rarely observed in animals younger than postnatal day 5 (Del Negro, 2002, 2005).

The *hybrid pacemaker* model then suddenly came under scrutiny with the observation that the rhythm persisted in vitro even after I_{NaP} and I_{CAN} were blocked, provided that the network excitability was increased with agents like SP and thyrotropin-releasing hormone (TRH) (Del Negro, 2005). Considerable controversy arose about the dependence of rhythm on these pacemaker currents and it largely centered on the concentration of blockers used and whether efforts were made to reactivate rhythm once the pacemaker currents were blocked. At higher concentrations of riluzole, rhythm could be irreversibly blocked but at these higher concentrations riluzole interferes with glutamergic transmission and repetitive firing, both of which are required for network activity. For example, 10 μM riluzole had no effect on

rhythm nor inspiratory drive potentials when added locally to the preBötC, suggesting that pacemakers are not essential for rhythmogenesis in the mouse medulla (Pace *et al.*, 2007b). In contrast, another study revealed that 10 μM riluzole, when microinfused locally to the preBötC for extended periods halted inspiratory rhythm in rats (Koizumi & Smith, 2008). However, these authors did not attempt to reactivate rhythm. The debate remains unresolved but the data suggest that higher concentrations or prolonged exposure are required to block rhythm and under these conditions non-specific drug actions are a concern.

The possibility that pacemaker currents are not necessary for rhythm generation gave increased credibility to a model that had appeared a few years earlier, referred to as *group pacemaker hypothesis* (Rekling & Feldman, 1998; Feldman & Del Negro, 2006). In this model, recurrent excitation recruits neurons through positive feedback evoking intrinsic currents, resulting in a burst. Intrinsic conductances and perhaps synaptic inhibition cause burst termination, a short afterhyperpolarization, and a post burst quiescent period when synapses are silent. Inspiratory neurons then begin to discharge, and this excitation percolates through the recurrent network leading to the next burst. In this model, which is gaining further experimental support, pacemaker membrane conductances I_{NaP} and I_{CAN} play an important, yet non-essential role, in amplifying depolarizations (Feldman & Del Negro, 2006; Del Negro *et al.*, 2010). The *group pacemaker hypothesis* is still under investigation, with novel experiments and computational techniques being applied to better understand the mechanisms underlying burst generation. These approaches have revealed new perspectives on the inspiratory network. For example, one proposal is that inspiratory neurons cannot be categorized into discrete categories in a traditional, static ball-and-stick model, but rather, their firing patterns vary along several continua of phase-dependent activity (Carroll *et al.*, 2013). Alternate approaches also have attempted to separate rhythm and pattern generating mechanisms to better characterize the internal 'clock' within the preBötC. Varying external potassium of the rhythmic slice preparation to modulate inspiratory rhythm, caused a multimodal change in burst period as an integer multiple of the shortest observed period

(Kam *et al.*, 2013a). This modulation revealed smaller "burstlets" measured from population preBötC activity, intermingled with XII bursts measured from the hypoglossal nerve. Burstlets, unlike bursts, had lower variability in frequency and were unaffected by the I_{CAN} blocker Cd^{2+}, suggesting that they were the result of a fundamental process responsible for maintaining basic rhythm, and that pacemaker like properties are important for transforming preBötC burstlets to XII bursts (Kam *et al.*, 2013a). These observations are in agreement with the *group pacemaker hypothesis*, which suggests that pacemaker properties are non-essential, but important for the amplification of neuronal excitation.

The *inhibitory ring hypothesis*, proposes an alternative view on the generation of rhythm, that the normal three-phase breathing pattern is based on mutual inhibition of compartmentalized groups of neurons (Smith *et al.*, 2012). In this model, the expiratory phase is based on a tonic drive supplied from the pons to the BötC, which forms an inhibitory ring of BötC post-inspiratory and augmenting expiratory neurons with early inspiratory and inspiratory neurons of the preBötC. Indeed, transectioning of the in situ preparation revealed that the removal of the pons caused loss of excitation to the BötC, changing the normal breathing pattern from a three-phase to a two-phase breathing pattern. Finally, removing the BötC isolated the remaining preBötC pre-inspiratory and inspiratory neurons into generating a one-phase breathing pattern (Smith *et al.*, 2007). A compartmentalized computational model based on the inhibitory ring hypothesis was impressively effective in simulating a normal three-phase breathing pattern and reproducing the experimental findings (Smith *et al.*, 2009). However, this model is based on a large number of unknown parameters and unconstrained parameter space, and so its true validity remains unclear. A direct prediction of the *inhibitory ring hypothesis* is that synaptic inhibition from the BötC is essential for generation of a two and three phase rhythm. As a direct test of this model, Feldman and colleagues blocked inhibition in the BötC of adult in vivo preparations. Local injections of a bicuculine and strychnine cocktail into the BötC (and preBötC) did not disrupt normal breathing, supporting that inhibition does not play a fundamental role in the generation of

normal breathing rhythm. Moreover, ablation of the BötC in vivo did not change frequency of breathing in vagus intact animals, and was non-essential in generating normal breathing, in direct opposition to the *inhibitory ring hypothesis* (Janczewski *et al.*, 2013). Although evidence supports that inhibition is not essential for generating the rhythm of breathing, there is little argument that inhibition plays an important role in pattern formation and in modulating the basic rhythm. As a result, while the mechanisms underlying rhythm generation are under intense investigation, increasing attention is being devoted to defining the role of inhibition (and other mechanisms) in the shaping of inspiratory output by premotor and motor networks; i.e., the process of pattern formation.

Both rhythm and pattern forming networks are fundamental for adaptation of normal breathing patterns to a various stimuli, processed via mechanosensory feedback and chemosensory feedback. Mechanosensory feedback allows respiratory networks to respond to the status of the muscles and lungs, allowing it to adapt to mechanical and functional changes in the respiratory apparatus that can accompany development, aging and disease. However, unlike most other motor systems, the respiratory system has an additional constraint; its output is matched to metabolic demand. This task is facilitated by chemosensory feedback, that can affect all levels of the respiratory network, producing changes in respiratory output. Following a brief discussion of glia in section 1.4, due to the growing evidence of astrocyte involvement in modulation of respiratory networks, in section 1.5, I provide an overview of the main focus of this thesis,: the role of purinergic modulation in these homeostatic reflexes.

1.4 Astrocytes as major contributors within neural networks

My thesis explores the purinergic modulation of respiratory networks. ATP is a major gliotransmitter, and glia feature prominently in the chemical control of breathing. I will therefore provide a brief description of the main types of glia and their functions, alluding to

the increasing evidence of astrocytic signalling, and its significant role in modulating the activity of neuronal networks.

Glia are a diverse class of cells that were classically identified as support cells for neurons. Glia contribute considerably to CNS homeostasis, maintaining structure, metabolism, extracellular space, protection and control of blood gases (Funk *et al.*, 2015), depending on their type and location. Glial cells consist of three main types located within the CNS: microglia, oligodendrocytes, and astrocytes.

Microglia, often termed immune cells of the brain, constantly survey the CNS for signs of cellular damage or disturbances and threats to normal brain function (Hanisch & Kettenmann, 2007). Once activated in an adaptable and graded manner, microglia become macrophage-like, consuming dead and foreign matter (Parkhurst & Gan, 2010). Although the microglia have neuroprotective roles in various models of inflammation, uncontrolled or impaired microglial function can also contribute to neurotoxicity, making their activation a major focus in the pathology of neurodegenerative diseases (Biber *et al.*, 2007)

Oligodendrocytes are myelin-forming cells, enhancing signalling conduction for long-range signalling through the CNS (Verkhratsky & Butt, 2013), making them a significant subject of diseases involving loss of myelin, such as ALS (Robberecht & Philips, 2013). More recently, they have been proposed as support cells for neuronal metabolism, contributing to lactate transport for neurons and axons (Lee *et al.*, 2012).

Astrocytes traditionally were viewed as a non-excitable 'supporting cast' of cells to neurons, providing metabolic support and neurochemical and ionic balance of the extracellular space (Parpura & Verkhratsky, 2011). However, astrocytes have been recently elevated to the title of "homeostatic cells of the brain" , directly modulating neuronal activity through Ca^{2+}-mediated exocytotic or gap junction-dependent release of gliotransmitters (Parpura & Verkhratsky, 2012). Although initially controversial, this role has garnered support, due to use of markers for the moderately-specific glial fibrillary acidic protein (GFAP) in identifying putative astrocytes actively participating in networks critical for

homeostasis. Of major relevance to this thesis, is the proximity and interaction of astrocytes with blood vasculature (Attwell *et al.*, 2010; Funk, 2013), prevalence of ATP as a gliotransmitter (Montana *et al.*, 2006), and CO_2/pH and O_2 sensitivity of astrocytes (Gourine *et al.*, 2010; Huckstepp *et al.*, 2010; Mulkey & Wenker, 2011; Angelova *et al.*, 2015), characteristics that place them at the center of respiratory network modulation. The contribution of these are highlighted in the context of purinergic modulation of respiratory networks in the following section.

1.5 Purinergic modulation of respiratory networks

Since the discovery that ATP is co-released with norepinephrine at the smooth neuromuscular junction (Burnstock *et al.*, 1972; Burnstock, 1986), the ubiquitous role of ATP in neuronal and glial signalling throughout the CNS has expanded greatly. Cloning of purinergic receptors revealed a large receptor family that are found throughout the CNS where they contribute to a variety of physiological functions. The purinergic P2 receptor family comprises seven ionotropic $P2X_{1\text{-}7}$ and eight metabotropic $P2Y_{1,2,4,6,11\text{-}14}$ receptor subtypes (Abbracchio *et al.*, 2006). Fast ATP-mediated excitatory responses occur through P2X receptors, which are trimeric ligand-gated, non-selective cation channels with significant Ca^{2+} permeability. P2Y receptors couple to G-proteins and mediate slower responses to ATP, uridine triphosphate (UTP), and adenosine diphosphate (ADP). $P2Y_{1,2,4,6}$ receptors signal primarily through $G\alpha_{q/11}$, $P2Y_{12,13}$ receptors through $G\alpha_i$, $P2Y_{14}$ receptors through $G\alpha_i/G\alpha_o$ and $P2Y_{12}$ receptors through $G\alpha_{q/11}$ and $G\alpha_s$ (Abbracchio *et al.*, 2006). P2X and P2Y receptors are found throughout the CNS on astrocytes and neurons, with the exception of $P2Y_{11}$ receptors that are only found in neurons, and $P2X_7$ receptors, which appear limited to immune cells and glia (Burnstock, 2007).

Purinergic signalling, however, is not limited to the excitatory actions of ATP and ADP at P2 receptors. ATP and ADP can be broken down further into the end-product adenosine (ADO) by a diverse family of enzymes called ectonucleotidases. ADO acts at P1

receptors comprising of four types of metabotropic receptors, including A1, A2a, A2b and A3 receptors. Interestingly, A1 receptors, which are found throughout the CNS (Burnstock *et al.*, 2011) are typically inhibitory, coupling through G proteins to inhibit adenylyl cyclase, decrease cyclic AMP (cAMP) production, and either presynaptically inhibit transmitter release or decrease excitability postsynaptically (Fredholm *et al.*, 2001). The resulting tripartite signalling system has unique implications for control networks in the CNS in that the final actions of ATP will depend on the degree to which ATP is degraded to ADP, adenosine monophosphate (AMP) or ADO, and this balance is determined not only by the local pattern of P2 and P1 receptors, but the subtype and concentration of ectonucleotidases.

Ectonucleotidases exist in four major families, each with numerous subtypes. These families are ectonucleotidase triphosphate diphosphohydrolases (E-NTPDases, 8 subtypes), ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP, 6 subtypes), alkaline phosphatases, and ecto-5'nucleotidases (Funk, 2013). The expression and activity of these ectonucleotidases can vary by age, species, and perhaps sex, providing unique responses to ATP signalling. For example, a different complement of ectonucleotidases in rats and mice appears to be a significant factor the in very different responses of their preBötC inspiratory networks to ATP. The preBötC of mice is seemingly insensitive to ATP, which conversely, causes a robust frequency increase when locally applied to the rat preBötC in vitro. This lack of response in mice is attributed to a higher expression of the ectonucleotidase, tissue-nonspecific alkaline phosphatase (TNAP), which favours the production of ADO and the subsequent activation of inhibitory A_1 receptors. (Zwicker *et al.*, 2011). On the other hand, the primary constituent in rat is ENTPDase2, which favours production of excitatory ADP, contributing to the robust excitatory response to ATP (Zwicker *et al.*, 2011).

The first recognition that ATP plays a modulatory role in respiratory networks was the observation that ATP increased hypoglossal inspiratory motor outflow to the genioglossus (GG) muscle (Funk *et al.*, 1997). Soon after, ATP was suggested to play an important role in mediating central CO_2 chemoreception within the VLM, including within

the preBötC (Thomas *et al.*, 1999; Thomas & Spyer, 2000). The $P2X_2$ receptor, based on potentiation of its currents by reductions in pH (King *et al.*, 1996), was originally proposed as the actual pH sensor responsible for excitation of inspiratory neurons and inspiratory network activity with increased CO_2 or reduced pH. This hypothesis was ultimately rejected based on findings that P2 receptors did not contribute to central chemosensitivity in the brainstem-spinal cord preparation in vitro (Lorier *et al.*, 2004), and that central chemosensitivity was unchanged in $P2X_2$ receptor knockout mice (Rong *et al.*, 2003). However, interest in the potential role of P2 receptor signalling in chemoreception within respiratory networks did not diminish. Instead, the focus switched from the $P2X_2$ receptor as the putative chemosensor, to the hypothesis that ATP is released in response to chemoreceptor activation, which then stimulates P2 receptors, contributing to the appropriate ventilatory responses. This hypothesis gained support with the observations in 2005, using ATP biosensors, that ATP is released from the ventral surface of the medulla in response to CO_2 and hypoxia and that block of P2 receptors (with the non-selective antagonist PPADS) attenuated the hypercapnic and hypoxic ventilatory responses (Gourine *et al.*, 2005a, b).

Attention also switched from the $P2X_2$ receptor subtype to $P2Y_1$ receptors with the demonstration that the ATP-mediated excitation of preBötC rhythm generating networks in vitro was not mediated by P2X receptors but by $P2Y_1$ receptors (Lorier *et al.*, 2007; Huxtable *et al.*, 2009; Gourine *et al.*, 2010; Huxtable *et al.*, 2010) which are expressed throughout the brainstem (Fong *et al.*, 2002a). These findings motivated significant exploration into the role of purinergic signalling in the homeostatic ventilatory responses to elevated CO_2 (reduced pH) and hypoxia. While the focus of my thesis research is on the role of ATP in contribution of the CNS to the ventilatory response to hypoxia, I first review the role of purinergic signalling in central chemoreception since this reflex is better understood and it may provide insight into potential mechanisms via which glia and ATP participate in the hypoxic ventilatory response.

1.5.1 Purinergic signalling in central chemoreception

The typical ventilatory response to hypercapnia consists of a robust increase in ventilation that results from an increase in both breath frequency and tidal volume, depending on species and stage of development (Putnam *et al.*, 2005). Peripheral chemoreceptors contribute ~20% of this response and the remainder is attributed to multiple chemosensitive sites within the CNS including the RTN, locus coeruleus (LC), NTS, medullary raphe and preBötC. The relative roles of these different areas remains a topic of debate (Nattie & Li, 2009), but the RTN is emerging as a key site, or perhaps a site that integrates chemoreceptor information from other central sites as well as the carotid body (Guyenet *et al.*, 2008; Guyenet *et al.*, 2010). The role of ATP in chemoreception is also best studied in the RTN. Therefore, I briefly outline what is currently known about purinergic receptor signalling and its contribution to chemosensitivity in other chemoreceptive areas of the brainstem before focusing on the RTN.

The medullary raphe comprises the raphe magnus most rostrally, raphe pallidus and raphe obscurus most caudally. Raphe magnus and the rostral half of raphe pallidus are most strongly implicated in central chemoreception (Iceman *et al.*, 2013). Interestingly, P2 receptor expression and contribution of purinergic signalling to chemoreception also varies between these subnuclei (Cao & Song, 2007). $P2Y_1$, $P2X_1$ and $P2X_3$ receptor immunolabeling is particularly strong in the raphe magnus, while $P2X_{1\text{-}6}$ receptors are expressed in raphe obscurus neurons (Yao *et al.*, 2000). P2 antagonist PPADS (20 mM) injected locally into the rostral raphe magnus attenuates the ventilatory response to CO_2 in conscious rats (da Silva *et al.*, 2012), while the same antagonist injected into the caudal raphe obscurus (da Silva *et al.*, 2012) and raphe pallidus (Sobrinho *et al.*, 2014), has little effect on the hypercapnic ventilatory response. As the raphe site most strongly implicated in chemoreception overlaps with the region where purinergic signalling appears to significantly contribute and with that of $P2Y_1$ receptor expression, it is possible that $P2Y_1$ receptors

contribute to the CO_2 ventilatory response of the medullary raphe, but this needs to be tested directly.

The nucleus tractus solitarius (NTS) is a fundamental site for central integration of sensory information from peripheral inputs with critical autonomic function (Lawrence & Jarrott, 1996), lung mechanoreceptive afferents and carotid body chemoreceptor feedback. As a result, a large number of neurotransmitters and neuromodulators have been proposed to be involved in the complex roles of the NTS, including ATP and its byproducts. Purinergic receptors, specifically $P2Y_1$ receptors, are expressed abundantly throughout the NTS (Fong *et al.*, 2002a), mediating actions through modulation of L, N, and P/Q type voltage-dependent calcium channels (Aoki *et al.*, 2004; Endoh, 2004). Although ATP signalling in the NTS plays a key role in cardiovascular reflexes (Ergene *et al.*, 1994), integration of peripheral chemosensory signals (Accorsi-Mendonça *et al.*, 2013) and respiratory mechanoreflexes (Gourine *et al.*, 2008), purinergic signalling does not appear to contribute significantly to the chemoreceptive function of the NTS (Sobrinho *et al.*, 2014).

The RTN plays a major role in central chemoreception as both a primary sensor and perhaps also as an integrator of all peripheral and central chemosensory information (Guyenet *et al.*, 2010). P2 receptor signalling plays a significant role in chemoreception within the RTN. There are at least two cell types that contribute to RTN chemosensitivity. The main contributors are intrinsically CO_2/pH-sensitive, phox2B positive RTN neurons (Mulkey *et al.*, 2004). These neurons are sensitive to ATP, but their intrinsic sensitivity to CO_2/pH is not dependent on P2 receptors (Mulkey *et al.*, 2006). The second cell type are specialized CO_2/pH-sensitive astrocytes that line the ventral surface of the medulla (Gourine *et al.*, 2010). These cells release ATP in response to changes in CO_2/pH via Ca^{2+}-dependent vesicular release (Gourine *et al.*, 2010) and CO_2-sensitive connexin 26 hemichannels (Huckstepp *et al.*, 2010; Meigh *et al.*, 2013). Whether individual astrocytes employ both mechanisms of release is not known. The relative roles of these two release mechanisms are also not known. However, the release of ATP via connexin 26 may only be a factor in

response to small changes in CO_2 because these hemichannels are inhibited with progressive reductions in pH (Huckstepp *et al.*, 2010). Based on the effects of P2 receptor antagonists in the RTN (Gourine *et al.*, 2005a), purinergic signalling accounts for ~20% of the RTN-mediated respiratory response to CO_2. It is proposed that CO_2/pH evokes astrocytic ATP release, which excites pH-sensitive RTN neurons, increasing their output and potentiating the ventilatory response to CO_2/pH. Purinergic transmission between astrocytes and RTN neurons and its involvement in chemoreception is supported by several observations. RTN neurons are sensitive to P2 receptor activation; P2Y receptors appear excitatory while P2X receptors appear to underlie presynaptic inhibition (Mulkey *et al.*, 2006). In organotypic culture, optogenetic activation of astrocytes transfected to express channel rhodopsin ChR2(H134R) via an adenoviral vector under the control of a GFAP promoter, depolarizes neighbouring phox2b^{+} RTN neurons and drives phrenic nerve activity in vivo. Thus, RTN astrocytes are able to increase respiratory activity (Gourine *et al.*, 2010). pH-evoked depolarization of RTN neurons in organotypic culture is also reduced by apyrase, which degrades ATP (Gourine *et al.*, 2010).

Recent data have shown a role for $P2Y_1$ receptor specific signalling within the RTN, though their contribution to the purinergic component of central chemosensitivity in the RTN is not clear. In organotypic culture, the MRS 2179-mediated (10 μM) reduction in RTN neurons of pH-induced membrane depolarizations, pH-induced Ca^{2+} increases, and depolarizing responses evoked by optogenetic activation of ChR2-expressing astrocytes all suggest $P2Y_1$ receptor involvement (Gourine *et al.*, 2010). However, these actions may also reflect antagonism of $P2X_{1,3,4}$ receptor subtypes (Jacobson & Knutsen, 2001). Consistent with P2X receptor actions, in acute slices only 12% of CO_2-sensitive RTN neurons respond to $P2Y_1$ receptor agonists (Wenker *et al.*, 2013). In addition, block of $P2Y_1$ receptors in vivo has no effect on CO_2-evoked increases in phrenic nerve activity or arterial blood pressure responses (Wenker *et al.*, 2012). Thus, $P2Y_1$ receptors do not appear to be major contributors to the ATP excitation of chemosensitive RTN neurons.

Despite the minimal role of $P2Y_1$ receptors in the responses of chemosensitive RTN neurons and the RTN network to CO_2, local activation of $P2Y_1$ receptors in the RTN increases mean arterial pressure and phrenic nerve amplitude and frequency (Wenker *et al.*, 2012). These responses are most likely mediated by CO_2 insensitive RTN neurons, ~70% of which respond to $P2Y_1$ agonist MRS 2365 with robust increases in firing rate. Interestingly, ~50% of MRS 2365-sensitive neurons are C1 neurons. Thus, $P2Y_1$ receptors may contribute to the increase in breathing and sympathetic nerve activity that results from activation of the reflex pathway from peripheral chemoreceptors to NTS to C1 neurons (Wenker *et al.*, 2013). The role of the $P2Y_1$ receptor-sensitive RTN neurons that are neither C1 nor chemosensory remains to be resolved.

1.6 ATP and the hypoxic ventilatory response

The ventilatory response to acute hypoxia is biphasic, comprising an initial rapid increase in ventilation, followed by a secondary depression (Moss, 2000). While there are several hypoxia sensitive regions in the CNS that could contribute to the initial increase, including the caudal hypothalamus, the NTS, C1 and the preBötC (Solomon, 2000; Neubauer & Sunderram, 2004) it is primarily attributed to the peripheral carotid body chemoreceptors. The secondary depression, on the other hand, is mainly centrally mediated. Purinergic signalling features prominently in both phases of the response, at central and peripheral levels.

1.6.1 Mechanisms underlying the initial hypoxia-induced increase in ventilation

The peripheral carotid body chemoreceptors contribute significantly to hypoxia sensitivity (Prabhakar, 2000), and underlie the first phase in the hypoxic ventilatory response. They are conveniently located at the bifurcation of the carotid artery to sense changes in blood oxygen. The carotid bodies consist of two types of cells, the glomus (type I) cells and

glial-like sustentacular (type II) cells. Based on recent evidence, glomus cells appear to respond directly to hypoxia by decreasing the inhibition of cystathionine-γ-lyase. The resulting production of hydrogen sulfide (H_2S) appears fundamental to the stimulation of the carotid body (Yuan *et al.*, 2015). An alternate hypothesis is that glomus cells respond to hypoxia with the closing of two-pore domain acid-sensitive K^+ (TASK) channels, causing a depolarization of membrane potential and an increase in intracellular calcium via activation of voltage gated calcium channels (VGCCs) (Xu, 2006). The resulting exocytotic release of ATP activates $P2X_2$ receptors on carotid sinus neuron terminals that relay this information to the NTS, which integrates this information and, through connections to the preBötC via the PRG and pFRG/RTN, evokes increases in ventilation (Prasad *et al.*, 2001; Rong *et al.*, 2003). Released ATP also acts via autocrine-paracrine interactions through $P2Y_2$ receptors on sustentacular cells which are assumed to cause the further release of ATP and glutamate (Xu *et al.*, 2003). In addition, activation of A2A receptors by ADO, generated from the degradation of ATP, inhibits TWIK-related acid-sensitive K^+ (TASK) conductances and contributes to the chemosensory response (Xu, 2006). At the same time, autocrine/paracrine activation of $P2Y_1$ receptors on glomus cells causes hyperpolarization and appears to act as a negative feedback mechanism (Xu *et al.*, 2005). This complex purinergic cross-talk between type I and II cells in the carotid body produces oscillations in $[Ca^{2+}]$ signalling, allowing for repeated release of transmitters from glomus cells during prolonged hypoxia (Tse *et al.*, 2012; Nurse, 2014; Murali & Nurse, 2015).

While the initial increase in ventilation during hypoxia is primarily attributed to carotid body chemoreceptor activation, some question whether there is a central component. Carotid body denervated rats (Martin-Body *et al.*, 1985), cats (Miller & Tenney, 1975), dogs (Davenport *et al.*, 1947), goats (Sorensen & Mines, 1970) and ponies (Bisgard *et al.*, 1980) still show a small increase in ventilation in response to hypoxia. Whether centrally-acting ATP contributes to this initial increase is not known. Evidence in which block of P2 receptors with PPADS affected ventilation only during the secondary depressive phase

suggests that ATP does not contribute to the initial increase. This question will be further explored in Chapter 2.

1.6.2 Central mechanisms shaping the secondary hypoxic depression

During the second phase of the hypoxic ventilatory response, the secondary depression, breathing falls from its peak over the first few minutes and stabilizes at a steady-state level of ventilation that in adults is above baseline, The secondary depression is much greater in premature and newborn infants where ventilation can fall to levels significantly below baseline (Fig. 1.2). The mechanisms underlying the secondary depression are still under debate, but central mechanisms are strongly implicated (Teppema & Dahan, 2010). A role for P2 receptor signalling in the hypoxic ventilatory response first emerged in 2005 with the use of biosensors that detected hypoxia-induced ATP release at the ventral surface of the medulla in anesthetized rats. The peak ATP signal followed the initial increase in ventilation, while application of the P2 receptor antagonist, PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid, 100 μM; i.e., concentrations that could affect glutamatergic transmission) over the ventral medullary surface caused a greater respiratory depression without affecting the initial increase in ventilation. These data suggested that the ATP is released during hypoxia and that its excitatory actions attenuate the secondary hypoxic depression (Gourine *et al.*, 2005b). Several questions remain. First, concerns over selectivity of the biosensors and specificity of PPADS raised doubts about the involvement of ATP. Additional questions included: (i) the site and (ii) source (neuronal or glial) of ATP; (iii) the mechanism of hypoxia sensing and ATP release; and (iv) the mechanism by which ATP increases frequency. Substantial progress has been made in addressing these questions. My thesis will provide insight into questions (i), (ii) and (iv).

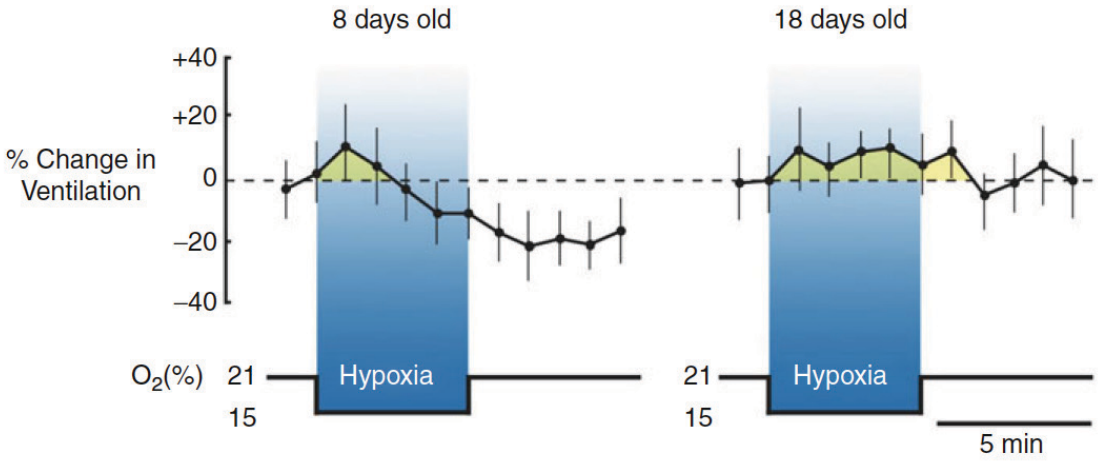

Figure 1.2 The hypoxic ventilatory response
The response to hypoxia (15% O_2, 5 min) in premature infants compared to that of older infants. In younger infants the initial increase is followed by a secondary depression which is attenuated in older infants. Adapted from Waters and Gozal (2003) *Respir Physiol & Neurobiol*. 136:115-129

(i) *Site of ATP actions*

Application of biosensors to hypoxia-exposed horizontal medullary slices revealed, at least in vitro, that hypoxia evokes ATP release from the VRC, including the preBötC (Gourine *et al.*, 2005b). The exact site at which ATP is released to increase breathing during hypoxia in vivo is unknown and will be explored here. However, the preBötC, a critical site for inspiratory rhythm generation is a strong candidate. Expression of TMPAP (transmembrane prostatic acid phosphatase) on neurons and glia of the preBötC and surrounding regions (via injection of a lentiviral vector expressing TMPAP and enhanced green fluorescent protein (eGFP) under the control of elongation factor 1α promoter), increased the secondary hypoxic respiratory depression similar to P2 receptor antagonists (Angelova *et al.*, 2015). In addition, mapping of the VLM in rhythmic medullary slices using local injections of P2 receptor agonists and antagonists indicates that ATP acts predominantly via $P2Y_1$ receptors in the preBötC to increase inspiratory frequency in the rat (Lorier *et al.*, 2007; Zwicker *et al.*, 2011).

(ii) *Source of ATP release*

Glia were first implicated in the response to ATP via in vitro experiments in the neonatal rhythmic slice showing that the frequency increase evoked by ATP, but not SP, in the preBötC was markedly reduced following incubation of the slice with glial toxins (Huxtable *et al.*, 2010). In addition, glial cells cultured specifically from the preBötC express $P2Y_1$ receptors and show $P2Y_1$ receptor-dependent, ATP-evoked increases in intracellular Ca^{2+}. These data led to the hypothesis that hypoxia evokes ATP release from unknown sources, and that the ATP acts in part on glial cells via ATP-evoked ATP (or glutamate) release to increase frequency (Huxtable *et al.*, 2010). More recent data from primary dissociated glial cultures from multiple brain regions suggest that astrocytes are the source of ATP in hypoxia. Total internal reflection fluorescence microscopy (TIRF) imaging revealed loss of ATP-containing vesicles (marked with fluorescent quinacrine or tagged vesicular

nucleotide transporter) upon exposure to hypoxia (Angelova *et al.*, 2015). However, autocrine, ATP-evoked ATP release did not contribute to the glial response to hypoxia; the rate of hypoxia-induced vesicle exocytosis was not affected by the $P2Y_1$ receptor antagonist, MRS 2179, or apyrase (Angelova *et al.*, 2015). Thus, the ATP-evoked ATP release proposed based on data from rhythmic slices does not occur in cultured astrocytes. Whether this mechanism contributes to the hypoxic ventilatory response remains to be determined.

(iii) *Mechanisms of hypoxia sensing*

Hypoxia triggers increases in intracellular Ca^{2+} in astrocytes in organotypic brain slice cultures, acute slices, primary dissociated glial cultures and in cortical astrocytes in vivo, suggesting that astrocytes are ubiquitously capable of sensing hypoxia (Angelova *et al.*, 2015). New data from dissociated glial cultures implicate mitochondria as the hypoxia sensor. Hypoxia inhibited mitochondrial respiration, which led to increases in reactive oxygen species (ROS), phospholipase C (PLC) activation, inositol triphosphate (IP_3)-induced increase in intracellular Ca^{2+}, and ultimately ATP release (Angelova *et al.*, 2015). ATP release from glia appears to occur via Ca^{2+}-dependent vesicular mechanisms (Angelova *et al.*, 2015). TIRF imaging of fluorescently labeled ATP-containing vesicles was blocked by Ca^{2+}chelators. Moreover, interfering with vesicular release mechanisms specifically in preBötC astrocytes (via an adenoviral vector that expressed tetanus light chain protein (TeLC) under the control of an enhanced GFAP (eGFAP) promoter) reduced the steady-state ventilatory response to hypoxia in unanesthetized adult rats in vivo (Angelova *et al.*, 2015). Note that a reduced steady-state increase in ventilation is equivalent to the PPADS- and TMPAP-mediated potentiation of the secondary respiratory depression discussed above. Demonstrating that the hypoxia sensitivity of glial mitochondria contributes to the hypoxic ventilatory response in vivo is a future challenge, but identification of a putative sensor mechanism and ATP release mechanism are exciting advances.

(iv) The excitatory actions of ATP

The mechanisms underlying excitatory actions of ATP on inspiratory rhythm remains a major unanswered question. $P2Y_1$ receptor signalling is strongly implicated in vitro, where the preBötC network, inspiratory neurons and cultured preBötC glia (Huxtable *et al.*, 2010) are excited by $P2Y_1$ receptor agonists and the effects of ATP and P2Y agonists on frequency are inhibited by MRS 2279. Whether $P2Y_1$ receptors mediate the actions of ATP within the preBötC during the hypoxic ventilatory response in vivo remains to be established and will be examined in Chapter 2. In addition, while $P2Y_1$ receptors typically activate the $G\alpha_{q/11}$-signalling pathway through PLC-IP_3 signalling, the second messenger cascades and ion channels through which $P2Y_1$ receptors signal on preBötC neurons and glia to increase frequency are not known and will be examined in Chapter 3.

1.7 Thesis objectives

This introduction has briefly outlined: the organization of central respiratory control networks; models of inspiratory rhythm generation in the preBötC, and; current views of purinergic signalling and its contributions to homeostatic chemoreflexes, including some of the major questions that remain regarding the role of purinergic signalling in the hypoxic ventilatory response. The overarching objective of my thesis is to advance understanding of the role played by ATP in the central component of hypoxic ventilatory response.

I will test two major hypothesis by addressing several specific aims.

Hypothesis 1. The exocytotic release of ATP from preBötC astrocytes attenuates the secondary hypoxic depression via $P2Y_1$ receptors in the preBötC.

Specific aims are to determine whether:

(a) gliotransmission in the VLM contributes to the hypoxic ventilatory response in vivo (Chapter 2);,

(b) $P2Y_1$ receptor activation in the preBötC increases respiratory frequency in vivo,

(c) ADO in the preBötC slows respiratory rhythm in vivo,

(d) ATP released during hypoxia acts via $P2Y_1$ receptors in the preBötC to attenuate the secondary hypoxic respiratory depression.

Hypothesis 2. The $P2Y_1$ receptor-evoked excitation of the preBötC inspiratory networks is dependent on an IP_3-mediated increase in intracellular Ca^{2+} derived from intracellular stores and downstream activation of protein kinase C (PKC).

Specific aims are:

(a) to determine the necessity of increases in intracellular calcium in mediating the $P2Y_1$-evoked frequency increase in the rhythmic slice preparation in vitro,

(b) to determine whether downstream activation of PKC contributes to the $P2Y_1$ mediated frequency increase in vitro,

(c) as a first step toward identifying the cellular targets on which ATP acts to increase frequency, to determine whether the signalling cascades responsible for the $P2Y_1$-receptor mediated increases in inspiratory frequency are functional in preBötC astrocytes.

Chapter 2: ATP acts via $P2Y_1$ receptors in the preBötzinger Complex to attenuate the secondary hypoxic depression

2.1 Introduction

The ventilatory response to hypoxia is biphasic, consisting of an initial increase in ventilation, followed by a secondary depression (Moss, 2000) that can be life-threatening in premature infants. During hypoxia, ATP is released in the VLM, including the preBötC, where it activates P2 receptors and attenuates the secondary respiratory depression (Gourine *et al.*, 2005b). Recent evidence suggests that, as originally hypothesized (Gourine *et al.*, 2005b; Huxtable *et al.*, 2010), the ATP released during hypoxia is of astrocytic origin (Angelova *et al.*, 2015). Cultured brainstem astrocytes respond to physiologically relevant decreases in PaO_2 with a reduction in mitochondrial respiration, increased production of ROS leading to lipid peroxidation (Angelova *et al.*, 2015) which activates a PLC-IP_3-mediated release of intracellular calcium and exocytotic release of ATP. In addition, the viral expression within astrocytes of the VLM of TeLC protein to block vesicular release or TMPAP, a potent ectonucleotidase, to reduce endogenous ATP levels, both cause a greater hypoxic respiratory depression in unanesthetized rats. These data suggest that the exocytotic release of ATP from astrocytes and its excitatory actions directly contribute to this key homeostatic ventilatory reflex (Angelova *et al.*, 2015).

These data have answered several of the major questions regarding the mechanisms by which ATP increases ventilation during hypoxia to counteracts the secondary respiratory depression. Specifically, they have identified the likely source of ATP, a putative mechanism of hypoxia sensing; and the mechanism of ATP release. A major unanswered question is how does ATP, once released, cause ventilation to increase? Where within the VLM does ATP exert its excitatory actions and via which P2 receptor subtype? It is well-established in the rhythmic medullary slice from neonatal rats and mice that ATP, and its by product ADP, act most potently in the preBötC via $P2Y_1$ receptors to increase inspiratory frequency (Funk *et al.*, 1997; Lorier *et al.*, 2004; Lorier *et al.*, 2007; Lorier *et al.*, 2008; Huxtable *et al.*, 2009; Zwicker *et al.*, 2011). The objective of this study is to test, in anesthetized, vagotomized adult rats, the hypotheses that: (i) gliotransmission contributes to the hypoxic ventilatory

response; (ii) $P2Y_1$ receptors underlie the central, ATP-mediated component of the hypoxic ventilatory response, and; (iii) these actions are mediated, at least in part, within the preBötC.

2.2 Methods

All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Alberta Animal Ethics Committee.

2.2.1 Adult in vivo preparations

Adult male Sprague-Dawley rats (250-350 g) were initially anesthetized in isofluorane (3% in 100% O_2) while the femoral vein was cannulated. Isofluorane anesthesia was replaced with urethane (1.5-1.7 g/kg), which was gradually delivered intravenously (i.v.) Additional doses of urethane were given to maintain anesthesia if necessary. The femoral artery was cannulated to monitor blood pressure (BP) and blood gases. The trachea was cannulated, and the vagus nerves resected bilaterally at the mid-cervical level to eliminate confounding effects induced by vagal reflex stimulation. From this point there were two variations of the preparation.

For hypoxia experiments, animals were paralyzed with gallamine triethoiodide or pancuronium bromide (10 mg/kg i.v.; 1 mg/kg) and pump ventilated with a mix of 20% or 30% O_2, balance N_2 (1 L/min, 60 breaths/min at ~3.5 ml/breath). Gases were measured and mixed using a GSM-3 programmable gas mixer (CWE, Inc.) Paralysis and pump ventilation was used to open chemoreflex loops so that changes in central respiratory drive would not affect blood gases. End-tidal O_2 and CO_2 were monitored from a small port in the tracheal tube (PowerLab gas analyzer, ML206, AD Instruments) to ensure that end-tidal CO_2 remained constant. Blood gases were also taken before and during hypoxic challenges to ensure that arterial PaO_2, $PaCO_2$, and pH were within physiological ranges and consistent

between trials.

Once the animal was on the ventilator, the brachial plexus was exposed dorsolaterally behind the right shoulder blade. The phrenic nerve was isolated, cut distally, placed over dual platinum electrodes and embedded in Kwik-Sil adhesive (World Precision Instruments, Sarasota, FL) for long-term stability and to prevent nerve dessication. Animals were then placed in prone position in a stereotaxic device (Kopf Instruments, Tujunga, CA) and the hypoxia protocol performed.

Experiments that tested the acute effects of locally applying purinergic agonists into the preBötC used spontaneously breathing animals. Airflow was measured via a pneumotachometer attached to the tracheal tube. Electrodes made from Teflon coated wires were placed in the GG and diaphragm (DIA) muscles to measure electromyographic activity (GG_{EMG} and $DIA_{EMG,}$ respectively). The animal was then positioned on a stereotaxic frame in prone position and body temperature maintained at 37°C with a servo-controlled heating pad (Harvard Apparatus).

2.2.2 Viral gene transfer in vivo

Adult male rats (200-250 g) were anesthetized with an interperitoneal (i.p.) injection of a mixture of ketamine (90 mg/kg i.p.) and xylezine (10 mg/kg, i.p). They were then placed in a stereotaxic frame, where a small craniotomy was performed to expose the obex. Using coordinates described below, adenoviral vectors were injected bilaterally into the preBötC. Glial expression of TeLC and eGFP was achieved using a bicistronic adenoviral vector (AVV) construct as described previously described, where eGFP expression was linked to TeLC via a "SKIP" sequence (Angelova *et al.*, 2015). The bicistronic construct, making use of the viral ribosomal skipping mechanism, allows for a high expression of eGFP while attenuating TeLC expression to avoid cell toxicity. Vectors contained enhanced shortened GFAP promoter to drive expression of TeLC and eGFP (AVV-sGFAP-eGFP-TeLC). The

control virus contained eGFP only (AVV-sGFAP-eGFP). A schematic of the AVV-sGFAP-eGFP-TeLC vector is shown in Figure 2.2A (bottom).

Virus (500 nl) was diluted in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and slowly injected into the preBötC under direct visualization over 2 minutes. Following injection, the pipette remained stationary for 5 minutes before withdrawal. The skin was sutured and the animal was put in post-operative care and closely observed throughout recovery from anesthesia. Animals were left to recover for 6-8 days before the hypoxia experiments to ensure strong viral expression. Virus dilution and post-injection incubation period were established through a series of experiments that involved a series of 10-fold dilutions and incubation periods that varied from 5-9 days. Animals were euthanized at the end of the respective incubation period, perfused and tissue examined to define the dilution and incubation period that provided the strongest glial expression with no histological evidence of evidence of cell damage. High virus concentrations produced bright yet grainy fluorescent expression and transfected cell bodies near the injection site were hard to locate, consistent with cell death/damage. Low virus concentrations yielded weaker eGFP fluoresence, but individual cell bodies and processes were clearly identifiable. Viruses were synthesized and supplied by Sergey Kasparov at the University of Bristol, using an AVV clone donated by Dr. J. Gogos (Columbia University, New York, NY).

2.2.3 Immunohistochemistry

At the end of each in vivo experiment, animals were perfused transcardially (10% saline (100 ml), followed by 4% paraformaldehyde (PFA, 100 ml). The brainstem was extracted and submersion fixed for at least 24 hours in 4% PFA solution. The brainstems were sectioned serially into 50 μm slices in phosphate buffered saline (PBS) solution using a vibratome (VT 1000S, Leica). The preBötC was identified based on anatomical landmarks; ~800 μm caudal to caudal border of the facial nucleus, at the same level as the lateral loop of

the principal subnucleus of the inferior olive and the semi-compact nucleus ambiguus, just caudal to compact nucleus ambiguus (Paxinos & Watson, 2007; Ruangkittisakul *et al.*, 2008). The well-established preBötC marker, NK1 receptor immunolabeling, was also used to define the preBötC. Serial sections were examined for the region of most intense labeling indicative of the preBötC (Guyenet & Wang, 2001; Guyenet *et al.*, 2002). MRS 2279 injection sites were identified via fluorescent microspheres (0.1 μm, yellow-green, 2% solids, Life Technologies). NK1 receptor immunolabeling was examined using a rabbit anti-NK1 receptor primary antibody (1:1000, Millipore) with a Cy3-conjugated donkey anti-rabbit secondary (1:200, Jackson ImmunoResearch). To assess viral expression, eGFP fluorescence was enhanced with AlexaFluor 488 anti-chicken secondary antibody (Life Technologies) in conjunction with a chicken anti-GFP primary antibody (1:1000, Aves Labs, Inc.). To check glial specificity of viral expression I used Cy3-conjugated NeuN rabbit antibody (1:200, Millipore) to stain neuronal nuclei. NeuN also faintly revealed neuronal cell bodies. Slices were mounted on slides and observed with a fluorescent microscope (DM5500, Leica) and a Hamamatsu digital camera. Low magnification images were acquired through MetaMorph (Molecular Devices) acquisition software, and the extent of viral expression analyzed using ImageJ (NIH) and Adobe Photoshop. To assess whether viral expression was specific to astrocytes, high magnification images were taken with a confocal microscope (Leica TCS SP5). Regions of interest were delineated by identification of neuronal cell bodies stained by NeuN. By first placing regions of interest (ROI) markers over neurons visible under Cy3 fluoresence, I then switched to GFP fluorescence and checked for any eGFP overlap in these original ROIs that would indicate neuronal expression of virally-transfected protein.

2.2.4 Recording of muscle and nerve activity

In vivo recordings of EMG and phrenic nerve signals were amplified using a differential AC amplifier (model 1700, AM-systems, Sequim WA) and sampled at 2 kHz

using a PowerLab 16/30 data acquisition system (AD Instruments Inc.). Phrenic nerve activity was rectified, bandpass filtered (300 Hz – 1 kHz) and the raw signal integrated (time constant = 0.08s) to indicate respiratory frequency and phrenic nerve inspiratory burst amplitude as an index of ventilatory output.

2.2.5 Drugs and their application

DL-Homocysteic acid (DLH)(1-10 mM) and Gallamine triethoiodide (10 mg/kg) were obtained from Sigma-Aldrich, while MRS 2365 (1 mM), ADO (500 μM) and MRS 2279 (500 μM) were obtained from Tocris Biosciences. Pancuronium bromide (1 mg/kg) was obtained from Alomone Labs. Drugs were made up in HEPES-buffer (containing in mM; 137 NaCl, 5.4 KCl, 0.25 Na_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2 \bullet 2H_2O$, 1.0 $MgSO_4 \bullet 7H_2O$, 4.2 $NaHCO_3$, 10 HEPES) to maintain neutral pH.

For preBötC drug injections, the head was tilted in the stereotaxic frame such that bregma was 5 mm below lambda. Drugs or vehicle (HEPES-buffer) were pressure injected (Picospritzer III, Parker, Pine Brook, NJ) into the preBötC through a reticuled, sharp glass pipette (40 μm tip). The preBötC was located using stereotaxic coordinates (in mm) that, relative to obex, were 0.9 rostral, 2.0 lateral and 2.8 ventral. Injection volume was monitored by watching the movement of the meniscus relative to the reticule. An excitatory response to DLH (1-10 mM) confirmed positive location of the preBötC (Monnier *et al.*, 2003). MRS 2279 solution also contained fluorescent microspheres (0.1 μm, yellow-green, 2% solids, Life Technologies) for post mortem identification of injection sites, and their overlap with NK1 receptor immunolabeling.

2.2.6 Data analysis

Signals for airflow, GG_{EMG}, DIA_{EMG}, and phrenic nerve activity ($\int$PN) were acquired and integrated in LabChart (ADInstruments). For spontaneously breathing rats, airflow was

used to obtain frequency, tidal volume (V_T) and minute ventilation ($\dot{V}_E$). Parameters during drug application were compared with the average value during a two-minute control period preceding drug application. Maximum drug effect was defined as the maximum value measured in a moving average of three consecutive bursts, during the first minute after injection. For paralyzed animals, relative frequency, integrated phrenic nerve amplitude (∫PNA) and ventilatory output (frequency × ∫PNA) were calculated in LabChart, and exported to Excel for analysis. Average frequency, ∫PNA and ventilatory output were calculated from 30-second time bins. Group averages were calculated from these values. Hypoxic ventilatory responses were compared based on the values of frequency, ∫PNA and ventilatory output obtained during the initial hypoxia induced increase in ventilation (Phase I) and after the secondary depression when ventilation had plateaued (Phase II). The peak value during phase I represents the group average of individual peak values obtained from a moving average of frequency, ∫PNA and ventilatory output, calculated based on the first 1.5 min of hypoxia. Phase II values represent the average measured over the last 2 minutes of hypoxia exposure (shaded areas in Figs. 2.1, 2.6 and 2.7).

All values are reported as means ± standard error of the mean (SEM). Differences between means were compared using either GraphPad Prism (Version 4, GraphPad Software Inc.) or SPSS (Version 20, IBM). For comparison of two groups, paired or unpaired t-tests were used as appropriate. For comparison of more than two groups, ANOVA was used in conjunction with a Tukey *post hoc* multiple comparison test. Group data were represented by boxplots created by the web-application 'BoxPlotR', recently featured in *Nature Methods* (2014). Each boxplot details the spread of data points, which are superimposed on each box. Whiskers are defined by the lowest point, 1st quartile, median, 3rd quartile and highest point, denoted by the lower whisker, bottom, middle, and top of box, and top whisker respectively. The mean for each group is denoted by "+".

2.3 Results

2.3.1 The exocytotic release of gliotransmitters in the ventrolateral medulla contributes to both phases of the hypoxic ventilatory response in vivo

As functional oxygen sensors in the CNS, astrocytes respond directly to physiological reductions in the partial pressure of O_2 with increases in intracellular calcium, and the fusion of vesicular compartments containing ATP (Angelova *et al.*, 2015). Moreover, the expression of TeLC in glia of the VLM (including, but not limited to the preBötC) to block vesicular release attenuates the hypoxia-induced steady-state increase in ventilation in freely behaving adult rats (Angelova *et al.*, 2015). This reduced ventilatory response indicates a significant contribution of medullary glia to the steady state increase in ventilation. The hypoxic ventilatory response, however, is biphasic, comprising an initial increase (phase I) and a secondary depression to a new steady-state (phase II). To assess the role of astrocyte-derived transmitters in the phase I and II components of the response, I compared the hypoxic responses of three groups of rats: naïve, viral controls expressing eGFP in glia, and rats expressing eGFP and TeLC

Five to seven days after viral injection, rats were anesthetized, paralyzed, vagotomized and pump ventilated. ∫PN, blood pressure (BP) and blood gases were monitored and the hypoxic ventilatory response compared between naïve, control virus, TeLC rats. Animals were exposed to normoxia (30% O_2/70% N_2) for two minutes, followed by 5.5 minutes of hypoxia (10% O_2, 90% N_2) and then returned to normoxia for two minutes. Sample traces from individual control and TeLC animals as well as time courses calculated for group data are shown in Fig. 2.1A, 2.1B. Comparisons of phase I and II parameters for the three groups are reported in 2.1C. During phase I, the frequency. and ∫PNA of naïve animals (n = 10) increased to 1.41 ± 0.10 and 1.30 ± 0.04 of control respectively, giving a ventilatory output of 1.79 ± 0.13. Rats injected with the control virus (n = 6) showed similar phase I increases in frequency, ∫PNA and ventilatory output of 1.35 ± 0.04, 1.44 ± 0.09 and 1.83 ± 0.09, respectively. The phase I response of the TeLC group was significantly reduced.

Frequency and ∫PNA were 1.25 ± 0.04 (n = 6) and 1.25 ± 0.04 (n = 6) of control. Ventilatory output increased to 1.35 ± 0.06 (n = 6) of baseline, which was significantly smaller than naïve and viral control animals (p = 0.038, p=0.041 One-way ANOVA, Tukey post-hoc test).

During Phase II of the hypoxic ventilatory response, the increases in frequency and ∫PNA were not significantly different between any of the three groups. Frequency was 1.16 ± 0.07, 1.15 ± 0.04 and 0.97 ± 0.04, and ∫PNA was 1.28 ± 0.04, 1.29 ± 0.08 and 1.17 ± 0.04 for naïve, viral control and TeLC expressing rats, respectively. Ventilatory output, however, was significantly lower in the TeLC expressing rats (1.12 ± 0.04) compared to naïve (1.49 ± 0.09) and viral control rats (1.48 ± 0.09) (p = 0.024, 0.048, One-way ANOVA, Tukey post-hoc test). These data suggest that vesicular release of gliotransmitter contributes to the ventilatory increase in both phases of the hypoxic ventilatory response.

Postmortem analysis of serial 50 μm transverse brainstem sections from the viral control and TelC groups revealed the extent of viral expression across the VRC (a schematic sagittal section is depicted in Fig. 2.2A). Viral expression was similar in both groups. It was strongest at the level of the preBötC where the injection pipette tracks were visible. The injection sites were located ~750-850 μm caudal to the caudal border of the facial nucleus at the same level as anatomical markers of the preBötC (lateral loop of the principal subnucleus of the inferior olive and the semi-compact division of nucleus ambiguus, just caudal to compact nucleus ambiguus (Paxinos & Watson, 2007; Ruangkittisakul *et al.*, 2008). The strongest viral expression also overlapped with the highest density of NK1 receptor immunolabeling (Fig. 2.2B) an established marker of the preBötC. Viral expression dropped off substantially 500 μM rostral and caudal to the injection site and almost disappeared ~1 mm away from the preBötC (Fig. 2.2C).

The morphology of eGFP labeled cells was consistent with glial labeling. The selectivity of the viral vector for driving expression in glia has been demonstrated previously (Liu *et al.*, 2008; Gourine *et al.*, 2010; Tang *et al.*, 2014). To ensure specificity in my study, I examined 253 cells from 3 sections from 3 different animals for coexpression of eGFP

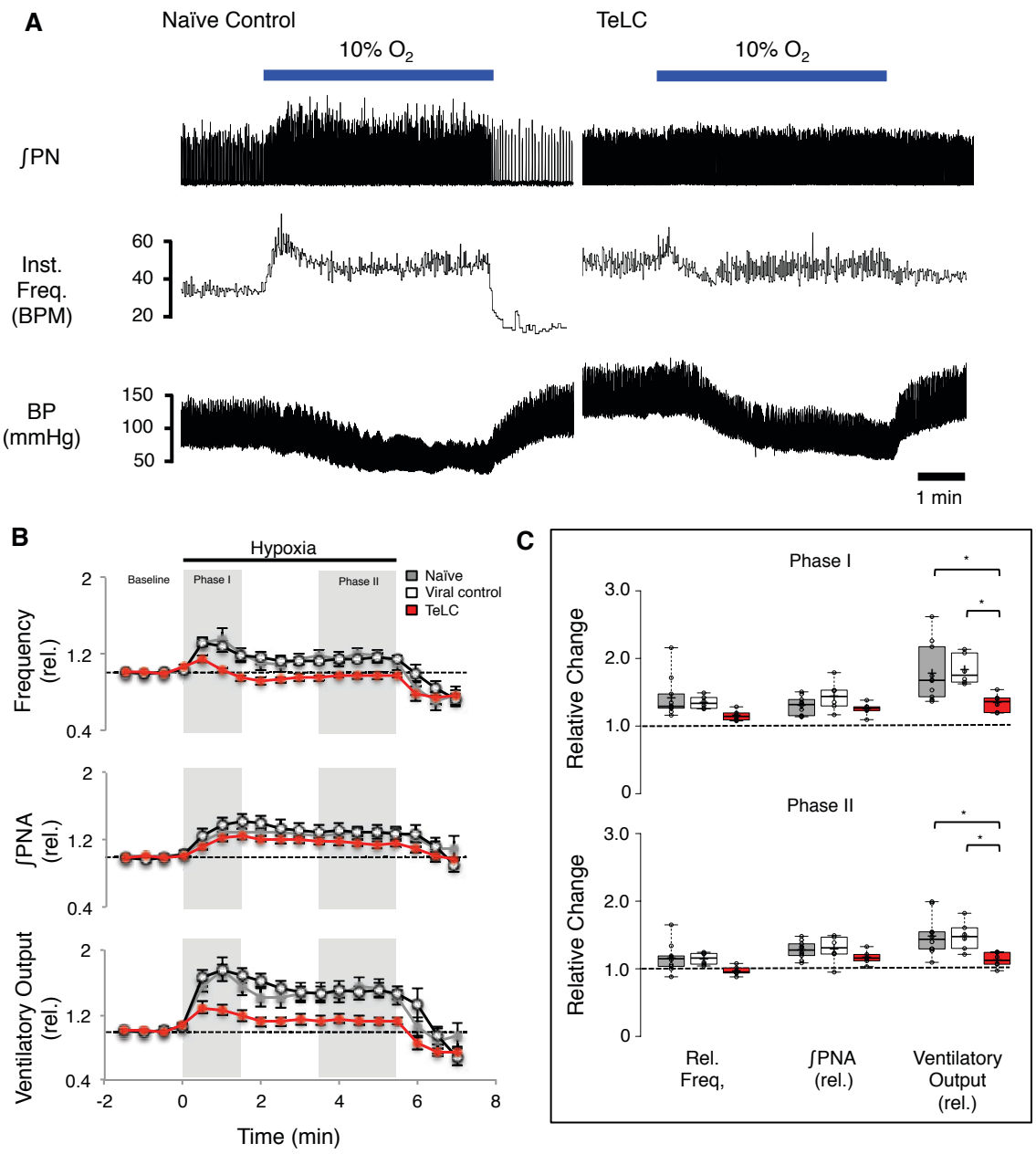

Figure 2.1 Expression of tetanus light chain (TeLC) protein specifically in glia attenuates the hypoxic ventilatory response.

(A) Representative traces from naïve control and TeLC-expressing animal showing changes in integrated phrenic nerve activity (∫PN), instantaneous frequency (inst. freq.) in breaths per minute (BPM) and blood pressure during 5.5 minutes of hypoxic exposure. (B) Time courses of relative frequency, integrated phrenic nerve amplitude (∫PNA) and ventilatory output (frequency × ∫PNA) calculated for the three groups: naïve controls (n=10), viral controls (n=6) expressing eGFP, and rats expressing TeLC (n=6). Phases I and II of the hypoxic ventilatory response are shaded in grey. All data are plotted relative (rel.) to baseline (prehypoxia) levels. (C) Comparisons of phase I and II parameters across the three groups reveals a significant decrease in ventilatory output of TeLC expressing rats during both phase I and II ($p<0.05$, One-way ANOVA, Tukey post-hoc test).

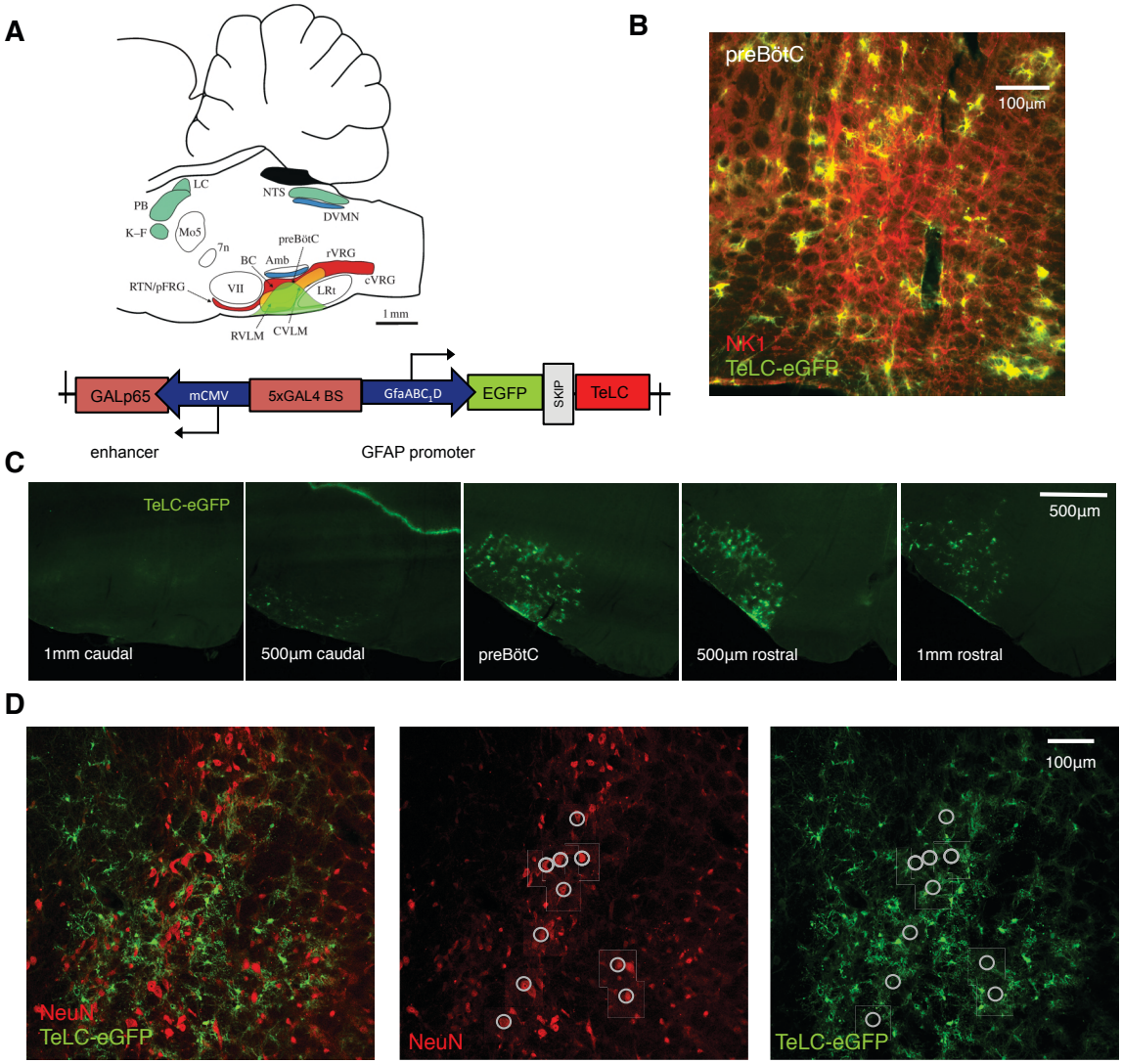

Figure 2.2 Viral expression was centered at the level of the preBötC and limited to glia.
(A) (Top) A sagittal view of the rat brainstem identifying relative locations of main groups of CNS neurons that contribute to respiratory, sympathetic and parasympathetic activities, and an estimation of the extent of viral expression. VII, facial nucleus; Amb, nucleus ambiguus; BC, Bötzinger complex; cVRG, caudal ventral respiratory group; CVLM, caudal ventrolateral medulla; DVMN, dorsal vagal motonucleus; K–F, Kölliker–Fuse nucleus; LC, locus ceruleus; LRt, lateral reticular nucleus; Mo5, motor trigeminal nucleus; NTS, nucleus of the solitary tract; PB, parabrachial nucleus; preBötC, preBötzinger complex; RTN/pFRG, retrotrapezoid nucleus/parafacial respiratory group; rVRG, rostral ventral respiratory group; RVLM, rostral ventrolateral medulla. Adapted from Spyer and Gourine (2009) *Phil. Trans. R. Soc. B* vol. 364 no. 1529 2603-2610I. (Bottom) Schematic of the bicistronic AVV-sGFAP-eGFP-TeLC vector. The eGFAP promoter ($GfaABC_1D$) controls the expression of eGFP and TeLC separated by a ribosomal "SKIP" sequence. As a bidirectional vector, the production of GaL4p65 enhancer binds to 5xGAL4 BS, promoting further synthesis. (B-D) eGFP (green B-D), NK1 receptor (red, B) and NeuN (red, D) expression in the ventrolateral medulla of AVV-sGFAP-eGFP-TeLC. (B) PreBötC neurons ($NK1^+$) are present in the region expressing TeLC-eGFP adenovirus. (C) Viral expression dropped off substantially 500 µM rostral and caudal to the injection site and almost disappeared ~1 mm away from the preBötC. (D) NeuN staining reveals no NeuN labeled cells that co-expressed eGFP. Ten NeuN labeled cells are circled to facilitate comparison between images).

with the neuron specific marker, NeuN. eGFP and NeuN staining and their overlay are shown for a sample section Fig. 2.2D. Ten NeuN labeled cells are circled to facilitate comparison. I did not find any NeuN labeled cells that coexpressed eGFP.

2.3.2 $P2Y_1$ receptor activation in the preBötC increases respiratory frequency in adult rats in vivo

The viral injection experiments described above do not define the excitatory gliotransmitter, but substantial evidence suggests that it is ATP. The next objective was to assess the P2 receptor subtype that mediates the action of ATP in the preBötC in vivo. ATP injected locally into the preBötC of neonatal rhythmically-active medullary slices causes a site-specific, $P2Y_1$ receptor-mediated increase in inspiratory-related frequency (Lorier *et al.*, 2004; Lorier *et al.*, 2007). I therefore tested whether $P2Y_1$ receptor activation also excites preBötC inspiratory rhythm in adult rats in vivo by injecting the $P2Y_1$ receptor agonist, MRS 2365 (1 mM, n = 8), locally and unilaterally into the preBötC of anesthetized, vagotomized spontaneously-breathing adult rats (250-300g), while measuring respiratory airflow, DIA_{EMG} and $GG_{EMG.}$

The preBötC was targeted stereotaxically, but also functionally identified based on the stereotypical response to local injection of DLH (10 mM) that comprises a rapid-onset increase in inspiratory frequency and decrease in burst amplitude (Monnier *et al.*, 2003), as shown in Fig. 2.3A. If the initial site did not evoke such a response, the pipette was moved in a grid-like manner until the expected response was observed. The excitatory effect of DLH was quite specific and helped guide the mapping. Injections 200 μm rostral to the preBötC (presumably in the BötC) reduced inspiratory frequency (Fig. 2.3B) while injections 300 μm caudal had minimal effect on inspiratory rhythm (Fig. 2.3C).

The response of one animal to preBötC injection of MRS 2365 is shown in Fig. 2.4A. This trace, its corresponding time course (Fig. 2.4B) and group data (Fig. 2.4C) show that MRS 2365 in the preBötC in vivo caused frequency to increase significantly to 1.44 ± 0.09 of

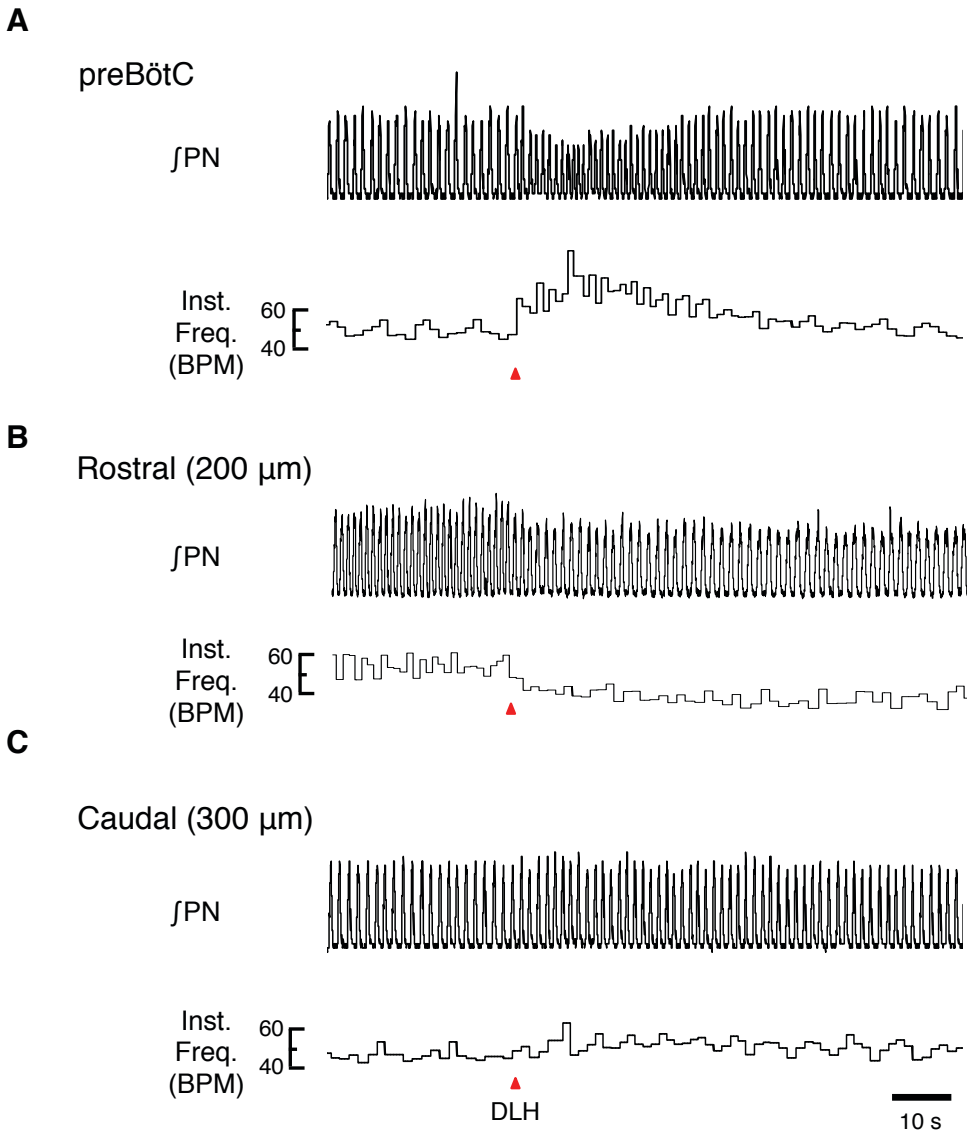

Figure 2.3: Functional identification of preBötC via local injections of DLH
Unilateral injection of DLH (10mM) into the VLM of urethane anesthetized, paralyzed adult rats while measuring instantaneous frequency (inst. freq.) of phrenic nerve activity (∫PN). (A) Unilateral injection of DLH (10mM) into the preBötC produces a robust increase in respiratory frequency. (B) In the same rat, injection 200 µm rostral to the preBötC (presumably in the BötC) reduced inspiratory frequency while, (C) injection 300 µm caudal had minimal effect on inspiratory rhythm.

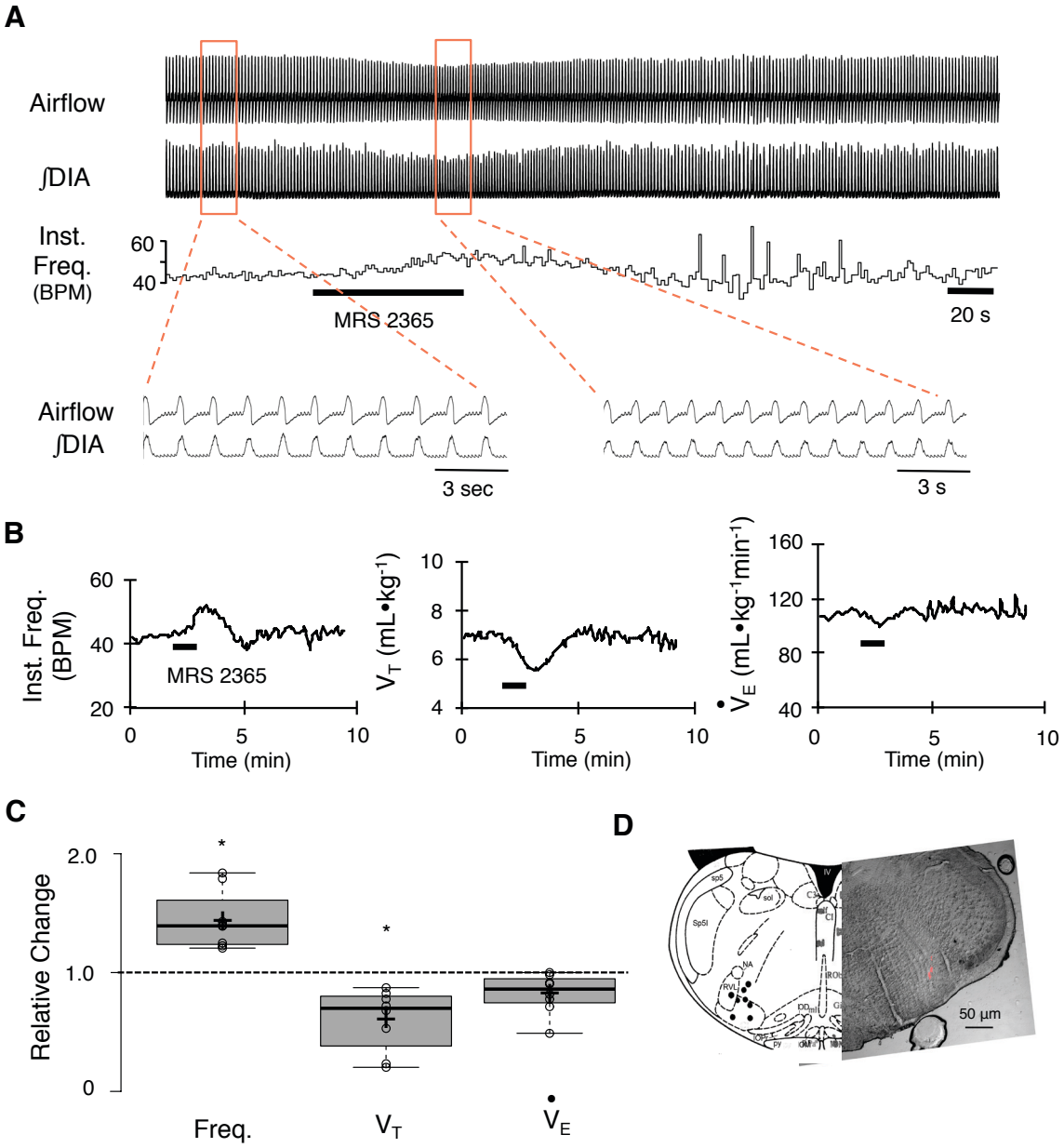

Figure 2.4 Activation of P2Y1R in preBötC of adult rat in vivo with MRS 2365 (1 mM) evokes an increase in respiratory frequency and a decrease in tidal volume (V_T)

(A) Airflow and diaphragm (DIA) EMG recordings show the response of an adult anesthetized vagotomized rat when stereotaxically injecting MRS 2365 into the preBötC. (B) Time course of instantaneous frequency (inst. freq.), V_T, and minute ventilation ($\dot{V}_E$) for a single injection. (C) Group data (n=8) illustrating the change in frequency, and $\dot{V}_E$ relative to control values significantly different from predrug controls ($p< 0.05$, paired T test). (D) Schematic showing 8 injection sites (left) and overlay of tissue section and injection site, marked with fluorescent microspheres (right). Inferior olive dorsal (IOD), inferior olive medial (IOM), inferior olive principal (IOP), 4th ventricle (IV), nucleus ambiguous (NA), pyramidal tract (PY), rostral ventrolateral medulla (RVL), spinal trigeminal tract (Sp5), spinal trigeminal interpolar (Sp5I), solitary tract (Sol).

control ($p = 0.0013$, paired t-test). V_T decreased to 0.60 ± 0.09 of control ($p = 0.0054$, paired t-test). The amplitude of $\int DIA_{EMG}$ and $\int GG_{EMG}$ (not shown) inspiratory bursts decreased similarly to 0.60 ± 0.10 ($p = 0.0049$) and 0.60 ± 0.05 ($p = 0.0002$ paired t-test) of control, respectively (Fig. 2.4C). The increase in frequency and decrease in V_T offset each other such that minute ventilation (V_E) was not significantly affected by MRS 2365 (representative time series are shown in Fig. 2.4B), as expected in an actively breathing animal with intact chemoreceptor feedback. Control saline injections into the same sites had no significant effects on frequency, V_T or $\dot{V}_E$.

Histological analysis of brain tissue confirmed the functional DLH mapping in that the fluorescent microspheres that marked the MRS 2365 injection sites were localized in a region corresponding to the preBötC based on anatomical criteria and the relationship to NK1 receptor immunolabeling as described above (Fig. 2.4D).

2.3.3 ADO injection into the preBötC has no effect on ventilation in adult rats in vivo

Extracellular ATP is degraded rapidly by ectonucleotidases ultimately into ADO, which acts via A1 receptors to inhibit respiratory activity. However, sites, mechanisms and effects appear to be species- and age-dependent (Huxtable *et al.*, 2009; Zwicker *et al.*, 2011). In the rat in vitro, ADO in the preBötC inhibits inspiratory frequency from embryonic stages until P2-3 (Herlenius *et al.*, 1997; Huxtable *et al.*, 2009). To determine whether the generation of ADO from hydrolyzed ATP could influence responses evoked by ATP in the preBötC of adult rats, I injected ADO (500 nl, 500 μM) locally and unilaterally into the preBötC of adult, anesthetized, spontaneously breathing, vagotomized rats while recording airflow, DIA_{EMG} and GG_{EMG} activity. The preBötC was located as described above (Fig. 2.3). The representative traces and group data (Fig. 2.5A and 2.5C) show that injection of ADO had no significant effect on ventilation. Frequency, V_T and $\dot{V}_E$ were 1.09 ± 0.03, 1.02 ± 0.01

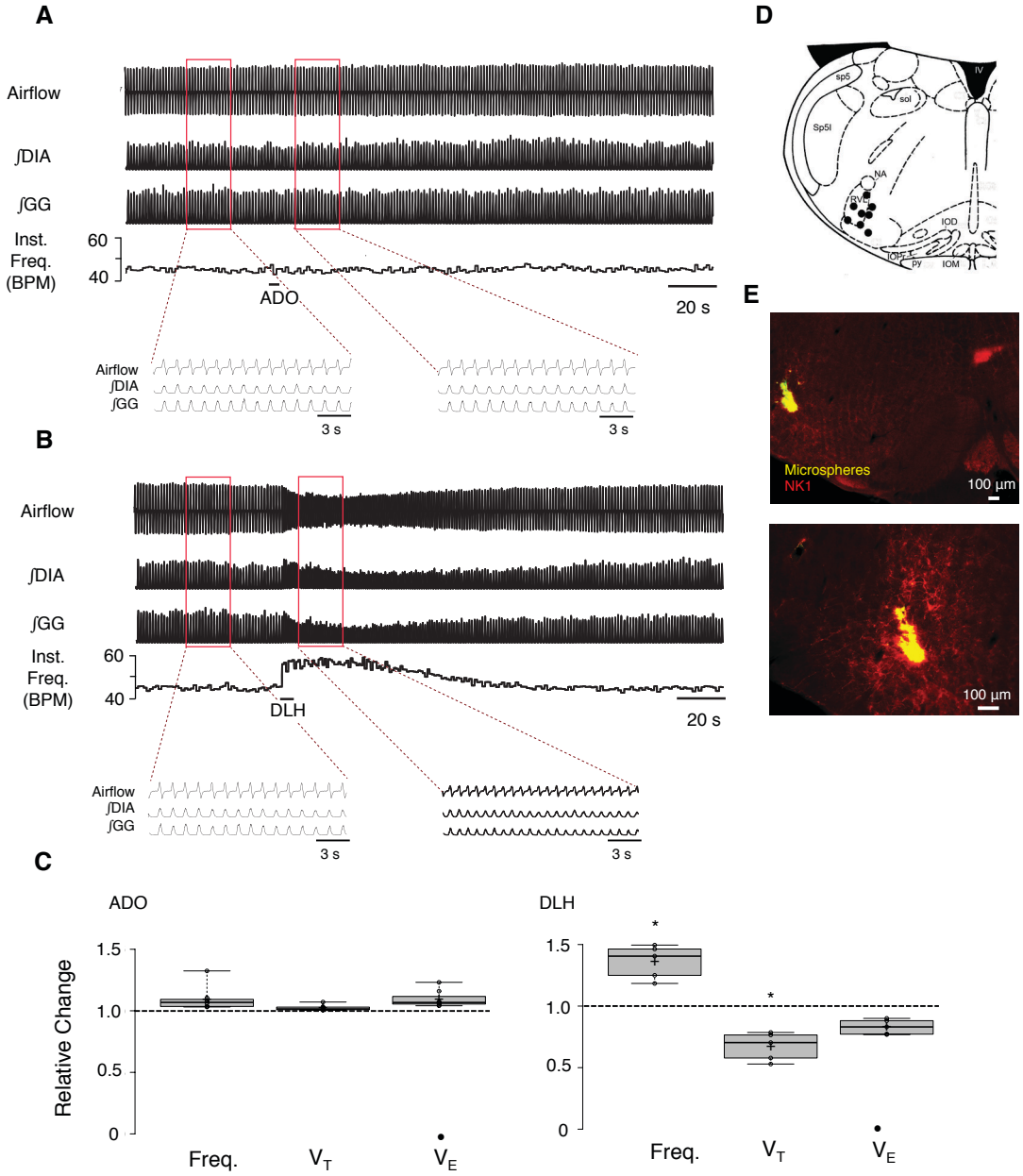

Figure 2.5 ADO (500 µM) injected into the preBötC of adult rat in vivo has no effect on respiratory frequency or tidal volume (V_T).
(A) Airflow, diaphragm (DIA) and genioglossus (GG) EMG recordings show the response of an adult anesthetized vagotomized rat when stereotaxically injecting ADO into the preBötC. (B) Airflow, diaphragm (DIA) and genioglossus (GG) EMG recordings show the response of an adult anesthetized vagotomized rat when stereotaxically injecting DLH (1mM) into the preBötC**.** (C) Group data illustrating the change in frequency and V_T relative to control (n=8), * values significantly different from predrug controls (paired T test $P< 0.05$) (D) Schematic showing 8 injection sites. Inferior olive dorsal (IOD), inferior olive medial (IOM), inferior olive principal (IOP), 4th ventricle (IV), nucleus ambiguus (NA), pyramidal tract (PY), rostral ventrolateral medulla (RVL), spinal trigeminal tract (Sp5), spinal trigeminal interpolar (Sp5I), solitary tract (Sol). (E) Tissue section with injection site, marked with fluorescent microspheres (yellow). PreBötC neurons ($NK1^+$) are present in the region of the injection site.

and 1.09 ± 0.02 of control, respectively ($p > 0.05$, paired t-test, n=7). Responses to injection of DLH into the same sites are included to verify injection within the preBötC (Fig. 2.5C). DLH caused a significant and rapid 1.36 ± 0.05 fold ($p = 0.004$, paired t-test) increase in frequency and parallel decrease in V_T to 0.67 ± 0.04 of control ($p = 0.002$, paired t-test) (Fig. 2.5C). Fluorescently-marked ADO injection sites mapped to the preBötC (Fig. 2.5D) based on anatomical landmarks and NK1 immunostaining (Fig. 2.5E), as described above.

2.3.4 $P2Y_1$ receptor inhibition in the preBötC of adult rats increases the secondary hypoxic respiratory depression

Given that ATP released along the VRC during hypoxia counteracts the secondary respiratory depression (Gourine *et al.*, 2005b), and that ATP evokes a $P2Y_1$ receptor mediated frequency increase in the preBötC in vitro (Lorier *et al.*, 2007) and in vivo (Fig. 2.4), I tested the hypothesis that the secondary hypoxic depression is attenuated by the activation of $P2Y_1$ receptors in the preBötC. I compared the hypoxic ventilatory responses of adult, urethane-anesthetized, paralyzed, ventilated and vagotomized rats following local unilateral injection of vehicle (0.2 µl HEPES buffer) or the $P2Y_1$ receptor antagonist, MRS 2279 (0.2 µl, 500 µM) into the preBötC, two minutes prior to the onset of hypoxia (n=6). ∫PN was monitored as an index of respiratory activity for two minutes in normoxia (20% O_2 - 70% N_2), five min in hypoxia (10% O_2 - 90% N_2) and then during the return to normoxia. Vehicle and MRS 2279 hypoxia trials were separated by one hour to ensure that animals had fully recovered from the first hypoxic event. Representative recordings of ∫PN in Fig. 2.6A show the hypoxic responses of one animal after vehicle and MRS 2279. Antagonism of $P2Y_1$ receptors with MRS 2279 had no effect on the initial phase I increase in frequency, ∫PNA or ventilatory output. Frequency and ventilatory output were numerically reduced but not statistically different from the vehicle control. In the control (vehicle) hypoxia trials, frequency, ∫PNA and ventilatory output increased in the first minute (phase I) to levels that were 1.42 ± .08, 1.52 ± 0.11 fold, and 1.83 ± 0.23 greater than control, respectively. In MRS

2279, hypoxia evoked initial increases in frequency, ∫PNA and ventilatory output that were 1.17±.06, 1.23 ± 0.06 and 1.36±0.11 of control. Only the change in ∫PNA was significantly reduced compared to control during phase I ($p=0.038$, paired t-test).

In contrast, MRS 2279 significantly reduced the ventilatory response observed during phase II. In the vehicle trials, during phase II, frequency fell to 0.95 ± 0.07 of baseline and ∫PNA remained elevated at 1.34 ± 0.09 of baseline such that ventilatory output remained above baseline normoxia levels (1.28 ± 0.15). In contrast, after MRS 2279 application, frequency and ventilatory output fell to levels significantly below values seen in the vehicle controls. Frequency fell to 0.62 ± 0.10 ($p = 0.0446$, paired t-test, $n = 6$) while ventilatory output was 0.73 ± 0.14 ($p = 0.0270$, paired t-test, $n = 6$) of baseline. Group data are summarized in Fig. 2.6B. Injection sites marked with fluorescent microspheres localized to the preBötC anatomically and based on the pattern of NK1 receptor immunolabeling (Fig. 2.6C).

To ensure that the greater hypoxic depression in MRS 2279 was not due to time-dependent changes in the hypoxic ventilatory response with repeated hypoxic exposures, I performed a control series in which the hypoxia protocol was repeated at a 1 hour interval but the first and second hypoxic trials were preceded by a vehicle injection. The frequency, ∫PNA or ventilatory output responses to the two consecutive hypoxic responses were indistinguishable (Fig. 2.7, $n = 5$). Comparison of the second hypoxia response in this time control series with the hypoxia response in MRS 2279 from the previous series (i.e., unpaired data) also showed that during phase II MRS 2279 caused a significant reduction in both frequency ($p = 0.01$, independent samples t-test) and ventilatory output ($p = 0.03$, independent samples t-test).

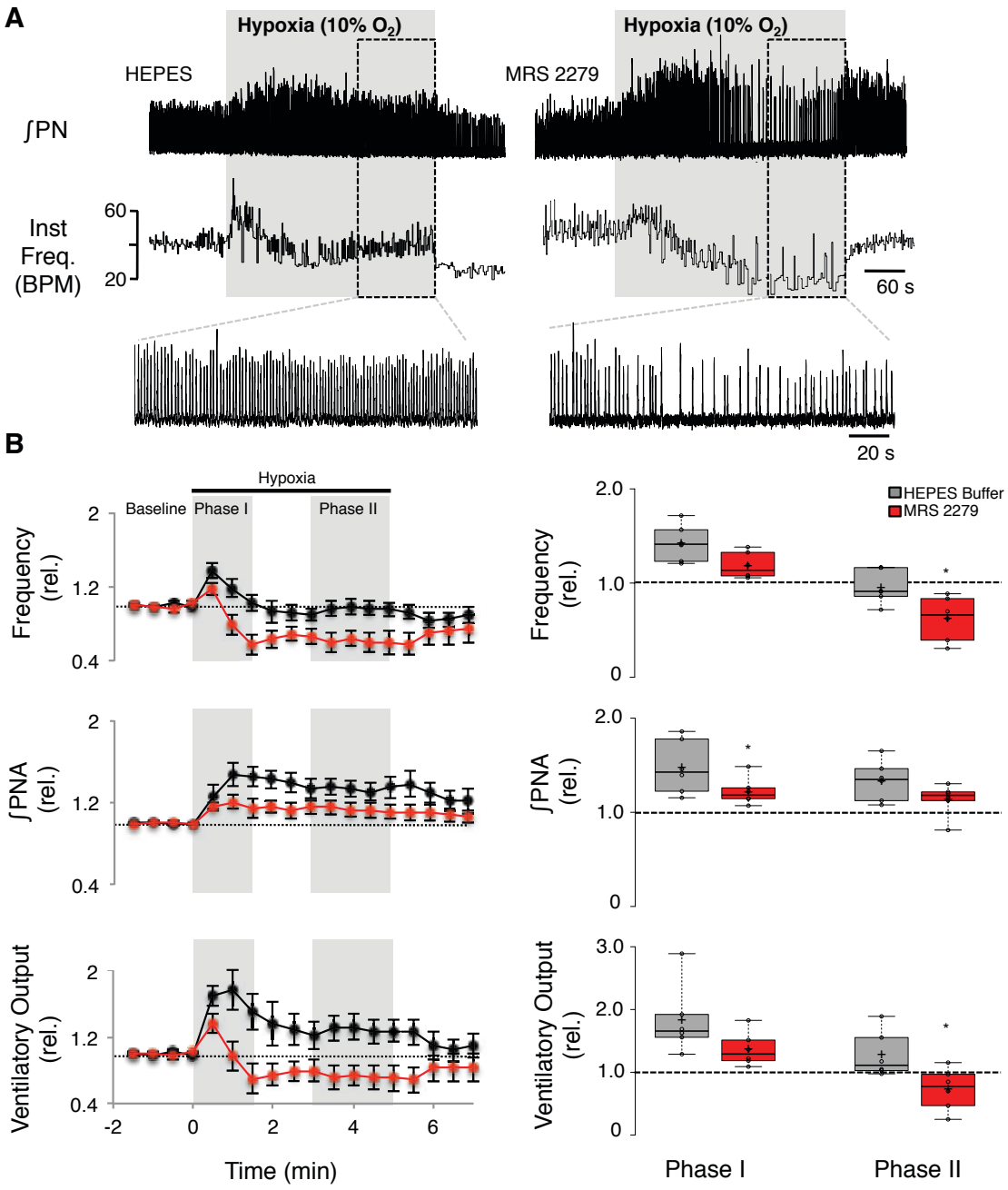
A
Hypoxia (10% O2)
HEPES
MRS 2279
Hypoxia (10% O2)
∫PN
Inst Freq. (BPM)
60
20
60 s
20 s
B
Hypoxia
Baseline
Phase I
Phase II
Frequency (rel.)
∫PNA (rel.)
Ventilatory Output (rel.)
HEPES Buffer
MRS 2279
Time (min)
Phase I
Phase II
C
100 µm
50 µm
Microspheres
NK1

Figure 2.6 Inhibition of $P2Y_1$ receptors in the preBötC of adult paralyzed rats in vivo with MRS 2279 increases the secondary hypoxic respiratory depression.
(A) Representative traces from the same animal showing changes in integrated phrenic nerve activity (∫PN) and instantaneous frequency (inst. freq.) in breaths per minute (BPM) during 5 minutes of hypoxic exposure following stereotaxic injection of vehicle (HEPES) or MRS 2279 into the preBötC. (B) *(Left)* Time courses of relative frequency, integrated phrenic nerve amplitude (∫PNA) and ventilatory output (frequency × ∫PNA) calculated before and after injection of MRS 2279 (n=6). All data are plotted relative (rel.) to baseline (prehypoxia) levels. Phases I and II of the hypoxic ventilatory response are shaded in grey. *(Right)* Comparisons of phase I and II parameters between responses reveals a significant decrease in ventilatory output and frequency during phase II of the hypoxic response, and a significant decrease in (∫PNA) during phase I. ($p < 0.5$, paired t-test). (C) (*Left)* Schematic showing 6 injection sites. Inferior olive dorsal (IOD), inferior olive medial (IOM), inferior olive principal (IOP), 4^{th} ventricle (IV), nucleus ambiguus (NA), pyramidal tract (PY), rostral ventrolateral medulla (RVL), spinal trigeminal tract (Sp5), spinal trigeminal interpolar (Sp5I), solitary tract (Sol). *(Right)* Tissue section with injection site, marked with fluorescent microspheres (yellow). PreBötC neurons ($NK1^+$) are present in the region of the injection site

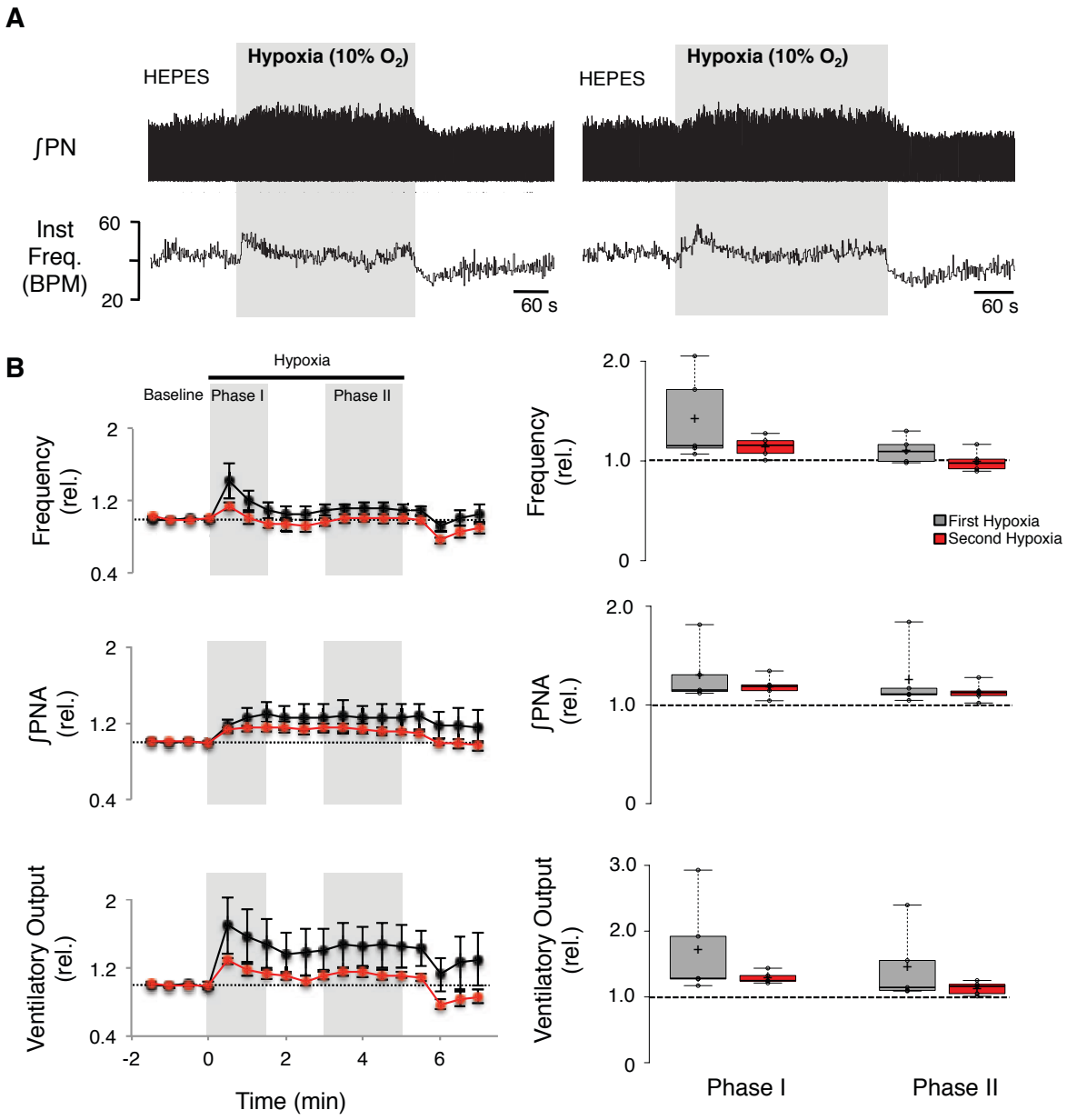

Figure 2.7 Time control data: hypoxic ventilatory response does not change over time
(A) Representative traces from the same animal showing changes in integrated phrenic nerve activity (∫PN) and instantaneous frequency (inst. freq.) in breaths per minute (BPM) during two subsequent hypoxic exposures following stereotaxic injection of vehicle (HEPES) into the preBötC. (B) *(Left)* Time courses of relative frequency, integrated phrenic nerve amplitude (∫PNA) and ventilatory output (frequency × ∫PNA) calculated before and after injection of MRS 2279 (n=6). All data are plotted relative (rel.) to baseline (prehypoxia) levels. Phases I and II of the hypoxic ventilatory response are shaded in grey. *(Right)* No significant differences of phase I and II parameters between responses were observed ($p>0.5$, paired t-test).

2.4 Discussion

During hypoxia, astrocytes in the VLM appear to sense low oxygen and release ATP (Angelova *et al.*, 2015) which increases ventilation and attenuates the secondary hypoxic respiratory depression (Gourine *et al.*, 2005b). In this study I advance understanding of the sites and mechanisms by which ATP acts to bring about this increase in ventilation. I make three main contributions. First, by expressing TeLC in astrocytes bilaterally in the preBötC and surrounding areas, I provide evidence suggesting that astrocyte mediated transmitter release contributes not only to the ventilatory increase that occurs in phase II of the hypoxic ventilatory response but also to the initial phase I increase in breathing. Second, I show that the preBötC inspiratory network is excited by $P2Y_1$ receptor activation but unaffected by ADO, receptor activation in vivo. Third, via unilateral injection of a $P2Y_1$ receptor antagonist into the preBötC, my data indicate that the ATP component of the phase II hypoxic ventilatory response is mediated in part by $P2Y_1$ receptors within the preBötC. Taken together, these data suggest that ATP released from astrocytes during hypoxia increases ventilation by activating $P2Y_1$ receptors in the preBötC.

2.4.1 The role of glia in the phase I and phase II components of the hypoxic ventilatory response in vivo

Glia have recently been identified as hypoxia sensors that play a significant role in the steady-state ventilatory increase evoked by hypoxia in unrestrained animals (Angelova *et al.*, 2015). My data from urethane-anesthetized, paralyzed, ventilated animals expressing TeLC bilaterally in astrocytes of the preBötC and vicinity confirmed a significant contribution of gliotransmitter release to the phase II component of the hypoxic ventilatory response. Evidence obtained here in TeLC rats that gliotransmitter release contributes to the initial phase I component of hypoxic ventilatory response is novel. It is also surprising given that the initial increase in ventilation is primarily attributed to activation of the carotid body chemoreceptors (Prabhakar, 2000). Indeed, the initial experiments examining the role of ATP

in the hypoxic ventilatory response detected ATP release from the ventral medullary surface only after the phase I increase had peaked and the phase II respiratory depression had begun (Gourine *et al.*, 2005b). Moreover, antagonism of P2 receptors reduced ventilation only during phase II of the hypoxic response (Gourine *et al.*, 2005b). My observation here that local antagonism of $P2Y_1$ receptors in the preBötC only reduced the phase II response further questions a role for gliotransmitters and ATP in the phase I, hypoxic ventilatory increase. Detection of a phase I contribution in the TeLC but not the MRS 2279 experiments may simply reflect that glial transmission was impaired over a wider area. TeLC injections were bilateral and, while centered in the preBötC, TeLC expression extended more than 1 mm rostrocaudally. Injection of $P2Y_1$ receptor antagonists was unilateral and antagonism likely limited to the preBötC, based on limited DLH responses rostral and caudal to the preBötC injection site. Thus, a contribution of gliotransmission to the phase I increase in ventilation, cannot be excluded.

My conclusion that gliotransmission contributes to the phase I or phase II component of the hypoxic ventilatory response depends completely on the selectivity of the viral vector for expressing TeLC in astrocytes and that once in astrocytes, it interferes with exocytotic release. I examined this issue by analyzing tissue sections for evidence of NeuN- labeled neurons that expressed eGFP, I found no cells that expressed both markers, suggesting that viral expression of TeLC was specific to glia. Glial specificity of this bidirectional sGFAP-based vector system has been previously established via immunohistochemical staining in multiple applications (Liu *et al.*, 2008). It has been used to express a genetically encoded Ca^{2+} indicator *Case12* and light-sensitive channel rhodopsin (ChR2) in astrocytes of the ventral surface of the medulla (Gourine *et al.*, 2010), and light-sensitive opsin-β_2-adrenergic receptors (optoβ_2ARs) receptors in astrocytes of the LC (Tang *et al.*, 2014). In all cases viral expression was specific to glia. Efficacy of TeLC in blocking exocytosis has been established using total TIRF microscopy of quinacrine-labeled vesicular compartments in cultured astrocytes (Angelova *et al.*, 2015). Vesicle fusion induced by ionomycin (Ca^{2+} ionophore) or

sodium dithionite (O_2 scavenger), and calcium waves that propagate between astrocytes (Angelova *et al.*, 2015) via exocytotic ATP release (Bowser & Khakh, 2007) were all blocked by expression of TeLC. Taken together, these findings suggest that the expression of TeLC under the control of the bidirectional sGFAP promoter interferes with exocytosis, specifically in glia.

Although the ribosomal skipping mechanism within the vector allows for the reduced expression of TeLC in comparison to eGFP, one caveat is that this implies that eGFP expression does not quantitatively correlate with the expression of TeLC. However, a cell that expresses eGFP does indicate successful viral gene transfer in vivo, and the likely expression of TeLC within that cell. The efficacy of TeLC in eGFP-expressing cells has been previously demonstrated (Angelova *et al.*, 2015).

2.4.2 ATP acts via $P2Y_1$ receptors to increase ventilation in vivo

Application of general P2 receptor antagonists to the ventral surface of the medulla provided the initial evidence that endogenous purinergic signalling contributes to the hypoxic ventilatory response by reducing the secondary hypoxic depression (Gourine *et al.*, 2005b). Extracellular ATP can act at a diverse array of P2 receptor subtypes within the CNS, including seven ionotropic P2X (North, 2002) and eight metabotropic P2Y (Abbracchio *et al.*, 2003) receptor subtypes. Here I provide evidence that the $P2Y_1$ subtype of P2 receptor is a major contributor to this ATP-mediated increase in ventilation in vivo.

$P2Y_1$ receptors are prevalent throughout the VLM. They overlap with NK1 receptor expression and are responsible for a "hot-spot" for ATP-evoked excitation of preBötC activity in vitro (Lorier *et al.*, 2007). In this paper, I translate these findings to the in vivo preparation, revealing that unilateral injection of $P2Y_1$ receptor specific antagonist into the preBötC increases the secondary hypoxic respiratory depression. These data suggest that $P2Y_1$ receptors contribute to the excitatory actions of endogenously released ATP during

hypoxia. The absolute magnitude of this $P2Y_1$ receptor-mediated effect is unclear as I only applied antagonists unilaterally. It is possible that my measurements underestimate the significance of the $P2Y_1$ receptor mediated increase in ventilation.

It is also uncertain whether $P2Y_1$ receptors are the only P2 receptor subtype involved in the hypoxic ventilatory response. Certainly, they are a major contributor to the frequency increase evoked by ATP in the preBötC in vitro, because this is almost completely blocked by MRS 2179 (Lorier *et al.*, 2007). Interestingly, MRS 2279 appears to be a bit less effective in blocking the actions of ATP in vitro compared to MRS 2179 (unpublished observations) which may reflect that MRS 2179 (a previous iteration of the MRS 2279) has low affinity interactions with other P2 receptor subtypes ($P2X_{1,2,3,4}$ and $P2Y_{2,4,6}$). MRS 2279 and its chemical derivatives are more selective. At 30 μM, they do not block $P2Y_{2,4,6,11,12}$ receptors and are inactive at most P2X receptor subtypes (Brown *et al.*, 2000b; Boyer *et al.*, 2002). Experiments here used pipette concentrations ranging from 10 to 500 μM for local application. These represent maximum values found in the pipette. Concentrations in the local tissue are ~10 fold lower due to diffusion, as demonstrated by the fact that to obtain similar effects, pipette drug concentrations need to be ~10 fold higher than the concentration in the bath (Liu *et al.*, 1990). Thus, the concentrations I used were not excessively high, and off-target effects of MRS 2279 are unlikely. As a result, MRS 2279-sensitivity of ATP responses and ATP involvement in the hypoxic ventilatory response is therefore good evidence for $P2Y_1$ receptor involvement.

Despite this, I cannot exclude involvement of other P2 receptor subtypes. MRS 2279-insensitive components of ATP responses of the preBötC network in rhythmic slices and inspiratory preBötC neurons in vitro (unpublished observations) indicate either incomplete block of $P2Y_1$ receptors or a role for other receptor subtypes. Certainly a variety of P2 receptors are expressed in the preBötC and other respiratory regions of the brainstem (Thomas *et al.*, 2001). $P2X_2$ receptors have been studied most extensively (Lorier *et al.*, 2004; Lorier *et al.*, 2007).

P1 receptors must also be considered. If ATP is released, it will most certainly be degraded by ectonucleotidases into ADO, which acts at A1 receptors to inhibit preBötC activity in rodents (Funk *et al.*, 2008; Huxtable *et al.*, 2009; Zwicker *et al.*, 2011). ADO receptors are also implicated in the secondary hypoxic respiratory depression (Moss, 2000). However, my data indicate that ADO in the preBötC does not affect respiratory activity in adult rats. Similarly, analysis of neonatal rat brainstem spinal cord preparations shows that the inhibitory actions of ADO on the respiratory network disappear after postnatal day 2-3 (Herlenius *et al.*, 1997; Huxtable *et al.*, 2009). Also, A_1 receptor blocker dipropylcyclopentylxanthine (DPCPX) had no effect on the hypoxic secondary depression produced by lentiviral vectors expressing a potent ectonucleotidase (TMPAP) in the preBötC of adult rats in vivo (Angelova *et al.*, 2015). Thus the potential production of ADO from ATP breakdown should not be a concern in my experiments using adult or neonatal rats. In more general terms, however, ADO signalling needs to be considered in discussions of ATP signalling as there are clear developmental and species differences (Zwicker *et al.*, 2011).

2.4.3 ATP acts within the preBötC to increase ventilation during hypoxia

During hypoxia, ATP is released throughout the VRC (Gourine *et al.*, 2005b) where it attenuates the hypoxic respiratory depression. My local injection experiments have greatly increased spatial resolution by demonstrating in vivo that a major site at which endogenously-released ATP acts during hypoxia is in the preBötC. I am confident that my injections were within the preBötC. First, the preBötC was functionally identified in all in vivo experiments prior to all test injections by the generation of a stereotypical response to DLH injection that comprised an immediate and robust increase in phrenic motor output activity (Solomon *et al.*, 1999). Second, the injection site was histologically verified postmortem by mapping the injection sites (marked with fluorescent microspheres) in relation to anatomical and immunohistochemical markers (specifically NK1 receptor

immunolabeling) that define the preBötC (Gray *et al.*, 1999; Guyenet & Wang, 2001). The injection site only marks the centre of the injection site and does not providing insight as to how far the diffusing agent will influence respiratory activity. However, I think that drug actions did not extend very far, if at all, outside the preBötC because the frequency increase evoked by DLH (the functional definition of the preBötC) was not observed when the pipette was moved just 200 μm away. DLH injections just 200 μm rostral produced inhibition, likely through activation of the Bötzinger complex (Monnier *et al.*, 2003), while injections 300 μm caudal had no effect.

I am also confident that viral transfection included the preBötC since injections sites were again mapped relative to anatomical and immunohistochemical markers. Injections were centered in the preBötC, but viral expression extended over a broader area than was likely affected by the local injections. Thus, other regions may have contributed to the effects of TeLC on the hypoxic ventilatory response, which may account for the observation that phase I initial increase in ventilation was reduced in TeLC animals but not after local injection of P2Y1 antagonists. The significance of this difference, however, is difficult to interpret because MRS 2279 was injected only unilaterally while TeLC was expressed bilaterally. In addition, my data may underestimate the disrupting gliotransmission in the preBötC on hypoxic responses because transfection extended outside the preBötC. TeLC expression in the BötC may have effects that counteract the actions of TeLC in the preBötC, as seen for DLH which is excitatory in the preBötC but inhibitory just 200 μm rostral in the presumptive BötC. Thus, while I cannot address the potentially confounding effects of TeLC in other regions in the VLM, the marked increase in the secondary depression by in TeLC animals and following local, unilateral antagonism of $P2Y_1$ receptors suggest that ATP actions are significant in the preBötC.

2.5 Summary

During hypoxia, astrocytes sense decreases in PaO_2 (Angelova *et al.*, 2015), causing a release of ATP within the VRC, attenuating the secondary hypoxic respiratory depression (Gourine *et al.*, 2005b). My data suggest that these excitatory actions of ATP are mediated through $P2Y_1$ receptors within the preBötC. Determining the site of action and receptor underlying the excitatory actions of ATP in the hypoxic ventilatory response are essential in understanding the mechanisms of the secondary hypoxic respiratory depression that is potentially lethal in premature infants. Moreover, this evidence has advanced appreciation for the role of glia and their contribution to increases in ventilation during hypoxia.

Chapter 3: The signalling pathway underlying $P2Y_1$ receptor modulation of respiratory networks in vitro

3.1 Introduction:

Chapter 2 provided new information about the source, site of action, and the subtype of P2 receptor that contribute to the excitatory actions of ATP in the hypoxic ventilatory response. Specifically, data suggest that in response to hypoxia in vivo, ATP is released from glia and acts at $P2Y_1$ receptors in the preBötC to attenuate the secondary hypoxic respiratory depression. However, the signalling cascades that underlie the excitatory actions of $P2Y_1$ receptors on preBötC neurons and glia are still unknown.

Throughout the CNS, $P2Y_1$ receptors couple primarily through the $G\alpha_{q/11}$-signalling pathway in neurons and glia (Abbracchio *et al.*, 2006). The conventional $G\alpha_{q/11}$-signalling pathway involves activation of the heterotrimeric G protein, and the subsequent dissociation of α_q and $\beta\gamma$ subunits. The α_q subunit activates PLC, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). IP_3 then binds to IP_3 receptors on the endoplasmic reticulum, triggering a release of intracellular calcium ($[Ca^{2+}]_i$). Ca^{2+} and DAG can then activate PKC. $[Ca^{2+}]_i$ and DAG have short- and long-term effects on neuronal excitability through processes that include ion channel (Veale *et al.*, 2007) and receptor modulation (Krishek *et al.*, 1994), receptor trafficking (Lan *et al.*, 2001) and protein synthesis (Hu *et al.*, 2007), remodeling of synaptic structure (Fong *et al.*, 2002b) and regulation of gene transcription (Bito *et al.*, 1996). The $\beta\gamma$ subunit has independent signalling actions by interacting directly with ion channels (Ikeda, 1996), or regulation of gene transcription (Spiegelberg & Hamm, 2005). Presynaptic $P2Y_1$ receptors most typically inhibit transmitter release (Goncalves & Queiroz, 2008), but potentiating actions have also been reported (Chandaka *et al.*, 2011).

To dissect the signalling pathway underlying $P2Y_1$ receptor mediated excitation within the preBötC, I turn to the in vitro rhythmic slice preparation where it is well-established that ATP, and its by product ADP, act most potently in the preBötC via $P2Y_1$ receptors to increase ventilation, in a glial-dependent manner (Funk *et al.*, 1997; Lorier *et al.*, 2004; Lorier *et al.*, 2007; Lorier *et al.*, 2008; Huxtable *et al.*, 2009; Huxtable *et al.*, 2010;

Zwicker *et al.*, 2011). Based on these data and evidence that the $P2Y_1$ receptor couples primarily through a $G\alpha_{q/11}$-signalling pathway (Abbracchio *et al.*, 2006), I will test the hypothesis that activation of the $P2Y_1$ receptor initiates a release of IP_3-mediated release of calcium from intracellular stores, and the downstream activation of PKC to increase respiratory frequency in vitro, and that astrocytes are excited via a common mechanism.

3.2 Methods:

All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Alberta Animal Ethics Committee.

3.2.1 Preparations

Rhythmically active medullary slice preparations. Rhythmically active medullary slices were obtained from neonatal rats (P0-P4) as described in detail previously (Smith *et al.*, 1991; Ruangkittisakul *et al.*, 2006; Zwicker *et al.*, 2011). Briefly, rats were anesthetized through inhalation of isofluorane and decerebrated. The brainstem spinal cord was then isolated in cold artificial cerebrospinal fluid (aCSF) containing (in mM): 120 NaCl, 3 KCl, 1.0 $CaCl_2$, 2.0 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 20 D-glucose, and bubbled with 95% O_2 – 5% CO_2. The brainstem-spinal cord was pinned to a wax chuck, and serial 100-200 μm sections were cut in the rostral to caudal direction using a vibrating microtome (VT1000S, Leica, Nussloch, Germany) and trans-illuminated to identify anatomical landmarks. A 700 μm rhythmic transverse slice containing the preBötC was obtained. Special care was made to ensure that the preBötC was at the rostral surface of the slice (~0.35mm caudal to the caudal aspect of the facial nucleus) (Smith *et al.*, 1991; Ruangkittisakul *et al.*, 2006; Lorier *et al.*, 2007). Rhythmic slices were pinned rostral surface up on Sylgard resin in a 5 ml recording chamber and perfused with aCSF at a flow rate of 15 ml/min (dead space of perfusion system

including inflow and outflow lines and chamber was ~20 ml). The concentration of K^+ in the aCSF ($[K^+]_e$) was raised from 3 to 9 mM at least 30 min before the start of data collection to produce prolonged stable rhythm (Ruangkittisakul *et al.*, 2006). Rhythmic slice experiments were conducted at room temperature.

Primary cultures of glia isolated from the preBötC. Cultures were prepared from 300 μm preBötC slices as described previously (Huxtable et al., 2010). The preBötC region was collected using 21 gauge tissue punches to produce preBötC cultures. Culture media were used at 37°C. Tissues were transferred to separate 15 ml conical tubes, washed (2×) with Dulbecco's PBS (2 ml) (Invitrogen), and centrifuged (1500 × g, 1 min). PreBötC tissue was plated directly on Thermanox plastic coverslips (NUNC Brand products) and placed in a T25 flask containing BME-glucose (1 ml) that was produced by adding to 1× basal medium Eagle solution (BME; Invitrogen): 3.3 mM glucose (final concentration of 8.8 mM), 2 mM L-Glutamine (Sigma-Aldrich), penicillin/ streptomycin (10,000 U of penicillin/10,000 μg of streptomycin per ml; Invitrogen), and 10% rat serum (RS).

PreBötC coverslips were incubated in 1 ml of BME-glucose for the first 24 h and 1 ml of BME-sorbitol (10% RS) thereafter. BME-sorbitol was equivalent to BME-glucose except that D-glucose was replaced with sorbitol (2.5 mM, Fisher Scientific) to select for glia. After 48 h, fresh media (with 2.5% RS) was added and changed every 3 days. Tissues were grown for 6 d, smeared, maintained in a 24-well plate for 2 weeks, and then imaged.

Ca^{2+} imaging of glial cultures. Cultures were loaded for 45 min (35°C) with the membrane-permeant Ca^{2+}-sensitive dye, fluo-4-AM (0.01 mM) in aCSF, containing the following (in mM): 117 NaCl, 5 KCl, 1 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, 26 $NaHCO_3$, and 6 glucose. Coverslips were then moved to the recording chamber (3 ml volume, 28°C, 6 ml/min flow rate) containing aCSF gassed with 20% O_2, 5% CO_2, 75% N_2. Fluorescence intensity was measured using an upright microscope (Zeiss Axioskop2 FS Plus) fitted with a 40× water-immersion objective (NA = 0.8), xenon arc lamp (175 W, Sutter Instruments), and

a Sensi-Cam QE (Cooke Corporation). Imaging Workbench 6.0 (INDEC Bio- Systems) was used to control a Lambda 10-2 shutter system (Sutter Instruments). Images were acquired at 1 Hz (20 ms exposure). Drugs (ATP 10 μM and thapsigargin 50 nM) were applied from triple-barreled micropipettes using a controlled pressure source. Consecutive agonist applications were 10 min apart. The locally-applied ATP concentration was 10-fold lower in these experiments compared to slice experiments because of improved drug access to glial monolayers.

3.2.2 Electrophysiology

In vitro recordings of inspiratory activity were made using glass suction electrodes (A-M Systems, Carlsborg, WA) placed on the XII (hypoglossal) nerve rootlets and directly from the ventrolateral surface of the slice. Field potentials were recorded from the surface of the slice using a four-axis manual manipulator to place a suction electrode (120 μm inside diameter) on the surface of the slice over the approximate region of the ventral respiratory cell column (Ramirez *et al.*, 1996). The pipette was systematically moved in 60 μm steps until the most robust signal was detected**.** This technique was used to establish the approximate location of the preBötC to assist local drug injection. Signals were amplified, bandpass filtered (300 Hz to 1 kHz) , rectified, integrated and displayed using Axoscope 9.2 (Molecular Devices, Sunnyvale Ca, USA). Data were saved using a Digidata 1322 A/D board and Axoscope 9.2 software (Molecular Devices) for offline analysis.

3.2.3 Drugs and their application

ATP (10 μM), MRS 2365 (10 μM), EGTA-AM (1 mM), 2-APB (100 μM), chelerythrine chloride (10 μM), PMA (PKC agonist, 500nm), thapsigargin (4μM, 200μM), and SP ([Sar^{9}-$Met(O_2)^{11}$]-SP (1μM) were obtained from Tocris Biosciences. Drugs were prepared as stock solutions in aCSF and frozen in aliquots. For drugs added in vitro, the final concentration of

K^+ in the drug solution was matched to that of the aCSF. When required, drugs were made up in dimethyl sulfoxide (DMSO) and, apart from thapsigargin, diluted such that the final concentration of DMSO never exceeded 0.01%. For thapsigargin experiments, a higher concentration of DMSO (0.4%) was required, so control injections of 0.4% DMSO were included in this experimental series.

Drugs were injected unilaterally into the preBötC via triple-barrelled pipettes (5-6 μm outer diameter). Drug microinjections were controlled by a programmable stimulator (Master-8; A.M.P.I., Jerusalem, Israel) connected to a picospritzer (Spritzer4 Pressure Micro-Injector, 18 psi). Consecutive agonist (MRS 2365) applications were separated by a minimum of 15 min. To establish the location of the preBötC for injections in vitro, a suction electrode was placed on the rostral surface of the slice and moved in 60 μm steps medially/laterally and dorsal/ventrally to find the maximum signal arising from the preBötC. This location was used to guide placement of the drug pipette. SP (1 μM) was then locally applied in order to functionally the preBötC based on a characterstic immediate, 3-4 fold increase in inspiratory frequency (Lorier *et al.*, 2007; Zwicker *et al.*, 2011). The injection site was moved in a grid-like fashion until the characteristic response was observed. When drugs were added directly to the circulating aCSF, they were given 20 minutes to equilibrate.

3.2.4 Additional second messenger blockers

Experiments to define the second messenger cascade that mediates the effects of $P2Y_1$ receptor activation on the preBötC network included additional series that are not included in the main body of the thesis. These were not included for two reasons; poor solubility and/or a lack of positive control. Poor solubility of the agent caused it to precipitate upon application or required high concentration of vehicle to dissolve the agent, which disrupted rhythm. Some drugs had no effect, and I was not able to design a positive control to verify that the agent was working. Very little is known in the rhythmic slice about second

messenger signalling, making it is difficult to design a positive control. The drugs that were without effect on the excitatory response of ATP or MRS2365 work from inside cells so access to the inside of cells throughout the network is problematic. Thus a negative effect reveals nothing without a positive control. My focus in developing the thesis was on the information that was instructive and insightful. Thus, I have not included the data from the in vitro experiments with second messenger blockers that had no effect. These agents are listed in Table A.2 of the appendix to document that these compounds have no effect under the in vitro conditions used here.

3.2.5 Data Analysis

XII nerve activity from rhythmic slices were analyzed offline in Clampfit (pClamp 9.2, Axon Instruments) and Microsoft Excel. To assess the effects of a specific agent on the frequency response evoked by local injection of P2 receptor agonists into the preBötC, we compared the relative increase in peak frequency evoked by MRS 2365 in control and in the presence of drug. Baseline frequency was the average frequency measured over the 2 minutes immediately prior to drug application. Peak frequency was the peak value in the first min of drug application obtained from a moving average calculated based on three consecutive XII bursts (Huxtable *et al.*, 2010; Zwicker *et al.*, 2011).

Ca^{2+} imaging data from glial cultures were analyzed offline using Imaging Workbench 6.0 (INDEC BioSystems). Cells were selected as ROIs, and baseline fluorescence calculated for each ROI by taking the average intensity of three images before drug administration. Drug effects were assessed by comparing the maximum, relative increase in fluoresence evoked by ATP in control and in the presence of the antagonist.

Mean values were compared using either GraphPad Prism version 4 (GraphPad Software Inc.) or SPSS version 20 (IBM). For comparison of two groups, paired or unpaired t-tests were used as appropriate. For comparison of more than two groups, ANOVA was used in conjunction with the Tukey *post hoc* multiple-comparison test. Group data were represented by boxplots created by the web-application 'BoxPlotR', recently featured in *Nature Methods* (2014).

3.3 Results

3.3.1 IP_3-mediated release of calcium from intracellular stores contribute to the excitatory actions of $P2Y_1$ receptor activation on preBötC rhythm

Despite the recognized limitations of using in vitro models to study hypoxic homeostatic reflex control mechanisms at the network level (Funk & Greer, 2013), the rhythmic slice preparation has been useful in discerning receptor subtypes and possible cell-types involved in the ATP excitation of the preBötC in vitro (Lorier *et al.*, 2007; Huxtable *et al.*, 2009; Huxtable *et al.*, 2010; Zwicker *et al.*, 2011). Having established that $P2Y_1$ receptor mechanisms in the preBötC contribute to the hypoxic response, I used rhythmic slice preparations to examine the signalling pathways via which $P2Y_1$ receptor activation excites the preBötC network. I compared the responses of the preBötC network to MRS 2365 that were evoked after local application of vehicle with those evoked after injection of putative $P2Y_1$ receptor signalling blockers, and again during washout.

I first tested the hypothesis that increases in $[Ca^{2+}]_i$ are essential for the frequency increase evoked by $P2Y_1$ receptor activation in the. I locally applied the vehicle or the Ca^{2+} chelator EGTA-AM (1 mM) to the preBötC for 5 minutes (5 sec on, 5 sec off), prior to local application of MRS 2365 (10s, 100 μM) into the preBötC. As shown for a single slice preparation and group data in Fig. 3.1, EGTA-AM significantly reduced the MRS 2365 evoked increase from a 2.1 ± 0.14 fold increase in control to a 1.5 ± 0.08 fold increase in EGTA-AM (n=6, p = 0.0047, paired t-test).

To assess whether the increase in intracellular Ca^{2+} that contributes to $P2Y_1$ receptor evoked frequency increase is derived from intracellular sources, I next compared control MRS 2365 responses (evoked after vehicle application) with those after local application to the preBötC of thapsigargin, a sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor that depletes from $[Ca^{2+}]_i$ stores. Since the site of action for thapsigargin is intracellular, additional time was allowed for diffusion into the tissue and across cell membrane. Thus, MRS 2365 applications were preceded by 15 min pre-applications of vehicle (5s on, 5s off) or thapsigargin (200 μM). Two consecutive MRS 2365 trials were performed in thapsigargin at 15 min intervals to maximize time for diffusion. The response of a single preparation and group data are shown in Fig. 3.2. Thapsigargin reduced the MRS 2365-evoked frequency increase from a peak of 4.11 ± 0.41 fold in vehicle to 2.62±0.17 at 15 min (not significant, $p=0.088$) and to 2.26 ± 0.49 on the second application after 30 min of thapsigargin ($n=4$, $p=0.024$, repeated measures ANOVA, Tukey post-hoc test) in frequency. EGTA-AM and 2-APB act from the intracellular space, which can impair washout. EGTA-AM is membrane permeable but upon entering the intracellular compartment it is cleaved to EGTA, which in non permeable. Thus, its effects will not washout. Attempts to washout thapsigargin were not effective after 30 min. Thus, I did not attempt washout in subsequent experiments using agents that work on intracellular targets.

The thapsigargin-sensitivity of the MRS 2365-evoked frequency increase suggests that a significant source of the calcium increase needed for the frequency response is from

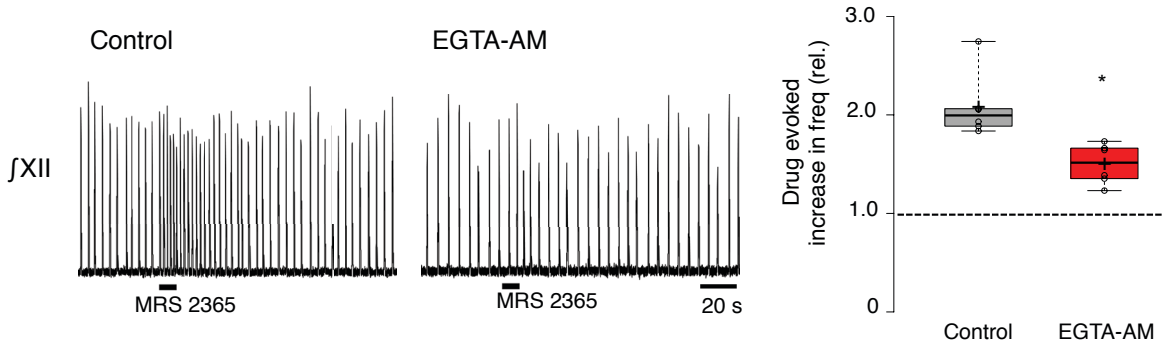

Figure 3.1 Chelation of calcium by EGTA-AM attenuates the $P2Y_1$ receptor mediated frequency increase in vitro.
Representative traces of integrated hypoglossal nerve output (∫XII) in response to $P2Y_1$ agonist MRS 2365 (10s, 100 μM) before and after the addition of cell permeable calcium chelator EGTA-AM (1 mM) locally to the preBötC. Group comparison between responses revealed a significant reduction of the $P2Y_1$ receptor mediated frequency increase in vitro ($n = 6$, $p < 0.05$, paired t-test). All data are plotted relative (rel.) to baseline levels.

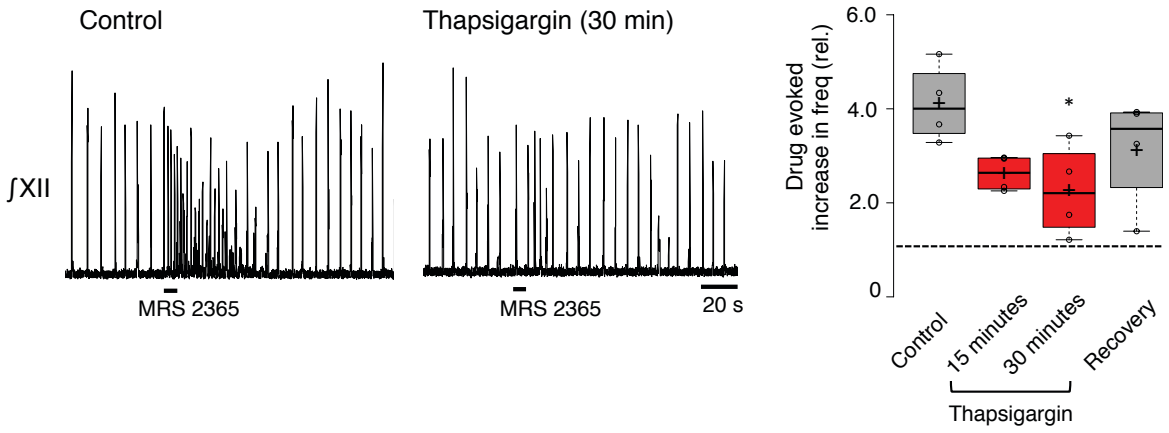

Figure 3.2 Calcium store depletion by thapsigargin attenuates the $P2Y_1$ receptor mediated frequency increase in vitro.
Representative traces of integrated hypoglossal nerve output (∫XII) in response to $P2Y_1$ agonist MRS 2365 (10s, 100 μM) before and after a 30 minute application of SERCA inhibitor thapsigargin (200 μM) locally to the preBötC. Group comparison between responses revealed a non-significant reduction of the response after 15 minutes of application and a significant reduction of the response after 30 minutes. (n=4, $p<0.5$, repeated measures ANOVA, Tukey post-hoc test). All data are plotted relative (rel.) to baseline levels.

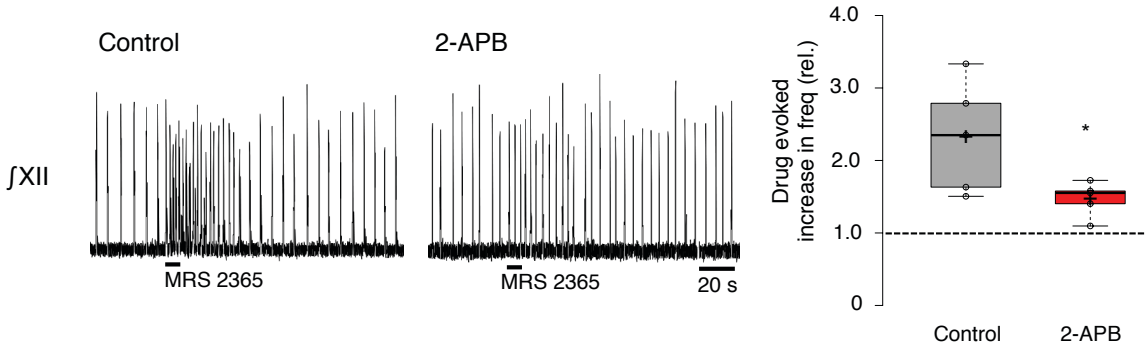

Figure 3.3 IP_3 receptor blocker 2-APB attenuates the $P2Y_1$ receptor mediated frequency increase in vitro. Representative traces of integrated hypoglossal nerve output (∫XII) in response to $P2Y_1$ agonist MRS 2365 (10s, 100 μM) before and after bath application of IP_3 receptor blocker 2-APB (100 μM). Group comparison between responses revealed a significant reduction of the $P2Y_1$ receptor mediated frequency increase in vitro (n=5, $p<0.05$, paired t-test). All data are plotted relative (rel.) to baseline levels.

intracellular stores.

Given that $G\alpha_{q/11}$ signals primarily through activation of IP_3 receptors, I next compared the MRS 2365 frequency increase evoked in before and bath application of the IP_3-receptor blocker, 2-APB (100 μM). 2-APB caused a significant attenuation of the MRS 2365-evoked peak frequency increase from a 2.32±0.34 to 1.47±0.11 (n=5, p=0.0425, paired t-test) fold increase, suggesting that IP_3 receptor-gated stores are an important source of $[Ca^{2+}]_i$ in mediating the network response (Fig. 3.3).

3.3.2 Protein kinase C activation contributes to the $P2Y_1$ receptor mediated frequency increase in vitro

PKC is a ubiquitous and divergent signalling molecule that is part of the $G\alpha_{q/11}$ initiated cascade and responsible for activating or modifying through phosphorylation the function of a large number of intracellular proteins, including ion channels (Veale *et al.*, 2007). Therefore, I tested whether activation of PKC contributes to the $P2Y_1$ receptor-mediated frequency increase in vitro. Bath application of the PKC inhibitor, chelerythrine chloride (10 μM), significantly reduced the MRS 2365-evoked peak frequency increase from a 2.37 ± 0.13 fold increase to a 1.62 ± 0.10 fold rise (Fig. 3.4A, n = 8, p = 0.0003, paired t-test). To further explore the potential involvement of PKC in the modulation of preBötC activity, I tested whether local application of PKC activator, phorbol-12-myrsitate-13-acetate (PMA, 500 nM, 10 s) can influence rhythm. PMA evoked an immediate increased frequency (2.26 ± 0.16, n = 5) similar in magnitude to that evoked by MRS 2365 (2.17 ± 0.18, n=5 Fig. 3.4B).

I next performed an occlusion test to determine whether MRS 2365 and PMA produce their actions on rhythm through a common pathway. I compared the MRS 2365 evoked frequency increase in control and after a 1 minute pre-application of PMA prior to local injection of $P2Y_1$ receptor agonist MRS 2365 in the preBötC. Pre-application of PMA produced a small but significant occlusion of the MRS 2365 effect from a 2.32 ± 0.24 fold

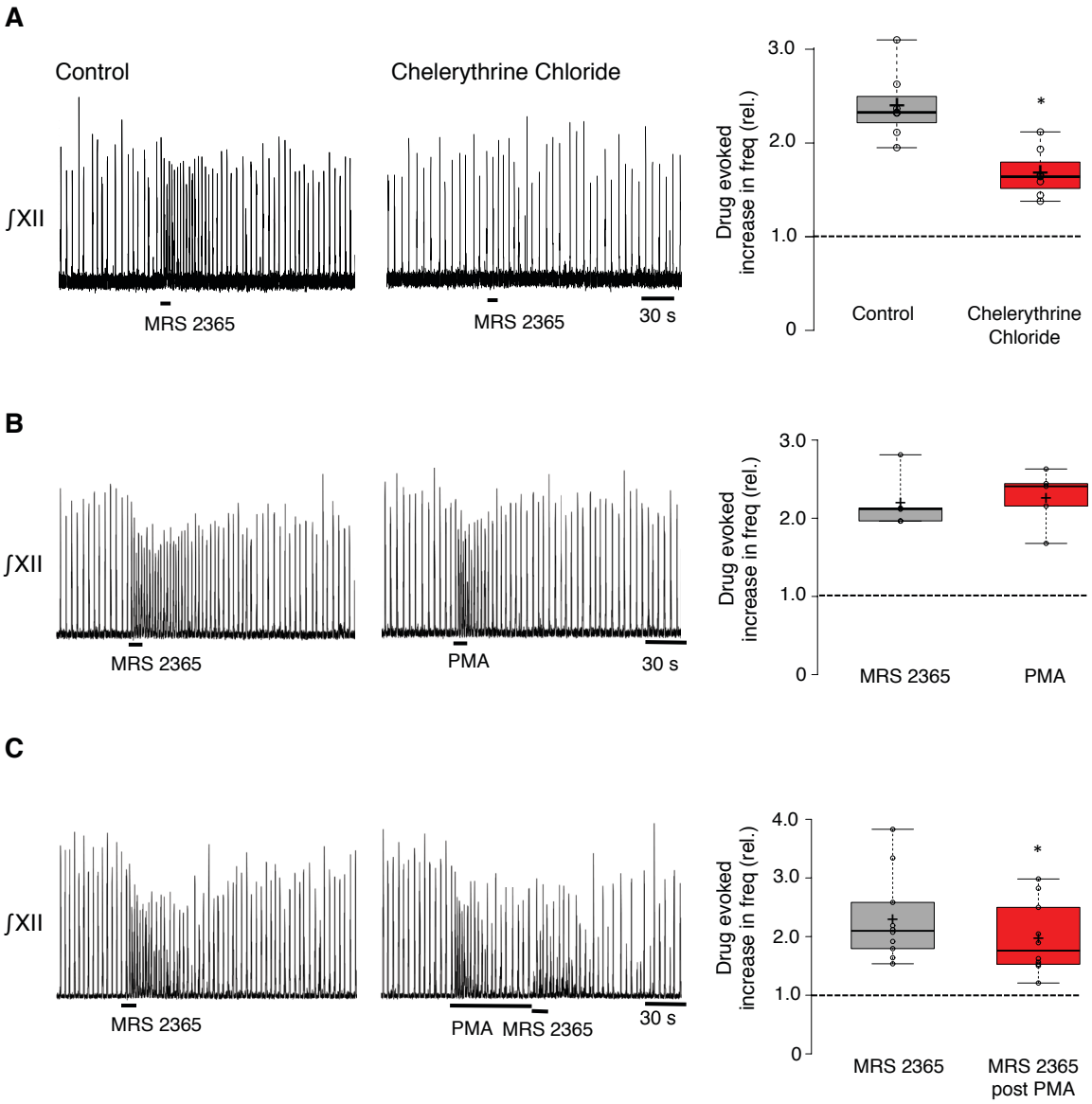

Figure 3.4 PKC activation contributes to the $P2Y_1$ receptor mediated frequency increase in vitro.
(A) Representative traces of integrated hypoglossal nerve output (∫XII) in response to $P2Y_1$ agonist MRS 2365 (10s, 100 μM) before and after local application of PKC inhibitor chelerythrine chloride (10 μM) to the preBötC. Group comparison between responses revealed a significant reduction of the $P2Y_1$ receptor mediated frequency increase in vitro (n=8, $p<0.05$, paired t-test). (B) Representative traces of ∫XII in response to $P2Y_1$ agonist MRS 2365 (10s, 100 μM) and PKC activator PMA (10s, 500 nM) to the preBötC. Group comparison between responses revealed that the frequency increases were similar in magnitude (n=5). (C) Representative traces of ∫XII in response to $P2Y_1$ agonist MRS 2365 (10s, 100 μM) before and after a 1 minute application of PMA (500 nM) to the preBötC. Group comparison between responses of MRS 2365 (before and after occlusion) revealed that the frequency increases were similar in magnitude (n=10, $p = 0.0097$, paired t-test). All data are plotted relative (rel.) to baseline levels.

increase in control to a 1.97 ± 0.19 (Fig. 3.4C, n = 10, p = 0.0097, paired t-test) fold increase after PMA.

3.3.3 Responses of preBötC astrocytes to ATP are sensitive to thapsigargin

My working hypothesis is that ATP is released from astrocytes during hypoxia and that one mechanism through which it contributes to the $P2Y_1$ receptor-mediated increase in frequency is via the autocrine/paracrine activation of astrocytes. PreBötC inspiratory neurons and glia cultured from the preBötC are both sensitive to $P2Y_1$ receptor activation (Lorier *et al.*, 2008; Huxtable *et al.*, 2010). Thus, both neurons and glia are potential targets for the actions of ATP. Fig. 3.2 suggests that the frequency increase evoked by $P2Y_1$ receptors in the preBötC is thapsigargin-sensitive and partly dependent on the release of calcium from intracellular stores. However, these data do not distinguish the contribution of neurons vs astrocytes. To determine if astrocytes could contribute to network response I tested whether the responses of cultured glia and the preBötC network to ATP share sensitivity to thapsigargin.

To test glial sensitivity, primary glia cultures isolated from the preBötC (Huxtable *et al.*, 2010) were loaded with the calcium indicator Fluo-4-AM to compare changes in $[Ca^{2+}]_i$ evoked by ATP (locally applied, 10 μM, 10s) in control and after bath application of thapsigargin (50 nm, 30 min). Images of fluorescence at baseline, and at the peak ATP response during control and in the presence of thapsigargin are shown for a group of cultured cells in Fig. 3.5A, along with the time course of fluorescence changes for four of these cells in Fig. 3.5B. Thapsigargin significantly reduced the ATP-evoked, calcium-related fluorescence response from a 3.08 ± 0.2-fold increase in control to a 1.18 ± 0.03 fold increase (Fig. 3.5B, n = 54 cells, $p < 0.001$, paired t-test).

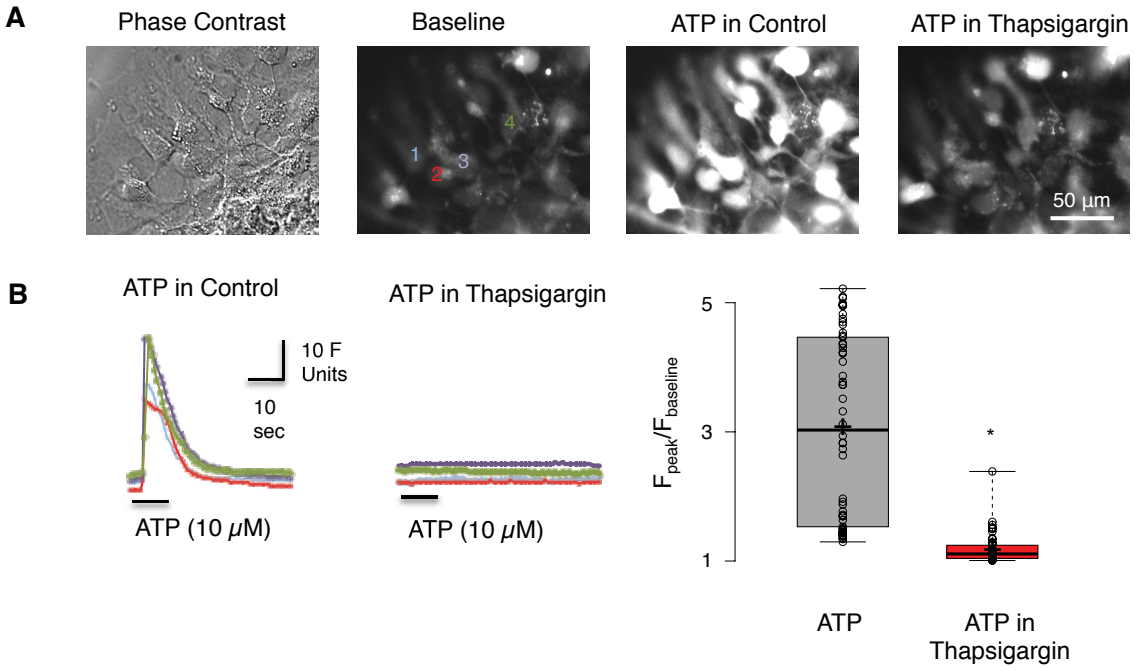

Figure 3.5 Responses of cultured preBötC glia to ATP are sensitive to depletion of intracellular calcium stores.
(A) Epifluorescence images showing, a phase contrast image of cultured glia (far left panel), the fluo-4 signal under baseline conditions (middle-left panel), during local application of ATP (100 µM, 10 s,) during control conditions (middle-right panel), and after pre-application of SERCA inhibitor thapsigargin (50 nm, 30 min) (far right panel). (B) Time course of ATP-evoked fluorescence changes measured from 4 ROIs (numbered in A) in control (left), and after thapsigargin application (middle). Group data (n=54) showing relative changes in fluorescence ($F_{peak}/F_{baseline}$) in response to ATP and to ATP in the presence of thapsigargin (right) reveal that thapsigargin significantly reduces the Ca^{2+} fluorescence response to ATP (n=53, $p<0.001$, paired t-test).

3.4 Discussion

Based on evidence from recombinant $P2Y_1$ receptors (von Kugelgen & Wetter, 2000) and $P2Y_1$ receptor signalling in a variety of brain regions (Abbracchio *et al.*, 2006), $P2Y_1$ receptors are thought to primarily act through a $G\alpha_{q/11}$ coupled receptor pathway. In this chapter, I show via analysis of network responses, that $P2Y_1$ receptor mediated excitation of the preBötC involves the IP_3-gated increase in $[Ca^{2+}]_i$ and that the downstream activation of PKC, at least in part, contributes to this response. Moreover, cultured preBötC glial responses to ATP also depend on the release of calcium from internal calcium stores, suggesting they could be cellular targets of ATP released during hypoxia. These data, in line with previous work (Huxtable *et al.*, 2010), suggest that ATP released from preBötC astrocytes, acting in an autocrine/paracrine manner to amplify astrocyte activation, contributes to the increase in ventilation observed in vivo.

3.4.1 Cellular mediators of the $P2Y_1$ receptor mediated increases in ventilation; neurons vs glia

Like the inspiratory network in vitro, cultured preBötC glia respond to ATP via $P2Y_1$ receptors that signal via a thapsigargin-sensitive pathway that involves release of Ca^{2+} from intracellular stores. Thus, glia and neurons are candidate cellular mediators of the ATP-evoked frequency increase. The only identified source of ATP during hypoxia is from astrocytes (Angelova *et al.*, 2015). Thus I propose that during hypoxia, astrocyte-derived ATP increases inspiratory frequency via two mechanisms, excitation of preBötC inspiratory neurons and autocrine/paracrine excitation of astrocytes causing further gliotransmitter release.

The roles of neurons versus glia in the $P2Y_1$ receptor mediated excitation of inspiratory rhythm is not known. A potential role for glia was first suggested with the demonstration that glial toxins attenuate the response evoked by ATP in the preBötC in vitro (Huxtable *et al.*, 2010). In contrast, recent analysis of cultured astrocytes using TIRF imaging

showed that hypoxia-evoked release of ATP containing vesicles is not affected by ATPases or $P2Y_1$ receptor antagonists, arguing against paracrine/autocrine actions of glial ATP (Angelova *et al.*, 2015). However, these cultures were sparse which could have compromised the ability to detect autocrine actions.

The observations that thapsigargin reduces the preBötC network response to $P2Y_1$ receptors while the astrocyte response in culture is blocked completely, could indicate that there are non-thapsigargin sensitive components in neurons that contribute to the network ATP response. However, while possible, a major caveat precludes making this conclusion; glial responses were studied in culture where thapsigargin has much greater access to the cells. In the end, while the relative importance of $P2Y_1$ receptors on neurons versus glia is unresolved, it is clear that both cell types are involved in the hypoxic ventilatory response. Astrocytes appear to sense hypoxia, release ATP and may also respond to the ATP. Neurons clearly respond to something released by astrocytes, most likely ATP, and transduce the glial signal into an increase in ventilation. In a more physiological context, it is important to emphasize that despite evidence of ATP release in hypoxia and its contribution to the hypoxic ventilatory response, direct purinergic activation of preBötC neurons by astrocytes during hypoxia in vivo remains to be demonstrated.

3.4.2 Signalling pathways underlying the $P2Y_1$ receptor-mediated frequency increase

Analysis of recombinant receptors suggest that $P2Y_1$ receptors signal through the $G\alpha_{q/11}$ pathway, which involves the activation of PLC, the release of $[Ca^{2+}]_i$ from intracellular stores, and the DAG-mediated activation of PKC (Simon *et al.*, 1995a; Simon *et al.*, 1995b; von Kugelgen & Wetter, 2000; Sak & Illes, 2005). Increases in $[Ca^{2+}]_i$ are fundamental to cellular interactions, including exocytosis of gliotransmitters (Parpura & Zorec, 2010; Verkhratsky *et al.*, 2012; Zorec *et al.*, 2012), neurotransmitters, and the activation of downstream ion channels. Here I show that $[Ca^{2+}]_i$ increases derived from thapsigargin- and

2-APB-sensitive internal stores contribute to the $P2Y_1$ mediated frequency increase in vitro. However, whether the responsible pathway is through $G\alpha_{q/11}$ and the activation of PLC is still unclear.

Neither thapsigargin nor 2-APB completely blocked the responses evoked by $P2Y_1$ agonist in slices. Whether this reflects a contribution of additional signalling pathways or an incomplete block of the pathway is not clear. While most evidence supports that $P2Y_1$ receptors primarily cause PLC activated, IP_3-mediated release from intracellular stores, $P2Y_1$ receptors have been reported to signal through $G\alpha_i$ which alters ion channel activity through actions of the membrane-delimited $\beta\gamma$ subunit rather than changes in intracellular Ca^{2+} rises (Brown *et al.*, 2000a; Filippov *et al.*, 2000a; Aoki *et al.*, 2004). As a result, I propose further study of the $P2Y_1$-receptor pathway in vitro, to further delineate mechanisms underlying $P2Y_1$ receptor activation in neurons and glia.

An important caveat with these in vitro experiments designed to define the signalling cascades through which $P2Y_1$ receptors signal in the preBötC, is that I am studying the cascades activated by exogenously applied ATP. The temporal, spatial and concentration profiles of ATP the develop following exogenous application of ATP may differ from those produced when ATP is released endogenously during hypoxia. My experiments define a catalogue of effects that could be mediated by ATP when it is released endogenously. Data will guide the much more challenging exercise of determining in vivo which of these the putative mechanisms is evoked in response to hypoxia.

Another limitation of this study, that is common in studies designed to characterize second messenger cascades, is that most agents used to manipulate specific elements in the pathway have off-target actions. 2-APB, in addition to blocking IP_3-gated calcium stores, has also been suggested to modulate plasma membrane cation channels from the transient receptor potential (TRP) family via inhibition of store-operated calcium entry. Of particular interest is the off-target blocking of TRPC3/7 channels (Zagranichnaya *et al.*, 2005) which produces rhythm irregularity in vitro (Ben-Mabrouk *et al.*, 2012). TRPC3/7 channels, in

addition to TRPM4/5 channels (Pace & Del Negro, 2008), may underlie an I_{CAN} current (Ben-Mabrouk & Tryba, 2010; Ben-Mabrouk *et al.*, 2012). However, whether $P2Y_1$ receptor activation leads to the downstream activation of an I_{CAN} current, is still unknown. This possibility is discussed in Chapter 4.

While PKC inhibitor chelerythrine chloride had a large impact on the $P2Y_1$ receptor mediated frequency increase in vitro, pre-application of PMA to occlude the MRS 2365 evoked response showed a significant, but much smaller reduction in frequency. A key part of the occlusion experiment was to apply MRS 2365, activating the $P2Y_1$ receptor pathway, immediately following the maximal activation of PKC with PMA. After 30 seconds of application, the PMA-induced frequency increase showed decay, likely reflecting a desensitization process. Although the PMA should still be activating the PKC pathway, it is difficult to determine how desensitization to PMA affected the potential of MRS 2365 to activate the PKC pathway and stimulate the network. Thus, while data indicate partial occlusion and suggest that PMA and MRS 2365 share a signalling pathway, it is more difficult to interpret the significance of the MRS 2365 effect that was not occluded. One possibility that might explain the differences in chelerythrine chloride and PMA occlusion is presence of specific PKC isoforms that may be differentially modulated by PMA. PKC isoforms exist in three groups, conventional (Ca^{2+} and DAG dependent), novel (Ca^{2+} independent and DAG dependent) and atypical (Ca^{2+} and DAG independent), and vary in their signalling pathways, and distribution (Way *et al.*, 2000). While all of these groups are blocked indiscriminately by chelerythrine chloride, atypical PKCs have a reduced DAG and phorbol ester binding site (Way *et al.*, 2000) and thus I speculate that they could have a weaker response to PMA. However, this would imply that the non-occluded MRS 2365 response might involve atypical PKC isoforms, which has yet to be investigated.

In testing the necessity of $[Ca^{2+}]_i$, the application of chelator EGTA-AM had a much greater effect on the $P2Y_1$ receptor evoked network response than thapsigargin. One interpretation of this difference is that intracellular Ca^{2+} chelation is a more complete block

of the necessary calcium for the $P2Y_1$-receptor mediated response, and that other sources of calcium such as extracellular entry through VGCCs, or thapsigargin-insensitive stores may contribute. However, thapsigargin is also a less direct test of Ca^{2+} release from intracellular stores, as it depends on the block of SERCAs, preventing the recovery of active or passive Ca^{2+} release into the cytosol. As a result, the success of thapsigargin in this respect depends on two factors, the presence of pumps on intracellular stores that are sensitive to thapsigargin, and that internal calcium stores have been sufficiently depleted to prevent re-release during subsequent MRS 2365 evoked responses (Treiman *et al.*, 1998). Problems with either of these factors could contribute to a reduced effect of thapsigargin on the Ca^{2+}-dependent $P2Y_1$ receptor mediated network response.

3.5 Summary

The data presented in this chapter suggest that the $P2Y_1$ receptor mediated frequency increase in vitro is dependent on increased $[Ca^{2+}]_i$, part of which is dependent on an IP_3-mediated, thapsigargin-sensitive mechanism, and the downstream activation of PKC. Given my findings, it is likely that an alternative mechanism (Ca^{2+} , IP_3 independent or thapsigargin insensitive) component contributes to the frequency increase. However, further investigation into the $P2Y_1$ receptor signalling pathways in both glia and neurons is required. The physiological and clinical significance of these observations and of characterizing detailed signalling cascades is considered in Chapter 4.

Chapter 4: General Discussion

"The essence of verification is multiple lines of reasoning that converge at a single point."

- Ian Malcolm, *The Lost World* by Michael Crichton

4.1 Summary of contributions

The general objective of this thesis was to explore the role of purinergic signalling and glia in the biphasic, homeostatic ventilatory response to acute hypoxia, and advance understanding of underlying signalling cascades. A working model in figure 4.1 describes how, during hypoxia, released ATP excites the preBötC network. At the synapse of two preBötC inspiratory neurons, volleys of action potentials in the presynaptic neuron evoke glutamate (Glu) release exciting postsynaptic neurons via ionotropic (GluR) and metabotropic (mGluR) glutamate receptors on preBötC inspiratory neurons (1). During hypoxia, astrocytes sense changes in O_2, causing increases in intracellular calcium (2), resulting in the exocytotic release of gliotransmitters including ATP (3). Released ATP acts via $P2Y_1$ receptors located on preBötC neurons (4), modulating downstream ion channels via the activation of PKC (5) and through autocrine/paracrine signalling on glia (6). ATP can also be broken down by ectonucleotidases into ADP, AMP and ADO (7), which can have inhibitory effects via presynaptic (8) and postsynaptic (9) P1 receptors.

Data presented in this thesis make five major contributions to our understanding of the process depicted in Fig. 4.1.

(i) Hypoxia evokes vesicular release of an unidentified transmitter(s) from glia within the preBötC (and vicinity) that contribute to the increases in ventilation seen during phase I and phase II of the hypoxic ventilatory response in vivo. (Fig 4.1, event 3)

(ii) ATP released during hypoxia in vivo occurs, at least in part, through activation of $P2Y_1$ receptors in the preBötC to excite the inspiratory network and increase ventilation during phase II of the hypoxic ventilatory response, thereby reducing the secondary hypoxic respiratory depression. (Fig 4.1, event 4)

(iii) The excitation of the preBötC network by $P2Y_1$ receptors in vitro is mediated in part through an increase in IP_3- mediated Ca^{2+} release from

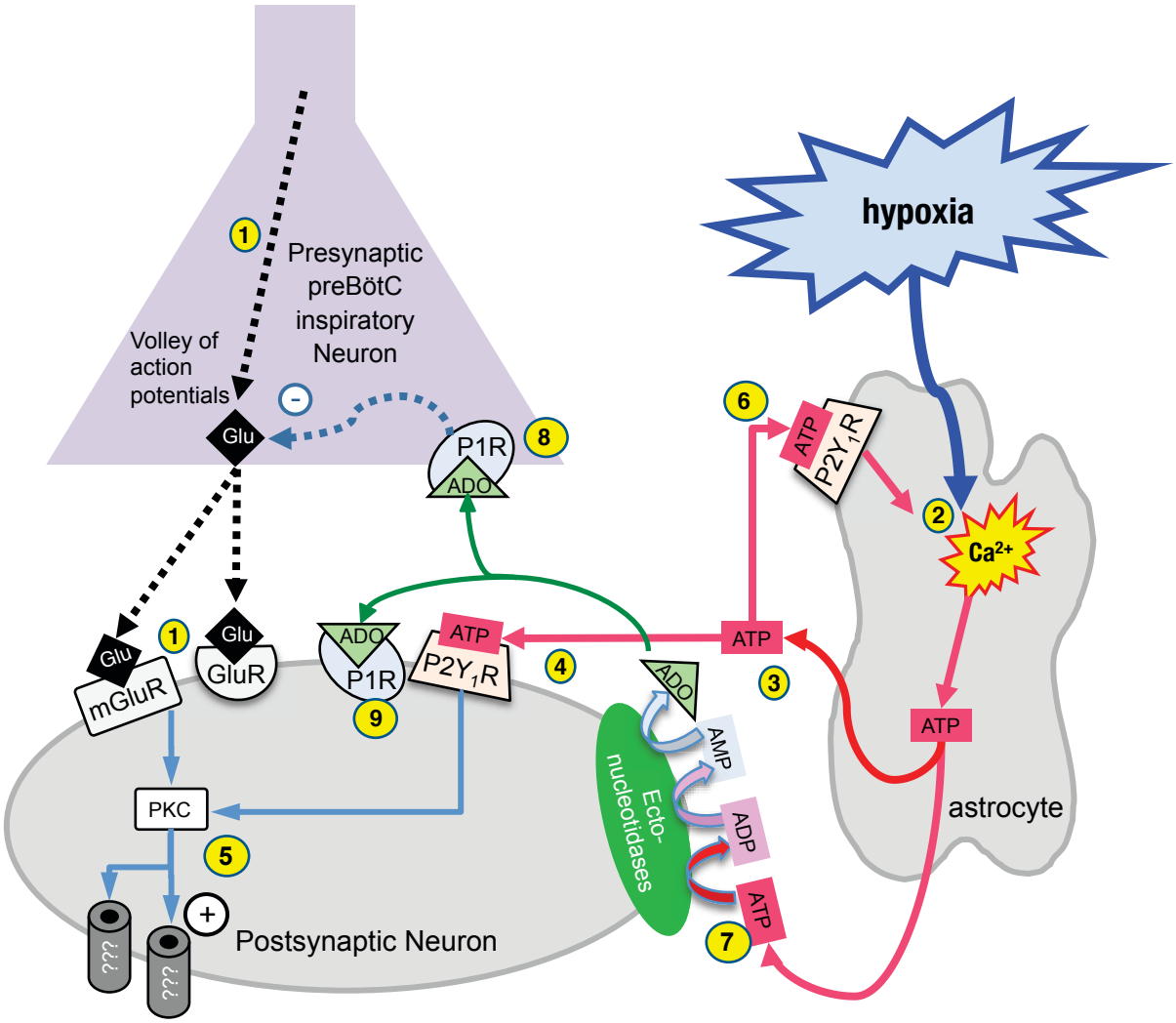

Figure 4.1 Working model describing the mechanisms underlying purinergic modulation of respiratory rhythm

(A) At the synapse of two preBötC inspiratory neurons, volleys of action potentials in the presynaptic neuron evoke glutamate (Glu) release exciting postsynaptic neurons via ionotropic (GluR) and metabotropic (mGluR) glutamate receptors on preBötC inspiratory neurons (1). During hypoxia, astrocytes sense changes in O_2, causing increases in intracellular calcium (2), resulting in the exocytotic release of gliotransmitters including ATP (3). Released ATP acts via $P2Y_1$ receptors located on preBötC neurons (4), modulating downstream ion channels via the activation of PKC (5) and through autocrine/paracrine signalling on glia (6). ATP can also be broken down by ectonucleotidases into ADP, AMP and ADO (7), which can have inhibitory effects via presynaptic (8) and postsynaptic (9) P1 receptors. Adapted from Funk et al (2015) Comp Biochem Physiol Part A Mol Intgr Physiol. 186:83-95.

thapsigargin-sensitive intracellular stores. (Fig 4.1, event 2)

(iv) The downstream activation of PKC contributes, in part to the $P2Y_1$ mediated excitation in the preBötC. (Fig 4.1, event 5)

(v) The responses of preBötC astrocytes (in culture) and the preBötC inspiratory network to $P2Y_1$ receptor activations are sensitive to thapsigargin. Thus preBötC glia, are candidate cellular mediators of the frequency increase evoked by ATP in the preBötC. (Fig. 4.1, event 6)

In sections 4.2 and 4.3, I discuss the physiological and clinical significance of these findings, pointing out experimental or technical limitations that influence the strength of these conclusions. In section 4.4, I reflect on these limitations and outline what I consider to be the next major steps that must be taken to understand the contribution of gliotransmission to respiratory control networks, the underlying mechanisms of $P2Y_1$ receptor modulation of the preBötC during hypoxia, and the clinical implications of my findings.

4.2 Physiological significance

4.2.1 $P2Y_1$ receptor modulation of preBötC rhythm

The observation that during hypoxia, ATP is released within the VLM in vivo (Gourine *et al.*, 2005b) triggered a number of studies in the in vitro rhythmic slice preparation exploring the mechanism underlying the excitatory actions of ATP. The preBötC was identified as a "hotspot" of these excitatory actions, overlapping with NK1 receptor activation in the preBötC (Lorier *et al.*, 2007). Deep pharmacological investigation involving agonist sensitivity and available P2 receptor antagonists revealed that the excitatory actions occurred specifically through the $P2Y_1$ receptor (Lorier *et al.*, 2007). However, the functional significance of these findings to the hypoxic ventilatory response in vivo had yet to be tested in vivo. In the second chapter of this thesis, I translate these in vitro findings to the in vivo

preparation, testing the hypothesis that during hypoxia, ATP acts via $P2Y_1$ receptors in the preBötC to offset the secondary depression.

The in vivo identification that the preBötC is a major site of ATP action is significant, as it increases our understanding of a brain region critical for respiratory rhythm generation, the mechanisms underlying its modulation, its potential importance in maintaining oxygen homeostasis and perhaps even in protecting against the potentially lethal effects of the secondary hypoxic respiratory depression.

4.2.2 Glia as contributors in maintaining oxygen homeostasis

Understanding of the role of neuroglia in the CNS has evolved tremendously since their initial depiction in the 1900s as 'brain glue' by Ramón y Cajal, providing a structural framework for neuronal distribution during development and cross-talk (Volterra & Meldolesi, 2005), and 'housekeepers' providing metabolic support and maintaining homeostasis of the extracellular space (Funk *et al.*, 2015). The current view of glia places them as major participants within signalling networks, responding to released neurotransmitters, and releasing chemical messengers of their own. This view was initially controversial, as the ability of astrocytes to participate in signalling and evidence of gliotransmission was limited to studies in culture, where cells can undergo changes in receptor expression, incurring differences in calcium responses when compared to acute slices (Kimelberg *et al.*, 1997). However, evidence supporting glial signalling has extended to more intact preparations including acute slices (Pascual *et al.*, 2005) and in vivo (Xie *et al.*, 2015). Evidence provided here with the TeLC experiments adds to this important database and shows that gliotransmission is important in modulating breathing to maintain blood gas homeostasis. The finding that glial toxins impair the $P2Y_1$ receptor-evoked frequency increase in vitro (Huxtable *et al.*, 2010), suggested a significant role for glia in the frequency increase evoked by exogenous ATP in the preBötC, particularly as contributors to

the ATP-mediated changes in ventilation during hypoxia (Gourine *et al.*, 2005b). The recent, and exciting observations that astrocytes sense changes in oxygen, releasing ATP-containing vesicles through exocytosis is a major step forward, suggesting that they indeed contribute to the hypoxic ventilatory response in vivo (Angelova *et al.*, 2015). In this thesis, I verify findings (Angelova *et al.*, 2015) that exocytotic release of gliotransmitters contributes to phase II of the hypoxic ventilatory response in vivo, and suggest that they also contribute to phase I. The significance of these findings is threefold. First, they provide key evidence that glia, through an exocytotic process (presumably gliotransmission) modulates the activity of the preBötC inspiratory network. Second, data support a role for glia in the the ATP-mediated increases in ventilation during hypoxia. Finally, they support the contribution of a central component to the initial increase in ventilation (phase I) of the hypoxic ventilatory response. This third remark suggests that centrally-acting ATP released from astrocytes may contribute to the small increase in ventilation observed in carotid body denervated animals (discussed in section 1.6.1).

In addition to the in vivo contribution of astrocytes during hypoxia, I also show that the ATP-mediated, MRS 2279-sensitive (Huxtable *et al.*, 2010) increases in intracellular calcium of cultured preBötC glia are dependent on internal calcium stores, implicating them in the $P2Y_1$ receptor-mediated excitation within the preBötC. These data support the contribution of glia to the hypoxic ventilatory response, via auto/paracrine interactions involving ATP-evoked ATP release, and are a step towards understanding their cell-type specific contribution (as opposed to that of neurons) to ATP mediated excitation during hypoxia.

4.3.3 The $P2Y_1$ receptor signalling cascade

Based on analysis of recombinant $P2Y_1$ receptors, $P2Y_1$ receptors typically couple through a $G\alpha_{q/11}$ pathway, to activate second messengers that cause the downstream release

of neuro- and gliotransmitters, or the activation of a host of ion channels (Abbracchio *et al.*, 2006; Rajani *et al.*, 2015). In this thesis, I have begun to explore the second messenger pathway activated by $P2Y_1$ receptors in the preBötC. Data indicate that the excitatory actions are partially dependent on IP_3-mediated release of calcium from intracellular stores the downstream activation of PKC. The reliance on calcium and activation of PKC in vitro strongly suggest that the $P2Y_1$ receptor pathway is mediated via $G\alpha_{q/11}$ rather than $G\alpha_{i/o}$ or $G\alpha_s$ (Wettschureck & Offermanns, 2005), and likely mediate the $P2Y_1$ receptor mediated increase in ventilation observed during hypoxia in vivo. Evidence outlining the $P2Y_1$ signalling pathway in this thesis, is limited to the $P2Y_1$ mediated frequency increase in vitro. As a result, the contribution of the $G\alpha_{q/11}$ signalling pathway and signalling molecules identified in vitro, to the in vivo hypoxic ventilatory response remains to be verified. In addition, the ion channel(s) underlying the excitatory response to ATP during hypoxia remain to be identified. As the these signalling cascades are also spatially located within the critical site of inspiratory rhythm generation, I propose that understanding the pre- and postsynaptic outcomes of these mechanisms may also greatly contribute to our understanding of how respiratory rhythm is generated.

4.3 Clinical relevance

The major treatment for apnea of prematurity is the administration of methylxanthines, primarily caffeine, which as ADO receptor antagonists, have therapeutic actions which are believed to be achieved through antagonism of an A1-receptor mediated inhibition respiratory activity (Funk, 2009). An increased understanding of the excitatory contribution of ATP, its underlying mechanism of action and the brain region where these actions take place, are critical in understanding the role of ADO in the context of the larger tripartite signalling pathway, involving ATP breakdown by ectonucleotidases. Not only do these findings enhance our view of the homeostatic response to hypoxia, but also provide an increased understanding of the mechanisms that can greatly influence therapies that are

currently used in the clinical setting. Insight into balancing the excitatory actions of ATP and the inhibitory actions of ADO in respiratory networks are key in improving current therapies targeting the inhibitory actions of ADO, and the development of new therapies that might enhance the excitatory actions of ATP or target the actions of specific ectonucleotidases. However, merely understanding the interplay between ATP and ADO signalling actions could not yet directly inform treatment development, as there are multiple hurdles and missing components in translating the findings in this thesis to the clinical setting.

A limitation to the clinical translation of the in vivo experiments presented here, is that they are conducted in adult rats due to their size and resiliency, allowing for ease of surgical techniques, reliable anesthesia, accuracy in targeting fundamental brain regions, and lack of extraneous developmental factors including temperature, nourishment, and stress, which could all contribute to variation in respiratory behaviour. One missing component in translating mechanisms of purinergic modulation during hypoxia to the clinical setting, particularly in premature infants who suffer frequent apneas, is how the excitatory actions of ATP in the preBötC manifest in the neonatal or premature hypoxic ventilatory response in vivo. Another component is how these findings from the adult rat translate to humans. Previous findings have shown that the effects of ADO vary across age (Herlenius *et al.*, 1997; Funk *et al.*, 2008) and species (Zwicker *et al.*, 2011). Variation across species is suggested to be a product of ATP breakdown and variations in ectonucleotidase expression (Zwicker *et al.*, 2011). These provoke future explorations of the tripartite signalling pathway (and the excitatory actions of ATP during hypoxia) at various neonatal developmental stages, and across higher mammalian species. I discuss possible approaches in applying the findings of this thesis to earlier developmental stages in section 4.4.

It is important to note that while my discussion has focused on the clinical relevance of the secondary hypoxic depression in premature infants, our analysis of mechanisms underlying the secondary phase of the hypoxic ventilatory response in adults also has clinical relevance. For example, the hypoxia and the hypoxic respiratory depression are ommon

features of obstructive sleep apnea and many other forms of sleep-disordered breathing (White, 2005). In addition, central apneas are implicated in sudden unexplained death in epilepsy (SUDEP). One of the major theories for the apneas is SUDEP is the accumulation of adenosine, which may be derived from the degradation of ATP released in response to hypoxia. Thus understanding the hypoxic ventilatory response and the role of ATP and glial signaling may help understand the underlying causes of SUDEP (Blum, 2009).

Other missing components regarding the clinical significance of my findings include the extent of glial involvement and the full characterization of the $P2Y_1$ receptor pathway in reference to ATP mediated excitation of respiratory networks. Due to the diversity in receptors and signalling pathways, and cell-specific activation of signalling pathways, characterization of second messengers is important in developing therapies with limited side-effects, and can greatly impact the optimization of drug development to produce a preferred physiological outcome. The key is to identify drug targets that will specifically affect only the cells/network of interest. The diversity of receptors and signalling molecules is the substrate on which specific therapies might evolve, so understanding expression patterns specifically in the respiratory network is important information. Future steps in understanding the contribution of glia and underlying mechanisms are discussed in the following section.

4.4 Future work

The model outlined in Fig 4.1 suggests that during hypoxia preBötC astrocytes sense the low oxygen causing an increase in intracellular calcium and the release of ATP and glutamate. ATP acts via $P2Y_1$ receptors on glia and neurons to increase ventilation. In this thesis, I have established that the exocytotic release of gliotransmitters contributes to the hypoxic ventilatory response in vivo via the activation of $P2Y_1$ receptors in the preBötC. Below, I discuss possible directions of future work that could address gaps in our understanding and limitations of the present experiments. These next steps would advance

understanding of purinergic/glial signalling in respiratory control and inform translational research.

4.4.1 The contribution of glia to modulation of respiratory networks

My data suggest that glia play an important role in the hypoxic ventilatory response by releasing gliotransmitters that act on preBötC neurons to excite the respiratory network. However, unanswered questions regarding the role of glia remain. I describe these gaps below, outlining questions that, if answered, would advance our knowledge regarding the contribution of glia to increasing ventilation during hypoxia.

(i) What are the identities of the gliotransmitters released from glia?

While the data presented here support that the exocytotic release of gliotransmitters from astrocytes contributes to the hypoxic ventilatory response in vivo, the identity of gliotransmitters released by glia are still not known. Evidence suggests ATP may be released exocytotically from astrocytes upon sensing low oxygen, however, glutamate, d-serine and tumor necrosis factor-α (TNF-α) are also possible candidates (Huxtable *et al.*, 2010; Oberheim *et al.*, 2012; Angelova *et al.*, 2015). An approach to determining the identity of gliotransmitters released by preBötC astrocytes could be using mixed cultures of preBötC neurons and glia. By triggering astrocyte activation with either mechanical stimulation or pharmaco/optogenetic tools, neuronal responses could be measured and identified in reference to blockers of hypothetical chemical messengers. The major caveat with this approach is the reliance on cultured cells that can undergo changes in gene transcription and receptor expression (Kimelberg *et al.*, 1997). How to identify the involvement of specific gliotransmitters in vivo is still an open question. However, an answer may lie in targeting specific proteins involved in gliotransmitter packaging and vesicular release. Storage compartments containing chemical messengers are heterogeneous in morphology and

mobility, and contain various sets of soluble N-ethylmaleimide factor attachment protein receptor (SNARE) proteins (such as VAMP2 and VAMP3) that could facilitate exocytosis in specific types of vesicles (Ropert *et al.*, 2015; Zorec *et al.*, 2015). Further characterization of these proteins may yield targets to specifically modulate different types of gliotransmitter release in vivo.

(ii) What is the contribution of astrocytes as hypoxia sensors?

As supported by two-photon imaging of cortical astrocytes in vivo and TIRF imaging of primary dissociated glial cultures from a variety of brain regions, astrocytes respond to decreases in oxygen with increases in intracellular calcium, and the subsequent release of ATP-containing vesicles (Angelova *et al.*, 2015). While the contribution of this gliotransmission to the hypoxic ventilatory response is supported by TeLC experiments in anesthetized preparations in vivo, these experiments do not directly demonstrate that the gliotransmitter release from the astrocytes was triggered by their oxygen sensitivity. Thus a functional role of astrocytes as oxygen sensors that contribute to a homeostatic reflex remains to be demonstrated directly. Although the centrally-mediated contributions of the oxygen sensitivity can be isolated by denervating the peripheral carotid bodies, isolating various oxygen sensitive components thought to underlie the centrally mediated component of the hypoxic ventilatory response is much more challenging.

The contribution of astrocytes as hypoxia sensors may also depend on their close association with the vascular system. The neuron-glia-vascular unit is important in controlling blood flow via "end-feet" between glia and neighboring blood vessels, making glia ideal candidates for information processing in reference to pH, PaO_2, and $PaCO_2$ levels, and modulating the activity of neighboring neurons (Funk *et al.*, 2015). However, much of the evidence for hypoxic sensitivity of astrocytes is limited to reduced and non-vascular preparations, so this component of glial hypoxic sensitivity remains unexplored.

(iii) Do glial auto/paracrine interactions contribute to the ATP-mediated excitation in vivo?

Cultured preBötC astroctyes have been observed to respond to exogenously applied ATP, causing increases in intracellular calcium, and previous findings suggest that astrocytic activation by ATP is necessary for the $P2Y_1$-mediated frequency increase in vitro (Huxtable *et al.*, 2010). These findings support that astrocytes respond to ATP. If they are the source of ATP in hypoxia, then autocrine/paracrine actions could amplify the astrocytic response. In contrast, recent findings from primary dissociated glial cultures suggest that ATP released from astrocytes does not have paracrine or autocrine actions (Angelova *et al.*, 2015). The discrepancy in these findings may have been due to differences in preparation. Discussions with the authors of Angelova et al., (2015), revealed that dissociated glial cultures were not confluent. Distance between cells would greatly reduce chances of detecting paracrine interactions between distant cells. In addition, due to the vast extracellular space in monolayers, diffusion of released compounds is much quicker when compared to actual brain tissue, further reducing chances of detecting autocrine actions. To address this issue requires a close examination of gliotransmitter release from an individual glial cell, in a more intact preparation, and the identification of cellular targets activated by released gliotransmitters. Holographic photolysis of caged-compounds, a technique recently applied to activate individual preBötC neurons in the in vitro rhythmic slice preparation (Kam *et al.*, 2013b), has a finer spatial selectivity than other opto- or pharmacogenetic methods and could be useful in approaching this question. Photolysis of caged calcium or ATP could selectively activate single astrocytes, provoking the activation of nearby astrocytes and neurons, measured via fluorescent imaging of calcium increases from intracellular stores. This would, however, only illustrate the capability of astrocytes to act via auto/paracrine interactions and would not provide evidence for the functional contribution of these signalling actions. Ultimately, functional significance would require evidence that these actions contribute to changes in

ventilation during hypoxia, which might be better understood after delineating the contribution of astrocytes as hypoxia sensors, and modulators of neuronal activity in vivo.

(iv) How do glia modulate neuronal excitability?

Evidence presented here supports the possibility that astrocytes (which may be oxygen sensors) are a source of extracellular ATP during hypoxia. PreBötC inspiratory neurons respond to ATP with MRS 2179-sensitive inward currents, and in the model of Fig. 4.1, I suggest that they are key in transforming ATP release into $P2Y_1$ receptor-mediated network excitation. However, neither the second messenger cascade nor the ion channels that are ultimately modulated in the inspiratory neurons to achieve the increase in frequency have been identified. I have begun to explore second messenger involvement in the ATP mediated network response. These experiments (presented in Chapter 3), however, do not distinguish between neurons and astrocytes. Whole-cell recording of identified neurons in rhythmic slices offers a viable, approach to explore the signalling pathways and ion channels through which $P2Y_1$ receptors affect neuronal excitability independently of glia. ATP causes a $P2Y_1$ mediated inward current in inspiratory neurons (Lorier *et al.*, 2008), which can be analyzed before and application of specific second messenger or ion channel blockers in vitro. Similarly, glial cultures or imaging approaches to measure responses of single astrocytes, can be used to explore signalling mechanisms in glia. Signalling molecules found to be critical in mediating the effects of ATP on single neurons or astrocytes could then be directly tested in vivo for their role in the hypoxic ventilatory response by locally applying agonists or antagonists to the preBötC, as done here to establish a role for $P2Y_1$ receptors. While I have started to explore second messenger pathways, virtually nothing is known about the identity of downstream ion channels that are modulated by $P2Y_1$ receptor signalling to bring about the frequency increase. This is the main subject of the next section.

4.4.2 Ion channels underlying the $P2Y_1$-receptor mediated increase in respiratory frequency

Downstream from the $G\alpha_{q/11}$ pathway, $P2Y_1$ receptors in the preBötC are hypothesized to open or close ion channels in inspiratory neurons producing a robust change in frequency (Rajani *et al.*, 2015). A strategy to identify these ion channels is to determine from the literature the ion channels that are known to be modulated by $P2Y_1$ receptors, as well as those that modulate changes in respiratory frequency. Channels that satisfy both criteria represent a list of logical targets to test in rhythmic slices for their role in producing the increase in frequency evoked by ATP in the preBötC. Any ion channels that contribute to the ATP evoked frequency increase in vitro would then be examined in vivo for its effects on baseline respiratory rhythm, ATP responses and the hypoxic ventilatory response. Data would be important not only for understanding purinergic modulation of inspiratory neurons, they may reveal interesting cellular properties of basic rhythm generation.

I have recently authored a review on $P2Y_1$ receptor signalling and have identified a number of ion channels that are modulated by $P2Y_1$ receptor signalling. These, include large conductance and small conductance calcium activated potassium channels (Schicker *et al.*, 2010; Coppi *et al.*, 2012; Sanders *et al.*, 2014), L, N, P/Q type channels (Brown *et al.*, 2000a; Aoki *et al.*, 2004), KCNQ channels (Filippov *et al.*, 2006) and TRPM4/5 channels (Alvares *et al.*, 2014). However, many of these channels have only modest effects in modulating respiratory frequency in vitro, or have effects on preBötC rhythm that still need to be characterized (Rajani *et al.*, 2015). Other channels, such as TASK1/3 channels, modulate respiratory frequency and are suspected to be modulated by via the $G\alpha_{q/11}$ pathway, but have not been associated with $P2Y_1$ receptors specifically. Below, I discuss each of these candidate channels, in reference to their association with $P2Y_1$ receptors, and, if any, their role in respiratory rhythm generation or modulation. The putative pathways by which these might be activated by $P2Y_1$ receptors are detailed in Fig. 4.2.

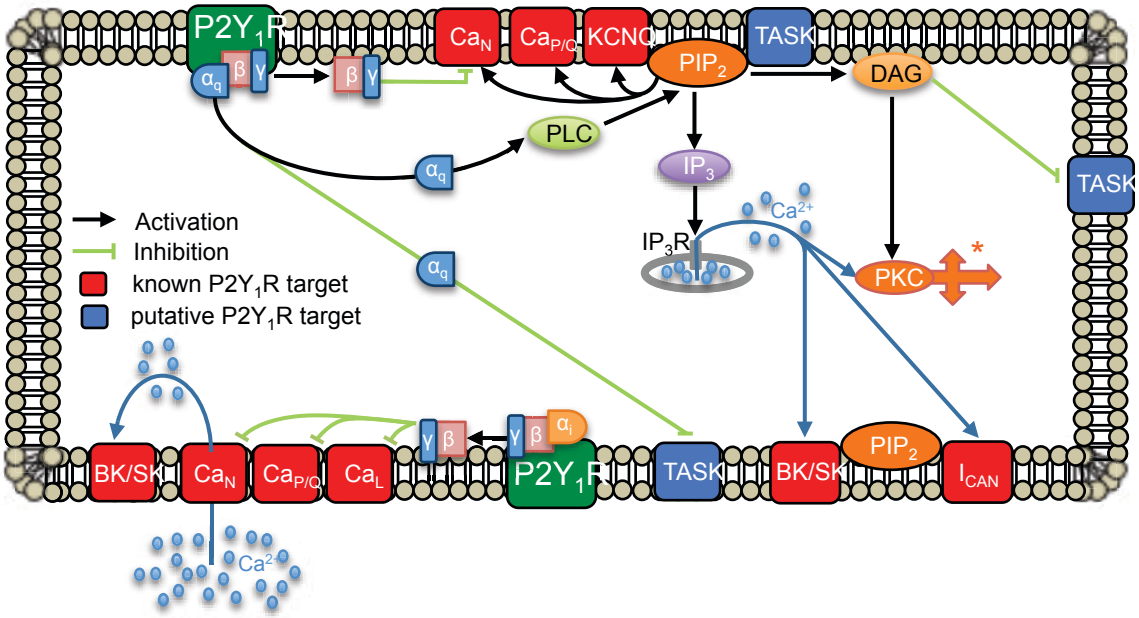

Figure 4.2 Putative pathways and ion channels through which $P2Y_1$ receptors might act to modulate preBötC rhythm.

Channels in red depict ion channels that are known targets of $P2Y_1$ receptor that also affect rhythm. Channels in blue depict ion channels that are sensitive to $G\alpha_q$ that also affect rhythm, but sensitivity to $P2Y_1$ receptors is not established. $P2Y_1$ receptors signal through $G\alpha_q$ and $G\alpha_i$ second messenger pathways. $P2Y_1$ receptor activation causes dissociation of $G\alpha_q$ and $G\alpha_i$ from $G\beta\gamma$ subunits. $G\alpha_q$ directly inhibits TASK channels (Chen, 2006). $G\alpha_q$ also signals through PLC, PIP2, IP3 and DAG. PIP2 is membrane delimited and can independently regulate the activity of KCNQ (Ford, 2003), TASK (Lopes, 2005), BK/SK (Vaithianathan, 2008; Zhang, 2014), P/Q-type (Perez-Burgos, 2010; Wu, 2002) and N-type Ca^{2+} channels (Gamper, 2004) and I_{CAN} (Crowder, 2007; Liu, 2003). IP3 binds to IP3 receptors on endoplasmic reticulum and triggers intracellular Ca^{2+} release. $[Ca^{2+}]_i$ activates BK/SK channels (Clements, 2013; Yamada, 2004), I_{CAN} (Zhang, 2007) and PKC, the latter of which is also activated by DAG. DAG and PKC inhibit TASK channels (Wilke, 2014; Veale, 2007). *Arrows are not included but PKC can modulate all channels depicted [KCNQ (Lee, 2010; Shen, 2005; Hoshi, 2003), BK/SK (van Welie, 2011; Zhou, 2010; Buchanan, 2010), Ca_N (Yang, 1993; García-Ferreiro, 2001; Anthony, 2004; Perez-Burgos, 2008), $Ca_{P/Q}$ (Perroy, 2000; Herlitze, 2001), Ca_L (Sarkar, 2008; Yang, 1993; Perez-Burgos, 2008) and I_{CAN} (Nilius, 2005; Earley, 2007)]. The $G\beta\gamma$ subunits is membrane delimited (Herlitze, 1996; Ikeda, 1996) and inhibits N-type Ca^{2+} channels (Alexander, 2000; Brown, 2000). $G\beta\gamma$ derived from the $G\alpha_i$ signaling pathway inhibits P/Q-, N- and L-type Ca^{2+} channels (Aoki, 2004). Inhibition of N-type Ca^{2+} indirectly inhibits BK/SK channels ((Neil, 1998; Sun, 2003; Wong, 2013). Adapted from Rajani et al. (2015) Respir Physiol Neurobiol. In press.

(i) BK/SK channels

Neuronal Ca^{2+}-activated potassium currents ($I_{K(Ca)}$) are carried through two main groups of channels, large-conductance (BK) and small conductance (SK) channels. BK channels require Ca^{2+} and membrane depolarization for activation. SK channels have 10-fold lower conductance than BK channels, and are gated solely by rises in Ca^{2+} (Sah & Faber, 2002). $P2Y_1$ receptor activation of Ca^{2+}-activated potassium currents ($I_{K(Ca)}$) causes rapid membrane hyperpolarization in striatal, (Coppi *et al.*, 2012) hippocampal, (Schicker *et al.*, 2010), and enteric neurons (Sanders *et al.*, 2014), through a PLC-dependent release of intracellular Ca^{2+}.

The effects of modulating $I_{K(Ca)}$ on action potential properties and the inspiratory drive potential/discharge of individual inspiratory neurons have been examined (Zavala-Tecuapetla *et al.*, 2008). The consequence of these cellular effects for network behavior is hard to predict. At the network level blocking $I_{K(Ca)}$ does not abolish respiratory rhythm in vitro, suggesting it is not essential for respiratory rhythmogensis (Onimaru *et al.*, 2003). Selective block of BK and SK channels has only minor effects on network rhythm and these vary depending on species, age and experimental preparation. Rhythm is, however, sensitive to activation of SK channels with channel opener 1-EBIO; frequency and amplitude decrease in vitro (100μM) and in vivo (Zavala-Tecuapetla *et al.*, 2008). Activation of BK in vivo increases amplitude but has no effect on burst frequency (Zavala-Tecuapetla *et al.*, 2008). Thus, under baseline conditions, neither BK nor SK are likely effectors of the $P2Y_1$ receptor mediated frequency increase. The role of these channels in the generation or modulation of rhythm, however, may not be constant. For example, block of BK channels in rats in vivo has no effect on rhythm. However, when glycinergic inhibition is reduced, block of BK results in loss of respiratory rhythm, suggesting that in the absence of inhibition, BK is important in rhythm generation, perhaps through a role in burst termination (Büsselberg *et al.*, 2003; Zhao *et al.*, 2006). Given that glycinergic inhibition decreases during hypoxia (St-John & Leiter, 2002), the ATP released during hypoxia may act through BK channels to

increase frequency. In addition, BK channels also play a role in gasping-like behaviour and autoresuscitation (Zavala-Tecuapetla *et al.*, 2008), both situations that arise specifically during acute and prolonged hypoxia. This 'situational' significance of BK channels to respiratory rhythm generation makes it a strong candidate for mediating the excitatory actions of $P2Y_1$ receptors in the preBötC

(ii) M-currents

The M-current, carried by Kv7 channel subunits and coded for by KCNQ genes, was first described in frog sympathetic ganglion neurons (Brown & Adams, 1980; Wang *et al.*, 1998). It is a K^+ current that activates at ~-60 mV, increases activity with depolarization and does not inactivate. Thus, the M-current is important in regulating neural excitability around resting membrane potential (Marrion, 1997; Brown & Passmore, 2009). It is inhibited by several $G\alpha_{q/11}$ coupled receptors in a number of different neuron types including muscarinic acetylcholine receptors, and some P2Y receptors (reviewed in (Marrion, 1997). Recombinant $P2Y_1$ receptors inhibit the M-current in dissociated rat superior cervical ganglion neurons (Brown *et al.*, 2000a), while endogenous $P2Y_1$ receptor activity inhibits the M-current in primary hippocampal cell culture (Filippov *et al.*, 2006). The specific cascade appears to differ between receptors and neurons. P2Y receptor modulation of the M-current can occur via IP_3-mediated release of Ca^{2+} from intracellular stores (Hernandez *et al.*, 2008) or IP_3-independent, direct PIP_2 inhibition (Stemkowski *et al.*, 2002; Suh & Hille, 2002).

Whether the M-current modulates preBötC inspiratory rhythm is uncertain. Endogenous ACh release modulates rhythm primarily via nicotinic receptors in the rhythmic medullary slice (Shao & Feldman, 2005), but bath application of muscarine (50 μM) increases frequency by 50% above control frequency, most likely via the M3 receptor subtype (Shao & Feldman, 2000). M3 receptor activation inhibits the M-current in neuroblastoma-glioma hybrid cells (Fukuda *et al.*, 1988), but it is not clear whether this occurs in preBötC neurons. The prominent current evoked in preBötC inspiratory neurons by

muscarinic receptors is a mixed cation current that reverses near zero mV. However, it is possible that this current obscures an underlying M-current. The effects on respiratory rhythm of selectively blocking the M-current are not known, but an increase in frequency is possible. Alternately, since the M-current activates progressively during depolarization, it may normally play a role in burst termination, in which case its block may also increase burst duration. The M-current therefore is a possible target through which $P2Y_1$ receptors act in the preBötC to increase frequency.

(iii) I_{CAN}

I_{CAN} is likely mediated by the gene that encodes TRPM4/5 (Crowder *et al.*, 2007) or TRPC3/7 channels (Ben-Mabrouk & Tryba, 2010), in the preBötC. Whether one type (TRPM or TRPC) or both types underlie I_{CAN} has not yet been resolved. I_{CAN} activation is both voltage- and calcium-dependent, and passes sodium and potassium ions, but not Ca^{2+} (Guinamard *et al.*, 2011). Whether $P2Y_1$ receptors modulate I_{CAN} in preBötC inspiratory neurons is presently unknown, although $P2Y_1$ receptors do potentiate I_{CAN} in XII motoneurons (Funk *et al.*, 2011; Alvares *et al.*, 2014). In preBötC rhythmic slices, activation of I_{CAN} by $G\alpha_{q/11}$ coupled, group 1 metabotropic glutamate receptors (mGluR1/5) dramatically potentiates inspiratory burst amplitude (Del Negro, 2005; Pace *et al.*, 2007a; Mironov, 2008). Alternatively, block of TRPC3/7 channels produces irregularity in rhythm in the preBötC in vitro. I_{CAN} activation results from the breakdown of PIP_2, and an IP_3-mediated increase in intracellular Ca^{2+}, coupled with membrane depolarization. PIP_2 can also modulate I_{CAN} directly in inspiratory preBötC neurons (Crowder *et al.*, 2007). Given that $P2Y_1R$ activation increases inspiratory frequency (Lorier *et al.*, 2007) while I_{CAN} activation primarily affects amplitude, I_{CAN} is unlikely the target of $P2Y_1$ receptor signalling in the preBötC.

(iv) TASK channels

TASK channels are acid-sensitive members of two-pore domain potassium channels that carry background currents important in regulating neuronal excitability. TASK-1, TASK-2 and TASK-3 are encoded by *KCNK3*, *KCNK5* and *KCNK9* genes, respectively. TASK channels are inhibited by multiple $G\alpha_{q/11}$-coupled receptors; e.g., in hypoglossal motoneurons, TASK-1 channels are inhibited by serotonin, norepinephrine, SP, TRH and group I metabotropic glutamate receptors (Talley *et al.*, 2000; Mathie, 2007). Although the mechanism underlying $G\alpha_q$-mediated inhibition of TASK channels is not well-established, $G\alpha_{q/11}$ (Chen *et al.*, 2006), PLC (Czirják *et al.*, 2001), PIP_2 (Lopes *et al.*, 2005), DAG (Wilke *et al.*, 2014) and PKC (Veale *et al.*, 2007) involvement have been independently reported. TASK-1 and TASK-3 channels are expressed in many respiratory neurons including preBötC inspiratory neurons (Koizumi *et al.*, 2010), hypoglossal neurons (Talley *et al.*, 2000) and raphe nuclei (Washburn *et al.*, 2002), while TASK-2 channels are found in phox2b expressing RTN neurons. Activation of TASK-1 and TASK-3 channels in rhythmic slices by bath application or injection into the preBötC of halothane significantly reduces frequency, indicating sensitivity of the preBötC to TASK-1 and TASK-3 activation (Koizumi *et al.*, 2010). To my knowledge, there is no evidence that $P2Y_1$ receptors modulate TASK channels. However, $P2Y_1$ receptors signal through $G\alpha_{q/11}$ cascades that modulate TASK channels. Thus, inhibition of TASK channels is a potential mechanism via which $P2Y_1$ receptor activation increase preBötC frequency.

(v) P/Q-, N- and L-type voltage gated Ca^{2+} channels

P/Q-, N- and L-type Ca^{2+} channels belong to $Ca_v2.1$, $Ca_v2.2$ and Ca_v1 subfamilies of VGCCs, respectively (Catterall, 2011). They carry high voltage-activated currents and are modulated by $G\alpha_{q/11}$-signalling (Beech *et al.*, 1992; Mathie *et al.*, 1992; Perez-Burgos *et al.*, 2010). $P2Y_1$ receptors inhibit N-type Ca^{2+} channels in superior cervical ganglion neurons via the $G\alpha_q$ pathway, but the inhibition is dependent on the $\beta\gamma$- rather than the α_q subunit

(Herlitze *et al.*, 1996; Ikeda, 1996; Brown *et al.*, 2000a; Filippov *et al.*, 2000b). Unconventional actions of $P2Y_1$ receptors in NTS neurons through the $G\alpha_i$ pathway produce a βγ-dependent inhibition of N-, P/Q- and L- type Ca^{2+} channels (Brown *et al.*, 2000a; Filippov *et al.*, 2000b; Aoki *et al.*, 2004). N- and L-type Ca^{2+} currents are present in all classes of respiratory neurons while P/Q-type Ca^{2+} currents are limited to pre-inspiratory neurons and a subset of inspiratory neurons (Onimaru *et al.*, 1996). Establishing a unique role for postsynaptic P/Q-, N- and L-type Ca^{2+} currents in inspiratory rhythm generation or shaping the inspiratory drive potential is challenging because these channels are located pre and postsynaptically (Takahashi & Momiyama, 1993; Aoki *et al.*, 2004). Inhibition of presynaptic Ca^{2+} currents will reduce the postsynaptic potential. With this caveat in mind, P/Q-, N- and L-type Ca^{2+} conductances all appear to contribute to the inspiratory drive potential (Onimaru *et al.*, 2003). Inhibition of N-type Ca^{2+} currents *in vitro* has only minor effects (<15%) on inspiratory rhythm (Onimaru *et al.*, 2003; Lieske & Ramirez, 2006; Morgado-Valle *et al.*, 2008; Koch *et al.*, 2013). Progressive inhibition of P/Q-type Ca^{2+} channels in rhythmic slices decreases and ultimately blocks rhythm (Lieske & Ramirez, 2006). Inhibition of L-type Ca^{2+} also slows rhythm, while potentiation of L-type Ca^{2+} currents by mGluR1/5 increases frequency (Mironov & Richter, 2000). mGluR1/5 and $P2Y_1$ receptors both signal through $G\alpha_{q/11}$. Thus, it is conceivable that potentiation of L-type currents contributes to the $P2Y_1$ receptor mediated frequency increase. However, the only documented action of $P2Y_1$ receptors on VGCC is inhibition, which would decrease rather than increase frequency.

4.4.3 Developmental characterization of purinergic signalling in the hypoxic ventilatory response

Characterizing the development of the centrally-mediated components of the hypoxic ventilatory response are essential in understanding the clinical relevance of my findings in terms of the increased secondary depression in neonates, which can be life threatening in

premature infants who suffer frequent apneas. In this thesis, I have suggested cellular targets and receptors and cell mechanisms that can now be investigated in younger animals to determine how purinergic signalling within the preBötC changes during development, and how these changes contribute to the hypoxic ventilatory response in vivo. Our current knowledge of purinergic signalling in the hypoxic ventilatory response have thus far been limited to adult animal models, and examination of the response of the in vitro rhythmic slice preparation to ATP and hypoxia. While useful for understanding the organization and neuromodulation of the networks involved in basic rhythm generation, the in vitro rhythmic slice preparation has limitations in translating the meaning of these behaviours in vivo (Funk & Greer, 2013). Below, I suggest the next steps in translating my findings to more clinically relevant developmental stages, to lend insight to the pathology of the impaired secondary hypoxic ventilatory depression in newborns.

(i) The contribution of $P2Y_1$ receptors to the neonatal hypoxic ventilatory response

In this work, I show that the excitatory actions of ATP act via $P2Y_1$ receptors in the preBötC to increase ventilation during hypoxia in adult rats in vivo. In addition, the robust frequency increase when ATP is exogenously applied to the preBötC has been established to occur primarily through $P2Y_1$ receptors in the neonatal rhythmic slice preparation (Lorier *et al.*, 2007). However, the significance of $P2Y_1$ receptor signalling in the hypoxic ventilatory response in neonates in vivo is still unclear. Size and fragility of neonatal rodents present major challenges in manipulating cellular receptors in in vivo neonatal preparations. I propose using a transgenic mouse preparation to understanding the role of $P2Y_1$ receptors during hypoxia. A $P2Y_1$ receptor knockout (KO) mouse has been developed, and applied to understanding purinergic signalling in a variety of scenarios, including atherosclerosis (Leon *et al.*, 1999; Hechler *et al.*, 2008), cerebral stroke (Abe *et al.*, 2013) and purinergic neurotransmission in the gastrointestinal tract (Gil *et al.*, 2013). Examination of the respiratory patterns and hypoxic responses of neonatal $P2Y_1$ KO mice, in comparison to wild

type (WT) may increase understanding of the contribution of $P2Y_1$ receptors during hypoxia in reference to neonatal respiratory behaviour. Conditional $P2Y_1$ receptor knockout strategies, especially those where the genetic deletion can be targeted to specific brain regions would of course be optimal, though these have yet to be developed.

(ii) The role of glia in hypoxia: developmental considerations

Experiments using adenoviruses to express TeLC specifically in astrocytes supports that exocytotic gliotransmission significantly contributes to the hypoxic ventilatory response in adult rats in vivo. Though glia play an important role in the $P2Y_1$ receptor mediated frequency increase in vitro (Huxtable *et al.*, 2010), understanding the contribution of gliotransmission to the in vivo response in younger animals may provide insight to the increased secondary depression experienced by newborns and premature infants. As viral expression often takes 1-2 weeks for expression, the method of selectively targeting vesicular release machinery of astrocytes is not feasible in younger animals. One approach could be to apply a similar strategy as indicated in (i), utilizing transgenic animals, particularly, those with impaired vesicular release mechanisms in astrocytes, like the dominant-negative SNARE (dn-SNARE) mouse developed by Haydon and colleagues (Pascual *et al.*, 2005). This strain is inducible by doxycycline, which means that it takes days for a change in glial phenotype. Thus inducible approaches are a challenge for developmental studies. Non-inducible strains targeting dn-SNARE may also prove difficult, since interference of glial activity may affect CNS development. Other transgenic mice have been engineered to facilitate optogenetic and pharmacogenetic manipulation of astrocytes. Pharmacogenetic approaches provide the potential to activate or inhibit astrocytic activity by pharmacologically bypassing the blood brain barrier without invasive surgery, a more realistic approach for neonatal animals. These approaches, however, are still under development (Xie *et al.*, 2015). I spent 12 months trying to develop optogenetic approaches to manipulate glia function using variants of light sensitive channel channelrhodopsin2

(AVV-sGFAP-hCHR2(H134R)-mKate2, AVV-sGFAP-CatCh-eYFP, and AVV-sGFAP-Optoβ2-eYGP). These efforts were not successful, reflecting that the vectors, while very effective in transfecting glia in organotypic culture, were minimally effective in vivo. The importance of glia as hypoxia sensors, a source of ATP, and contributors to the hypoxic ventilatory response in normal physiology and pathophysiology and in prematurity are important areas of inquiry.

(iii) Ectonucleotidase expression at different developmental stages

As critical components to the tripartite signalling system, ectonucleotidases are at the center of the balance between the excitatory action of ATP and ADP and the inhibitory effects of ADO within the respiratory network. Ectonucleotidases are a diverse family of proteins with differential substrate affinities and end products. As a result, their differential expression can underlie differences in responses to ATP neuromodulation between species (e.g. rats vs mice) (Zwicker *et al.*, 2011), during development as is seen in embryonic compared to postnatal rats (Herlenius *et al.*, 1997; Herlenius & Lagercrantz, 1999; Huxtable *et al.*, 2009). While these studies clearly illustrate the impact of ectonucleotidase isoform expression on ATP actions, quantifying the relative contributions of various isoforms and assessing their contribution to the in vivo hypoxic ventilatory response is non-trivial. ATP and ADO biosensors, used in the rhythmic slice (Huxtable *et al.*, 2009) and in vivo (Gourine *et al.*, 2005b, a; Dale & Frenguelli, 2011), present an opportunity to measure diffusion and breakdown profiles of ATP release during hypoxia, where ectonucleotidase blockers could help isolate the relative contributions of different ectonucleotidase isoforms to ATP breakdown, and ADO production. While ATP biosensor assay experiments have been conducted in vivo at the surface of the ventral lateral medulla during hypoxia in adult rats (Gourine *et al.*, 2005b), conducting the same experiments in younger animals in vivo could present many challenges. However, given that ectonucleotidase expression and resulting ATP sensitivity varies across species, I propose that much can be inferred from a comparative

physiology perspective. Variations in brainstem ectonucleotidase expression across phylogeny may contribute the different hypoxic responses observed across small mammalian species (Frappell *et al.*, 1992), and possibly, though not yet examined, in precocial versus altricial rodents. Species differences in ectonucleotidase expression and resulting hypoxic responses could offer insight into those evident at different developmental stages (newborn vs adult) in a single species. Theoretically, if enzyme activity could be reduced specifically in the preBötC, it could both increase ventilation through $P2Y_1$ receptor excitation and minimize depression by slowing the breakdown of ATP to ADO, thereby reducing the A1 mediated respiratory depression.

4.5 Conclusions

Purinergic signalling is integral to the maintaining respiratory homeostasis, particularly during changes in environmental O_2 and CO_2. During hypoxia, astrocytes sense changes in O_2, and release of ATP within in the VLM, offsetting the secondary hypoxic respiratory depression (Gourine *et al.*, 2005b; Angelova *et al.*, 2015). In this thesis, I test hypotheses derived from the investigation of ATP excitation in the preBötC in vitro, and determine that ATP activates $P2Y_1$ receptors in the preBötC, reducing the secondary hypoxic depression in vivo; i.e. I have identified a site and receptor subtype that mediates the excitatory actions of ATP on ventilation during hypoxia. I also suggest that the underlying mechanism of $P2Y_1$ receptor activation in the preBötC involves an IP_3-mediated release of intracellular calcium from internal stores, and the downstream activation of PKC. Data also suggest that preBötC glia contribute to the changes in ventilation during hypoxia, and are dependent on internal calcium stores for activation.

These findings are essential steps in understanding the contribution of the tripartite purinergic signalling system in the secondary hypoxic respiratory depression, and are clinically relevant to conditions such as apnea of prematurity. Identifying the relative contributions of various cellular mediators and activated ion channels will greatly impact the

development of pharmacological therapies, and yield insight into the mechanisms by which rhythm generating networks can be modulated.

Appendix

A.1 Hypoxia experiments in spontaneously breathing rats

As a first approach to determining the role of $P2Y_1$ receptors during hypoxia, MRS 2279 was stereotaxically injected into the preBötC of anesthetized, vagotomized, non-paralyzed, spontaneously breathing adult rats while measuring airflow and diaphragmatic and genioglossus EMGs. These experiments revealed a significant increase in secondary depression following the injection of MRS 2279 (n=6). However, I encountered a number of difficulties during these experiments, including

(i) adequate ventilation while measuring airflow through a pneumotachometer which extended the trachea tube, causing airflow resistance that became life threatening during acute hypoxia, and

(ii) confounding effects of central respiratory drive on blood gases due to compensatory changes in ventilation.

As a result, these experiments became meaningless in the context of the questions under investigation. For this reason I have not included them here. As a result, I modified my approach to paralyzed, ventilated animals, so that I could preserve animal health and open chemoreflex loops so that changes in respiratory drive would not affect blood gases.

A.2 Optogenetic activation of preBötC astrocytes

Establishing the appropriate protocol for viral transfection is key to the successful expression of proteins to modulate cell behaviour. However, inclusion of such information in publications is not standard. I am therefore including that information in this appendix.

In addition to viral experiments presented in this thesis, through a collaboration with Dr. Sergey Kasparov (University of Bristol, UK), I devoted significant time to the development of vectors for the optogenetic manipulation of astrocytes. An initial objective of my thesis was to use these tools to test the effects of selectively driving astrocytes in the preBötC on respiratory behaviour. I felt that the potential for novel information with this approach justified the time invested. There were no tools, and still are no effective tools for

the selective activation of astrocytes in a physiological manner to explore their functional significance. Although these efforts were unsuccessful, I am documenting the constructs tested and experiments performed in this appendix.

Table 1: Constructs used to characterize optogenetic activation of astrocytes

Construct	Purpose	Number of Animals
AVV - sGFAP - optoβ_2 - eYFP	protocol development	2
AVV - sGFAP - optoβ_2 - eYFP	functional tests	4
AVV - sGFAP - hChR2(H134R) - mKate2	protocol development	4
AVV - sGFAP - hChR2(H134R) - mKate2	functional tests	14
AVV - sGFAP - CatCh - eYFP	protocol development	4
AVV - sGFAP - CatCh - eYFP	functional tests	18

A.3 Characterization of second messenger signalling cascades

Experiments designed to dissect the second messenger cascades were more extensive than indicated in this thesis. The limitation is that because so little is known in our system about second messenger signalling, it is difficult to design a positive control. Many of the drugs I tried produced no effect. However, these drugs typically work from inside cells so diffusion is an issue. They are also very insoluble. Thus a negative effect reveals nothing without a positive control. My focus in developing the thesis was on the information that was instructive and insightful. Thus, I have not included the data from the in vitro experiments with second messenger blockers that had no effect. I list them here to document that these compounds have no effect under the in vitro conditions used here.

Table 2: Second messenger blockers without effect on responses evoked by MRS 2365 or ATP into the preBötC of rhythmic slices

Drug	Target	Application	Experimental Issue	Number of Attempts
BAPTA-AM (1-10 μM)	$[Ca^{2+}]_i$ buffer	bath/local	A	10
U73122 (25 μM)	PLC	local	B	2
Xestospongin C (10 μM)	IP_3 receptor	local	B	5
GF109203X (100 μM)	PKCα, βI	local	B, C	3
CGP53353 (100-500 μM)	PKCβII	local	B, C	6
Gö6976 (10-100 μM)	PKCα, βI	bath/local	B, C	5
Ro 32-0432 Hydrochloride (100 μM)	PKCα, βI, βII, γ, ε	local	B	3

A. Agent disrupted baseline rhythm
B. Lack of positive control; no way to assess that the agent diffused successfully into the cells within the slice
C. Solubility; agent precipitated in solution upon application

References

(2014). Kick the bar chart habit. *Nat Methods* **11,** 113.

Abbracchio MP, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA & Burnstock G. (2003). Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* **24,** 52-55.

Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA & Weisman GA. (2006). International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacological reviews* **58,** 281-341.

Abe Y, Terazono Y, Hiroyuki O & Kato F. (2013). Involvement of glial P2Y1 receptors in cognitive deficit after focal cerebral stroke in a rodent model. *Journal of Neuroinflammation* **10,** 95.

Accorsi-Mendonça D, Zoccal DB, Bonagamba LGH & Machado BH. (2013). Glial cells modulate the synaptic transmission of NTS neurons sending projections to ventral medulla of Wistar rats. *Physiol Rep* **1,** e00080.

Alheid GF & McCrimmon DR. (2008). The chemical neuroanatomy of breathing. *Respir Physiol Neurobiol* **164,** 3-11.

Alvares TS, Revill AL, Huxtable AG, Lorenz CD & Funk GD. (2014). P2Y 1receptor-mediated potentiation of inspiratory motor output in neonatal rat in vitro. *The Journal of Physiology* **592,** 3089-3111.

Angelova PR, Kasymov V, Christie I, Sheikhbahaei S, Turovsky E, Marina N, Korsak A, Zwicker J, Teschemacher AG, Ackland GL, Funk GD, Kasparov S, Abramov AY & Gourine AV. (2015). Functional Oxygen Sensitivity of Astrocytes. *J Neurosci* **35,** 10460-10473.

Aoki Y, Yamada E, Endoh T & Suzuki T. (2004). Multiple actions of extracellular ATP and adenosine on calcium currents mediated by various purinoceptors in neurons of nucleus tractus solitarius. *Neurosci Res* **50,** 245-255.

Attwell D, Buchan AM, Charpak S, Lauritzen M, MacVicar BA & Newman EA. (2010). Glial and neuronal control of brain blood flow. *Nature* **468,** 232-243.

Beech DJ, Bernheim L & Hille B. (1992). Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. *Neuron* **8,** 97-106.

Ben-Mabrouk F, Amos LB & Tryba AK. (2012). Metabotropic glutamate receptors (mGluR5) activate transient receptor potential canonical channels to improve the regularity of the respiratory rhythm generated by the pre-Botzinger complex in mice. *Eur J Neurosci* **35,** 1725-1737.

Ben-Mabrouk F & Tryba AK. (2010). Substance P modulation of TRPC3/7 channels improves respiratory rhythm regularity and ICAN-dependent pacemaker activity. *Eur J Neurosci* **31,** 1219-1232.

Biber K, Neumann H, Inoue K & Boddeke HW. (2007). Neuronal 'On' and 'Off' signals control microglia. *Trends Neurosci* **30,** 596-602.

Bisgard GE, Forster HV & Klein JP. (1980). Recovery of peripheral chemoreceptor function after denervation in ponies. *J Appl Physiol Respir Environ Exerc Physiol* **49,** 964-970.

Bito H, Deisseroth K & Tsien RW. (1996). CREB phosphorylation and dephosphorylation: a Ca 2+-and stimulus duration–dependent switch for hippocampal gene expression. *Cell* **87,** 1203-1214.

Blum AS. (2009). Respiratory Physiology of Seizures. *J Clin Neurophysiol* **26,** 309-315.

Bowser DN & Khakh BS. (2007). Vesicular ATP is the predominant cause of intercellular calcium waves in astrocytes. *J Gen Physiol* **129,** 485-491.

Boyer JL, Adams M, Ravi RG, Jacobson KA & Harden TK. (2002). 2-Chloro N(6)-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate is a selective high affinity P2Y(1) receptor antagonist. *Br J Pharmacol* **135,** 2004-2010.

Brown DA & Adams PR. (1980). Muscarinic suppression of a novel voltage-sensitive K+ current in a vertebrate neurone. *Nature* **283,** 673-676.

Brown DA, Filippov AK & Barnard EA. (2000a). Inhibition of potassium and calcium currents in neurones by molecularly-defined P2Y receptors. *Journal of the Autonomic Nervous System* **81,** 31-36.

Brown DA & Passmore GM. (2009). Neural KCNQ (Kv7) channels. *Br J Pharmacol* **156,** 1185-1195.

Brown SG, King BF, Kim Y-C, Jang SY, Burnstock G & Jacobson KA. (2000b). Activity of novel adenine nucleotide derivatives as agonists and antagonists at recombinant rat P2X receptors. *Drug Dev Res* **49,** 253-259.

Brown TG. (1914). On the nature of the fundamental activity of the nervous centres; together with an analysis of the conditioning of rhythmic activity in progression, and a theory of the evolution of function in the nervous system. *The Journal of Physiology* **48,** 18-46.

Burns BD. (1963). The central control of respiratory movements. *Br Med Bull* **19,** 7-9.

Burnstock G. (1986). Autonomic neuromuscular junctions: current developments and future directions. *J Anat* **146,** 1-30.

Burnstock G. (2007). Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87,** 659-797.

Burnstock G, Dumsday B & Smythe A. (1972). Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br J Pharmacol* **44,** 451-461.

Burnstock G, Fredholm BB & Verkhratsky A. (2011). Adenosine and ATP receptors in the brain. *Curr Top Med Chem* **11,** 973-1011.

Büsselberg D, Bischoff AM & Richter DW. (2003). A combined blockade of glycine and calcium-dependent potassium channels abolishes the respiratory rhythm. *Neuroscience* **122,** 831-841.

Cao Y & Song G. (2007). Purinergic modulation of respiration via medullary raphe nuclei in rats. *Respiratory Physiology & Neurobiology* **155,** 114-120.

Carroll MS, Viemari JC & Ramirez JM. (2013). Patterns of inspiratory phase-dependent activity in the in vitro respiratory network. *Journal of Neurophysiology* **109,** 285-295.

Catterall WA. (2011). Voltage-Gated Calcium Channels. *Cold Spring Harbor Perspectives in Biology* **3**.

Chandaka GK, Salzer I, Drobny H, Boehm S & Schicker KW. (2011). Facilitation of transmitter release from rat sympathetic neurons via presynaptic P2Y(1) receptors. *Br J Pharmacol* **164,** 1522-1533.

Chen X, Talley EM, Patel N & Gomis A. (2006). Inhibition of a background potassium channel by Gq protein α-subunits. *Proc Natl Acad Sci U S A* **103,** 3422-3427.

Coppi E, Pedata F & Gibb AJ. (2012). P2Y1 receptor modulation of Ca2+-activated K+ currents in medium-sized neurons from neonatal rat striatal slices. *Journal of Neurophysiology* **107,** 1009-1021.

Crowder EA, Saha MS, Pace RW, Zhang H, Prestwich GD & Del Negro CA. (2007). Phosphatidylinositol 4,5-bisphosphate regulates inspiratory burst activity in the neonatal mouse preBotzinger complex. *J Physiol* **582,** 1047-1058.

Czirják G, Petheő GL & Spät A. (2001). Inhibition of TASK-1 potassium channel by phospholipase C. *Am J Physiol Cell Physiol* **281,** C700-C708.

da Silva GSF, Moraes DJA, Giusti H, Dias MB & Glass ML. (2012). Purinergic transmission in the rostral but not caudal medullary raphe contributes to the hypercapnia-induced ventilatory response in unanesthetized rats. *Respir Physiol Neurobiol* **184,** 41-47.

Dale N & Frenguelli BG. (2011). Measurement of purine release with microelectrode biosensors. *Purinergic Signalling* **8,** 27-40.

Davenport HW, Brewer G & et al. (1947). The respiratory responses to anoxemia of unanesthetized dogs with chronically denervated aortic and carotid chemoreceptors and their causes. *Am J Physiol* **148,** 406-416.

Del Negro CA. (2002). Persistent Sodium Current, Membrane Properties and Bursting Behavior of Pre-Botzinger Complex Inspiratory Neurons In Vitro. *Journal of Neurophysiology* **88,** 2242-2250.

Del Negro CA. (2005). Sodium and Calcium Current-Mediated Pacemaker Neurons and Respiratory Rhythm Generation. *Journal of Neuroscience* **25,** 446-453.

Del Negro CA, Hayes JA, Pace RW, Brush BR, Teruyama R & Feldman JL. (2010). Synaptically activated burst-generating conductances may underlie a group-pacemaker mechanism for respiratory rhythm generation in mammals. *Prog Brain Res* **187,** 111-136.

Dobbins EG & Feldman JL. (1994). Brainstem network controlling descending drive to phrenic motoneurons in rat. *J Comp Neurol* **347,** 64-86.

Dutschmann M & Herbert H. (2006). The Kölliker-Fuse nucleus gates the postinspiratory phase of the respiratory cycle to control inspiratory off-switch and upper airway resistance in rat. *Eur J Neurosci* **24,** 1071-1084.

Endoh T. (2004). Extracellular ATP both inhibits and facilitates calcium channel currents in acutely dissociated rat nucleus tractus solitarius. *Bull Tokyo Dent Coll* **45,** 59-63.

Ergene E, Dunbar JC, O'Leary DS & Barraco RA. (1994). Activation of P2-purinoceptors in the nucleus tractus solitarius mediate depressor responses. *Neurosci Lett* **174,** 188-192.

Feldman JL & Del Negro CA. (2006). Looking for inspiration: new perspectives on respiratory rhythm. *Nat Rev Neurosci* **7,** 232-241.

Feldman JL & Smith JC. (1989). Cellular mechanisms underlying modulation of breathing pattern in mammals. *Ann N Y Acad Sci* **563,** 114-130.

Feldman JL, Smith JC, Ellenberger HH, Connelly CA, Liu GS, Greer JJ, Lindsay AD & Otto MR. (1990). Neurogenesis of respiratory rhythm and pattern: emerging concepts. *Am J Physiol* **259,** R879-886.

Filippov AK, Brown DA & Barnard EA. (2000a). The P2Y1 receptor closes the N-type Ca2+ channel in neurones, with both adenosine triphosphates and diphosphates as potent agonists. *Br J Pharmacol* **129,** 1063-1066.

Filippov AK, Brown DA & Barnard EA. (2000b). The P2Y1 receptor closes the N-type Ca2+ channel in neurones, with both adenosine triphosphates and diphosphates as potent agonists. *British Journal of Pharmacology* **129,** 1063-1066.

Filippov AK, Choi RCY, Simon J, Barnard EA & Brown DA. (2006). Activation of P2Y1 nucleotide receptors induces inhibition of the M-type K+ current in rat hippocampal pyramidal neurons. *Journal of Neuroscience* **26,** 9340-9348.

Fong AY, Krstew EV, Barden J & Lawrence AJ. (2002a). Immunoreactive localisation of P2Y1 receptors within the rat and human nodose ganglia and rat brainstem: comparison with [alpha 33P]deoxyadenosine 5'-triphosphate autoradiography. *Neuroscience* **113,** 809-823.

Fong DK, Rao A & Crump FT. (2002b). Rapid synaptic remodeling by protein kinase C: reciprocal translocation of NMDA receptors and calcium/calmodulin-dependent kinase II. *J Neurosci* **22,** 2153-2164.

Frappell P, Lanthier C, Baudinette RV & Mortola JP. (1992). Metabolism and ventilation in acute hypoxia: a comparative analysis in small mammalian species. *Am J Physiol* **262,** R1040-1046.

Fredholm BB, AP IJ, Jacobson KA, Klotz KN & Linden J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacological reviews* **53,** 527-552.

Fukuda K, Higashida H, Kubo T, Maeda A, Akiba I, Bujo H, Mishina M & Numa S. (1988). Selective coupling with K+ currents of muscarinic acetylcholine receptor subtypes in NG108-15 cells. *Nature* **335,** 355-358.

Funk G, Huxtable A & Lorier A. (2008). ATP in central respiratory control: A three-part signaling system. *Respiratory Physiology & Neurobiology* **164,** 131-142.

Funk GD. (2009). Losing sleep over the caffeination of prematurity. *The Journal of Physiology* **587,** 5299-5300.

Funk GD. (2013). Neuromodulation: Purinergic Signaling in Respiratory Control. *Comprehensive Physiology* **3,** 331-363.

Funk GD & Greer JJ. (2013). The rhythmic, transverse medullary slice preparation in respiratory neurobiology: Contributions and caveats. *Respiratory Physiology & Neurobiology* **186,** 236-253.

Funk GD, Kanjhan R, Walsh C, Lipski J, Comer AM, Parkis MA & Housley GD. (1997). P2 receptor excitation of rodent hypoglossal motoneuron activity in vitro and in vivo: a molecular physiological analysis. *J Neurosci* **17,** 6325-6337.

Funk GD, Rajani V, Alvares TS, Revill AL, Zhang Y, Chu NY, Biancardi V, Linhares-Taxini C, Katzell A & Reklow R. (2015). Neuroglia and their roles in central respiratory control; an overview. *Comp Biochem Physiol, Part A Mol Integr Physiol*.

Funk GD, Zwicker JD, Selvaratnam R & Robinson DM. (2011). Noradrenergic modulation of hypoglossal motoneuron excitability: developmental and putative state-dependent mechanisms. *Archives italiennes de biologie* **149,** 426-453.

Gil V, Martinez-Cutillas M, Mane N, Martin MT, Jimenez M & Gallego D. (2013). P2Y(1) knockout mice lack purinergic neuromuscular transmission in the antrum and cecum. *Neurogastroenterol Motil* **25,** e170-182.

Goncalves J & Queiroz G. (2008). Presynaptic adenosine and P2Y receptors. *Handb Exp Pharmacol***,** 339-372.

Gourine AV, Dale N, Korsak A, Llaudet E, Tian F, Huckstepp R & Spyer KM. (2008). Release of ATP and glutamate in the nucleus tractus solitarii mediate pulmonary stretch receptor (Breuer-Hering) reflex pathway. *The Journal of Physiology* **586,** 3963-3978.

Gourine AV, Kasymov V, Marina N, Tang F, Figueiredo MF, Lane S, Teschemacher AG, Spyer KM, Deisseroth K & Kasparov S. (2010). Astrocytes Control Breathing Through pH-Dependent Release of ATP. *Science* **329,** 571-575.

Gourine AV, Llaudet E, Dale N & Spyer KM. (2005a). ATP is a mediator of chemosensory transduction in the central nervous system. *Nature* **436,** 108-111.

Gourine AV, Llaudet E, Dale N & Spyer KM. (2005b). Release of ATP in the ventral medulla during hypoxia in rats: role in hypoxic ventilatory response. *Journal of Neuroscience* **25,** 1211-1218.

Gray PA, Hayes JA, Ling GY, Llona I, Tupal S, Picardo MCD, Ross SE, Hirata T, Corbin JG, Eugenin J & Del Negro CA. (2010). Developmental Origin of PreBotzinger Complex Respiratory Neurons. *Journal of Neuroscience* **30,** 14883-14895.

Gray PA, Janczewski WA, Mellen N, McCrimmon DR & Feldman JL. (2001). Normal breathing requires preBötzinger complex neurokinin-1 receptor-expressing neurons. *Nat Neurosci* **4,** 927-930.

Gray PA, Rekling JC, Bocchiaro CM & Feldman JL. (1999). Modulation of respiratory frequency by peptidergic input to rhythmogenic neurons in the preBötzinger complex. *Science* **286,** 1566-1568.

Guinamard R, Salle L & Simard C. (2011). The non-selective monovalent cationic channels TRPM4 and TRPM5. *Advances in experimental medicine and biology* **704,** 147-171.

Guyenet PG, Sevigny CP, Weston MC & Stornetta RL. (2002). Neurokinin-1 Receptor-Expressing Cells of the Ventral Respiratory Group Are Functionally Heterogeneous and Predominantly Glutamatergic. *The Journal of Neuroscience* **22,** 3806-3816.

Guyenet PG, Stornetta RL & Bayliss DA. (2008). Retrotrapezoid nucleus and central chemoreception. *The Journal of Physiology* **586,** 2043-2048.

Guyenet PG, Stornetta RL & Bayliss DA. (2010). Central respiratory chemoreception. *J Comp Neurol* **518,** 3883-3906.

Guyenet PG & Wang H. (2001). Pre-Bötzinger Neurons With Preinspiratory Discharges "In Vivo" Express NK1 Receptors in the Rat. *Journal of Neurophysiology* **86,** 438-446.

Hanisch UK & Kettenmann H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* **10,** 1387-1394.

Hechler B, Freund M, Ravanat C, Magnenat S, Cazenave JP & Gachet C. (2008). Reduced atherosclerotic lesions in P2Y1/apolipoprotein E double-knockout mice: the contribution of non-hematopoietic-derived P2Y1 receptors. *Circulation* **118,** 754-763.

Herlenius E & Lagercrantz H. (1999). Adenosinergic modulation of respiratory neurones in the neonatal rat brainstem in vitro. *J Physiol* **518,** 159-172.

Herlenius E, Lagercrantz H & Yamamoto Y. (1997). Adenosine modulates inspiratory neurons and the respiratory pattern in the brainstem of neonatal rats. *Pediatr Res* **42,** 46-53.

Herlitze S, Garcia DE, Mackie K, Hille B & Scheuer T. (1996). Modulation of Ca2+ channels by G-protein ßy subunits. *Nature* **380,** 258-262.

Hernandez CC, Zaika O, Tolstykh GP & Shapiro MS. (2008). Regulation of neural KCNQ channels: signalling pathways, structural motifs and functional implications. *J Physiol* **586,** 1811-1821.

Hu JY, Chen Y & Schacher S. (2007). Protein kinase C regulates local synthesis and secretion of a neuropeptide required for activity-dependent long-term synaptic plasticity. *J Neurosci* **27,** 8927-8939.

Huckstepp RTR, id Bihi R, Eason R, Spyer KM, Dicke N, Willecke K, Marina N, Gourine AV & Dale N. (2010). Connexin hemichannel-mediated CO2-dependent release of ATP in the medulla oblongata contributes to central respiratory chemosensitivity. *The Journal of Physiology* **588,** 3901-3920.

Huxtable AG, Zwicker JD, Alvares TS, Ruangkittisakul A, Fang X, Hahn LB, Posse de Chaves E, Baker GB, Ballanyi K & Funk GD. (2010). Glia Contribute to the Purinergic Modulation of Inspiratory Rhythm-Generating Networks. *J Neurosci* **30,** 3947-3958.

Huxtable AG, Zwicker JD, Poon BY, Pagliardini S, Vrouwe SQ, Greer JJ & Funk GD. (2009). Tripartite Purinergic Modulation of Central Respiratory Networks during Perinatal Development: The Influence of ATP, Ectonucleotidases, and ATP Metabolites. *Journal of Neuroscience* **29,** 14713-14725.

Iceman KE, Richerson GB & Harris MB. (2013). Medullary serotonin neurons are CO2 sensitive in situ. *J Neurophysiol* **110,** 2536-2544.

Ikeda SR. (1996). Voltage-dependent modulation of N-type calcium channels by G-protein β γsubunits. *Nature* **380,** 255-258.

Iscoe S. (1998). Control of abdominal muscles. *Prog Neurobiol* **56,** 433-506.

Jacobson KA & Knutsen LJS. (2001). P1 and P2 purine and pyrimidine receptor ligands. In *Purinergic and Pyrimidinergic Signalling I: Molecular, Nervous and Urogenitary System Function*, ed. P AM & M W. Springer.

Janczewski WA, Tashima A, Hsu P, Cui Y & Feldman JL. (2013). Role of inhibition in respiratory pattern generation. *Journal of Neuroscience* **33,** 5454-5465.

Kam K, Worrell JW, Janczewski WA, Cui Y & Feldman JL. (2013a). Distinct inspiratory rhythm and pattern generating mechanisms in the preBötzinger complex. *Journal of Neuroscience* **33,** 9235-9245.

Kam K, Worrell JW, Ventalon C, Emiliani V & Feldman JL. (2013b). Emergence of Population Bursts from Simultaneous Activation of Small Subsets of preBotzinger Complex Inspiratory Neurons. *Journal of Neuroscience* **33,** 3332-3338.

Kimelberg HK, Cai Z, Rastogi P, Charniga CJ, Goderie S, Dave V & Jalonen TO. (1997). Transmitter-induced calcium responses differ in astrocytes acutely isolated from rat brain and in culture. *J Neurochem* **68,** 1088-1098.

King BF, Ziganshina LE, Pintor J & Burnstock G. (1996). Full sensitivity of P2X2 purinoceptor to ATP revealed by changing extracellular pH. *Br J Pharmacol* **117,** 1371-1373.

Koch H, Zanella S, Elsen GE, Smith L, Doi A, Garcia AJ, 3rd, Wei AD, Xun R, Kirsch S, Gomez CM, Hevner RF & Ramirez JM. (2013). Stable Respiratory Activity Requires Both P/Q-Type and N-Type Voltage-Gated Calcium Channels. *J Neurosci* **33,** 3633-3645.

Koizumi H, Smerin SE & Yamanishi T. (2010). TASK channels contribute to the K+-dominated leak current regulating respiratory rhythm generation in vitro. *J Neurosci* **30,** 4273-4284.

Koizumi H & Smith JC. (2008). Persistent Na+ and K+-dominated leak currents contribute to respiratory rhythm generation in the pre-Bötzinger complex in vitro. *Journal of Neuroscience* **28,** 1773-1785.

Krishek BJ, Xie X, Blackstone C, Huganir RL & Moss SJ. (1994). Regulation of GABA A receptor function by protein kinase C phosphorylation. *Neuron* **12,** 1081-1095.

Kubin L, Alheid GF, Zuperku EJ & McCrimmon DR. (2006). Central pathways of pulmonary and lower airway vagal afferents. *J Appl Physiol* **101,** 618-627.

Lan J, Skeberdis VA, Jover T, Grooms SY & Lin Y. (2001). Protein kinase C modulates NMDA receptor trafficking and gating. *Nat Neurosci* **4,** 382-390.

Lawrence AJ & Jarrott B. (1996). Neurochemical modulation of cardiovascular control in the nucleus tractus solitarius. *Prog Neurobiol* **48,** 21-53.

Lee Y, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, Liu Y, Tsingalia A, Jin L, Zhang PW, Pellerin L, Magistretti PJ & Rothstein JD. (2012). Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* **487,** 443-448.

Leon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, Dierich A, LeMeur M, Cazenave JP & Gachet C. (1999). Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest* **104,** 1731-1737.

Lieske SP & Ramirez JM. (2006). Pattern-Specific Synaptic Mechanisms in a Multifunctional Network. I. Effects of Alterations in Synapse Strength. *J Neurophysiol* **95,** 1323-1333.

Lipski J & Merrill EG. (1980). Electrophysiological demonstration of the projection from expiratory neurones in rostral medulla to contralateral dorsal respiratory group. *Brain Res* **197,** 521-524.

Liu B, Paton JF & Kasparov S. (2008). Viral vectors based on bidirectional cell-specific mammalian promoters and transcriptional amplification strategy for use in vitro and in vivo. *BMC Biotechnol* **8,** 49.

Liu G, Feldman JL & Smith JC. (1990). Excitatory amino acid-mediated transmission of inspiratory drive to phrenic motoneurons. *Journal of Neurophysiology* **64,** 423-436.

Lopes C, Rohács T, Czirják G & Balla T. (2005). PIP2 hydrolysis underlies agonist-induced inhibition and regulates voltage gating of two-pore domain K+ channels. *J Physiol (Lond)* **564,** 117-129.

Lorier AR, Huxtable AG, Robinson DM, Lipski J, Housley GD & Funk GD. (2007). P2Y1 Receptor Modulation of the Pre-Botzinger Complex Inspiratory Rhythm Generating Network In Vitro. *J Neurosci* **27,** 993-1005.

Lorier AR, Lipski J, Housley GD, Greer JJ & Funk GD. (2008). ATP sensitivity of preBötzinger complex neurones in neonatal rat in vitro: mechanism underlying a P2 receptor-mediated increase in inspiratory frequency. *The Journal of Physiology* **586,** 1429-1446.

Lorier AR, Peebles K, Brosenitsch T, Robinson DM, Housley GD & Funk GD. (2004). P2 receptors modulate respiratory rhythm but do not contribute to central CO2 sensitivity in vitro. *Respir Physiol Neurobiol* **142,** 27-42.

Marrion NV. (1997). Control of M-current. *Annu Rev Physiol* **59,** 483-504.

Martin-Body RL, Robson GJ & Sinclair JD. (1985). Respiratory effects of sectioning the carotid sinus glossopharyngeal and abdominal vagal nerves in the awake rat. *J Physiol* **361,** 35-45.

Mathie A. (2007). Neuronal two-pore-domain potassium channels and their regulation by G protein-coupled receptors. *J Physiol (Lond)* **578,** 377-385.

Mathie A, Bernheim L & Hille B. (1992). Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. *Neuron* **8,** 907-914.

McKay LC & Feldman JL. (2008). Unilateral ablation of pre-Botzinger complex disrupts breathing during sleep but not wakefulness. *Am J Respir Crit Care Med* **178,** 89-95.

Meigh L, Greenhalgh SA, Rodgers TL, Cann MJ, Roper DI & Dale N. (2013). CO2 directly modulates connexin 26 by formation of carbamate bridges between subunits. *Elife* **2,** e01213.

Mellen NM, Janczewski WA, Bocchiaro CM & Feldman JL. (2003). Opioid-induced quantal slowing reveals dual networks for respiratory rhythm generation. *Neuron* **37,** 821-826.

Miller MJ & Tenney SM. (1975). Hypoxia-induced tachypnea in carotid-deafferented cats. *Respir Physiol* **23,** 31-39.

Mironov SL. (2008). Metabotropic glutamate receptors activate dendritic calcium waves and TRPM channels which drive rhythmic respiratory patterns in mice. *J Physiol* **586,** 2277-2291.

Mironov SL & Richter DW. (2000). Hypoxic modulation of L-type Ca 2+ channels in inspiratory brainstem neurones: intracellular signalling pathways and metabotropic glutamate receptors. *Brain Res* **869,** 166-177.

Monnier A, Alheid GF & McCrimmon DR. (2003). Defining ventral medullary respiratory compartments with a glutamate receptor agonist in the rat. *J Physiol* **548,** 859-874.

Montana V, Malarkey EB, Verderio C, Matteoli M & Parpura V. (2006). Vesicular transmitter release from astrocytes. *Glia* **54,** 700-715.

Morgado-Valle C, Beltran-Parrazal L, DiFranco M, Vergara JL & Feldman JL. (2008). Somatic Ca2+ transients do not contribute to inspiratory drive in preBötzinger Complex neurons. *J Physiol* **586,** 4531-4540.

Moss IR. (2000). Respiratory responses to single and episodic hypoxia during development: mechanisms of adaptation. *Respir Physiol* **121,** 185-197.

Mulkey DK, Mistry AM, Guyenet PG & Bayliss DA. (2006). Purinergic P2 receptors modulate excitability but do not mediate pH sensitivity of RTN respiratory chemoreceptors. *Journal of Neuroscience* **26,** 7230-7233.

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA & Guyenet PG. (2004). Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat Neurosci* **7,** 1360-1369.

Mulkey DK & Wenker IC. (2011). Astrocyte chemoreceptors: mechanisms of H+ sensing by astrocytes in the retrotrapezoid nucleus and their possible contribution to respiratory drive. *Experimental physiology* **96,** 400-406.

Murali S & Nurse CA. (2015). Purinergic signaling mediates bidirectional crosstalk between chemoreceptor type I and glial-like type II cells of the rat carotid body. *J Physiol*. In press.

Nattie E & Li A. (2009). Central chemoreception is a complex system function that involves multiple brain stem sites. *J Appl Physiol* **106,** 1464-1466.

Neubauer JA & Sunderram J. (2004). Oxygen-sensing neurons in the central nervous system. *J Appl Physiol (1985)* **96,** 367-374.

North RA. (2002). Molecular physiology of P2X receptors. *Physiol Rev* **82,** 1013-1067.

Nurse CA. (2014). Synaptic and paracrine mechanisms at carotid body arterial chemoreceptors. *The Journal of Physiology* **592,** 3419-3426.

Oberheim NA, Goldman SA & Nedergaard M. (2012). Heterogeneity of astrocytic form and function. *Methods Mol Biol* **814,** 23-45.

Onimaru H, Ballanyi K & Homma I. (2003). Contribution of Ca2+-dependent conductances to membrane potential fluctuations of medullary respiratory neurons of newborn rats in vitro. *J Physiol* **552,** 727-741.

Onimaru H, Ballanyi K & Richter DW. (1996). Calcium-dependent responses in neurons of the isolated respiratory network of newborn rats. *J Physiol* **491,** 677-695.

Onimaru H & Homma I. (2003). A novel functional neuron group for respiratory rhythm generation in the ventral medulla. *J Neurosci* **23,** 1478-1486.

Pace RW & Del Negro CA. (2008). AMPA and metabotropic glutamate receptors cooperatively generate inspiratory-like depolarization in mouse respiratory neurons in vitro. *European Journal of Neuroscience* **28,** 2434-2442.

Pace RW, Mackay DD, Feldman JL & Del Negro CA. (2007a). Inspiratory bursts in the preBotzinger complex depend on a calcium-activated non-specific cation current linked to glutamate receptors in neonatal mice. *J Physiol* **582,** 113-125.

Pace RW, Mackay DD, Feldman JL & Del Negro CA. (2007b). Role of persistent sodium current in mouse preBotzinger Complex neurons and respiratory rhythm generation. *J Physiol* **580,** 485-496.

Parkhurst CN & Gan WB. (2010). Microglia dynamics and function in the CNS. *Curr Opin Neurobiol* **20,** 595-600.

Parpura V & Verkhratsky A. (2011). The astrocyte excitability brief: From receptors to gliotransmission. *Neurochemistry international*. **64,** 610-621.

Parpura V & Verkhratsky A. (2012). Homeostatic function of astrocytes: Ca(2+) and Na(+) signalling. *Transl Neurosci* **3,** 334-344.

Parpura V & Zorec R. (2010). Gliotransmission: Exocytotic release from astrocytes. *Brain Research Reviews* **63,** 83-92.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K & Haydon PG. (2005). Astrocytic purinergic signaling coordinates synaptic networks. *Science* **310,** 113-116.

Paxinos G & Watson C. (2007). *The rat brain in stereotaxic coordinates*. Academic Press/Elsevier, Amsterdam. Boston.

Pena F, Parkis MA, Tryba AK & Ramirez JM. (2004). Differential contribution of pacemaker properties to the generation of respiratory rhythms during normoxia and hypoxia. *Neuron* **43,** 105-117.

Perez-Burgos A, Prieto GA, Galarraga E & Bargas J. (2010). CaV2.1 channels are modulated by muscarinic M1 receptors through phosphoinositide hydrolysis in neostriatal neurons. *Neuroscience* **165,** 293-299.

Prabhakar NR. (2000). Oxygen sensing by the carotid body chemoreceptors. *Journal of Applied Physiology* **88,** 2287-2295.

Prasad M, Fearon IM, Zhang M, Laing M, Vollmer C & Nurse CA. (2001). Expression of P2X2 and P2X3 receptor subunits in rat carotid body afferent neurones: role in chemosensory signalling. *J Physiol* **537,** 667-677.

Putnam RW, Conrad SC, Gdovin MJ, Erlichman JS & Leiter JC. (2005). Neonatal maturation of the hypercapnic ventilatory response and central neural CO2 chemosensitivity. *Respiratory Physiology & Neurobiology* **149,** 165-179.

Rajani V, Zhang Y, Revill AL & Funk GD. (2015). The role of P2Y receptor signaling in central respiratory control. *Respir Physiol Neurobiol***,** In press.

Ramirez JM, Quellmalz UJ & Richter DW. (1996). Postnatal changes in the mammalian respiratory network as revealed by the transverse brainstem slice of mice. *The Journal of physiology* **491 (Pt 3),** 799-812.

Rekling JC & Feldman JL. (1998). PreBotzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. *Annu Rev Physiol* **60,** 385-405.

Richter DW. (1982). Generation and maintenance of the respiratory rhythm. *J Exp Biol* **100,** 93-107.

Richter DW, Ballantyne D & Remmers JE. (1986). How Is the Respiratory Rhythm Generated? A Model. *Physiology* **1**, 109-112.

Robberecht W & Philips T. (2013). The changing scene of amyotrophic lateral sclerosis. *Nat Rev Neurosci* **14,** 248-264.

Rong W, Gourine AV, Cockayne DA, Xiang Z, Ford APDW, Spyer KM & Burnstock G. (2003). Pivotal role of nucleotide P2X2 receptor subunit of the ATP-gated ion channel mediating ventilatory responses to hypoxia. *Journal of Neuroscience* **23,** 11315-11321.

Ropert N, Jalil A & Li D. (2015). Expression and cellular function of vSNARE proteins in brain astrocytes. *Neuroscience*. **323**, 76-83.

Ruangkittisakul A, Schwarzacher SW, Secchia L, Ma Y, Bobocea N, Poon BY, Funk GD & Ballanyi K. (2008). Generation of eupnea and sighs by a spatiochemically organized inspiratory network. *J Neurosci* **28,** 2447-2458.

Ruangkittisakul A, Schwarzacher SW, Secchia L, Poon BY, Ma Y, Funk GD & Ballanyi K. (2006). High sensitivity to neuromodulator-activated signaling pathways at physiological [K+] of confocally imaged respiratory center neurons in on-line-calibrated newborn rat brainstem slices. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **26,** 11870-11880.

Sah P & Faber ES. (2002). Channels underlying neuronal calcium-activated potassium currents. *Prog Neurobiol* **66,** 345-353.

Sak K & Illes P. (2005). Neuronal and glial cell lines as model systems for studying P2Y receptor pharmacology. *Neurochemistry international* **47,** 401-412.

Sanders KM, Ward SM & Koh SD. (2014). Interstitial Cells: Regulators of Smooth Muscle Function. *Physiol Rev* **94,** 859-907.

Schicker KW, Chandaka GK, Geier P, Kubista H & Boehm S. (2010). P2Y 1receptors mediate an activation of neuronal calcium-dependent K +channels. *J Physiol* **588,** 3713-3725.

Schwarzacher SW, Rub U & Deller T. (2010). Neuroanatomical characteristics of the human pre-Botzinger complex and its involvement in neurodegenerative brainstem diseases. *Brain* **134,** 24-35.

Shao XM & Feldman JL. (1997). Respiratory Rhythm Generation and Synaptic Inhibition of Expiratory Neurons in Pre-Bötzinger Complex: Differential Roles of Glycinergic and GABAergic Neural Transmission. *Journal of Neurophysiology* **77,** 1853-1860.

Shao XM & Feldman JL. (2000). Acetylcholine modulates respiratory pattern: effects mediated by M3-like receptors in preBötzinger complex inspiratory neurons. *J Neurophysiol* **83,** 1243-1252.

Shao XM & Feldman JL. (2005). Cholinergic neurotransmission in the preBotzinger Complex modulates excitability of inspiratory neurons and regulates respiratory rhythm. *Neuroscience* **130,** 1069-1081.

Simon J, Webb TE, King BF, Burnstock G & Barnard EA. (1995a). Characterisation of a recombinant P2Y purinoceptor. *European journal of pharmacology* **291,** 281-289.

Simon J, Webb TE, King BF, Burnstock G & Barnard EA. (1995b). Characterisation of a recombinant P2Y purinoceptor. *European Journal of Pharmacology: Molecular Pharmacology* **291,** 281-289.

Smith J, Ellenberger H, Ballanyi K & Richter D. (1991). Pre-Botzinger complex: a brainstem region that may generate respiratory rhythm in mammals. *Science*. **254**, 726-729.

Smith JC, Abdala APL, Borgmann A, Rybak IA & Paton JFR. (2013). Brainstem respiratory networks: building blocks and microcircuits. *Trends Neurosci*. **36**, 152-162.

Smith JC, Abdala APL, Koizumi H, Rybak IA & Paton JFR. (2007). Spatial and functional architecture of the mammalian brain stem respiratory network: a hierarchy of three oscillatory mechanisms. *Journal of Neurophysiology* **98,** 3370-3387.

Smith JC, Abdala APL, Rybak IA & Paton JFR. (2009). Structural and functional architecture of respiratory networks in the mammalian brainstem. *Philos Trans R Soc Lond, B, Biol Sci* **364,** 2577-2587.

Sobrinho CR, Wenker IC, Poss EM, Takakura AC, Moreira TS & Mulkey DK. (2014). Purinergic signaling contributes to chemoreception in the retrotrapezoid nucleus but not the nucleus of the solitary tract or medullary raphe. *The Journal of Physiology*. **592**, 1309-1323.

Solomon IC. (2000). Excitation of phrenic and sympathetic output during acute hypoxia: contribution of medullary oxygen detectors. *Respir Physiol* **121,** 101-117.

Solomon IC, Edelman NH & Neubauer JA. (1999). Patterns of phrenic motor output evoked by chemical stimulation of neurons located in the pre-Botzinger complex in vivo. *J Neurophysiol* **81,** 1150-1161.

Sorensen SC & Mines AH. (1970). Ventilatory responses to acute and chronic hypoxia in goats after sinus nerve section. *J Appl Physiol* **28,** 832-835.

Spiegelberg BD & Hamm HE. (2005). Gβγ binds histone deacetylase 5 (HDAC5) and inhibits its transcriptional co-repression activity. *Journal of Biological Chemistry* **280,** 41769-41776.

St-John WM & Leiter JC. (2002). Gasping is elicited by briefer hypoxia or ischemia following blockade of glycinergic transmission. *Respir Physiol Neurobiol* **133,** 167-171.

Stemkowski PL, Tse FW, Peuckmann V, Ford CP, Colmers WF & Smith PA. (2002). ATP-inhibition of M current in frog sympathetic neurons involves phospholipase C but not Ins P(3), Ca(2+), PKC, or Ras. *J Neurophysiol* **88,** 277-288.

Stornetta RL, Rosin DL & Wang H. (2003). A group of glutamatergic interneurons expressing high levels of both neurokinin-1 receptors and somatostatin identifies the region of the pre-Bötzinger complex. *Journal of Comparative Neurology* **455,** 499-512.

Suh BC & Hille B. (2002). Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron* **35,** 507-520.

Suzue T. (1984). Respiratory rhythm generation in the in vitro brain stem-spinal cord preparation of the neonatal rat. *The Journal of Physiology* **354,** 173-183.

Takahashi T & Momiyama A. (1993). Different types of calcium channels mediate central synaptic transmission. *Nature* **366,** 156-158.

Talley EM, Lei Q, Sirois JE & Bayliss DA. (2000). TASK-1, a two–pore domain K+ channel, is modulated by multiple neurotransmitters in motoneurons. *Neuron* **25,** 399-410.

Tan W, Janczewski WA, Yang P, Shao XM, Callaway EM & Feldman JL. (2008). Silencing preBötzinger complex somatostatin-expressing neurons induces persistent apnea in awake rat. *Nat Neurosci* **11,** 538-540.

Tang F, Lane S, Korsak A, Paton JFR, Gourine AV, Kasparov S & Teschemacher AG. (2014). Lactate-mediated glia-neuronal signalling in the mammalian brain. *Nat Commun* **5,** 3284.

Teppema LJ & Dahan A. (2010). The ventilatory response to hypoxia in mammals: mechanisms, measurement, and analysis. *Physiol Rev* **90,** 675-754.

Thoby-Brisson M & Ramirez JM. (2001). Identification of two types of inspiratory pacemaker neurons in the isolated respiratory neural network of mice. *Journal of Neurophysiology* **86,** 104-112.

Thomas T, Ralevic V, Bardini M, Burnstock G & Spyer KM. (2001). Evidence for the involvement of purinergic signalling in the control of respiration. *Neuroscience* **107,** 481-490.

Thomas T, Ralevic V, Gadd CA & Spyer KM. (1999). Central CO2 chemoreception: a mechanism involving P2 purinoceptors localized in the ventrolateral medulla of the anaesthetized rat. *The Journal of Physiology* **517 (Pt 3),** 899-905.

Thomas T & Spyer KM. (2000). ATP as a mediator of mammalian central CO2 chemoreception. *The Journal of Physiology* **523.2,** 441-447.

Treiman M, Caspersen C & Christensen SB. (1998). A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca(2+)-ATPases. *Trends Pharmacol Sci* **19,** 131-135.

Tryba AK & Ramirez JM. (2004). Background sodium current stabilizes bursting in respiratory pacemaker neurons. *J Neurobiol* **60,** 481-489.

Tse A, Yan L, Lee AK & Tse FW. (2012). Autocrine and paracrine actions of ATP in rat carotid body. *Can J Physiol Pharmacol* **90,** 705-711.

Veale EL, Kennard LE, Sutton GL & MacKenzie G. (2007). Gαq-mediated regulation of TASK3 two-pore domain potassium channels: the role of protein kinase C. *Mol Pharmacol* **71,** 1666-1675.

Verkhratsky A & Butt A. (2013). Neuroglia: Definition, Classification, Evolution, Numbers, Development. *Glial Physiology and Pathophysiology***,** 73-104.

Verkhratsky A, Rodríguez JJ & Parpura V. (2012). Calcium signalling in astroglia. *Mol Cell Endocrinol* **353,** 45-56.

Volterra A & Meldolesi J. (2005). Astrocytes, from brain glue to communication elements: the revolution continues. *Nat Rev Neurosci* **6,** 626-640.

von Kugelgen I & Wetter A. (2000). Molecular pharmacology of P2Y-receptors. *Naunyn-Schmiedeberg's archives of pharmacology* **362,** 310-323.

Wang HS, Pan Z, Shi W, Brown BS, Wymore RS, Cohen IS, Dixon JE & McKinnon D. (1998). KCNQ2 and KCNQ3 potassium channel subunits: molecular correlates of the M-channel. *Science* **282,** 1890-1893.

Wang X, Hayes JA, Revill AL, Song H, Kottick A, Vann NC, LaMar MD, Picardo MCD, Akins VT, Funk GD & Del Negro CA. (2014). Laser ablation of Dbx1 neurons in the pre-Bötzinger complex stops inspiratory rhythm and impairs output in neonatal mice. *Elife* **3,** e03427.

Washburn CP, Sirois JE, Talley EM, Guyenet PG & Bayliss DA. (2002). Serotonergic raphe neurons express TASK channel transcripts and a TASK-like pH- and halothane-sensitive K+ conductance. *J Neurosci* **22,** 1256-1265.

Way KJ, Chou E & King GL. (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* **21,** 181-187.

Wenker IC, Sobrinho CR, Takakura AC, Moreira TS & Mulkey DK. (2012). Regulation of ventral surface CO2/H+-sensitive neurons by purinergic signalling. *The Journal of Physiology* **590,** 2137-2150.

Wenker IC, Sobrinho CR, Takakura AC, Mulkey DK & Moreira TS. (2013). P2Y1 receptors expressed by C1 neurons determine peripheral chemoreceptor modulation of breathing, sympathetic activity, and blood pressure. *Hypertension* **62,** 263-273.

Wettschureck N & Offermanns S. (2005). Mammalian G proteins and their cell type specific functions. *Physiol Rev* **85,** 1159-1204.

White DP. (2005). Pathogenesis of Obstructive and Central Sleep Apnea. *Am J Respir Crit Care Med* **172,** 1363-1370.

Wilke BU, Lindner M, Greifenberg L & Albus A. (2014). Diacylglycerol mediates regulation of TASK potassium channels by Gq-coupled receptors. *Nat Commun* **5,** 5540.

Wilson RJ, Vasilakos K, Harris MB, Straus C & Remmers JE. (2002). Evidence that ventilatory rhythmogenesis in the frog involves two distinct neuronal oscillators. *J Physiol* **540,** 557-570.

Xie AX, Petravicz J & McCarthy KD. (2015). Molecular approaches for manipulating astrocytic signaling in vivo. *Front Cell Neurosci* **9,** 144.

Xu F. (2006). Adenosine stimulates depolarization and rise in cytoplasmic [Ca2+] in type I cells of rat carotid bodies. *Am J Physiol Cell Physiol* **290,** C1592-C1598.

Xu J, Tse FW & Tse A. (2003). ATP triggers intracellular Ca2+ release in type II cells of the rat carotid body. *The Journal of Physiology* **549,** 739-747.

Xu J, Xu F, Tse FW & Tse A. (2005). ATP inhibits the hypoxia response in type I cells of rat carotid bodies. *J Neurochem* **92,** 1419-1430.

Yao ST, Barden JA, Finkelstein DI, Bennett MR & Lawrence AJ. (2000). Comparative study on the distribution patterns of P2X(1)-P2X(6) receptor immunoreactivity in the brainstem of the rat and the common marmoset (Callithrix jacchus): association with catecholamine cell groups. *J Comp Neurol* **427,** 485-507.

Yuan G, Vasavda C, Peng YJ, Makarenko VV, Raghuraman G, Nanduri J, Gadalla MM, Semenza GL, Kumar GK, Snyder SH & Prabhakar NR. (2015). Protein kinase G-regulated production of H2S governs oxygen sensing. *Sci Signal* **8,** ra37.

Zagranichnaya TK, Wu X & Villereal ML. (2005). Endogenous TRPC1, TRPC3, and TRPC7 proteins combine to form native store-operated channels in HEK-293 cells. *J Biol Chem* **280,** 29559-29569.

Zavala-Tecuapetla C, Aguileta MA, Lopez-Guerrero JJ, González-Marín MC & Peña F. (2008). Calcium-activated potassium currents differentially modulate respiratory rhythm generation. *Eur J Neurosci* **27,** 2871-2884.

Zhao MG, Hulsmann S, Winter SM, Dutschmann M & Richter DW. (2006). Calcium-regulated potassium currents secure respiratory rhythm generation after loss of glycinergic inhibition. *Eur J Neurosci* **24,** 145-154.

Zorec R, Araque A, Carmignoto G, Haydon PG, Verkhratsky A & Parpura V. (2012). Astroglial excitability and gliotransmission: an appraisal of Ca2+ as a signalling route. *ASN Neuro* **4**.

Zorec R, Verkhratsky A, Rodriguez JJ & Parpura V. (2015). Astrocytic vesicles and gliotransmitters: Slowness of vesicular release and synaptobrevin2-laden vesicle nanoarchitecture. *Neuroscience***,** In Press.

Zwicker JD, Rajani V, Hahn LB & Funk GD. (2011). Purinergic modulation of preBötzinger complex inspiratory rhythm in rodents: the interaction between ATP and adenosine. *J Physiol* **589,** 4583-4600.