Identification of the mechanisms through which ATP excites the inspiratory network in vitro

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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Abstract

The ventilatory response to hypoxia comprises an initial peripheral chemoreceptor-mediated increase in ventilation followed by a centrally-mediated secondary depression that can be life-threatening in premature infants with apnea. ATP appears to be released from astrocytes in the preBötzinger Complex (preBötC, critical site for inspiratory rhythm generation), during hypoxia where it attenuates the secondary depression via a $P2Y_1$ receptor-dependent excitation of inspiratory neurons. However, the underlying mechanism(s) coupled to $P2Y_1$ receptors is unknown. Here we apply nerve and whole-cell recording methods to rhythmic medullary slices (700 μm) from neonatal rat to test the hypothesis that the $P2Y_1R$ operates through 1) the $G\alpha_q$-signalling pathway and 2) elevation of cAMP levels and potentiation of the hyperpolarization-activated inward current, I_h, to excite the inspiratory neurons and network. We found that blocking the individual steps of the $G\alpha_q$-signalling pathway via intracellular dialysis of the second messenger blockers attenuated the 5 mM ATP- or MRS 2365 (P2Y1 receptor agonist, 100 μM)-induced inward currents by 20% - 30% and ~55%, respectively. At the network level, blocking the $G\alpha_q$-signalling pathway reduced the MRS 2356 ($P2Y_1$ receptor agonist)-induced network excitation by up to ~60%. These data suggest that the $G\alpha_q$-signalling contributes to about 50 - 60% of the $P2Y_1$ receptor-meditated excitation. We also discovered that the MRS2365 current reversed between -60 and -40 mV, suggesting activation of I_h. ZD7288 (open channel blocker of I_h, 100 μM) attenuated he MRS 2365-induced inward current by ~35% (15 min) and –network excitation by ~60% (1^{st} trial). $P2Y_1$ receptor activation induced an ~9.8 mV depolarizing shift in $V_{1/2}$ (membrane potential at which 50% of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which underlie I_h, are open). Moreover,blocking cAMP production by SQ 22536

(adenylyl cyclase inhibitor) attenuated the MRS 2365-induced currents by ~60 % (15 min, in 9 of 18 cells) and -network excitation by ~50% (60 min, bath). These data suggest that the $P2Y_1$ receptor-mediated excitation of the preBötC network is produced via 1) activation of the $G\alpha_q$-signalling pathway, and 2) an elevation in cAMP level and potentiation of I_h in a subpopulation of inspiratory neurons.

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 2019).

Collaborations:
All the experiments were designed and completed in Dr. Funk's lab at the University of Alberta. There is no collaboration with other labs or individuals involved.

I was the primary experimentalist in this study and was involved in all aspects of experiment design. I was responsible for data analysis, figure preparation and writing the manuscript. I also generated the majority of the data, especially the whole-cell recording experiments that used various blockers/antagonists to assess the contribution of different signaling molecules to the MRS 2365-evoked inward currents. A number of individuals, including summer students and beginning graduate students whom I helped supervise, performed some experiments that tested the effects of these same agents on the network frequency increase evoked by injecting MRS 2365 directly into the preBötC. The work done by Alexis Katzell, Vishaal Rajani and Ana Miranda Tapia (the extracellular recording experiment) was overseen by me. Contributions of others to data collection are:

- The experiments that tested the effects of U73122, chelerythrine and paxilline (4 μM and 1 μM) on the MRS 2365-induced inward currents were done by Ana Miranda Tapia (Fig. 2.7; Fig. 2.9C – F).
- The experiments that tested the effects of 2-APB and chelerythrine on the MRS 2365-induced frequency increase were done by Vishaal Rajani (Fig. 2.8C – F).
- The experiments that tested the effects of paxilline, $BaCl_2$ and apamin on the MRS 2365-induced frequency increase were done by Venkatesh Jalubula (Fig. 2.9A).
- The experiments that tested the effect of glibenclamide on the MRS 2365-induced frequency were done by Alexis Katzell (Fig. 2.9A).
- The experiments that tested the effects of 9-phenanthrol and flufenamic acid on the MRS 2365-induced frequency increase were done by Robert Reklow (Fig. 2.9A).

Data analysis and concluding discussion of these experiments are my original work.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Gregory Funk for his support. To me Greg is an excellent mentor who has always been encouraging and his enthusiasm and commitment is inspiring. He likes to guide me through my thoughts on the experimental designs and to help me grasp the big picture of my projects in the process. He never pushed me to finish the experiments nor did he forced me to do the experiments that we didn't agree on. Greg is also very adaptive. He is willing to tailor his way of mentoring to satisfy the need of his mentees individually. One prime example is that I had an irregular work schedule (started at 5 P.M. and went home past midnight) when doing the whole-cell patch recording experiments so I didn't get to talk to him very often. In the beginning, Dr. Funk was concerned about the potential issue of lack of communication. After realizing that I came late to avoid distraction and disturbances, he accepted it and set up the meetings with me on a regular basis so we could discuss about the progress and be on the same page. Moreover, Greg was always available whenever I encountered a problem and would sit down and troubleshoot with me. He is very insightful so his suggestions usually turned out to be constructive, which ended up saving me a lot of time and effort. At last, Greg never acts like he is superior to you. He is so easy-going and humorous that it's a pleasure to work with him.

I would also like to thank the members of my supervisory committee; Dr. John Greer and Dr. Silvia Pagliardini, Dr. Po-Yin Cheung for their guidance throughout my PhD, as well as external examining members, Dr. Declan Ali and Dr. Daniel Mulkey and Dr. Zamaneh Kassiri for their role in the examination process.

I would like to thank my lab members and friends for their help throughout my PhD study including Vishaal Rajani, Ann Revill, Jennifer Zwicker, Nathan Chu, Venkatesh Jalubula, Alexis Katzell, Robert Reklow, Vivian Biancardi, Ana Miranda Tapia, Alexander Toohey, Vladimir Rancic, Toka Abu Jaib, Ren Jun, Xiuqing Ding, Wei Zhang, Li Ma and vising students; Glauber da Silva, João Paulo Jacob Sabino and Camila Linhares-Taxini. A special thanks goes to Tucaauê Alvares who has been helping me since the moment I arrived in Canada. All the techniques I used to generate the data included in the thesis, I learned from Tuca. He also took his time going through what our lab was focused on and what the past lab members had done with me when I first joined the lab, which helped me get a better understanding of the my project. Thanks to him, my transition into the new lab was painless. To me, Tuca is not only a friend but also a mentor.

Finally, I would like to thank my beloved wife Jie Zeng for her support through these years. I wouldn't have had such a great PhD career and met the aforementioned lovely people without her. I also want to thank my father Fugen Zhang and mother Xiulan Liu for being considerate and for all the love they give me.

Funding sources: CIHR, AIHS, NSERC, CFI, and WCHRI

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List of abbreviations

Abbreviation	**Definition**
[D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin	DAMGO
AC	Adenylyl cyclase
ADK	Adenosine kinase
AMPA receptor	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
AOP	Apnea of prematurity
AP5	DL-2-Amino-5-phosphonopentanoic acid
ATP	Adenosine triphosphate
BK channel	Big conductance Ca^{2+}-activated potassium channel
BötC	Bötzinger complex
cAMP	Cyclic AMP
CCAP	2-Chloro-N^6-cyclopentyladenosine
CHE	Chelerythrine chloride
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CPA	Cyclopiazonic acid
DAG	Diacylglycerol
DBX1	Developing brain homeobox protein 1
DMSO	Dimethyl sulfoxide
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
ENT	Equilibrative nucleoside transporter

EPSP	Excitatory postsynaptic potential
FA	Fluoroacetate
FFA	Flufenamic acid
GABA	Gamma-aminobutyric acid
GIRK channel	G protein-coupled inwardly-rectifying potassium channel
HCN channel	Hyperpolarization-activated cyclic nucleotide-gated channel
HVR	Hypoxic ventilatory response
Hyperpolarization-activated inward current, h-current	I_h
IBI	Interburst interval
I_{CAN}	Calcium-activated nonspecific cationic current
I_{NaP}	Persistent sodium current
IP_3	Inositol trisphosphate
K_{ATP} channel	ATP-sensitive potassium channel
L-PIA	N^6-L-2-phenylisopropyl adenosine
MSO	Methionine sulfoximine
NTS	Nucleus of the solitary tract
P1R	P1 receptor
P2R	P2 receptor
$P2Y_1R$	$P2Y_1$ receptor
PCA	Posterior cricoarytenoid
pFRG	Parafacial respiratory group
PiCo	Postinspiratory complex
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C

PPADS	Pyridoxal phosphate-6-azo tetrasodium salt hydrate
preBötC	Pre-Bötzinger complex
R-PIA	N^6-R-2-phenylisopropyl adenosine
RTN	Retrotrapezoid nucleus
SK channel	Small conductance Ca^{2+}-activated potassium channel
SubP	Substance P
THG	Thapsigargin
TIRF	Total internal reflection fluorescence
TLC	Tetanus toxin light chain
TRPM4/5 channel	Transient receptor potential cation channel subfamily M member 4 and 5
VDCCs	Voltage-dependent Ca^{2+} channels
VRC	Ventral respiratory column

Chapter 1. Introduction

1.1 Overview

Breathing is a vital behavior that moves air in and out of the lungs through rhythmic contraction of respiratory muscles to maintain a constant level of arterial blood gases (O_2 and CO_2) and pH (Greer & Funk, 2013). The mammalian breathing cycle is comprised of three phases: inspiration, post-inspiration and expiration. Inspiration is achieved via contraction of the inspiratory pump muscles (the diaphragm and external intercostals). Unlike inspiration, expiration is usually passive at rest; the expiratory muscles (the abdominal and internal intercostals) are only recruited when respiratory drive increases during exercise or temporary exposure to high-altitude environments (Greer & Funk, 2013). Active expiration empties lung of more air than passive expiration, creating a more negative pressure at the sites of alveoli which facilitates air intake for the next inspiration and as a consequence increases tidal volume and improves gas exchange (Feldman *et al.*, 2013). Post-inspiratory activities involve lengthening contraction of the diaphragm and adduction of laryngeal muscles, which slow passive expiration, increasing time for gas exchange. It is during postinspiration that swallowing and vocalization occur so post-inspiratory activities are also important in the coordination of breathing with other orofacial behaviors (Del Negro *et al.*, 2018). The phasic composition and motor pattern of breathing are highly adaptive to the changes in the blood O_2/CO_2 levels (Greer & Funk, 2013). In acute hypoxia, reduction in arterial partial pressure of O_2 triggers the biphasic hypoxic ventilatory response (HVR), which consists of an initial increase in ventilation followed by a secondary depression (Pamenter & Powell, 2016). In adults, the level of ventilation remains higher than baseline throughout the HVR to combat hypoxia. However, in most premature and some newborn mammals and preterm infants, ventilation falls below baseline during the secondary hypoxic ventilatory

depression, which exacerbates the hypoxia, causing even greater depression (Moss, 2000). This vicious cycle can be problematic or even life-threatening especially to premature infants who suffer from apnea of prematurity (AOP). In Canada, about 8% of infants are born prematurely (Shah *et al.*, 2018) and about 85% of infants born at less than 34 weeks of gestation will undergo AOP (Barrington & Finer, 1991). Due to their immature respiratory network, those newborns will be subject to spontaneous apneic sessions, a condition clinically referred to as the primary apnea. Apnea-induced reduction in the blood O_2 level (hypoxia) will lead to respiratory depression which further suppresses breathing and prolongs the length of apneic session. If asphyxia is allowed to continue, infant will stop responding to any approaches of breathing stimulation after some time and mechanical ventilation is needed for resuscitation. This second phase of apnea is called secondary apnea (Kalaniti *et al.*, 2018). Methylxanthines such as aminophylline, theophylline and caffeine are used clinically for treatment of the primary apnea, among which caffeine citrate is considered to be the "silver bullet in neonatology" due to its effectiveness, larger therapeutic index, longer serum half-life, wider safety margin and fewer side effects (Henderson-Smart & Steer, 2010). However, about 20% of AOP patients are insensitive to caffeine (Schmidt *et al.*, 2012; Lista *et al.*, 2016) and they are more likely to develop bronchopulmonary dysplasia tested at a postmenstrual age of 36 weeks (Schmidt *et al.*, 2006) and have a reduced survival rate without neurodevelopmental disabilities (e.g., cerebral palsy and cognitive delay) at a corrected age of 18 to 21 months (Schmidt *et al.*, 2007).

Despite the clinical relevance, the mechanism(s) underlying the secondary hypoxic ventilatory depression is not well understood. Dogma holds that the depression is primarily central origin and that there is no central excitatory mechanism that contributes to the hypoxic ventilatory response (Funk & Gourine, 2018a). Recent evidence, however, suggests that ATP is released in

the pre-Bötzinger complex, the center of the inspiratory rhythm generation in the ventrolateral medulla, where it offsets the secondary hypoxic ventilatory depression via its action on $P2Y_1$ receptors. The ultimate objective of my study is to identify the signaling pathway(s) and ion channel(s) through which $P2Y_1$ receptors excite the inspiratory neurons and the inspiratory network. Here I review the literature on the following topics, which provide the background information necessary to understand the rationale and significance of my thesis research:

1) Respiration: a three-phase behavior;

2) Hypothesized mechanisms of respiratory rhythm generation;

3) The hypoxic ventilatory response;

4) Role of ATP signaling in central respiratory chemosensing of hypercapnia and hypoxia.

Topics 1 and 2 produce evidence that reveals the importance of inspiration to breathing and how it is produced in the brainstem, in particular the pre-Bötzinger complex (preBötC). It is also established that purinergic modulation of the activity of the preBötC changes ventilatory output, which suggests that the preBötC may be the place where ATP acts to offset the secondary hypoxic ventilatory depression. Topic 3 covers the knowledge on the mechanisms underlying the biphasic HVR which largely supports the dogma that the initial increase in ventilation of the HVR is mediated by the peripheral chemoreceptors whereas the secondary depression is mediated purely by central inhibition. Topic 4 presents evidence demonstrating that ATP-mediated excitation of neurons or network contributes to CO_2 and O_2 respiratory chemoreception. In the context of hypoxia, astrocytes detect a reduction in the level of O_2 in blood and in turn release ATP which excites the preBötC network through its actions on $P2Y_1$ receptors to offset the secondary hypoxic ventilatory depression (Angelova *et al.*, 2015b; Rajani *et al.*, 2018), which suggests that the dynamics of the secondary depression is shaped by not only inhibitory mechanisms but also

excitatory ones. The main objective of my thesis is to identify the signalling cascade through which $P2Y_1$ receptor excite the inspiratory network in vitro, which would complement our understanding of the $P2Y_1$ receptor/ATP signalling. The candidate pathways tested were chosen based on the studies reviewed under topic 4. Identification of the in vitro signalling pathway will provide insight into how ATP excites the inspiratory network during hypoxia in vivo and may open up new ATP-based treatment possibilities for AOP patients who do not respond to caffeine.

1.2 Centers of the respiratory rhythm generation

1.2.1 Inspiration

The notion that breathing is generated in the brainstem was first proposed by Galen, the physician to the gladiators, who noticed that he could still see breathing movements in the mouth and neck of gladiators who had been injured in the cervical region and that these movements were absent in those with brainstem injuries. In the early 19th century, French physiologist Julien Jean Cesar Legallois (Finger, 1994; Cheung, 2013) reported that breathing persisted in rabbits after removal of cerebrum and cerebellum but ceased following a transection made at a certain level in the medulla where the vagus nerve originates. Another French physiologist Marie Jean Pierre Flourens took Legallois' research a step forward by demonstrating that the lower part of the medulla (which he referred to as *noeud vital*) alone was able to produce breathing as its puncture terminated breathing (Finger, 1994). However, it was not until the late 20th century that the exact location *noeud vital*, the source of breathing, was identified. Smith *et al.* (1991) first demonstrated that a rat medullary slice as thin as 350 μm taken at the level just caudal to the retrofacial nucleus continued to generate the inspiratory-related motor output in cranial nerve XII that innervates the genioglosssus muscle, and that neurons with inspiratory-related activity (in phase with the

population nerve activity) were concentrated in a region identified as part of the ventral respiratory column located ventrolaterally to the semi-compact nucleus ambiguus, immediately caudal to the compact nucleus ambiguus (Smith *et al.*, 1991). It was considered very important at the time that the region contained inspiratory neurons with voltage-dependent pace-maker properties, which was the "inspiration" behind the conditional inspiratory pacemaker network hypothesis. This region was named the pre-Bötzinger complex (preBötC). Its role in inspiratory rhythmogenesis has been extensively investigated both in vitro and in vivo. Increasing the excitability of the preBötC through pharmacological intervention (e.g., local injection of high concentration of KCl, excitatory substances such as glutamate, substance P and thyrotropin-releasing hormone and ATP) (McCrimmon *et al.*, 1986; Smith *et al.*, 1991; Greer *et al.*, 1996; Lorier *et al.*, 2007; Rajani *et al.*, 2018) or optogenetic photoactivation (Alsahafi *et al.*, 2015) enhances breathing. In contrast, silencing the preBötC in vitro via pharmacological manipulation of preBötC neurons (e.g., local application of CNQX or DAMGO) (Smith *et al.*, 1991; Funk *et al.*, 1993; Gray *et al.*, 1999) silenced rhythm. Even single preBötC "islands" isolated from the rhythmic slice preparations are able to independently produce rhythmic respiratory-related activity (Johnson *et al.*, 2001; Vandam *et al.*, 2008). However, it was not until NK1 receptor, μ-opioid receptor, somatostatin and DBX1(developing brain homeobox 1) expression were identified as markers of preBötC neurons (Xia & Haddad, 1991; Gray *et al.*, 1999; Stornetta *et al.*, 2003; Gray *et al.*, 2010) that crucial in vivo tests of preBötC function became possible. Selective inhibition of NK1 and μ-opioid receptor-expressing neurons in the preBötC via local application of saporin-conjugated substance disrupted breathing (Gray *et al.*, 2001; McKay *et al.*, 2005). Consistent with a critical role of preBötC neurons in the inspiratory rhythm generation, allatostatin-mediated inhibition of somatostatin-expressing neurons in vivo and photoinhibition/laser ablation of DBX1 preBötC neurons in vitro

lead to suppression or even termination of breathing (Tan *et al.*, 2008; Wang *et al.*, 2014; Vann *et al.*, 2016). In contrast, prolonged photostimulation of preBötC DBX1 neurons stimulates breathing (Vann *et al.*, 2018).

Initial connectivity studies revealed that the preBötC was unique compared to neighbouring regions of the ventral respiratory column in that it contained a high proportion of local interneurons and very few projection neurons (Smith *et al.*, 1991). This was interpreted to reflect its hypothesized role as a local microcircuit generating inspiratory rhythm rather than a pool of neurons relaying that rhythmic output to other premotor and motor areas. More tracing studies that investigated the projection of the preBötC conducted later on established that, in addition the contralateral preBötC, the preBötC has extensive projections to other regions of the ventral respiratory column including the Bötzinger Complex (BötC), ventral respiratory group, nucleus of the solitary tract (Clements *et al.*), parahypoglossal nucleus, parafacial respiratory group (pFRG)/the retrotrapzoid nucleus (RTN), parabrachial and Kölliker-Füse nuclei, the locus coeruleus, as well as major projections to the midbrain periaqueductal gray (Tan *et al.*, 2010; Yang & Feldman, 2018). The preBötC also receives inputs from key chemosensitive regions such as the RTN and raphe nuclei that adjust breathing in proportion to increased respiratory drive (Bochorishvili *et al.*, 2012; Morinaga *et al.*, 2019). These afferent and efferent connections of the preBötC provide an anatomical basis for coordination between breathing and other behaviors, physiological processes and higher brain functions such as cognition and emotion. Furthermore, the preBötC has been identified in multiple species including cats, rabbits, mice and human (Schwarzacher *et al.*, 1995; Wenninger *et al.*, 2004; Bongianni *et al.*, 2010; Pantaleo *et al.*, 2011; Schwarzacher *et al.*, 2011; Tupal *et al.*, 2014). Taken together, the evidence suggests that the preBötC is both necessary and sufficient for the generation of the inspiratory rhythm.

1.2.2 Expiration

With evolution of the diaphragm, a horizontal dome-like sheet of muscle that separates the thorax from the abdomen, expiration primarily became a passive event in mammals. During inspiration, contraction of the diaphragm and the external intercostals expands the volume of the thorax. The resultant negative intrathoracic pressure causes air to move into the lung along the pressure gradient. Upon cessation of the inspiratory phase, the elastic energy stored in the chest wall and abdomen through active inspiration causes the lung to recoil back to its equilibrium position, driving air out of the lung (Greer & Funk, 2013). Under metabolic challenges (e.g. exercise) expiration becomes active with rhythmic recruitment of the expiratory muscles, mainly the abdominal and internal intercostal muscles. Active expiration further decreases the lung volume at end expiration, which augments the tidal volume of the next breath. This increase in tidal volume, along with increases in frequency, underlie the increases in ventilation that occur in response to increased ventilatory drive (Greer & Funk, 2013). Active expiration also occurs during active sleep in neonates and rapid eye movement sleep in adults, which stabilizes breathing and increases ventilation (Andrews & Pagliardini, 2015; Saini & Pagliardini, 2017). A common view of the respiratory rhythm generator was that it consisted of inspiratory and expiratory half center oscillators, similar to that proposed for locomotor networks (Brown, 1914) that produced rhythm via tonic drive, reciprocal inhibition and accommodative properties that caused the half centers to self-terminate. This construct came under close scrutiny with the discovery of the preBötC that could generate the inspiratory rhythm on its own (Smith *et al.*, 1991), suggesting that expiration was simply the manifestation of the preBötC refractory period. The discovery of a potentially separate oscillator more rostrally in the VRC once again changed perspective (Mellen *et al.*, 2003). The initial evidence for a second oscillator was indirect but compelling. Bath application of μ-

opioid receptor antagonist DAMGO gradually slowed and then abolished the inspiratory rhythm in the rhythmic slice preparation, whereas in *en bloc* brainstem-spinal cord preparation, DAMGO caused rhythm to slow in a quantal nature (Mellen *et al.*, 2003). The most parsimonious explanation was that a second, opioid-insensitive oscillator in the brainstem spinal cord preparation but absent in the slice is coupled to the preBötC. As the preBötC oscillator is inhibited by opioids, it occasionally fails, leaving quantized gaps in the output, and can only generate bursts when it receives input from the second, opioid insensitive oscillator. Optical recordings of neurons labeled with voltage-sensitive dye in the perinatal (embryonic days 20 and 21, postnatal days 0 and 1) rat brainstem-spinal cord preparation later revealed that rhythmic neuronal activity first emerged in the region ventrolateral to the facial nucleus (referred to the parafacial respiratory group/pFRG or in some studies, the retrotrapezoid nucleus/RTN) prior to the onset of activity in the preBötC and inspiratory motoneuron pools (Onimaru & Homma, 2003, 2005), suggesting that the pFRG couples with the preBötC to facilitate generation of the inspiratory rhythm during the perinatal stage of rat development. On the contrary to these findings, rhythmic activity in the preBötC and pFRG were observed simultaneously at as early a stage as E14.5 in mice; i.e., high- and low-frequency rhythmic burstings were recorded from the pFRG and the preBötC respectively at E14.5 in the transverse mouse slices (Thoby-Brisson *et al.*, 2005). Moreover, Ca^{2+} imaging experiments illustrated a rhythmic fluctuation of the fluorescent signal of Ca^{2+}-sensitive dye (Calcium Green 1AM) in the pFRG which entrained the weak preBötC reflected by irregular XII bursting with failures at E14.5 in the mouse brainstem-spinal cord preparation (Thoby-Brisson *et al.*, 2009). The role of these two oscillators during perinatal development, how their coupling changes over the embryonic stage and why it varies between species remain an important question in respiratory control. In adults, however, a leading hypothesis is that the pFRG/RTN is the active expiratory

oscillator and the preBötC generates the inspiratory rhythm; moreover, the pFRG/RTN is dependent on inputs from the preBötC for its rhythmicity since hyperpolarization of preBötC neurons prevented generation of active expiration evoked by depolarization of lateral pFRG neurons (Huckstepp *et al.*, 2016). This possibility first emerged from experiments in vivo where, Janczewski and Feldman (Janczewski & Feldman, 2006) used fentanyl to uncouple inspiratory from expiratory motor output in juvenile rats and found that fentanyl-induced quantal slowing was only evident in the inspiratory but not expiratory rhythm. They also found that removal of the pFRG/RTN effectively eliminated the spontaneous expiratory motor output in juvenile rats (Janczewski & Feldman, 2006) and hypercapnia-induced expiratory motor activity in brainstem-spinal cord prepration (Abdala *et al.*, 2009). Moreover, pharmacological disinhibition or photostimulation of the pFRG/RTN induced active expiration in adult anesthetized rats (Pagliardini *et al.*, 2011). HM3D DREADD (Designer Receptors Exclusively Activated by Designer Drugs) receptor-mediated depolarization and bicuculline/strychnine-mediated disinhibition of the lateral part of the pFRG elicit active expiration (Pagliardini *et al.*, 2011; Huckstepp *et al.*, 2016). Consistent with the essential role of the pFRG in the expiratory rhythm generation, chemical inhibition of the pFRG (Abdala *et al.*, 2009) or allatostatin/ HM4D DREADD receptor-mediated hyperpolarization of neurons specifically in the lateral pFRG (pF_L) attenuated hypercapnia-induced recruitment of the active expiration (indicated by rhythmic abdominal muscle activity)(Huckstepp *et al.*, 2015). Since inhibition of neurons in the ventral pFRG (pF_v) but not in the pF_L attenuated the hypercapnia-induced changes in inspiratory activity, it suggests that the pF_v may at least overlap with the chemoreceptive RTN and that the pF_L and RTN are not the same brain region (Huckstepp *et al.*, 2015).

Embryological studies also support the concept that the RTN and pFRG are distinct entities. The pF_V/RTN and pF_L neurons derive from two distinct groups of progenitor cells and exert different physiological functions. Transcription factors paired-like homeobox 2B (PHOX2B)(Dubreuil *et al.*, 2009) and atonal homologue 1 (ATOH1)(Rose *et al.*, 2009) have so far only been found in pF_V progenitor cells while genetic identification of pF_L neurons remains to be elucidated. Therefore, it's likely that pF_V neurons correspond to chemoreceptors in the RTN and neurons that facilitate the generation of the inspiratory rhythm during the perinatal stages (Onimaru & Homma, 2003; Onimaru *et al.*, 2008) and drive to respiration in vivo (Huckstepp *et al.*, 2015). pF_L neurons are the "expiratory" neurons which underlie active expiration. The projection of the pF_L to the expiratory premotor neurons in the caudal ventral respiratory group has been established (Janczewski *et al.*, 2002) and the coupling between pF_L and the preBötC has been shown to be necessary for active expiration to occur (Huckstepp *et al.*, 2016). In summary, the pFRG, the lateral portion in particular, appears to be responsible for generation of active expiration.

1.2.3 Postinspiration

Postinspiration is the last one of the three phases of breathing during which some respiratory-related reflexes and behaviors like swallowing and singing are coordinated to occur. Despite its prevalence in the breathing cycle, postinspiratory activity does not seem to be essential for tidal breathing (Dutschmann *et al.*, 2014) or respiratory rhythmogenesis *per se* as transection at the level just rostral to the Bötzinger complex of a brainstem-spinal cord preparation eliminated the postinspiratory activity but not the inspiratory rhythm (Smith *et al.*, 2007). Historically, the pneumotaxic center which contains the pontine respiratory group has been thought to underlie the postinspiratory activity (Poon & Song, 2014). A recent study (Anderson *et al.*, 2016) suggests that

a conditional oscillator approximately 400 μm rostral to the preBötC, dorsal to the BötC, caudal to the facial nucleus and dorsomedial to the nucleus ambiguus may underlie the generation of postinspiratory activity. The region is named the postinspiratory complex (Vaithianathan *et al.*) and is rhythmically active in isolated horizontal or transverse slices. Bath-applied norepinephrine greatly enhances the PiCo rhythm, which can outpace that of the preBötC and photostimulation of ChAT-positive, PiCo neurons elicits postinspiratory activity in slices and in vivo. In contrast, bath application in slices and local application in the PiCo in anesthetized mice of DAMGO or somatostatin abolishes postinspiratory activity. Note that at the same concentrations, DAMGO and somatostain only slightly slow down the preBötC inspiratory rhythm in slices, suggesting that the PiCo is more sensitive to DAMGO- and somatostatin-modulation than the preBötC (Anderson *et al.*, 2016). Considering that norepinephrine also has differential effects on the PiCo and preBötC rhythms, the data suggest that the PiCo and preBötC rhythms can be separately modulated and that the PiCo is an independent oscillator for the generation of postinspiratory activity. The relationship between the PiCO and preBötC was examined in the same paper and found to be mutually inhibitory so that postinspiration does not coincide with inspiration. Whether there is a connection between the PiCo and the pF_L which is responsible for generation of active expiration remains unclear. Further work is required to confirm the existence of the PiCo since there has been only one study from one lab that suggests its existence.

1.3 Hypotheses of respiratory rhythm generation

1.3.1 Half-center model

Inhibition has long been proposed to play an essential role in the generation of rhythmic movements in invertebrates due to the prevalence of inhibitory central pattern generator neurons.

The role of inhibition in generation of rhythmic locomotor activity in mammals was, however, largely neglected since the doctrine in the late nineteenth and early twentieth centuries was that locomotive pattern generation relied on sensory inputs. The paradigm was challenged by Thomas Graham Brown who found that anesthetic-mediated deprivation of proprio- and extero-ception did not eliminate basic pattern of stepping produced by anaesthetized cats, rabbit and guinea-pigs that had their spinal cords transected at the lower thoracic level (Brown, 1914). The result suggests that locomotor activity can be generated entirely in the spinal cord independent of sensory afferent inputs, based on which Brown proposed what he called the "half-center" model for central pattern generation of locomotion. The idea is that two groups of spinal cord neurons that are not necessarily rhythmic reciprocally inhibit each other to produce rhythm. Although the roles of synaptic inhibition in the generation of locomotive rhythm has yet to be definitively established, blockade of fast inhibitory transmission disrupts the left-right alternating pattern of rhythmic motor activity recorded from isolated neonatal rat spinal cords and in some cases, produces a synchronous pattern of left-right motor activity (Cowley & Schmidt, 1995). The inspiratory off-switch model for respiratory rhythmogenesis was proposed by von Euler and modified after the "half-center" concept. According to it, inspiratory neuron population is slowly depolarized presumably through recurrent excitation. Upon reaching a threshold, an inspiratory burst is generated which subsequently actives a powerful inhibitory network that "switches off" inspiratory activity. Inhibition is relieved over time (expiratory phase) before the next inspiratory phase resumes (von Euler, 1983). In agreement with the hypothesis, inhibitory postsynaptic currents are detected in inspiratory-related neurons of anesthetized and paralyzed cats during inspiration and are postulated to underlie the termination of inspiratory bursts (Richter, 1982).

1.3.2 Three-phase respiratory network model

The three phase respiratory network model was initially proposed by Richter (Richter *et al.*, 1986; Funk & Feldman, 1995). It initially featured inhibitory interconnections between six classes of respiratory neurons located in the ventral respiratory column which are early-, late-, ramp-, post- and premotor inspiratory neurons and expiratory neurons. Ramp- and early-I neurons also produce excitatory outputs. Similar to the half-center model, the reciprocal inhibition between early-inspiratory (early-I) neurons and post-inspiratory (post-I) neurons comprises the primary component of the respiratory rhythm generator. The other four types of respiratory neurons constitute the pattern-generating circuits and pre-motorneuron neurons that receive and process the rhythmic activity to generate appropriate respiratory motor outputs. Some of these neurons also contribute to the shaping of the final motor outputs via their direct inhibitory influence on early-I and post-I neurons. One important aspect of this model is that it produced all three phases of breathing (inspiration, expiration and post-inspiration) compared to the aforementioned half-center model which could only simulate inspiration and expiration. With the better delineation of the ventral respiratory group including the discovery of the preBötC, the three-phase model was updated to allocate different classes of respiratory neurons into the BötC, preBötC and rostral ventral respiratory group (Smith *et al.*, 2007). Following incorporation of inputs from the pFRN and pons, the latest iteration of the three-phase model can reproduce several experimentally observed behaviors such as quantal slowing of phrenic activity with progressive suppression of pre-BötC excitability (Molkov *et al.*, 2010). According to the model, the preBötC inspiratory, BötC postinspiratory and pFRG/RTN expiratory neurons are all presumed to be tonically active in the absence of inhibitory influence and only obtain their corresponding rhythmicity when inhibitory modulation from the preBötC and BötC populations is present (Smith *et al.*, 2007; Rubin *et al.*, 2009; Molkov *et al.*, 2010). This hypothesis is supported by the evidence that 1) low [Cl^-]

perfusate-induced reduction in global inhibition in *in situ* preparations transformed the activity of the preBötC from inspiratory-modulated to tonically firing (Smith *et al.*, 2007) and 2) blocking inhibition locally in the preBötC via gabazine ($GABA_AR$ antagonist) and strychnine (GlyR antagonist) converted the respiratory rhythm from rhythmic to tonic in *in situ* preparations and in vivo (Marchenko *et al.*, 2016).

1.3.3 Role of synaptic inhibition in respiratory rhythmogenesis

Despite the proposed role of synaptic inhibition in the half-center and three-phase respiratory network models for respiratory rhythmogenesis, there is strong evidence that suggests otherwise. For example, global disruption of Cl^--dependent synaptic inhibition by removal of extracellular Cl^- as well as blockade of $GABA_A$ or $GABA_B$ receptors via bath application of bicuculline or phaclofen respectively did not affect the inspiratory rhythm recorded from C4 nerve of the brainstem-spinal cord preparation (Feldman & Smith, 1989). Similarly, blocking $GABA_A$ receptor- and glycine receptor-mediated synaptic inhibition through bath application of bicuculline and strychnine respectively did not abolish the rhythm in the neonatal rat brainstem-spinal cord preparation (Brockhaus & Ballanyi, 1998). In the mouse rhythmic slice, blockade of $GABA_B$ receptors with CGP55845A increased the respiratory frequency at P0 – P3 and decrease it at P7 – P15. Note that the respiratory rhythm still persisted in the presence of CGP55845A in the older age group (Zhang *et al.*, 2002). Moreover, systemic administration of strychnine synchronized inspiratory and postinspiratory activities recorded from phrenic and vagus nerve respectively without eradicating the respiratory rhythm in the working heart-brainstem preparation (Dutschmann & Paton, 2002). Consistent with a lack of involvement of inhibition in the rhythmogenesis, inspiratory-phase photoinhibition of glycinergic neurons in the preBötC increases tidal volume without altering inspiratory duration (Sherman *et al.*, 2015). Finally, injection of a

mixture of bicuculline and strychnine in both the preBötC and BötC slowed down the respiratory rhythm in vagotomized rats but had no effect on the rhythm in vagus-intact rats (Janczewski *et al.*, 2013). This is a key experiment because the level of disinhibition mediated by bicuculline and strychnine was carefully controlled so that it suppressed the lung inflation-induced Breuer-Hering inspiratory reflex without causing the underlying network to be tonically active. The control was necessary as removal of inhibition may lead to an excessive increase in excitability of a network and events that are specific to hyperexcitability of the network but not the intervention that causes it. Yet at the same concentrations effective for blunting the Breuer-Hering reflex, microinjection of bicuculline and strychnine in the preBötC failed to affect the inspiratory rhythm in vagus-intact rats, which suggests that the synaptic inhibition is not required for inspiratory rhythmogenesis. In conclusion, the evidence, when examined together, raises a doubt as to the essential role of synaptic inhibition in generation of the inspiratory rhythm.

1.3.4 Pacemakers

Intrinsic pacemaker-like activity (voltage-dependent rhythmic bursting) of preBötC neurons was reported together with identification of the preBötC as the inspiratory rhythm generator (Smith *et al.*, 1991). Efforts to discern the mechanism of the respiratory rhythmogenesis then focused on the characterization of the conductances that underlie the rhythmic bursting. Activation of fast-activating persistent sodium (Na^+) current (I_{NaP}) was hypothesized to be the underlying ionic mechanism due to the implication of I_{NaP} in bursting behavior in rat hippocampal, hypothalamic, and cortical neurons (Llinas, 1988; Franceschetti *et al.*, 1995; Li & Hatton, 1996). The voltage-dependent model of rhythmic bursting of preBötC neurons was based on I_{NaP} and K^+-dominated leak currents (I_{leak}, which plays a role in burst termination) whose simulated outputs matched the dynamics of oscillatory bursting activity recorded from preBötC neurons in vitro

(Butera *et al.*, 1999). Both I_{NaP} and I_{leak} are experimentally detected in a subpopulation of preBötC neurons. Blocking I_{NaP} with riluzole (I_{NaP} blocker) consistently abolished rhythmic bursting of a subpopulation of preBötC neurons (Del Negro *et al.*, 2002b; Del Negro *et al.*, 2005; Koizumi & Smith, 2008). However, the effect of riluzole on the inspiratory rhythm varies between the studies. Del Negro et al. reported that the inspiratory rhythm in rat slice preparations persisted following bath application of riluzole up to 200 μm (Del Negro *et al.*, 2005) whereas Koizumi and Smith demonstrated that bilateral microinfusion into the preBötC of riluzole at as low as 5 μM was able to abolish the inspiratory rhythm in rat rhythmic slices (Koizumi & Smith, 2008). The different in thickness of the rhythmically active slices used in these two groups may account for the inconsistent network effects of riluzole. The slices used in Koizumi's paper were 250 – 350 μm thick while the ones in Del Negro's paper were about 550 μm thick. It is possible that the preBötC was kept more intact in the preparations in Del Negro's paper and therefore more resilient in response to the reduction in the general excitability of its constituent neurons caused by riluzole.

The pacemaker properties of preBötC neurons are not solely mediated by the I_{NaP} mechanism. Discovery of another mechanism starts with identification of riluzole-insensitive and Ca^{2+}-dependent pacemaker activity in a subset of preBötC neurons (Thoby-Brisson & Ramirez, 2001; Pena *et al.*, 2004; Del Negro *et al.*, 2005). These neurons remain rhythmically-active in the presence of CNQX and riluzole. Their regenerative bursting activity can be blocked by cadmium. This Ca^{2+}-dependent current is presumed to be the Ca^{2+}-activated nonspecific cationic current (I_{CAN}) as flufenamic acid (FFA, I_{CAN} blocker) also eliminated the riluzole-insensitive neuronal rhythmic bursting (Pena *et al.*, 2004; Del Negro *et al.*, 2005). Similar to I_{NaP}, I_{CAN} has been associated with neuronal oscillatory activity in various brain regions (Kramer & Zucker, 1985; Swandulla & Lux, 1985; Partridge *et al.*, 1994; Ghamari-Langroudi & Bourque, 2002), including

respiratory-related nuclei such as the nucleus ambiguus (Rekling & Feldman, 1997). Unlike I_{NaP}-bursting, I_{CAN}-mediated bursting is less voltage-sensitive (Thoby-Brisson & Ramirez, 2001; Del Negro *et al.*, 2005; Ramirez *et al.*, 2011) and is influenced by neuromodulation (Viemari & Ramirez, 2006; Tryba *et al.*, 2008). On the contrary to its hypothesized network effect, blocking I_{CAN} with cadmium or FFA did not affect the inspiratory rhythm recorded in VRC islands (Tryba *et al.*, 2008) or slices (Pena *et al.*, 2004; Del Negro *et al.*, 2005; Viemari & Ramirez, 2006), suggesting that I_{CAN}-mediated bursting activity does not appear to be required for generation of the inspiratory rhythm. The lack of effects of I_{CAN} blockers on the rhythm can be due to the rare occurrence of I_{CAN}-mediated bursting in preBötC neurons in the neonatal preparations used in these studies. Only 0.6% of inspiratory neurons have I_{CAN}-dependent bursting property in P0-P5 mouse slices and its relative prevalence increases developmentally to about 7.5% in P8-P10 slices (Del Negro *et al.*, 2005). However, considering the findings that breathing persists in the presence of riluzole in some cases (Del Negro *et al.*, 2002b; Pace *et al.*, 2007; St-John *et al.*, 2007; St-John, 2008; Fong *et al.*, 2009), a concern should be raised regarding the essential role of pacemaker-like activity in respiratory rhythmogenesis. What further confounds the literature is the expression profiles of I_{NaP} and I_{CAN} and the side effects of the I_{NaP} and I_{CAN} blockers. I_{NaP} and I_{CAN} are expressed in all types of preBötC neurons including inhibitory neurons (Morgado-Valle *et al.*, 2010; Koizumi *et al.*, 2013). Therefore, the perturbation of the respiratory rhythm induced by the I_{NaP} and I_{CAN} blockers may be due to their off-target actions on non-rhythmogenic inhibitory neurons. Combined with the fact that riluzole and FFA may affect other membrane properties (e.g., rluzole at 3 μM reduces transient Na^+ currents evoked in preBötC neurons) (Doble, 1996; Ptak *et al.*, 2005; Guinamard *et al.*, 2013), it is possible that a global depression of excitatory synaptic transmission underlies the inhibitory effects of riluzole and FFA on the respiratory rhythm. The hypothesis is

supported by data showing that when rhythm is silenced by riluzole and FFA, it can rescued by increasing preBötC excitability through application of Substance P or TRH (Del Negro *et al.*, 2005). In addition, riluzole and FFA are often applied in the bath and this route of drug administration lacks spatial and temporal specificity. Drug effects outside of the preBötC can also alter the inspiratory rhythm. For example, local injection of riluzole in the raphe obscurus causes rhythm to cease because under baseline conditions in the slice tonic serotonin release in the preBötC increases excitability (Pace *et al.*, 2007; Ptak *et al.*, 2009). Notably, the apnea evoked by riluzole in the raphe can be rescued by once again increasing preBötC excitability. Therefore, riluzole-induced blockade of rhythm could be due to its action in reducing excitatory modulatory drive to the preBötC from regions like the raphe obscurus. To address this issue, riluzole and FFA were applied together in the preBötC of a rhythmic slice and their effects on the inspiratory rhythm were assessed. Local application of riluzole and FFA failed to eliminate the rhythm, which suggests that I_{NaP} and I_{CAN} are not involved in the respiratory rhythmogenesis (Pace *et al.*, 2007). Although some researchers still try to build their models under the framework, the experimental results, when examined together, question the involvement of pacemakers in the generation of the inspiratory rhythm.

1.3.5 Emergent network and burstlet models

The emergent network model of respiratory rhythmogenesis advocates that the inspiratory rhythm is generated in the preBötC via recurrent excitation of inspiratory neurons and features the following steps: 1) spontaneous spiking of several preBötC neurons; 2) recurrent excitation through which more interneurons are recruited; 3) Intrinsic conductances such as I_{NaP} and I_{CAN}-mediated augmentation of synaptic excitation which promotes inspiratory bursts; 4) transmission of bursts to pattern-forming preBötC neurons, premotor neurons and motor neurons (Del Negro *et*

al., 2008). The early study on the electroresponsive properties and membrane potential trajectories classified inspiratory neurons into three types (Rekling *et al.*, 1996). Type-1 inspiratory neurons are the earliest to fire burst of spikes before hypoglossal nerve discharge and display regular and/or pre-inspiratory spiking, and the burst is followed by an afterhyperpolarization. Type-2 neurons have delayed spiking activity compared to type-1 neurons and occasionally fire regular spikes without ramp potential or remain silent between two inspiratory bursts. Type-3 neurons are the last to fire spikes with the similar behaviors to those of type-2 neurons during interburst interval (IBI) (Rekling *et al.*, 1996). The hypothesis is that type-I neurons are the first ones to regain spontaneous spiking activity presumably through Na^+ leak channel (NALCN)-mediated tonic depolarization (Lu *et al.*, 2007; Lu *et al.*, 2010) following the refractory period and their activities are synchronized and enhanced through recurrent excitation before propagating to type-2 neurons. The depolarizing ramp potential that gives rise to burst-like spiking during interburst intervals may reflect excitatory postsynaptic potentials (EPSPs) resulting from recurrent excitation between type-1 neurons. The spontaneous IBI spiking of type-2 neurons may underlie percolation of activity among type-1 and type-2 neurons which leads to a greater and greater synchrony among these rhythmogenic neurons. Summation of EPSPs increases progressively and, once surpassing threshold (of I_{NaP} and I_{CAN} perhaps), produces inspiratory bursts. Type-3 neurons are postulated to be motor neurons due to comparably smaller input resistance and late spiking timing. The emergent network model was later replaced by the burstlet model following the discovery of burstlets. According to the model, burstlets are the basic rhythmogenic component instead of bursts and bursting neurons are not necessarily rhythmogenic. The existence of burstlets was uncovered in rhythmic slice preparations in the presence of 6 mM or 3 mM extracellular K^+ (Kam *et al.*, 2013a). High-amplitude preBötC bursts are concurrent with hypoglossal nerve discharges in 9 mM K^+-

containing aCSF, which emerge with a stable and regular pattern. Reduction in neuronal excitability by lowering the concentration of extracellular K^+ resulted in failures of hypoglossal bursts, thus highly variable and quantally-distributed interburst periods in which low-amplitude preBötC burstlets were revealed at times when a hypoglossal burst was expected. In addition, the rising phase of burstlets had similar dynamics to the ramp potentials that preceded the inspiratory bursts. The results are consistent with a burstlet-based rhythmogenic mechanism which involves: 1) spontaneous spiking of several preBötC neurons; 2) recurrent excitation between active neurons which generates low-amplitude pre-inspiratory activity; 3) percolation of activity through rhythmogenic population which underlies burstlets; 4) transformation of burstlets into bursts and transmission of bursts to pattern-forming preBötC neurons, premotor neurons and motor neurons. Although inspiratory bursts do not underlie rhythmogenesis, they play an important role in forming patterns of final motor output. The model predicts that some of inspiratory-modulated neurons are part of the pattern generator. Indeed, laser ablation of about 15 DBX1-expressing neurons that received inspiratory modulation in the preBötC decreased hypoglossal motor output by 50% (Wang *et al.*, 2014). Moreover, short-pulse photostimulation of DBX1- or somatostatin-expressing neurons in the preBötC in early inspiration augmented inspiratory bursts, reflected by a larger amplitude and longer duration. Long-pulse photostimulation of DBX1+ neurons also produced augmented inspiratory bursts (Cui *et al.*, 2016). In agreement with the hypothesis proposed by the model that spontaneous spiking of several preBötC neurons is able to initiate a burstlet generation which then turns into an inspiratory burst, simultaneous activation of as few as 4 inspiratory-modulated preBötC neurons via photo-uncaging of glutamate mid expiratory phase produces an ectopic, endogenous-like bursts (Kam *et al.*, 2013b). Finally, a computational model of a randomly connected network developed with only spiking conductances (i.e., I_{NaP}, I_{CAN} and high voltage-

dependent I_{Ca} were not incorporated) demonstrated that nondeterministic spiking and dynamic synaptic strengths (i.e., synaptic fluctuation between facilitation and depression) were sufficient to initiate rhythmic population activity, simulate the burstlet-based rhythmogenesis, and replicate experimental results (Guerrier *et al.*, 2015). In light of these findings, the emergent network model appears to be valid and the framework should be taken into consideration when designing experiments or interpreting data regarding the inspiratory rhythm generation/modulation.

1.4 The hypoxic ventilatory response (HVR)

Hypoxic ventilatory responses vary widely depending on severity of hypoxia as well as pattern and length of hypoxic exposure. Acute, prolonged and intermittent hypoxia can have differential effects (potentiation vs attenuation) on ventilation with different onsets (during hypoxia vs after hypoxia) and time scales (seconds vs years). In this section, I will focus on the ventilatory response to acute hypoxia (referred to the "hypoxic ventilatory response" or HVR hereafter) because this is the response that is involved in the homeostatic control of breathing under normal physiological conditions and is most relevant to processes that occur in premature infants who experience acute hypoxia caused by frequent apneas due to the immature state of their central networks. The adaptive, plastic responses that can be evoked by exposure to chronic hypoxia or acute and chronic intermittent hypoxia will not be discussed.

1.4.1 Overview of the hypoxic ventilatory response

The physiological manifestation of the HVR is a biphasic response comprising an initial increase in ventilation (phase 1) followed by a secondary depression (phase 2). The level of ventilation peaks about 1 min following the onset of hypoxic exposure and gradually falls down

to an above-baseline plateau in the next 5 min in adults (Moss, 2000). The initial increase is primarily attributed to a classic reflex response to sensory input from the carotid body (a peripheral chemoreceptor located at the bifurcation of the carotid artery). ATP plays an important role in relaying hypoxic signals. Upon hypoxia, ATP is released from the chemosensitive glomus cells of the carotid body and contributes to the excitation of the sinus nerve afferent fibers, primarily through activation of $P2X_2$ (but also $P2X_3$) receptors (Zhang *et al.*, 2000; Prasad *et al.*, 2001). Consistent with the in vitro data, $P2X_2$ knockout mice but not $P2X_3$ knockout mice showed a significant attenuation of the HVR, but the P2X2/3 double knockout have a more attenuated response (Rong *et al.*, 2003). The carotid sinus nerve synapses on neurons of the NTS (Clements *et al.*, 2013), a medullary region that receives and processes visceral afferent information (Andresen & Kunze, 1994; Boscan *et al.*, 2002; Zoccal *et al.*, 2014). ATP is released in the NTS during hypoxia (Mizusawa *et al.*, 1994) from afferent terminals or astrocytes (Accorsi-Mendonca *et al.*, 2013) and, along with other transmitters such as glutamate, activates postsynaptic NTS neurons (Braga *et al.*, 2007; Gourine *et al.*, 2008). Hypoxia-sensitive NTS neurons then activate the RTN via excitatory projection (Stornetta *et al.*, 2006; Takakura *et al.*, 2006), which in turn excites the preBötC (Bochorishvili *et al.*, 2012) and evokes the homeostatic increase in ventilation. While carotid bodies are the primary contributor to the phase I increase in ventilation evoked by hypoxia, other peripheral chemoreceptors likely contribute, most likely aortic bodies. Combined section of glossopharyngeal and carotid sinus nerves produced a greater reduction in the ventilatory response to a brief hypoxia (2 min) given one day after the surgery (note the potential long-term compensation for the loss of carotid body was ruled out) than section of carotid sinus nerve alone (Martin-Body *et al.*, 1985), supporting the hypothesis that additional peripheral mechanisms (in this case glossopharyngeal inputs) are involved in the hypoxic ventilatory

potentiation. These other, non-carotid body peripheral mechanisms may always contribute or they may become functional with time following carotid body denervation.

As mentioned above, the initial phase 1 ventilatory increase does not last throughout the entire period of hypoxic exposure. In adults, after the initial increase in the first minute or so of hypoxic exposure, ventilation decreases over the next 4-5 min to new phase 2, steady state level that remains higher than baseline. This secondary depression to the phase 2 steady state is referred to as the secondary hypoxic respiratory depression, or HRD (Pamenter & Powell, 2016). In premature and most newborn infants, while there is a potent phase 1 response (although less than in the adult)(Lemke *et al.*, 1996; Martin *et al.*, 1998), the secondary hypoxic depression is much more powerful such that ventilation falls well below baseline during the secondary depressive phase, which can exacerbate the hypoxia with life-threatening consequences for premature infants with apnea (Moss, 2000). Hence, understanding the factors that shape the HVR, especially the secondary hypoxic ventilatory depression, is an important question of high clinical relevance, especially for the pediatric population. Dogma has held for decades that there are two main mechanisms involved in the HVR, a carotid body-mediated phase 1 increase and a centrally-mediated secondary hypoxic depression; i.e. the only contribution of the CNS to the HVR is inhibition. However, recent data suggest that in addition to the peripheral chemoreceptors, the dynamics of the secondary hypoxic depression are determined by an interaction between central inhibitory and excitatory mechanisms. The contributions of peripheral and central mechanisms in shaping the HRD are discussed below.

1.4.2 Mechanisms that shape the secondary hypoxic ventilatory depression

The role of peripheral chemoreceptors in the secondary hypoxic ventilatory depression. A common property of many types of sensory receptors is adaptation, which is defined as a reduction in output over time in the presence of a constant stimulus. Adaptation of carotid body chemoreceptor output to a constant hypoxic stimulus could contribute to the secondary ventilatory depression. Consistent with this hypothesis, in ~50% of pentobarbital-anesthetized kittens (< 7 days old), carotid sinus nerve activity responded to hypoxia with a biphasic pattern in which discharge peaked within 30s and then declined (Marchal *et al.*, 1992; Carroll *et al.*, 1993). The time course of the carotid sinus nerve discharge doesn't fit perfectly with that of minute ventilation, which begins to decline after ~ 1 min of hypoxia, but the decrease in the activity of carotid sinus nerve could contribute the secondary hypoxic ventilatory depression. Studies in piglets between P1 and P20 also support a role for carotid body adaptation in the HRD (Mulligan, 1991). Carotid sinus nerve activity in piglets exposed to hypoxia increased rapidly but then declined over a time course that matched the decrease in minute ventilation shown in a separate piglet study (Lawson & Long, 1983). Moreover, carotid body denervation of fetal sheep delayed the onset of the hypoxia-induced apnea, which indicates that carotid body activation depresses the respiratory networks (Bureau *et al.*, 1985). However, there is also evidence suggesting that carotid body adaptation does not underlie the secondary hypoxic ventilatory depression. For example, carotid body activity recorded in 2- to 3-day-old lambs, assessed by measuring the reduction ventilation evoked by rapid inspiration of 100% O_2 (the DeJours test (Dejours, 1962 #3330)) increased initially in response to 8% hypoxia and only decline after 15 min of hypoxia (Carroll & Bureau, 1987). Peripherally chemodenervated lambs at 4 days of age showed reduced ventilation in both initial and secondary phases of the ventilatory response to 7% hypoxia compared with 2-day-old intact lambs, suggesting that the carotid body plays a role in the initial potentiation and stimulating

ventilation during phase 2 (Burr & Sinclair, 1988). A closer look at the response of the carotid body to hypoxia using in vitro isolated preparations reveals that the delayed roll-off of the carotid body activity is only evident during extreme hypoxia (Vidruk *et al.*, 2001; Cummings & Wilson, 2005; Donnelly *et al.*, 2009). For example, action potential activity recorded from the soma of a petrosal neuron that projects to the carotid body increased during the entire duration of 12% hypoxia whereas it became silent following the initial 1 min increase in response to anoxia (Donnelly *et al.*, 2009). Taken together, it appears that the involvement of carotid body adaptation in the secondary hypoxic ventilatory depression depends on the level of hypoxia; its role appears to increase as the level of hypoxia becomes more severe.

Central contribution to the secondary hypoxic ventilatory depression. The potential central mechanisms of the secondary depression involve a direct depressive effect of hypoxia on the metabolism of neurons and glial cells (Mortola, 1993) and hypoxia-induced neuromodulation of the respiratory network. Several neuromodulators are implicated in the modulation of the respiratory network during hypoxia. The roles of adenosine, opioids, GABA and norepinephrine at alpha 2-adrenergic receptors in the secondary hypoxic ventilatory depression are discussed here.

Adenosine and its effect on basal breathing. Adenosine binds to P1 receptors which are further divided in A1, A2A, A2B and A3 receptor subtypes (Haas & Selbach, 2000; Sebastiao & Ribeiro, 2009a). Adenosine has long been proposed as the neurotransmitter responsible for the hypoxic ventilatory depression. To begin, blockade of P1 receptors potentiates baseline breathing (Eldridge *et al.*, 1985), suggesting that breathing networks are under tonic, P1 receptor-mediated inhibition. In addition, exogenous adenosine, through its action in the CNS, depresses breathing in all the species tested including lambs (Koos & Matsuda, 1990; Bissonnette *et al.*, 1991), piglets (Wilson *et al.*, 2004), rabbits (Lagercrantz *et al.*, 1984; Runold *et al.*, 1986), cats (Eldridge *et al.*,

1984, 1985; Schmidt *et al.*, 1995), neonatal (Herlenius *et al.*, 2002) and adult rat whether awake, anesthetized, carotid body intact or denervated (Burr & Sinclair, 1988). This adenosine-mediated inhibition can be prevented or reversed by treatment with the A1 adenosine receptor (P1R) antagonist (8-Cyclopentyl-1,3-dipropylxanthine, DPCPX). More importantly, the profound hypoxic respiratory depression observed in neonatal mammals is dramatically reduced by non-specific adenosine receptor antagonists, such as theophylline, aminophylline and caffeine (Darnall, 1985; Runold *et al.*, 1986; Runold *et al.*, 1989; Bissonnette *et al.*, 1990; Bissonnette *et al.*, 1991; Yan *et al.*, 1995a; Koos *et al.*, 2001; Koos *et al.*, 2005). The adenosine inhibition is age-dependent at least in rats and rabbits where it decreases perinatally (Runold *et al.*, 1986; Herlenius *et al.*, 2002), reflecting a developmental change in the responsiveness of the respiratory network to A1 receptor signaling. Interestingly, the adenosine-induced ventilatory depression spontaneously recovers during a prolonged intracarotid injection. This recovery may be mediated by adaptation of the respiratory network to adenosine stimulation over time through desensitization/internalization of P1 receptors or up-regulation of adenosine transporters which enhance adenosine uptake, reducing the level of extracellular adenosine (Koos & Matsuda, 1990). Whether the levels of extracellular adenosine produced in these infusions studies, and the evoked "recovery" mechanisms are activated during hypoxia, however, remains to be established. Nonetheless, the evidence suggests that adenosine contributes the secondary hypoxic ventilatory depression.

Site of action for adenosine during hypoxia. Tremendous efforts have been made to identify the region responsible for the hypoxic ventilatory depression. The involvement of the pons was first investigated by Dawes and colleagues who showed that hypoxia (8.5% O_2) depressed breathing in the fetal sheep that underwent a brainstem transverse section at the level of optic

chasma or decerebration, while it increased both frequency and amplitude of breathing in those with a more caudal section at the upper pontine/ midcollicular level (Dawes *et al.*, 1983). This section-induced hyperpnoea was also observed in rabbit pups (Martin-Body & Johnston, 1988). Transection at the level of pontine-midbrain junction attenuated the hypoxic ventilatory depression (8% O_2) through a sustained increase in tidal volume and a stronger drive to ventilation reflected by higher inspiratory and expiratory flow rates compared with the control intact pups. In the newborn lambs, which show the typical biphasic response to hypoxia (12% O_2) under control conditions, focal unilateral cooling of the locus coeruleus reversed the secondary hypoxic ventilatory depression but did not affect breathing in normoxia. Importantly, removal of the cold block during hypoxia saw respiratory output fall to levels similar to those seen during the hypoxic respiratory depression in control conditions (Moore *et al.*, 1996). C-fos staining showed that the neurons in the subcoeruleus region of the pons, a noradrenergic cell group like the locus coerruleus, were activated by hypoxia in fetal but not in newborn sheep, suggesting that the subcoeruleus may be responsible for the hypoxia-induced fetus-specific cessation of breathing movements and muscle atonia (Breen *et al.*, 1997). Furthermore, electrolytic lesion of the upper lateral pons at the level of the middle cerebral peduncle in the region of and slightly rostral to the principal sensory and motor nuclei of the trigeminal nerve caused an increase in breathing rate during a 15-minute hypoxic exposure (9% O_2) in fetal lamb while lesion of other pontine regions didn't affect the secondary depression (Gluckman & Johnston, 1987).

While the upper lateral pons is strongly implicated in the hypoxic ventilatory depression, more rostral brain regions are also involved. Bilateral electrolytic lesion of the red nucleus (RN), a structure located in the rostral midbrain, in vagotomised, pre-collicular decerebrated young rabbits (~26-days old) attenuated the hypoxia-induced depression of the phrenic nerve activity

while unilateral RN lesion and lesion of non-RN regions had no effect on the depression. In addition, electrical stimulation of the RN depressed phrenic nerve activity (Waites *et al.*, 1996). Similarly, in the region of the thalamus encompassing the parafascicular nuclei, ibotenic acid lesion attenuated in 7 of 11 sheep (120 days of gestation) the secondary ventilatory depression induced by hypoxia (9% O_2) (Koos *et al.*, 1998). These lines of evidence suggest brain regions from rostral pons to forebrain contribute to the hypoxic depression of ventilation. A role for adenosine accumulation within these rostral regions was explored via microdialysis studies that monitored levels of adenosine in the brains of fetal sheep during hypoxia (9% O_2). Hypoxia increased the concentration of adenosine in the microdialysate collected from the midbrain (Koos *et al.*, 1994) and the subthalamic area (Koos *et al.*, 1997) by ~2-fold (in the presence of adenosine uptake inhibitors, dipyridamole, lidoflazine and 4-nitrobenzylthioinosine). Interestingly, adenosine release was also detected in the NTS in the medulla of piglets (20-25 days old)(Yan *et al.*, 1995a) using microdialysis during hypoxia. However, adenosine sensor data showed that in adult rats, the level of adenosine only increased in the NTS during re-oxygenation after termination of anoxia, which is too late for adenosine to mediate the hypoxia-induced depression of the respiratory activity (Gourine *et al.*, 2002). Another ibotenic acid lesion study revealed that the thalamic parafascicular nuclear complex that has a moderate density of A2A receptors (Koos *et al.*, 2005; Yan *et al.*, 2006) also played an important role in the secondary hypoxic ventilatory depression as its lesion caused a significantly weaker secondary depression (Koos *et al.*, 2016). Consistent with this finding, intracisternally applied CGS21680 (A2A receptor agonist) caused respiratory depression, reflected by a decrease in frequency of the phrenic nerve recording in P5-10 piglets, that was blocked by bicuculline locally injected in the rostral ventrolateral medulla by (Wilson *et al.*, 2004). In addition, application of adenosine specifically in the preBötC reduced

frequency of the XII nerve activity recorded from the rhythmic slices cut from mice aged from P0 - P5 (Zwicker *et al.*, 2011), suggesting that the preBötC may be the site of action of adenosine in the rostral ventrolateral medulla. In spite of the supporting evidence, the relative contribute and importance of these brain regions to the adenosine-mediated ventilatory depression during hypoxia remains to be elucidated.

Source of adenosine. Three mechanisms have been proposed to underlie the accumulation of extracellular adenosine: 1) ectonucleotidase-mediated degradation of extracellular ATP derived from neurons via synaptic release or from astrocytes via multiple release mechanisms; 2) intracellular adenosine that accumulates from metabolism of intracellular ATP and is then transported to the extracellular space down its concentration gradient via the equilibrative nucleoside transporter (ENT); 3) direct release from astrocytes (Martin *et al.*, 2007). The sources and releasing mechanisms for extracellular adenosine vary depending on stimulus (e.g., energy depletion, hypoxia and oxidative stress), brain region and cell type (i.e., neuron vs glia)(Latini & Pedata, 2001). A large body of evidence indicates that adenosine is produced intracellularly before being exported into the extracellular space under ischemic or hypoxic conditions (Meghji *et al.*, 1989; Lloyd *et al.*, 1993; Brundege & Dunwiddie, 1996). However, most of the studies were performed in reduced preparations such as cell culture neurons or hippocampal slices. In contrast to the observations in vitro, an in vivo functional study demonstrated that during hypoxia, adenosine accumulated through breakdown of ATP in the thalamic parafascicular nuclear complex of fetal sheep where it depressed ventilation via A2A receptors as inhibition of ecto-5'-nucleotidases (which degrades ATP into adenosine) but not ENTs prevented the extracellular accumulation of adenosine (Koos *et al.*, 1997). A similar process may also occur in the preBötC. During hypoxia, a delayed release of ATP was detected in the ventrolateral medulla including the

preBötC which coincided with the depressive phase of the HVR (Gourine *et al.*, 2005b). Application of ATP in the preBötC in vitro causes a biphasic response consisting of an initial increase in frequency of the XII nerve bursting followed by a decrease in rat slices which is mediated by adenosine that likely resulted from ATP breakdown (Lorier *et al.*, 2007). Imaging experiments demonstrated that somatosensory astrocytes imaged in an anesthetized rat are sensitive to hypoxia and cultured astrocytes responded to hypoxia with exocytosis of ATP (Angelova *et al.*, 2015a). Such exocytotic release of ATP could also be induced in the RTN astrocytes by acidosis, which presumably contributes to the hypercapnic ventilatory depression (Gourine *et al.*, 2010). Given that activity of ectonucleotidases was detected at the early postnatal ages at least in the preBötC of rats (Huxtable *et al.*, 2009), we propose that adenosine is produced primarily through the degradation of extracellular ATP during hypoxia to mediate the secondary hypoxic ventilatory depression, which also marks the importance of astrocytes as an adenosine sink. Adenosine deaminase and adenosine kinase (ADK) inside astrocytes convert intracellular adenosine into inosine and AMP, respectively, which sets up a concentration gradient for extracellular adenosine accumulated during hypoxia to move across cell membrane into cytoplasm through ENTs. The removal of extracellular adenosine may contribute to the termination of the secondary hypoxic ventilatory depression. The role of the astrocytic adenosine clearance mechanism in shaping the HVR has not been determined yet.

The inhibitory mechanisms: Adenosine, opioids, norepinephrine and GABA

Adenosine, once present in extracellular space, will bind to its receptors on neurons and glia to exert its modulatory effects. Adenosine receptors consist of 4 subtypes of G-protein-coupled receptors namely A1, high affinity A2A and low affinity A2B and A3 receptors. A1, A2A and A3

receptors are predominant types in the CNS. A1 and A2A receptors are reportedly involved in the depressive effect of adenosine on breathing in both normoxia and hypoxia.

A1 receptor-mediated depression of breathing in normoxia and hypoxia. Adenosine A1 receptors are coupled to the Gi-signalling pathway and present on both the pre- and post-synaptic membranes. Activation of pre-synaptic A1 receptors usually leads to inhibition of voltage-dependent Ca^{2+} channels in a cAMP-independent fashion, which usually results in suppression of neurotransmitter release (Mynlieff & Beam, 1994; Cunha, 2001; Sebastiao & Ribeiro, 2009b). On the other hand, activation of post-synaptic A1 receptor causes neuronal hyperpolarization via activation of ion channels such as G protein-coupled inwardly rectifying K^+ channels (Luscher *et al.*, 1997; Dunwiddie & Masino, 2001; James *et al.*, 2018). Activation of A1 receptors signaling pathway in the CNS through intraventricular or sometimes systemic injection depresses breathing in various species in normoxia. N^6-L-2-phenylisopropyl adenosine (L-PIA, A1 receptor agonist) injected in the fourth ventricle decreased the percentage of time during which fetal breathing occurred and reduced the inspiratory slope in unanesthetized fetal sheep (Bissonnette *et al.*, 1991). A similar N^6-R-2-phenylisopropyl adenosine (R-PIA, A1 receptor agonist, i.p. injection)-induced respiratory depression was seen in unanesthetized newborn rabbits (Runold *et al.*, 1986) and anesthetized preterm and newborn rabbit (Hedner *et al.*, 1984; Wessberg *et al.*, 1984). In anesthetized adult rats, intracerebroventricular and intraperitoneal injection of L-PIA reduced the respiratory frequency, tidal volume and minute ventilation in a dose-dependent manner (Wessberg *et al.*, 1984). Similarly in cats, intravenous injection or intraventricular injection into the third ventricle of L-PIA depressed breathing reflected by a reduction in phrenic nerve activity (Eldridge *et al.*, 1985). On the other hand, blocking A1 receptor signaling potentiated basal breathing in adult cats, fetal sheep and lambs (Schmidt *et al.*, 1995; Koos *et al.*, 2001; Koos *et al.*, 2005), indicating

A1 receptors contribute to the tonic inhibition of basal breathing in normoxia. The fact that A1 receptor antagonism also increases inspiratory frequency in reduced brainstem-spinal cord preparations (Kawai *et al.*, 1995) or medullary slices (Wang *et al.*, 2005) suggests that, at least in neonates, a significant component of A1 receptor inhibition originates from the brainstem. It remains possible that A1 receptor-mediated modulation of the inspiratory rhythm is age-dependent as R-PIA inhibition of the inspiratory-related activity in the rat brainstem–spinal cord preparation diminished with postnatal development and disappeared around P3 (Herlenius *et al.*, 1997). In agreement with this finding, work from our lab also demonstrated that, possibly due to developmental changes in the dynamics of the purinome in favor of a reduced buildup of extracellular adenosine, tonic inhibition of breathing by A1 receptors in rhythmic slices from rat was only present in fetal but not postnatal ages and A1 receptor blockade potentiated the ATP-mediated excitation of the inspiratory network only in fetal preparations (Huxtable *et al.*, 2009).

Aside from its baseline effect on breathing, the role of A1 receptor activation in the hypoxic ventilatory depression has also been investigated in vitro and in vivo. In rhythmically-active medullary slices cut from P0-P4 mice, CCPA (2-Chloro-N^6-cyclopentyladenosine, A1 receptor agonist) shortened the duration of hypoxic augmentation of hypoglossal nerve activity and made the hypoxic depression occur earlier, which was reversed by DPCPX (Mironov *et al.*, 2000). In anesthetized cats, systemic application of DPCPX delayed the hypoxia (5% O_2)-induced apnea (Schmidt *et al.*, 1995). Moreover, adult mice in which A1 receptors were knocked out showed an attenuated secondary respiratory depression during severe hypoxia (6-10%)(Heitzmann *et al.*, 2016). These results suggest that A1 receptor signaling contributes to the secondary hypoxic ventilatory depression.

The cells that may mediate the putative A1 receptor inhibition of breathing in hypoxia are not well defined. R-PIA (A1 receptor agonist) decreases the discharge of biphasic expiratory neurons and inspiratory neurons located in medial region of the retrofacial nucleus (a region immediately caudal to the facial nucleus) of neonatal rat in vitro (Wang *et al.*, 2005). Similarly, R-PIA decreased the discharge duration and frequency of inspiratory neurons in the ventral respiratory group of the rat brainstem-spinal cord preparation and hyperpolarized expiratory neurons with a reduction in input resistance (Herlenius & Lagercrantz, 1999) (likely reflecting an activation of K^+ conductance). In mouse rhythmic slices, bath application of CCPA (A1 receptor agonist) depressed the inspiratory rhythm, reduced the amplitude of inspiratory inputs in inspiratory neurons and increased the K_{ATP} open probability. These CCPA effects were later reversed by a forskolin-induced increase in cAMP levels. Consistent with an adenosine A1 receptor-cAMP signalling pathway, a reduction in cAMP level by sodium fluoride (NaF) increased the open probability of K_{ATP} channels (Mironov *et al.*, 1999). The activity of the stage 2 expiratory neurons in anesthetized cats is also modulated pre- and post-synaptically by A1 receptors. DPCPX (A1 receptor antagonist) increased the amplitude of evoked PSPs, and potentiated the inspiratory drive potentials (Schmidt *et al.*, 1995). In contrast to these results demonstrating A1 receptor activation suppressed the respiratory neurons, CCPA failed to modulate the activity of most of inspiratory neurons in the brainstem-spinal cord preparation cut from P0-P4 mice (Brockhaus & Ballanyi, 2000). It will remain unclear whether A1 receptor-modulation of respiratory neurons in the ventral respiratory group, in particular the preBötC, contributes to the secondary hypoxic ventilatory depression until more definite data are available. The involvement of the neurons outside of the ventral respiratory group in the A1 receptor-modulation of breathing also cannot be ruled out.

A2A receptor-mediated depression of breathing in normoxia and hypoxia. Activation of A2A receptors also inhibits the basal breathing in normoxia in various species. In fetal sheep, intraarterial injection of CGS-21680 (A2A receptor agonist) causes a biphasic response in breathing frequency measured through tracheal pressure. The initial increase is mediated by peripheral adenosine A2A receptors as sinoaoritic-denervation abolished it. The secondary depression caused by CGS-21680 is centrally mediated as it was not affected by the denervation (Koos & Chau, 1998). Moreover, the adenosine-mediated reduction of basal breathing frequency was completely abolished by ZM-241385 (A2A receptor antagonist). ZM-241385 when infused also increased the basal frequency of breathing (Koos *et al.*, 2001). Activation of central A2A receptors has similar inhibitory actions on breathing in piglets (Wilson *et al.*, 2004) and rats (Mayer *et al.*, 2006). Injection of CGS-21680 into the fourth ventricle of 5-10 day old piglets caused apnea or reduced the amplitude and frequency of inspiratory bursts recorded from the phrenic nerve. Similarly, intracisternal CGS-21680 abolishes diaphragm EGM activity in P14 and P21 rats but not adults. In a separate study, intracerebroventricular injection of NECA (a different A2A receptor agonist) reduced the respiratory frequency, tidal volume and minute ventilation of adult rats (Wessberg *et al.*, 1984).

A2A receptor activation typically excites neurons through a postsynaptic mechanism involving activation of the Gs-signalling pathway, which leads to an increase in cAMP levels, activation of PKA and phosphorylation of downstream effectors such as ion channels and transcription factors (Fredholm *et al.*, 2011; Sheth *et al.*, 2014). Therefore, the mechanism via which A2A receptor activation inhibits breathing appears to occur through excitation of inhibitory neurons (Wilson *et al.*, 2004; Mayer *et al.*, 2006). Intracisternal injection of bicuculline prior to CGS21680 in rats prevented the A2A receptor-mediated abolition of the diaphragm EMG activity.

Moreover, pre-appliation of $GABA_A$ receptor antagonist, bicuculline, in the rostral ventrolateral medulla of the piglets abolished the CGS21680-induced respiratory depression, suggesting that the location of the A2A receptor-mediated excitation of GABAergic neurons lies rostral to the preBötC. Consistent with these findings, neither CGS21680 nor NECA had an effect on inspiratory rhythm recorded from rhythmically active medullary slices of P4-P14 mice in vitro which do not contain regions rostral to the preBötC (Mironov *et al.*, 1999). It is also possible that A2A receptor inhibition of breathing involves an action in even more rostral brain regions than the medulla, which is supported by the expression profile of A2A receptors (Rosin *et al.*, 1998).

A2A receptor mechanisms appear to contribute to the hypoxic ventilatory depression. Intravascular injection of ZM-241385 (A2A receptor antagonist) blunts the secondary hypoxic ventilatory depression through increasing tidal volume but not inspiratory frequency in piglets (Koos & Chau, 1998) and abolishes the hypoxia-induced inhibition of eye movements and breathing in fetal sheep (Koos *et al.*, 2002). The cells responsible for A2A receptor inhibition of breathing are not known. GABAergic neurons in the rostral ventrolateral medulla that express A2A receptors (Zaidi *et al.*, 2006) are candidates due to the dependency of the A2A receptor-mediated respiratory depression of basal breathing on $GABA_A$ receptor, at least in rats (Mayer *et al.*, 2006) and piglets (Wilson *et al.*, 2004). The relative contribution of A2A receptor mechanism vs other putative mechanisms to the hypoxic ventilatory depression is unclear.

Opioids. Evidence for a role of opioids in the hypoxic respiratory depression is three-fold. First, μ- and δ-opioid receptor antagonists, naltrexone and naltrindole respectively, stimulate breathing in piglets (aged 4-11 and 26-33 days) in both normoxia and hypoxia (Moss *et al.*, 1993b)). In 4-11 day old piglets, only naltrexone increased baseline respiratory frequency and diaphragm activity. In 26-33 day old piglets, naltrexone caused a weaker stimulation of respiratory

timing while naltrindole enhanced the activity of one of the upper airway muscles (the posterior cricoarytenoid, PCA) (Moss *et al.*, 1993b). During hypoxia, naltrindole stimulated PCA activity in both age groups, and naltrexone increased the breathing frequency and slope of diaphragm activity in the older group (Moss *et al.*, 1993a). The second piece of evidence is that hypoxia causes the release of the δ-opioid peptide, met-enkephalin, in the NTS along with adenosine (Yan *et al.*, 1995b) where it may inhibit breathing. The third piece of evidence is that μ- and δ-opioid ligands and receptors are expressed in the respiratory-related brainstem regions (Yan *et al.*, 1995b; Laferriere *et al.*, 1999; Liu *et al.*, 2000). However, more data are needed to definitively establish a contribution of μ- and δ-opioids to the hypoxic ventilatory depression, including establishing their site(s) of action and cellular mechanisms.

α2-adrenergic receptor signaling. Evidence for a role of α2-adrenergic receptor signaling in the HRD in fetal sheep includes that injection of L-657,743 (α2-adrenergic receptor antagonist) into the lateral cerebral ventricles prevented hypoxia-induced apnea in 5 of 6 fetal sheep (Bamford & Hawkins, 1990). Ventricular injection of the α2-adrenergic agonist, clonidine, reduced the incidence of fetal breathing movements by 66% and this inhibition was reversed by L-657,743 (α2-adrenergic antagonist) (Bamford & Hawkins, 1990). Neither systemic administration nor ventricular injection of L-657,743 affected the basal breathing, suggesting that these receptors do not provide a tonic inhibition to breathing under basal conditions and that the α2-adrenergic receptor-meditated inhibitory mechanism was specifically activated during hypoxia. In contrast, aortic injection of idazoxan (α2-adrenergic antagonist) stimulates fetal breathing movements in lambs in normoxia (Bamford *et al.*, 1986). However, this excitation most likely reflects disinhibition of drive from peripheral chemoreceptors. Precise sites in the brain at which the inhibitory action occur are not clear. However, in vitro experiments using the brainstem spinal

cord from rat and mouse have revealed an interaction between α1 excitatory and α2 inhibitory mechanisms within the ventral respiratory column. An excitatory drive comes from the caudal medulla and an inhibitory input comes from the A5 noradrenergic cell group. In rats the inhibitory mechanism dominates while the reverse is true in mice. Whether these medullary mechanisms are activated by hypoxia is not known (Errchidi *et al.*, 1991; Zanella *et al.*, 2006). More work needs to be done to identify the specific site of action for the α2-adrenergic receptor-mediated inhibition of breathing and its underlying cellular mechanism.

GABA. Central administration of GABA or GABA receptor agonists depresses breathing. Injection of GABA or $GABA_A$ receptor agonist muscimol into the cisterna magna or onto the ventral surface of the medulla of anesthetized cats decreases tidal volume and eventually causes apneic events, an effect that could be reversed by intracisternal injection of the $GABA_A$ receptor antagonist, bicuculline (Yamada *et al.*, 1981; Yamada *et al.*, 1982). $GABA_B$ receptor agonization in the ventral medulla caused a similar depression in ventilation in anesthetized cats (Dasilva *et al.*, 1987). On the other hand, blocking $GABA_A$ receptors by systemic application of bicuculline enhanced baseline breathing (Melton *et al.*, 1990). The respiratory depressing nature of GABA makes it a candidate molecule that may mediate the hypoxic ventilatory depression. In agreement with this hypothesis, i.v. administration of midazolam, a drug that increases the affinity of GABA for its receptors, greatly potentiated the secondary hypoxic ventilatory depression induced in human subjects without affecting the initial increase in ventilation (Dahan & Ward, 1991). Moreover, systemic application of bicuculline attenuated the secondary hypoxic ventilatory depression in anesthetized and vagotomized cats (Melton *et al.*, 1990) and piglets (Xiao *et al.*, 2000). Similar to the effect of the $GABA_A$ receptor antagonist, $GABA_B$ receptor antagonization by i.v. injection CGP-35348 during hypoxia phase restored the minute ventilation (Huang *et al.*,

1994). The NTS appears to be one of the sites responsible for the GABA-mediated ventilatory depression during hypoxia. Microdialysates collected from the NTS of awake rats showed that hypoxia increased the extracellular concentration of GABA. Consistent with a respiratory depressing effect of GABA, local application of GABA receptor agonists muscimol and, the $GABA_B$ receptor agonist, baclofen, in the NTS further reduced ventilation during hypoxia whereas microinjection of GABA receptor antagonists, bicuculline and saclofen ($GABA_B$ receptor antagonist), in the NTS attenuated the hypoxic ventilatory depression (Tabata *et al.*, 2001). Interestingly, this GABA-mediated hypoxic ventilatory depression is dependent on peripheral chemoreceptor stimulation as hypoxia-induced GABA release in the NTS and GABA receptor antagonization-mediated attenuation of the secondary hypoxic ventilatory depression were absent in rats with carotid body denervation. Whether other brain regions such as those in the ventrolateral medulla where GABA depresses breathing (Dasilva *et al.*, 1987; Wilson *et al.*, 2004) also contribute to the GABA-mediated hypoxic ventilatory depression is not clear.

1.5 Role of ATP signaling in central respiratory chemosensing of hypercapnia and hypoxia

The conventional view of the HVR is that the secondary hypoxic depression reflects a gradually (2-5 min) developing central inhibitory mechanism superimposed on the carotid-body mediated excitation; i.e., it reflects two primary processes, a peripheral excitation and a central inhibition (involving adenosine and perhaps other transmitter systems)(Pamenter & Powell, 2016). Data have emerged over the last 14 years that directly challenge this dogma; these data suggest that in addition to the peripheral chemoreceptor excitation, a central excitatory mechanism is activated by hypoxia that attenuates the hypoxic ventilatory depression. The transmitter ATP plays a central role in this novel, central excitatory component to the homeostatic ventilatory response

to hypoxia, the HVR. ATP signaling in the brain also appears to play an important part in the homeostatic ventilatory response to CO_2. Thus, the following sections will provide a brief overview of the purinergic signaling system, with a primary focus on P2 receptors. This is followed by a brief overview of the role played by P2 signaling in central CO_2 chemoreception and finally its role in the HVR, with a specific focus on ATP signaling through the metabotropic $P2Y_1$ receptor subtype most strongly implicated in the HVR.

1.5.1 Purinergic signalling: a brief overview

Purinergic signalling refers to a complex system encompassing the signaling actions of extracellular purine nucleotides at P2 receptors (primarily ATP) and nucleosides (adenosine) at P1 receptors. The concept was first proposed by Burnstock in 1972 (Burnstock, 1972) after the discovery that ATP was responsible for the non-adrenergic, non-cholinergic (NANC) component of synaptic signalling to neurons in the gut (Burnstock *et al.*, 1970). Purinergic signalling features interaction between signalling molecules and their receptors that were not cloned until the early 1990's. Purinergic receptors can be classified into two groups: P1 and P2 receptors. P1 receptors are selective for adenosine and consist of four subtypes: A1, A2A, A2B and A3. P2 receptors are sensitive to purine and pyrimidine nucleotides and can be further divided into two groups: ligand-gated ion channel receptor/ionotropic P2X receptors that have seven subtypes ($P2X_{1\text{-}7}$) and G protein-coupled receptor/metabotropic P2Y receptors that have eight subtypes ($P2Y_{1/2/4/6/11/12/13/14}$) (Burnstock, 2018).

Other important player in purinergic signalling is ecto-nucleotidases, which determines the relative contribution of P1- vs P2- receptor-mediated mechanisms by breaking ATP/ADP down to adenosine (Deaglio & Robson, 2011). There are four types of ectonucleotidases with different preferred substrates and end products including: ectonucleoside triphosphate

diphosphohydrolases, nucleotide pyrophosphatase/phos-phodiesterases, alkaline phosphatases, 5′-nucleotidase and monoamine oxidase. Once adenosine is produced, additional components of the purinergic signalling system come into play that are important in clearing extracellular adenosine and terminating its actions. These include equilibrative nucleoside transporters, ENTs, that passively transport adenosine across cell membranes down its concentration gradient. There are also the intracellular enzymes, adenosine kinase and adenosine deaminase, that convert adenosine into AMP or inosine, respectively, which maintain intracellular adenosine levels below extracellular levels.

In the context of the response of the preBötC network to hypoxia, the working model on the underlying purinergic signalling involves a release of ATP from O_2 sensitive astrocytes in the preBötC (Angelova *et al.*, 2015a). ATP then depolarizes inspiratory neurons via its action on $P2Y_1$ receptors (Lorier *et al.*, 2007; Huxtable *et al.*, 2009; Zwicker *et al.*, 2011; Rajani *et al.*, 2018). ATP, once present in the extracellular space, will be broken down to adenosine by ecto-nucleotidases. The resultant adenosine will bind to P1 receptors on both pre- and post-synaptic membranes of inspiratory neurons to suppress synaptic transmission and directly hyperpolarize inspiratory neurons, respectively. The inhibitory effect of adenosine will be terminated once excessive extracellular adenosine is cleared through the ENT-dependent mechanisms mentioned above. Intracellular adenosine clearing mechanisms are important since they ensure an influx of adenosine into the intracellular space down its concentration gradient via ENTs by create an intracellular sink for adenosine.

1.5.2 P2 receptor signaling in central chemoreception (CO_2)

Central chemoreception is the regulatory process in the CNS that excites breathing in response to an increased CO_2 stimulation (Guyenet *et al.*, 2010). Chemosensitivity has been

documented in multiple sites including the caudal NTS, RTN, preBötC, medullary raphe and locus coeruleus collectively mediates central chemoreception (Nattie & Li, 2009). Whether neurons in those regions are all chemosensitive and contributes equally (Nattie & Li, 2009; Dean & Putnam, 2010; Gargaglioni *et al.*, 2010) or the RTN is the only pH-sensitive chemoreceptive region that receives and integrates chemosensory information from the other brain regions (Guyenet *et al.*, 2010) is under debate. Neuronal chemosensitivity is often assessed based on their response to pH which serves as a proxy of PCO_2. Considerable efforts have been put to discern the pH sensing mechanisms which lead to the discovery of background K^+ current (Mulkey *et al.*, 2004), two-pore-domain potassium 2 (TASK2)(Wang *et al.*, 2013) and G-protein coupled receptor 4 channel (GPR4)(Kumar *et al.*, 2015) as potential molecular identities of the neuronal CO_2 sensors. Double knockout of TASK2 and GPR4 specifically in Phox2b-expressing RTN neurons almost completely blocked the hypercapnic ventilatory response, suggesting that the RTN plays a critical role in CO_2 chemoreception (Kumar *et al.*, 2015). Data also reveal the possibility that astrocytes can have their own intrinsic pH sensor such as heteromeric inwardly rectifying potassium (Koch *et al.*) 4.1 - Kir 5.1 channels (Wenker *et al.*, 2010) and contribute to the CO_2 chemoreception likely via a purinergic signalling-mediated neuronal depolarization (Mulkey *et al.*, 2006; Gourine *et al.*, 2010; Wenker *et al.*, 2010).

The Role of P2 receptor signaling in the CO_2 chemoreception started to receive attention due to the findings in Xenopus oocytes (King *et al.*, 1996) and HEK293 cells (Stoop *et al.*, 1997) that exogenously expressed $P2X_2$ receptors produced inward ATP currents that increased several fold in response to physiologically relevant changes in pH. $P2X_2$ receptors are expressed in medullary regions including the rostral ventrolateral medulla (Gourine *et al.*, 2003) and preBötC (Thomas *et al.*, 2001) and this was initially hypothesized to underlies the ATP sensitivity of

neurons in the rostral ventrolateral medulla (Ralevic *et al.*, 1998). Consistent with this hypothesis, desensitizing P2X receptors with αβ-methylene in the ventrolateral medulla attenuated the hypercapnic ventilatory response (Thomas *et al.*, 1999), suggesting that a $P2X_2$ receptor-dependent mechanism in the ventrolateral medulla may contribute to the CO_2 chemoreception. However, this hypothesis was later refuted as $P2X_2$ and $P2X_2/P2X_3$ double knockout mice had normal CO_2 sensitivity (Rong *et al.*, 2003). Although the involvement of $P2X_2$ receptors as the CO_2 sensors was ruled out, it remained possible that ATP contributes to CO_2 chemoreception through a CO_2 sensing mechanism that caused ATP release and its subsequent activation of other P2 receptors on chemosensitive neurons. Indeed, it was later shown that hypercapnia induces an ATP release in regions of the ventrolateral medulla which correspond to the chemosensitive RTN and medullary raphe (Gourine *et al.*, 2005a). Two mechanisms have been proposed that underlie the CO_2-induced ATP release. In the RTN, it is found that astrocytes respond to a 0.2 decrease in pH with an exocytosis of ATP which can be blocked by brefeldine A or bafilomycin A, two drugs that interference with vesicular release (Gourine *et al.*, 2010). On the other hand, the CO_2-induced ATP release in the ventrolateral medulla is found to be mediated by connexin 26 hemichannels as blockade of these channels prevented the CO_2-induced ATP release and attenuated the hypercapnic ventilatory depression (Huckstepp *et al.*, 2010a; Huckstepp *et al.*, 2010b). It is possible that these two mechanisms coexist and both contribute to the ATP-mediated CO_2 chemoreception.

Whether ATP receptor activation of RTN neurons is responsible for RTN chemosensitivity has been controversial. The majority of data indicate that RTN neurons are intrinsically chemosensitive (Mulkey *et al.*, 2004; Stornetta *et al.*, 2006) and that this is not dependent on ATP (Mulkey *et al.*, 2006). In agreement with this view, local application of PPADS in the RTN only attenuated the CO_2-induced firing rate increase in RTN neurons by 30% (Wenker *et al.*, 2012). In

contrast, blocking $P2Y_1$ receptor with MRS 2179 abolished the acidosis (from pH 7.4 to 7.0)-induced depolarization of chemosensitive RTN neurons. The most parsimonious explanation is that there are two CO_2 sensing mechanisms, neuronal and glial, and that the glial mechanism converges on chemosensitive RTN neurons to increase their excitability and CO_2 response. However, this discrepancy may also reflect a poor specificity of the PRSx8 promoter by by Gourine et al. (2010) for labeling chemosensitive RTN neurons, which also labels $P2Y_1$ receptor-sensitive, non-chemosensitive catecholaminergic C1 neurons (Card *et al.*, 2006). Thus, the blockade of pH sensitivity of RTN neurons by Gourine et al. (2010) may reflect that they were recording from C1, rather than Phox2b chemosensitive neurons.

The medullary raphe may also contribute to CO_2 chemoreception via activation of RTN neurons (Wu *et al.*, 2002; Mulkey *et al.*, 2007; Depuy *et al.*, 2011). Despite immunolabeling for multiple P2 receptors (Close *et al.*, 2009), local application of ATP in the medullary raphe did not affect the baseline rhythm or the cardiorespiratory response to hypercapnia in anesthetized vago-sino-aortic denervated rats (Sobrinho *et al.*, 2014). In contrast, injection of PPADs in the rostral medullary raphe attenuated the hypercapnic ventilatory response in unanesthetized rats (da Silva *et al.*, 2012). The opposite results most likely reflect that PPADS has off target actions when used at concentrations higher than 10 μM, including glutamate receptor antagonism (Motin & Bennett, 1995), which would reduce respiratory activity independent of any action at P2 receptors. (anesthetized vs unanesthetized rats). Other potential chemoreceptive sites include the preBötC (Kawai *et al.*, 1996; Lorier *et al.*, 2008) and the locus coeruleus (Gargaglioni *et al.*, 2010). However, there are currently no data supporting a role of P2 signalling pathway in CO_2 chemoreception in these regions.

1.5.3 P2 receptor signaling in the HVR

The conventional view of the HVR is that the secondary hypoxic depression is an interplay between a central inhibition and the carotid-body mediated excitation. However, recent evidence from our lab and others challenges this dogma by showing that there is a centrally mediated, $P2Y_1$ receptor-dependent excitation of the inspiratory network that occurs during hypoxia and offsets the secondary hypoxic ventilatory depression. This hypothesis first emerged in 2005 with the demonstration that during hypoxia, a slow onset release of ATP was detected on the ventral medullary surface of anesthetized rats. Blockade of ATP receptors via application of the non-selective P2 receptor antagonist, PPADS, on the surface of the ventrolateral medulla significantly decreased ventilation during the secondary depressive phase in anesthetized rats, suggesting that ATP excites ventilation and offsets the secondary hypoxic ventilatory depression (Gourine *et al.*, 2005b). To assess where in the medulla it might have this excitatory effect, ATP was injected within the ventrolateral medulla in a three-dimensional grid and the network effects evoked were compared. It was found that ATP caused the strongest network excitation when injected in the area corresponding to the preBötC, suggesting that the preBötC is a potential site of action for the ATP-mediated attenuation of the secondary hypoxic ventilatory depression (Lorier *et al.*, 2007). In the same study, a variety of P2 receptor agonists were applied in the preBötC and their effects on the inspiratory rhythm were assessed to explore the receptor subtype through which ATP excites the inspiratory network. The response profile suggested that $P2Y_1$ receptor might underlie the ATP-mediated excitation. In agreement with the hypothesis, pre-application of the $P2Y_1$ receptor selective antagonist MRS 2179 abolished the ATP-induced frequency increase (Lorier *et al.*, 2007). The involvement of the $P2Y_1$ receptor-dependent mechanism in the hypoxic ventilatory response was later examined in anesthetized and vagotomized adult rats (Rajani *et al.*, 2018). The

evidence that unilateral blockade of $P2Y_1$ receptors specifically in the preBötC potentiated the secondary hypoxic ventilatory depression strongly suggests that ATP offsets the secondary depression through a $P2Y_1$ receptor-mediated excitation of the inspiratory network. ATP, however, is broken down by ectonucleotidases into adenosine, which exerts inhibitory actions via P1 receptors. Indeed, the actions of ATP in the preBötC in vitro are determined by an interaction between magnitude of the ATP excitation and the adenosine inhibition (Huxtable *et al.*, 2009). This interaction may also be important in shaping the secondary hypoxic ventilatory depression. The contribution of glia to the network effect of ATP was first implicated in the study where the frequency increase evoked in hypoglossal nerve discharge by local injection of ATP into the preBötC was abolished after the slices had been incubated in the glial toxins, fluoroacetate (FA, glial aconitase enzyme inhibitor) or methionine sulfoximine (MSO, glial glutamine synthetase enzyme inhibitor) while the substance P effect remained unchanged (Huxtable *et al.*, 2010). In vivo imaging of the somatosensory cortex of anesthetized rats showed that calcium-sensitive dye OGB1-labeled astrocytes respond to hypoxia with an increase in intracellular Ca^{2+} concentration. Combined with the finding that hypoxia triggers vesicular release of ATP from cultured astrocytes (Angelova *et al.*, 2015b), data suggest that astrocytes are local O_2 sensors in the preBötC that detect hypoxia and respond with ATP release. The mechanism underlying hypoxia-induced astrocytic ATP release includes inhibition of mitochondrial respiration, consequent mitochondrial depolarization, production of free radicals, lipid peroxidation, activation of phospholipase C and inositol triphosphate receptors and the Ca^{2+} release from the intracellular stores (Angelova *et al.*, 2015a). Blockade of glial ATP release (via expression of tetanus toxin light chain) or purinergic signaling (via expression of a potent ectonucleotidases, TMPAP) in the preBötC and surrounding areas potentiated the secondary hypoxic ventilatory depression in the carotid body-intact

(Angelova *et al.*, 2015a) and peripherally chemodenervated rats (Rajani *et al.*, 2018). These data support the hypothesis that astrocytes in the preBötC are central O_2 sensors independent of the carotid body (Funk & Gourine, 2018a) and argue against the view held by some that the excitatory component of the HVR is solely mediated by the peripheral chemoreceptors (Funk & Gourine, 2018b; Teppema, 2018). Taken together, the evidence suggests that preBötC astrocytes release ATP in response to hypoxia which attenuates the hypoxic ventilatory depression by exciting the inspiratory network through its action on $P2Y_1$ receptors. How ATP, following its release, depolarizes inspiratory neurons and ultimately excites the network is not known and is the focus of my thesis.

1.5.4 $P2Y_1$ receptor-coupled signaling pathways

The $G\alpha_q$-signaling pathway. The coupling of $P2Y_1$ receptors to the $G\alpha_q$ protein was first postulated in the studies where activation of heterologously expressed $P2Y_1$ receptors in various expression systems was shown to stimulate inositol phosphate accumulation, a characteristic feature of Gαq- pathway activation, without affecting the level of cAMP (Simon *et al.*, 1995; Schachter *et al.*, 1996). Such coupling was later confirmed by the reconstitution experiments where activation of $P2Y_1$ receptors reconstituted in proteoliposomes together with $G\alpha_q$ and $G\beta_1\gamma_2$ caused an increase in GTP hydrolysis, indicating a $P2Y_1$ receptor-mediated activation of $G\alpha_q$ protein (Waldo & Harden, 2004). Numerous studies now support that the $G\alpha_q$-signaling pathway is part of the $P2Y_1$ receptor signal transduction mechanism in both neurons (Usachev *et al.*, 2002; Filippov *et al.*, 2004; Song *et al.*, 2007; Milenkovic *et al.*, 2009) and glia (Schachter et al., 1996; Schachter et al., 1997; Weissman et al., 2004; Weng et al., 2008; Lipp et al., 2009; Jacob et al., 2014). The canonical $G\alpha_q$-signaling pathway (see Fig. 2.3 for the overview) starts with activation of the heterotrimeric G protein which leads to dissociation of α_q and βγ subunits (Wettschureck &

Offermanns, 2005). The α_q subunit activates phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to IP_3Rs on the endoplasmic reticulum, triggering Ca^{2+} release. Protein kinase C (PKC) can be activated by Ca^{2+} and DAG. Intracellular Ca^{2+} and DAG have short- and long-term effects on neuronal excitability through processes that include ion channel (Vergara et al., 1998; Veale et al., 2007) and receptor modulation (Krishek et al., 1994; Lan et al., 2001), receptor trafficking (Park *et al.*, 1999; Chung *et al.*, 2000; Hayashi *et al.*, 2000; Lan *et al.*, 2001; Boehm *et al.*, 2006) and protein synthesis (Hu et al., 2007), remodeling of synaptic structure (Matsuoka *et al.*, 1996; Chung *et al.*, 2000; Hayashi *et al.*, 2000; Fong *et al.*, 2002; Star *et al.*, 2002; Rabenstein *et al.*, 2005) and regulation of gene transcription (Bito *et al.*, 1996). The $\beta\gamma$ subunit has independent signaling actions by interacting directly with ion channels (Herlitze *et al.*, 1996b; Ikeda, 1996), or by regulating gene transcription (Park et al., 1999; Spiegelberg & Hamm, 2005). PIP_2 can also modulates activity of some ion channels directly (Suh & Hille, 2002; Wu *et al.*, 2002; Ford *et al.*, 2003; Lopes *et al.*, 2005; Suh & Hille, 2008; Vaithianathan *et al.*, 2008). A review of the literature for $P2Y_1$ receptor-modulated ion channels/currents that are capable of influencing the inspiratory rhythm has identified Ca^{2+}-activated potassium channels (BK/SK), the M-current (KCNQ), I_{CAN}, K_{ATP} channels, TASK channels and N-type voltage-gated Ca^{2+} channels as potential targets of the $G\alpha_q$-signaling pathway coupled to $P2Y_1$ receptors (Rajani *et al.*, 2016).

The $G\alpha_i$-signalling pathway. In addition to $G\alpha_q$, there is limited evidence that $P2Y_1$ receptors couple to the $G\alpha_i$ pathway and inhibit P/Q-, N- and L- type voltage-gated Ca^{2+} channels via $\beta\gamma$ subunits (Brown *et al.*, 2000a; Filippov *et al.*, 2000; Aoki *et al.*, 2004). ATP-induced inhibition of Ca^{2+} currents (I_{Ca}) carried by P/Q-, N- and L-type Ca^{2+} channels evoked in NTS

neurons was prevented by bath application of the $P2Y_1$ receptor antagonist, MRS2179, or intracellular dialysis of an α_i, but not α_q or α_s, subunit antibody, suggesting a link between $P2Y_1$ receptors and the $G\alpha_i$-signaling pathway. The I_{Ca} inhibition could be relieved by pre-pulses of strong depolarizing voltage, which is consistent with a voltage-dependent $\beta\gamma$ subunit-dependent mechanism (Herlitze *et al.*, 1996b; Ikeda, 1996; Aoki *et al.*, 2004). Inhibition of N-type Ca^{2+} currents via activation of heterologously expressed $P2Y_1$ receptors in superior cervical ganglion (SCG) neurons involves a pertussis toxin (PTX)-sensitive component, which again suggests an involvement of $G\alpha_i$ (Brown *et al.*, 2000a). N-type and P/Q type Ca^{2+} channels are present in both pre- and post-synaptic terminals and carry high voltage-activated Ca^{2+} currents (Catterall, 2011). However, unlike the ubiquitous expression of N-type Ca^{2+} channels in respiratory neurons, P/Q-type Ca^{2+} channels are only detected on pre-inspiratory neurons and a subset of inspiratory neurons (Onimaru et al., 1996). Although Ca^{2+} entry through N-type voltage-gated Ca^{2+} channels is required for breathing in vivo (Ramirez *et al.*, 1998b), blocking N-type Ca^{2+} channels in vitro has minor effects (<15%) on rhythm (Onimaru *et al.*, 2003; Lieske & Ramirez, 2006; Morgado-Valle *et al.*, 2008; Koch *et al.*, 2013). The inconsistency can be attributed to the discrepancy in experimental conditions. P/Q-type Ca^{2+} channel knockout mice have reduced synaptic efficacy and a progressive respiratory perturbation during postnatal development that is eventually lethal (Koch *et al.*, 2013). L-type Ca^{2+} channels carry a high voltage-activated Ca^{2+} current with a slower rate of inactivation. They are expressed in all classes of respiratory neurons (Onimaru et al., 1996). Inhibition of L-type Ca^{2+} channels also slows rhythm, while potentiation of L-type Ca^{2+} currents by mGluR1/5 increases frequency (Mironov & Richter, 2000a). mGluR1/5 and $P2Y_1$ receptors both signal through $G\alpha_{q/11}$. Taken together, it is conceivable that potentiation of Ca^{2+} currents contributes to the $P2Y_1$ receptor-mediated frequency increase. However, the only documented

action of $P2Y_1$ receptors on voltage-dependent Ca^{2+} channels (VDCCs) is inhibition (Brown *et al.*, 2000a; Filippov *et al.*, 2000; Aoki *et al.*, 2004), which would decrease rather than increase frequency and argues against a role of the $G\alpha_i$-signaling pathway in $P2Y_1$ receptor-excitation of the inspiratory network.

The $G\alpha_s$-signaling pathway. The $G\alpha_s$-signaling cascade starts with the activation of adenylyl cyclase (AC), which increases cyclic AMP (cAMP) level leading to cAMP-dependent activation of protein kinase A (PKA). The evidence for the coupling between $P2Y_1$ receptor and the $G\alpha_s$-signaling pathway is extremely scarce. To my knowledge, there are only three studies that document $P2Y_1$ receptor-activation of the $G\alpha_s$-signaling pathway and none of these are associated with alterations in neuronal excitability. 1) ATP protects pancreatic duct epithelial cells from alcohol-induced damage through its action on $P2Y_1$ receptors, which increases the cAMP level and consequently activates PKA (Seo *et al.*, 2016). 2) In xenopus kidney cells (A6 cells), $P2Y_1$ receptor-induced Cl^- efflux is dependent on PKA activation (Guerra *et al.*, 2004). 3) During development, adenylyl cyclase 5 activation and increased cytoplasmic cAMP levels are essential for $P2Y_1$ receptor-induced axonal elongation of hippocampal neurons (del Puerto *et al.*, 2012). We have found no evidence of a $P2Y_1$ receptor-mediated change in neuronal excitability that is produced through activation of the $G\alpha_s$-signaling pathway.

1.6 Thesis objectives

Newborn (especially preterm) infants experience frequent hypoxia which evokes the HVR. The secondary ventilatory depression of the biphasic response is a lot greater in infants than in adults which exacerbates the hypoxia, causing an even greater respiratory depression, with life-threatening consequences. Caffeine treatment is ineffective in about 20% of patients who suffer from apnea of prematurity. Developing an alternate means of stimulating breathing for this

population is of great clinical importance. Puringergic signaling in the preBötC is a strong candidate in that ATP is released in the preBötC during hypoxia where it offsets the secondary hypoxic ventilatory depression via its action on $P2Y_1$ receptors. The signaling pathway coupled to $P2Y_1$ receptors and its final effector (ion channels in this cases) are not known. The overarching objective of my thesis is to identify the signaling pathway(s) and ion channel(s) through which $P2Y_1$ receptor signaling excites inspiratory neurons and the inspiratory network. We will test the hypothesis that $P2Y_1$ receptors excite inspiratory neurons and the inspiratory network in vitro via activation of the $G\alpha_q$-signaling pathway and ion channels that are reportedly sensitive to modulation by the $G\alpha_q$-signalling pathway and capable of affecting the inspiratory rhythm. Candidates include ATP-sensitive potassium (K_{ATP}) channel, G protein-coupled inwardly-rectifying potassium (GIRK) channel, transient receptor potential cation channel subfamily M member 4 (TRPM4), small conductance calcium-activated potassium (SK) channel and big conductance calcium-activated potassium (BK) channel.

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Chapter 2. ATP excitation of the preBötzinger Complex inspiratory rhythm generating network in vitro is mediated by $P2Y_1$ receptors acting through $G\alpha_q$ GPCR signalling pathway.

2.1 Abstract

Hypoxia ATP offsets the hypoxic ventilatory depression via its actions on $P2Y_1$ receptors in the preBötC in vivo, which challenges the long-lasting view that only central contribution to the hypoxic ventilatory depression is inhibition. Here we first developed in neonatal rat slices an in vitro protocol that evoked a reproducible, biphasic ventilatory response to anoxia similar to that reported in juvenile mice, in order to directly examine mechanisms underlying this ATP excitation of the inspiratory network during hypoxia. However, we found that local application of MRS 2279 ($P2Y_1$ receptor antagonist, 500 μM) in the preBötC did not alter the anoxia-induced ventilatory depression in the 700 μm thick, rhythmically-active medullary slices, suggesting that purinergic signalling mechanisms involved in the in vitro anoxic ventilatory response differ from those involved in the response to moderate hypoxia in vivo. Therefore, based on the established contribution of preBötC $P2Y_1$ receptor signalling to the HVR in vivo and that the frequency increase evoked by ATP in the preBötC in vitro is mediated by $P2Y_1$ receptors, we focused on defining the signalling pathway(s) via which exogenously applied ATP/$P2Y_1$ receptor agonists excite the preBötC inspiratory network in rhythmic medullary slices. Given the conventional coupling of $P2Y_1$ receptors with the $G\alpha_q$-signalling pathway, we examined the role of the $G\alpha_q$-signalling pathway in $P2Y_1$ receptor-mediated excitation of inspiratory neurons and the network. On the cellular level, intracellular dialysis of the $G\alpha_q$ second messenger blockers U73122 (phospholipase C blocker, 2 μM), 2-APB (inositol triphosphate receptor blocker, 50 μM), chelerythrine (protein kinase C inhibitor, 10 μM), thapsigargin (sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, 4 μM) or cyclopiazonic acid (SERCA inhibitor, 20 μM) attenuated the 5 mM ATP-induced inward currents by 20% - 30%. Blocking $P2Y_1$ receptors with MRS 2279 caused a similar 30% - 35% reduction in the ATP (5mM) current. In addition, U73122 and

chelerythrine attenuated the MRS 2365 ($P2Y_1$ receptor agonist, 100 μM)-induced inward currents by 50 ± 7.7% and 55 ± 3.5%. On the network level, bath application of 2-APB (50 μM) and chelerythrine (10 μM) reduced the MRS 2365-induced frequency increase of the inspiratory-related activity recorded from the hypoglossal (XII) nerve rootlets of the rhythmic slices by 59 ± 13% and 55 ± 7% respectively. Lastly, in trying to identify the ion channel through which $P2Y_1$ receptor activation increases frequency, we ruled out involvement of the: i) ATP-sensitive potassium channel (K_{ATP}); ii) G protein-coupled inwardly-rectifying potassium channel (GIRK); iii) transient receptor potential cation channel subfamily M member 4 (TRPM4); iv) small conductance calcium-activated potassium channel (SK); and, v) big conductance calcium-activated potassium channel (BK). Taken together, these results suggest that ATP excitation of the inspiratory network is produced, at least in part, via $P2Y_1$ receptor activation of the $G\alpha_q$-signalling pathway in inspiratory neurons in the preBötC.

2.2 Introduction

Breathing is a complex, robust and dynamic behavior responsible for gas exchange and the homeostatic control of blood gases. This control is achieved by adaptive adjustment of ventilation to changes in the metabolic, environmental and behavioral demands under a wide range of physiological and pathological conditions. Such adjustment results from the interplay between excitation and inhibition of the respiratory network mediated by neuromodulators like adenosine triphosphate (ATP). In mammals, the inspiratory rhythm is generated in the pre-Bötzinger complex (preBötC), a brain region located in the ventrolateral medulla (Smith *et al.*, 1991; Del Negro *et al.*, 2018). Hypoxia (low oxygen) induces a biphasic ventilatory response comprising an initial increase in ventilation followed by a secondary depression (Moss, 2000). The initial increase is primarily mediated by the carotid bodies (peripheral chemoreceptors located at the bifurcation of the carotid artery) as denervation of the carotid body greatly attenuates the potentiation (Favier & Lacaisse, 1977; Martin-Body *et al.*, 1985). The secondary hypoxic ventilatory depression is not completely understood but is primarily attributed to central mechanisms (Boddy *et al.*, 1974; Dawes *et al.*, 1983; Gluckman & Johnston, 1987; Martin-Body & Johnston, 1988). The mechanisms underlying the hypoxic respiratory depression are very relevant clinically because the depression is most severe in premature infants (~8% of births in Canada)(Shah *et al.*, 2018) with immature respiratory networks (Moss, 2000). Despite a strong initial increase, the level of ventilation in premature mammals falls well below baseline during the secondary depressive phase, which produces greater hypoxia, greater respiratory depression and can result in a life-threatening positive feedback loop. Caffeine, an adenosine receptor antagonist, is used as a respiratory stimulant to treat children with AOP. It is very effective, but ~20% of infants do not respond or suffer significant side effects, including seizure (Lista *et al.*, 2016). Thus, there is great

interest in understanding the mechanisms underlying this ATP-mediated excitation for its potential as an alternate means of stimulating breathing during hypoxia.

The depressing effect on breathing of adenosine, a metabolite of ATP, has been identified in multiple species (Eldridge *et al.*, 1984; Lagercrantz *et al.*, 1984; Eldridge *et al.*, 1985; Runold *et al.*, 1986; Burr & Sinclair, 1988; Koos & Matsuda, 1990; Bissonnette *et al.*, 1991; Schmidt *et al.*, 1995; Herlenius *et al.*, 2002; Wilson *et al.*, 2004). Consistent with the hypothesis that adenosine underlies the hypoxic ventilatory depression, accumulation of extracellular adenosine is detected during hypoxia. Adenosine protects brain from brief periods of hypoxia by reducing brain activity and consequently metabolic demand. This depression is adaptive in all brain regions except in the respiratory network and cardiovascular centers where it becomes maladaptive as depression of activity of these regions impairs ventilatory and cardiac efforts needed to recover from hypoxia. However, central hypoxia does not just evoke inhibitory mechanisms. Specific brain regions involved in cardiorespiratory control are excited by hypoxia. For instance, C1 neurons critical in control of heart rate and blood pressure are potently excited by hypoxia (Koshiya *et al.*, 1993; Sun & Reis, 1993; Erickson & Millhorn, 1994; Sun & Reis, 1994; Hirooka *et al.*, 1997; Guyenet *et al.*, 2013). In addition, recent data suggest that ATP is released in the preBötC during the secondary depressive phase where it stimulates breathing and offsets the secondary depression (Gourine *et al.*, 2005b; Angelova *et al.*, 2015a; Rajani *et al.*, 2018). Thus, the preBötC appears to be one of a few unique brain regions that mount an excitatory response to hypoxia. This is in direct contrast to the dogma that the only contribution of the central nervous system to the hypoxic ventilatory (HVR) is inhibition (Funk & Gourine, 2018a).

A revised view of central processes evoked by central hypoxia is that astrocytes in the preBötC are hypoxia sensors that release ATP in responses to a hypoxia-induced inhibition of

astrocytic mitochondrial respiration (Angelova *et al.*, 2015a). ATP then excites the inspiratory network via activation of $P2Y_1$ receptors on inspiratory neurons in the preBötC, which leads to an increase in ventilation that reduces the secondary hypoxic ventilatory depression (Lorier *et al.*, 2007; Rajani *et al.*, 2018). Neither the second messenger signalling cascades nor the ion channels through which $P2Y_1$ receptors excite inspiratory neurons and the inspiratory network during hypoxia are known. $P2Y_1$ receptors signal through the $G\alpha_q$-second messenger pathway in other brain regions (Usachev *et al.*, 2002; Song *et al.*, 2007; Milenkovic *et al.*, 2009) so this pathway and ion channels downstream of its activation that excite breathing were the focus of this investigation.

To address these questions, we used rhythmically-active medullary slice preparations that greatly facilitate the pharmacological exploration of signalling pathways and their effector ion channels. We compared the network frequency responses evoked by ATP (or $P2Y_1$ receptor agonists) injected into the preBötC and currents evoked in inspiratory preBötC neurons by ATP (or $P2Y_1$ receptor agonists), before and after bath application, local application or intracellular dialysis of specific second messenger/ion channel blockers. We first established in rat a protocol that produced a repeatable biphasic ventilatory response similar to that reported previously in mice (Ramirez *et al.*, 1998a; Telgkamp & Ramirez, 1999) to examine responses to hypoxia and endogenous ATP release. However, unlike the ATP-mediated excitation of the preBötC network in vitro (Lorier *et al.*, 2007) and the ATP-mediated excitation of the preBötC during hypoxia in vivo (Rajani *et al.*, 2018), which are both sensitive to $P2Y_1$ receptor antagonism, the ATP-mediated excitation of the preBötC during anoxia in vitro involved a PPADS/suramin-sensitive mechanism that was insensitive to $P2Y_1$ receptor antagonists. Given potential limitations of studying homoeostatic HVRs in vitro (Funk & Greer, 2013) and the observation that P2 receptor

mechanisms activated in vitro under anoxic conditions differed from those in the oxygenated slice in vitro and hypoxic animal in vivo, we did not pursue further mechanistic studies using the in-vitro anoxia model. As an alternative, we explored the signalling cascades and ion channels that underlie excitation of the preBötC by exogenously applied ATP to identify the potential mechanisms that could be activated by endogenous ATP released during hypoxia. 16 of 35 recorded neurons responded to ATP with a $P2Y_1$ receptor phenotype (i.e., slow inward current sensitive to MRS 2279 but not PPADS/Suramin). Second, inhibition of specific steps in the $G\alpha_q$-signalling pathway reduced the $P2Y_1$ receptor-mediated excitation of the preBötC network and inspiratory neurons by 55 to 59% and 45 to 50% respectively. Finally, we eliminated several ion channels as potential mediators of the $P2Y_1$ receptor network excitation, but were unable to identify the final effector. These data suggest that the $P2Y_1$ receptor excitation of the preBötC is mediated by a subpopulation of inspiratory neurons and that the $G\alpha_q$-signalling pathway contributes, but is not the only mechanism. Results advance our understanding of the molecular mechanism underlying the ATP-induced excitation of the preBötC inspiratory network and provide insight into how ATP may act centrally to offset the secondary hypoxic ventilatory depression in vivo.

2.3 Methods

2.3.1 Animals

Timed-pregnant Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, Massachusetts, United states) or BioScience Animal Services of University of Alberta (Edmonton, Alberta, Canada) and received at the animal facility 1 or 2 weeks prior to the date of birth. Dames were single-housed under a 12-h light-dark cycle (light on from 7 a.m. to 7

p.m.) with access to food and water *ad libitum*. Pups used in the study ranged in age from postnatal day 0-4 (P0-P4). All animal studies and experimental procedures were conducted in accordance with the guideline of Canadian Council on Animal Care and were approved by the University of Alberta Animal Ethics Committee.

2.3.2 Rhythmically-active medullary slice preparations

The procedures to obtain rhythmically active medullary slices have been described elsewhere (Smith *et al.*, 1991; Ruangkittisakul *et al.*, 2006; Zwicker *et al.*, 2011; Rajani *et al.*, 2018). In brief, P0 - P4 pups were anesthetized through isoflurane inhalation and decerebrated. A transection was made at the level of the diaphragm to isolate the thorax and head. Skin was then removed and the thorax/head immersed in cooled (4 °C) carbogen (95% O_2 and 5% CO_2)-bubbled 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. An additional transection was then made at the midpontine level, cerebellum removed and a spinal laminectomy performed to expose the brainstem and cervical spinal cord, which were subsequently isolated and pinned to a wax chuck. The chuck was then placed in the vice of a vibratome and the brainstem sectioned serially in the rostral-to-caudal direction using a vibrating microtome (VT1200S, Leica, Nussloch, Germany) until the structures of inferior olive dorsal and inferior olive principal nuclei were observed. Once these landmarks were visible, a single, 700 μm thick, rhythmic slice containing the preBötC was collected and moved to the recording chamber.

2.3.3 Extracellular hypoglossal (XII) nerve recordings

Rhythmically active medullary slices were either pinned on Sylgard resin in a 5 ml recording chamber for the anoxia and extracellular recording experiments or secured under a silver

harp in a 2.5 ml chamber for the whole-cell patch recording experiments. The slices were placed rostral surface up and perfused with aCSF at flow rates of 15 ml/min and 2 ml/min for the extracellular and whole-cell recording experiments respectively. The extracellular K^+ ($[K^+]_e$) was elevated from 3 mM to either 8 mM for the anoxia experiments and 9 mM for all others at least 30 min before data collection to produce prolonged, stable rhythm (Ruangkittisakul *et al.*, 2006). Inspiratory-related activity was recorded from the XII nerve roots on the ventral surface of rhythmic slices through a suction electrode (A-M Systems, Carlsborg, WA, USA). Signals were amplified, bandpass filtered (300 Hz to 1 kHz), full-wave rectified, integrated using a leaky integrator (τ = 25 or 50 ms), and displayed using Axoscope 9.2 (Molecular Devices, Sunnyvale, CA, USA). Data were saved to computer using a Digidata 1322 A/D board and AxoScope 9.2 software (Molecular Devices) for off-line analysis. Anoxia experiments were performed at 29 ± 0.5°C; all other experiments were performed at 24°C.

2.3.4 Anoxia experiments

The protocol used to induce the in vitro anoxic ventilatory response was similar to that described previously for mice (Ramirez *et al.*, 1997; Ramirez *et al.*, 1998a; Telgkamp & Ramirez, 1999). Rhythmically-active medullary slices were placed in the 5 ml recording chamber circulated with aCSF containing 3 mM K^+. K^+ concentration was then increased to 8 mM for the rest of experiment. 30 min later, bath temperature was increased over 25 min to 29 ± 0.5°C. Baseline activity was recorded for 5 min and then the slices were exposed to the first anoxia by bubbling the circulating through the chamber aCSF bubbled with anoxic gas (95% nitrogen + 5% CO_2) for 3 min. Recovery was monitored for 20 min. This protocol was repeated three times in the time-control experiments (with 40 min total between consecutive anoxic exposures) to ensure that responses were repeatable. In pharmacological experiments, pyridoxal phosphate-6-azo

tetrasodium salt hydrate (PPADS) and/or suramin were bath applied 20 min prior to the second anoxic exposure. PPADS/suramin were washed out for 20 min before a third anoxic exposure was applied to assess recovery of the anoxic ventilatory response. The effects of MRS 2279 on the anoxic response were compared after bilateral microinjection of aCSF (control) or MRS 2279 locally into the preBötC (bath-application was cost-prohibitive). aCSF and MRS 2279 were injected for 5 min starting after 2 min of switching to anoxic gas (the time it takes for anoxic gas to reach the recording chamber).

2.3.5 Whole-cell patch recordings

Whole-cell recordings from inspiratory neurons located in the preBötC were made in rhythmically active slices using patch pipettes (4-6 MΩ) pulled from borosilicate glass (Harvard Apparatus, Catalog No. 30-0044) that were filled with intracellular solution (ICS) containing (in mM): 140 K-gluconate, 5 NaCl, 0.1 EGTA, 10 HEPES, 1 $MgCl_2$ and 1 glucose (liquid junction potential: -14.5 mV). Osmolarity of intracellular solutions was adjusted to 290–300 mOsm with sucrose and pH adjusted to 7.2–7.3 with KOH. Membrane potentials were not corrected for liquid junction potentials. PreBötC inspiratory neurons were selected based on their location ventral or ventrolateral the semicompact division of nucleus ambiguus, the presence of rhythmic inspiratory-related synaptic currents that were in phase with the inspiratory–related rhythm recorded from the XII nerve rootlets. Inspiratory neurons with a resting membrane potential of -45 mV or more hyperpolarized, were included in the analysis. If access resistance changed by more than 20% during a voltage clamp protocol (i.e., between control, test and recovery conditions), or if the holding current was not stable between control and test conditions, data were excluded from analysis.

Cells were visualized with infrared and DIC optics on an upright microscope (Axioskop2

FS plus, Carl Zeiss, Oberkochen, Germany). The patch pipette was moved through the tissue toward the target neuron along the electrode axis to minimize tissue compression. Gigaseal formation and membrane rupture were performed in voltage-clamp mode. Whole-cell experiments were conducted at 24°C. Whole-cell signals were amplified, low pass filtered at 5 kHz, digitized (sampled at 20 kHz) and saved to computer using a MultiClamp 700A amplifier, Digidata 1322 A/D board and AxoScope 9.2 software (Molecular Devices, Union City, CA) for off-line analysis. Series resistance (Rs) and whole-cell capacitance (Cm) were estimated as done previously (Adachi *et al.*, 2005; Pagliardini *et al.*, 2005) using the Rs and Cm compensation features of MultiClamp Commander software (Axon Instruments) to manually correct the current response to square-wave voltage pulses (100 Hz,−10 mV, 3 ms) under voltage-clamp conditions. Neuronal input resistance (R_N) was calculated based on the current response to a voltage ramp (1.5 s duration, from −90 to −40 mV) applied before, during and after drug application. The inverse slope of the linear portion (usually between -70 mV to -60 mV) of the current–voltage (I–V) relationship was used to estimate R_N.

The membrane potential was held at -60 mV in all experiments except those that investigated currents evoked by 100 μM ATP, when it was held at -80 mV to enhance evoked current amplitudes. P2 receptor currents were evoked via local pressure application of ATP or MRS 2365 from a triple-barrel pipette placed in close proximity (about 80 -120 μm) to the targeted neuron. To assess the P2 receptor subtypes, or the contribution of a specific second messenger signalling molecules or ion channels to the ATP/MRS 2365-evoked currents, we compared the current induced first under control conditions, again during application of P2 receptor, $G\alpha_q$, or ion channel blockers and, when possible, after washout of the various blockers. Consecutive agonist applications were always separated by at least 15 min. Blockers were bath-applied, locally applied

via triple barrel pipettes, or added to the ICS (see below for method of application for each drug). Effects on P2 receptor-evoked currents of agents loaded via the ICS were assessed by comparing currents evoked within 2 min of cell rupture (considered the baseline current) and after 15-min of intracellular dialysis of the specific agent. Time-matched control experiments compared the P2 receptor current evoked within the first 2 min of obtaining whole-cell configuration and that evoked 15 min later using ICS containing the relevant vehicle (DMSO).

2.3.6 Drugs

PPADS, suramin, apamin, 9-Phenanthrol, flufenamic acid, glibenclamide and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St Louis, MO, USA). MRS 2365, MR S2279, substance P (Sub P), ATP, U73122, 2-APB, cyclopiazonic acid, thapsigargin, and chelerythrine chloride were obtained from Tocris Biosciences (Bristol, UK). Paxilline was obtained from Alomone Labs (Jerusalem, Israel).

2.3.7 Drug preparation

Drugs were prepared as stock solutions in 100% dimethyl sulfoxide (DMSO) (9-Phenanthrol, flufenamic acid, glibenclamide, U73122, 2-APB, cyclopiazonic acid, thapsigargin and paxilline) or aCSF (Sub P, MRS 2365 and MRS 2279) or ddH_2O (PPADS, suramin, chelerythrine chloride, ATP and apamin). The stock solutions were diluted to the final concentration before use. The final concentration of DMSO ranged from 0.02% to 0.2% and was increased to 0.4% in one case to push the concentration of U73122. All time controls were performed with matched vehicle concentrations.

2.3.8 Drug applications

Drugs were either applied to the solution bathing the preparation (PPADS, 10 or 50 μM;

suramin, 100 μM; U73122, 20 μM; 2-APB, 50 μM; chelerythrine chloride, 10 μM; apamin, 3 μM; 9-Phenanthrol, 100 μM; flufenamic acid, 100 μM), injected into or above the preBötC (Sub P, 1 μM; MRS 2365, 100 μM; MRS 2279, 100 and 500 μM; ATP, 100 μM or 5 mM; U73122, 10 μM; glibenclamide, 10 μM; paxilline, 1 μM), or added to the intracellular solution (U73122, 2 μM; 2-APB, 50 μM; thapsigargin, 4 μM; cyclopiazonic acid, 20 μM; chelerythrine chloride, 10 μM; paxilline, 4 μM and 1 μM). Local injection of drugs was achieved through a triple-barrelled pipette (5-6 μm outer diameter per barrel). The injections were controlled by a programmable stimulator (Master-8; A.M.P.I., Jerusalem, Israel) connected to a picospritzer (Spritzer4 Pressure Micro-Injector, 18 psi).

For the extracellular recording experiments designed to examine the mechanisms underlying the ATP excitation of the preBötC network, Sub P injections (10 sec, 1 μM) were used to help locate the preBötC. SubP injection sites were moved in a grid-like fashion until a site was located at which substance P caused more than a two-fold increase in the frequency of inspiratory bursts recorded from the XII nerve. This site was defined as the preBötC and all subsequent P2 receptor agonist injections were made at the same site (Lorier *et al.*, 2007; Zwicker *et al.*, 2011).

Two challenges with the extracellular experiments designed to define the intracellular signaling pathways or ion channels activated by P2 receptors located on cell membranes are that the agents must cross cellular membranes to reach their intracellular sites of action, and it is often impossible to include a positive control to demonstrate that the delivered agent has reached its target. Thus, it is difficult to interpret negative responses (i.e., when the delivered agent has no effect on the ATP/MRS 2365-evoked network response). To partially address this limitation, second-messenger/ion channel blockers were first bath-applied, and if minimal effects were observed, drugs were also locally-applied to the preBötC (usually via pulsatile injection) to

maximize access of the blocking agent to the preBötC. In such cases, drug tests were compared with identical control experiments that involved administration of vehicle.

2.3.9 Data Analysis

The extracellular and whole-cell patch recording data were analyzed offline using Clampfit (pClamp 9.2, Axon Instruments). The instantaneous frequency of rhythmic XII nerve bursts was generated in Clampfit and normalized relative to the baseline frequency calculated based on the average frequency during the two min preceding drug injection or the five min preceding anoxic exposure. The maximum excitatory effect of an injected drug on frequency was determined as the maximum value measured in the moving average of instantaneous inspiratory frequencies of five consecutive bursts during the first minute after injection compared to baseline. The maximum excitatory effect of anoxia on frequency was determined as the maximum value observed in the moving average between the onset of anoxia and the onset of the secondary anoxic depression. The minimum frequency value of the moving average recorded within 10 min of the onset of anoxia was taken as the minimum frequency and used to calculate (relative to baseline) the magnitude of the anoxia-induced depression.

The roles of P2 receptor subtypes in the ATP-evoked frequency increase respectively were assessed by comparing the peak frequencies (calculated relative to pre-ATP baseline levels of 100%) evoked by ATP in control and following application of P2 receptor antagonists (Fig. 2.2). This comparison provides insight into the magnitude of the ATP-evoked frequency increase and how it is affected by different drugs applied in tandem in the same experiment. All the data examining the effects of $G\alpha_q$ second messenger blockers and ion channel blockers on network frequency were plotted as a ratio of the change in relative frequency evoked by the second MRS 2365 application (whether time control or drug test) over the change in relative frequency evoked

by the first MRS 2365 application. The advantage of this representation is that it if there is no time dependent run-down or no drug effect, the ratio is close to 1. Ratio values less than one indicate run-down (in the case of the time control) or that the drug had an inhibitory effect.

For the whole-cell recording data, the effects of ATP/MRS 2365 on whole-cell currents and inspiratory synaptic currents were measured in control trials and again after bath, local or intracellular application of receptor antagonists, second messenger blockers or ion channel blockers. For the U73122 bath application experiments, the amplitudes of ATP currents and synaptic inputs were reported and compared in absolute terms. For experiments involving intracellular dialysis of second messenger blockers or paxilline, values measured 15 min after the initial control trials (conducted in the first 2 min of obtaining whole-cell configuration) are reported relative to the initial baseline (control trial) measurements.

Differences between means were compared using GraphPad Prism (Version 6.01, GraphPad Software Inc.). Paired or unpaired t-tests were used as appropriate for comparison of two groups. For comparison of more than two groups, one-way or two-way ANOVA was used in conjunction with either a Tukey or Bonferroni post-hoc multiple comparison test. P values < 0.05 were considered significant. Note that to address potential concerns about time-dependent rundown of ATP/MRS 2365 effects, the majority of statistical comparisons are made between experimental groups and separate time control groups where the agonist was applied repeatedly in the presence of vehicle. For this reason, time control values are not always 1.0; time control values > 1 indicate that the second agonist response was greater than the first, while values < 1.0 indicate that the second consecutive agonist response was less than the first. Group data are presented as boxplots created by the web-application 'BoxPlotR' (Spitzer *et al.*, 2014). Spread of data points, the lowest point, 1st quartile, median, 3rd quartile and highest point are superimposed on each box

as open circles, the lower whisker, bottom, middle, and top of box, and top whisker respectively. The mean for each group is denoted by "+".

2.4 Results

2.4.1 P2 receptor mechanisms evoked by anoxia in vitro differ from those evoked by hypoxia in vivo

Rhythmically-active medullary slices prepared from mice ranging in age from P0-22 responded to perfusion with aCSF bubbled with 95% N_2/5%CO_2 with a biphasic ventilatory response that resembles the biphasic ventilatory response evoked by hypoxia in vivo (Ramirez *et al.*, 1997; Ramirez *et al.*, 1998a; Telgkamp & Ramirez, 1999). Because most is known about P2 receptor signalling in the preBötC and HVR of rat (Funk, 2013; Funk & Gourine, 2018a), we reasoned that a similar preparation from rat could be useful for interrogating the roles of ATP and various signalling mechanisms in the HVR. We tested if a similar in vitro biphasic anoxic response could be reproduced in rhythmically-active slices from P0-4 rats that was reliable between slices, but also repeatable within a slice. Reliability and repeatability were key if this preparation was to be useful for pharmacological interrogation of signalling mechanisms as this would allow control, treatment and recovery trials in the same slice. The duration of anoxic perfusion (2-5 min) and aCSF temperature (24-29°C) were systematically varied until we established that when slices were held at a bath temperature of 29°C (but not 24-28°), exposure to anoxia for 3 min induced a biphasic anoxic ventilatory response very similar to that reported in mice (Ramirez *et al.*, 1997; Telgkamp & Ramirez, 1999), and that the response was highly repeatable at least 3 times (highest number attempted) when the stimulus was presented at 40 min intervals. The frequency of XII inspiratory-related burst activity increased to a peak that was 2.1 ± 0.4-fold greater than baseline

2.7 ± 0.3 min following introduction of anoxia. This was accompanied by an increase in tonic discharge (apparent in the upward shift in the baseline of the integrated XII nerve recording, Figs. 2.1A and B) and was followed by a gradual decrease in frequency that reached a nadir (i.e., lowest frequency) 6.5 ± 0.4 min after the onset of hypoxia. Rhythm gradually recovered to baseline levels 9.8 ± 0.7 min following hypoxia onset (Fig. 2.1A). The repeatability of this response is illustrated in Fig. 2.1A, which shows the response of one slice to three consecutive anoxic exposures delivered at 40 min intervals. To quantitatively assess the repeatability of these anoxic responses across all time control experiments, and ultimately assess the effects of P2 receptor antagonists on the biphasic response, we compared in consecutive trials the peak frequencies obtained during phase 1 of the biphasic anoxic response (when frequency was increasing) and the lowest, or nadir, frequency during the phase 2 depression. For ease of comparison, the peak frequency measured relative to baseline in phase I and the minimum frequency recorded relative to baseline in Phase II of the second anoxic exposure (i.e. that recorded in the second trial of the time control or the drug trial) are reported as a ratio over the peak relative frequency observed in phase I of the first anoxic exposure or minimum frequency observed during phase II of the first anoxic exposure, respectively. A ratio of 1.0 indicates that phase 1 and 2 responses in the second time-control anoxic exposure (or in the presence of drug) were the same as the first; i.e. that hypoxic responses were consistent (or there was no drug effect). A ratio below 1.0 indicates that the frequency recorded in the second time-control anoxic exposure (or in the drug) was lower than during the first anoxic exposure; i.e., that phase 1 frequency increase was lower and the phase 2 depression was greater during second response.

From this analysis, the consistency of time control responses is apparent in Figs. 2.1C and 1D which show the ratios of the frequencies during phase 1 and 2 of the second anoxic response

over the same value recorded during the first anoxic response are very near to 1; i.e., the peak and nadir frequencies were very similar.

Having established a reliable and repeatable biphasic anoxic response in vitro, the next step was to assess the role of P2 receptors in shaping this response. We compared the phase I and phase II values obtained in the initial control anoxic trial with values obtained in the subsequent test anoxic trial during which the slices were exposed to different P2 receptor antagonists including: PPADS alone (at 10 or 50 μM, bath-applied for 20 min pre-anoxia, non-selective ATP receptor antagonist), suramin alone (100 μM, bath-applied for 20 min pre-anoxia, non-selective ATP receptor antagonist); PPADS (10 μM or 50 μM) plus suramin (100 μM, bath-applied together for 20 min pre-anoxia), and MRS 2279 (500 μM, microinjected bilaterally into the preBötC for 5 min starting 2 min after onset of anoxia, $P2Y_1$ receptor antagonist).

None of the P2 receptor antagonists or their combinations affected the phase I, anoxia-induced increase in inspiratory frequency (Fig. 2.1C); i.e., ratios of peak frequency in Phase 1 of the drug trial over peak frequencies in the initial control trial were near one. Only the combination of PPADS at 50 μM and suramin at 100 μM significantly altered the phase II response; the ratio of the nadir frequency in this antagonist combination over that in the control trial was near 0.5 reflecting an increase in the magnitude of the secondary depression (Figs. 2.1B and D). Most surprisingly, bilaterally application of MRS 2279 (500 μM) in the preBötC did not affect the secondary depression (Fig. 2.1D). This result was unexpected based on previous data showing that the increase in frequency evoked by ATP in the preBötC is blocked by MRS 2179 ($P2Y_1$ receptor antagonist), and completely insensitive to PPADS/Suramin (Lorier *et al.*, 2007). It is also inconsistent with data in anesthetized, vagotomized, mechanically-ventilated adult rats in vivo

where local application of MRS 2279 (500 μM) in the preBötC potentiates the secondary hypoxic ventilatory depression (Rajani *et al.*, 2018).

2.4.2 ATP excites the inspiratory network via activation of preBötC $P2Y_1$ receptors

Our observation above (Fig. 2.1D) that MRS 2279 did not, but PPADS/Suramin did, alter the anoxic ventilatory response forced us to revisit our previous work on which we based the conclusion that ATP excitation of the preBötC in vitro is through activation of $P2Y_1$ receptors (Lorier *et al.*, 2007). This conclusion was based on the observation that the excitation of the preBötC evoked by local injection of ATP was only blocked by MRS 2179; none of the other antagonists tested, including PPADS and Suramin, had any effect. The potential limitation with this earlier experiment is that although the competitive antagonist MRS 2179 has much higher affinity for $P2Y_1$ receptors (equilibrium dissociation constant K_B = 100 nM), it is not completely selective, with lower affinity for other P2 receptors including $P2X_1$ (IC_{50} = 1.15 μM), $P2X_3$ (IC_{50} = 12.9 μM), $P2X_2$, $P2X_4$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Boyer *et al.*, 1998; Brown *et al.*, 2000b; Jacobson *et al.*, 2006; Jacobson & Muller, 2016). MRS 2179 was used at 100 μM in the previous study (Lorier *et al.*, 2007). Thus, the inhibitory effect of MRS 2179 on the ATP-evoked frequency increase may not have been limited to its actions at $P2Y_1$ receptors. In addition, while the previous work of Lorier et al. (2007) showed that PPADS and suramin alone had no effect on the ATP-evoked frequency increase, PPADS and suramin were not tested together. Note that only when applied together did PPADS and suramin affect the anoxic respiratory response in vitro (Fig. 2.1). Based on these concerns, we retested the exclusive role of $P2Y_1$ receptors in this ATP excitation using the more selective, more potent $P2Y_1$ receptor antagonist, MRS 2279. 2-min pre-application of MRS 2279 (500 μM) attenuated the ATP (100 μM)-induced network excitation by 96.7 ± 2.1%

while bath application of PPADS (50 μM) together with suramin (100 μM) had no effect (Fig. 2.2). These data confirm that excitation of the preBötC by ATP in vitro is primarily mediated by $P2Y_1$ receptors.

2.4.3 The $G\alpha_q$-signalling pathway contributes to the ATP excitation of preBötC inspiratory neurons and the preBötC network

The data presented in Figs. 2.1 and 2.2 strongly suggest that the purinergic mechanisms that shape the anoxic ventilatory depression in vitro differ from those that operate in an oxygenated rhythmic slice and also from those that shape the HVR in vivo (Rajani *et al.*, 2018). For these reasons and additional concerns with using the rhythmic slice preparation to study homeostatic responses to hypoxia, which are discussed in detail elsewhere (Funk & Greer, 2013), we did not use the in vitro anoxia model to study purinergic signalling mechanisms. Instead, based on compelling evidence that ATP is released in the preBötC during hypoxia where it stimulates breathing via $P2Y_1$ receptors (Angelova *et al.*, 2015a; Rajani *et al.*, 2018), we used the oxygenated rhythmic slice to study the mechanisms by which exogenous ATP excites preBötC inspiratory neurons and the preBötC network. The underlying assumption with this approach is that the mechanisms activated by exogenous ATP will be the same as those activated by ATP when it is released endogenously during hypoxia.

In other CNS and peripheral nervous system (PNS) regions, $P2Y_1$ receptors are conventionally considered to couple to the $G\alpha_q$-signalling pathway (Usachev *et al.*, 2002; Abbracchio *et al.*, 2006; Song *et al.*, 2007; Chandaka *et al.*, 2011; Rajani *et al.*, 2016). Activation of the $G\alpha_q$-signalling pathway starts with activation of PLC by the α_q subunit of the heterotrimeric G protein (Fig. 2.3). Upon activation, PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-

bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to IP_3 receptors on the endoplasmic reticulum, triggering release of calcium (Ca^{2+}) from intracellular stores. Both DAG and elevated intracellular Ca^{2+} can activate protein kinase C (PKC), leading to ion channel phosphorylation and modulation of network activity. We hypothesized that ATP, via $P2Y_1$ receptors, depolarizes inspiratory preBötC neurons by activating the $G\alpha_q$-signalling pathway, and this in turn underlies the $P2Y_1$ receptor-mediated excitation of the inspiratory network. Thus, we separately tested the contribution of multiple steps in $G\alpha_q$ pathway to the ATP-evoked excitation of individual preBötC inspiratory neurons and the preBötC network.

2.4.4 ATP excitation of preBötC inspiratory neurons involves $G\alpha_q$ signalling

We tested the contribution of the $G\alpha_q$-signalling pathway to the ATP-mediated excitation of preBötC inspiratory neurons using whole-cell recording techniques to compare the effects of locally-applied ATP on membrane current and inspiratory synaptic currents before and during application (via the bath, local injection or intracellular dialysis) of agents that block specific steps of the $G\alpha_q$-signalling pathway. In the whole-cell recording experiments, the concentration of ATP in the drug injection pipette used to stimulate single neurons was increased from 100 μM, as used in Fig. 2.2 to evoke network responses, to 5 mM. To study network responses, ATP is microinjected directly into the preBötC because rapid degradation of ATP by ectonucleotidases hinders its diffusion into tissue; e.g., measurements of ATP concentration indicate that when applied to the perfusion solution, the concentration of ATP in the preBötC is ~100-fold less than in the perfusion solution (Funk *et al.*, 2008; Huxtable *et al.*, 2009). Pressure injection of drugs into the tissue, however, often destabilizes whole-cell recordings. Thus, drug pipettes were placed above the slice surface and ATP concentration increased to compensate for its degradation and for the exponential decrease in concentration that occurs with increasing distance from the pipette tip.

Thirty sec local application of 5 mM ATP over inspiratory preBötC neurons evoked consistent (repeatable) inward currents that in most neurons peaked within 5.4 ± 0.7 sec of drug onset and then decreased over the next 14 ± 0.9 sec to a steady-state level that was less than half the peak amplitude in most cases, as shown for an example neuron in Fig. 2.4C. ATP currents were stable over the time frame of this experiment. A time-control group showed that the first control ATP current was the same as the second control ATP current evoked 15 min later (n=6, p = 0.85, paired t-test). The peak current evoked by 5 mM ATP for all inspiratory preBötC neurons averaged -118 ± 15 pA (n = 61). Input resistance was not measured during the peak component of the current but during the steady state component it was 102 ± 9 MΩ in ATP compared to 104 ± 8 MΩ in control (pre-ATP, n = 54).

Phospholipase C. Bath application of the phospholipase C blocker, U73122 (20 μM), for 30 min had no effect on the amplitude of inward currents induced by local application of ATP (5 mM, 30 sec) (Figs. 2.4A and B, n = 6). This was unexpected given that the actions of ATP on rhythm are via $P2Y_1$ receptors, which in other brain regions signal via $G\alpha_q$ that couples to PLC (Usachev *et al.*, 2002; Filippov *et al.*, 2004; Milenkovic *et al.*, 2009). Due to the lack of a positive control to ensure diffusion of U73122 to, and into, the recorded neuron, we next added U73122 directly to the ICS and compared ATP currents evoked immediately after rupturing the membrane with those evoked 15 minutes later. Under these conditions, 15 min intracellular dialysis of U73122 (2 μM) via the recording pipette into the inspiratory neurons significantly attenuated the amplitude of the ATP-induced inward currents, as shown for a single neuron and group data (Figs. 2.4C and E). To control for potential time-dependent changes in the amplitude of ATP currents evoked in U73122 at 15 min (reported relative to the initial ATP current in control trial) were compared to a separate time control group in which ATP was applied alone at 15 min intervals. In

the time control group, the second ATP current was 93.6 ± 4.7% of the initial ATP current. Compared to the time control group, 15 min of dialysis with U73122 significantly reduced the ATP current to 62 ± 4.3% of the initial response (n=7, p=0.0002). Inhibition of the ATP current by dialyzed, but not bath-applied, U73122 data point to the challenge of getting drugs across membranes to their sites of action. To avoid this issue in subsequent experiments, second messenger and ion channel blockers were added to the ICS.

IP_3 receptors. Similar to the effect of U73122, compared to the time control group blockade of IP_3 receptors by intracellular dialysis of 2-APB (IP_3 receptor inhibitor) significantly reduced the amplitude of the ATP currents in inspiratory neurons to 67 ± 13% of the initial response (Figs. 2.4G and I, n = 5, p=0.045).

Intracellular Ca2+ stores. Excitation of the inspiratory network by MRS 2365 in vitro is partially blocked by Ca^{2+} chelation with EGTA-AM and by depleting intracellular Ca^{2+} stores with thapsigargin (Wells *et al.*) or cyclopiazonic acid (CPA) that block Ca^{2+}-ATPase in the sarco /endoplasmic reticulum (Rajani *et al.*, 2018). ATP currents evoked in inspiratory neurons before and after 15 min of dialysis with ICS containing THG (4 μM, Figs. 2.5A and C, n = 10) or CPA (20 μM, Figs. 2.5D and F, n = 9) were 77.4 ± 5.2 (p=0.0411) and 78.4 ± 3.5% (p=0.0135) of the initial response, respectively, which were significantly reduced compared to time control values.

Protein kinase C. Intracellular dialysis of the PKC inhibitor, chelerythrine chloride (CHE, 10 μM), had a similar effect on the ATP-current, reducing it to 79.4 ± 4.6% of the initial response, which was significantly greater than the reduction in the time control group (Figs. 2.5G and I, n = 8, p = 0.0411).

5 mM ATP significantly reduced the amplitude of inspiratory synaptic currents from -186 pA to -174 pA (n = 40, p = 0.0365). However, inspiratory synaptic currents were not affected by

any of the second messenger blockers (U73122, 2-APB, THG, CPA and CHE; Figs. 2.4D, H; 5B, E, H). In summary, intracellular dialysis of various signalling blockers suggest that $G\alpha_q$-signalling contributes between 20 and 30% of the inward current evoked in preBötC inspiratory neurons by 5 mM ATP.

Differential responses of preBötC inspiratory neurons to ATP. The incomplete block of the 5 mM ATP current in inspiratory neurons by $G\alpha_q$ signalling blockers was surprising given that $P2Y_1$ receptors signal through the $G\alpha_q$ pathway and that the frequency effects of ATP are blocked by $P2Y_1$ receptor antagonists. This could reflect that the blocker concentrations were too low (which is unlikely given the concentrations used and IC_{50} values for the various agents (Herbert *et al.*, 1990; Smallridge *et al.*, 1992; Wootton & Michelangeli, 2006; Togashi *et al.*, 2008). The incomplete block could also reflect that P2 receptors other than the $P2Y_1$ subtype contribute to the current evoked by 5 mM ATP. ATP was used at a concentration of 100 μM to evoke the network frequency increase in extracellular recording experiments (Fig. 2.2) while 5 mM was used in whole-cell experiments for reasons described above. We initially assumed that the same P2 receptors would be activated with 100 μM and 5 mM ATP, since the $P2Y_1$ receptor-mediated effects of ATP in the preBötC on frequency are dose-dependent between 0.01 and 1 mM (Lorier *et al.*, 2007) and the frequency effects are only sensitive to $P2Y_1$ receptor antagonists.

To test this assumption that currents evoked by 5 mM ATP are mediated solely by $P2Y_1$ receptors, we compared currents evoked by 5 mM ATP before and following 2 min local pre-application of MRS 2279 at concentrations of 100 (n = 6) and 500 μM (n = 9). All inspiratory neurons tested had an MRS 2279-sensitive component to the 5 mM ATP current, but it was only a fraction of the total. 50 and 100 μM MRS 2279 had similar effects on the peak ATP current, reducing it to 67.1 ± 8.3% and 65.5 ± 6.3% of control, respectively (Figs. 2.6A and C). Subsequent

co-application of the general P2 receptor antagonists, PPADS (50 μM) and suramin (100 μM), to the bath almost completely blocked the MRS 2279-insensitive component of the 5 mM ATP current (Figs. 2.6A and C). Next, local application of $P2Y_1$ receptor agonist MRS 2365 (100 μM) revealed a slow-onset inward current with a peak amplitude of -28 ± 4 pA (n=25) that did not significantly affect input resistance (90 ± 9 MOhm in control and 91 ± 9 MOhm in MRS 2365), and that was almost completely blocked by MRS 2279 (500 μM), but was unaffected by co-application of PPADS (50 μM) with suramin (100 μM) (Fig. 2.6B). These data suggest that the inward current induced by 5 mM ATP involves a $P2Y_1$ receptor-mediated current characterized by relatively slow onset kinetics and a larger PPADS/suramin-sensitive current with rapid onset and slow but incomplete desensitization (Fig. 2.6C).

Since our interest primarily lies in understanding the mechanisms underlying the ATP-mediated increase in preBötC frequency, which between 10 μM and 1 mM ATP is mediated almost entirely by $P2Y_1$ receptors, we next examined the currents evoked in inspiratory preBötC neurons by 100 μM ATP. Neurons responded with one of two distinct types of currents (Figs. 2.6D, E, F). The first type, observed in 54% of neurons (19 of 35), was slow in onset resembling the MRS 2365-evoked current, averaged -35 ± 4 pA (n = 19), had no effect on input resistance, was reduced to 26.2 ± 2.9% of control by locally-applied MRS 2279 (500 μM, n = 19) and was insensitive to bath applied PPADS/suramin. The second type of current evoked by 100 μM ATP (observed in 16 of 35 neurons) was similar in profile to the currents evoked by 5 mM ATP (but smaller in amplitude). These currents had a rapid onset that peaked at -74 ± 25 pA and then desensitized to a lower steady-state level, were not associated with a change in input resistance (measured during steady-state), and were almost completely blocked by PPADS/suramin (to 22.9 ± 4.4% of control,

Figs. 2.6D, E, F). In addition, these desensitizing currents were not inhibited by MRS 2279; if anything they were potentiated (144 ± 12% of control, Fig. 2.6E, n = 16).

Taken together, these data suggest that there are at least two subpopulations of inspiratory neurons, one that predominantly expresses MRS 2279-sensitive, PPADS/suramin insensitive presumably $P2Y_1$ receptors, and one that predominantly expresses non-$P2Y_1$, PPADS/suramin-sensitive P2 receptors.

2.4.5 $G\alpha_q$ signalling contributes to the $P2Y_1$ receptor-mediated excitation of preBötC inspiratory neurons

Our observations that 20-35% of the current evoked by 5 mM ATP is blocked by the $P2Y_1$ receptor antagonist and that blockers of the $G\alpha_q$ signalling pathway block 20-30% of the same current suggests indirectly that the $P2Y_1$ receptor component of the 5 mM ATP current is mediated almost entirely via the $G\alpha_q$–signalling pathway. To directly test this hypothesis, we next assessed the contribution of the $G\alpha_q$-signalling pathway specifically to $P2Y_1$ receptor currents; i.e., those evoked by MRS 2365. We retested 2 of the 5 agents used in the 5 mM ATP experiments; U73122 which is at the beginning of the cascade and reduced the 5 mM ATP current to 62 ± 4.7% of control and chelerythrine chloride which is at the end of the cascade and reduced the 5 mM ATP current to 79.4 ± 4.6% of control. As described previously, MRS 2365 evoked slow onset currents that in this subset of cells had an average peak amplitude of -27 ± 4 pA (n=11). 15 min of dialysis with U73122 and chelerythrine reduced the MRS 2365 current to 50.1 ± 7.7% (n = 6, Figs. 2.7A and B) and 45.2 ± 3.5% (n = 5, Figs. 2.7C and D) of the initial response, respectively. Note that the block was still incomplete, suggesting alternate signalling pathways contribute to the $P2Y_1$ receptor-mediated excitation of inspiratory neurons.

2.4.6 $P2Y_1$ receptor excitation of the preBötC network: contribution of the $G\alpha_q$-signalling pathway.

Given that the network excitation of preBötC frequency by ATP is mediated almost exclusively by $P2Y_1$ receptors but only ~50% of the $P2Y_1$ receptor-mediated current in inspiratory neurons is mediated via the $G\alpha_q$-signalling pathway, the next key question is how much of the $P2Y_1$ receptor-mediated network excitation is via $G\alpha_q$ signalling? To test this, we compared the frequency increase evoked by local injection of MRS 2365 into the preBötC before and during bath and/or local application of various $G\alpha_q$ signalling blockers.

Phospholipase C. Similar to our findings with whole-cell recording, bath application of U73122 had no effect on the 2.5 fold increase in frequency evoked by 10 sec local application of MRS 2365 into the preBötC (n = 9, Figs. 2.8A and B). To facilitate access of U73122 to the preBötC and increase the likelihood of loading cells with the drug, we next compared the control response to MRS 2365 with that evoked after simultaneous bath application (10 μM) and locally pulsed injection (20 μM, pulse injection: 10 sec on and 30 sec off) of U73122 into the preBötC. The effects of this combined antagonist application were somewhat variable but overall there was no significant effect of U73122. The peak frequency evoked by MRS 2365 was 2.5 ± 0.1 and 2.5 ± 0.2 fold above baseline in control and after U73122 respectively. Whether this reflects that U73122 did not sufficiently access its intracellular targets, as suggested by the whole-cell data, or that PLC is not involved on the $P2Y_1$ receptor mediated effects on rhythm is uncertain.

IP3 receptor. The involvement of the IP_3 receptor in the MRS 2365-evoked frequency increase was assessed by comparing the increase in control and after bath application of 2-APB

(50 μM). 2-APB significantly reduced the MRS 2365-induced frequency increase in by 59 ± 13% (Figs. 2.8C and D).

Protein kinase C. Previous work from our lab, using EGTA-AM and thapsigargin to chelate intracellular Ca^{2+} and deplete intracellular Ca^{2+} stores respectively, has demonstrated that increases in intracellular Ca^{2+} are important in the MRS 2365-evoked frequency increase; i.e. local application of EGTA or thapsigargin greatly reduced the frequency increase in vitro (Rajani *et al.*, 2018). Both DAG activation and increased intracellular Ca^{2+} can activate PKC. To investigate whether or not PKC activation is required for $P2Y_1$ receptor -mediated network excitation, chelerythrine chloride (PKC inhibitor, 10 μM) was bath applied and its effect on the MRS 2365-induced frequency increase was assessed. Chelerythrine chloride reduced the MRS 2365-induced frequency increase by 55± 7% (Figs. 2.8E and F).

2.4.6 $P2Y_1$ receptor excitation of the preBötC network and neurons: ion channels

To alter network rhythm, $P2Y_1$ receptor activation, in part through the $G\alpha_q$-signalling pathway must alter neuronal and network excitability. Candidate ion channels were identified via their documented sensitivity to $P2Y_1$ receptor or $G_{\alpha q}$ modulation and that they also affect inspiratory rhythm when manipulated experimentally, as summarized elsewhere (Rajani *et al.*, 2016). Ion channels tested included: G protein-coupled inwardly-rectifying potassium (GIRK) channel (Lei *et al.*, 2001; Filippov *et al.*, 2004; Montandon *et al.*, 2016), ATP-sensitive potassium (K_{ATP}) channel (Takashi *et al.*, 1999; Krey *et al.*, 2010), small-conductance Ca^{2+}-activated K^+ (SK) channel (Bissonnette, 2002; Zavala-Tecuapetla *et al.*, 2008; Coppi *et al.*, 2012), big-conductance Ca^{2+}-activated K^+ (BK) channel (Zavala-Tecuapetla *et al.*, 2008; Coppi *et al.*, 2012; Qian *et al.*, 2018) and transient receptor potential cation channel subfamily M member 4 and 5 (TRPM4/5)

(Pena *et al.*, 2004; Del Negro *et al.*, 2005; Zhang *et al.*, 2005; Alvares *et al.*, 2014; Leitner *et al.*, 2016).

Antagonizing GIRK channels with $BaCl_2$ (400 μM, bath, n = 4), K_{ATP} channels with glibenclamide (10 μM, injection pulse: 10 sec on and 10 sec off; injection duration: 15 min, n = 3), small-conductance Ca^{2+}-activated K^+ (SK) channels with apamin (3 μM, bath, n = 4), and transient receptor potential cation channel subfamily M member 4 and 5 (TRPM4/5) with 9-Phenanthrol (100 μM, bath, n = 5) or flufenamic acid (100 μM, bath, n = 6), had no effect on the MRS 2365-induced frequency increase (Fig. 2.9A). 9-Phenanthrol slowed, while $BaCl_2$ slowed/destabilized baseline rhythm, which accounts for the apparent increase in MRS 2365-evoked peak relative frequency under these two conditions. The peak absolute frequency evoked by MRS 2365 was unaffected by 9-Phenanthrol or $BaCl_2$.

Local application of the BK channel blocker, paxilline (1 μM; injection pulse: 5 sec on and 5 sec off; injection duration: 30 min) to the preBötC did not affect the MRS 2365-evoked frequency increase when all the data were examined together (Fig. 2.9A, n = 10). Note that the effects of paxilline were highly variable. In a subgroup of 5 these 10 preparations, paxilline caused an up to a 75% reduction in the MRS 2365-induced frequency increase (see Fig. 2.9B for representative traces). One explanation for the inconsistency of the paxilline effects is that, like U71322, access of paxilline to the network may be limited and vary between preparations. One option to address this was to increase the concentration of paxilline. The only potential limitation with this approach is that at higher concentrations paxilline can also block SERCA, which will inhibit the MRS 2365-evoked frequency increase. To assess whether increasing paxilline concentration was an option, paxilline (1 μM or 4 μM) was delivered into inspiratory neurons via intracellular dialysis and its effect on the MRS 2365-induced inward currents were assessed. At a holding potential of -60 mV

under voltage-clamp conditions, blocking BK should have no ability to affect the MRS 2365 current because BK activation requires depolarization and an increase in intracellular Ca^{2+}. Any effect of paxilline on the MRS 2365 current would indicate inhibition of SERCA. As expected, intracellular dialysis of paxilline (1 μM, 15 min) had no effect on the MRS 2365 currents (Figs. 2.9C and D) because at this concentration it does not affect SERCA (Bilmen *et al.*, 2002). In contrast, intracellular dialysis of paxilline at 4 μM (15 min) reduced the amplitude of the MRS 2365-induced inward currents by 47 ± 6% of the control response (Figs. 2.9E and F). Note that 4 μM paxilline could have a minor off-target action on SERCA as IC50 of paxilline for SERCA inhibition is 5 - 50 μM (Bilmen *et al.*, 2002). Due to the potential side effect, we were unable to further increase the concentration of paxilline to conclusively exclude the possibility that BK contributes to the MRS 2365 evoked increase in preBötC frequency.

2.5 Discussion

For decades the convention has been that the sole contribution of the CNS to the biphasic HVR is as the source of the secondary hypoxic respiratory depression. Recent data, however, have challenged this dogma with strong evidence that astrocytes detect hypoxia and release ATP in the preBötC where it acts, at least in part, through $P2Y_1$ receptor-mediated neuronal excitation to increase ventilation and attenuate the secondary hypoxic depression of breathing (Angelova *et al.*, 2015a; Rajani *et al.*, 2018). In this study we used rhythmically active, medullary slice preparations from neonatal rat to explore the second messenger signalling pathways and ion channels via which ATP and $P2Y_1$ receptor activation excite preBötC inspiratory neurons and the preBötC network. Main findings are that:

i. The biphasic response of the rhythmic slice preparation to anoxia is not a useful model to study $P2Y_1$ receptor mechanisms contributing to the HVR, because, unlike in the response of

the preBötC network to ATP in vitro and the HVR in vivo, $P2Y_1$ receptors are not involved in the anoxic ventilatory response in vitro.

ii. ATP excites preBötC neurons through $P2Y_1$ and non-$P2Y_1$ receptor-mediated mechanisms. $P2Y_1$ receptor mechanisms mediate the increase in frequency evoked by 100 μM ATP.

iii. There are two subpopulations of preBötC inspiratory neurons. One predominantly expresses $P2Y_1$ receptors whereas the other does non-$P2Y_1$ receptors.

iv. The $P2Y_1$ receptor-mediated excitation of preBötC network is mediated in part by the $G\alpha_q$-signalling pathway, involving activation of PLC and IP_3 receptors, release of Ca^{2+} from intracellular stores and activation of PKC. However, this mechanism contributes at most 60% of the overall network effect, meaning that an as yet unidentified signalling pathway(s) remains to be identified.

v. Multiple targets of $P2Y_1$ receptor signalling, including K_{ATP}, GIRK, SK, TRPM4/5 and BK channels, do not appear to contribute to the $P2Y_1$ receptor-mediated excitation of the preBötC.

2.5.1 The biphasic ventilatory response to anoxia in vitro; questionable relevance to the homeostatic biphasic ventilatory response to hypoxia in vivo

Hypoxia induces a biphasic ventilatory response consisting of an initial increase, which is mediated by the peripheral carotid body, followed by a centrally mediated secondary depression. The secondary hypoxic ventilatory depression is more profound in neonates in which ventilation falls even below baseline. Astrocytes release ATP in response to hypoxia through exocytosis (Angelova *et al.*, 2015a). Blockade of glial vesicular release or ATP actions specifically in the preBötC potentiates the secondary hypoxic ventilatory depression, suggesting that ATP is released in the preBötC where it offsets the depression (Angelova *et al.*, 2015a; Rajani *et al.*, 2018). The

strength of the data stems from the fact that these experiments were done in awake and carotid body-intact animals, which rules out the potential confounding factors including 1) anesthesia-induced cortical inhibition and 2) carotid body denervation-induced adaptation of aortic bodies, neuroplastic changes in chemoreceptive mechanisms and respiratory network reconfiguration. Despite the compelling evidence that there is a central, glial and ATP-mediated contribution, the underlying mechanisms through which ATP excites the inspiratory network during the secondary depressive phase remain to be elucidated. An in vitro approach to this problem would greatly facilitate mechanistic studies. Rhythmically active medullary slices from P0 – P22 mice (Ramirez *et al.*, 1998a; Telgkamp & Ramirez, 1999) respond to anoxia with a biphasic ventilatory response, with some features similar to that observed in vivo. The key assumption of using this in vitro model is that the in vitro anoxic ventilatory response is mediated by the same mechanism(s) as the HVR in vivo. This assumption, however, was directly challenged by the finding that blockade of $P2Y_1$ receptors in the preBötC with MRS 2279, which potentiates the secondary depression in anesthetized rats (Rajani *et al.*, 2018), has no effect on the anoxic ventilatory response observed in rhythmic slices. This discrepancy suggests that the in vitro and in vivo HVRs do not share the same underlying mechanism(s). Numerous factors may account for the different responses (Funk & Greer, 2013), beginning with differences in the oxygenation profiles in slices compared to the perfused brain in vivo. Oxygen in aCSF bubbled with carbogen reaches the core of slice through passive diffusion. Upon anoxia introduction, cells close to the surface of slice may undergo a more drastic change in oxygen level than those in deeper layers as superficial cells are likely to experience a change from hyperoxia to hyoxia while deeper cells will see a change from normoxia to anoxia. This is further confounded by the fact that individual neurons with extensive dendritic branching will experience a wide range of oxygen stimuli at differing sections of their membranes.

In contrast, cells in a functioning brain are supplied with oxygen through blood vessels, and the entire brainstem respiratory network will experience a more-or-less similar hypoxic challenge. The age of preparation may also be a factor in the different effects of $P2Y_1$ receptors antagonist on the hypoxic/anoxic ventilatory responses observed in neonatal slices in vitro and adults in vivo (Moss, 2000; Robinson *et al.*, 2000; Liu *et al.*, 2006). Regardless of the mechanisms responsible for differences in the role of $P2Y_1$ receptors in the hypoxic/anoxic responses in vitro and in vivo, the presence of these differences discouraged us from using the in vitro anoxic model to investigate purinergic mechanisms on the HVR.

2.5.2 ATP excitation of preBötC inspiratory neurons is mediated in part by the $G\alpha_q$-signalling pathway

ATP offsets the secondary hypoxic ventilatory depression through excitation of the inspiratory network (Gourine *et al.*, 2005b; Angelova *et al.*, 2015a; Rajani *et al.*, 2018). Here we exploited the rhythmically active medullary slice preparation to investigate the mechanisms underlying the excitation of preBötC inspiratory neurons by exogenously applied ATP. Note that the average input resistance (109 ± 9 MOhm) of the inspiratory neurons in my thesis is lower than those reported in other studies which are usually above 200 MOhm (Rekling *et al.*, 1996; Mironov *et al.*, 1998; Del Negro *et al.*, 2002a; Lorier *et al.*, 2008). The lack of ATP in the intracellular solution may cause reduction in input resistance due to the opening of K_{ATP} channels. We chose not to add ATP in the intracellular solution to avoid desensitization of $P2Y_1$ receptors as we approach the cell with recording pipette. Activation and inhibition of K_{ATP} channels decrease and increase input resistance of neurons in different brain regions, respectively, suggesting that modulation of the activity of K_{ATP} channels can potentially change input resistance in certain neurons which may include preBötC inspiratory neurons (Pierrefiche *et al.*, 1996; Fujimura *et al.*,

1997; De Bernardis Murat & Leao, 2019). Several observations suggest that the ATP currents in preBötC neurons are mediated, in part, by the $G\alpha_q$-signalling pathway. Currents evoked by local application of 5 mM ATP were significantly attenuated by up to 35% following intracellular dialysis of several blockers of the $G\alpha_q$-signalling pathway, including 2-APB, thapsigargin, cyclopiazonic acid and chelerythrine. Similarly, MRS 2279, a $P2Y_1$ receptor antagonist attenuated the ATP current by ~35%, and $P2Y_1$ receptor-mediated currents were attenuated by 50 and 55 % following inhibition of phospholipase C or protein kinase C.

Our conclusion that ATP currents in preBötC neurons are mediated, in part, by the $G\alpha_q$-signalling pathway is based solely on pharmacological evidence and therefore its veracity depends on the specificity of agents used to block specific steps in the $G\alpha_q$ pathway. Like most drugs, none of the blockers used in this study is perfectly selective for the desired target; all have off-target actions. Thus, while this is a limitation when considering evidence for each step in the $G\alpha_q$ cascade, which is based on one antagonist (except SERCA), when data are considered in their entirety with respect to the involvement of $G\alpha_q$, the case is much stronger. The antagonists used have different off target actions, but all share the $G\alpha_q$ cascade as a target. Thus, the consistent attenuation of ATP currents by all agents, similar inhibition of ATP currents by the $P2Y_1$ antagonist MRS 2279, and the inhibition of $P2Y_1$ receptor currents by U73122 and chelerythrine lend strong support to the conclusion that ATP signals partly through $P2Y_1$ receptors and the $G\alpha_q$ cascade in a subpopulation of preBötC inspiratory neurons.

Another important point is that we have likely underestimated the degree to which the various steps in the $G\alpha_q$ signaling cascade contribute to the ATP current. Drugs were delivered to their sites of actions via intracellular dialysis from the whole-cell recording pipette. All efforts were made to ensure the control ATP currents were evoked within minutes of cell rupture.

However, even in this short time blockers within the pipette would have already begun to have their effect. Thus, the initial ATP current is not likely to represent the peak current, which means that we have underestimated the degree of inhibition. The perforated patch clamp recording technique (Lippiat, 2008; Linley, 2013) could be used to address this limitation in the future.

2.5.3 ATP activates preBötC inspiratory neurons via $P2Y_1$ and non-$P2Y_1$ receptor mechanisms

We confirmed the observation of Lorier et. al. (2007) that the frequency increase evoked by ATP in the preBötC (in vitro) is mediated entirely by $P2Y_1$ receptors. An important finding of this study is that while the network excitation is exclusively mediated by $P2Y_1$ receptors, only a fraction, 54%, of preBötC neurons had a 100 μM ATP current with a $P2Y_1$ receptor phenotype (slow onset, sensitive to MRS 2279). The remaining 46% of inspiratory neurons responded to 100 μM ATP with a PPADS/Suramin sensitive, non $P2Y_1$ receptor-mediated current. This is not surprising given that the preBötC is a highly heterogeneous region containing multiple types of inspiratory neurons, including excitatory (glutamatergic), inhibitory, pacemaker (inhibitory and excitatory)(Morgado-Valle *et al.*, 2010), type I rhythmogenic and type II nonrhythmogenic neurons, each with distinct functions in respiratory (Gray *et al.*, 2001; VanDunk *et al.*, 2011) (Wang *et al.*, 2014; Sherman *et al.*, 2015; Cui *et al.*, 2016; Baertsch *et al.*, 2018) and respiratory-related activities such as sighing (Li *et al.*, 2016; Del Negro *et al.*, 2018).

A key future objective will be to determine the inspiratory neuron type that expresses $P2Y_1$ receptors and mediates the ATP-induced increase in network frequency. We are currently labeling all $P2Y_1$-sensitive and non$P2Y_1$-sensitive neurons during whole-cell recording for subsequent analysis with RNAScope to assess whether the $P2Y_1$-sensitive neurons are glutamatergic or GABA/glycinergic. One possible challenge is this regard is that our data with 5 mM ATP suggest

that the P2 receptor expression may not be completely discrete. Currents evoked by 5 mM ATP almost always had a $P2Y_1$ and a PPADS/Suramin component, suggesting that cells may differ in their relative expression of $P2Y_1$ vs non-$P2Y_1$ receptors, with some primarily expressing $P2Y_1$ receptors and other primarily expressing non-$P2Y_1$ receptors.

Neither the P2 receptor subtype(s) underlying the PPADS/suramin-sensitive ATP currents nor their physiological significance is known. The rapid onset, partial-desensitization is consistent with a $P2X_2$ receptor contribution (Coddou *et al.*, 2011). Immunolabeling for the $P2X_2$ subunit is particularly strong in the ventrolateral medulla and in the preBötC, but multiple P2X subunits are expressed in the region (Yao *et al.*, 2000; Thomas *et al.*, 2001; Brosenitsch *et al.*, 2005; Lorier *et al.*, 2007). Thus, it is reasonable to conclude that the non-$P2Y_1$ receptor currents involves P2X receptors, with a likely contribution of the $P2X_2$ subunit. Regarding the physiological significance of the non$P2Y_1$, PPADS/Suramin sensitive current, it is possible that under conditions of high respiratory drive and higher concentrations of ATP that non$P2Y_1$ components contribute to the ATP-mediated increase in inspiratory frequency. However, this is unlikely because the non$P2Y_1$ currents are clearly activated by 100 μM (e.g. 46% of inspiratory neurons responded to 100 μM ATP with a PPADS/Suramin-sensitive ATP current), yet their blockade does not alter the network response to ATP. The non-$P2Y_1$ receptor currents could contribute to other respiratory-related behaviors including sighs or gasps but this is entirely speculative. What will be required to determine their role is to first identify the type of inspiratory neuron that expresses the non $P2Y_1$ current, establish its transcriptome, perhaps using single-cell RNA sequencing (Hayes *et al.*, 2017; Hwang *et al.*, 2018), and then designing genetic tools to specifically manipulate either the expression of non-$P2Y_1$ receptors in this pool of neurons or manipulate the activity of these cells via opto- or chemogenetic approaches.

2.5.4 The $P2Y_1$ receptor-mediated excitation of preBötC network is mediated in part by the $G\alpha_q$-signalling pathway

Attenuation of the frequency effect evoked by ATP or the $P2Y_1$ receptor agonist MRS 2365 by multiple blockers that act on the $G\alpha_q$-signalling pathway, indicate that the $G\alpha_q$ pathways contributes to the $P2Y_1$ receptor effect. None of the blocking agents, however, completely blocked the $P2Y_1$ network excitation. There are two possible interpretations of these data. Either there is a non-$G\alpha_q$ mechanism that contributes, or the entire response is $G\alpha_q$-mediated but the incomplete block reflects incomplete access of the blocking agents to their intracellular sites of action. Our observation that U73122 attenuated ATP currents in the inspiratory neurons following intracellular dialysis but not bath application suggests that the latter is possible. However, intracellular dialysis of U73122 and chelerythrine, which removed all issues of drug access, still failed to abolish MRS 2365 currents in preBötC inspiratory neurons. These data strongly suggest that $P2Y_1$ receptors signal through a $G\alpha_q$ and a non-$G\alpha_q$-signalling pathway(s) to excite the inspiratory network.

Candidates for a non-$G\alpha_q$, $P2Y_1$ receptor activated signalling pathway are few. A thorough literature review revealed that $P2Y_1$ receptors may act in a Gs/cAMP/Protein kinase A-dependent manner to mediate axonal elongation of hippocampal neurons (del Puerto *et al.*, 2012), protect pancreatic duct epithelial cells from alcohol-induced damage (Seo *et al.*, 2016) and induce Cl^- efflux in renal A6 cell line (Guerra *et al.*, 2004). $P2Y_1$ receptors can also couple to the G_i-signalling pathway to inhibit voltage-dependent Ca^{2+} channels in neurons of nucleus tractus solitarius (Aoki *et al.*, 2004) and superior cervical ganglion (Brown *et al.*, 2000a; Filippov *et al.*, 2000). N-type Ca^{2+} channels are not essential for the generation of the baseline inspiratory rhythm in vitro (Onimaru *et al.*, 2003; Lieske & Ramirez, 2006; Morgado-Valle *et al.*, 2008; Koch *et al.*, 2013), but modulation of these channels may alter the network excitability and its responses to

modulatory inputs. Therefore, the link between $P2Y_1$ receptors and the Gs/G_i-signalling pathway needs to be examined for its potential contribution to the $P2Y_1$ receptor-mediated excitation of the inspiratory network.

2.5.5 Search for the ion channel targets underlying the $P2Y_1$ receptor-mediated frequency increase

We are operating from the premise that the effects of $P2Y_1$ receptor activation on preBötC frequency are ultimately mediated by the modulation of an ion channel and an increase in neuronal/network excitability. A list of candidate ion channels was generated recently based on their reported modulation by $P2Y_1$ receptors or the $G\alpha_q$-signalling pathway, and that their activation/inhibition modulates inspiratory rhythm (Rajani *et al.*, 2016). We excluded five ion channels from the list of candidates, including K_{ATP}, GIRK, SK and TRPM4/5 channels. Bath or local application of blockers of these channels consistently had no effect on the MRS 2365-evoked frequency increase.

A limitation of these experiments is that we do not have positive controls to establish that the each drug blocked the channel in question. This is offset in several cases in that the drug altered baseline respiratory network activity, providing evidence that it had accessed the network. This was the case for $BaCl_2$, which increased tonic activity and slowed baseline rhythm, and flufenamic acid and 9-phenanthrol, which also slowed baseline rhythm. These data strengthen the conclusion that GIRK and TRPM4/5 channels are not involved. Regarding K_{ATP} and SK channels, we have no direct evidence that glibenclamide and apamin, respectively, blocked these channels, but these drugs have been used successfully at similar or lower concentrations in similar conditions to inhibit the relevant ion channel (Hallworth *et al.*, 2003; Krey *et al.*, 2010). Thus, we do not think that the

inability of any of these agents to alter the $P2Y_1$ receptor mediated frequency increase is a false negative finding.

The only ion channel blocker that showed some efficacy in blocking the $P2Y_1$ receptor-mediated frequency increase was paxilline, but its actions were highly variable, which is not surprising considering that BK channel blockage has mixed baseline effects on breathing (Rajani *et al.*, 2016). It had no effect in 5 of 10 experiments, but produced up to a 75% reduction in the MRS 2365-induced frequency increase in the remaining 5. One explanation for the inconsistency is that the concentration of paxilline was at the threshold of what is necessary to block the actions of $P2Y_1$ receptors on BK. The IC_{50} of paxilline for BK channel inhibition in cultured cells is in the 10 - 20 nM range (Sanchez & McManus, 1996; Zhou & Lingle, 2014), suggesting that the concentration should have been high enough to reveal an effect. However, one can never be certain about the concentration required in more complex environments of thick tissue slices. Increasing the concentration of paxilline, however, is complicated by the fact that it inhibits SERCA with an IC_{50} between 5 μM and 50 μM (Bilmen *et al.*, 2002), and we have established that inhibition of SERCA will inhibit $P2Y_1$ receptor-mediated effects on frequency (Rajani *et al.*, 2018). The option of increasing paxilline concentration was explored, but abandoned based on whole-cell recording experiments that demonstrated that while 1 μM paxilline did not affect the baseline MRS 2365 current, 4 μM paxilline did. At -60 mV under voltage clamp conditions, the only way that paxilline could affect the MRS 2365 current is through inhibition of SERCA. BK channels are Ca^{2+}-activated and voltage-gated. In order to activate BK channels at -60 mV, the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) needs to be higher than 50 μM (Wei *et al.*, 1994; Wang *et al.*, 2009; Li *et al.*, 2018). $[Ca^{2+}]_i$ is about 50-100 nM in neurons at rest and only increase to levels as high as 1000 nM following activity-dependent Ca^{2+} release from intracellular Ca^{2+} stores or Ca^{2+} influx

through voltage-gated Ca^{2+} channels (Berridge *et al.*, 2000; Grienberger & Konnerth, 2012). While $[Ca^{2+}]i$ can reach levels in excess of 1000 nM in microdomains close to voltage-gated Ca^{2+} channels where BK channels are often found (Gola & Crest, 1993; Marrion & Tavalin, 1998; Womack *et al.*, 2004; Berkefeld *et al.*, 2006), the likelihood that $[Ca^{2+}]i$ was high enough to activate BK channels in our voltage-clamp recordings at -60 mV is remote. A direct $P2Y_1$ receptor-mediated activation of voltage-gated Ca^{2+} channels and indirect activation of BK is conceivable, but the only documented action of $P2Y_1$ receptors on voltage-gated Ca^{2+} channels is inhibition (Brown *et al.*, 2000a; Filippov *et al.*, 2000; Aoki *et al.*, 2004). γ subunits of BK channel are reported to modulate its voltage dependency by producing a hyperpolarizing shift of the activation curve (Zhang & Yan, 2014). There are four types of γ subunits (Li & Yan, 2016) and only γ3 subunit is expressed in brain tissues (Yan & Aldrich, 2012; Zhang *et al.*, 2018). Binding of γ3 subunit to BK channel leads to an about -50 mV shift of the activation curve of BK channel when $[Ca^{2+}]_i$ is 0 μM in HEK293 cells (Yan & Aldrich, 2012). The γ3 subunit-mediated shift becomes relatively smaller with higher $[Ca^{2+}]_i$. Even under the condition where BK channel is modulated by γ3 subunit and the $[Ca^{2+}]_i$ is fairly high at 2 μM (which may not be achievable by $P2Y_1$ receptor activation as discussed above), the activation threshold for BK channel (between 0 mV and 20 mV) is still far away from the resting membrane potentials (-45 to -52 mV) measured in the inspiratory neurons (Yan & Aldrich, 2012). Consistent with this argument, $P2Y_1$ receptor activation did not evoke BK currents (or any currents) in rat striatal neurons held near -60 mV. However, at more depolarized levels $P2Y_1$ receptor activation potentiated BK (Coppi *et al.*, 2012).

Given that paxilline at 4 μM inhibits SERCA, we did not examine the effect of higher paxilline concentrations on the $P2Y_1$ receptor-mediated frequency increase. Thus, it remains possible BK channels are a target of $P2Y_1$ receptor signaling and that they contribute to the

frequency effect. $P2Y_1$ receptors activate BK channels in striatal (Coppi *et al.*, 2012), hippocampal (Schicker *et al.*, 2010) and enteric neurons (Sanders *et al.*, 2014). Activity of BK channels can also be modulated by PIP_2 (Vaithianathan *et al.*, 2008; Zhang *et al.*, 2014) as well as intracellular Ca^{2+} (Sah & Faber, 2002) which are both affected by $P2Y_1$ receptors. The effects of BK inhibition on basal respiratory rhythm is highly variable (Onimaru *et al.*, 2003; Zhao *et al.*, 2006; Zavala-Tecuapetla *et al.*, 2008; Zhang *et al.*, 2010; Rajani *et al.*, 2016), but their activation is necessary for the rhythmogenesis when glycinergic inhibition is removed (St-John & Leiter, 2002) and for the generation of gasping and autoresuscitation in hypoxia (Zavala-Tecuapetla *et al.*, 2008). Gasping is evoked under conditions of extreme hypoxia, thus it is possible that the positive network effect of 1 μM paxilline in some experiments was due to a very hypoxic preparation. This is not considered likely since the protocols lasted hours and hypoxic slices tend not to remain stable for such long periods. Thus, while we can't definitively exclude a contribution of BK to the $P2Y_1$ receptor mediated frequency increase, a major involvement is unlikely.

In summary, we have presented evidence demonstrating that ATP-excitation of the inspiratory network in vitro is mediated by $P2Y_1$ receptor-mediated activation of the $G\alpha_q$-signalling pathway in a subpopulation of preBötC inspiratory neurons. The findings of this study hold potential translational value in that ATP may operate through a similar mechanism to offset the secondary hypoxic ventilatory depression in humans. If so, the signalling pathway used by $P2Y_1$ receptors may serve as a pharmaceutical target for treatment of apnea of prematurity because its enhancement may stimulate breathing.

Figures

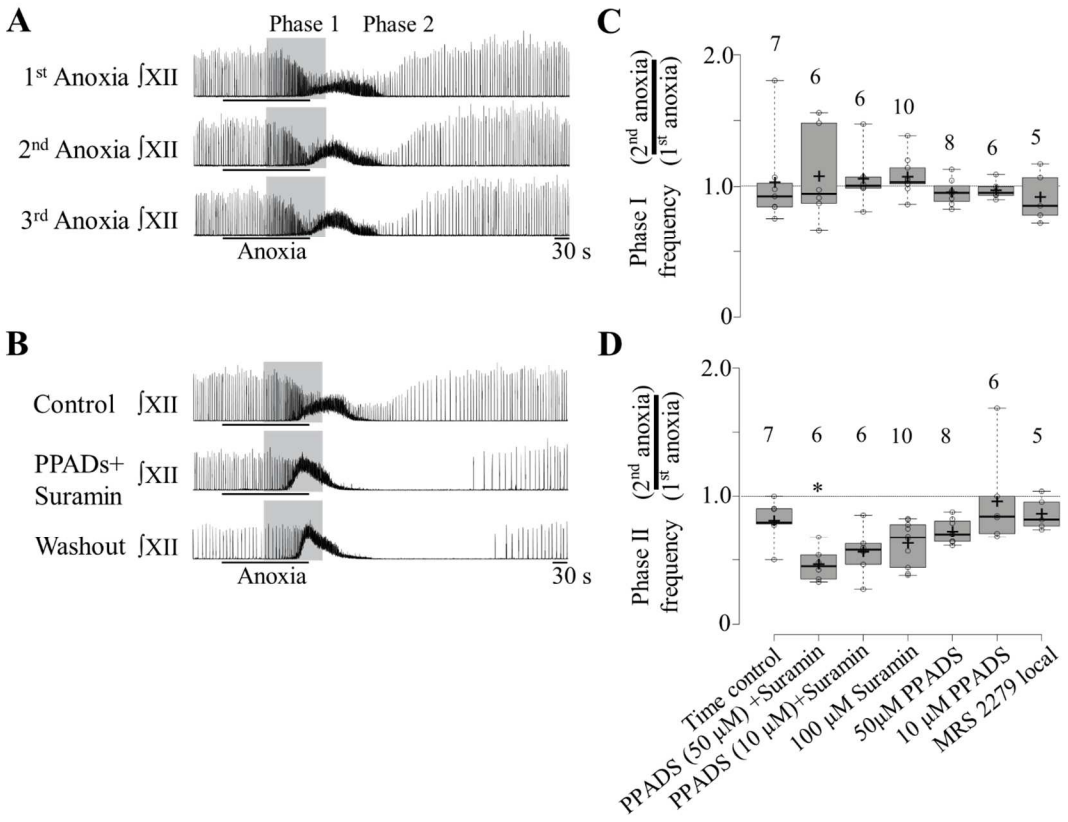

Figure 2.1 Anoxia-induced respiratory depression in vitro is attenuated by bath-applied PPADs and suramin but not MRS 2279 (500 µM, 5 min) locally applied in the pre-Bötzinger complex

Biphasic responses of rhythmic medullary slices to three consecutive anoxic episodes (3 min, 20 min intervals) in: (A) control and (B) before and after bath application of the general P2 receptor antagonists PPADS and Suramin. Group data show the effects of purinergic receptor antagonists on the initial anoxia-induced frequency increase (C) and the secondary depression (D). Sample sizes of the groups are shown above their corresponding boxes in (C) and (D). PPADS (50 µM) in combination with suramin (100 µM) significantly increased the secondary depression compared with time-matched control ($p = 0.0156$, One-way ANOVA with Bonferroni post-hoc test).

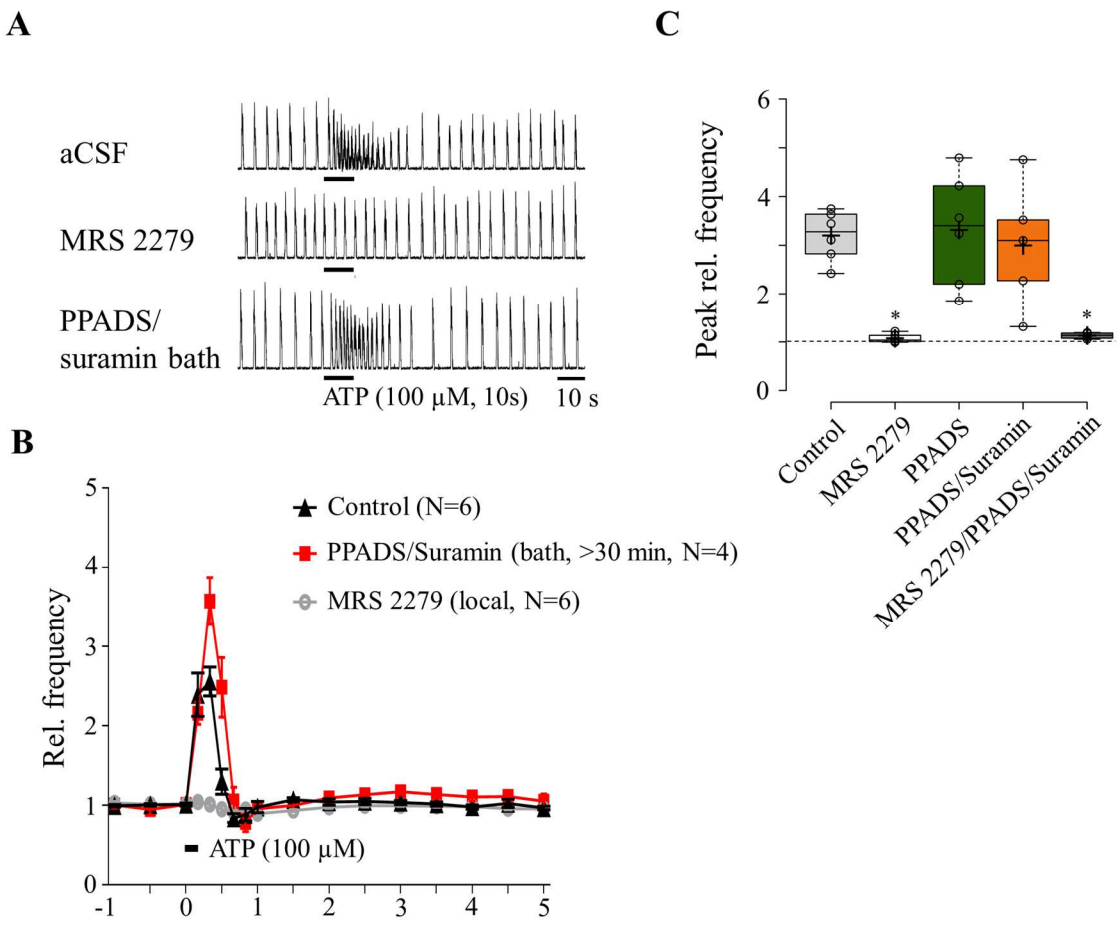

Figure 2.2 ATP-induced excitation of the inspiratory network is blocked by MRS 2279

Representative traces (A), time-courses of relative frequency of XII nerve activity (B) and group data (C) showing the effects on the ATP (100 µM, 10 sec)-evoked frequency increase of MRS 2279 (500 µM, 2 min, local), PPADS alone (50 µM, bath), PPADS (50 µM, bath) in combination with suramin (100 µM, bath) for 15 min and all antagonists together (MRS 2279 local, PPADS 50 µM and Suramin 100 µM). Sample sizes were indicated above the boxes. B) $P < 0.0001$ between the ATP control response and MRS 2279 groups; $P < 0.0001$ between the ATP control response and PPADS/suramin groups; $P < 0.0001$ between the ATP control response and MRS 2279 groups. C) One-way ANOVA with Bonferroni post-hoc test. * $P < 0.01$ between the control and MRS 2279 groups; $P < 0.01$ between the control and MRS 2279/PPADS/suramin groups.

A

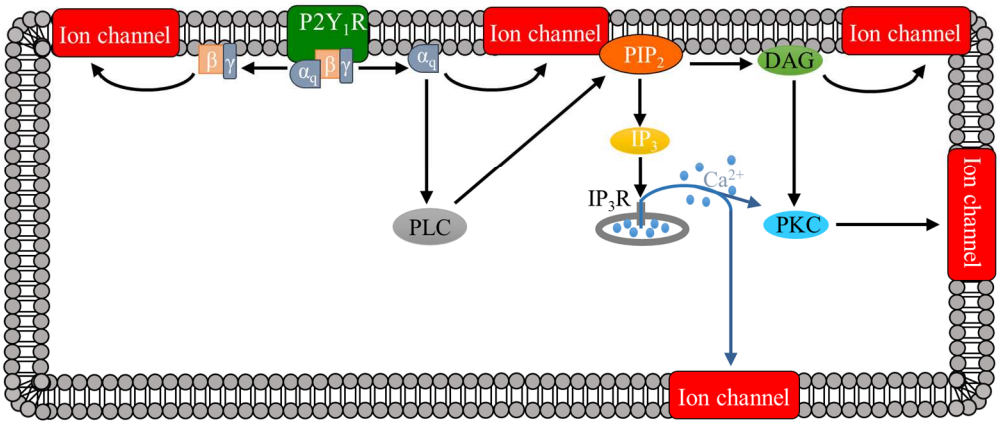

Figure 2.3 Overview of the P2Y$_1$ receptor-coupled Gα_q-signalling pathway

Upon activation of P2Y$_1$ receptors, α_q subunit disassociates from the βγ subunit and activates phospholipase C (PLC). PLC then catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptors on the endoplasmic reticulum and triggers intracellular Ca^{2+} release. DAG and free Ca^{2+} then activate protein kinase C (PKC). The α_q and βγ subunits can also modulate the activity of ion channels directly, likely in a membrane-delimited manner (Herlitze *et al.*, 1996a; Ikeda, 1996; McCudden *et al.*, 2005; Chen *et al.*, 2006). Figure 2.3 is reproduced based on figure 1 of the article with the title of "The role of P2Y$_1$ receptor signaling in central respiratory control". As one of the authors, I retain the copyright to reuse the figure.

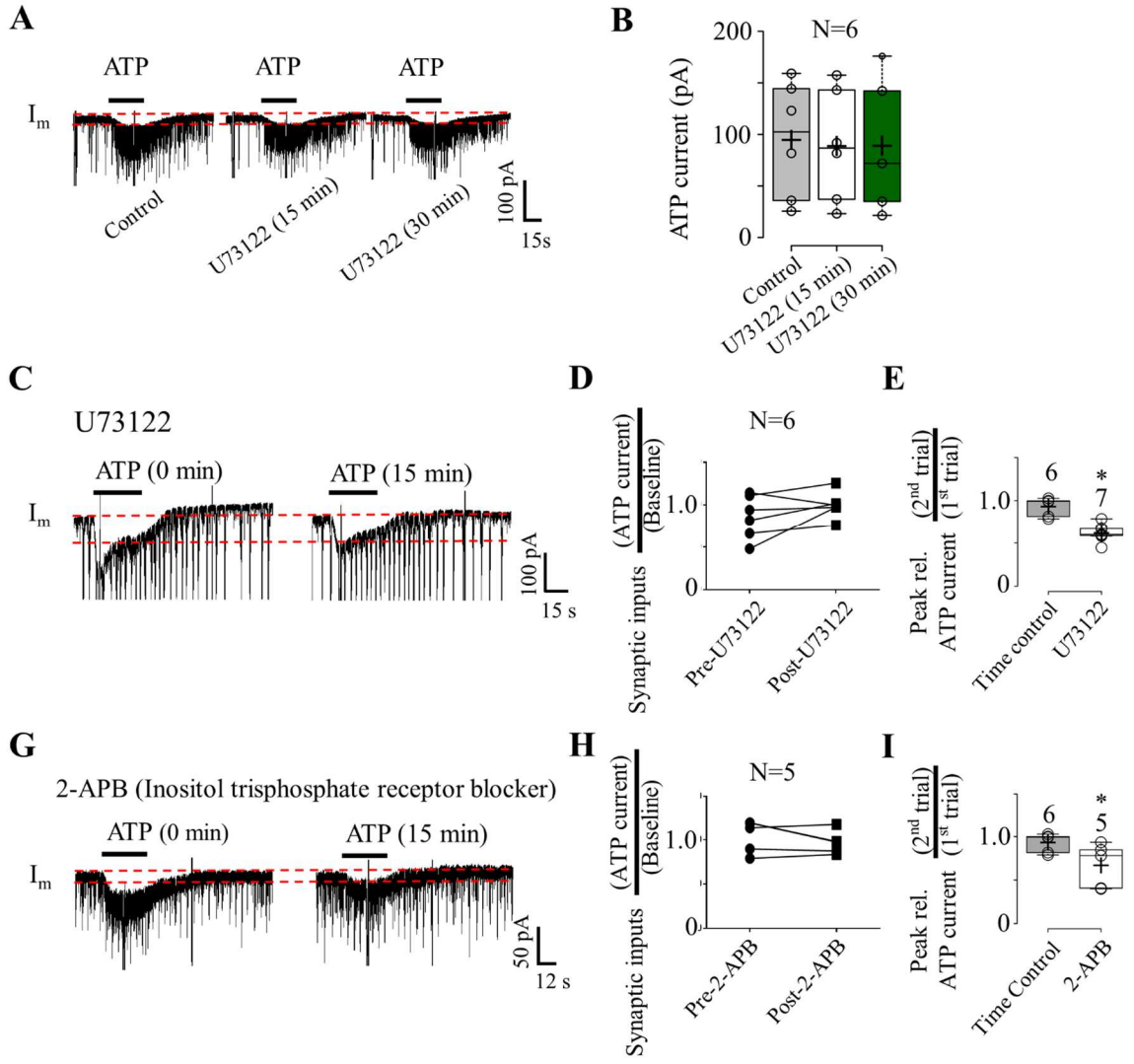

Figure 2.4 Intracellular dialysis of U73122 or 2-APB, but not bath applied U73122, attenuates inward currents evoked in preBötC inspiratory neurons by ATP

Representative traces (A) and group data (B) showing the effect of bath applied U73122 (20 µM, 30 min) on 5 mM ATP-induced inward currents evoked in inspiratory neurons. Representative traces (C) and pooled data (E) demonstrating the effect of 15-min intracellular dialysis of U73122 (2 µM) on the amplitude of ATP currents. P=0.0002, unpaired student t-test. D) Effect of U73122 on the synaptic inputs in ATP current. P > 0.05, paired student t-test. Sample sizes are indicated above the boxes. Representative traces (G) and group data (I) showing the effect of intracellular

dialysis of 2-APB (50 μM) on ATP currents. P = 0.0445, unpaired student t-test. H) Effect of 2-APB on the synaptic inputs in ATP current. P > 0.05, paired student t-test.

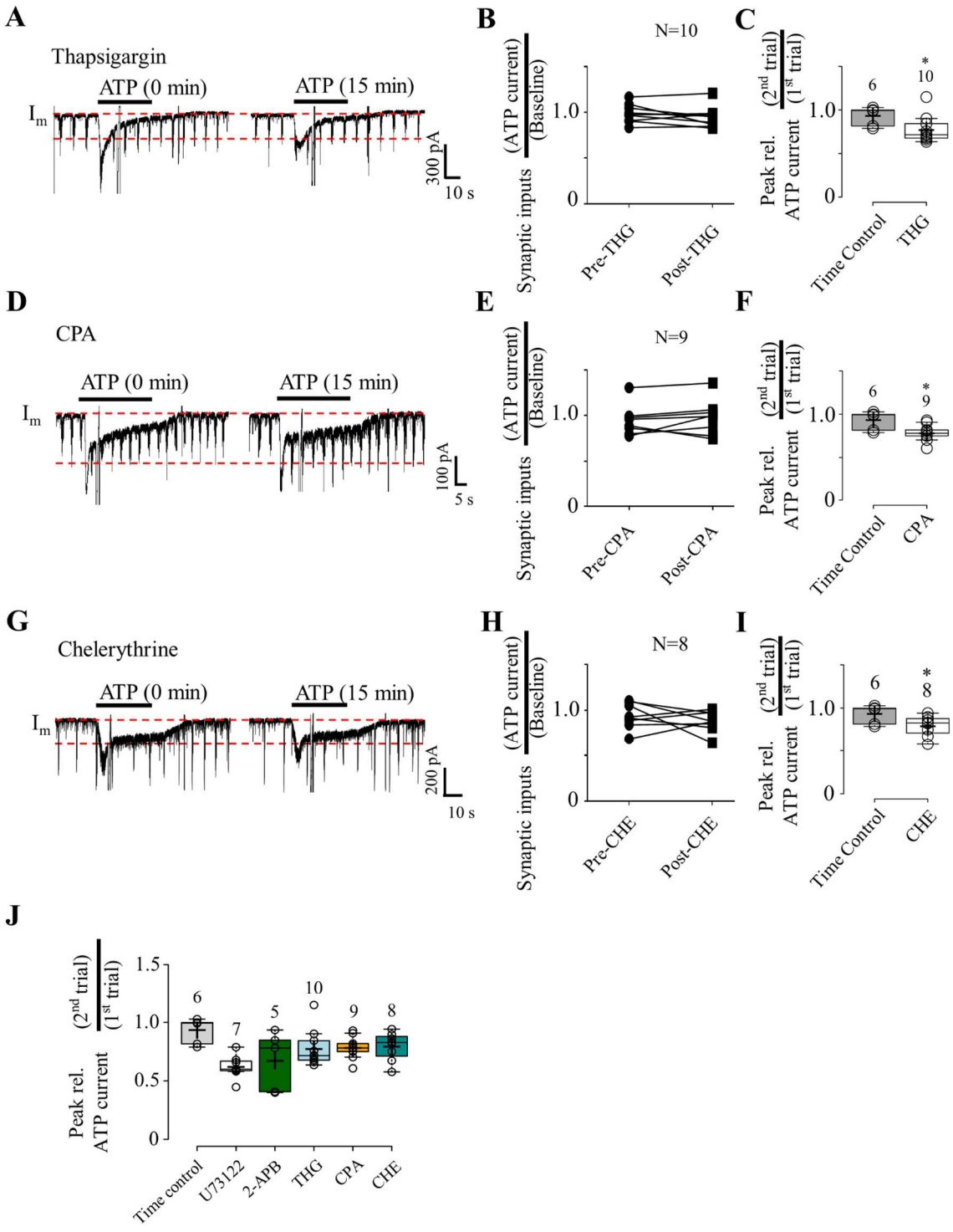

Figure 2.5 Depletion of intracellular calcium stores or PKC inhibition attenuates inward currents evoked in preBötC inspiratory neurons by ATP

Representative traces (A) and group data (C) showing that the effect of 15-min intracellular dialysis of thapsigargin (4 μM) on the amplitude of ATP currents evoked in inspiratory neurons. P = 0.0411, unpaired student t-test. B) Effect of thapsigargin on the synaptic activity in ATP current. Representative traces (D) and group data (F) showing the effect of 15-min intracellular dialysis of cyclopiazonic acid (20 μM) on the amplitude of ATP currents evoked in inspiratory neurons. P = 0.0135, unpaired student t-test. E) Effect of cyclopiazonic acid on the synaptic activity in ATP current. P > 0.05, paired student t-test. Representative traces (G) and group data (I) demonstrating the effect of 15-min intracellular dialysis of chelerythrine chloride (10 μM) on the amplitude of ATP currents. P = 0.0411, unpaired student t-test. H) Effect of chelerythrine chloride on the synaptic activity in ATP current. P > 0.05, paired student t-test. J) Pooled data summarizing the effects of all the drugs used in this study on ATP-induced inward currents evoked in inspiratory neurons. Note that blocking the $G\alpha_q$-signalling pathway does not completely block ATP current. Sample sizes are denoted by the numbers above the histograms.

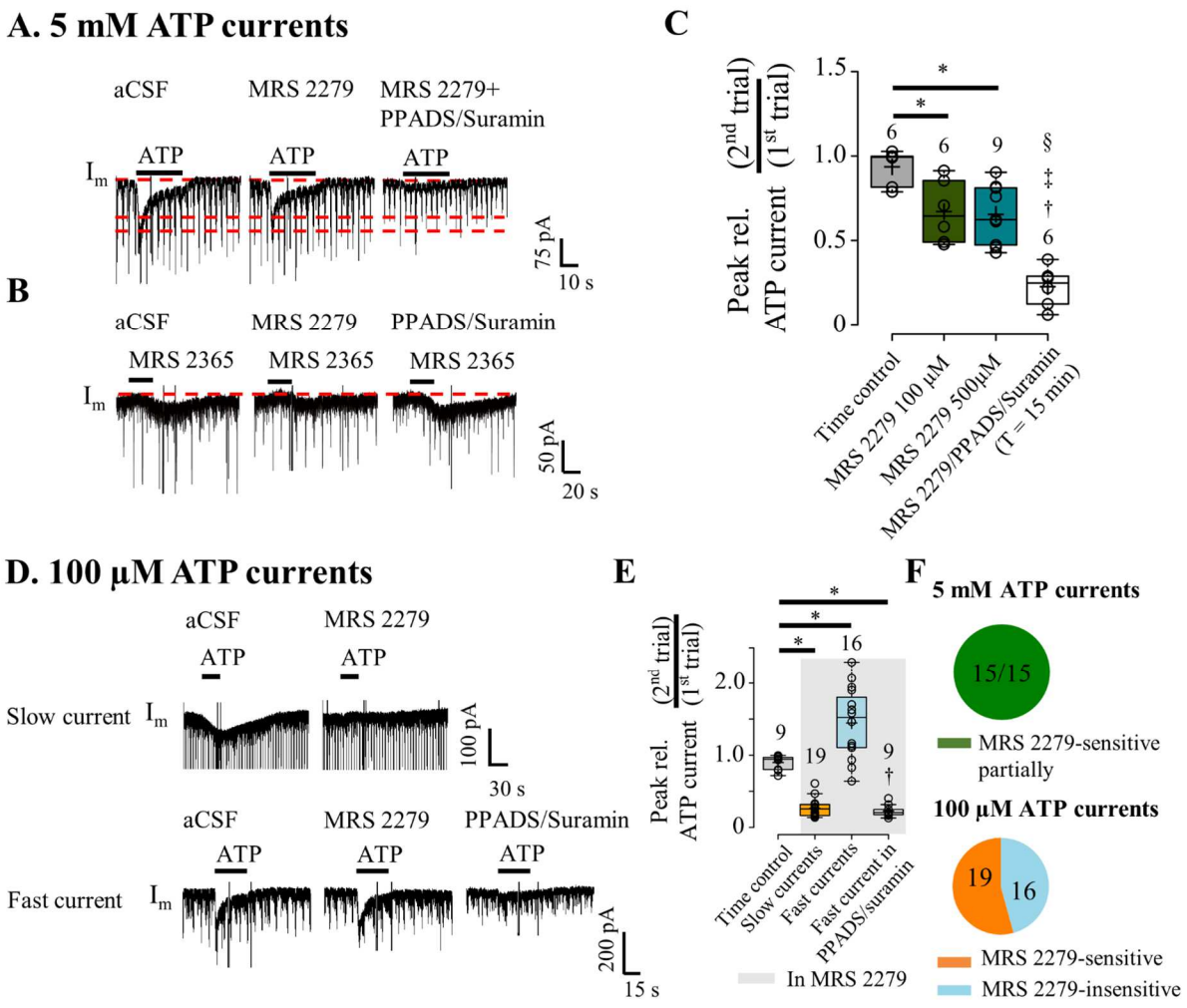

Figure 2.6 Inward currents induced by 5 mM ATP in inspiratory neurons involves both $P2Y_1$ receptor-dependent and -independent components while ATP at 100 µM activates either one of the two types of currents in different inspiratory neurons

Representative traces (A) and group data (C) showing the effects of MRS 2279 (500 µM, local, 2 min), PPADS (50 µM, bath, 15 min) and suramin (100 µM, bath, 15 min) on inward currents induced by 5 mM ATP. * $P < 0.05$, † $P < 0.0001$ between time-matched control and MRS 2279/PPADS/suramin groups, ‡ $P < 0.0001$ between MRS 2279 (100 µM) and MRS 2279/PPADS/suramin groups, § $P < 0.0001$ between MRS 2279 (500 µM) and MRS 2279/PPADS/suramin groups. One-way ANOVA, Bonferroni post-hoc test. B) Representative

traces showing the effects of MRS 2279, PPADS and suramin on the MRS 2365 (100 μM)-induced inward currents. Representative traces (D) and group data (E) showing the effect of MRS 2279 on slow currents induced by 100 μM in a subset of inspiratory neurons and the effects of MRS 2279, PPADS and suramin on fast currents evoked in another group of neurons. * $P < 0.0001$, † $P < 0.0001$ between the "fast current" and "fast current in PPADS/suramin" groups. One-way ANOVA, Bonferroni post-hoc test. F) Pie charts illustrating the proportion of the inspiratory neurons which responded to 5 mM ATP with inward currents that contained a $P2Y_1$ receptor-dependent component (top) and the proportions of the inspiratory neurons that responded to 100 μM ATP with MRS 2279-dependent slow inward currents and those with PPADS/suramin-dependent fast currents (bottom).

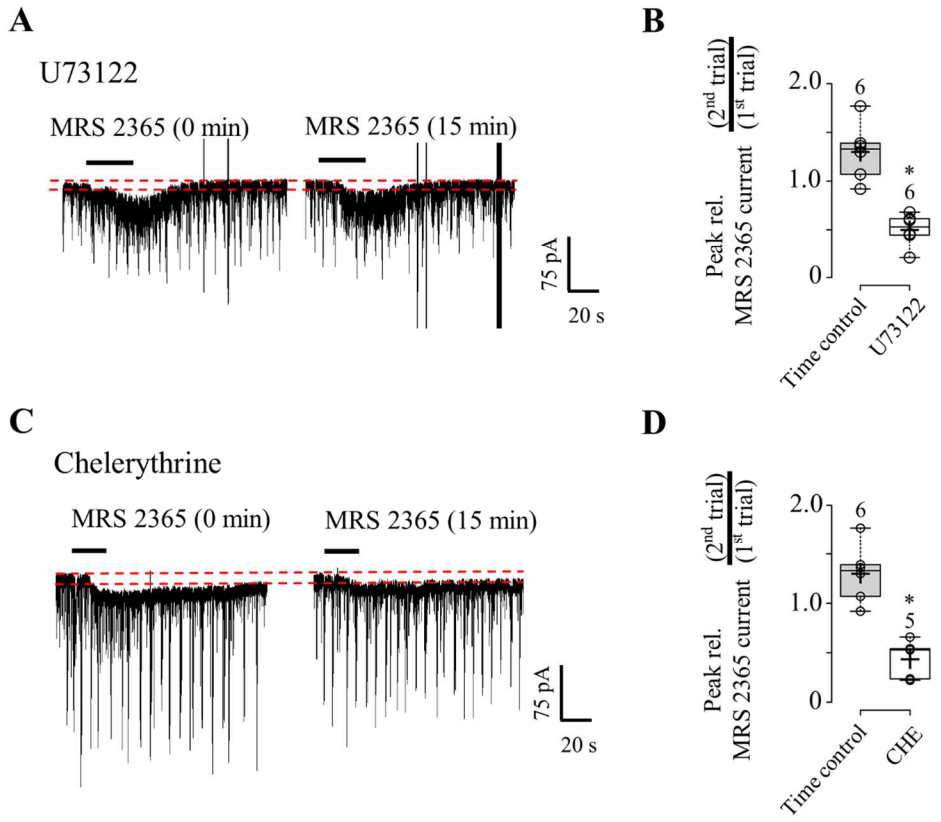

Figure 2.7 U73122 and chelerythrine attenuate MRS 2365-induced inward currents

Representative traces (A) and group data (B) showing the effect of 15-min intracellular dialysis of U73122 (4 μM) on the MRS 2365-induced inward currents evoked in the inspiratory neurons. * P=0.00018, unpaired student t-test. Representative traces (C) and group data (D) showing the effect of 15-min intracellular dialysis of chelerythrine (10 μM) on the MRS 2365-induced inward currents evoked in the inspiratory neurons. * P=0.00059, unpaired student t-test.

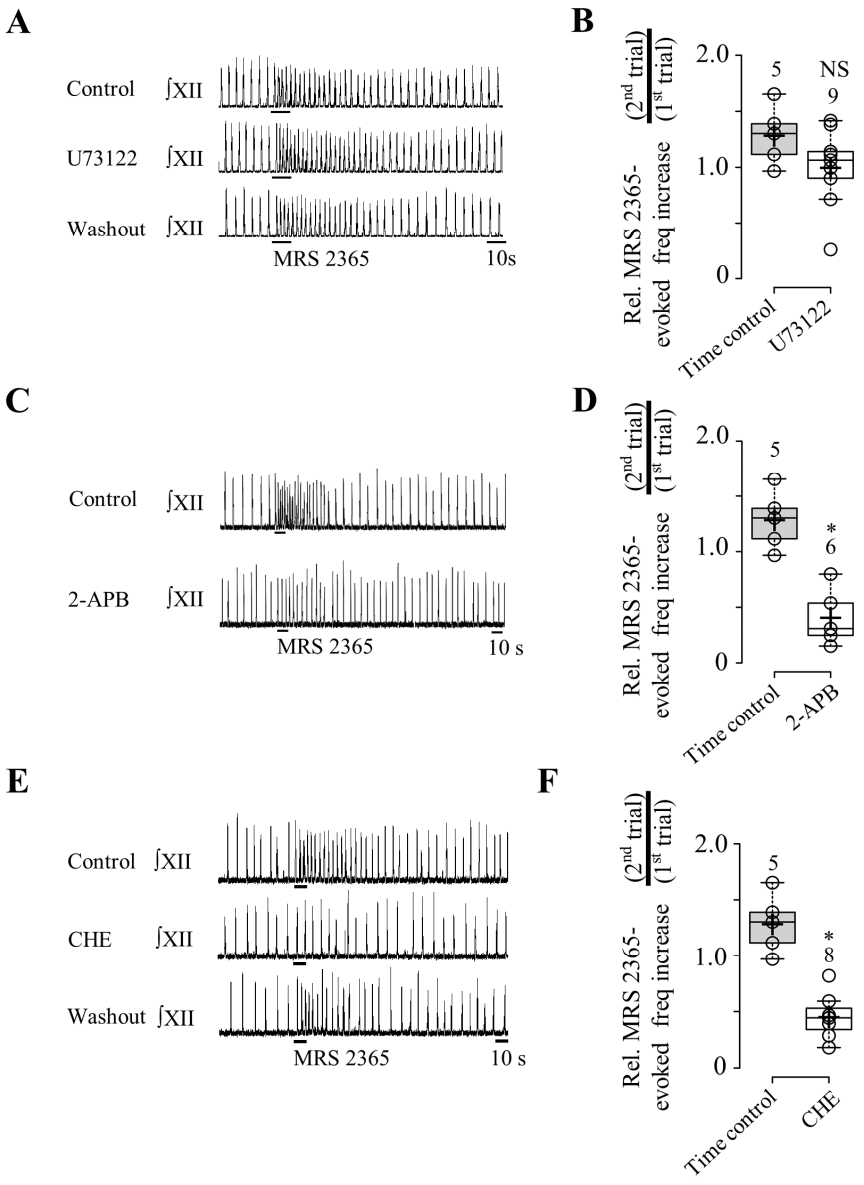

Figure 2.8 2-APB and chelerythrine, but not U73122, reduce MRS 2365-induced frequency increase in inspiratory-related activity

Representative traces (A) and group data (B) showing that U73122 had no effect on the MRS 2365-induced frequency increase. U73122 was applied in the bath (20 µM, 40 min) and the preBötC (10 µM, pulse injection: 10 sec on and 30 sec off, total duration: 40 min) simultaneously. $P > 0.05$, unpaired student t-test. Representative traces (C) and group data (D) demonstrating the effect of 2-APB (50 µM, bath) on the MRS 2365-induced excitation of the inspiratory network. *

P=0.0007, unpaired student t-test. Representative traces (E) and group data (F) demonstrating the effect of chelerythrine (10 μM, bath, 30 min) on the MRS 2365-induced excitation of the inspiratory network. * P = 0.0006 unpaired student t-test.

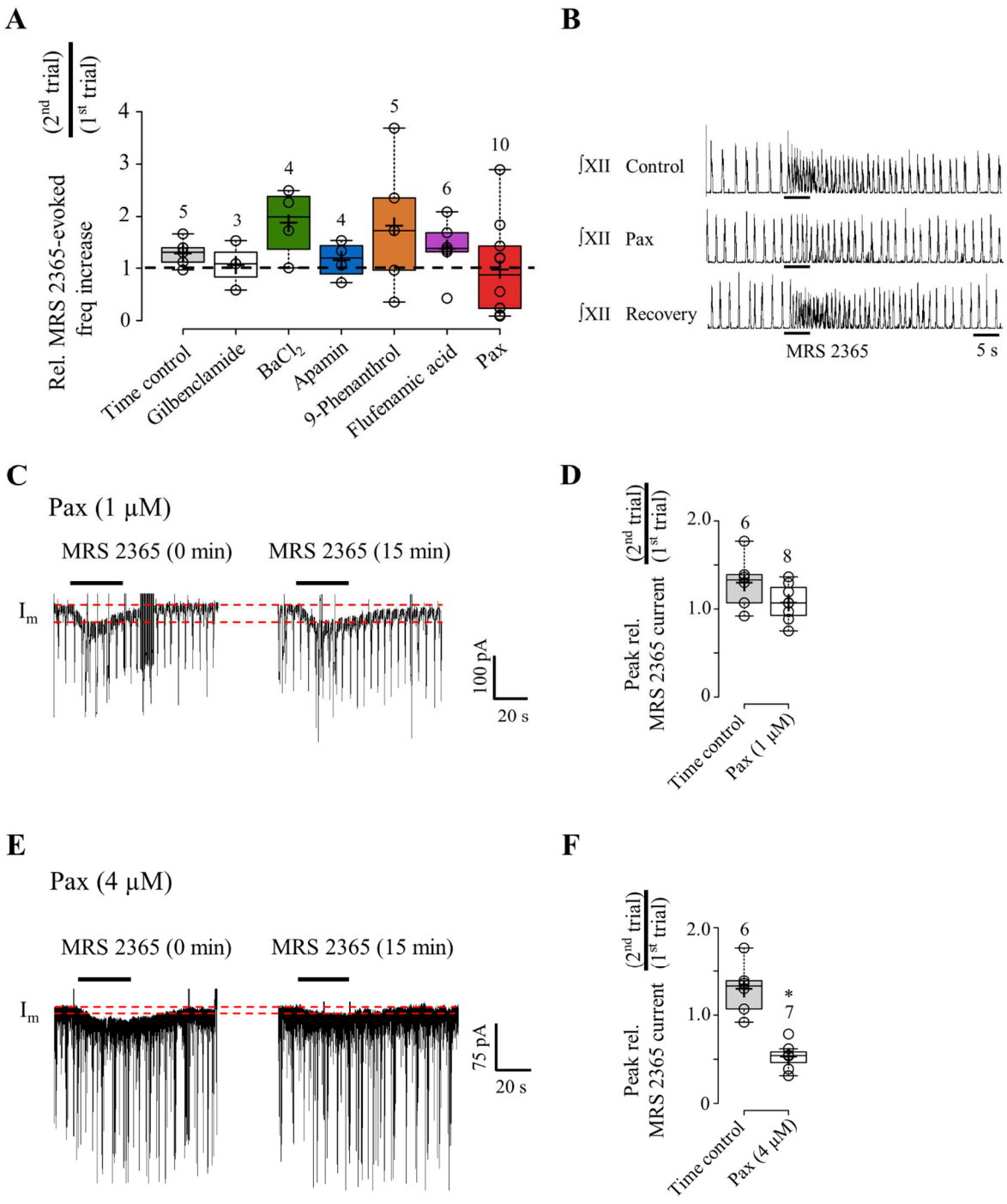

Figure 2.9 None of KATP, GIRK, SK, TRPM4/5 and BK channels contribute to the $P2Y_1$ receptor-mediated excitation of the inspiratory network

A) Effects of glibenclamide, $BaCl_2$, apamin, 9-phenanthrol, flufenamic acid and paxilline on MRS2365-induced frequency increase. $P > 0.05$, one-way ANOVA with Bonferroni post-hoc test. B) Representative traces showing that paxilline attenuated the MRS 2365-induced frequency increase. Representative traces (C) and group data (D) showing a lack of paxilline (1μM, local, 30 min) effect on MRS2365-induced frequency increase. Representative traces (E) and group data (F) showing the effect of 15-min intracellular dialysis of paxilline (4μM) on MRS2365-induced inward currents in the inspiratory neurons. * $P<0.0001$, unpaired student t-test

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Chapter 3. $P2Y_1$ receptor excitation of the pre-Bötzinger complex inspiratory network in vitro is partially mediated by elevation of cAMP levels and potentiation of I_h in a subset of inspiratory neurons

3.1 Abstract

ATP excites the inspiratory network via activation of the $G\alpha_q$-signalling pathway and an unknown ion channel. However, blocking the $G\alpha_q$-signalling pathway does not abolish the $P2Y_1$ receptor-mediated excitation of inspiratory neurons or the inspiratory network, suggesting that a second mechanism is involved. In this study we tested the hypothesis that the $P2Y_1$ receptor-mediated excitation of the preBötC network is mediated via an increase in cAMP levels and potentiation of the hyperpolarization-activated inward current, I_h, in inspiratory neurons. In Chapter 2, we established that ~54% of preBötC inspiratory neurons expressed an ATP-current (100 μM) with a large $P2Y_1$ receptor component. Here we found that 57% (45/79) of preBötC inspiratory neurons responded to MRS 2365 ($P2Y_1$ receptor agonist, 100 μM) with an obvious inward current greater than -15 pA. In 11 of 11 neurons, this current increased with membrane hyperpolarization and reversed between -60 and -40 mV (n = 10/11) or -40 and – 20 mV (n = 1/11), suggesting activation of I_h. The MRS 2365-induced inward current was markedly attenuated in 9 of 9 neurons by local pre-application of ZD7288 (open channel blocker of I_h, 100 μM). Progressively hyperpolarizing voltage-steps from -50 to -110 mV or -140 mV (-10 mV increments) revealed a sag current, diagnostic of I_h, in 38 of 46 inspiratory neurons. The MRS 2365 sensitivity of the I_h current was then examined with various protocols in a total of 45 inspiratory neurons. This sag current was blocked by ZD 7288 in 8 of 8 neurons and potentiated by MRS 2365 in 7 of 7 neurons (28 ± 6.1% potentiation measured at -100 mV) with a large MRS 2365 current (measured at -60 mV), but also in 11 inspiratory neurons without a noticeable MRS 2365 current at a holding potential of -60 mV (30.4 ± 5% potentiation measured at -100 mV). 23/45 inspiratory preBötC neurons did not respond to MRS 2365 in any way; i.e. no inward current and no potentiation of I_h whereas MRS 2365 attenuated I_h in the rest 4/45 cells. Comparison of I_h activation curves,

produced via analysis of tail currents evoked by a series of 10 mV hyperpolarizing steps from -50 mV to -140 mV, in control and MRS 2365, revealed an average 9.8 mV, MRS 2365-induced depolarizing shift in $V_{1/2}$ (n=6). At the network level, pre-application of ZD7288 at 25 μM (n=10) and 100 μM (n=6) attenuated the MRS 2365-induced frequency increase in inspiratory-related activity by 67 ± 12% and 90 ± 2% after three trials, respectively. $P2Y_1$ receptors typically operate through the $G\alpha_q$ second messenger pathway, whereas I_h is typically modulated via Gs activation of cAMP. We therefore examined whether cAMP was involved in the $P2Y_1$ receptor modulation of preBötC neurons and rhythm. 15-min intracellular dialysis of SQ 22536 (adenylyl cyclase inhibitor, 100 μM) from the whole-cell pipette significantly attenuated the MRS 2365 currents by 60 ± 4% (n = 9 out of 18 neurons). Similarly, SQ 22536 (100 μM) attenuated the MRS 2365-induced frequency increase by 51 ± 13% following a 60 min bath application (n = 8). These data suggest that the $P2Y_1$ receptor-mediated excitation of the preBötC network is produced in part via an elevation in cAMP level and potentiation of I_h in a subpopulation of inspiratory neurons.

3.2 Introduction

Breathing in mammals is a rhythmic behavior that moves air into and out of the lungs through the airways and is largely responsible for the homeostatic control of arterial O_2 and CO_2/pH levels. The respiratory rhythm is generated by a neural network comprising two critical oscillators located in the brainstem– the oscillator responsible for generating inspiratory rhythm in the preBötzinger Complex (preBötC)(Smith *et al.*, 1991; Funk *et al.*, 1993; Gray *et al.*, 2001) and the oscillator responsible for generating active expiration located in the parafacial respiratory group (pFRG), at least in adults (Onimaru & Homma, 2003, 2005; Janczewski & Feldman, 2006; Abdala *et al.*, 2009; Pagliardini *et al.*, 2011; Huckstepp *et al.*, 2016). From one perspective the respiratory network is very much like other rhythmic motor networks in that its main function is to produce a rhythmic motor behavior, breathing. The unique feature of the respiratory network is that its level of activity is tightly controlled to match metabolic rate by sensory feedback relaying information about levels of oxygen and CO_2/pH in the arterial blood and brain.

As mentioned in chapter 1, hypoxia evokes a biphasic ventilatory response comprising an initial increase in ventilation followed by a secondary depression, which is largely attributed to central inhibitory mechanisms mediated in part through the actions of adenosine (Darnall, 1985; Runold *et al.*, 1986; Runold *et al.*, 1989) (Koos & Chau, 1998; Koos *et al.*, 2002) and other transmitters such as GABA (Melton *et al.*, 1990; Dahan & Ward, 1991; Huang *et al.*, 1994; Xiao *et al.*, 2000) . In adults, ventilation remains above baseline throughout the hypoxic exposure whereas ventilation falls below the baseline during the secondary depressive phase in most neonatal mammals (Moss, 2000), including human infants, especially those born prematurely (~8% of births in Canada)(Shah *et al.*, 2018). These infants who are susceptible to apnea of prematurity (AOP) due to their immature respiratory network are given caffeine, an adenosine

receptor antagonist, as a respiratory stimulant to stabilize breathing. While effective, ~20% of infants do not respond to caffeine (Lista *et al.*, 2016) and this underlies the interest in searching for an alternative means of stimulating breathing.

Despite the long-standing view that the only respiratory hypoxia sensor resides in the carotid bodies (or other peripheral chemoreceptive structures) (Youngson *et al.*, 1993; Prabhakar, 2000)(Funk, 2018 #3076, recent studies have identified astrocytes in the preBötC as local O_2 sensors (Angelova *et al.*, 2015a) that contribute to the ventilatory response evoked by acute hypoxia, via their release of adenosine triphosphate (ATP). ATP, a phylogenetically ancient molecule long associated with sensory function (Chen *et al.*, 1995; Lewis *et al.*, 1995; Cook *et al.*, 1997), is a neurotransmitter/gliotransmitter in the central nervous system (Gourine *et al.*, 2003; Pascual *et al.*, 2005; Jourdain *et al.*, 2007) where it acts via ionotropic $P2X_{1\text{-}7}$ receptors (North, 2002) and metabotropic $P2Y_{(1,\,2,\,4,\,6,\,11\text{-}14)}$ receptors (Illes *et al.*, 1996; Burnstock, 2007) to mediate fast excitatory transmission and modulate neuronal excitability. ATP released by brainstem astrocytes, including those in the preBötC, during hypoxia excites breathing and counteracts the secondary hypoxic respiratory depression (Angelova *et al.*, 2015a; Sheikhbahaei *et al.*, 2018). The ATP excitation in the preBötC is mediated by $P2Y_1$ receptors in vitro (Lorier *et al.*, 2007; Huxtable *et al.*, 2009; Huxtable *et al.*, 2010; Zwicker *et al.*, 2011)(Chapter 2, Fig. 2) and also in vivo (Rajani *et al.*, 2018) during exposure to hypoxia .

$P2Y_1$ receptors are conventionally thought to signal through the $G\alpha_q$ second messenger pathway (Usachev et al., 2002; Abbracchio et al., 2006; Song & Vijayaraghavan, 2007; Chandaka et al., 2011; Rajani et al., 2016). However, in Chapter 2 application of multiple inhibitors of the $G\alpha_q$ signalling pathway to the bath in network studies or inside single inspiratory neurons in whole-cell recording studies never reduced the ATP- or MRS 2365-mediated excitation by more than

60%. Our previous data similarly showed that depletion of intracellular Ca^{2+} stores with THG and CPA attenuated the MRS 2365-induced frequency increase by less than 50% (Rajani *et al.*, 2018). Collectively, these data suggest that an unidentified, non-$G\alpha_q$ signalling cascade(s) targeting an unknown ion channel(s) contributes to the $P2Y_1$ receptor-mediated excitation of the inspiratory network.

The objective of this study was to identify these unknown contributors. To do so we took the opposite approach from Chapter 2, in which we started at the beginning of the signalling cascade with the identification of the P2 receptor subtype and then pursued the G-protein coupled receptor pathway through which $P2Y_1$ receptors are known to signal. In this study, we started at the end of signalling cascade by trying to characterize the current and ion channel(s) through which $P2Y_1$ receptors excite preBötC inspiratory neurons, and worked backwards from that point to identify components of signalling cascade. Whole-cell recording analyses of preBötC inspiratory neurons in the rhythmically-active medullary slice preparations of neonatal rat revealed that in a subpopulation of inspiratory neurons (18 of 45) MRS 2365 potentiated the hyperpolarization-activated inward current, I_h, that is mediated by hyperpolarization-activated, cyclic nucleotide-gated (HCN) ion channels (Ludwig *et al.*, 1998) and has been previously identified in preBötC inspiratory neurons (Mironov *et al.*, 2000). The blockade of I_h does not significantly affect baseline rhythm in rhythmic slices in vitro (Mironov *et al.*, 2000), but this does not exclude the possibility that its activation could increase rhythm. Further investigation revealed that MRS 2365 potentiated the sag current evoked by hyperpolarizing pulses, a signature of I_h, by causing a ~10 mV depolarizing shift of the activation curve of HCN channels and that the I_h blocker ZD7288 significantly attenuated the MRS 2365 current. At the network level, ZD7288 significantly attenuated (60 - 90%) the frequency increase evoked by activation of $P2Y_1$ receptors in the

preBötC, while reduction of cAMP levels, the cyclic nucleotide that gates/potentiates HCN channels, with SQ 22536 (an adenylyl cyclase inhibitor) reduced the amplitude of the MRS 2365-induced inward currents by 60 ± 4% in 9 out of 18 preBötC inspiratory neurons, and halved the MRS 2365-induced frequency increase. Taken together, these data suggest that ATP-mediated excitation of the preBötC network is produced in part via a $P2Y_1$ receptor-mediated increase in cAMP and potentiation of I_h in a subpopulation of inspiratory neurons, both of which may serve as targets for development of a novel breathing stimulant for treatment of AOP, especially in caffeine-resistant infants.

3.3 Methods

3.3.1 Animals

Timed-pregnant, Sprague Dawley rats were obtained from Charles River Laboratories (Wilmington, Massachusetts, United states) or BioScience Animal Services of University of Alberta (Edmonton, Alberta, Canada) and received at the animal facility 1 or 2 weeks prior to the date of birth. Dames were single-housed under a 12-h light-dark cycle (light on from 7 a.m. to 7 p.m.) with access to food and water *ad libitum*. Pups used in the study ranged in age from postnatal day 0-4 (P0-P4). All animal studies and experimental procedures 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 (Protocols AUP255 and AUP256).

3.3.2 Rhythmically-active medullary slice preparations

Rhythmically active medullary slices were prepared as described in previous studies (Smith *et al.*, 1991; Ruangkittisakul *et al.*, 2006; Zwicker *et al.*, 2011; Rajani *et al.*, 2018). In brief, P0 –

P4 pups were decerebrated under anesthesia maintained via isoflurane inhalation. The thorax and head were isolated through a transection at the level of the diaphragm and perfused following removal of skin in cooled (4^0C) carbogen (95% O_2 and 5% CO_2)-bubbled 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. Another transection was made at the midpontine level and the cerebellum was removed. The brainstem and cervical spinal cord were then exposed via a spinal laminectomy, isolated and pinned to a wax chuck which was then placed in the vice of a vibratome (VT1200S, Leica, Nussloch, Germany) set for serial sectioning. The brainstem was sectioned in the rostral-to-caudal direction in 200 μM steps. Once the structures of inferior olive dorsal and inferior olive principal nuclei were observed, a 700 μm thick slice containing the preBötC was collected and moved to one of two recording chambers, depending on the experiment.

3.3.3 Extracellular hypoglossal nerve (XII) recordings

Rhythmically-active medullary slices were either pinned on the Sylgard resin in a 5 ml recording chamber for the extracellular recording experiments that examined network effects or secured under a silver harp in an 2.5 ml chamber for the whole-cell recording experiments. The slices were placed rostral surface up and perfused with aCSF at flow rates of 15 ml/min and 2 ml/min for the extracellular and whole-cell recording experiments respectively. The extracellular K^+ ($[K^+]_e$) was elevated from 3 mM to 9 mM at least 30 min before data collection to produce prolonged, stable rhythm (Ruangkittisakul *et al.*, 2006). Inspiratory-related activity was recorded from the hypoglossal nerve roots via a suction electrode (A-M Systems, Carlsborg, WA, USA). Signals were amplified, bandpass filtered (300 Hz to 1 kHz), full-wave rectified, integrated using a leaky integrator (τ = 25 or 50 ms), and displayed using Axoscope 9.2 (Molecular Devices, Sunnyvale, CA, USA). Data were saved to computer using a Digidata 1322 A/D board and

AxoScope 9.2 software (Molecular Devices) for off-line analysis. All recordings were conducted at 24°C.

3.3.4 Whole-cell recordings

Whole-cell recordings from inspiratory neurons located in the preBötC were made in rhythmically active slices using whole-cell pipettes (4-6 MΩ) pulled from borosilicate glass that were filled with intracellular solution (ICS) containing (in mM): 140 K-gluconate, 5 NaCl, 0.1 EGTA, 10 HEPES, 1 $MgCl_2$ and 1 glucose (liquid junction potential: −14.5 mV). Osmolarity of intracellular solutions was adjusted to 290–300 mOsm with sucrose and pH adjusted to 7.2–7.3 with KOH. Membrane potentials were not corrected for liquid junction potentials. PreBötC inspiratory neurons were selected based on their location ventral to the semicompact division of nucleus ambiguus, the presence of rhythmic inspiratory-related synaptic currents that were in phase with the inspiratory–related rhythm recorded from the XII nerve rootlets. Inspiratory neurons with a resting membrane potential of -45 mV or more hyperpolarized, were included in the analysis. If access resistance changed by more than 20% during a voltage clamp protocol (i.e., between control, test and recovery condition), or if the holding current was not stable between control and test conditions, data were excluded from analysis.Cells were visualized with infrared and DIC optics on an upright microscope (Axioskop2 FS plus, Carl Zeiss, Oberkochen, Germany). The whole-cell pipette was moved through the tissue toward the target neuron along the electrode axis to minimize tissue compression. Gigaseal formation and membrane rupture were performed in voltage-clamp mode. The membrane potential was held at -60 mV except during the voltage-clamp protocols designed to examine properties of the MRS 2365-evoked current and also I_h. Details of these protocols are included in relevant portions of the Results Section.

Whole-cell experiments were conducted at 24°C. Whole-cell signals were amplified, low

pass filtered at 5 kHz, digitized (sampled at 20 kHz) and saved to computer using a MultiClamp 700A amplifier, Digidata 1322 A/D board and AxoScope 9.2 software (Molecular Devices, Union City, CA) for off-line analysis. Series resistance (Rs) and whole-cell capacitance (Cm) were estimated as done previously (Adachi *et al.*, 2005; Pagliardini *et al.*, 2005) using the Rs and Cm compensation features of MultiClamp Commander software (Axon Instruments) to manually correct the current response to square-wave voltage pulses (100 Hz,−10 mV, 3 ms) under voltage-clamp conditions. Neuronal input resistance (R_N) was calculated based on the current response to a voltage ramp (1.5 s duration, from −90 to −40 mV) applied before, during and after drug application. The inverse slope of the linear portion (usually between -70 mV and -60 mV) of the current–voltage (I–V) relationship was used to estimate R_N.

3.3.5 Drugs

Substance P (SubP), MRS 2365, ZD7288, forskolin and SQ 22536 were obtained from Tocris Biosciences (Bristol, UK). Forskolin and SQ22536 were prepared as stock solutions in 100% dimethyl sulfoxide (DMSO), while Substance P, MRS 2365 and ZD7288 were prepared as stock solutions in 3 mM K^+-containing aCSF. The stock solutions were diluted by at least 2 orders of magnitude to their final concentrations before use. The final concentration of DMSO was 0.2% in forskolin and SQ 22536 solutions used for local application as well as in SQ 22536-containing ICS. DMSO concentration was 0.2% in the bath where SQ 22536 was applied.

3.3.6 Drug applications

MRS 2365 (100 µM, 1 mM), ZD7288 (25µM, 100 µM), forskolin (10 µM) and SQ 22536 (1 mM) were applied locally via pressure injection into or above the preBötC. SQ 22536 was also applied to the bath (100 µM) or added to the intracellular solution (100 µM). Triple-barrelled pipettes (5-6 µm outer diameter per barrel) were used for local injection of drugs into or above the

tissue. Injections were controlled by a programmable stimulator (Master-8; A.M.P.I., Jerusalem, Israel) connected to a picospritzer (Spritzer4 Pressure Micro-Injector, 18 psi). Substance P (10 sec, 1 μM) was injected to help locate the preBötC, as described previously (Lorier *et al.*, 2007; Zwicker *et al.*, 2011). The injection site was moved in a grid-like fashion until a site was located at which Substance P caused more than a two-fold increase in the frequency of inspiratory bursts recorded from the hypoglossal nerve. This site was then defined as the preBötC (Lorier *et al.*, 2007; Zwicker *et al.*, 2011). SQ 22536 was added in the ICS to ensure its access to the intracellular site of action in the whole-cell recording experiments. The cells in which SQ 22536 had no effect on the MRS 2365-induced inward currents were treated as time control. The ZD7288 effect on the MRS 2365-induced inward currents evoked in the inspiratory neurons was also compared with this time control to assess the I_h contribution.

3.3.7 Data Analysis

The extracellular and whole-cell data were analyzed offline using Clampfit (pClamp 9.2, Axon Instruments) and Microsoft Excel. The instantaneous frequencies of rhythmic hypoglossal nerve activity were calculated in Clampfit and normalized relative to the baseline frequency, which was the average frequency during the two minute period immediately preceding drug application. The maximum effects of MRS 2365 on inspiratory frequency was measured as the peak frequency value recorded in the moving average of instantaneous inspiratory frequency (calculated based on the moving average of five consecutive bursts) during the first two minute after MRS 2365 injection. The effect of the various blockers on the ability of MRS 2365 to increase network frequency was assessed by comparing the peak frequency evoked by MRS 2365 in control (aCSF), drug and washout trials (aCSF). The effects of forskolin and SQ 22536 on baseline rhythm were measured by taking the value from the moving average of inspiratory frequency during the 5 min

of drug application that was maximally different from the baseline frequency (the average frequency during the 2 min prior to drug application).

The contribution of I_h and cAMP signalling to the MRS 2365 current evoked in inspiratory neurons was assessed by comparing the peak MRS 2365-evoked inward current in control and again 15 min and 30 min later after application of ZD7288 or SQ 22536. In this case of SQ 22536, the control MRS 2365 current was measured within 2 min of obtaining whole-cell recording configuration and again after 15 or 30 min dialysis of SQ 22536 into the neuron. To ensure that any antagonist-mediated reduction in the MRS 2365 current was not the result of current run down (which can occur when using whole-cell recording methods to measure currents evoked via second messenger signalling cascades), antagonist experiments were compared with time control experiments. The current evoked by the second application of MRS 2365 (whether in the time control or antagonist experiments) was reported as a percentage of the first response (i.e. $I_{MRS\ 2365\ \#2}$/ $I_{MRS\ 2365\ \#1}$) x 100). A value of 1.0 indicates that the agent had no effect on the MRS 2365 current; while values less than or greater than 1.0 indicates that the agent inhibited or potentiated the response to the indicated percentage of the control response. For the time control experiments, a value less than 1.0 would indicate some run down in the MRS 2365 current. Statistically we then compared the magnitude of change between first and second MRS 23656 responses in the time control group with the change observed over the same time frame in the presence of the antagonists, ZD7288 or SQ 22536. Note in some cases MRS 2365 was applied several times in the presence of a blocker, in which case the value of the MRS 2365 response measured in the nth response is reported relative to the initial control response (($I_{MRS\ 2365\ \#n}$/$I_{MRS\ 2365\ \#1}$) x 100%).

To assess the effects of $P2Y_1$ receptor signalling on the voltage-dependence of I_h activation in preBötC inspiratory neurons, we analyzed the tail-currents evoked by a voltage clamp protocol

in which neurons, normally held at a Vm of -60, were given a 1 sec pre-pulse to -50 mV, followed by a series of 1s hyperpolarizing steps from -50 mV to -140 mV in -10 mV increments. I_h activates and inactivates slowly and is maximally activated at -140 mV (Ludwig *et al.*, 1998). When the membrane is next stepped directly to -60 mV for at least 1 sec, the maximally activated I_h produces a tail current that can be measured and this is referred to as I_{Max} which is set at 100%. Hyperpolarizing pulses less than -140 mV will not maximally activate I_h so the tail current measured with the step back to -60 mV from the hyperpolarizing steps were calculated as a fraction of I_h activated at a certain potential (I/I_{Max} * 100). These I/I_{Max} data were plotted against the hyperpolarizing potentials and fitted with the Boltzmann function: $I/I_{Max} = 1/(1+ \exp((V \pm V_{1/2})/k))$ where $V_{1/2}$ is the potential at which half of HCN channels are open and k is the slope factor (Ludwig *et al.*, 1998; Wainger *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002). Tail current analyses were performed under baseline conditions (4 min local application of aCSF) and in the presence of MRS 2365 (4 min local application).

Differences between means were compared using GraphPad Prism (Version 6.01, GraphPad Software Inc.). Paired or unpaired t-tests were used for comparison of two groups. For comparison of more than two groups, one-way or two-way ANOVA was used in conjunction with either a Bonferroni post-hoc multiple comparison test. P values < 0.05 were considered significant. Group data are presented as boxplots created using 'BoxPlotR' (Spitzer *et al.*, 2014). Spread of data points, the lowest point, 1st quartile, median, 3rd quartile and highest point are superimposed on each box as open circles, the lower whisker, bottom, middle, and top of box, and top whisker respectively. The mean for each group is denoted by "+".

3.3.8 Neuronal numbers

In total 83 preBötC inspiratory neurons from the region immediately ventral or

ventrolateral to the nucleus ambiguus were exposed to MRS 2365 and their responses studied using whole-cell recording methods. 45 of the 83 neurons had a noticeable MRS 2365-evoded current that was greater than -15 pA (on average -29 ± 2 pA). In 11 of these 45 neurons with large MRS 2365 currents we measured only the reversal potential of the MRS 2365 current. In 9 different neurons with large MRS 2365 currents, we measured only the sensitivity of the MRS 2365 current to ZD7288. We then began the specific exploration of I_h and the sensitivity of I_h to MRS 2365. For this part of the study we recorded 45 neurons (of the total 83 neurons mentioned above). All 45 neurons had an I_h, based on the presence of inward sag currents evoked by the hyperpolarizing command potentials. Of this group of 45 neurons, 7 had large MRS 2365 currents and all 7 cells had an I_h that was potentiated by MRS2365. 14 of the 45 inspiratory neurons had very small or non-detectable responses to MRS 2365 at -60 mV but expressed an I_h that was potentiated by MRS 2365. In 4 of 45 neurons MRS 2365 attenuated the I_h without inducing inward currents. The rest 20 of 45 neurons did not response to MRS 2365 in any way; i.e. there was no obvious inward current at -60 mV and there was no effect of MRS 2365 on the I_h current. In the experiment where we tested the involvement of cAMP in the MRS 2365-induced inward current, we recorded 18 inspiratory neurons, all of which responded to MRS 2365 with large inward currents (18 of 45). However, the MRS 2365 current was only cAMP-dependent in 9 of the 18 neurons.

3.4 Results

3.4.1 $P2Y_1$ receptors activate h-currents in the inspiratory neurons

Efforts to identify the ion channel through which $P2Y_1$ receptors excite the inspiratory network from a list of candidates with documented sensitivity to $P2Y_1$ receptor/$G\alpha_q$ modulation that also modulate inspiratory rhythm (Rajani *et al.*, 2016)(Chapter 2, Fig. 2.9A) were unsuccessful. In this study we took the reverse approach by first characterizing properties of the

MRS 2365-evoked current, then identifying the likely ion channel and finally components of the second messenger pathway through which $P2Y_1$ receptors might modulate the ion channel to increase network frequency. We began by measuring the reversal potential of $P2Y_1$ receptor-mediated inward currents evoked in inspiratory neurons as well as the associated change in neuronal input resistance. 11 inspiratory neurons were held, in random order, at membrane potentials of -80 mV, -60 mV and -40 mV and the MRS 2365 currents measured at 15 minute intervals were -41 ± 15 pA, -15 ± 2 pA and 21 ± 7 pA, respectively (Figs. 3.1A and B, n = 11); i.e., the $P2Y_1$ receptor-activated current increased with hyperpolarization and reversed at a potential between -60 mV and -40 mV. The MRS 2365 currents had no significant effect on neuronal input resistance, which under control conditions measured 82.4 ± 13.2, 94.7 ± 4.2 and 139.9 ± 17.8 MOhms at -80, -60 mV and -40 mV respectively, and in MRS 2365 measured 82.9 ± 13.5, 94.5 ± 4.7 and 128.7 ± 15.7 MOhms. The reversal potential of the MRS 2365 current and its increase with hyperpolarization were suggestive of an h-current (I_h), which has been described previously in a subpopulation of preBötC inspiratory neurons in rhythmic slices from neonatal mice (Mironov *et al.*, 2000).

Based on these similarities and the observation in mesencephalic trigeminal neurons that $P2Y_1$ receptor activation facilitates I_h (Huang *et al.*, 2010), we hypothesized that $P2Y_1$ receptor activation excites the inspiratory network by potentiating I_h in a subpopulation of preBötC inspiratory neurons. To test this, we compared the MRS 2365 inward currents evoked in inspiratory neurons in control conditions and again 15 min later after 3 min local application of ZD7288 (I_h blocker). ZD7288 reduced the amplitude of the MRS 2365-induced inward currents to 63.8 ± 3.7% (n = 9, Figs. 3.1C and D) of the initial response; i.e., it reduced the response by ~36% and 58%. This ZD7288-mediated attenuation was significantly greater than the time-dependent

change of the MRS 2365 current measured in time control experiments where the second control MRS 2365 current was 104.3 ± 5.1% (n = 9) of the initial control MRS 2365 current; i.e., the MRS 2365 current did not run down. While ZD7288 reduced the MRS 2365 currents, the block was incomplete and the ZD7288 effect plateaued after two trials (Fig. 3.1D). To test whether the incomplete block might reflect that ZD7288 is an open channel blocker (Shin *et al.*, 2001) and that it has limited access to its site of action during the MRS 2365 trials performed at -60 mV (where I_h is not fully activated), we repeated ZD7288/MRS 2365 trial three times to facilitate access of ZD7288 to its binding site in the pore of HCN channels. We found that there was an additional significant decrease in the MRS 2365 current in the second ZD7288 trial where the current fell to 42 ± 5 % of control and no further decrease was seen between the second and third ZD7288 trials (Fig. 3.1D). The progressive inhibition of the MRS 2365 current is due to ZD7288 but not run down because the MRS 2365 current did not change over 30 min in the time-matched control experiments. These data suggest that between 36 and 58% of the MRS 2365 current is sensitive to ZD7288. Another important point is that ZD7288 never completely blocked the MRS 2365 current, even after 3 consecutive trials.

The reversal potential and pharmacological data in Fig. 3.1 suggest that I_h contributes to the currents evoked in inspiratory neurons by $P2Y_1$ receptor activation. To more directly test whether $P2Y_1$ receptor activation affects I_h in inspiratory neurons, we used two slightly different voltage clamp protocols to evoke I_h in aCSF and MRS 2365. Protocol 1 involves holding the neurons at -60 mV, stepping to -50 mV for a 1 sec prepulse followed by a series of five, 1-sec hyperpolarizing voltage steps starting at -70 mV to -110 mV (-10 mV increments)(Mironov *et al.*, 2000). Protocol 2 consists of the same 1 sec, -50 mV prepulse and 1 sec hyperpolarizing steps in -10 mV increments with the exception of a broader voltage range from -50 mV to -140 mV (Ludwig

et al., 1998). Protocol 2 allows for maximum activation of I_h so it was also used to assess the effect of $P2Y_1$ receptor activation on the activation curve of HCN channels. Data collected from the experiments using protocol 1 (n = 32) and 2 (n = 13) were pooled together for analysis. If I_h is present, these hyperpolarizing steps evoke a characteristic inward sag current that develops slowly and reaches a steady state after several 100 msec; the magnitude of I_h (and the sag) also increases with progressive hyperpolarization. We compared the magnitude of these sag currents during local application of aCSF and then MRS 2365 (n = 45) or aCSF and then ZD7288 (n = 8). A sag current indicative of I_h was observed in 51 of 60 neurons. MRS 2365 (at 100 μM, n = 15; and 1 mM, n = 3) was applied to 45 inspiratory neurons with an I_h; MRS 2365 potentiated the sag current in 18 of these 45 preBötC neurons from -89.9 ± 15.7 pA to -121.71 ± 17.8 pA (Fig. 3.2B for the summarized effect of 100 μM MRS 2365, n = 15), as can be seen for 100 μM MRS 2365 in a single inspiratory neuron in Fig. 3.2A. The 45 inspiratory neurons can be classified into four groups based on whether MRS 2365 induced inward currents and potentiated I_h. MRS 2365 induced inward currents and potentiated I_h in 7 of 45 neurons (group 1). MRS 2365 potentiated I_h but had not effect on the holding currents in 14 of 45 neurons (group 2). In contrast, MRS 2365 attenuated I_h in 4 of 45 neurons (group 3). Finally, 20 of 45 neurons did not respond to MRS 2365 with potentiation of I_h or inward currents (group 4). One example of a group 1 neuron illustrated that the cellular effects of MRS 2365 could be blocked by ZD7288 (Fig. 3.3A). Another example neuron from group 2 was shown in Fig. 3.3B. There is no difference in input resistance between neurons of groups 1, 2 and 3 (109.4 ± 25.6 MOhms vs 113.5 ± 22.5 MOhms vs 108.4 ± 41.2 MOhms).

We confirmed that the sag currents evoked by the hyperpolarizing voltage step protocols were indeed carried by I_h with the group data demonstrating that 2 and 3 min of locally applied

ZD7288 (100 μM) reduced the sag current evoked by the steps from -50 to -100 mV from -100 ± 12 pA at baseline to -44 ± 8 pA and -19 ± 6 pA, respectively. In other words, sag currents were reduced to 43.7 ± 7.5% and 19.9 ± 5.9% of the control sag current after locally applying ZD7288 for 2 min and 3 min, respectively (Fig. 3.2C and D, n = 8).

3.4.2 $P2Y_1$ receptor activation potentiates I_h in preBötC inspiratory neurons via a depolarizing shift in its voltage-activation curve

As their name indicates, HCN channels that carry I_h currents are gated by cyclic nucleotides and they are therefore highly modulated. A major mechanism of HCN modulation that enhances the influence of I_h on membrane excitability occurs via depolarizing shifts in the voltage dependence of I_h activation (Kase & Imoto, 2012). To test whether $P2Y_1$ receptors share this mode of action on I_h, we measured the voltage-dependence of I_h under control conditions and in the presence of MRS 2365 by analyzing the tail-currents evoked by the voltage protocol 2 that began with a 1 sec pre-pulse to -50 mV from a holding potential of -60 mV, followed by a series of 1-sec hyperpolarizing voltage steps from -50 mV to -140 mV (-10 mV increments) and then returning to -60 mV (where the membrane was held between steps to ensure the same driving force for I_h during the I_{tail} measurements). I_h activates and inactivates with slow kinetics and the degree of activation increases with hyperpolarization, reaching maximum activation at -140 mV. Thus, at the end of the 1 sec hyperpolarizing pulse to -140, I_h is maximally activated. With the step back to -60 mV, I_h begins to inactivate very slowly such that the inward tail current measured immediately after the step back to -60 mV provides a measure of the maximum I_h, referred to as I_{max}, and this current is set at 100%. The tail currents measured after the smaller hyperpolarizing currents will be some fraction of I_{max}. Then, by plotting the size of the tail currents evoked at each potential relative to I_{max} against the hyperpolarizing pulse potential and fitting these data with the Boltzmann

function (see Methods for details), one obtains the voltage-activation curve for I_h. As shown for one neuron in Fig. 3.4A and for group data in Fig. 3.4B, MRS 2365 (100 μM, 4 min) potentiated the tail currents and caused a significant, 9.8 mV depolarizing shift in V1/2 of the voltage-activation curve of I_h from -112.8 ± 3.4 mV in control to -103 ± 1.6 mV in MRS 2365 (n = 6).

3.4.3 $P2Y_1$ receptor evoked inward currents in a subpopulation of inspiratory neurons are mediated in part through increases in intracellular cAMP

HCN channels, by definition, are cyclic nucleotide gated ion channels, and a major effect of cAMP on I_h is that it causes a depolarizing shift in its voltage activation curve (Ludwig *et al.*, 1998; Wainger *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002) similar to that just demonstrated for the $P2Y_1$ receptor agonist, MRS 2365. We therefore hypothesized that the $P2Y_1$ receptor-mediated inward current and excitation of inspiratory neurons is dependent, at least in part, on cAMP. To test this, we used the adenylyl cyclase inhibitor, SQ 22536, to reduce the intracellular cAMP levels and assessed whether this had the predicted inhibitory effect on currents evoked in preBötC inspiratory neurons by MRS 2365.

The amplitude of the MRS 2365-induced inward currents evoked in the inspiratory neurons was measured before and after 15-min intracellular dialysis of SQ 22536 (100 μM). Interestingly, the SQ 22536 effect varied between cells. SQ 22536 had no effect on the MRS 2365-induced currents in 9 out of 18 inspiratory neurons, but reduced the amplitude of the MRS 2365 currents in the remaining 9 neurons. As shown for one neuron in Fig. 3.5B, the MRS 2365 current fell from to 50.3% and 38.2% of the initial response after 15 and 30 min of SQ 22536 dialysis, respectively. Group data indicate that on average the MRS 2365 current fell to 62 ± 2.9% of the initial response after 15 min of SQ 22536 dialysis (Fig. 3.5C). The cells that did not respond to SQ 22536 were named as “non-responders” and treated as the time controls (Fig. 3.5A). There is no difference in

input resistance between the responders (83.3 ± 6.4 MOhms) and non-responsders (78.6 ± 14.9 MOhms). These data suggest that in a subpopulation of preBötC inspiratory neurons, $P2Y_1$ receptor-mediated excitation involves an elevation of intracellular cAMP levels.

3.4.4 $P2Y_1$ receptors excite the preBötC inspiratory network by potentiating I_h

To test whether the activation of I_h in the preBötC contributes to the $P2Y_1$ receptor-mediated excitation of the inspiratory network, we compared the effects of MRS 2365 on the frequency of the inspiratory-related activity after a 5 min pre-application of aCSF in the control trial, after a 5 min pre-application of ZD7288 at 100 μM or 100 μM and after 5 min pre-application of aCSF again during the washout trial to assess whether the effects of ZD7288 were reversible. Consecutive MRS 2365 applications were always separated by 15 min, which we have shown previously allows for repeatable responses. Single application of ZD7288 reduced the MRS 2365-induced frequency increase to 51.6 ± 13.6% of the control response, which recovered after a 45 min washout (back to 98.3 ± 6.1% of control). The slow washout reflects a poor access of ZD7288 into and out of tissue. Therefore, to ensure ZD7288 reaches its site of action, the ZD7288 – MRS 2365 trial was repeated three times. ZD7288 is an open channel blocker so it can only block HCN channels when they are open. Since HCN channels are minimally activated under baseline conditions in the rhythm slice preparation (Mironov *et al.*, 2000)(because they are usually more depolarized than the activation threshold of I_h), we reasoned that ZD7288 would primarily have access to the open channels in the presence of MRS 2365 (which depolarizes the activation threshold). As shown for a single preparation in Fig. 3.6A and group data in Fig. 3.6B, 100 μM ZD7288 reduced the MRS 2365-induced frequency to 38 ± 8%, 26 ± 8% and 10 ± 2% of the initial control response in the first, second and third ZD7288 trial, respectively. The MRS 2365 effect recovered to 64 ± 27% of the control response following a 60 min ZD7288 washout (Fig. 3.6B).

We then repeated this experiment using 25 μM ZD7288 to address potential off-target actions of 100 μM ZD7288. Similar to 100 μM ZD7288, the MRS 2365-induced frequency increase was progressively inhibited with repeated application, falling to 48 ± 15% ($P = 0.0705$, $n = 7$), x ± x% ($P = 0.2057$, $n = 5$), and 32 ± 12% ($P < 0.05$, $n = 5$) of the control response during the first, second and third ZD7288 trials, respectively, as can be seen in a single preparation (Fig. 3.6C) and group data (Fig. 3.6D). The ZD7288-mediated inhibition slowly washed out such that after 45 min the MRS 2365-induced frequency increase was back to 72 ± 24% of the initial response (Figs. 3.5C and D). These data suggest that $P2Y_1$ receptors excite the inspiratory network via activation of I_h in the preBötC.

3.4.5 $P2Y_1$ receptors excite the inspiratory network via an elevation of intracellular cAMP level

Given the importance of cAMP in the ability of MRS 2365 to evoke inward currents in 50% of preBötC inspiratory neurons (Fig. 3.5C), that I_h blockers attenuated MRS 2365-evoked currents and increases in network frequency and presumed connection between cAMP and I_h in inspiratory neurons, we next assessed the importance of cAMP in the MRS 2365-evoked increase in preBötC frequency. We began with a proof of principle experiment by testing the effects of bath-applied forskolin, an activator of adenylyl cyclase, on baseline preBötC rhythm. If MRS 2365 works through cAMP to increase preBötC frequency, then forskolin should also increase preBötC frequency. In contrast to the local application of aCSF into the preBötC, which had minimal effect on rhythm (13.1 ± 0.7 burst/min in control to 13.5 ± 0.5 burst/min after aCSF injection, Figs. 3.7A and B), local application of forskolin (10 μM, 5 min) increased baseline frequency of the inspiratory-related activity by almost 30% from 14.4 ± 0.6 burst/min to 18.4 ± 0.8 burst/min (Figs. 3.7A and B).

We next tested whether there basal cAMP tone in the preBötC was responsible for an elevation of frequency. Bath application of 100 μM SQ 22536 had no effect on basal rhythm. To help ensure that we were not missing an effect, we then compared the effects locally applied aCSF (5 min into the preBötC) with the effects of 100 μM SQ 22536 applied to the bath in combination with SQ 22536 locally-applied to the preBötC (1 mM, 5 min). This combined treatment had no effect on basal rhythm, which was 14.6 ± 1.1 burst/min during local application of aCSF and 15.4 ± 1.2 burst/min in SQ 22536 (Figs. 3.7C and D, P = 0.36, n = 8).

Finally, we tested the involvement of cAMP in the MRS 2365-evoked frequency increase by comparing the frequency increase evoked by MRS 2365 under control conditions and after 60 min bath application of SQ 22536 (100 μM). As shown for a single preparation and group data in Fig. 3.7E and 7F, the 2.5 fold increase in frequency evoked by MRS 2365 under control conditions was reduced to 49 ± 12% of control by SQ 22536. The SQ 22536-mediated inhibition washed out after 30 min when the MRS 2365-induced frequency increase recovered to 118 ± 10% of the initial control response (Figs. 3.7E and F, n = 5).

3.5 Discussion

ATP excites the inspiratory network in vitro (Lorier *et al.*, 2007; Huxtable *et al.*, 2009; Zwicker *et al.*, 2011) and attenuates the secondary hypoxic ventilatory depression in vivo via activation of $P2Y_1$ receptors in the preBötC (Rajani *et al.*, 2018). Understanding the mechanism underlying the $P2Y_1$ receptor-mediated excitation of breathing is of clinical significance since it may prove to be a target for an alternative means of stimulating breathing for those who are insensitive to the commonly used respiratory stimulant, caffeine (Lista *et al.*, 2016). We demonstrated in Chapter 2 that $P2Y_1$ receptors excite the inspiratory network and neurons in part via activation of the $G_{\alpha q}$-signalling pathway in the preBötC, which is in agreement with the

conventional notion that $P2Y_1$ receptors couple to the $G_{\alpha q}$-signalling pathway (Usachev *et al.*, 2002; Abbracchio *et al.*, 2006; Song *et al.*, 2007; Chandaka *et al.*, 2011; Rajani *et al.*, 2016). However, this mechanism contributes at most 60% of the $P2Y_1$ receptor-mediated excitation. We therefore hypothesized that $P2Y_1$ receptors operate through at least two different mechanisms to excite inspiratory neurons and the network. Thus, the purpose of this study was to identify the ion channel and second messenger system underlying the non $G\alpha_q$-mediated excitatory actions of $P2Y_1$ receptors on inspiratory neurons and the inspiratory network.

The main findings are:

i. $P2Y_1$ receptor-mediated excitation of the preBötC inspiratory network involves $P2Y_1$-receptor-mediated potentiation of I_h in a subpopulation of inspiratory neurons that occurs via a depolarizing shift in the activation curve of HCN channels;

ii. $P2Y_1$ receptor excitation the preBötC inspiratory network involves elevation of intracellular cAMP levels in a subpopulation of preBötC inspiratory neurons;

iii. Based on these observations, we propose that the increase in preBötC frequency evoked by $P2Y_1$ receptor activation involves the activation in a subpopulation of inspiratory neurons of a signalling pathway that increases cAMP and causes a depolarizing shift in the activation curve of HCN channels.

3.5.1 $P2Y_1$ receptors potentiate I_h in a subpopulation of preBötC inspiratory neurons

In Chapter 2 we demonstrated that $P2Y_1$ receptors signal in part via the $G_{\alpha q}$-signalling pathway to increase preBötC inspiratory frequency. In addition, while we established that K_{ATP},

GIRK, SK, TRPM4/5 and BK channels are not the ion channels downstream of the $G_{\alpha q}$-signalling pathway that underlie the frequency increase, we were unable to identify the ion channel(s) that is the ultimate effector of the $P2Y_1$ receptor-initiated network response. Here we provide compelling evidence that the hyperpolarization activated inward current, I_h, contributes to the $P2Y_1$ receptor-mediated excitation of a subpopulation of inspiratory preBötC neurons and the inspiratory network itself. At the neuronal level, evidence includes that the MRS 2365 current increased with hyperpolarization, reversed between -60 mV and -40 mV in 10 of 11 inspiratory neurons and between -40 and -20 mV in 1/11 neurons, and was sensitive to ZD7288. This range of reversal potentials for the MRS 2365 current is slightly more hyperpolarized than the reversal potential reported previously for I_h in inspiratory neurons of a similar preparation in mouse (-40 mV) (Mironov *et al.*, 2000), but this may reflect that the total $P2Y_1$ receptor-mediated current is likely a mixture of currents that includes I_h. Inhibition of the MRS 2365-induced inward currents by the I_h blocker ZD7288 in nine of nine inspiratory neurons also supports that I_h contributes to the $P2Y_1$ receptor-mediated depolarization of inspiratory neurons. The strength of this pharmacological evidence depends on the selectivity of ZD7288 for I_h under our specific experimental conditions. The concentration of ZD7288 in the drug pipette, which is places 50-100 μm from the recorded neuron above the tissue slice, was 100 μM. When drugs are applied locally, concentrations cannot be compared with those in experiments where the same agents are bath-applied as the concentration of drug decreases exponentially with distance from the pipette tip (Nicholson, 1985), which accounts for observations in similar preparations that the concentration of drug in the pipette must be ~10-fold greater than the bath-applied concentration to produce similar effects (Liu *et al.*, 1990). Thus, the concentration of ZD7288 that reduced the MRS 2365 currents was in the range of 10 μM or less. The I_h measured in inspiratory neurons of medullary slices from mouse appears

to be completely blocked by bath concentrations of 1 μM ZD7288 (Mironov *et al.*, 2000). However, IC_{50} values reported for ZD7288 at I_h vary between neurons. IC_{50}s of 15 μM, 1.4 μM, 0.2 μM and 1.8 μM are reported in dorsal root ganglion cells (DRG)(Wu *et al.*, 2012), basket cells of the rat dentate gyrus (Aponte *et al.*, 2006), rat facial motoneurons (Larkman & Kelly, 2001) and rat supraoptic neurons (Ghamari-Langroudi & Bourque, 2000). Thus, we have used an appropriate concentration of ZD7288 in terms of using it to block I_h. Off target actions include the potential inhibition of voltage-gated Na^+ channels. This has been documented in DRG neurons where ZD7288 blocked the Na^+ current with an IC_{50} of 1.7 μM (Wu *et al.*, 2012). However, this is unlikely to be an issue in our experiments. First, small diameter DRG neurons express a unique profile of TTX-resistant Nav1.8 and Nav1.9 subunit-containing voltage-gated Na^+ channels (Ho & O'Leary, 2011) that may underlie the ZD7288 sensitivity of these Na^+ currents. In addition, if ZD7288 affected voltage-gated Na^+ channels in the preBötC in our experiments, inspiratory rhythm would have been disrupted and in no cases did ZD7288 alter basal inspiratory rhythm. Similarly, Mironov et al (2000) report that 1 μM bath-applied ZD7288 has no effect on basal rhythm in rhythmic medullary slices from mouse.

ZD 7288 can also inhibit voltage-gated Ca^{2+} channels (Felix *et al.*, 2003; Sanchez-Alonso *et al.*, 2008), but only at very high concentrations (i.e., with an IC_{50} of over 100 μM) that are at least 10-fold higher than what would have been achieved in our local injection experiments. Side effects of ZD7288 at high concentrations may potentially affect inspiratory rhythm in a different manner from normal actions of low concentrations of ZD7288. For example, inhibition of I_h typically reduces the frequency of rhythmic behaviors (Yue & Huguenard, 2001; Xu *et al.*, 2004; DiFrancesco & Borer, 2007; Montandon & Horner, 2013), but at bath concentrations of 100 μM ZD7288 paradoxically increases inspiratory rhythm (Thoby-Brisson *et al.*, 2000). Given the low

effective concentration of locally applied ZD7288 (10 μM), and that local applications of ZD7288 at 100 μM and 25 μM, an even lower concentration that produces an effective concentration of 2.5 μM, had similar effects on the $P2Y_1$ receptor-mediated excitation of the inspiratory network in general, we are confident that the actions of ZD7288 that we have observed are due to the inhibition of I_h.

Our conclusion that the actions of ZD7288 are mediated through inhibition of I_h is further supported by the observation that the ZD7288-mediated inhibition of the MRS 2365 current increased from ~36% after the first application to ~58% after the second. As mentioned in the results, this is expected when examining the actions of an antagonist that is open channel blocker (Shin *et al.*, 2001) with minimal access to its binding site that also takes a long time to washout. Under baseline conditions in the rhythmic medullary slice, inspiratory neurons are depolarized relative to the activation voltage of I_h, meaning that ZD7288 will have minimal access to it binding site inside the channel pore (Mironov *et al.*, 2000). We propose that ZD7288 would begin to block I_h with application of MRS 2365 due to the associated depolarizing shift in its voltage-activation curve. However, MRS 2365 was only applied for 30 sec, which is unlikely to provide sufficient time for complete block. As seen here (Fig. 3.6D), ZD7288 washes out very slowly and incompletely from slices (Harris & Constanti, 1995), such that with the second application of ZD7288 and MRS 2365 15 min later, block of additional channels in combination with those that remained blocked from the first application resulted in a greater inhibition of the MRS 2365 current.

Additional evidence to support that the MRS 2365 excitation of a subpopulation of inspiratory neurons involves I_h is that the slowly-activating, inward sag currents evoked by hyperpolarizing voltage-steps, which are a diagnostic feature of I_h in many types of neurons

(hippocampal interneurons, (Halliwell, 1982 #3434)(Bergles *et al.*, 1996; Maccaferri & McBain, 1996); spinal sensory ganglion neurons (Mayer & Westbrook, 1983; Duchen, 1990); facial motoneurons (Larkman & Kelly, 1992); hypoglossal motoneurons (Bayliss *et al.*, 1994); bulbospinal neurons in the nucleus solitarius (Dekin, 1993)), were potentiated by MRS 2365 in all inspiratory neurons in which MRS 2365 evoked an inward current (at -60 mV) and even in some neurons in which an MRS 2365 current was not noticeable at -60 mV. These sag currents increased with hyperpolarization and were blocked by ZD7288, confirming that they represent I_h. The MRS 2365-mediated potentiation of the sag currents evoked by the hyperpolarizing pulses was also blocked by ZD7288. The only other current aside from I_h known to be activated in preBötC inspiratory neurons with progressive hyperpolarization is that mediated by K_{ATP} channels (Mironov *et al.*, 2000). However, it is highly unlikely that K_{ATP} has any role in our experiments because the K_{ATP} current is extremely small in oxygenated slices, it is not affected by ZD7288 and it does not contribute to sag currents (Mironov *et al.*, 2000). When considered together, these data strongly support the conclusion that $P2Y_1$ receptor activation excites a subpopulation of inspiratory preBötC neurons by potentiating I_h.

3.5.2 $P2Y_1$ receptor excitation of the preBötC inspiratory network involves modulation of I_h

I_h is not necessary for the generation of inspiratory-related rhythm in vitro, nor does it play a role in setting inspiratory rhythm under baseline conditions in vitro; i.e., block of I_h does not alter basal rhythm (Mironov *et al.*, 2000). This does not, however, exclude the possibility that inspiratory rhythm can be modulated by I_h. In addition to compelling data at the cellular level that $P2Y_1$ receptors potentiate I_h in a subpopulation of inspiratory neurons, we provide pharmacological evidence at the network level that this same effect contributes to the $P2Y_1$ receptor-mediated increase in preBötC frequency. The main evidence that I_h is involved in the $P2Y_1$ receptor-

mediated frequency increase is that the frequency increase is antagonized by ZD7288. The selectivity of ZD7288 for I_h has been discussed above in the context of MRS 2365-evoked inward currents. The same arguments apply at the network level. Just as the inhibitory effect of 100 μM ZD7288 on the currents increased with repeated application, ZD7288 reduced the MRS 2365-induced frequency to 38 ± 8%, 26 ± 8% and 10 ± 2% of the initial control response in the first, second and third ZD7288 trial, respectively. Importantly, for the network experiments we reduced the concentration of ZD7288 in the pipette to 25 μM, which corresponds to ~2.5 μM in the tissue, which also reduced the excitatory effect of MRS 2365 on the network with successive applications. As discussed above, it is highly unlikely that the effects of ZD7288 at these concentrations on the MRS 2365-evoked frequency increase are due to off-target actions on voltage-gated Na^+ or Ca^{2+} channels (Felix *et al.*, 2003; Sanchez-Alonso *et al.*, 2008; Wu *et al.*, 2012). The possibility that the progressive decrease in both the MRS 2365-induced inward currents was due to rundown of the MRS 2365 response over time was addressed here with time controls showing that MRS 2365 responses are highly repeatable if separated by 15 min intervals. In addition, the ZD7288 inhibition of the MRS 2365 plateaued after the second ZD7288/MRS 2365 application (Fig. 3.1D), which would not be expected if the MRS 2365 current was running down. Pharmacological data that indirectly support I_h involvement in the $P2Y_1$ receptor-mediated frequency increase include that forskolin, which increases cAMP, an activator of I_h, increases basal inspiratory-related frequency and that SQ 22536, which decreases cAMP, inhibits the excitatory actions of MRS 2365 on preBötC rhythm. Taken together, these data support an important role for I_h in the $P2Y_1$ receptor-mediated excitation of the inspiratory network.

3.5.3 Mechanisms underlying the $P2Y_1$ receptor-mediated modulation of I_h

Depolarizing shift in the voltage-dependence of Ih activation. I_h is a mixed Na^+/K^+ current carried by hyperpolarization activated, cyclic nucleotide gated (HCN) channels (Ludwig *et al.*, 1998). Its slow kinetics of activation and inactivation combined with its unique voltage gating properties underlie the signature inward sag currents that increase in magnitude with progressive hyperpolarizing pulses to peak at approximately -140 mV. Every preBötC inspiratory neuron with an MRS 2365 current >15 pA (at a holding potential of -60 mV) had an I_h that was potentiated by MRS 2365. An additional 14 inspiratory neurons with negligible MRS 2365 currents at -60 mV also had an I_h current that was enhanced by MRS 2365; i.e. MRS 2365 potentiated I_h in 47% (21/45) neurons. Further analysis of the voltage-activation curves of I_h channels in 6 of these neurons revealed that $P2Y_1$ receptor activation causes a depolarizing shift of the activation curve of ~ 9.8 mV and we propose that this is one mechanism via which $P2Y_1$ receptor activation excites inspiratory neurons and the preBötC network. Note that the hyperpolarizing step to -140 mV does not appear to have maximally activated I_h since the control activation curve does not plateau below -130 mV as reported in other studies (Ludwig *et al.*, 1998; Wainger *et al.*, 2001). Failure to maximally activate I_h, however, should not impact our conclusion that $P2Y_1$ receptor activation depolarizes the activation threshold for I_h by about 10 mV unless we dramatically underestimate $I_{h\text{-}max}$, in which case the magnitude of the shift would be smaller than 10 mV. . In theory, we could inject more currents to ensure a 100% activation of I_h. However, it may prove to be a technical issues given that few cells survived the current "-140 mV" hyperpolarizing voltage step and injecting more currents may drastically decrease of survival chance of a µµpatched cell. Whether $P2Y_1$ receptor activation increases HCN channel conductance was not assessed. The only way to address this question for HCN channels is through nonstationary noise analysis (Barrow & Wu, 2009). The conventional patch-clamp method for recording single-channel conductance cannot be

applied on HCN channels because their small conductance is below the thermal noise threshold of any physically realizable patch-clamp amplifier.

cAMP. The voltage-dependence of I_h activation, and therefore its influence on the integrative firing behavior of neurons, is highly modulated, primarily by transmitters that signal through Gs/Gi and adenylyl cyclase to increase or decrease the intracellular levels of cAMP; indeed elevations in cAMP can cause a depolarizing shift in the activation curve of I_h by as much as 30 mV (Ludwig *et al.*, 1998; Wainger *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002). $P2Y_1$ receptor activation caused a 9.8 mV depolarizing shift in the activation curve of I_h, Although we did not directly demonstrate that this MRS 2365-mediated potentiation of I_h or the shift in the activation curve were dependent on cAMP (i.e., that they were SQ 22536-sensitive), we did show that the MRS 2365 current evoked in 50% (9/18) of inspiratory neurons was strongly inhibited by the adenylyl cyclase, inhibitor SQ 22536. It is difficult to reconcile the observation that intracellular dialysis of 100 μM SQ 22536 attenuated the MRS 2365 inward currents in only 50% of cells, with the observation that the I_h blocker, ZD7288, reduced the MRS2365 current in every neuron tested. It suggests either that $P2Y_1$ signalling alters I_h via a non cAMP-dependent pathway in some neurons, or that SQ 22536 was ineffective in half of the recorded inspiratory neurons. Given that the SQ 22536 was delivered via the whole-cell recording pipette, we have not been able to identify an experimental factor that could have accounted for this differential response, such as poor intracellular access in some cells due to high access resistance. The series resistances in the 9 cells that were sensitive or insensitive to SQ 22536 averaged 83.3 ± 6.4 and 78.6 ± 14.9 MOhms, respectively.

Never the less, our suggestion that cAMP is activated as part the $P2Y_1$ receptor signalling cascade in the preBötC is further supported by the observation that the ZD7288-sensitive, MRS

2365-evoked increase in network frequency was inhibited by SQ 22536 in every preparation. This inhibition took time to develop but this is entirely consistent with time required for SQ 22536 to diffuse into neurons throughout the preBötC network. Moreover, that the SQ 22536 inhibition was partially reversible after 30 min argues convincingly that the SQ 22536 effect was not due to run-down of the MRS 2365 response.

These data therefore suggest that $P2Y_1$ receptors operate in part by increasing cAMP, which causes a depolarizing shift in the activation voltage of I_h in a subpopulation of inspiratory neurons, to excite the inspiratory network.

The $G\alpha_q$- vs the G_s-cAMP signalling pathway. As mentioned above, $P2Y_1$ receptors primarily signal through the $G\alpha_q$ pathway (Usachev *et al.*, 2002; Abbracchio *et al.*, 2006; Song *et al.*, 2007; Chandaka *et al.*, 2011; Rajani *et al.*, 2016), while I_h is typically modulated by Gs or Gi-dependent changes in cAMP levels (Ludwig *et al.*, 1998; Wainger *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002). Thus, our data suggesting that $P2Y_1$ receptors signal through cAMP and I_h to modulate the excitability of inspiratory neurons and the inspiratory network are very surprising. These data also raise some very important questions, including how do $P2Y_1$ receptors access cAMP and I_h? Do $P2Y_1$ receptors activate Gs or another G protein coupled receptor (GPCR) that can access cAMP or I_h? Is there an alternative mechanism via which $G\alpha_q$ signalling can access cAMP and I_h? At present we do not have answers to these questions. The only documented effect of $G\alpha_q$ signalling on I_h is a protein kinase C (PKC)- or phosphatidylinositol 4,5-biphosphate (PIP2)-mediated inhibition (Pian *et al.*, 2006; Williams *et al.*, 2015), which completely contrasts with the actions reported here.

However, it is premature to exclude the possibility of positive cross-talk between these two GPCR signalling pathways. The $G\alpha_q$-signalling pathway can increase the activity of different isoforms of adenylyl cyclase through PKC activation (Kawabe *et al.*, 1994; Tabakoff *et al.*, 2001; Schallmach *et al.*, 2006), which may subsequently activate I_h via increased cAMP level (i.e., $P2Y_1$ receptor activation of adenylyl cyclase and I_h is mediated by PKC instead of the Gs subunit).

In addition, while $P2Y_1$ receptors primarily signal through $G\alpha_q$, $P2Y_1$ receptor coupling through the G_i-signalling pathway has been documented. However, data are limited to neurons of rat superior cervical sympathetic ganglia and nucleus tractus solitarius, where $P2Y_1$ receptors inhibit voltage-dependent Ca^{2+} channels (Brown *et al.*, 2000a; Filippov *et al.*, 2000; Aoki *et al.*, 2004). A similar action in the inspiratory network would most likely reduce inspiratory frequency (Ramirez *et al.*, 1998b; Mironov & Richter, 2000b; Koch *et al.*, 2013). Furthermore, activation of the G_i-signalling pathway conventionally inhibits adenylyl cyclase and I_h (Ingram & Williams, 1994; Rainnie *et al.*, 1994; Frere & Luthi, 2004). Taken together, it appears unlikely that $P2Y_1$ receptors excite the inspiratory network via activation of the G_i-signalling pathway. $P2Y_1$ receptors may also act through the G_s-signalling pathway, cAMP and directly modulate I_h. Evidence of such actions, however, is scarce. To the best of our knowledge, the only evidence of a $P2Y_1$ receptor-mediated increase in cAMP levels in the CNS is in hippocampal neurons, where $P2Y_1$ receptor-induced increases in cAMP are important in axonal elongation (del Puerto *et al.*, 2012). Note that in this example the $P2Y_1$ receptor-mediated increase in cAMP did not alter neuronal excitability. cAMP directly modulates I_h, but it also activates protein kinase A (PKA), which can independently modulate I_h (Vargas & Lucero, 2002; Liao *et al.*, 2010). Whether $P2Y_1$ receptors can access PKA to potentiate I_h remains to be tested. Thus, at present we are left with the conclusion that $P2Y_1$ receptors, through an unknown GPCR system, increase cAMP and potentiate I_h in a subpopulation

of preBötC inspiratory neurons, which contributes to a $P2Y_1$ receptor-mediated increase in breathing frequency.

3.5.4 Which preBötC inspiratory neurons underlie the $P2Y_1$ receptor-mediated network excitation?

Multiple subtypes of ATP-sensitive inspiratory neurons. An important theme that has arisen from our analysis of ATP responses in preBötC inspiratory neurons is that the $P2Y_1$ receptor-mediated excitation of the inspiratory network does not come from a ubiquitous sensitivity of all inspiratory neurons to ATP. In Chapter 2 we demonstrated that not all preBötC inspiratory are sensitive to ATP and that ATP-sensitive neurons fall into at least two categories. One subpopulation (16/35 neurons) featured ATP (100 μM) responses with fast kinetics that were sensitive to PPADS/Suramin, suggesting P2X pharmacology, while a second subpopulation (19/35) responded with slower kinetics and predominant sensitivity to $P2Y_1$ receptor antagonists. The heterogeneity of inspiratory neuron responses to ATP is not at all surprising given the diversity of inspiratory neurons in the preBötC (Gray *et al.*, 2001; Gray *et al.*, 2010; VanDunk *et al.*, 2011). The challenge is identifying the type of inspiratory neuron that underlies the $P2Y_1$ receptor-mediated excitation of the preBötC network and the increase in inspiratory frequency.

Inspiratory neurons with $P2Y_1$ receptor sensitivity. The effects of $P2Y_1$ receptor activation on the inspiratory neurons appeared to be cell-type specific. First, among the neurons with $P2Y_1$ receptor-mediated currents (n = 18), SQ 22536 attenuated the MRS 2365 currents only in half of them (Fig. 3.5). The SQ 22536-sensitive and –insensitive neurons showed similar input resistances (83.3 ± 6.4 MOhms vs 78.6 ± 14.9 MOhms) and MRS 2365-induced inward currents (-36 ± 5 pA vs 36 ± 5 pA). Second, as another key property which helps us categorize the inspiratory neurons, MRS 2365 potentiated I_h in 21 of 45 preBötC inspiratory neurons. However, despite the consistent

potentiating effect on I_h, MRS 2365 did not induced inward currents in all the neurons. In fact, MRS 2365 had no effect on the holding current in 14 of 21 neurons. Analysis of these 14 vs the rest 7 neurons in which MRS 2365 induced inward currents, again, showed that there is no difference in input resistance or amplitude of control I_h between the "inward current" and "no inward current" groups (-93 ± 38 pA vs -88 ± 24 pA; 109 ± 26 MOhms vs 113 ± 23 MOhms). These data suggest that $P2Y_1$ receptor-sensitive inspiratory neurons can be further classified into subgroups, which raises another important question as to whether the $P2Y_1$ receptor-mediated excitation is dependent on specific subtypes of $P2Y_1$ receptor-sensitive inspiratory neurons or all subtypes contribute. To address this, we first need to distinguish between the subpopulations.

Classification of respiratory neurons into functionally meaningful subgroups is an ongoing challenge. This began in the 30's based on pattern of discharge and neuron location (Gesell *et al.*, 1936). The pattern of inhibitory synaptic input (based on in vivo intracellular recording and Cl^- iontophoresis) was added to the mix starting in the mid 70's (Richter *et al.*, 1975). Beginning in the late 80's, development of rhythmically-active in vitro preparations and whole-cell recording methods allowed detailed analysis of synaptic and intrinsic membrane properties (Suzue, 1984; Smith & Feldman, 1987; Feldman & Smith, 1989). A decade long search for molecular markers that would identify key populations of respiratory neurons involved in rhythm generation, fuelled by the hope of translating in vitro findings into methods to manipulate key neuronal populations in vivo, identified in 1999 NK1 receptors and μ-opioid receptors (on Type I inspiratory neurons) as likely markers of rhythmogenic neurons (Gray *et al.*, 1999). This discovery marked a transition in the field where the key hypotheses about respiratory network function, based largely on in vitro experiments, could be directly tested in vivo (Gray *et al.*, 2001). These efforts, combined with continued in vitro experiments led to identification of additional markers, including SST (Gray *et*

al., 2010), transcription factors (e.g., DBX1, (Gray, 2010 #3241)) and specific genes that identified transmitter phenotype (glutamate vs GABA/Glycine)(Hayes *et al.*, 2017) and ion channel expression patterns (Picardo, 2019 #3455). Previous work from our lab established that at least a portion of 2MeSADP-sensitive neurons (the best $P2Y_1$ agonist available at the time) are also NK1 and μ-opioid sensitive (Lorier *et al.*, 2007; Lorier *et al.*, 2008) and that $P2Y_1$ and NK1 receptor co-localized on preBötC inspiratory neurons (Lorier *et al.*, 2007). These data agree with a potential role of $P2Y_1$ receptor-sensitive neurons in the modulation of inspiratory rhythm but failed to differentiate between the subtypes of $P2Y_1$ receptor-sensitive neurons, which requires better characterization of those neurons for their specific neuronal properties.

So, aside from $P2Y_1$ receptor sensitivity, what neuronal properties will help identify the subpopulation of neurons that underlies the $P2Y_1$ receptor-mediated excitation? Additional properties that helps classify inspiratory neuron phenotype, first presented in 1996 (Rekling *et al.*, 1996), combined a detailed analysis of discharge pattern, subthreshold membrane potential trajectory and intrinsic membrane properties. Based on these properties, Rekling et al. described type-1, -2 and -3 inspiratory neurons, all of which fired action potentials (AP) during inspiration. The differences between these three types of neurons are: 1) Type-1 inspiratory neurons had pre-inspiratory discharge in which APs were clustered into small burst-like discharge and AP frequency increased towards the onset of inspiration. The inspiratory burst was followed by a prolonged afterhyperpolarization consisting of short fast- and long slow- repolarizing components; 2) Type-2 neurons had delayed spiking activity compared to type-1 neurons and occasionally fire regular spikes without ramp potential or remain silent between two inspiratory bursts. They also had afterhyperpolarization but with a shorter duration; 3) Type-3 displayed a low-threshold depolarization following current pulses and were the last to fire spikes with the similar behaviors

to those of type-2 neurons during interburst interval (IBI). One major difference between type-1/2 and type-3 neurons is that type-3 neurons did not express afterhyperpolarization but afterdepolarization following inspiratory potentials. Another difference between the three types of inspiratory neurons is input resistance, which is 306 ± 130, 296 ± 212 and 126 ± 34 MOhms for type-1, type-2 and type-3 neurons, respectively. Note that the input resistance of type-3 neurons was remarkably lower. Type 1 cells expressed NK1 and μ-opioid receptors (Gray *et al.*, 1999) and were proposed as key for rhythm generation, which was supported by the observations that that local application of Substance P and DAMGO in the preBötC greatly increases and decreases the basal inspiratory frequency in the rat rhythmically active medullary slices, respectively (Gray *et al.*, 1999), and that SP-saporin mediated killing of NK1R-expressing preBötC neurons in vivo led first to apnea during REM sleep followed days later by ataxic breathing in all states (Gray *et al.*, 2001; McKay *et al.*, 2005) and allatostatin-mediated inhibition of somatostatin-expressing preBötC neurons in vivo reversibly inhibits breathing (Tan *et al.*, 2008). Type-2 neurons only expressed NK1 receptors (Gray *et al.*, 1999) and are the only type that responded to hyperpolarizing current pulses with depolarizing sags and postinhibitory round, which was suggestive of activation of I_h. They were considered as "relay cells" that contributed to synchronization of type-1 and -2 neuronal excitation, amplification of rhythmogenic bursts/burstlets initiated by type-1 neurons and percolation of this rhythmic activity to pattern-forming neurons (i.e., motoneurons and pre-motoneurons) which were hypothesized to be type-3 neurons due to their late spiking timing, smaller input resistances and lack of I_h that underlies rhythm generation in some systems (Yue & Huguenard, 2001; Xu *et al.*, 2004; DiFrancesco & Borer, 2007).

Another attempt to characterize inspiratory neurons was made by Thoby-Brisson et al. in 2000 ((Thoby-Brisson, 2000 #2992). Inspiratory neurons recorded from mouse rhythmic slices were classified into two groups mainly based on their spiking patterns during the interburst interval (IBI). Type-1 neurons displayed no tonic activity during the IBI while type-2 had tonic activity. I_h expression was detected in 9 of 31 type-2 neurons but not in all 5 type-1 neurons tested. If type-2 neurons were further classified as follower neurons vs pacemaker based on tonic vs bursting behavior after CNQX application, 36% (3/18) of the follower neurons had I_h. In contrast 6/7 or 86% of pacemaker type-2 neurons had an I_h. This suggests that the I_h may play an important role in the generation of respiratory pacemaker activity. The findings were, in most part, consistent with those from Rekling's paper except that they identified two subpopulations of "type-2" neurons. Note that while pacemaker properties of inspiratory neurons are no longer considered necessary for inspiratory rhythm generation, this does not mean that pacemaker neurons are not important in rhythm generation (Del Negro *et al.*, 2005).

In our experiments, presence of an MRS2365-sensitive I_h is a key property which helps us categorize the inspiratory neurons. MRS 2365 potentiated I_h in 21 of 45 preBötC inspiratory neurons. Given the type 2-specific expression of I_h reported by Rekling et al. (Rekling *et al.*, 1996), it is plausible to hypothesize that these 21 neurons belong to the type-2 subpopulation. To test this, it requires comparison of membrane potentials of inspiratory neurons and XII nerve activity under baseline conditions for each neuron. Unfortunately, we did not systematically perform current clamp recordings in all MRS 2365-sensitive neurons. Membrane depolarization was measures in 13 of 21 cells. Interestingly, 9 of 13 cells had an activity pattern similar to type-3 neurons; i.e., late onset, presence of afterdepolarization and few or no spiking during the IBI and. Consistent with their type 3-like neuronal activity, the average input resistance of the 9 cells was 90.1 ± 9 MOhms,

which was similar to that of type-3 neurons (126 ± 34 MOhms) but a lot lower than those of type-1 (306 ± 130 MOhms) and type-2 (296 ± 212 MOhms) cells (Rekling *et al.*, 1996). These data suggest that, similar to the type 2, there is a subtype of type-3 inspiratory neurons which was not identified in the Rekling's paper that expressed MRS 2365-sensitive I_h. MRS 2365 induced inward currents in 1 of the 9 cells. 4 of 13 cells appeared to receive both excitatory and inhibitory inspiratory inputs at the same time as they were hyperpolarized during inspiration under the current clamp mode (I = 0 pA, resting membrane potential: -46.3 ± 1.1 mV, n = 4) but received rhythmic inward currents when being held at -60 mV. MRS 2365 induced inward currents in all 4 cells. The difference (1/9 vs 4/4) in the percentage of cells that responded to MRS 2365 with both inward currents and potentiation of I_h suggests that these 4 neurons belong to a different population from the other 9 ones. Measurement of the reversal potential of Cl^-, which was estimated to be -75 mV with 131 mM $[Cl^-]_o$ and 7 mM $[Cl^+]_i$, are needed to verify the occurrence of the inhibitory modulation of inspiratory activity in this subpopulation of neurons before we can use it as a criterion to distinguish between neurons that respond to MRS 2365 with both inward currents and potentiation of I_h and those with only inward currents.

Percentage of I_h-expressing preBötC inspiratory neurons. Mironov and Richter (Mironov *et al.*, 2000) report that in mouse the percentage of preBötC inspiratory neurons with I_h is ~15%. But they defined a neuron as having an I_h only if the I_h was >50 pA. In our cases, we consider that a sag current with an amplitude of > 25 pA evoked at -100 mV was I_h. The lower cutoff may contribute to the higher percentage (84%) of I_h-containing preBötC inspiratory neurons in this study. In addition, we recorded the inspiratory neurons strictly in the area immediately ventral or ventrolateral to the nucleus ambiguus in rhythmic slices cut from P0-P3 rats while Mironov and Richter recorded the cells in the whole preBötC region in rhythmic slice from P6 – P11 mice. It is

possible that I_h-containing inspiratory neurons are not evenly distributed; i.e., some regions have higher density of I_h-containing neurons compared to others. If the same 50 pA cutoff is applied in the analysis for our data, the percentage of I_h-containing inspiratory neurons will decrease from 84% to 75%, which, however, is still far greater than 15% reported in Mironov's paper. Therefore, the differences in specie, age of prep and more importantly, population of inspiratory neurons recorded are more likely to be the reason why we had a higher chance of finding neurons with I_h.

Future directions. The key observation here is that the $P2Y_1$ receptor activation depolarized only a subset of the inspiratory neurons, suggesting that this specific group of inspiratory neurons mediate at least part of the $P2Y_1$ receptor excitation of the inspiratory network. To further test the role of this subset of inspiratory neurons in the $P2Y_1$ receptor-mediated excitation of the inspiratory network and ultimately the hypoxic ventilatory depression, we first need to characterize these neurons. One of the most important questions is whether the responsible neurons are glutamatergic (i.e., excitatory) or GABA/glycinergic (i.e. inhibitory). Our original assumption was that the $P2Y_1$ receptor-sensitive neurons that mediate excitation would be glutamatergic, since rhythm generation is dependent on glutamatergic transmission (Funk *et al.*, 1993) and activation of glutamatergic neurons increases network frequency (Vann *et al.*, 2018). However, recent data in rhythmic slice indicate that optogenetic stimulation of inhibitory, preBötC inspiratory neurons also increases network frequency and in fact may be more effective than stimulating glutamatergic neurons (Baertsch *et al.*, 2018). Experiments that will help us achieve this goal include: 1) RNAscope-based in-situ hybridization experiment where the cells exposed to MRS 2365 will be labeled with biocytin and hybridized with probes for excitatory and inhibitory neurons. Combined with their neuronal response to MRS 2365, this method allows us to identify the transmitter phenotype of the cells presumably underlying the $P2Y_1$ receptor-mediated network excitation, i.e.,

whether they are excitatory or inhibitory, and may shed light on the mechanisms via which $P2Y_1$ receptor-mediated excitation of inspiratory neurons is transformed into network excitation; 2) Whole-cell recording experiment in which inspiratory neurons from VGlut2-eGFP and VGAT-eGFP transgenic mice will be recorded for their response to MRS 2365. Using this method, we can immediately characterize a neuron based on its sensitivity to MRS 2365 and neurotransmitter phenotype. 3) Single-cell RNA sequencing experiment where the content of an inspiratory neuron will be extracted after testing its response to MRS 2365 and the entire transcrpitome reverse transcribed and quantified via sequencing. The advantage of this method over the previous two is that it allows for a high-throughput screening. We will be able to analyze hundreds of candidate mRNA sequences using RT-qPCR-based microarray, which will produce a myriad of cell markers for neurons that do or do not respond to MRS 2365 with inward currents. Some of the markers may be specific to one of the two subpopulations. Such information is highly valuable in that it will allow us to devise a strategy to selectively modulate activity of those neurons that respond to MRS 2365 with inward currents via optogenetic or chemogenetic tools, and eventually test the contribution of MRS 2365-sensitive vs. MRS 2365-insensitive inspiratory neurons to the secondary hypoxic ventilatory depression.

In summary, in the preBötC inspiratory network of neonatal rat we have presented evidence of a novel, $P2Y_1$ receptor signalling mechanism that, via an increase in cAMP levels and a depolarizing shift in the voltage-activation curve of I_h in a subset of preBötC inspiratory neurons, contributes to an ATP-mediated increase in inspiratory frequency. To the best of our knowledge, this is the only evidence that $P2Y_1$ receptor signalling pathways can potentiate I_h and operate through cAMP to alter neuronal and network excitability. We have not directly demonstrated that the $P2Y_1$ receptor-mediated potentiation of I_h in this subpopulation of preBötC neurons is mediated

by cAMP. However, given that $P2Y_1$ receptors and cAMP (Ludwig *et al.*, 1998; Wainger *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002) both cause depolarizing shifts in the voltage-dependence of I_h activation, and that $P2Y_1$ receptor currents and the $P2Y_1$ receptor-mediated increases in network frequency are inhibited by blockers of cAMP and I_h, it is highly likely that the effects of $P2Y_1$ receptors on I_h are mediated by cAMP. Important questions that remain to be answered include: through which GPCR signalling system do $P2Y_1$ receptors access cAMP; which subpopulation of preBötC inspiratory neurons underlies the $P2Y_1$ receptor-mediated excitation of the inspiratory network; is this putative $P2Y_1$ receptor-cAMP-I_h pathway unique to preBötC inspiratory neurons in the neonatal rat? A better understanding of these signalling pathways within functionally identified inspiratory neurons of the preBötC network may inform development of adenosine-independent respiratory stimulants to treat breathing instability and hypoxic respiratory depression in apnea of prematurity (Schmidt *et al.*, 2006; Lista *et al.*, 2016) and multiple neurodegenerative disorders that involve significant brainstem dysfunction/pathology such as Parkinson's disease (Torsney & Forsyth, 2017), multiple system atrophy (Iranzo, 2007; Gaig & Iranzo, 2012), amyotrophic lateral sclerosis (Niedermeyer *et al.*, 2019) and Alzheimer's disease (Brzecka *et al.*, 2018).

Figures

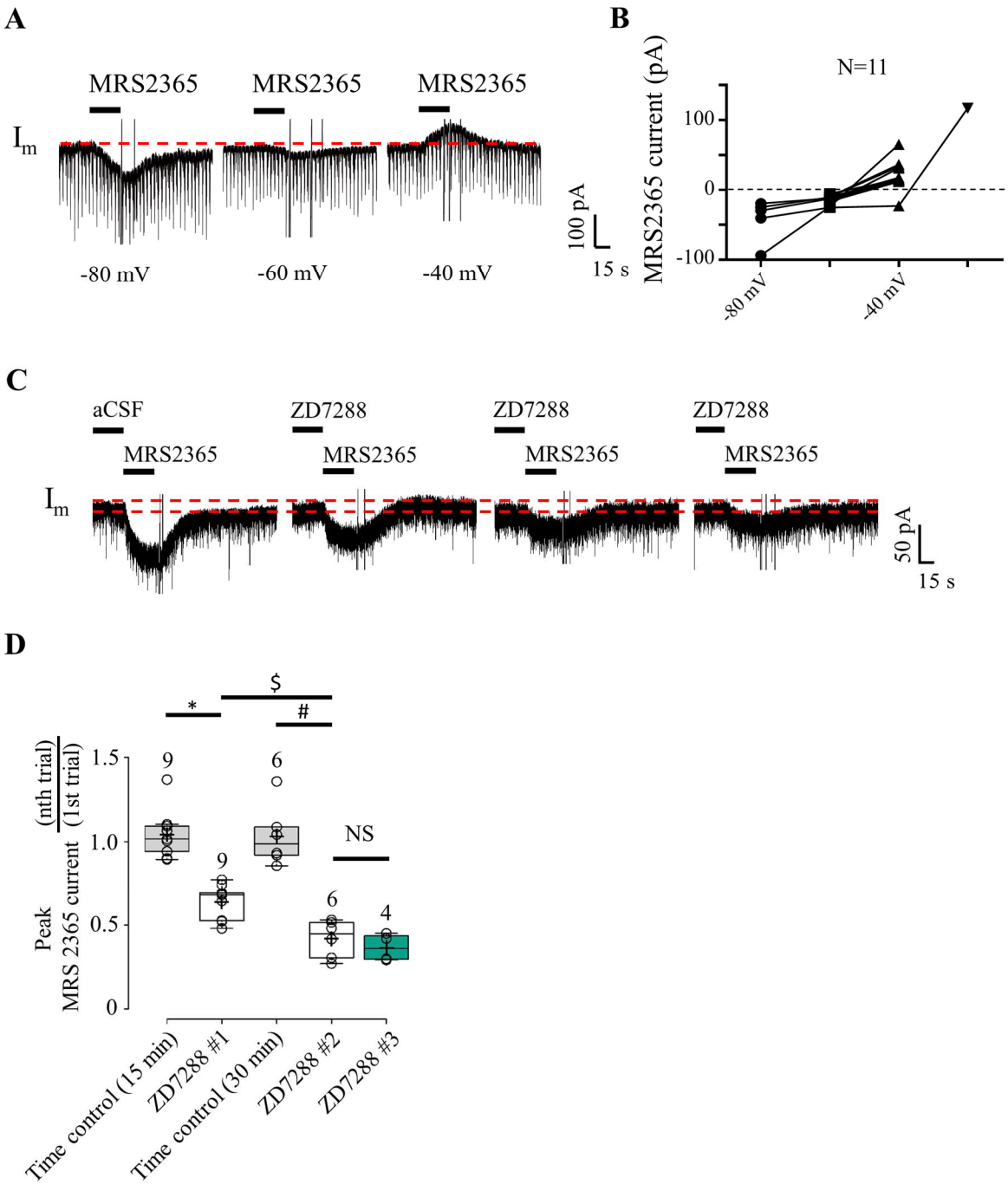

Figure 3.1 H-current activation contributes to the $P2Y_1$ receptor-mediated inward current

Representative traces (A) and group data (B) showing that the MRS 2365 (1 mM, 30 sec) currents reversed at a potential between -60 mV and -40 mV. C) Representative traces demonstrating that

local application of ZD7288 (100 μM, 3 min) attenuated the MRS 2365 (100 μM, 30 sec)-induced currents. D) Group data summarizing the effects on the MRS 2365 (100 μM, 30 sec) -induced currents of three consecutive ZD7288 (100 μM) applications conducted at intervals of 15 min. Samples sizes are denoted above the boxes. * indicates a significant difference in $I_{MRS\ 2365\ \#2}/I_{MRS\ 2365\ \#1}$ between time control and the ZD7288 trial 1 at 15 min, $p < 0.0001$, one-way ANOVA with Bonferroni's post-hoc test; # indicates a significant difference in $I_{MRS\ 2365\ \#3}/I_{MRS\ 2365\ \#1}$ between in time control and the ZD7288 trial at 30 min, $p < 0.0001$, one-way ANOVA with Bonferroni's post-hoc test; $ indicates a significant difference between $I_{MRS\ 2365\ \#2}/I_{MRS\ 2365\ \#1}$ and $I_{MRS\ 2365\ \#3}/I_{MRS\ 2365\ \#1}$ in the ZD7288 experiments, $p = 0.0359$, one-way ANOVA with Bonferroni's post-hoc test; NS indicates that there is no significant difference between $I_{MRS\ 2365\ \#2}/I_{MRS\ 2365\ \#1}$ and $I_{MRS\ 2365\ \#3}/I_{MRS\ 2365\ \#1}$, $p > 0.05$, one-way ANOVA with Bonferroni's post-hoc test. Samples sizes are denoted above the boxes.

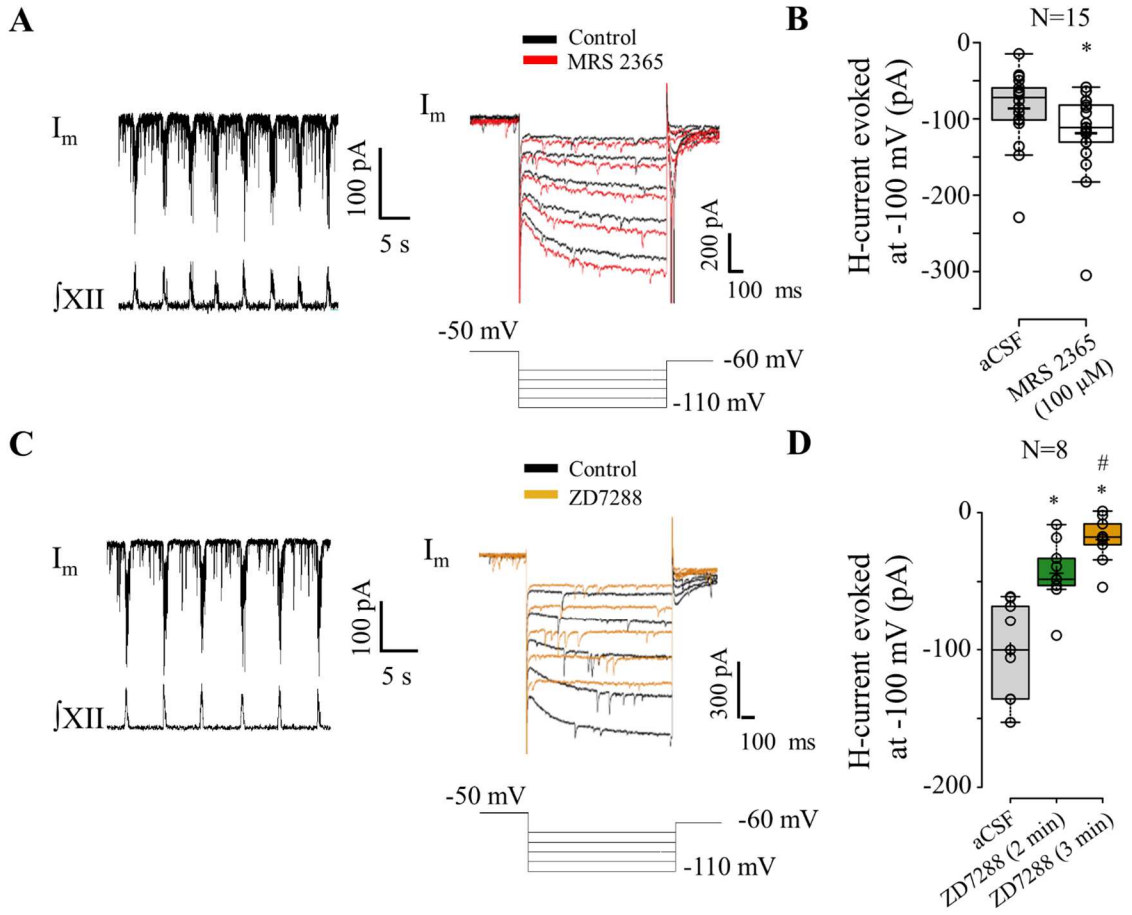

Figure 3.2 MRS 2365 potentiates h-currents evoked in a subset of the preBötC inspiratory neurons

A) Left panel, membrane current (top) recorded under the voltage-clamp mode showing that the neuron received inspiratory inputs which are in phase with the hypoglossal nerve bursting (bottom). Right panel, effect of MRS 2365 (local, 100 µM, 3 min) on the h-currents (top) evoked by the voltage protocol (bottom) in the same inspiratory neuron. B) Group data showing that 100 µM MRS 2365 potentiated the h-currents evoked at -100 µM in the inspiratory neurons. * $P < 0.0001$, paired student t-test. C) Left panel, membrane current recorded in another inspiratory neuron and the hypoglossal nerve activity (bottom). Right panel, effect of ZD7288 (local, 100 µM, 3 min) on the h-currents evoked in the same neuron. D) Group data showing the ZD7288-mediated

attenuation of the h-currents over time. * $P < 0.01$ between the ZD7288 (2 min) and time control groups and * $P < 0.001$ between the ZD7288 (3 min) and time control groups; # $P < 0.01$ between the ZD7288 (2 min) and ZD7288 (3 min) groups, one-way ANOVA with Bonferroni's post-hoc test.

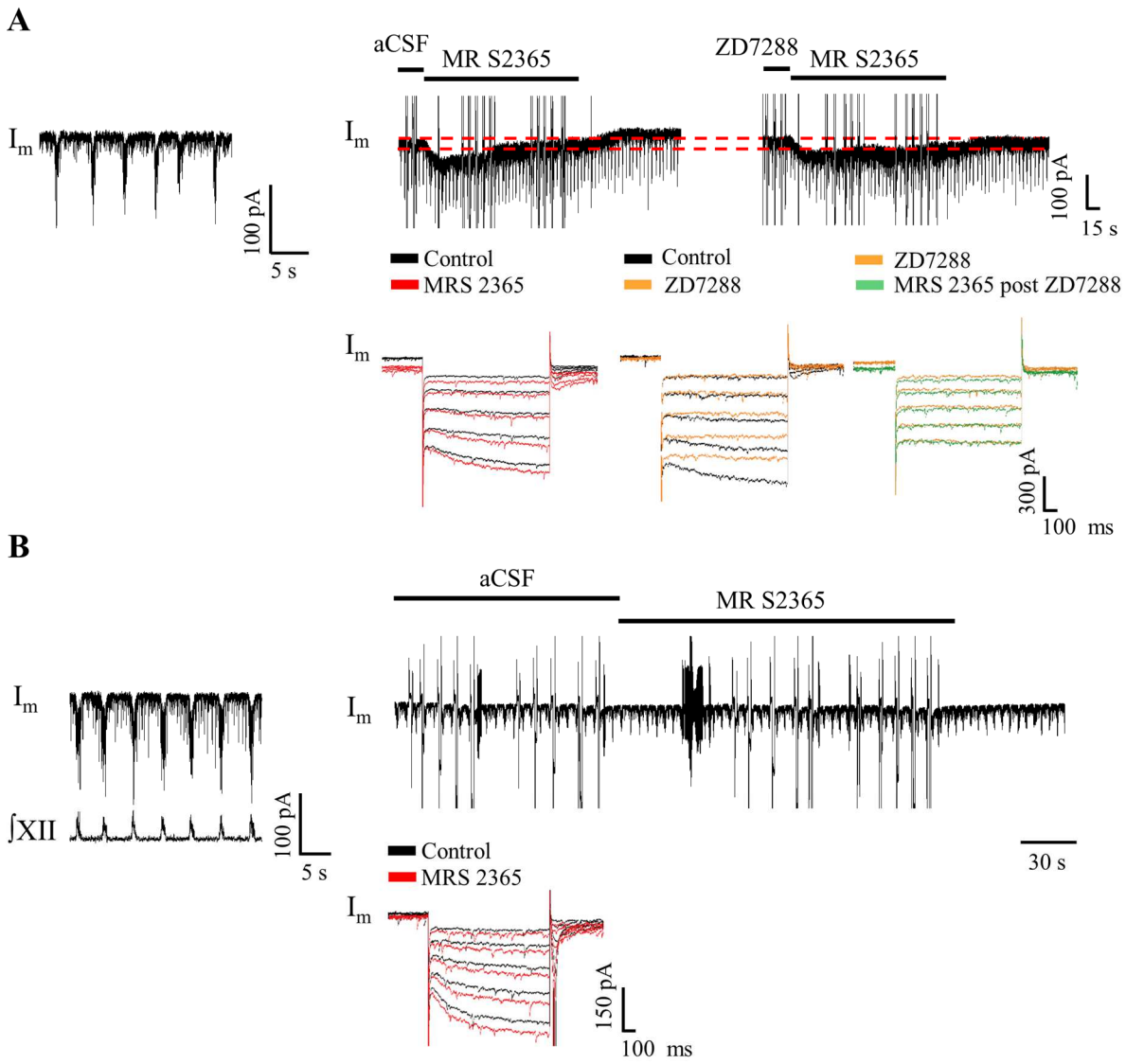

Figure 3.3 The cellular effects of P2Y1 receptor activation varies between inspiratory neurons

A) Left panel, membrane current recorded in an inspiratory neuron. Right panel, an example showing that 1) on the network level, ZD7288 attenuated the MRS 2365 (100 µM, 3 min)-induced inward current in the same inspiratory neurons (top) and 2) MRS 2365 (100 µM, 3 min) potentiated h-currents which were then blocked by ZD7288 (100 µM, 3 min) (bottom). B) Left panel, membrane current (top) recorded under the voltage-clamp mode showing that the neuron received

inspiratory inputs which are in phase with the hypoglossal nerve bursting (bottom). Right panel, an example showing that MRS 2365 (100 μM, 3 min) potentiated the I_h in this neuron (bottom) but did not induce inward current (top).

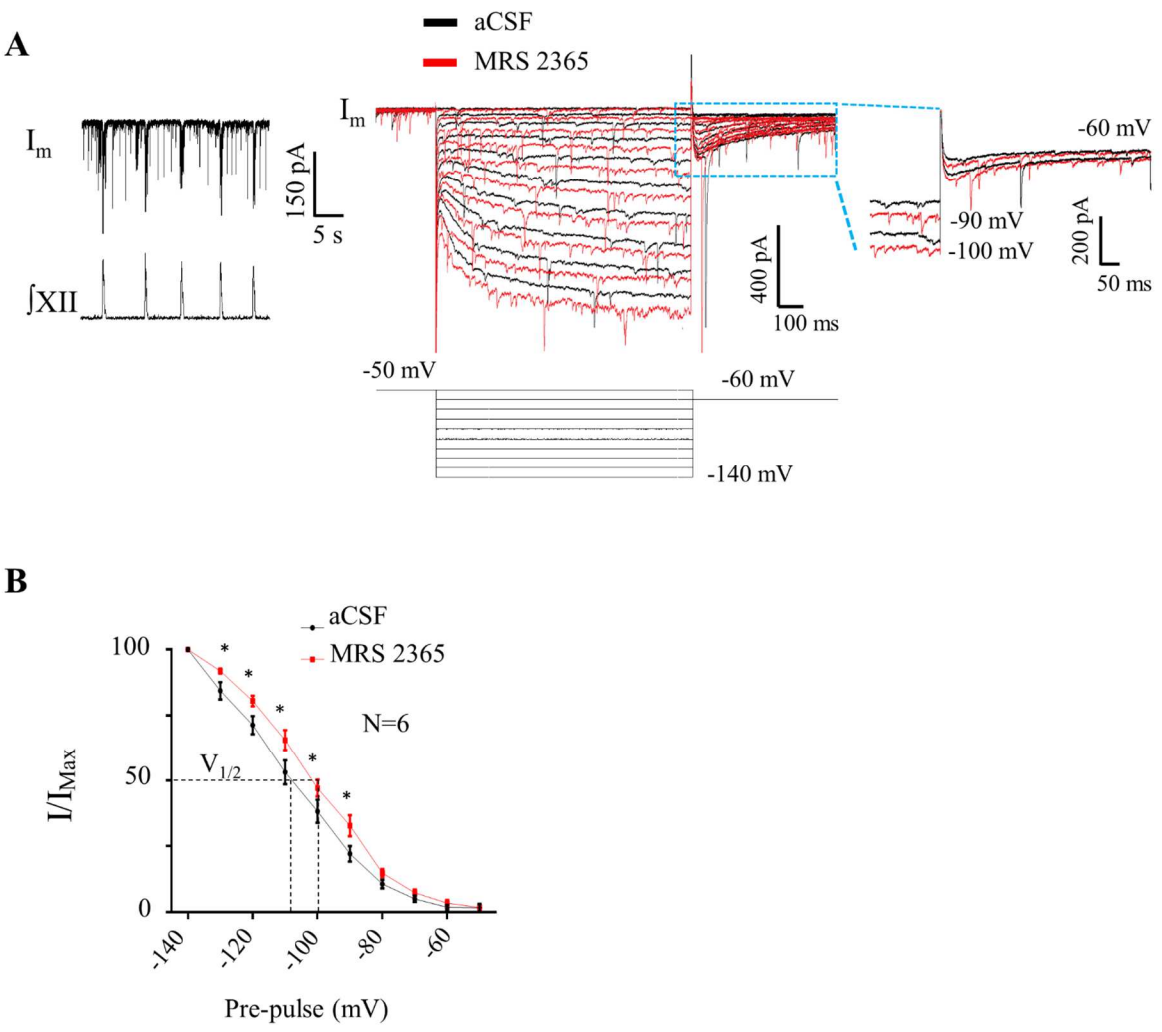

Figure 3.4 MRS 2365 potentiates h-currents evoked in the preBötC inspiratory neurons via shifting the activation curve of HCN channels to a more depolarized potential

A) Left panel, membrane current (top) recorded under the voltage-clamp mode showing that the neuron received inspiratory inputs which are in phase with the hypoglossal nerve bursting (bottom). Middle panel, effect of MRS 2365 (local, 100 µM, 4 min) on the tail currents (top) activated following the voltage protocol (bottom). Right panel, inset demonstrating that both sag currents evoked at -90 mV and -100 mV and tail currents activated at -60 mV following -90 mV

and -100 mV prepulses were potentiated in the presence of MRS 2365. B) Activation curves of HCN channels showing that the voltage dependence of the HCN channels in the preBötC inspiratory neurons was shifted towards a more depolarized potential in the presence of MRS 2365. * $P < 0.01$ at 130 mV and $P < 0.001$ from -90 mV to -120 mV between the time control and MRS 2365 groups, one-way ANOVA with Bonferroni's post-hoc test.

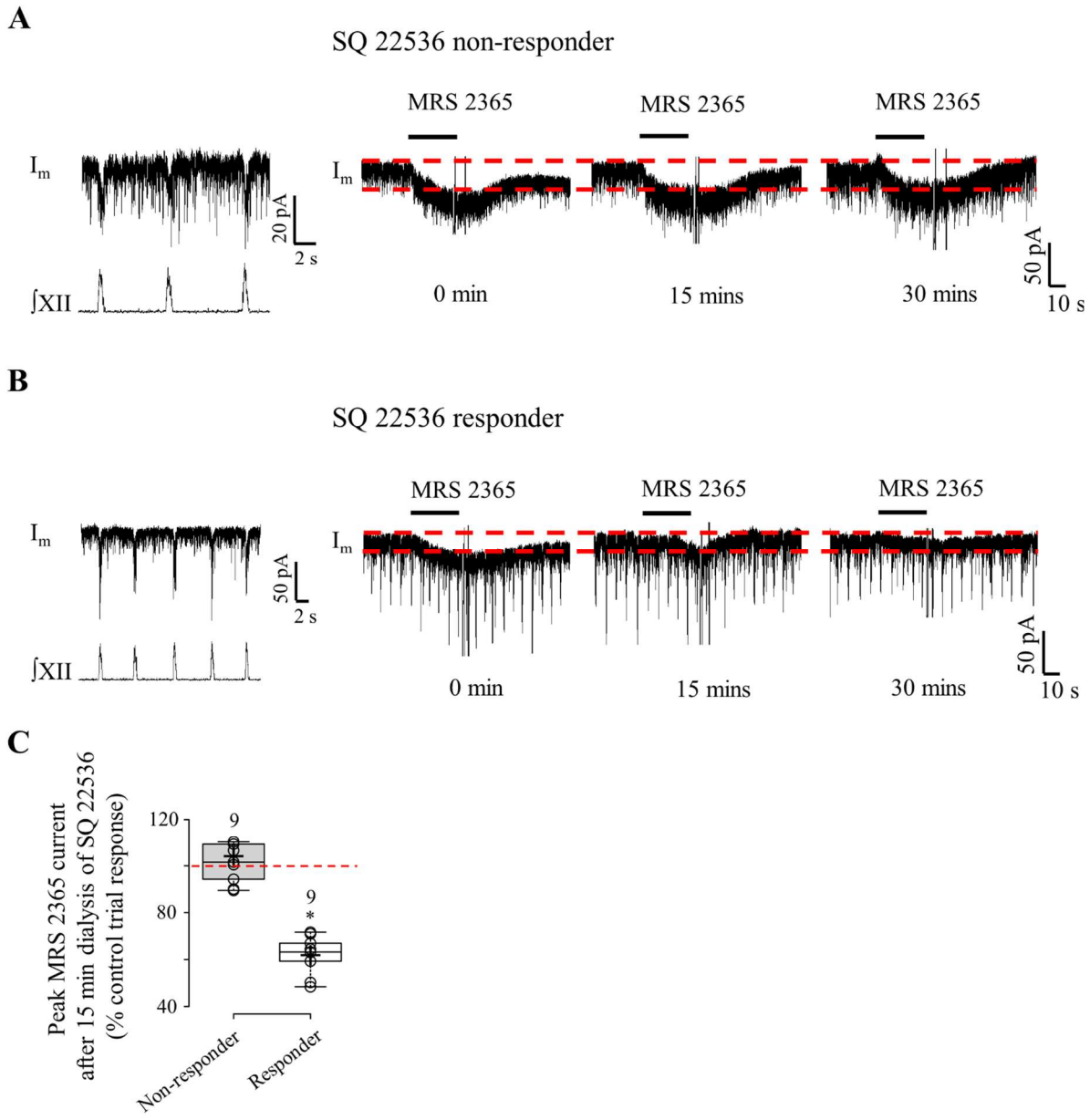

Figure 3.5 SQ22536 attenuates the MRS 2365-induced excitation of the preBötC inspiratory neurons

A) Left panel, membrane current (top) recorded under the voltage-clamp mode showing that the neuron received inspiratory inputs which are in phase with the hypoglossal nerve bursting (bottom). Right panel, representative traces showing that intracellular dialysis of SQ 22536 (100 μM) did not affect the MRS 2365-induced inward current in that cell. B) Left panel, membrane

current (top) recorded under the voltage-clamp mode showing that the neuron received inspiratory inputs which are in phase with the hypoglossal nerve bursting (bottom). Right panel, representative traces showing that intracellular dialysis of SQ 22536 (100 μM) reduced the amplitude of the MRS 2365-induced inward currents evoked in that cell. C) Group data comparing the effects of SQ 22536 on MRS 2365-induced inward currents in "responders" vs "non-responders". * $P<0.0001$, unpaired student t-test.

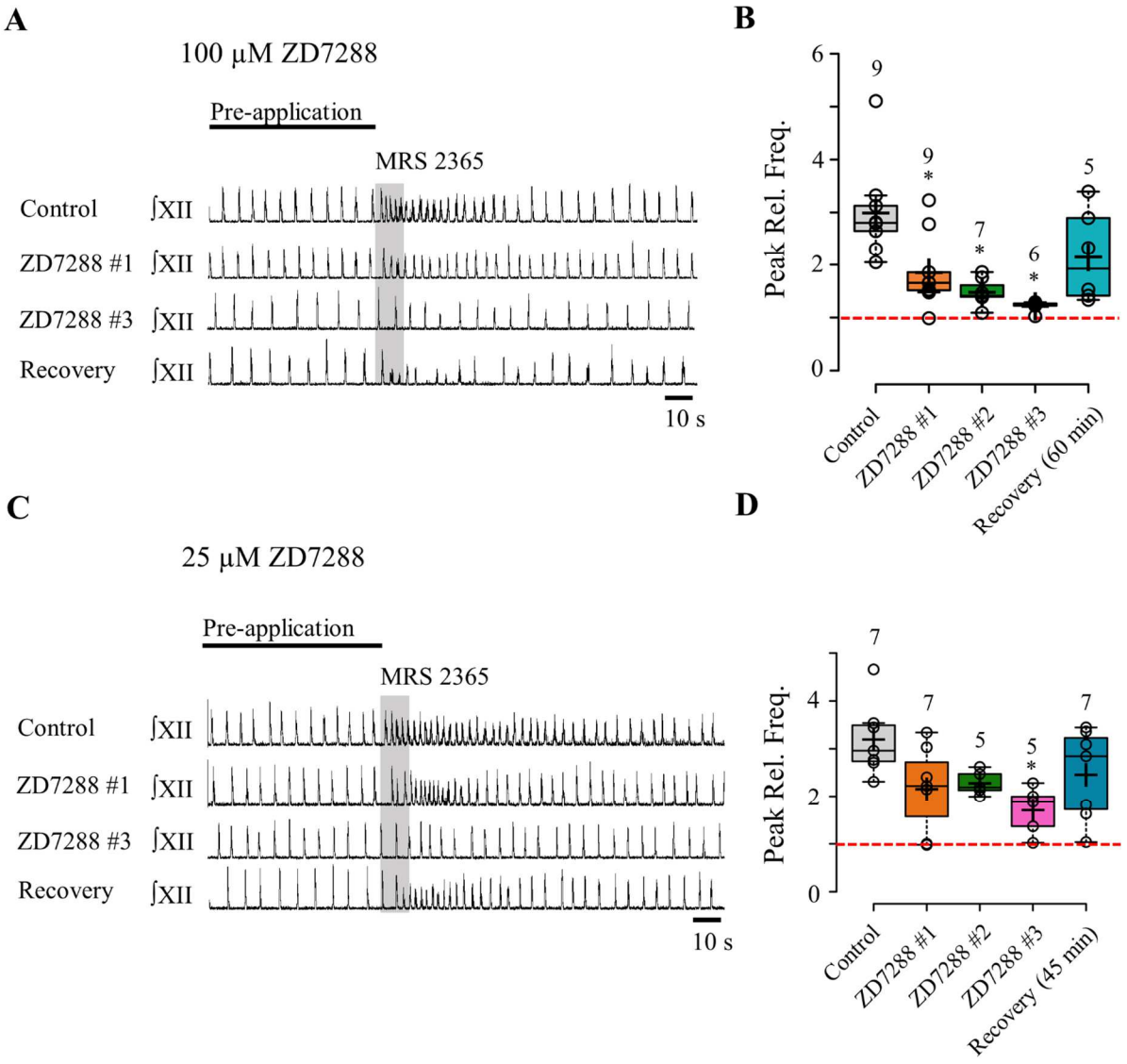

Figure 3.6 H-current activation mediates the MRS 2365-induced excitation of the inspiratory network

A) Representative traces showing that pre-application of ZD7288 at 100 µM progressively blocked the MRS 2365-induced frequency increase, an effect that could be washed out. B) Group data demonstrating the time course of the MRS 2365 (100 µM, 10 sec)-induced frequency increase in aCSF, three consecutive ZD7288 (100 µM, 5 min pre-application) and washout trials. * P = 0.01 between the time control and ZD7288 #1 groups, * P = 0.0009 between the time control and

ZD7288#2 groups, * P = 0.0002 between the time control and ZD7288#3 groups, one-way ANOVA with Bonferroni's post-hoc test. C) Representative traces showing that pre-application of ZD7288 at 25 μM progressively attenuated the network response of MRS 2365 and the attenuation was reversed following the washout session. D) Group data showing the time course of the MRS 2365 (100 μM, 10 sec)-induced frequency increase in aCSF, three consecutive ZD7288 (25 μM, 5 min pre-application) and washout trials. * P < 0.0108 between the time control and ZD7288#3 groups, one-way ANOVA with Bonferroni's post-hoc test.

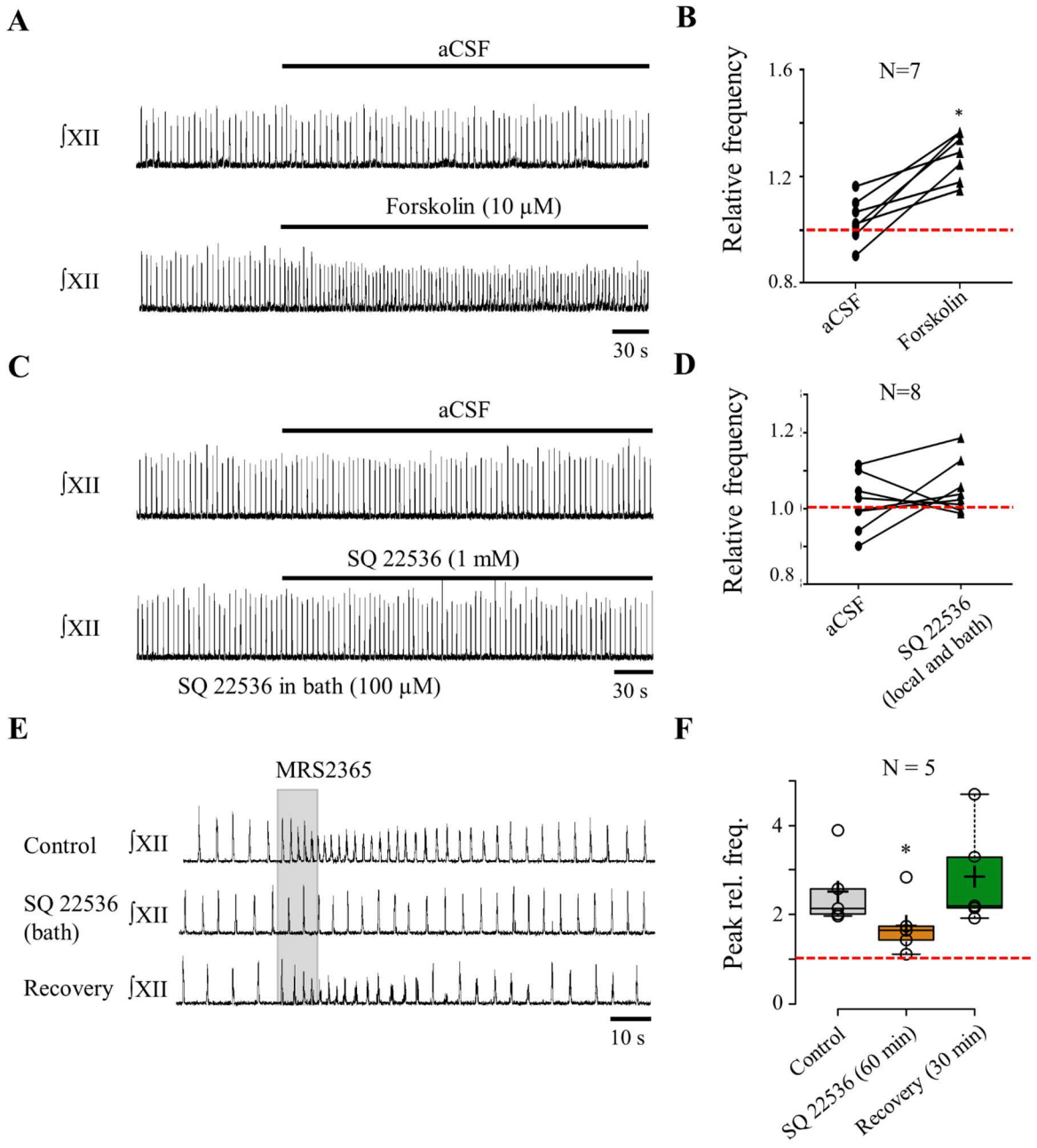

Figure 3.7 SQ22536 attenuates the MRS 2365-induced excitation of the inspiratory network

A) Representative traces showing that forskolin (10 µM, local, 5 min) increased the frequency of the inspiratory-related activity recorded from the hypoglossal nerve rootlet. B) Group data showing the effect of forskolin on baseline frequency. * $P < 0.01$, paired student t-test. Representative traces (C) and group data (D) showing that local (1 mM, 5 min) and bath application (100 µM) of SQ

22536 simultaneously had no effect of the baseline inspiratory rhythm. E) Representative traces showing that bath application of SQ 22536 (100 μM) reversibly attenuated the MRS 2365-induced frequency increase. F) Group data summarizing the effect of SQ 22536 on MRS 2365-induced network excitation. * $P < 0.05$ between the time control and SQ 22536 (60 min) groups, one-way ANOVA with Bonferroni's post-hoc test.

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Chapter 4. General Discussion

The respiratory network underlying the control of breathing is rhythmic motor network responsible for generating continuous (in mammals), efficient breathing movements, the primary function of which is to supply O_2 to support metabolism and to remove CO_2. Specialized chemoreceptors in the blood vessels and brain provide sensory feedback to the central network about acute changes in O_2 and CO_2/pH that elicit adaptive changes in breathing that restore O_2 and CO_2 homeostasis (Greer & Funk, 2013). Our interest lies in the mechanisms underlying the biphasic hypoxic ventilatory response that consists of an initial, carotid-body mediated increase in ventilation within the first minute followed by a secondary depression that develops over the next 4 – 5 min (Moss, 2000; Pamenter & Powell, 2016) that, has historically been attributed entirely to incompletely characterized, centrally-mediated inhibitory mechanisms (Darnall, 1985; Melton *et al.*, 1990; Koos *et al.*, 2005). In other words, the biphasic HVR has been viewed as the result of two mechanisms operating on different timescales – a peripheral excitation and central inhibition. Recent data from our lab and that of our colleagues (Gourine *et al.*, 2005b; Angelova *et al.*, 2015a; Rajani *et al.*, 2018) has challenged this dogma with evidence that the secondary depressive phase results from the interaction between central inhibitory mechanisms as well as an ATP-mediated, central excitatory mechanism that attenuates the depression. Specifically, the central hypoxia-induced excitation of breathing networks appears to result from the release of ATP from astrocytes in the preBötC (Angelova *et al.*, 2015a) that acts via neuronal $P2Y_1$ receptors to increase ventilation, which attenuates the secondary hypoxic ventilatory depression (Rajani *et al.*, 2018). Our goal is to advance basic understanding of this fundamental, but misunderstood, homeostatic respiratory reflex. A mechanistic understanding of this reflex is also of significant clinical interest because premature infants are much more sensitive the depressive effects of hypoxia. For example, unlike adults where the ventilation remains above baseline during the HRD, ventilation falls well

below baseline in premature mammals (Moss, 2000). This sensitivity, combined with the inherent instability of premature respiratory networks, sets up a potentially life-threatening positive feedback loop in which immature networks generate a breathing pattern featuring frequent apneas (primary apnea) that result in hypoxia, triggering the biphasic HVR response and a powerful respiratory depression, which leads to greater hypoxia, greater respiratory depression and so on (secondary apnea)(Barrington & Finer, 1991; Moss, 2000; Kalaniti *et al.*, 2018). Caffeine is used clinically to stimulate breathing in virtually all premature infants with apnea (Schmidt *et al.*) to reduce the risk of apnea and life-threatening hypoxia (Schmidt *et al.*, 2006; Henderson-Smart & Steer, 2010). Caffeine is generally very effective but alternative treatments are needed because ~20% of premature infants with apnea of prematurity either do not respond to caffeine are experience negative side-effects, including seizure (Schmidt *et al.*, 2012; Lista *et al.*, 2016). Understanding the mechanisms of the ATP excitation may inform development of alternate therapeutic/pharmacological approaches to increase breathing.

The general objective of this thesis was to characterize the intracellular signalling pathways and ionic mechanisms through which $P2Y_1$ receptors excite the preBötC inspiratory network. The main contributions of this thesis include the novel observations that:

1) The ATP excitation of the preBötC does not reflect ubiquitous sensitivity of preBötC inspiratory neurons to ATP – at least three subpopulation of inspiratory neurons were defined based on their ATP sensitivity: those that are ATP insensitive and those that respond predominantly with $P2Y_1$ and non-$P2Y_1$ receptor phenotypes. Those with the $P2Y_1$ receptor phenotype underlie the increase in frequency evoked by ATP;

2) The $P2Y_1$ receptor-mediated excitation of preBötC inspiratory neurons and network is mediated by at least two second-messenger systems;

3) One of the second-messenger systems, which underlies ~50% of frequency increase, involves the $G\alpha_q$-mediated modulation of an unknown effector/ion channel (that is not K_{ATP}, GIRK, SK, TRMP M4/5 or BK channels);

4) The second $P2Y_1$ receptor pathway involves an unknown second-messenger system that depends on an increase in cAMP and a depolarizing shift in the voltage-activation curve of I_h.

In summary, the data suggest that in the preBötC of neonatal rat in vitro, ATP signals through two independent $P2Y_1$ receptor-coupled pathways to excite preBötC inspiratory neurons and the inspiratory network. One involves $P2Y_1$ receptor activation of the $G\alpha_q$-signalling pathway and modulation of an unknown effector (ion channel), while the other involves $P2Y_1$ receptor activation of an unknown GPCR, cAMP elevation and a depolarizing shift in the voltage-dependence of I_h activation (see Fig. 4.1 for the working model). The significance of these finding is discussed in detail below in the section 4.2. Section 4.3 outlines future directions and specific experiments required to further advance our understanding of the mechanisms underlying ATP-mediated excitation of the preBötC inspiratory network and the translational potential of manipulating purinergic signaling to treat disorders of breathing that involve the nervous system.

4.1 Significance of advancing understanding of mechanisms that underlying the ATP-mediated excitation of the preBötC inspiratory network

4.1.1 ATP sensitivity defines multiple subtypes of preBötC inspiratory neuron

We have shown that a low concentration of ATP (100 μM) evokes either no current, a "fast" or a "slow" inward current in preBötC inspiratory neurons. The slow ATP currents are blocked by the $P2Y_1$ receptor antagonist MRS2279 and resemble those evoked by the $P2Y_1$ selective agonist, MRS2365. The fast currents, in contrast, are insensitive to MRS2279 and

inhibited by general P2R agonists, PPADS and suramin. Analysis of responses to high concentrations of ATP (5 mM) reveals in the majority of ATP sensitive neurons both $P2Y_1$ and non-$P2Y_1$ receptor components. Thus, data suggest that there is a differential expression pattern of $P2Y_1$ receptors and non-$P2Y_1$ P2 receptors in preBötC inspiratory neurons with $P2Y_1$ receptors predominantly expressed in one subset of inspiratory neurons and the PPADS/suramin-sensitive receptors in the other. It is also clear that the excitatory effect of ATP on the preBötC network is completely dependent on $P2Y_1$ receptor activation; the PPADS/suramin-sensitive currents, which are typically larger that the $P2Y_1$ receptor currents, do not contribute. The physiological significance of the "fast current" and the neurons that express theses currents remains to be elucidated. One hypothesis is that the neurons that predominantly express non-$P2Y_1$ receptors are the initial responders to ATP released from astrocytes upon hypoxic exposure which relay hypoxic signalling to the $P2Y_1$ receptor-sensitive "effector" neurons through ATP signalling that involves activation of $P2Y_1$ receptors on the "effector" neurons. According to the hypothesis, activation of non-$P2Y_1$ neurons is critical for the $P2Y_1$ receptor-mediated excitation of the inspiratory network that occurs during hypoxia but not necessary for the network excitation induced by exogenously applied ATP because ATP becomes accessible to "effector" neurons independent of the initial responders immediately after application.

4.1.2 The $G\alpha_q$-signalling pathway contributes to the $P2Y_1$ receptor-mediated excitation of the preBötC

Previous work from our lab demonstrated that $P2Y_1$ receptor-excitation of the inspiratory network underlies the ATP-mediated offsetting of the secondary hypoxic ventilatory depression (Rajani *et al.*, 2018). In this thesis, I verified the exclusive role of $P2Y_1$ receptor in 100 μM ATP-induced excitation of the inspiratory in vitro with MRS2279. Compared to the $P2Y_1$ receptor

antagonist MRS2179 used in the old study, MRS 2279 is about 5X more potent (EC50: 52 nM vs 330 nM)(Jacobson *et al.*, 2009) and more selective in that it does not block $P2X_1$ and $P2X_3$ (Boyer *et al.*, 2002) receptors while MRS 2179 does (Brown *et al.*, 2000b). In addition, the ribose on the adenosine biphosphate backbone was replaced by a cyclopentane ring in MRS 2279 to prevent a potential riboside biphosphate-mediated agonizing effect on $P2Y_1$ receptors (Jacobson *et al.*, 2009). The coupling of $P2Y_1$ receptors to the $G\alpha_q$-signalling pathway has been reported in both central and peripheral nervous systems such as dorsal root ganglion (Usachev *et al.*, 2002), supraoptic nucleus (Song *et al.*, 2007) and superior cervical ganglion neuron (Chandaka *et al.*, 2011). My work described in chapter 2 is the first to suggest that in the preBötC, ATP depolarizes inspiratory neurons and excite the network via $P2Y_1$ receptor activation of the $G\alpha_q$-signalling pathway. While this was indeed expected based on the body of data that $P2Y_1$ receptors signal through $G\alpha_q$, the actual demonstration that this pathway is involved in the preBötC network is very significant. Although $P2Y_1$ receptors are conventionally coupled to the $G\alpha_q$-signalling pathway, one cannot assume that the same will hold everywhere in the brain. Other signaling mechanisms have been reported (Aoki *et al.*, 2004; del Puerto *et al.*, 2012). Our own data show the importance of directly testing the involvement of $G\alpha_q$ pathway by revealing that it accounted for only ~50% of the $P2Y_1$ receptor excitation. This finding led to the search in Chapter 3 of other potential pathways and identification of a novel action of $P2Y_1$ receptors through cAMP and I_h (discussed further below). Also of significance is that while we were not able to identify the ion channel through which $G\alpha_q$ system signals to alter preBötC rhythm, we ruled out several candidates, which brought us closer to uncovering the actual effector.

4.1.3 $P2Y_1$ receptor-mediated increases in cAMP and modulation of h-current voltage-dependence contribute to the ATP-mediated excitation of inspiratory neurons and the preBötC network

The incomplete block of $P2Y_1$ receptor-mediated preBötC excitation by multiple blockers of the $G\alpha_q$ second messenger system strongly suggests that other mechanisms contribute. In addition to the $G\alpha_q$-signalling pathway, $P2Y_1$ receptor couples to the $G\alpha_i$-signalling pathway through which it inhibits N-, P/Q and L-type voltage-dependent Ca^{2+} channels (Brown *et al.*, 2000a; Filippov *et al.*, 2000; Aoki *et al.*, 2004). Considering that VDCCs contribute to synaptic transmission and the inspiratory drive potential of inspiratory neurons recorded in brainstem-spinal cord preparations (Ramirez *et al.*, 1998b; Mironov & Richter, 2000b; Onimaru *et al.*, 2003; Koch *et al.*, 2013), inhibition of VDCC by $P2Y_1$ receptor activation of the $G\alpha_i$-signalling pathway in the preBötC would disrupt the inspiratory rhythm. Thus, the observation that $P2Y_1$ receptors excite the inspiratory network excludes $P2Y_1$ receptor activation of the $G\alpha_i$-signalling in the preBötC. $P2Y_1$ receptors have also been reported to signal through the $G\alpha_s$-signalling pathway which involves activation of adenylyl cyclase, cAMP production and activation of protein kinase A, to modulate downstream targets including I_h. These reports, however, are sparse. We were able to find three papers that showed a relationship between $P2Y_1$ and $G\alpha_s$ pathway. Two of them were performed in non-neuronal cell lines; ATP works through $P2Y_1$ receptors, cAMP and PKA to protect pancreatic duct epithelial cells from alcohol-induced damage (Seo *et al.*, 2016) and in *Xenopus* kidney cells, $P2Y_1$ receptor activates PKA to induce Cl- efflux (Guerra *et al.*, 2004). The third was performed in hippocampal neurons where the $P2Y_1$-$G\alpha_s$ signaling pathway is important in axon elongation (del Puerto *et al.*, 2012). We have found no evidence that $P2Y_1$ receptors activate $G\alpha_s$-signalling pathway to bring about a change in neuronal excitability. Thus, our data

suggesting that $P2Y_1$ receptors activate $G\alpha_s$ to modulate neuronal excitability are highly novel. Given this novelty, it is key to point out that our evidence that $P2Y_1$ receptors activate $G\alpha_s$ is indirect and based on the fact that $P2Y_1$ receptor effects in the preBötC, like $G\alpha_s$ signaling, involve increases in cAMP and a depolarizing shift the voltage-dependence of I_h activation. One of the main effects of $G\alpha_s$ activation is elevation of cAMP levels, which causes up to a 30 mV depolarizing shift of activation curve of I_h (Ludwig *et al.*, 1998; Wainger *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002). It will therefore be important to establish directly that the cAMP- and I_h- dependent actions of $P2Y_1$ receptor activation depend on $G\alpha_s$. A second issue is that while it is clear that the $G\alpha_s$-signalling pathway can modulate I_h via cAMP, we have not demonstrated directly that the $P2Y_1$ receptor-mediated potentiation of I_h is dependent on cAMP – we have only shown that the effects of $P2Y_1$ receptor activation on inspiratory neurons and preBötC frequency are dependent on both cAMP and I_h. When considered in total, our data strongly suggest that $P2Y_1$ receptors excite the preBötC through a cAMP-dependent modulation of I_h.

4.1.4 Clinical relevance

As has mentioned on several occasions, the hypoxia-induced ventilatory depression can be problematic or even life-threatening to infants born prematurely. The most common respiratory stimulant used to treat apnea of prematurity is methylxanthines, primarily caffeine (Schmidt *et al.*, 2006; Henderson-Smart & Steer, 2010), which is a non-selective adenosine receptor antagonist. However, about 20% of neonates with apnea of prematurity are resistant to caffeine (Schmidt *et al.*, 2012; Lista *et al.*, 2016). Thus, there is a requirement for alternate treatments that are not based on adenosine receptors. ATP released in the preBötC during hypoxia (Gourine *et al.*, 2005b; Angelova *et al.*, 2015a) is a physiological respiratory stimulant that attenuates the secondary hypoxic ventilatory depression in a $P2Y_1$ receptor-dependent manner (Rajani *et al.*, 2018).

Enhancement of the ATP/$P2Y_1$ receptor signalling in the preBötC is therefore one potential strategy for exciting breathing and counteracting respiratory depression associated with hypoxia, opioids and other respiratory depressants (including barbiturates, ethanol)(Hunter *et al.*, 1968; Smith *et al.*, 1975), and neurodegenerative diseases (Iranzo, 2007; Torsney & Forsyth, 2017; Brzecka *et al.*, 2018; Niedermeyer *et al.*, 2019). Identification of the two independent, $P2Y_1$ receptor mechanisms that excite breathing further increases the therapeutic targets that might be manipulated to stimulate breathing. Our finding that inspiratory neurons are differentially sensitive to ATP and that a subset of $P2Y_1$ receptor-sensitive neurons most likely underlies the ATP mediated excitation offers additional hope that the $P2Y_1$ pathway, or its downstream signalling pathways, can be manipulated to selectively stimulate breathing with minimum unwanted side effects. This emphasizes the importance of characterizing the $P2Y_1$ receptor-sensitive preBötC inspiratory neurons (i.e., are they glutamatergic, GABAergic or glycinergic; are they SST positive, NK1 receptor-expressing…). This information is essential for understanding how activation of these neurons by ATP causes an increase in network rhythm and key to the development $P2Y_1$ receptor-based approaches that selectively stimulate breathing. Moreover, the rats used in this study aged from P0 to P4 which correspond to the gestational ages of 20 to 24 weeks in human based on several criteria (different parts of the body develop at different rates so multiple criteria are needed) (Liu *et al.*, 2013; Pressler & Auvin, 2013). Due to the similar ages, it is not too far of a stretch to assume that the $P2Y_1$ receptor signalling pathways identified here also operate in human preterm infants born before 24 weeks of pregnancy. Considering the fact that preterm babies born within that time window are highly predisposed to AOP (Barrington & Finer, 1991), $P2Y_1$ receptor-mediated of the inspiratory network may prove to be of particular importance as an adenosine receptor-independent way of stimulating breathing in AOP patients.

4.2 Future directions

Major contributions of this thesis include the discovery that, based on ATP sensitivity, there are multiple types of preBötC inspiratory neurons and that ATP operates through at least two independent pathways to excite preBötC inspiratory neurons and the inspiratory network in vitro. We demonstrate that in the preBötC, as elsewhere (Usachev et al., 2002; Abbracchio et al., 2006; Song & Vijayaraghavan, 2007; Chandaka et al., 2011; Rajani et al., 2016), $G\alpha_q$-signalling involves activation of PLC, IP_3Rs, intracellular Ca^{2+} release and activation of PKC. The second pathway involves increasing intracellular cAMP and potentiation of I_h. Several important questions, however, must be addressed to fully understand the mechanisms underlying the $P2Y_1$ receptor-mediated excitation of the preBötC network. Then, there are key questions that must be addressed to establish the relevance of these $P2Y_1$ receptor mechanisms to the hypoxic ventilatory response and the ATP-mediated excitation that counteracts the hypoxic respiratory depression.

4.2.1 Final verification of whether $P2Y_1$ receptor activation can modulate BK channels – is this a viable mechanism through which $P2Y_1$ receptors might modulate the preBötC

As discussed in chapter 2, the literature suggests that $P2Y_1$ receptors do not activate BK channel at -60 mV (Wei *et al.*, 1994; Wang *et al.*, 2009; Li *et al.*, 2018). However, $P2Y_1$ receptor may still potentiate BK channel activity in a freely firing inspiratory neuron which undergoes rhythmic depolarization of membrane potential. To test this, cells need to be recorded under the current-clamping technique and the effect of intracellular dialysis of 1 μM paxilline on afterhyperpolarization (AHP) activated following simulated burst will be measured under control condition. If paxilline reduces the amplitude of AHP, it suggests that BK channel activation plays a role in AHP and 1 μM paxilline is effective in blocking BK channels in rhythmically active inspiratory neurons. The next step is to examine the effect of $P2Y_1$ receptor agonization on AHP

and compare magnitudes of AHP before and after intracellular dialysis of 1 μM paxilline if $P2Y_1$ receptor potentiates AHP. A paxilline-induced attenuation of $P2Y_1$ receptor potentiation of AHP suggests that $P2Y_1$ receptors excites inspiratory neurons via activation of BK channels and the lack of effect of 1 μM paxilline on $P2Y_1$ receptor-mediated excitation of the inspiratory network is likely due to a poor access of paxilline into inspiratory neurons as it was locally applied in the extracellular space. Then similar experiments with a more selective and potent blocker (if available) will be conduct to confirm the modulation of BK channels by $P2Y_1$ receptor in inspiratory neurons. On the other hand, if paxilline application has no effect on $P2Y_1$ receptor potentiation of BK channels, it suggests that $P2Y_1$ receptors do not operate through BK channels. The other possibility is that neither paxilline nor $P2Y_1$ receptor activation influences basal AHP in the first place. In this case, it suggests that BK channels do not contribute to AHP or $P2Y_1$ receptors do not work through potentiation of AHP (regardless of whether there is BK channel component) to excite inspiratory neurons and the network respectively. To address the concern that paxilline at 1 μM is not effective in blocking BK channels, the experiments will be repeated with a different BK channel inhibitor such as iberiotoxin to verify the lack of BK channel contribution to AHP.

4.2.2 What is the ion channel/effector through which $G\alpha_q$ acts to excite the preBötC?

If the aforementioned experiments conclude that BK channels do not underlie the $P2Y_1$ receptor-mediated excitation of the inspiratory neurons, we will be left with the old question "what effector/ion channel does $G\alpha_q$ act to excite the preBötC?" Our data show that the $G\alpha_q$-signalling contributes to about 50% of the $P2Y_1$ receptor-mediated excitation of the inspiratory network. Although $P2Y_1$ receptor-potentiation of I_h is established in inspiratory neurons and it also contributes to the $P2Y_1$ receptor effects, it is unlikely that the $G\alpha_q$-signalling pathway targets I_h given that the activation of the $G\alpha_q$-signalling pathway generally inhibits I_h (Pian *et al.*, 2006;

Williams *et al.*, 2015). Therefore, the final effect of the $G\alpha_q$-signalling pathway remains to be revealed. We have specifically screened the ion channels that are reportedly modulated by $P2Y_1$ receptors/the $G\alpha_q$-signalling pathway and capable of modulating the inspiratory rhythm (Rajani et al., 2016). However, neither K_{ATP}, GIRK, SK, TRMP M4/5 nor BK channels appear to contribute to the $P2Y_1$ receptor-mediated excitation of the preBötC. The next steps will be to 1) test ion channels that meet only one of two criteria and 2) run single-cell RNA sequencing experiments on inspiratory neurons that show a $G\alpha_q$ excitation and then look for ion channels known to be $P2Y_1$ or $G\alpha_q$-sensitive. This will hopefully give us a new list of candidates to test for their involvement of $P2Y_1/G\alpha_q$ excitation. A differential screen of RNA seq data from $P2Y_1/G\alpha_q$ sensitive and insensitive neurons would also help to identify candidates.

4.2.3 Is the $P2Y_1$ receptor modulation of I_h dependent on cAMP?

We demonstrate in Chapter 3 that $P2Y_1$ receptor excitation of the inspiratory network is mediated in part by an increase in cAMP levels and activation of I_h in preBötC inspiratory neurons. Although the $P2Y_1$ receptor-mediated increase in cAMP level is likely what leads to activation of I_h as activity of HCN channels are tightly regulated by intracellular cAMP level (Ludwig *et al.*, 1998; Wainger *et al.*, 2001; Wang *et al.*, 2001; Wang *et al.*, 2002), we have not demonstrated this directly. To test if $P2Y_1$ receptor activation of I_h depends on cAMP, the excitatory effects of $P2Y_1$ receptor on I_h evoked in inspiratory neurons by the voltage protocol will be measured before and after intracellular dialysis of SQ 22536. A weaker $P2Y_1$ receptor potentiation of I_h in the presence of SQ 22536 suggests that $P2Y_1$ receptor activation of I_h is indeed dependent on cAMP. Note that a control experiment that tests the effect of SQ 22536 on I_h without $P2Y_1$ receptor agonist is critical since it is possible that SQ 22536 attenuates I_h by reducing cAMP level on its own and as a result the SQ 22536-mediated attenuation of $P2Y_1$ receptor potentiation of I_h could be non-specific. We

can also measure I_h in control and SQ 22536 after depleting cAMP. If I_h is still evident and potentiated by $P2Y_1$ receptors even in the absence of cAMP, it suggests that $P2Y_1$ receptor activation does not modulate I_h.

4.2.4 Is the $P2Y_1$ receptor modulation of I_h dependent on PKA?

cAMP activates PKA by binding to its regulatory units (Das *et al.*, 2007). Once bound by cAMP, the regulatory units of PKA dissociate from the catalytic units which then exert functions by phosphorylating target molecules, including HCN channels (Vargas & Lucero, 2002; Liao *et al.*, 2010). Therefore, it remains as a possibility that cAMP-dependent activation of PKA contributes to the $P2Y_1$ receptor-excitation of the inspiratory network. To test this hypothesis, MRS 2365-induced frequency increases before and after inhibiting PKA will be compared. If PKA inhibition causes a reduction in MRS2365-induced frequency increase, it indicates that PKA activation is required for the $P2Y_1$ receptor-mediated excitation of the inspiratory network. Since it has been reported that PKA activation is able to shift the voltage dependence of HCN channels to a more positive potential (Vargas & Lucero, 2002; Liao *et al.*, 2010), it is possible that PKA activation contributes to the $P2Y_1$ receptor excitation of the inspiratory network via activation of I_h. To test that, the MRS 2365-potentiation of I_h evoked by the voltage protocol will be measured in the presence and absence of PKA inhibitor. A reduction in MRS 2365-mediated potentiation suggests that $P2Y_1$ receptors potentiates I_h via activation of PKA. In contrast, if PKA inhibition does not affect the MRS 2365 effect on I_h, it suggests that PKA may act through other ion channels. The next step is to apply the same strategies as the one used to screen the potential $G\alpha_q$-signalling pathway-mediated ion channels but this time focus on ion channels that are sensitive to the $P2Y_1$ receptor/PKA and capable of modulating the inspiratory rhythm when manipulated.

4.2.5 What is the role of the $G\alpha_q$-signalling pathway in the $P2Y_1$-mediated excitation of the preBötC network in vivo?

Blocking the $G\alpha_q$-signalling pathway significantly attenuates $P2Y_1$ receptor-mediated network excitation in the rhythmic slices. However, whether or not the activation of the $G\alpha_q$-signalling pathway in inspiratory neurons contributes to $P2Y_1$ receptor-mediated breathing stimulation in vivo remains unknown. Local application of MRS 2365 in the preBötC increases respiratory frequency (Rajani *et al.*, 2018). If the $G\alpha_q$-signalling pathway underlies this $P2Y_1$ receptor-mediated frequency increase, selective activation of the $G\alpha_q$-signalling pathway in $P2Y_1$ receptor-sensitive preBötC using genetic tools (e.g., $G\alpha_q$-coupled DREADD) should mimic the MRS 2365 effect. The premise to this strategy, however, is that $P2Y_1$ receptor-sensitive and -insensitive neurons express different cell markers. The transcription factor of $P2Y_1$ receptor itself is not appropriate since our data demonstrate that 5 mM ATP induced MRS 2279-sensitive current in almost all inspiratory neurons recorded, suggesting that $P2Y_1$ receptors are present in most of inspiratory neurons with different expression levels. A detailed characterization of neurons that predominantly express $P2Y_1$ receptors vs those that predominantly express PPADS/suramin-sensitive receptors is required to discover cell-type specific markers (see Discussion of chapter 3 for information on subtypes of ATP/$P2Y_1$ receptor-sensitive inspiratory neurons). A comparison between the MRS 2365/hypxoia-induced increases in breathing frequency in anesthetized animals before and after blockade of the $G\alpha_q$-signalling pathway with the same blockers used in the in vitro study (U73122, 2-APB, chelerythrine etc.) will also provide insight into the relevance of the $G\alpha_q$ mechanism in in vivo settings. However, pharmacology is always limited by the specificity of drugs and their access to cells of interest. Therefore, to address the concerns, the protocol should be repeated with multiple blockers that share the same inhibitory effect on the $G\alpha_q$-signalling

pathway but have different cell permeability and side effects (e.g., thapsigargin and cyclopiazonic acid). In addition, whether or not the second messenger blockers affect the baseline rhythm needs to be tested as the baseline effect will be confounded with those specific to the $P2Y_1$ signalling.

4.2.6 What is the role of cAMP and I_h in the $P2Y_1$-mediated excitation of the preBötC network in vivo?

Our data suggest that $P2Y_1$ receptors excite the inspiratory network in part via activation of I_h in inspiratory neurons. The role of HCN channels in the inspiratory rhythm modulation is controversial. Thoby-Brisson et al. reported that blocking I_h increases the inspiratory rhythm in slice (Thoby-Brisson *et al.*, 2000). However, the concentration of ZD7288 used in their study is 100 μM in bath, which is about 100 times higher than what's required to block I_h in the same slice preparation (Mironov *et al.*, 2000). Mironov and Richter demonstrate that bath application of ZD 7288 at 0.3 – 3μM effectively blocks the I_h evoked by the voltage protocol in preBötC inspiratory neurons but did not affect the baseline inspiratory rhythm in mouse slice (Mironov *et al.*, 2000). An in vivo study shows that I_h together with I_{NaP} is essential for the inspiratory rhythm generation (Montandon & Horner, 2013). Note that the ZD7288 concentration is again 100 μM. Despite being applied in in vivo preparations where drugs are subject to faster clearance and metabolization, we cannot rule out the possibility that ZD7288 at this concentration exerts side effects. These data, when examined together, primarily point out that I_h does not modulate basal rhythm. However, they in no way rule out the possibility that activation of I_h under conditions such as hypoxia can excite the network and increase frequency so more definitive data are need. SQ 22536 can be applied locally in the preBötC of anesthetized animals to test the involvement of cAMP in MRS 2365-induced network excitation. A reduction in MRS 2365-induced increase of respiratory frequency indicates that $P2Y_1$ receptors excite the inspiratory network via an elevation of cAMP

levels, provided that SQ 22536 has no baseline effect. A similar strategy that involves blocking HCN channels with ZD7288 at low concentrations can be used to assess the I_h contribution. The arbiter of physiological significance of the cAMP level and HCN channels in ATP-mediated offsetting of the hypoxic ventilatory depression is whether or not blocking this pathway in the preBötC through micro-dialysis of SQ 22536 and/or ZD7288 will increase the magnitude of the secondary depression. A SQ22536/ZD7288-induced potentiation of the hypoxic ventilatory depression suggests that the cAMP and I_h potentiation play a role in the ATP-mediated offsetting of the hypoxic ventilatory depression. Note that it is best to use different drugs to block increase of cAMP levels and potentiation of I_h and then compare the effects on $P2Y_1$/hypoxia-mediated excitation of the inspiratory network of drugs with the same main target to address the concern that SQ22536 and ZD7288 are not specific. Genetic tools will not be available unless the attempts to characterize inspiratory neurons with different sensitivity to ATP/$P2Y_1$ receptor lead to discovery of cell type-specific markers (see Discussion of chapter 3 for the proposed experiments that may help identify neuronal properties we can use to distinguish between the subpopulations).

4.2.7 What is the role of the purinome in the $P2Y_1$-mediated excitation of the preBötC network?

In addition to the excitatory actions of ATP, one cannot forget that the overall actions of ATP are ultimately determined by a complex signaling system called the purinome. The purinome includes purine nucleotides and nucleosides, mainly ATP and adenosine (Burnstock, 1972), ecto-nucleotidases which break ATP/ADP down to adenosine (Deaglio & Robson, 2011) and nucleoside transporters which moves nucleoside substrates across cell membrane (Clements *et al.*, 2013). Thus, from a clinical perspective, one must consider how any of clinical manipulation targeted at $P2Y_1$ receptors will affect other components of the purinergic signaling system. The

purinome also presents alternate strategies to enhance ATP/$P2Y_1$ excitation and stimulate breathing (e.g., reducing ATP degradation by inhibiting local ectonucleotidases). Ecto-nucleotidases are widely expressed in the brain but there are many types with different properties (Langer *et al.*, 2008). There is substantial evidence that Ecto-nucleotidases expression in the brain varies between regions and event that there is differential expression in the preBötC (Zwicker *et al.*, 2011) - which raises the hope that ATP degradation could be specifically inhibited in the preBötC. Other strategies include mechanisms that increase clearance of extracellular adenosine. Astrocytes express adenosine deaminase and adenosine kinase which convert intracellular adenosine into inosine and AMP (Boison, 2013), respectively, effectively creating a concentration gradient across membrane for adenosine which facilitates clearance of extracellular adenosine by allowing it to move into astrocytes through ENTs. Removal of extracellular adenosine enhances the excitatory effect of ATP and promotes breathing. Therefore increasing activities of astrocytic adenosine deaminase, adenosine kinase and ENTs may prove as an alternative meanings of stimulating breathing.

4.3 Conclusions

The mammal brain and body operate on constant levels of blood O_2 and CO_2, which is achieved primarily by adjustment of breathing under different conditions, physiological or pathological. Neuromodulation of the respiratory networks is the neural substrate for many of the adaptive respiratory responses. Among those, our interest lies in the biphasic ventilatory response to hypoxia because the secondary hypoxic ventilatory depression is implicated in morbidity and mortality of neonates who are prone to hypoxic exposure, especially preterm ones who likely experience apnea of prematurity due to their immature respiratory network. A growing body of evidence is consistent with a role of ATP released from preBötC astrocytes in response to hypoxia

in offsetting the secondary hypoxic ventilatory depression (Gourine *et al.*, 2005b; Angelova *et al.*, 2015a; Rajani *et al.*, 2018). Previous work from our lab has shown that the excitatory effect of ATP is exclusively mediated by $P2Y_1$ receptors in the preBötC (Lorier *et al.*, 2007). However, the mechanism through which $P2Y_1$ receptors excite the inspiratory network remained elusive until the completion of this thesis. We have identified two separate pathways in inspiratory neurons that underlies $P2Y_1$ receptor-mediated excitation of the inspiratory network: 1) the $G\alpha_q$-signalling pathway and 2) elevation of cAMP levels and potentiation of I_h.

These findings filled the gaps in our understanding of the mechanisms underlying ATP-offsetting of the hypoxic ventilatory depression and contribute to the comprehensive understanding of the tripartite purinergic modulation of the inspiratory network which shapes the secondary hypoxic ventilatory depression. Such knowledge is clinically valuable as it provides targets for pharmacological therapies and basis for development of new medicines and may be applicable in other brain regions/systems that are subject to purinergic modulation.

Figure

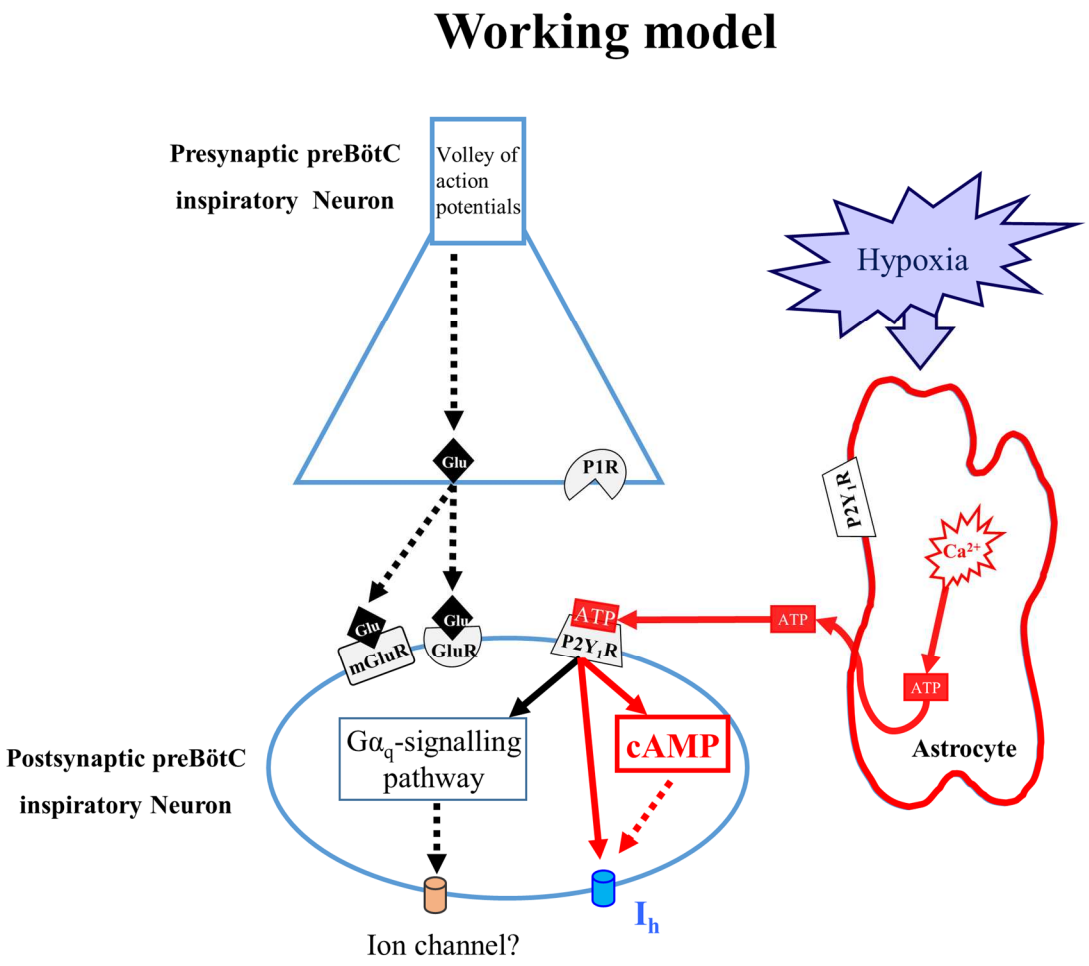

Figure 4.1 A working model on how ATP excites the inspiratory network during hypoxia.

In the preBötC, presynaptic inspiratory neurons are the first to be active during inspiration. Upon activation, they fire action potentials that trigger glutamate release. Glutamate then binds to postsynaptic ionotropic and metabotropic glutamate receptors, which excites postsynaptic neurons. Postsynaptic neurons likely send excitatory inputs back to presynaptic neurons to further increase or maintain their activity. Recurrent excitation between pre- and postsynaptic neurons eventually leads to generation of an inspiratory burst. During hypoxia, preBötC astrocytes respond to the reduction in O_2 level with an increase in the intracellular Ca^{2+} concentration which causes

vesicular release of ATP. ATP, after being released, depolarize inspiratory neurons through its action on $P2Y_1$ receptors. $P2Y_1$ receptors couple to two independent mechanisms in an inspiratory neuron. One involves activation of the $G\alpha_q$-signalling pathway and an unidentified ion channel while the other involves an increase in the intracellular cAMP levels and potentiation of I_h. Figure 4.1 is adapted from figure 4 of the article with the title of "Neuromodulation: Purinergic Signaling in Respiratory Control". I obtained the permission to reuse the figure from the publisher John Wiley and Sons. The license number is 4676580786728.

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