Molecular mechanisms involved in $P2Y_1$ receptor-mediated excitation of the inspiratory rhythm generating network

by

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ABSTRACT

Hypoxic ventilatory response is biphasic, comprising of an initial increase in ventilation followed by secondary depression. Initial increase in ventilation is mediated by peripheral carotid bodies where as central mechanisms were implicated in secondary depression. The secondary depression phase is more pronounced in premature infants which is life threatening. $P2Y_1$ receptors play an important role in shaping hypoxic ventilatory response. During hypoxia ATP is released in the ventral respiratory column, including the preBötzinger Complex (preBötC, key site of inspiratory rhythm generation), where it evokes a $P2Y_1$ receptor mediated increase in inspiratory frequency that attenuates the secondary depression. The molecular mechanisms involved in the network excitation by ATP are unknown. In other parts of the brain, $P2Y_1$ receptors are coupled to the Gαq-signaling pathway, which involves phospholipase C and inositol trisphosphate-mediated release of Ca^{2+} from intracellular stores. Thus, I predicted that preBötC inspiratory neurons would respond to $P2Y_1$ receptors activation with an increase in Ca^{2+} fluorescence. 18 inspiratory neurons from 5 slices showed a ~50% increase in baseline fluorescence to local applied $P2Y_1$ receptor agonist MRS 2365 (100µm, 10 Sec). Based on the high sensitivity of the network to $P2Y_1R$ activation, I hypothesized that a population of neurons that is important in rhythm generation or in providing direct excitatory drive to rhythm generating neurons, are very sensitive to $P2Y_1$ receptor activation. I tested the hypothesis that not all Inspiratory neurons are equally sensitive to MRS 2365 evoked increase in $Ca^{2+}_{i.}$ Responders, whose fluorescence change was obvious on visual inspection, constituted 12 neurons of 20 neurons screened. A detailed quantification revealed 47 ± 10% and 6.5 ± 2.8% increase of their baseline Ca^{2+} fluorescence in responders and non-responders respectively. I next tested the source of MRS 2365 evoked Ca^{2+} and its contribution to respiratory network excitation. Depleting Ca^{2+}_{i} with sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) blockers attenuated the MRS 2365-evoked increase in Ca^{2+}_{i} by 44 ± 7%. At network level SERCA blockers thapsigargin (200µM) and cyclopiazonic acid (CPA) attenuated the MRS 2365 evoked frequency increase by 27 ± 7% and 32 ± 8% respectively. I next screened G protein-coupled inwardly-rectifying potassium channel (GIRK; $BaCl_2$), SK (Apamin), Voltage gated K^+ channels (Tetraethylammonium) (TEA), type I BK (Iberiotoxin) channels as downstream effector of $P2Y_1$ receptor and found no effect on MRS 2365 evoked frequency increase, while Paxilline, a type I & II BK channel blocker at 1 µM attenuated MRS 2365 evoked frequency increase by 29 ± 13% suggesting a possible role of Ca^{2+}_{i} and type II BK channels in $P2Y_1$ receptor mediated network excitation.

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:

Study Title : “Neuromodulation of inspiratory network activity in vitro”,
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Collaborations:

Ca^{2+} imaging experiments in this thesis were performed in collaboration with Drs. Vladimir Rancic and Klaus Ballanyi in Dr. Ballany’s laboratory.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
Ca^{2+}_i	intracellular calcium
aCSF	artificial cerebral spinal fluid
ADO	adenosine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
$BaCl_2$	barium chloride
BK	large conductance calcium activated potassium currents
BötC	Bötzinger Complex
cAMP	cyclic adenosine monophosphate
CCHS	congenital central hypoventilation syndrome
CHO	chinese hamster ovary
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPA	cyclopiazonic acid
DAG	diacyl glycerol
DIA	diaphragm
DMSO	dimethyl sulfoxide
DRG	dorsal respiratory group
E-NPP	ectonucleotide pyrophosphatase
E-NTPDase	ecto-nucleoside triphosphate diphosphohydrolase
Epac	exchange protein directly activated by cAMP
GABA	gamma-aminobutyric acid
GPR-4	G-protein couple receptor-4
I_{CAN}	calcium activated non-specific cation current
Inst Freq	instantaneous frequency
IP3	inositol triphosphate
∫PNA	integrated phrenic nerve amplitude
MRS 2179/2279	selective $P2Y_1$ receptor antagonist
MRS 2365	selective $P2Y_1$ receptor agonist
NHERF2	sodium-hydrogen exchange regulatory cofactor-2
NK1	neurokinin1
NTS	nucleus of the solitary tract (c-caudal)
P1	adenosine receptor
P2X	purinergic receptor
P2Y	purinergic receptor
P_aCO_2	partial pressure arterial carbon dioxide
P_aO_2	partial pressure arterial oxygen
pFRG	parafacial respiratory group
PIP2	phosphatidylinositol 4,5-biphosphate

PKC	protein kinase C
PLC	phospholipase C
preBötC	preBötzinger Complex
PRG	pontine respiratory group
Rel Freq	relative frequency
ROI	region of interest
ROS	reactive oxygen species
RTN	retrotrapeziod nucleus
SD	sprague dawley
SK	small conductance calcium activated potassium currents
SP	substance P
SST	somatostatin
TASK	TWIK-related aid sensitive K^+ channel
TeLC	tetanus light chain protein
THG	Thapsigargin
TIRF	total internal reflection fluorescence
TMPAP	transmembrane prostatic acid phosphatase
TNAP	tissue-nonspecific alkaline Phosphatase
TNF	tumor necrosis factor
TRH	thyrotropin-releasing hormone
TRP	transient receptor potential cation channel
UTP	uridine triphosphate
VGCC	voltage gated calcium channels
VLM	ventrolateral medulla
VRC	ventral respiratory column
VRG	ventral respiratory group (r-rostral, c-caudal)

1.0 Literature Review

1.1 Respiratory system: an overview

In mammals, the respiratory system generates a complex, highly coordinated pattern of rhythmic activity called respiration. Respiration is important in number of functions. In vertebrates it supports metabolism by providing gas exchange, regulating blood PO_2, PCO_2 and pH, and, in many vertebrates it also contributes to temperature regulation.

Generating complex behaviors like breathing requires integration and coordination of multiple neuromuscular components. Breathing is both robust; it begins in fetal life and, in most mammals, continues almost uninterrupted until death despite dramatic changes in the brain and body that accompany growth and development, pregnancy, disease and aging. Also, to match the metabolic demand across various physiological states, respiration is also highly adaptable.

Like many other rhythmic motor behaviors, respiratory rhythm is also produced by a central pattern generator (CPG). CPGs are semi-autonomous neural networks that are capable of producing rhythmic patterned outputs without inputs from other central circuits and sensorimotor feedback signals (Grillner et al. 2005; Marder and Calabrese 1996). The respiratory CPG consists of a rhythm generator, the preBötzinger Complex (preBötC) (Smith et al. 1991) which produces the basic rhythm and a pattern generator comprising premotoneurons and motoneurons that transform the basic rhythm into required patterns of activity in all the inspiratory muscles that pump air, modulate airflow resistance and stabilize the rib cage (Lane 2011; Monteau and Hilaire 1991). The central network received mechanoreceptive feedback from a number of sources including slowly adapting pulmonary stretch receptors in the airways that underlie the Breuer-Herring reflexes important in respiratory timing and maintaining an efficient motor pattern (Widdicombe 2006).

Homeostatic control of blood gases via gas exchange in the lungs is the primary physiological role of breathing and the networks controlling breathing. To achieve this, the respiratory network receives chemosensory feedback about PO_2 and PCO_2/pH from the sensors located in the arteries and brain. Any change in arterial PaO_2, $PaCO_2$ or pH triggers ventilatory chemoreflexes that adjust ventilation to restore homeostasis (Cunningham et al. 1986).

The core components for respiratory rhythm generation and shaping inspiratory and expiratory motor patterns are located along an extensive column in the ventrolateral brainstem called the ventral

respiratory column (VRC) that runs from the caudal medulla to pons. Moving from caudal to rostral, the VRC is further divided into the following regions (1) the caudal ventral respiratory group (cVRG) corresponding to the level of nucleus retroambiguus (2) the rostral VRG (rVRG) extends from the level of obex to the rostral portion of the nucleus ambiguus (3) the preBötC located caudal to the retrofacial nucleus (4) the Bötzinger complex **(**BötC) located caudal to the facial (VII) nucleus and ventral to the nucleus ambiguus (5) the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFR), located ventral to the VII nucleus and the pontine respiratory group (PRG), which include the nucleus the parabrachialis medialis and the kölliker fuse. A schematic depicting the relative locations of brainstem respiratory groups is shown in figure 1.

Respiratory nuclei also include a region in the dorsal medulla called the dorsal respiratory group (DRG). The DRG is located in the caudal, ventrolateral nucleus of the solitary tract (cNTS) and mostly contains inspiratory neurons. The DRG contains pump or P-cells that receive input from pulmonary mechanoreceptors important in the Breuer-Hering reflexes (Kubin et al. 2006). It is much reduced in rodents compared to cats where it also contains a significant population of phrenic premotoneurons (Duffin and Lipski 1987; Lipski, Kubin, and Jodkowski 1983; Tian and Duffin 1998). The reduced size of the DRG in rat combined with the rise in prominence of rodents as the animal model of choice for respiratory control means that the DRG received little attention. The NTS, however, is an important afferent relay nucleus separate from the DRG that is as the first central synapse for a host of respiratory-relevant afferent signals, most importantly that from the carotid body chemoreceptors (Guyenet et al. 2010).

The PRG is implicated in controlling inspiratory and expiratory phase transitions (Dutschmann and Herbert 2006), and sensorimotor integration (Potts 2005; Song and Poon 2004). Pontine projections to medullary circuits are crucial for maintaining upper airway resistance during the initial phase of expiration, called postinspiration where a lengthening contraction of the diaphragm and airway muscle activation slow expiratory airflow maximizing gas exchange (Dutschmann and Herbert 2006).

The RTN/pFRG located near the medullary surface ventral to the facial nucleus, were first considered to be one large region that is chemosensitive in nature (Guyenet et al. 2010; Mulkey et al. 2004; Nattie and Li 2002). Based on the observation that disinhibition or activation of mostly lateral

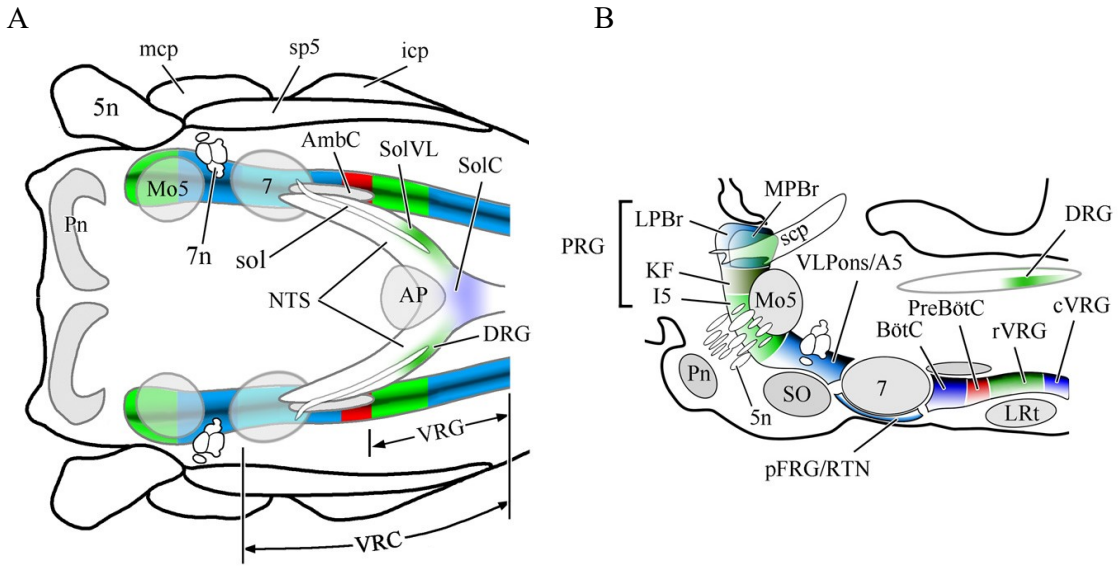

Figure 1. Anatomy of brain stem respiratory groups of the rat: (A) horizontal and (B) Sagittal view. Note that respiratory related regions comprise a nearly continuous column in the lateral rhombencephalon. The boundaries depicted between the various brainstem compartments reflect functional distinctions between adjacent regions relative to their impact on breathing.

Abbreviation: 5n, trigeminal nerve; 7, facial nucleus; 7n, facial nerve; A5, A5 noradrenergic neuronal group; AmbC, compact part of nucleus ambiguus; AP, area postrema; BötC, Bötzinger complex; cVRG, caudal division of ventral respiratory group; DRG, dorsal respiratory group; I5, intertrigeminal area; icp, inferior cerebellar peduncle; KF, Kölliker-Fuse nucleus; LPBr, lateral parabrachial region; LRt, lateral reticular nucleus; mcp, medial cerebellar peduncle; Mo5, motor nucleus of the trigeminal nerve; MPBr, medial parabrachial region; NTS, nucleus of the solitary tract; pFRG, parafacial respiratory group; Pn, basilar pontine nuclei; preBötC, preBötzinger complex; PRG, pontine respiratory group; RTN, retrotrapezoid nucleus; rVRG, rostral division of ventral respiratory group; scp, superior cerebellar peduncle; SO, superior olive; sol, solitary tract; SolC, commissural subdivision of the nucleus of the solitary tract; SolVL, ventrolateral subdivision of the nucleus of the solitary tract; sp5, spinal trigeminal tract; vlPons, ventrolateral pontine region; VRC, ventral respiratory column of the medulla; VRG, ventral respiratory group. (Adapted with permission from Alheid and McCrimmon , 2008).

parafacial neurons (pF_L) elicits active expiration suggests that the RTN/pFRG is separable into rhythmic active expiration generating pFRG and chemosensitive RTN, at least in adult rats (Huckstepp et al. 2015; Pagliardini et al, 2011). Apart from constantly monitoring brain CO_2 levels, the RTN is considered an integrating centre that receives input from peripheral oxygen and CO_2-sensitive chemoreceptors (Mulkey et al. 2004) as well other central chemoreceptive sites.

The RTN chemosensitive neurons express the transcription factor Phox2b (Stornetta et al. 2006). Mutation in Phox2b is implicated in congenital central hypoventilation syndrome. 'Pre-inspiratory' oscillatory neurons were identified in the pFRG region in neonatal rodent in vitro brainstem-spinal cord preparations (Onimaru et al. 1988; Onimaru et al. 2006). The pFRG provides rhythmic excitatory drive to the preBötC in the embryonic and early developmental stages (Huckstepp et al. 2015, 2016; Onimaru and Homma 2003); this reduces developmentally and in adult rodents at rest, late expiratory activity is completely absent in both the RTN and the pFRG regions (Huckstepp et al. 2015; Oku et al. 2007). Recent advances support the view of the pFRG as a conditional expiratory oscillator that generates active expiration during increased respiratory drive (e.g., exercise) and REM sleep (Huckstepp et al. 2015, 2016).

The BötC predominantly contains expiratory neurons (Lipski and Merrill 1980) and provides widespread inhibitory projections throughout the VRC. Two subtypes of expiratory neurons were identified in the BötC, namely E-AUG (augmenting expiratory neuron) and E-DEC (decrementing expiratory neuron). Another subtype of E-AUGs is found in the cVRG (Ezure et al. 2003) but these are expiratory premotoneurons. E-DEC inhibitory neurons of the BötC are believed to play an important role in expiratory and inspiratory phase transitions (Ezure et al. 2003; Smith et al. 2007). Activity of E-AUG neurons in the BötC can suppress upper airway motoneuronal activity which normally remains inactive during vocalization, swallowing and the compressive phase of coughing (Hisa 2016).

Caudal to the BötC is the preBötC, which is considered to be the kernel for inspiratory rhythm generation (Smith et al. 1991) in mammals. The preBötC is a heterogeneous neuronal network. A subset of neurons in the preBötC called pacemaker neurons have ability to express autonomous oscillating properties and were once considered important for respiratory rhythm generation (Koshiya and Smith 1999; Smith et al. 1991). Authors proposed that the calcium-activated nonselective cationic current (I_{CAN}) or the persistent sodium current (I_{NaP}), in combination with

excitatory synaptic interactions, might contribute for this intrinsic rhythmic activity (Butera1999; Butera et al. 1999; Koizumi and Smith 2008; Ramirez et al. 2004). However, surprisingly rhythm persisted even after pharmacological block of these currents suggesting these currents are not necessary for the generation of inspiratory rhythm (Del Negro et al. 2005). Role of inhibition in respiratory rhythm generation was also examined. Rhythm persisted despite of blocking inhibition in both preBötC and BötC regions of adult rats (Janczewski et al. 2013). Most of the preBötC neurons are glutamatergic and express Neurokinin-1 receptors (NK1-R) which is used as a marker of preBötC (Gray et al. 1999). Blockade of non-N-methyl-D-aspartate (non-NMDA) glutamate receptors by 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) abolished the rhythm suggesting that glutamatergic signaling is critical in respiratory rhythm generation (Funk et al. 1993; Smith et al. 1991). Selective and bilateral ablation of NK1-R expressing cells in the preBötC caused severely ataxic breathing patterns and abnormal responses to hypoxia and hypercapnia in vivo (Gray et al. 2001), while unilateral ablation of NK1-R expressing cells in the preBötC caused disruption of breathing that appeared first during sleep (Feldman and McKay 2008). Colocalized with NK1-R positive neurons, the preBötC neurons express somatostatin, a peptide hormone (Stornetta et al. 2003). Interestingly, silencing these somatostatin expressing cells in awake adult rats cause a persistent apnea (Tan et al. 2008). These experiments pointed out specific phenotypes of cells that are important in rhythm generation. Glutamatergic preBötC neurons possessing NK1 receptors and expressing somatostatin are necessary for rhythm generation. These glutamatergic, NK1 and SST expressing neurons were identified as being derived from Dbx1-expressing progenitor cells (Gray et al. 2010). Neurons derived from Dbx1 progenitor cells are expressed throughout the VLM. However, those present in the preBötC are essential for rhythm generation. This is supported by the fact that Dbx1 mutant mice showed severe respiratory deficits (Gray et al. 2010) and that selective, neuron by neuron deletion of the preBötC Dbx1-derived neurons rapidly disrupts inspiratory rhythm in vitro (Wang et al. 2014).

The rVRG comprises excitatory, glutamatergic bulbospinal inspiratory neurons that project monosynaptically to external intercostal and phrenic motoneurons (Dobbins and Feldman 1994; Stornetta et al. 2003). E-AUG neurons in the cVRG constitute excitatory bulbospinal neurons that project to expiratory abdominal and expiratory internal intercostal motoneurons (Ezure et al. 2003; Iscoe 1998). Inputs from multiple regions of the VRC, including the preBötC, BötC and the

pFRG/RTN regions are integrated to produce appropriate activation and inhibition of the various respiratory pump muscles (Alheid and McCrimmon 2008).

Advent of the rhythmically-active medullary slice preparation (Smith et al. 1991) significantly advanced our understanding of respiratory rhythm generation and the modulation of rhythm by various neurotransmitters and modulators. Relatively easy physical and pharmacological (i.e., no blood brain barrier) access to a defined area of interest, combined with the ability to apply a vast array of technologies to such a defined circuit made the medullary slice a versatile tool to study physiological mechanisms underlying rhythm generation, pattern formation and neuromodulation at network, synaptic, cellular and ultimately molecular levels. I exploited these advantages of the rhythmic medullary slice preparation to explore the role of purinergic signaling in modulating preBötC inspiratory rhythm

1.2 Purinergic modulation of respiratory networks

Physiological actions of extracellular adenosine triphosphate (ATP) were first demonstrated in 1929 on heart and blood vessels (Drury and Szent-Györgyi 1929). ATP was initially recognized as a neurotransmitter in non-adrenergic, noncholinergic inhibitory nerves of the guinea-pig taenia coli, and later as a co-transmitter in sympathetic and parasympathetic nerves (Burnstock 1976). Since then ATP's role in various physiological and pathophysiological pathways has been extensively investigated (Ackland et al. 2007; Burnstock 2007). The first P2 receptors were cloned in 1993 (Lustig et al. 1993; Webb et al. 1993). Since then numerous studies revealed wide distribution of purinergic receptor subtypes throughout the CNS (Burnstock 2007). Purinergic receptors can be broadly classified into P1 (adenosine) and P2 (ATP) receptors. The P2 receptors are again subdivided into P2X and P2Y receptors. P2X are ionotropic, ligand-gated non selective cation channels permeable to Na^+, K^+ and Ca^{2+}, that mediate fast excitatory responses to ATP. Seven genes, $P2X_1$ through $P2X_7$, code for seven P2X receptor subtypes. Eight P2Y receptors ($P2Y_{1,2,4,6,11\text{-}14}$) are G protein–coupled receptors (GPCRs) that mediate slower responses to ATP, adenosine diphosphate (ADP) and uridine triphosphate (UTP) (Abbracchio et al. 2006). Based on the second messenger pathways to which these receptors are coupled, P2Y receptor subtypes can be grouped according the G-protein coupled receptors they activate, which include: i) Gαq - $P2Y_{1,2,4,6}$; ii) G_i - $P2Y_{12,13}$, and; iii) multiple G-proteins: $P2Y_{14}$ to G_i/G_O and $P2Y_{12}$ to Gαq and G_S (Abbracchio et al. 2006). P2X and P2Y receptors are expressed throughout the CNS on astrocytes and neurons. However, $P2Y_{11}$

receptor expression is limited to the neurons where as $P2X_7$ receptor expression appears limited to immune cells and glia (Burnstock 2007).

Hydrolysis of ATP, ADP or AMP by ectonucleotidases generates adenosine (ADO) as an end-product, which acts as an endogenous ligand for P1 receptors. Four types of adenosine receptors (A_1, A_{2a}, A_{2b} and A_3) have been identified. Typically A_1 and A_3 are G_i coupled whose activation inhibits cyclic AMP (cAMP) production, whereas A_{2a} and A_{2b} stimulate cAMP production via Gs. A_1 receptors are highly expressed throughout the brain. A_{2a} receptors are highly expressed on striatopallidal GABAergic neurons of the brain (Fredholm et al. 2001). In general, A_1 receptor activation inhibits neurotransmitter release presynaptically and reduces neuronal excitability post synaptically (Fredholm et al. 2001).

The net effect of extracellular ATP on CNS networks results from a dynamic interaction between: i) the actions of ATP at P2X receptors; ii) ATP and ADP on P2Y receptors; (ii) ectonucleotidases that differentially degrade ATP, ADP and AMP ultimately into ADO; iii) the actions of ADO at P1 receptors (Funk 2013). Hence in addition to P2 and P1 receptor expression levels, the concentration and subtypes of ectonucleotidases will also influence the net effect of ATP on CNS networks.

Ectonucleotidases hydrolyze extracellular nucleotides to their respective nucleosides. Four families of ectonucleotidases have been identified. These include ectonucleotidase triphosphate diphosphohydrol-ases (ENTPDases). These are widely expressed ectonucleotidases in the CNS. Eight subtypes were identified. ENTPDase1 hydrolyzes ATP and ADP to AMP equally (Heine et al. 1999; Wang and Guidotti 1996). Both ENTPDase2 and ENTPDase3 preferentially hydrolyzes ATP rather than ADP, (Kegel et al. 1997; Mateo et al. 1999) but ENTPDase3 has an approximate tenfold higher affinity for ADP than ENTPDase2 (Kukulski et al. 2005; Kukulski et al. 2004; Smith and Kirley 1998). Ectonucleotide pyrophosphatase/phosphodiesterease (E-NPP) are a second class that hydrolyze ATP to AMP (Goding et al. 2003). Third, alkaline phosphatases hydrolyze ATP to ADO and ecto-5' nucleotidases hydrolyze AMP to ADO (Zimmermann 2000, 2001). Activity and expression of these ectonucleotidases can vary by age, species, and perhaps sex which may drastically influence the effect of ATP on CNS networks (Abbracchio et al. 2009; Kukulski et al. 2004; Zimmermann 2001). For example, ATP produces a very different effect on respiratory rhythm in the preBötC of neonatal rats vs. mice when applied in vitro. In rats, ATP causes a robust increase in respiratory frequency when ATP is injected into the inspiratory rhythm generator, the preBötC,

reflecting the sensitivity of respiratory rhythm to ATP acting at $P2Y_1$ receptors (Zwicker et al. 2011). In contrast, respiratory rhythm of mice is almost unaffected by ATP applied under virtually identical conditions (Zwicker et al. 2011) because the excitatory actions of ATP at $P2Y_1$ receptors are cancelled out by the inhibitory actions of its by product, adenosine, at A1 receptors. It's interesting to note that this difference in sensitivity is partially due to differential expression of ectonucleotidases in rats and mice as well as greater sensitivity of the mouse preBötC to adenosine. Mice express high levels of the ectonucleotidase, tissue nonspecific alkaline phosphatase (TNAP), which degrades ATP directly into ADO and the latter activates inhibitory A_1 receptors (Zwicker et al. 2011). Rats, on the other hand expressed primarily ENTPDase2, which preferentially hydrolyzes ATP to ADP, which is the preferred physiological agonist at excitatory $P2Y_1$ receptors (Zwicker et al. 2011).

Other important proteins that affect purinergic signaling cascades are equilibrative nucleoside transporters (ENTs), ENT 1 and ENT 2. ENTs are transporting nucleosides like ADO across the cell membranes down their concentration gradient. ENTs can either elevate or decrease extracellular ADO level by transporting accumulated intracellular ADO generated during hypoxia or by transporting ADO generated due to hydrolysis of ATP by ectonucleotidases into cells respectively. A schematic showing our working model of how purinergic signaling cascades operate in the preBötC during hypoxia is shown in figure 2.

The ability of exogenous ATP to modulate respiratory networks was first recognized in 1997 with the demonstration that ATP increases hypoglossal inspiratory motor outflow to the genioglossus (GG) in neonatal rat in vitro and adult rat in vivo (Funk et al. 1997). Further evidence of ATP modulation of inspiratory motor output emerged in 2002 by the demonstration in vitro of similar ATP-mediated potentiation of phrenic motoneuron excitability (Alvares et al. 2014; Miles et al. 2002). Based on the reversal potentials of ATP-evoked currents and membrane conductance changes, ATP actions on motoneurons were originally attributed to P2X receptors (Funk et al. 1997; Miles et al. 2002). At least at hypoglossal motoneurons, $P2Y_1$ receptors appear to contribute by augmenting the amplitude of inspiratory bursts recorded from the hypoglossal nerve (Alvares et al. 2014).

The role of ATP in respiratory control drew considerable attention in mid 90's. High expression levels of $P2X_2$ receptors in chemosensitive areas of the VLM (Kanjhan et al. 1999) and potentiation

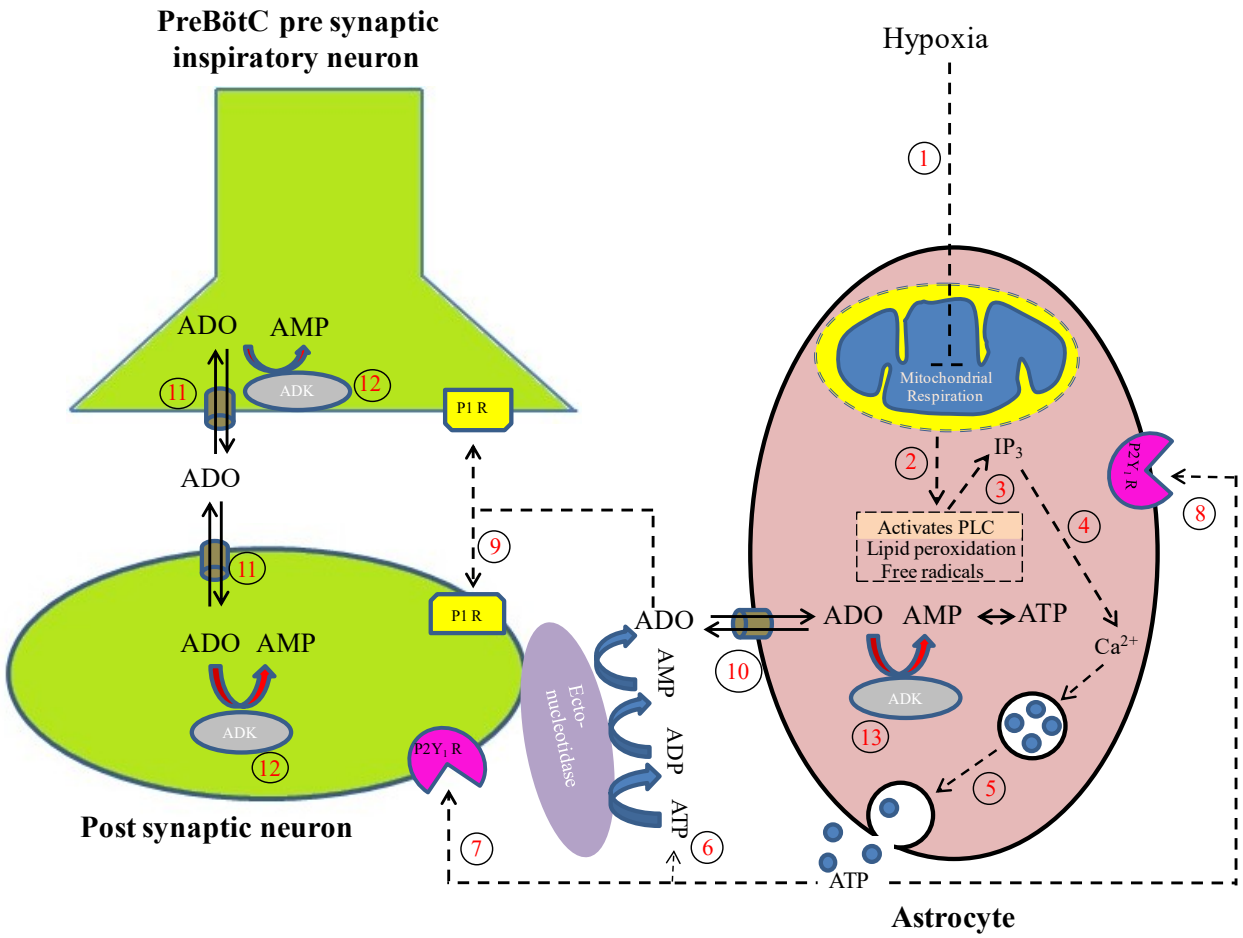

Figure 2. The Purinergic signaling cascade during hypoxia.

Hypoxia inhibits astrocyte mitochondrial respiration (1), resulting in production of free radicals, lipid peroxidation and activation of PLC (2). Activated PLC generates IP_3 (3) by hydrolysing PIP_2 (not shown). IP_3 elicit release of Ca^{2+} from intracellular stores (4). Increase intracellular Ca^{2+} triggers exocytotic release of ATP and other gliotransmitters into the extracellular space (5). Fate of the ATP depends partially on ectonucleotide expression levels. Unhydrolyzed ATP binds to the $P2Y_1$ receptors ($P2Y_1R$) on neurons (7) and glia (8). ATP released in to extracellular space is also degraded by ectonucleotidases (6), producing ADP (which activates $P2Y_1Rs$, not shown) and adenosine (ADO). ADO acts on presynaptic P1 receptors (P1Rs) and postsynaptic P1 receptors to inhibit glutamate release (not shown) and hyperpolarize post synaptic neuron respectively, causing an inhibition. The end product of hydrolysis by ectonucleotidases, ADO is transported bidirectionally either into the cells (glia (10) or neurons (11) or into extracellular space depending upon concentration gradient by equilibrative nucleoside transporters (ENTs). ADO entering neurons and glia is phosphorylated into AMP by adenosine kinase (ADK) (10, 11)

of ATP responses at $P2X_2$ receptors by reductions in pH (King et al. 1996, 1997) led to the hypothesis that $P2X_2$ receptors may be the actual CO_2 sensors involved in central respiratory chemoreception (Thomas et al. 1999; Thomas and Spyer 2000). This hypothesis was ultimately rejected as central chemosensitivity in $P2X_2$ knockout mice remained unaltered (Rong et al. 2003) and $P2X_2$ antagonists had no effect on CO_2 sensitivity in vitro (Lorier et al. 2004). However, these data did not exclude the potential role of ATP and P2 receptor signaling in chemoreception. Gourine et al. (2005), using ATP biosensors, demonstrated that ATP is released from the ventral surface of the medulla overlying the region of the retrotrapezoid nucleus (RTN) (CO_2 chemosensitive region of the brainstem) in response to hypercapnia. Also, blockade of P2 receptors with pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) attenuated the hypercapnic ventilatory response by 20-30% (Gourine et al. 2005). These data strongly supported the contribution of ATP and P2 receptors in central CO_2 sensitivity and led to a significant body of ongoing work described below that continues to explore the role of ATP and glia in central chemosensitivity.

ATP and P2R signaling are also involved in the hypoxic ventilatory response. Gourine and colleagues also demonstrated release of ATP from the ventral surface of the medulla in response to hypoxia. They hypothesized that this ATP attenuates the secondary depression phase of the hypoxic ventilatory response because the increase in ATP concentration was delayed and occurred during the secondary depression. Block of P2 receptors with PPADS also augmented the secondary depression. Further investigation into the source of the ATP revealed that ATP detected at the ventral surface originates from the VRC, including the preBötC (Gourine et al. 2005). The unanswered question that remained was the subtype of P2 receptor that mediated the excitatory effect of ATP on respiratory networks. ATP-mediated excitation of preBötC was abolished by pre-application of $P2Y_1$ receptor antagonist MRS 2179 in rat medullary slice preparation (Lorier et al. 2007). This has since been confirmed in mouse and rat (Zwicker et al. 2011), leading to the main objective of my research which is to understand how $P2Y_1$ receptor activation by ATP excites the preBötC inspiratory network. Specifically I am interested in the second messenger pathway(s) and ion channels though which $P2Y_1$ receptors signal to increase inspiratory frequency in vitro.

1.2.1 Purinergic signaling in central chemoreception

Respiratory chemoreception is the ability of an organism to sense changes in the levels of O_2, CO_2, or pH of the blood or cerebrospinal fluid. Continuous monitoring of these parameters is necessary

for homeostatic control of respiratory and cardiovascular systems. Both central and peripheral receptors are involved in chemoreception and work in coordination. Specialized cells within the brainstem that sense CO_2/H^+ contribute to central chemoreception (Guyenet et al. 2010; Moreira et al. 2011). Peripherally, carotid body chemoreceptors, which sense changes in $O_2/CO_2/H^+$, contribute ~20% of the ventilatory response to CO_2/H^+. Until recently, the increase in ventilation during hypoxia was attributed entirely to the carotid body chemoreceptors, but recently it has emerged that brainstem astrocytes are capable of detecting low PO_2 and exciting respiratory network and appear to play a role in the respiratory response to hypoxia by attenuating the secondary hypoxic respiratory depression (Angelova et al. 2015).

The hypercapnic ventilatory response consists of an increase in breathing frequency and tidal volume that contribute to the overall increase in ventilation. However this increase in ventilation due to hypercapnia varies between species and with development (Putnam et al. 2005). Multiple central chemosensitive sites including the RTN, the locus coeruleus (LC), the nucleus tractus solitarius NTS, medullary raphe and preBötC together contribute 80% of the ventilatory increase with carotid bodies contributing the remainder (Nattie and Li 2009). Among these various central sites, the RTN has emerged as perhaps the key site for chemoreception. I briefly outline current understanding of purinergic receptor signaling and its contribution to central chemosensitivity in each of above putative chemoreceptive areas.

The medullary raphe comprises the raphe magnus, raphe pallidus and raphe obscurus. Serotonergic medullary raphe neurons are considered by some to play an important role in central chemoreception (Ray et al. 2011; Richerson 2004), but whether they are major contributors or simply provide tonic excitatory modulatory drive to the RTN chemosensitive neurons remains controversial (Guyenet et al. 2010). The various raphe nuclei also show differential sensitivity to CO_2. The rostral raphe magnus contains a large population of serotonergic neurons with greater chemosensitivity than the caudal raphe obscurus (Dias et al. 2008; Gao and Mason 2001; Da Silva et al. 2011). Raphe magnus shows strong immunolabeling for $P2Y_1$, $P2X_1$ and $P2X_3$ receptors while $P2X_{1\text{-}6}$ receptors are expressed in raphe obscurus neurons (Yao et al. 2000). In anesthetized rats injection of ATP into raphe magnus and raphe pallidus excited and inhibited respiration, respectively; PPADS, a P2 receptor antagonist, had no effect on base line respiratory activity but partially blocked the ATP effects (Cao and Song 2007). In conscious rats, local application of PPADS (20 mM) into the rostral

raphe magnus blunted the ventilatory response to CO_2 whereas very little effect on the hypercapnic ventilatory response was observed following PPADS administration into caudal raphe obscurus (da Silva et. al. 2012) and raphe pallidus (Sobrinho et al. 2014) which provided evidence that P2 purinoceptors within the rostral but not caudal medullary raphe, exert an excitatory modulation of the ventilatory response to hypercapnia (Da Silva et al. 2012). PPADS at the concentration used in da Silva et al and Sobrinho et al. study could affect glutamatergic transmission making it hard to predict whether the results observed are due to P2X purinoceptor antagonism or inhibition of glutamatergic transmission.

The NTS in the dorsal medulla is a fundamental site for the integration of viscerosensory information involved in cardiovascular and respiratory control. Caudal portions of the NTS are implicated in chemoreception (Dean et al. 1990; Nattie and Li 2002). P2 receptors are widely expressed in NTS (Yao et al. 2000). Injection of ATP into the caudal NTS increases ventilation (Antunes et al. 2005), whereas PPADS (used in nanomolar concentration where it does not affect glutamate receptors) in the NTS blunted peripheral chemoreflex control of cardiorespiratory function (Braga et al. 2007; Paton et al. 2002). However, purinergic signaling does not appear to contribute significantly to the chemoreceptive function of the NTS as P2 receptor block in cNTS did not affect the firing rate response to CO_2/H^+ in vitro, or the ventilatory response to CO_2 in vivo (Sobrinho et al. 2014).

As already mentioned, the RTN is an important site of central chemoreception (Guyenet et al. 2009). pH-sensitive neurons (Mulkey et al. 2004) and glial cells (Gourine et al. 2010) both contribute to chemoreception in the RTN. pH-sensitive RTN neurons are glutamatergic, express the transcription factor Phox2b and project directly to key pontomedullary respiratory centers, including the pre-Bötzinger complex (Mulkey et al. 2004). The molecular mechanism by which these neurons sense pH change is of great interest. The ability of these neurons to respond to pH changes persisted even after blockade of ionotropic glutamate receptors with CNQX and P2 receptors with PPADS (Mulkey et al. 2006) and action potentials, indicating that they are intrinsically chemosensitive. These neurons expresses a pH-sensitive, voltage-independent K^+ conductance which is likely involved in sensing pH changes (Mulkey et al. 2004). This pH-sensitive current strongly suggests involvement of the TASK family of background K^+ channels, including TASK 2 channels (Bayliss et. al. 2015). Importantly, pH-sensitivity remained in a subpopulation of the RTN neurons from

TASK-2 knockout mice suggesting additional molecular sensor (Bayliss et. al. 2015). Recently GPR4, a proton-activated GPCR, has been shown to contribute to the pH sensitivity of a subset of the RTN neurons (Kumar et al. 2015). Mice lacking GPR4 showed diminished CO_2-evoked RTN neuronal activation and CO_2-stimulated breathing (Kumar et al. 2015).

pH/CO_2 sensitive astrocytes that line the ventral surface of the medulla also contribute to central chemosensitivity. pH-sensitive glial cells in the RTN release ATP in response to changes in CO_2/pH (Gourine et al. 2005) and contribute to the pH sensitivity of the RTN neurons by increasing their excitability. Extracellular acidification evokes elevations in intracellular Ca^{2+} and ATP release (Gourine et al. 2010). Optogenetic mimicking of pH-evoked $Ca^{2+}{}_i$ excitation in astrocytes causes release of ATP and a robust increase in breathing (Gourine et al. 2010). Depolarization-induced exocytosis is identified as one mechanism of ATP release by the RTN astrocytes (Kasymov et al. 2013). ATP is also released via a hemichannel sensitive mechanism. Connexin 26 (Cx26) hemichannels preferentially expressed on astrocytes near the ventral surface of medulla release ATP in response to elevated PCO_2 rather than reductions in pH (Huckstepp et al. 2010). Selective Cx26 hemichannel blockers in vivo attenuate the release of ATP and subsequent hypercapnic ventilatory response by ~20% (Huckstepp et al. 2010). Cx26 transfected HeLa cells, preloaded with ATP showed a CO_2-dependent ATP similar to that observed in brain slices (Huckstepp et al. 2010). In vivo studies demonstrated the release of ATP within the RTN when subjected to hypercapnia. Additionally, ATP application into the RTN increased respiratory output and the hypercapnia induced increase in respiratory frequency is attenuated about ~25% when P2 receptors are blocked with PPADS (100 μM i.e. concentrations that could affect glutamatergic transmission) in the same region (Gourine et al. 2005). It is proposed that changes in CO_2/pH evoke release of ATP from astrocytes which excites pH-sensitive RTN neurons. Even though sensitive to ATP, the pH-sensitivity of these RTN neurons is not dependent on P2 receptors (Mulkey et al. 2006). The RTN neurons are modulated by purinergic signaling. Postsynaptic P2Y receptor activation excites the RTN neurons where as presynaptic P2X receptor activation via interneuron inhibits the RTN neurons. (Mulkey et al. 2006).

Recent studies established role of $P2Y_1$ receptor mediated signaling in the RTN. However, its contribution to the purinergic component of central chemosensitivity in the RTN remains unclear. In organotypic culture, MRS 2179 (3 μM), PPADS (5 μM) or TNP-ATP (20 nM) significantly

attenuated the acidification induced increase in intracellular Ca^{2+} in astrocytes suggesting role of $P2Y_1$ signaling (Gourine et al. 2010). All the drugs used can also block ionotropic ATP receptors, in particular $P2X_1$ and $P2X_3$ receptors. The same study (Gourine et al. 2010) also demonstrated that acidification triggered a Ca^{2+} wave that propagated between ventral surface astrocytes due to intercellular ATP release acting on P2X and P2Y receptors, ultimately arriving at RTN neurons to depolarize them. Supporting P2X receptor role, Wenker et al 2012 demonstrated that purinergic signalling in the RTN contributes to ~30% of RTN chemoreception, a small portion that depends on hemichannel-mediated ATP release and that the $P2Y_1$ receptors don't mediated actions of endogenous ATP on RTN neurons (Wenker et al. 2012; Khakh and North 2012).

Another important group of the rostral VLM (RVLM) neurons that control sympathetic vasomotor tone at rest and during chemoreflexes are C1 neurons (Guyenet et al. 2013). C1 neurons receive excitatory glutamatergic inputs from peripheral chemoreceptors. These neurons express $P2Y_1$ receptors, but are not the pH sensitive neurons of the RTN (Wenker et al. 2013). This was established based on following observations: i) a $P2Y_1$ receptor agonist greatly promoted firing of pH-insensitive neurons whereas firing of pH sensitive neurons of the RTN is unaltered by $P2Y_1$ receptor activation (Wenker et al. 2013); ii) $P2Y_1$ receptor immunoreactivity colocalizes with tyrosine hydroxylase (TH), which is a marker of C1 neurons but not Phox2b positive chemosensitive neurons (Wenker et al. 2013); iii) $P2Y_1$ receptor activation in the RVLM increases phrenic and sympathetic nerve activity and mean arterial pressures (Wenker et al. 2013) in anesthetized adult rats and this response is blocked when C1 neurons are lesioned (Wenker et al. 2013).

1.3. The hypoxic ventilatory response and ATP

The hypoxic ventilatory response is biphasic, comprising an initial increase in ventilation, followed by a secondary depression (Cross 1951; Cross et al. 1954, 1957; Moss and Harding 2000), which can be life threatening in premature and newborn infants (Moss and Harding 2000; Waters and Gozal 2003). The initial increase in ventilation is mostly mediated by the peripheral chemoreceptors (Brady, Ia, and Verduzco 1975). The secondary depression is mostly attributed to the central mechanisms. Figure 3 compares the hypoxic ventilatory response in newborn and adult pigs. Purinergic signaling features prominently in both phases of the response at peripheral and central levels, respectively (Lorier et al. 2007; Prasad et al. 2001; Zhang et al. 2000).

1.3.1 The role of peripheral chemoreceptors in the hypoxic ventilatory response

Even though several CNS regions including the caudal hypothalamus, the NTS and the preBötC contribute to the initial increase in ventilation during hypoxia (Neubauer and Sunderram 2004; Solomon 2000), it is primarily mediated by the peripheral carotid body chemoreceptors (Prabhakar 2000). The carotid bodies are located at the bifurcation of the carotid artery and are highly sensitive to change in blood PO_2 levels. Two types of cells are present in carotid body, namely glomus type I, neuron-like cells and sustentacular type II glia-like cells. Type I glomus cells play an important role in hypoxia sensing. Recent studies suggest hydrogen sulfide (H_2S) as an important molecule in carotid body hypoxia sensing. During hypoxia H_2S production in glomus cells increases due to a decrease in the inhibition of cystathionine-γ-lyase, an enzyme which catalyzes break down of cystathionine into cysteine, increased H_2S levels stimulates carotid body neural activity and ultimately ventilation (Yuan et al. 2015). The molecular mechanisms underlying hypoxic sensitivity of the glomus cells appear to involve closure of hypoxia sensitive K^+ channels, leading to cell depolarization, activation of voltage-gated Ca^{2+} channels and vesicular release of ATP and acetylcholine which activate $P2X_2$ and $P2X_3$ receptors and acetylcholine receptors on axon terminals of the carotid sinus nerve. ATP actions on $P2X_3$ receptors also contribute to the activation of carotid sinus nerve as $P2X_2$ and $P2X_3$ knockout mice hypoxia responses are much more blunted than $P2X_2$ knockout mice alone.

Knockout mice lacking TASK 1 channels, a two-pore domain acid-sensitive K^+ channel, have carotid sinus nerve hypoxia responses that are reduced by ~50% compared to wild-type animals (Trapp et al. 2008). These data suggest that TASK 1 channels are a candidate for the hypoxia sensitive K^+ channel. Carotid sinus neuron terminals relay this information to the NTS, which integrates this information and, via the PRG and pFRG/RTN, connects to the preBötC to evoke an increase in respiration. ADO, which is produced from degradation of extracellular ATP and from intracellular stores inhibits TASK channels and contributes to the chemosensory response. Simultaneously, ATP activation of $P2Y_1$ receptors hyperpolarizes glomus cells and provides negative feedback to terminate the stimulus.

An alternate mechanism proposes that a heme and/or a redox-sensitive enzyme is the oxygen sensor and that a biochemical event associated with the heme protein triggers excitatory neurotransmitter(s) from the glomus cells (Prabhakar 2000). Mitochondrial cytochromes are one

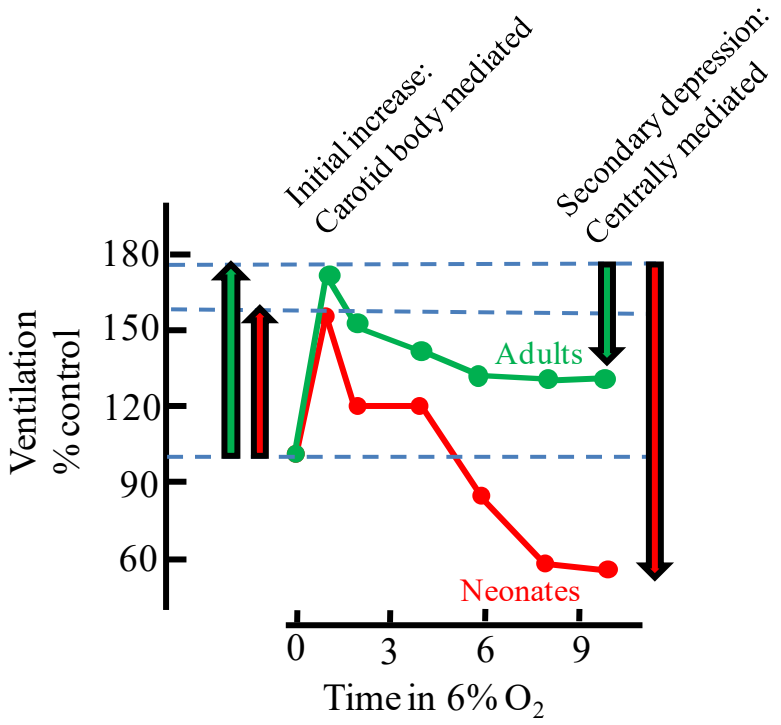

Figure 3. Comparison of the hypoxic ventilatory response in adult's vs. neonates.

The hypoxic response depicted above is from anesthetized piglets (red) and adult pigs (green) exposed to 6% inspired O_2. Hypoxic ventilatory response is biphasic, with initial increase in ventilation mediated by carotid bodies followed by secondary depression, which is centrally mediated. Note that the secondary depression phase is more pronounced in neonates compared to adults, where ventilation falls below baseline. (Even though the initial increase phase (upward arrows) is similar, compare to adults (green downward arrow), the secondary depression is more pronounced in neonates (red downward arrow) where ventilation falls below baseline). Adapted with permission from Moss, 2000).

type of heme-containing enzyme that resides within mitochondria. In this theory, mitochondria in glomus cells are involved in the oxygen sensing process and role of oxygen sensor is performed by a cytochrome which has a very low affinity for oxygen (Prabhakar 2000). Supporting this hypothesis, Biscoe et al. 1982 (Biscoe et al. 1982) reported that glomus cell mitochondria depolarize in hypoxia and are highly sensitive to changes in oxygen compared with other cells that depolarize in hypoxia (Prabhakar 2000). Also, inhibiting the mitochondrial respiratory chain increased basal sensory discharge and prevented the sensory response to hypoxia but not to CO_2. A role for nonmitochondrial heme-containing enzymes in oxygen sensing is also proposed (Prabhakar 2000).

1.3.2 Central mechanisms shaping the secondary depression

During the second phase of the hypoxic ventilatory response, respiratory frequency drops from the initial peak which occurs in the first minute to stabilize at a steady-state level typically by 3-4 minutes following the initial exposure to hypoxia (provided the hypoxic stimulus was delivered abruptly) (Moss and Harding 2000). The steady-state level of ventilation in adults remains above base line, but in premature and (some new born) infants ventilation falls well below baseline, which can lead to development of a life threatening positive-feedback loop whereby the immature respiratory neuronal network in neonates produces a breathing pattern that features frequency apneas, which lead to hypoxia, hypoxia-induced respiratory depression and more severe hypoxia ultimately death. The mechanisms underlying the secondary depression are still under debate, but central mechanisms are strongly implicated (Teppema and Dahan 2010). ADO is implicated in the secondary hypoxic depression. ADO suppresses respiration many mammals.(Funk 2013). Two mechanisms have been proposed for the ADO-mediated respiratory depression: i) A2a receptor-mediated excitation of inhibitory GABAergic neurons that inhibit respiratory networks,(Wilson et al. 2004) and ii) pre and post synaptic inhibition mediated by A1 receptors (Mironov et al. 1999). Thus, the biphasic hypoxic ventilatory response was considered to reflect an initial excitation from the carotid bodies followed by a secondary, centrally-mediated depression. The excitation was primarily attributed to the peripheral chemoreceptors. However, there was evidence in virtually all mammals tested that, given time, an excitatory hypoxic ventilatory response emerges following carotid body chemoreceptor denervation; i.e., there might be a central excitatory component to the hypoxic ventilatory response. Specifically, carotid body denervated dogs (Davenport et al. 1947), goats (Sorensen and Mines 1970), cats (Miller and Tenney 1975), ponies (Bisgard et al. 1980) and rats

(Martin-body et al. 1985) showed an increase in ventilation in response to hypoxia, indicating that either additional peripheral chemoreceptor (e.g., aortic bodies) came into play or there was a direct excitatory effect of hypoxia on ventilation.

In 2005, using ATP biosensors, Gourine and colleagues demonstrated ATP release from the ventral surface of the medulla during hypoxia in anesthetized rats (Gourine et al. 2005). Interestingly, the peak ATP concentration was recorded after the initial increase in ventilation. Also, application of the P2 receptor antagonist, PPADS, over the ventral medullary surface did not affect the initial increase in ventilation, but caused a greater secondary depression. These data suggested that ATP does not contribute to the initial increase in ventilation but that its delayed release is excitatory and attenuates the secondary depression. However, sensitivity of biosensors, selectivity of PPADS (at higher concentrations PPADS can inhibit glutamatergic transmission) raised doubts about the involvement of ATP. Many questions remained at this early stage (2005), but since then significant progress has been made regarding: i) the site of ATP release/action during hypoxia and receptor subtypes that underlie the excitatory actions of ATP; ii) whether the ATP released in hypoxia comes from neurons or astrocytes; and, iii) the mechanism of hypoxia sensing and ATP release. An area that remains most poorly investigated is the mechanisms by which ATP excites the inspiratory network once released during hypoxia. This latter issue is the main focus of my research. I will review progress in each of the three areas described above before concluding with the background and rational to my specific research questions.

(i) The site of ATP release/action and receptor subtypes that underlie the excitatory actions of ATP.

In anesthetized rats, ATP release during hypoxia was first detected at the ventral surface of medulla using sensors placed rostrocaudally that sampled from the entire length of the medulla (Gourine et al. 2005). Further investigation on source of the ATP in vitro revealed that the ATP originates from ventral respiratory column, including the preBötC (Gourine et al. 2005). The precise location where ATP acts to increase respiratory frequency within VLM remained unclear. The preBötC, the kernel of respiratory rhythm generator was a strong candidate. Mapping of the VLM in the rhythmic medullary slice preparation using local injections of ATP revealed the preBötC as the site of action of ATP-mediated excitation. Also, MRS 2179, a $P2Y_1$ receptor antagonist, significantly attenuated the ATP-evoked frequency increase in vitro. $P2Y_1$ receptor sensitivity was confirmed later using the $P2Y_1$ receptor agonist MRS 2365 (Lorier et al. 2007; Zwicker et al. 2011). These data suggest that

ATP released during hypoxia activates $P2Y_1$ receptors in the preBötC to cause a frequency increase. In addition, expression of TMPAP (transmembrane prostatic acid phosphatase, an ectonucleotidase that prevents accumulation of ATP in the extracellular space and in vesicles) in the preBötC in vivo augmented the secondary hypoxic respiratory depression (Angelova et al. 2015), as seen previously with application of PPADS to the ventral surface of the medulla (Gourine et al. 2005). Finally, unilateral injection of $P2Y_1$ receptor antagonist MRS 2279 (500 μM) into the preBötC of anesthetized adult rats augmented secondary hypoxic respiratory depression (unpublished data Rajani and Funk et al, 2017). These observations together suggest that during hypoxia the release of ATP in the preBötC activates $P2Y_1$ receptors which attenuate the hypoxic respiratory depression.

(ii) Source of ATP during hypoxia

Neurons and glia constitutes the cellular structure of brain. It is belived that glial: neuron ratio in brain is 10:1, study done in 2009 settled on glial: neuron ratio of 1:1 (Azevedo et al. 2009). Despite the ratio, glial cells play an important role in most central homeostatic processes and also during development. Glial cells can be classified into: microglia, oligodendrocytes, polydendrocytes, and astrocytes. Microglias are the primary resident immune cells of the CNS. Pathogens and injury activates microglia cells. Activated microglia secretes both pro- and anti-inflammatory mediators, phagocytose and destroy pathogens as well as remove damaged cells. Activation of microglia causes upregulation of P2X and P2Y receptor subtypes which plays an important role in chronic pain pathways. The role of microglia and P2 receptor signaling in modulating respiratory network activity in physiological and pathophysiological states received little attention. Inhibition of microglial activation in neonatal rats does not affect behavior of the rhythmic medullary slice in vitro (Zwicker et al., 2014.

Glia (presumably astrocytes) also appear to play a role in the respiratory frequency increase evoked by ATP in the preBötC of the rhythmically active medullary slice preparation from neonatal rats (Huxtable et al. 2010). Slices incubated with glial toxins showed a significant reduction in the ATP-evoked frequency increase. However, no significant changes were noticed in substance P responses, indicating that the toxins had not interfered with the network's ability to respond to excitatory modulation. In addition, cultured preBötC glial cells showed $P2Y_1$ receptor-dependent, ATP-evoked increases in intracellular Ca^{2+}. These data lead to formulation of the hypothesis that ATP released from an unknown source during hypoxia acts in part on glia to release glutamate and ATP, which in

turn excites preBötC inspiratory neurons causing an increase in frequency (Huxtable et al. 2010). Analysis of primary dissociated glial cultures from multiple brain regions suggests that astrocytes are a source of ATP in hypoxia (Marina et al. 2016). Total internal reflection fluorescence microscopy (TIRF) imaging of cultured astrocytes revealed that hypoxia causes exocytosis of ATP-containing vesicles (Angelova et al. 2015). However, in contrast to the data of Huxtable et al (2010) suggesting that glia release and respond to ATP through ATP-evoked ATP release, the $P2Y_1$ receptor antagonist MRS 2179 had no effect on hypoxia-induced vesicle exocytosis, suggesting that ATP-evoked ATP release does not occur in cultured astrocytes (Angelova et al. 2015). The only consideration with the cultured astrocyte data is that the astrocytes were cultured at low density, which could limit the ability detect autocrine or paracrine actions of ATP.

Disrupting astrocyte exocytosis using tetanus light chain protein (TeLC) in the preBötC and surrounding areas of anesthetized rats augmented the secondary hypoxic respiratory depression (Angelova et al. 2015). This provides evidence that exocytotic release of ATP from glia excites the preBötC network to increase ventilation and attenuate the secondary hypoxic respiratory depression.

(iii) Mechanism of hypoxia sensing

The ability of glial cells to sense hypoxia was examined recently by Angelova et al. (2015), again using cultured astrocytes. When cultured astrocytes were exposed to physiological relevant levels of hypoxia, they responded with an increase in intracellular Ca^{2+}. Mitochondrial depolarizations underlie astrocytic Ca^{2+} responses to hypoxia suggesting that astrocyte mitochondria are the hypoxia sensor (Angelova et al. 2015). Using fluorescent probes to measure reactive oxygen species (ROS) revealed immediate increases in mitochondrial ROS in response to hypoxia indicating inhibition of the mitochondrial respiratory chain, which lead to production of free radicals, lipid peroxidation, phospholipase C (PLC) activation, inositol triphosphate (IP_3)-mediated increases in intracellular Ca^{2+}, and ultimately vesicular release of ATP (Angelova et al. 2015). Ca^{2+} is considered important in the glial ATP release. Ca^{2+} chelators blocked the vesicular ATP release. Expression of tetanus toxin light chain, which can interfere with vesicular release mechanisms, in preBötC astrocytes reduced the hypoxia-induced increase in respiration in rats with denervated peripheral respiratory chemoreceptors (Angelova et al. 2015).

1.4 Background and rationale for current work

ATP is released in the preBötC during hypoxia where it acts on $P2Y_1$ receptors and increases ventilation, reducing the magnitude of the secondary hypoxic respiratory depression (Angelova et al. 2015)(unpublished data Rajani and Funk et al. 2017). The molecular mechanisms underlying the excitatory actions of ATP in the preBötC, however, are not known. The overall goal of my thesis is to advance understanding of these excitatory actions, in particular to further understanding of the GPCR signaling pathways through which $P2Y_1$ receptors act to excite the preBötC and the ion channels that are modulated to bring about the excitation of preBötC inspiratory rhythm. I will therefore review what is known of $P2Y_1$ receptor signaling pathways in the CNS and their known ion channel targets before presenting my specific aims.

1.4.1. GPCRs that mediate the actions of $P2Y_1$ receptors

$P2Y_1$ receptors are expressed throughout CNS. In most areas of brain $P2Y_1$ receptors are coupled to the $G_{\alpha q}$ signaling pathway. Conventionally, $G_{\alpha q}$-signaling pathway activation dissociates the heterotrimeric G protein into αq and βγ subunits. The αq subunit activates PLC which subsequently hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 promotes release of Ca^{2+} from intracellular stores by binding to IP_3 receptors on the endoplasmic reticulum. Ca^{2+} and DAG activate protein kinase C (PKC). Whether $P2Y_1$ receptors couple to $G_{\alpha q}$ in the preBötC or preBötC neurons is not known. We tested the effect of the specific $P2Y_1$ receptor agonist MRS 2365 on intracellular Ca^{2+} levels of inspiratory neurons.

Evidence also exists in other cells or regions of the brain that $P2Y_1$ receptors can couple through the $G_{\alpha i}$ signaling pathway. In CFTR-expressing Chinese hamster ovary cells, $P2Y_1$ receptor-mediated Ca^{2+} increases are sensitive to pertussis toxin (Marcet et al. 2004). In rat sympathetic neurons pertussis toxin abolishes $P2Y_1$ receptor-mediated closing of GIRK channels, further suggesting $G_{\alpha i}$ coupling (Erb and Weisman 2012). Studies have also demonstrated that $P2Y_1$ receptors can form heteromeric complexes with Adenosine (A_1) receptors. This hybrid receptor responds to ADPβS to activate both $G_{\alpha i}$ and $G_{\alpha q}$ (Yoshioka et al. 2001).

Similar to many GPRCs, $P2Y_1$ receptors also express c-terminal PDZ-binding domains. The PDZ-binding domain of the $P2Y_1$ receptor interacts with NHERF-2 (Hall et al. 1998), which can modulate the coupling of $P2Y_1$ receptors to ion channels. NHERF2 is present in the brain, where it is associated with both glia and neurons (Paquet et al. 2006). Co-expression of NHERF-2 in rat

superior cervical sympathetic ganglia (SCG) neurons with $P2Y_1$ receptors suppressed $P2Y_1$ receptor-mediated inhibition of Ca^{2+} currents but did not change the inhibition of the M-current (Filippov et al. 2010). Interestingly, Ca^{2+} signaling mediated by $P2Y_1$ receptor activation is significantly prolonged in C6 glioma cells when $P2Y_1$ receptors are co-expressed with NHERF-2, whereas disruption of the P2Y1 receptors–NHERF-2 interaction by point mutations attenuated the duration of P2Y1 receptor-mediated Ca^{2+} responses (Fam et al. 2005).

1.4.2. Ion channels that may contribute to the $P2Y_1$-receptor mediated increase in respiratory frequency

The downstream effector channels that mediate the excitatory actions of $P2Y_1$ receptor activation on the preBötC network are not known. Below I propose a series of candidate ion channels based on the fact that they are either modulated by $P2Y_1$ receptors, or the $G_{\alpha q}$ signaling pathway and that these channels when activated or inhibited alter preBötC rhythm.

(i) Ca^{2+} activated potassium channels (BK/SK)

Ca^{2+} activated potassium channels include large-conductance (BK) and small conductance (SK) channels. BK channels are high conductance K^+ channels with a conductance of 100–200 pS. BK channels are activated by both a rise in the intracellular Ca^{2+} and membrane depolarization (Calderone 2002; Gribkoff, Starrett, and Dworetzky 1997; Kaczorowski et al. 1996) and mainly contribute to the fAHP. Based on gating properties, BK channels are classified as Type I & II (Wang et al. 2014). BK channels have a tetrameric structure, containing an α (pore forming) subunit associated with a tissue-specific β subunit (β1 to β4) (Wang et al. 2014). Most CNS neurons express β2 and β4 as principal β subunits (Wang et al. 2014). The fate of BK channels depends upon how the α subunit interacts with the β subunits. In Type II BK channels, the α pore forming unit is expressed with a β4 subunit (Wang et al. 2014). The β4 subunit confers BK channels with slow gating kinetics and Iberiotoxin resistance (Wang et al. 2014). Many CNS regions including the posterior pituitary (Brenner et al. 2005) pyramidal neurons of the cortex, CA3 pyramidal neurons, dentate gyrus of the hippocampus, olfactory bulb, and purkinje neurons of the cerebellum (Brenner et al. 2005; Wang et al. 2014) express Type II BK channels.

Effects of BK channel blockade on respiratory rhythm depend on the species, preparation, drug and dose. In rat (P_0-P_4) brainstem-spinal cord preparations BK channel blockade decreases frequency

(Onimaru and Homma 2003), while frequency increases in medullary slice preparation when BK channels were blocked with paxilline (10 μM) a Type I & II BK channel blocker (Zhang et al. 2010). In mouse medullary slice preparations paxilline (1-3 μM) increases frequency while Iberiotoxin (blocker of Type I channels) has no influence on frequency (Zavala-Tecuapetla et al. 2008). Blockade of BK channels and glycine together abolished the respiratory rhythm (Büsselberg et al. 2003; Zhao et al. 2006) suggesting a role for BK channel in rhythm generation, perhaps through a role in burst termination (Rajani et al. 2016). During hypoxia ATP is released and glycinergic inhibition decreases (St.-John and Leiter 2002). Thus, it is possible that ATP enhances BK-mediated currents and increases frequency (Rajani et al. 2016).

SK channels have lower single-channel conductance (10–20 pS) compared to BK channels (100–200 pS). SK channel activation depends on increases in intracellular Ca^{2+} and contributes significantly to the medium afterhyperpolarization, which directly influences intrinsic excitability of neurons (Adelman et al. 2012).

Blockade of SK channels with Apamin in rat brainstem-spinal cord preparations has minimal effect on baseline rhythm or amplitude (Onimaru and Homma 2003). In mouse, however, in both medullary slice and in vivo preparations, blockade of SK channels with Apamine increases frequency and amplitude of the inspiratory rhythm (Zavala-Tecuapetla et al. 2008), while application of an SK channel activator 1-ethyl-2-benzimidazolinone reduces amplitude and frequency of respiratory bursts in mice medullary slice preparations (Zavala-Tecuapetla et al. 2008).

In summary, BK/SK channels are not essential for respiratory rhythm generation but can modulate rhythm, depending on species and perhaps preparation.

(ii) M current

The KCNQ gene encodes for the Kv7 K^+ channel subunit, four of which come together to form the functional channel that mediates the M current. The M current was first identified in frog sympathetic ganglion neurons (Brown and Adams 1980; Wang 1998). M channels begin to activate at -60mV, activate progressively with increasing depolarization and do not inactivate. Thus, they help maintain resting membrane potential by resisting depolarization (Brown and Passmore 2009; Marrion 1997). M channels are also inhibited by $G_{\alpha q}$ coupled receptors. Recombinant $P2Y_1$ receptors expressed in dissociated rat superior cervical ganglion neurons inhibit the M current (Brown et al.

2000). Also, in primary hippocampal cell culture, M channel currents are inhibited by $P2Y_1$ receptor activation (Filippov 2006). P2Y receptors can modulate M current in SCG neurons either by IP_3-dependent Ca^{2+} release (Hernandez et al. 2008) or IP_3-independent, direct PIP_2 inhibition (Stemkowski et al. 2002; Suh and Hille 2002).

Whether inspiratory rhythm is modulated by M currents is not known. Endogenous acetylcholine primarily acting via nicotine receptors modulates rhythm in the rhythmic medullary slice (Shao and Feldman 2005). Bath applied muscarine (50 μM), increases frequency in rhythmic medullary slices by 50%, most likely by acting on M_3 receptors (Shao and Feldman 2000). In neuroblastoma-glioma hybrid cells M_3 receptor activation inhibits the M-current (Fukuda et al. 1988). In rhythmic slice preparations of neonatal mice, blockade of M currents in the preBötC with XE991 increases burst duration and slowed burst frequency (unpublished data Katzel and Funk et al, 2017), while activation of M currents with retigabine slows rhythm and decreases burst duration. Thus, the role of M-channel modulation in setting inspiratory frequency is not clear, but its activation during an inspiratory burst may contribute to inspiratory burst termination, along with synaptic depression and activation of a outward Na^+-K^+ ATPase current that develops during the inspiratory burst (Del Negro et al. 2009). Efficacy of M currents in controlling burst duration in vivo remains to be examined.

(iii) Calcium-activated non-specific cation current (I_{CAN})

I_{CAN} contributes to burst generation (Pace et al. 2007) and is key in pacemaker behaviour in some inspiratory neurons. However, it is no longer considered to play an essential role in actual inspiratory rhythm generation (Pace and Del Negro 2008). TRPM 4 and 5 currents are believed to underlie the I_{CAN} current (Crowder et al. 2007), and are expressed in the preBötC, especially TRPM4 (Crowder et al. 2007). Both intracellular Ca^{2+} and depolarization can activate I_{CAN}, which is carried by Na^+ and K^+ ions but not Ca^{2+}. $P2Y_1$ receptor activation in hypoglossal motoneurons also appears to potentiate I_{CAN} (Alvares et al. 2014; Funk et al. 2011). Activation of I_{CAN} potentiates inspiratory burst amplitude in rhythmically active medullary slice preparations and the drive potential in individual inspiratory neurons, presumably by amplifying inspiratory synaptic currents (Mironov 2008; Del Negro et al. 2005; Pace et al. 2007). The observation that in the preBötC I_{CAN} affects amplitude rather than frequency suggests that I_{CAN} may not be the target of $P2Y_1$ receptors, since $P2Y_1$ receptor activation primarily increases frequency (Huxtable et al. 2009, 2010; Lorier et al. 2007).

(iv) TASK channel

TASK channels are two-pore domain, pH sensitive K^+ channels that mediate background K^+ currents. TASK-1, TASK-2 and TASK-3 are encoded by KCNK3, KCNK5 and KCNK9 genes, respectively. PreBötC inspiratory neurons (Koizumi et al. 2010), hypoglossal motoneurons (Talley et al. 2000) and raphe nuclei (Washburn et al. 2002) express TASK-1 and TASK-3 channels. TASK 2 channels are expressed in pH sensitive neurons of RTN. Halothane, a TASK-1 and 3 channel activator, significantly reduces respiratory frequency of medullary slices when bath applied or injected into the preBötC (Koizumi et al., 2010). $G_{\alpha q}$ coupled receptors can inhibit TASK channel (Mathie 2007; Talley et al. 2000). As $P2Y_1$ receptors are presumed to be $G_{\alpha q}$ coupled in preBötC, inhibition of TASK channels by $P2Y_1$ receptors is a possible mechanism through which ATP could increase frequency.

1.5 Study Objectives

ATP and $P2Y_1$ receptors play an important role in shaping the hypoxic ventilatory response. ATP released during hypoxia attenuates the secondary hypoxic respiratory depression by acting on $P2Y_1$ receptors (Rajani et al. 2015). The molecular mechanisms underlying ATP excitation of respiratory network are not known. The main objective of my thesis is to dissect the molecular mechanisms underlying $P2Y_1$ receptor mediated excitation of respiratory network.

The specific aims of my study are to:

(a) test the hypothesis that $P2Y_1$ receptor activation causes an increase in intracellular Ca^{2+} in inspiratory neurons,
(b) test the hypothesis that inspiratory preBötC neurons, which are both inhibitory and excitatory, are differentially sensitive to $P2Y_1$ receptor-mediated increases in intracellular Ca^{2+},
(c) determine whether the $P2Y_1$ receptor evoked increases in intracellular Ca^{2+} derive from intracellular stores, and
(d) identify the downstream ion channel on which $P2Y_1$ receptors act in the preBötC to increase frequency.

2.0 Introduction

The hypoxic ventilatory response is biphasic. Ventilation increases initially and then decreases to a steady-state level that remains above baseline in adults but falls well below baseline in premature/newborn mammals (Moss and Harding 2000). The mechanisms underlying the secondary depression are still under debate, but central mechanisms are strongly implicated (Teppema and Dahan 2010). In 2005 Gourine and colleagues, using ATP biosensors, demonstrated that during hypoxia ATP is released from the ventral medullary surface along the length of the VRC, including the preBötC (Gourine et al. 2005). Administration of PPADS, a P2 receptor antagonist, to the VLM augmented the secondary depression without affecting the initial increase in ventilation. These experiments suggested that ATP attenuates the secondary depression. Significant progress has been made since then in understanding (i) the site and the receptor(s) that underlie the excitatory actions of ATP (ii) the source of ATP and (iii) the mechanism of hypoxia sensing and ATP release.

Not all the sites where ATP acts to increase respiratory frequency are known, but growing data suggest that the preBötC is an important contributor. Mapping of the VLM in rhythmic medullary slice using local injections of ATP revealed that the preBötC is most sensitive to the excitatory actions of ATP. Also, MRS 2179, a $P2Y_1$ receptor antagonist virtually blocked the ATP- and MRS 2365 (a non-hydrolyzable, $P2Y_1$ receptor agonist.)-evoked increase in inspiratory frequency (Lorier et al. 2007; Zwicker et al. 2011). In addition, expression of TMPAP (transmembrane prostatic acid phosphatase, an ectonucleotidase that prevents accumulation of ATP in the extracellular space and in vesicles) in the preBötC in vivo augmented the secondary hypoxic respiratory depression (Angelova et al. 2015). In agreement with in vitro data, unilateral injection of a $P2Y_1$ receptor antagonist into the preBötC in vivo reveals that the ATP component of the hypoxic ventilatory response is mediated, at least in part, by $P2Y_1$ receptors within the preBötC (Rajani et al. 2015). These data suggest that ATP released during hypoxia activates $P2Y_1$ receptors of preBötC to increase frequency.

Total internal reflection fluorescence microscopy (TIRF) imaging revealed that hypoxia causes exocytosis of ATP-containing vesicles of astrocytes (Angelova et al. 2015). These experiments suggest glia as at least one source of ATP during hypoxia. The same group also provided insight on hypoxia sensing mechanism by astrocytes. Mitochondria within astrocytes act as the hypoxic sensor. A decrease in Po_2 inhibited the mitochondrial respiratory chain, which lead to production of free radicals, lipid peroxidation, PLC activation, IP_3 -induced increase in intracellular Ca^{2+}, and

ultimately release ATP. Disrupting exocytosis using tetanus light chain protein (TeLC) in astrocytes bilaterally in the preBötC and surrounding areas of anesthetized rats, provide in vivo evidence that exocytotic release of ATP, contributes to the increase in ventilation observed during the second phase of the hypoxic ventilatory response (Rajani et al. 2015).

One of the least studied questions is the molecular mechanisms underlying ATP excitation of respiratory network. The main objective of my thesis is to dissect the molecular mechanisms underlying the $P2Y_1$ receptor mediated excitation of respiratory network. Conventionally $P2Y_1$ receptors are coupled to Gαq. Whether $P2Y_1$ receptors couple to $G_{\alpha q}$ in the preBötC is not known and I examined this indirectly by monitoring changes in baseline fluorescence intensity of inspiratory neurons labeled with the Ca^{2+}-sensitive dye Fluo-4 in the presence of a MRS 2365, a $P2Y_1$ receptor agonist. The preBötC is a heterogeneous neuronal network. The high sensitivity of the network to $P2Y_1R$ activation suggested that preBötC neurons are differentially sensitive to $P2Y_1R$ activation. Certainly equivalent activation of excitatory and inhibitory mechanisms is less likely to result in powerful excitation than selective activation of excitatory neurons. This hypothesis is tested by bath applying MRS 2365 and monitoring for a visually detectable change in fluorescence intensity of inspiratory neurons located in same depth. To identify the source of Ca^{2+} and its contribution to respiratory network excitation SERCA blockers were used. Changes in baseline fluorescence intensity of inspiratory neurons to MRS 2365 in presence of SERCA blocker was compared to the control responses, similarly MRS 2365 evoked frequency in presence of SERCA blocker is compared to the control MRS 2365-evoked frequency to test the contribution of store released Ca^{2+} in network excitation. Finally to identify the downstream effector channel of $P2Y_1$ receptor I compared the network response to MRS 2365 before and after application of ions channel blockers that are known targets of $P2Y_1$ receptor signaling.

I used rhythmically active medullary slice preparation to address these questions. Direct physical and pharmacological (i.e., no blood brain barrier) access to this defined area of interest, combined with the ability to apply a vast array of technologies to the defined preBötC circuit were important factors in the selection of this approach to identify the molecular mechanisms underlying $P2Y_1$ receptor excitation of the preBötC inspiratory rhythm generating network.

3.0 Methods

All experiments and procedures were approved by the University of Alberta Animal Ethics Committee and performed in accordance with their guidelines for the care, handling and treatment of experimental animals.

3.1 Rhythmically active medullary slice preparation

Experiments were performed on rhythmically active medullary slice preparations from neonatal Sprague-Dawley rats (obtained from Charles River and the Biological Science Laboratories at the University of Alberta) ranging in age from postnatal day 0-4 (P_0-P_4). Rhythmic medullary slices preparations were generated as described previously (Ruangkittisakul et al. 2006; Smith et al. 1991; Zwicker et al. 2011). Briefly, the following steps are followed. Animals were deeply anesthetized through inhalation of isofluorane, decerebrated, transected caudal to the ribcage and the forelimbs were removed. The remaining preparation was then transferred to a dissection chamber containing room-temperature (~20ºC) artificial cerebrospinal fluid (aCSF) comprising of (in mM): 120 NaCl, 3 KCl, 1 $CaCl_2$, 2 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 20 D-glucose, equilibrated with carbogen, a mixture of 95% O_2 and 5% CO_2. The base of the dissection chamber was coated with Sylgard™ resin, to which the preparation was pinned in such a way that the ventral surface faced down, the skull was then cut along the midline and skull flaps were also pinned down. The cerebellum and the cerebellar peduncles were removed. Muscles of the back were removed to reveal the spinal column and a dorsal laminectomy performed to expose the dorsal surface of the spinal cord. The preparation was turned over and the jaw was removed. The ribcage was cut laterally and removed along with the viscera. Following the midline, hard and soft palates were cut and a ventral laminectomy was performed, exposing the ventral side of the spinal cord. Hypoglossal (XII) nerves were cut and spinal cord was exposed up to C4-C5 cervical segment. The spinal cord was then severed at the level of the 4-5th cervical segment (C4-C5), and pinned down. After carefully removing dura mater a final cut was made at the pontomedullary junction. The preparations therefore extended from the pontomedullary junction rostrally to approximately C4-C5 caudally. The brainstem-spinal cord preparation was transferred on to a wax chuck and pinned. The wax chuck containing the preparation is kept submerged in aCSF equilibrated with carbogen. Using a vibratome, (VT1000S, Leica, Germany) serial sections of 150-250 μm thick were cut in rostral to caudal directions. Sections were transilluminated and examined for specific anatomical landmarks. The thin slices were generated

until compact division of nucleus ambiguus (cNA) was no longer evident and the rostral margin of the inferior olive first appeared in the thin slice. After this thin slice a 700 μm thick rhythmically active slice was cut. The rhythmic slice contains the preBötC, rostral ventral respiratory group (rVRG), most of the XII motor nuclei, and the rostral XII nerve rootlets. The rhythmically active medullary slices were transferred in to a recording chamber (volume 7.0 mL) pinned down on Sylgard™ resin with the rostral surface up, and perfused with aCSF that was recirculated at a flow rate of 18 mL/min and maintained at room temperature (25°C).

At least thirty minutes prior to the start of data collection the concentration of K^+ in the aCSF was raised from 3 to 9 mM (Funk et al. 1993; Smith et al. 1991). Elevated $[K^+]_e$ is not necessary for rhythm generation. It is demonstrated that rat medullary slices cut with specific rostrocaudal boundaries are able to generate stable rhythm for a longer time even in 3mM K^+. (Ruangkittisakul et al. 2006). The reason for increasing the extracellular K^+ from 3 to 9 mM was to produce stable inspiratory-related rhythm for extended periods. The majority of protocols in this study involved multiple interventions and therefore required slices that produced stable inspiratory-related rhythm for longer periods of time. The mechanism by which elevated $[K^+]_e$ prolongs the rhythm is not clear. However, it is believed that elevated $[K^+]_e$ compensates for the loss of excitatory / modulatory input (Funk et al. 1993) that is lost during the slicing process.

3.2 – Recordings

3.2.1 – Extracellular nerve root recordings

Inspiratory related activity of the rhythmic slice preparations was recorded from the XII (hypoglossal) nerve roots using glass suction electrodes. Signals were amplified (10,000x), band-pass filtered (300Hz-1kHz) kHz; A-M Systems, Carlsborg, WA, USA), full-wave rectified, integrated using a leaky integrator (τ=50 ms), and displayed on a computer monitor using AxoScope 9.2 software (part of pClamp Suite, Molecular Devices, USA). Data were saved to the computer using Digidata 1322 A/D board (Molecular Devices, USA). Data was analyzed off-line using Axoscope software. All experiments were conducted at room temperature.

To assess the effects of a specific agent on the frequency response evoked by local injection of P2 receptor agonists into the preBötC, I compared the relative increase in peak frequency evoked by the agonist in control and in the presence of drug. Baseline frequency was the average frequency

measured over the 2 minutes immediately prior to drug application. Peak frequency was the peak value in the first min of drug application obtained from a moving average calculated based on three consecutive XII bursts (Huxtable et al. 2010; Zwicker et al. 2011).

For Ca^{2+} imaging experiments accompanying extracellular recordings, hypoglossal nerve activity was recorded as described earlier except that LabChart 7 (AD Instruments) was used instead of AxoScope 9.2.

3.2.2 Two-photon Ca^{2+} imaging recordings

Ca^{2+} oscillations of inspiratory neurons within the preBötC of rhythmically active medullary slices (described above and in (Ruangkittisakul et al. 2006, 2008) were visualized using Ca^{2+}-sensitive fluorophores loaded in the cytoplasm of preBötC cells to monitor Ca^{2+}-dependent changes in emitted fluorescence. Relative changes in the free cytosolic Ca^{2+} concentration (Ca^{2+}_i) was monitored in rhythmically active medullary slices obtained as described earlier (Ruangkittisakul et al. 2006, 2008), using an Olympus FV1000 MPE multiphoton scanning microscopy (Olympus, Markham, Ontario, Canada). The membrane-permeable Ca^{2+} sensitive dye fluo-4-AM was pressure-injected (25-50 mmHg, continuously for 10 min from the rostral surface into the preBötC using a broken patch pipette (outer diameter, 5-10μm) containing aCSF along with 0.5 mM fluo-4-AM dissolved in dimethyl sulfoxide and 20% pluronic acid (Stosiek et al. 2003). Rhythmic inspiratory activity was simultaneously recorded from the hypoglossal (XII) nerves rootlets (described above). A MaiTai-BBTi:sapphire (10 W Mira/Verdi; Coherent, Santa Clara, CA) femtosecond pulsed laser set to excite at 810 nm was used for fluorescence excitation and an Olympus X 20 1.0 NA immersion objective (20x XLUMPlanFl, numerical aperture, 0.95) was used to image the neurons (Nikolenko, Nemet, and Yuste 2003). Ten minutes was allowed post injection for the dye to diffuse. The whole stained area is scanned for identifying inspiratory neurons at various depts. If no inspiratory neurons were found, the pipette is moved to different location and the process is repeated. Stained areas were 150-300 μm in diameter and imaged cells were located between 30 and 75 μm from the surface of the slice which was mounted rostral surface up. The stained region was monitored using a 2x digital zoom at reduced field for y-axis scanning. Compared with full-frame acquisition (512 x 512 pixels), such "clipped mode" imaging sampled an area of 512 x 100-220 pixels and provided scan rates of 1.25-1.43 scans/s sufficient to detect 70-100% of the peak inspiratory Ca^{2+} rises as established empirically (Ruangkittisakul et al. 2006). Thapsigargin, CPA

and MRS 2365 were either bath applied or pressure injected (0.5-2 psi) using a broken patch pipette (outer diameter, 5-8µm).

Rhythmic slices were transferred in to 3 mL recording chamber and held in place with horizontal nylon threads stretched over a U-shaped platinum wire. Slices were continuously perfused with aCSF recirculated at a flow rate of 5 mL / min (dead space of perfusion system including inflow and outflow lines and chamber was ~20 ml).

Baseline and changes in baseline fluorescence of cells due to drug applications were recorded and saved using fluoroview software (Olympus, Markham, Ontario, Canada). These recordings were analyzed offline to quantify the changes in baseline fluorescence of cells due to various drug applications. A region of interest (ROI) was placed on each cell of interest (in most of the cases inspiratory neurons), which provided fluorescent intensity measurements (arbitrary units) for each cell over time, which was used to calculate $\Delta F/F_o$ values. ΔF indicates the difference between baseline fluorescence intensity and after drug application. The average intensity of 3-5 inspiratory bursts before drug administration was used to calculate baseline fluorescence (F_o). Similarly, the averages of 3-5 highest values obtained during the drug application were used to calculate F.

3.3 Drugs and drug application

All drugs used for my experiments were obtained from Tocris bioscience, except for thapsigargin which was obtained from Alomone Labs, Israel and $BaCl_2$ which was obtained from Sigma-Aldrich.

Thapsigargin, CPA and paxilline were dissolved in 100% DMSO. The DMSO concentrations in working solution were 0.2% for thapsigargin, 0.1% for CPA and 0.04% for Paxilline. All control applications of MRS 2365 were paired with application of vehicle containing the same concentration of DMSO as the drug.

Iberiotoxin, Apamine, MRS 2365 and Substance P were dissolved in aCSF. These drugs were prepared as concentrated stock solutions (10-fold higher than working concentrations) and frozen in 10 µL aliquots. During each experiment, the drugs were thawed and diluted with 90 µL aCSF to experimental concentrations. The final concentration of K^+ in the drug solutions was matched to that of the aCSF.

Drugs were either bath applied or microinjected into the preBötC. For bath application, drugs were added directly to the circulating aCSF and washed into the recording chamber at their required concentrations. For microinjection, triple barrel drug pipettes were used (4-5 μm outer diameter per barrel). These triple barrel drug pipettes were pulled from borosilicate glass capillaries (WPI, USA) and their tips were carefully broken to obtain desired diameter. Barrel tips greater than 5 μm can leak. A programmable stimulator (Master-8, AMPI, Israel) was used to control drug injection duration and length. Except MRS 2365 and Substance P, all other drugs were injected for 45 min, 5 sec on and 5 sec off allowing drug to diffuse. MRS 2365 and Substance P were injected for 10 sec. To produce a repeatable response two consecutive MRS 2365 injections were separated at least for 15 min apart.

3.4 Statistical analysis

Parameters are reported relative to control (pre-drug) levels, as mean ± standard error of the mean. All statistical analyses were performed on raw data. Means of two groups were compared using the Student's t-test. When the means of more than two groups were compared, repeated measure ANOVA was performed followed by Bonferroni's Multiple Comparison tests (Prism 4.0, GraphPad Software, USA). Values of $p < 0.05$ were assumed significant.

4.0 RESULTS

4.1 Intracellular signaling pathway that mediates the frequency effects of $P2Y_1$ receptor activation

4.1.1 $P2Y_1$ receptor activation increases Ca^{2+}_i in preBötC inspiratory neurons *in vitro*

$P2Y_1$ receptors play a critical role in shaping the hypoxic ventilatory response. ATP is released in the ventrolateral medulla during hypoxia, where it acts via $P2Y_1$ receptors to increase ventilation and attenuate the secondary depression. Conventionally, $P2Y_1$ receptors signal through the $G_{\alpha q}$ pathway that activate phospholipase C pathway and cause an increase in Ca^{2+}_i (generally by releasing Ca^{2+} from intracellular stores). My objective was to test this phenomenon in the preBötC neurons. To accomplish this, I labeled the preBötC neurons of rhythmic slices with the Ca^{2+}-sensitive dye Fluo-4 while inspiratory activity was electrophysiologically monitored from the hypoglossal (XII) nerves. Inspiratory neurons were identified by rhythmic increases in the Fluo-4 signal in phase with the rhythm recorded from the XII nerve. Five such neurons are marked with arrows in Fig. 4A and their baseline, inspiratory-related oscillations in Ca^{2+}_i are apparent in the recordings of fluorescence intensity (Fig. 4B). MRS 2365 (100 μM, 10 sec) was locally applied and changes in baseline fluorescence of inspiratory neurons were monitored. Local application of MRS 2365 near the surface of the slice evoked significant increases in the Fluo-4 signal in all 5 inspiratory neurons imaged in this slice (Fig. 4B). Group data taken from 18 inspiratory neurons in 5 slices showed a ~50% increase in the Fluo-4 Ca^{2+}_i signal (Fig. 4D).

4.1.2 Not all preBötC inspiratory neurons responded to $P2Y_1$ receptor activation with an increase in Ca^{2+}_i.

The preBötC is a heterogeneous neuronal network. Pre-inspiratory and inspiratory neurons are most common but occasionally neurons with expiratory discharge are also found. Inspiratory neurons can be further divided into pacemaker and non-pacemaker subtypes and can be either excitatory or inhibitory (Lieske et al. 2000; Smith et al. 1991; Winter et al. 2009). My experiments revealed that $P2Y_1$ receptor activation increases Ca^{2+}_i in preBötC inspiratory neurons. Based on the high sensitivity of the preBötC rhythm to $P2Y_1$ receptor activation, I predicted excitatory neurons are likely to be more sensitive than inhibitory neurons and that neurons involved in rhythm generation,

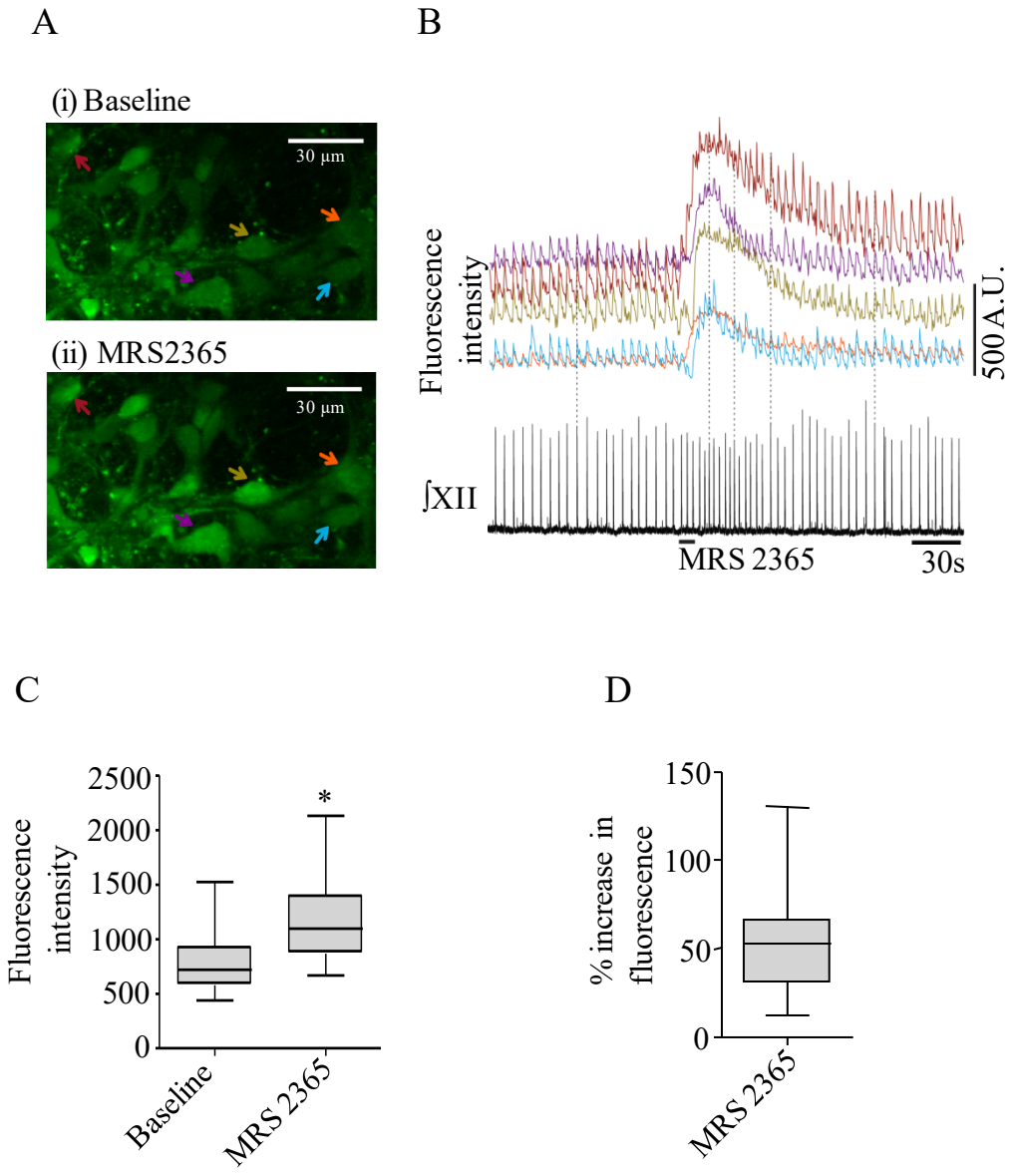

Figure 4. preBötC inspiratory neurons respond to locally applied MRS 2365 with an increase in $Ca^{2+}_{i.}$ A. Two-photon image of the preBötC inspiratory cells showing Fluo-4 fluorescence of multiple cells at baseline (i) and at the peak of the MRS 2365 response (ii). B. Fluorescence responses of five inspiratory neurons (marked with color coded arrows in A) to locally applied MRS 2365 (100 µM, 10 Sec) that showed oscillations in Ca^{2+} that were synchronous with XII nerve activity. Note the MRS 2365-evoked increase in frequency. C. Group data showing an increase compared to baseline in peak fluorescence in response to locally applied MRS 2365 (100 µM, 10 Sec) (n=18 from 5 slices). *p<0.0001, significant difference between baseline and MRS 2365. D. Group data showing an approximate ~50% increase in fluorescence response to locally applied MRS 2365 (100 µM, 10 Sec) (n=18 from 5 slices).

or controlling the excitability of rhythm generating neurons, are likely to more sensitive P2Y1 receptor activation than other neurons. I was not able to address this prediction directly, but if correct it is likely that inspiratory neurons are not equally sensitive to MRS 2365; i.e., some neurons, most likely the inhibitory inspiratory neurons, are likely to be insensitive. To address this question I applied MRS 2365 (10 μM for 1 min) to the bath and monitored Ca^{2+} fluorescence of inspiratory neurons. Bath application will affect all the inspiratory cells in the slice and should also provides uniform concentration of MRS 2365 to all the cells in a given plane. I did not use local application of MRS 2365 in these experiments because of variable distances from the injection pipette and the different inspiratory neurons. Inspiratory neurons lying farther from the drug pipette would receive a lower concentration of drug than nearby neuron due to dilution. In addition, local application allows stimulation of only a limited number of inspiratory neurons close to the pipette tip. I recorded the responses of 20 inspiratory neurons in 7 slices. When data from all neurons were grouped together, they showed an average increase in Ca^{2+} fluorescence of 31 ± 7% above baseline (Fig. 5D). However, it was clear from visual inspection of the imaging data that neurons showed variable responses. Some responded strongly while responses of other inspiratory neurons that were surrounded by strongly responding non-inspiratory neurons were not apparent on visual inspection. Thus, I arbitrarily divided neurons into two groups, non responders (those that responded to MRS 2365 with less than a 10% increase in Ca^{2+} fluorescence) and responders (those that responded to MRS 2365 with greater than a 10% increase in Ca^{2+} fluorescence). Twelve inspiratory neurons from 5 slices that were classified as responders showed an average 47 ± 10% increase in their $Ca^{2+}{}_i$ flourescence. Eight, nonresponding inspiratory neurons from 2 slices showed only an average 6.5 ± 2.8% increase in their Ca^{2+} fluorescence, suggesting that an increase in $Ca^{2+}{}_i$ in response to $P2Y_1$ receptor activation varies between preBötC neurons. As a positive control, data from experiments with non-responsive inspiratory neurons were only included in the analysis if there were neurons (inspiratory or non-inspiratory) in the same that showed a strong response to bath-applied MRS 23656 (Fig 5C). Inspiratory neurons were not categorized further.

4.1.3 SERCA blockers attenuate the MRS 2365-evoked $Ca^{2+}{}_I$ increase in the preBötC inspiratory neurons.

Ca^{2+} imaging experiments revealed that $P2Y_1$ receptor activation increases $Ca^{2+}{}_i$ in ~60% of preBötC inspiratory neurons. The Ca^{2+} could come from the extracellular space through voltage

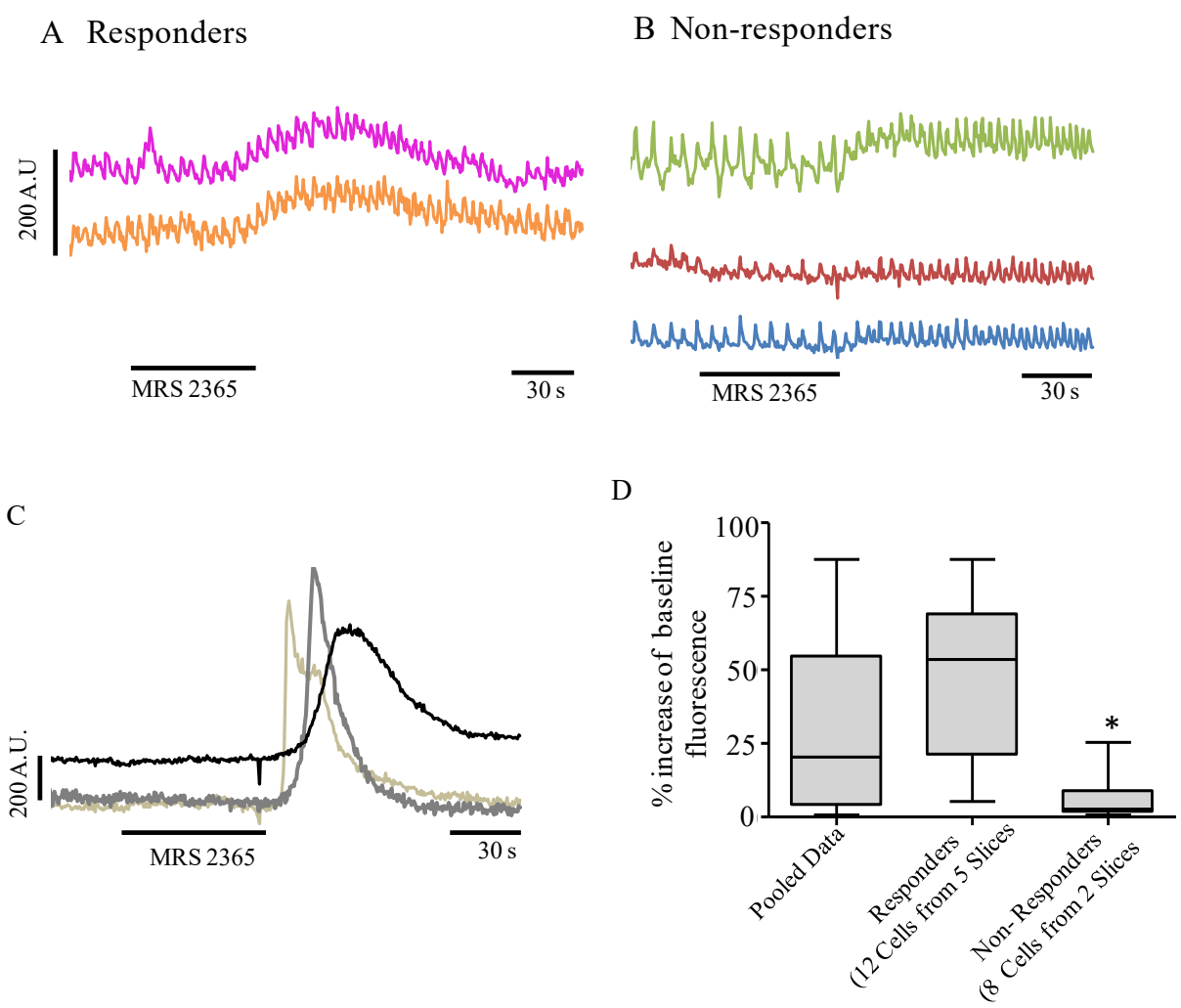

Figure 5. Not all preBötC inspiratory neurons respond to MRS 2365 with an increase in Ca^{2+}_i. A. Typical baseline fluorescence increase of the preBötC inspiratory neurons in response to bath applied MRS 2365 (10 µM, 1 min) (Responders) B. Three inspiratory neurons on a focal plane showing a minimal increase of their baseline fluorescence (Non-responders) C. Non inspiratory neurons from the same focal plane of non responders showing a large increase in their baseline Ca^{2+} fluorescence signal in response to bath applied MRS 2365. D. Group data showing MRS 2365-evoked (10 µM, 1 min) changes in Ca^{2+} fluorescence in all neurons examined and neurons that were classified as strong responders or weak/non-responders. (*p=0.0063, Paired t-test).

-gated ion channels or via release from intracellular stores. If the source of Ca^{2+} is intracellular, then depleting intracellular Ca^{2+} with SERCA blockers should attenuate the of MRS 2365-evoked Ca^{2+} increase. To test this I first measured the Ca^{2+} fluorescence responses evoked by MRS 2365 (10 μM, 1 min) in control. To control for possible time-dependent reductions on the MRS 2365 evoked Ca^{2+} response that could occur with bleaching, I performed a second control MRS 2365 trial 30 min later. After an additional 30 min recovery period, I applied MRS 2365 after the bath-application of SERCA blocker (thapsigargin, 100 μM, n = 8; CPA, 100 μM, n = 4) for 30 min. I then compared the magnitude of the 2nd control response relative to the first control response with the magnitude of the test (third) response (SERCA blocker) relative to the second control response. The effects of the two SERCA blockers were similar so data were pooled. Inspiratory neurons are identified as described above. Two inspiratory neurons are marked with arrows in Fig. 6A and their responses to two control MRS 2365 applications are shown in Fig. 6B and 6C respectively. The third application in the presence of thapsigargin is shown in Fig. 6D. Group data taken from 12 inspiratory neurons in 5 slices (same responsive neurons as in Fig. 2) showed that the second control application of MRS 2365 evoked an increase in Ca^{2+} fluorescence that was 95.0 ± 2.7% of the first control response (i.e., ~5% reduction over time). The MRS 2365 response evoked in the presence of SERCA blockers was significantly reduced from the previous application at only 44 ± 7% of the second MRS 2365 control response. In other words the decrease in fluorescence over the same time period was ~7 fold greater in the presence of thapsigargin/CPA. Data indicate minimal time-dependent run-down in the response and that a major source of the MRS 2365-evoked increase in Ca^{2+} fluorescence is from intracellular stores. Note that the MRS 2365-evoked increase in inspiratory frequency was also greatly attenuated by the SERCA blockers (Fig. 6D).

4.1.4 The $P2Y_1$ receptor-evoked increase of inspiratory frequency involves release of Ca^{2+} from intracellular stores

To further test whether the MRS 2365-evoked frequency increase involves release of Ca^{2+} from intracellular stores, I compared control MRS 2365 responses with those evoked after local application of two SERCA blockers to the preBötC. Since the site of action for these blockers is intracellular, additional time was allowed for diffusion into the tissue and across cell membrane. I first tested thapsigargin. MRS 2365 applications were preceded by 15 min pre-applications of vehicle (0.2% DMSO, 5s on, 5s off) or thapsigargin (200 μM in 0.2% DMSO). Two consecutive

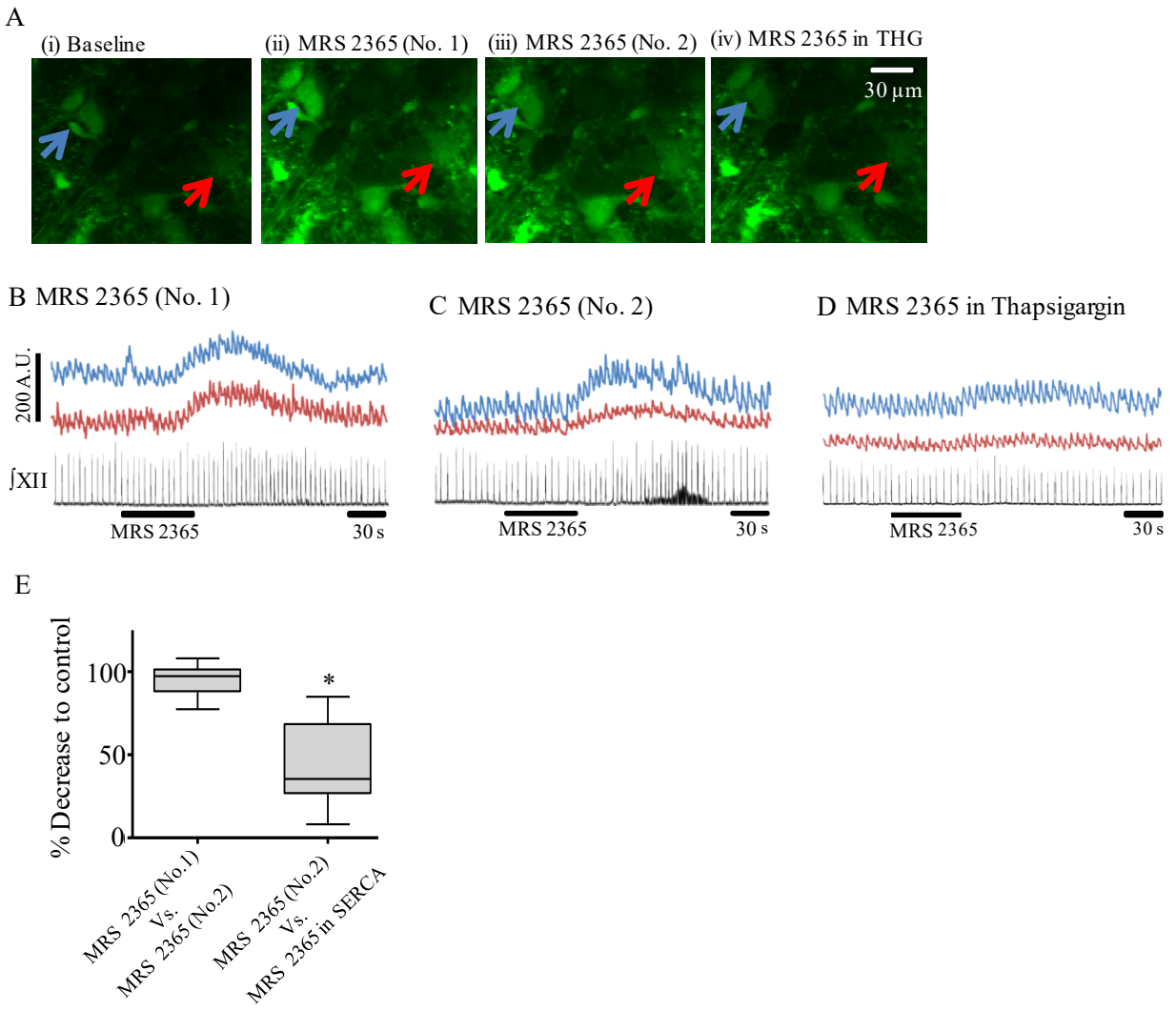

Figure 6. MRS 2365-evoked Ca^{2+} increase is sensitive to SERCA blockers. A (i),Two-photon image showing Fluo-4 fluorescence of 2 inspiratory neurons (marked with arrows under baseline condition (i), during the peak of the responses evoked by MRS 2365 (10 µM, 1 min) (ii and iii) and after bath application of the SERCA blocker, thapsigargin, for 30 min B, C, D. Recordings of Ca^{2+} fluorescence from inspiratory neurons (same neurons as in A) showing rhythmic oscillations in phase with inspiratory bursts recorded from the XII nerve and responses to two control MRS 2365 applications (B, C) and a third application in the presence of thapsigargin (D). E. Box plot of group data showing that the reduction in Ca^{2+} fluorescence evoked by MRS 2365 in the SERCA blocker compared to the second control MRS 235 response was significantly greater than the decrease between the two control MRS 2365 responses (n=12 neurons, 5 slices; *p<0.0001).

MRS 2365 trials were performed in thapsigargin at 15 min intervals. The response of a single preparation and group data are shown in Fig. 4. Thapsigargin reduced the MRS 2365 evoked frequency increase from a peak of 2.6 ± 0.15 fold in control to 2.11 ± 0.2 fold at 15 min ($p>0.05$) and to 1.9 ± 0.20 fold on the second application after 30 min of thapsigargin ($n = 6$, $p = 0.007$, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). In other words, 30 min of locally applied thapsigargin reduced the MRS 2365 potentiation by ~30%.

The concentration of DMSO required to dissolve thapsigargin to the indicated concentration was 0.2%. To ensure that long-term local application of 0.2% DMSO into the preBötC does not affect the ability of the network to respond to MRS 2365, I compared the response of the preBötC network to MRS 2365 at 15 min intervals during a continuous 75 min (5 sec on, 5 sec off) application of vehicle (aCSF with 0.2% DMSO). The MRS 2365 was stable for 5 consecutive applications. At min 15, 30, 45, 60, and 75, the peak MRS 2365-evoked frequency increase averaged 4.00 ± 0.83, 3.73 ± 0.51, 3.73 ± 0.55, 3.98 ± 0.54 and 4.07 ± 0.78 relative to baseline.

The second SERCA blocker Cyclopiazonic acid (CPA, 100μM) was tested using a protocol similar to that of Thapsigargin except that the vehicle contained 0.1% DMSO and CPA was applied for a total of 45 min as it took longer than thapsigargin to have a significant effect. The response of a single preparation and group data are shown in Fig. 5. CPA reduced the MRS 2365-evoked frequency increase by ~35% from a peak of 2.80 ± 0.28 fold in vehicle to 1.82 ± 0.12 on the third application after 45 min of Cyclopiazonic acid ($n = 6$, $p = 0.040$, paired t test).

4.2 Identification of the ion channel(s) underlying frequency effects of $P2Y_1$ receptor activation

Having demonstrated that release of Ca^{2+} from intracellular stores is part of the cascade through which $P2Y_1$ receptor activation increases preBötC frequency, my next objective was to identify the ion channel or channels that are responsible for the $P2Y_1$ receptor-mediated frequency increase. A survey of the literature identified a host of ion channels in various brain regions that are modulated by $P2Y_1$ receptors acting through the $G_{\alpha q}$ signaling pathway and involve thapsigargin-sensitive

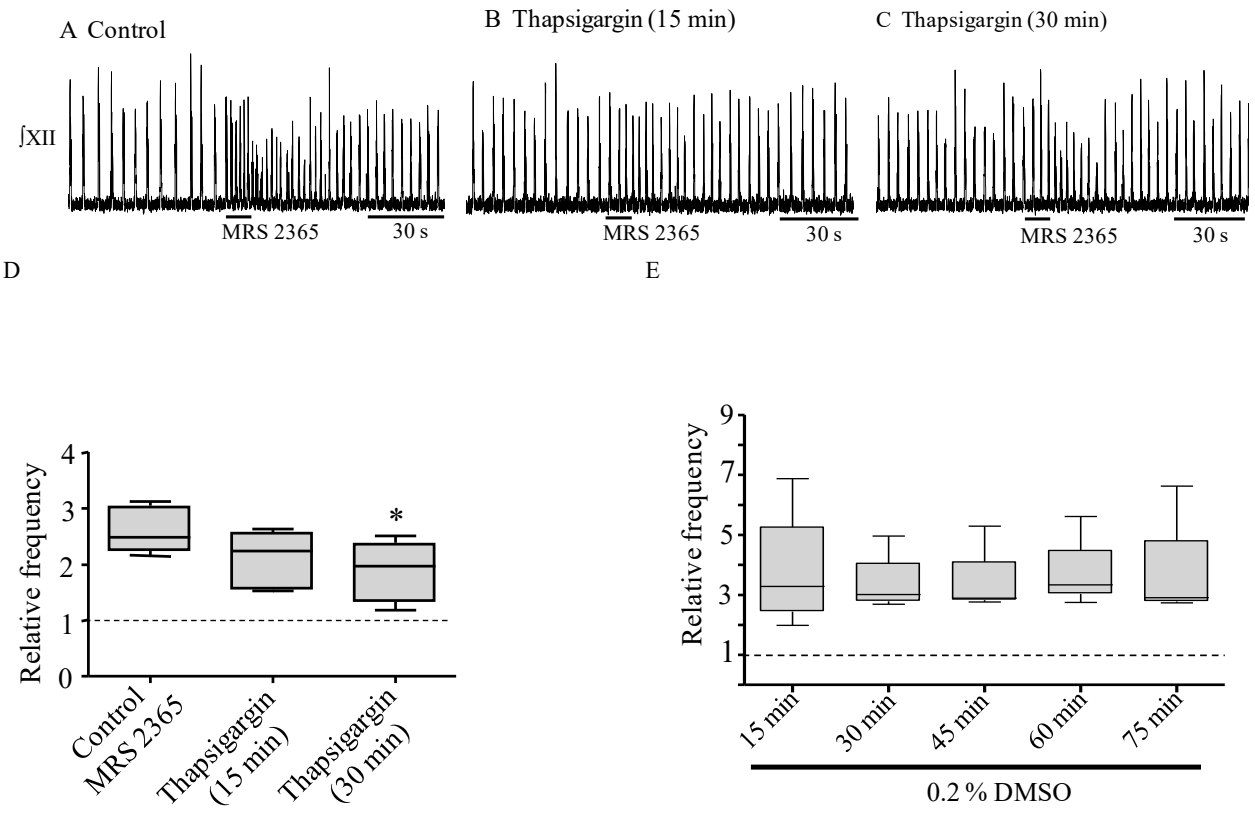

Figure 7. Depletion of intracellular Ca^{2+} stores by thapsigargin attenuates the MRS 2365-evoked frequency increase. Representative traces of integrated hypoglossal nerve output (∫XII) showing the frequency increase evoked in response to local injection of the $P2Y_1$ agonist MRS 2365 (100 µM, 10 Sec) into the preBötC in control conditions, and after 15 (B) and 30 min (C) application of thapsigargin (200 µM) locally to the preBötC. D, Group data showing peak frequency increase evoked by MRS 2365 in control and after 15 and 30 min of thapsigargin. (n=6, p=0.007, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). E, Group comparison responses showing non significant differences among 0.2% DMSO time controls. (n=5, p>0.05, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). All data are plotted relative (rel.) to baseline levels.

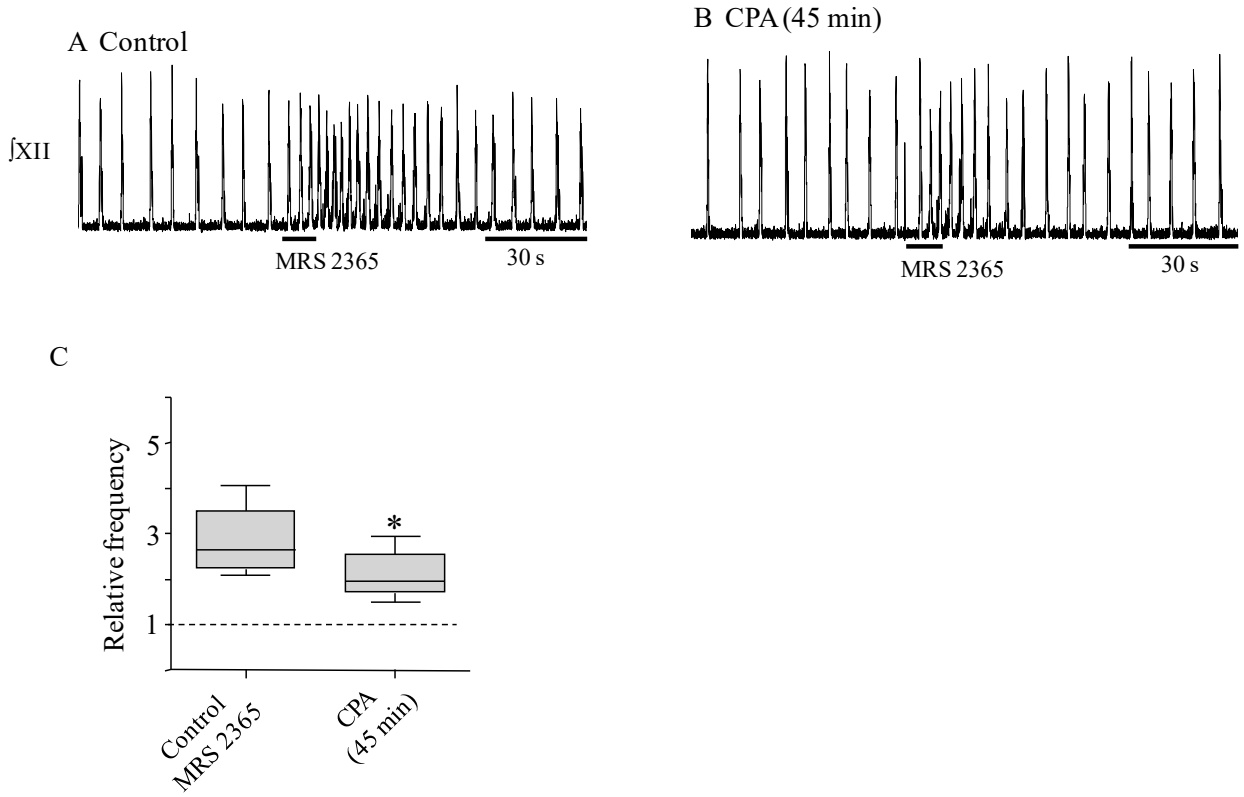

Figure 8. Depletion of intracellular Ca^{2+} stores by CPA attenuates the MRS 2365-evoked frequency increase. A, Representative traces of ∫XII showing the frequency increase evoked by MRS 2365 (100 µM, 10 Sec) in control (A) and after 45 min of locally applying CPA (100 µM) to the preBötC. C, Box plot of group data comparing the MRS 2365 evoked frequency increase in control and 45 minutes after introduction of CPA (n=6, p = 0.040, paired t test). All data are plotted relative to baseline.

increases in intracellular Ca^{2+} (Rajani et al. 2016). Potential targets included (i) GIRK, (ii) SK (iii) Voltage dependent $K^{+.}$ channels. (iv) BK. To isolate the ion channel involved in the $P2Y_1$ receptor -mediated frequency increase, I used the rhythmically-active medullary slice preparation and compared the peak frequency evoked by MRS 2365 in control and after bath or local application of blockers that target these candidate ion channels. A total of four candidate ion channels were tested. The first three, which had no apparent contribution to the $P2Y_1$ receptor -mediated frequency increase, are described first.

4.2.1 GIRK channels

The GIRKs are a family of inward-rectifier K^+ ion channels. $G_{\beta\gamma}$ protein interaction is required to activate these channels. GIRK channels are permeable to K^+ ions that result in membrane hyperpolarization when activated. I used barium chloride ($BaCl_2$) to block the GIRK channels. $BaCl_2$ was bath applied at a starting concentration of 400 μM and raised to 500 and 700 μM. At 400 μM, $BaCl_2$ was associated with a reduction in baseline frequency and an increase in tonic activity. The concentration of $BaCl_2$ was not increased beyond 700 μM because at these levels inspiratory-related rhythm was difficult to distinguish from background tonic activity. MRS 2365 was injected for 10 sec at 15 min intervals, first in control and again after 15 min in 400, 500 and 700 μM $BaCl_2$.

The response of a single preparation and group data are shown in Fig. 9. Control MRS 2365 evoked a 2.23 ± 0.18 fold increase in frequency while MRS 2365 injections in presence of $BaCl_2$ at 400 μM, 500 μM and 700 μM evoked 3.18 ± 0.28, 3.89 ± 0.29 and 4.88 ± 0.70 fold increases respectively ($n = 4$, $p > 0.05$, repeated measures ANOVA, Bonferroni's multiple comparison test). However, as the baseline frequency decreased progressively with increasing $BaCl_2$, the apparent potentiation of the MRS 2365-evoked frequency increase reflected the reduction in baseline more than an increase in absolute frequency evoked by MRS 2365 which peaked at 24 ± 1.2, 25.2 ± 1.8, 27 ± 4.8, 30.6 ± 1.8 beats per minutes (BPM) in control, 400, 500 and 700 μM $BaCl_2$ respectively.

4.2.2 Small conductance (SK) channels

SK channels are Ca^{2+}-activated K^+ channels that are activated solely by increases in intracellular Ca^{2+} and have 10-fold lower conductance than BK channels, hence the name small conductance channels. The contribution of SK channels to the MRS 2365-evoked frequency increase was

examined by comparing the frequency responses evoked in control with those evoked after local application of the SK channel blocker, Apamin (3 μM), to the preBötC.

The response of a single preparation and group data are shown in Fig. 10. MRS 2365 evoked a 2.62 ± 0.33 fold frequency increase in control. Apamin had no obvious effect on the MRS 2365-evoked frequency increase. At 15, 30 and 45 min of Apamin, MRS 2365 evoked 2.62 ± 0.10, 2.61 ± 0.21 and 2.72 ± 0.92 fold increases, respectively (n=4, $p > 0.05$, repeated measures ANOVA, Bonferroni's multiple comparison test). The absolute baseline frequency values of control and in 15, 30, 45 min of Apamin are 11.4 ± 0.6, 12 ± 1.2, 12 ± 1.2 and 11.4 ± 1.2 BPM respectively, whereas peak absolute frequency values are 29.4 ± 1.8, 31.2 ± 2.4, 31.2 ± 4.2 and 30.6 ± 3 BPM respectively. These data indicate that Apamin had minimal effect on baseline respiratory rhythm and the MRS 2365 evoked frequency increase.

4.2.3 Voltage-gated K^+ Channels

I screened voltage-gated K^+ channels by comparing control MRS 2365 responses with those evoked after local application of the generic K^+ channel blocker, TEA (1 mM), that also blocks a number of other channels including Ca^{2+}- activated K^+ channels and aquaporins.

The response of a single preparation and group data are shown in Fig. 11. Control MRS 2365 evoked a 2.70 ± 0.27 fold increase while MRS 2365 injections in presence of TEA at 15, 30 and 45 min evoked 2.48 ± 0.09, 2.94 ± 0.29 and 2.70 ± 0.25 fold increases, respectively ($n = 4, p > 0.05$, repeated measure ANOVA). The absolute baseline frequency values of control and in 15, 30, 45 min of TEA are 12 ± 1.2, 10.8 ± 0.6, 11.4 ± 0.6 and 11.4 ± 0.6 BPM respectively, where as peak absolute frequency values are 31.8 ± 1.2, 27.6 ± 1.8, 33 ± 3 and 31.2 ± 3.6 BPM respectively.

4.2.4 BK channels

BK channels, also referred to as Maxi-K or slo1 channels, are K^+ channels characterized by their large K^+ conductance and their activation by changes in membrane potential and increases in the concentration of intracellular Ca^{2+}. BK channels are classified as type 1 or type II based on differential pharmacology and the slow gating kinetics of Type II compared to Type I channels

I tested the role of BK channels in the MRS 2365-evoked frequency increase by comparing responses before and during local application of the general BK channel blocker, paxilline.

Following a control application of MRS 2365, paxilline (20 μM) was applied locally into the preBötC complex for 30 min (5 sec on, 5 sec off) and MRS 2365 was injected every 15 min (twice in total) followed by a recovery MRS 2365 injection after 15 min of paxilline washout. The response of a single preparation and group data are shown in Fig. 12. Paxilline at 20 μm reduced the MRS 2365-evoked frequency increase from a peak of 2.85 ± 0.39 fold to 1.51 ± 0.23 at 15 min ($p<0.005$) and 1.69 ± 0.33 on the second application after 30 min of paxilline ($n = 3$, $p < 0.05$, repeated measures ANOVA, Bonferroni's multiple comparison test) suggesting that BK channels may be the downstream effector channel of $P2Y_1$ receptors in inspiratory neurons. The absolute baseline frequency values of control and in 15 and 30 min of paxilline (20 μM) are 11.4 ± 0.6, 13.8 ± 1.2 and 12.6 ± 0.6 BPM respectively, where as peak absolute frequency values are 31.8 ± 0.6, 20.4 ± 0.6 and 21.6 ± 0.6 BPM respectively.

At 20 μM, however, paxilline has potential off-target actions at SERCA (Bilmen, Wootton, and Michelangeli 2002), which I have already shown to inhibit the MRS 2365 response. To assess whether this potential off-target action of paxilline was an issue in my experiments, I used the Ca^{2+} imaging approach described previously. In this case I locally injected 20 μM paxilline into the preBötC and monitored changes in the baseline fluorescence intensity of inspiratory neurons. After 15 min, the paxilline trial was followed by local injection of a SERCA blocker (either Thapsigargin or CPA as both a positive control to ensure drug injection was effective, but also to compare the paxilline-evoked response with that of a known SERCA blocker. Any increase in baseline fluorescence intensity by paxilline is suggestive of off-target SERCA blockade.

Paxilline (20 μM) increased baseline Ca^{2+} fluorescence of inspiratory neurons by 30 ± 5.6% above control. SERCA blockers evoked a similar increase of 22 ± 1.7% above base line ($n = 4$ inspiratory neurons from 2 slices $p = 0.26$, paired t test). These experiments suggest that at 20 μM paxilline is blocking SERCA and the attenuation of MRS 2365 response at network level by paxilline might be due to this off-target action on SERCA.

I next repeated the above experiments using 10 μM paxilline to assess whether decreasing the paxilline concentration would avoid its off-target actions on SERCA. When injected at a concentration of 10 μM, paxilline had no effect on baseline Ca^{2+} fluorescence in inspiratory neurons.

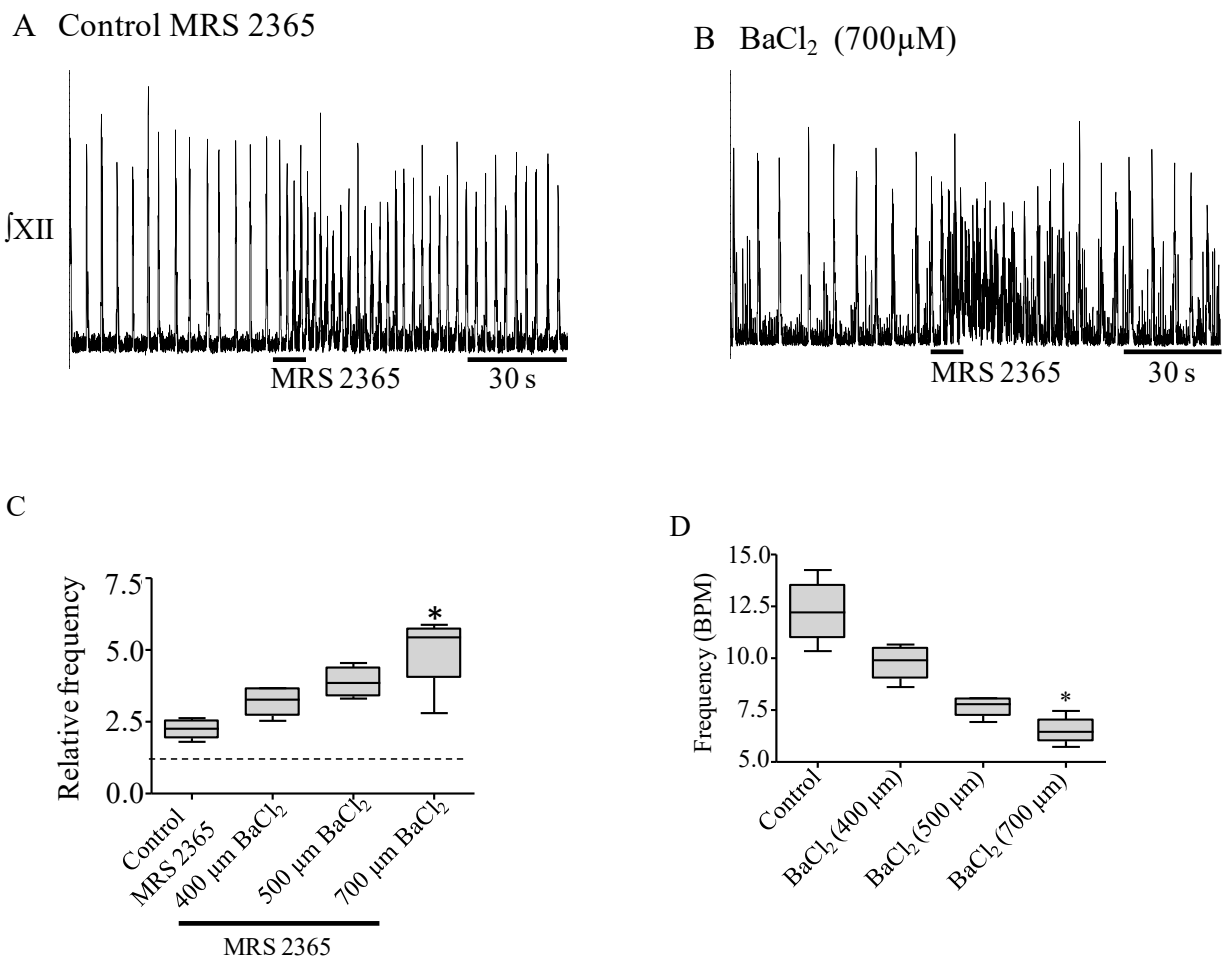

Figure 9. Barium chloride potentiates the MRS 2365-evoked frequency increase. Representative traces of ∫XII nerve activity showing the increase in relative frequency evoked by MRS 2365 (100 µM, 10 sec) in control (A) and after bath application of 700 µM of BaCl2 (B). C. Box plot of group data showing the effects of bath-applied $BaCl_2$ on the relative frequency increase evoked by MRS 2365-evoked (n=4, $p<0.05$, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). All data are plotted relative to baseline levels. D. Group data showing the effect of bath-applied $BaCl_2$ on baseline frequency. (n=4, $p<0.05$, repeated measures ANOVA, Bonferroni's Multiple Comparison Test).

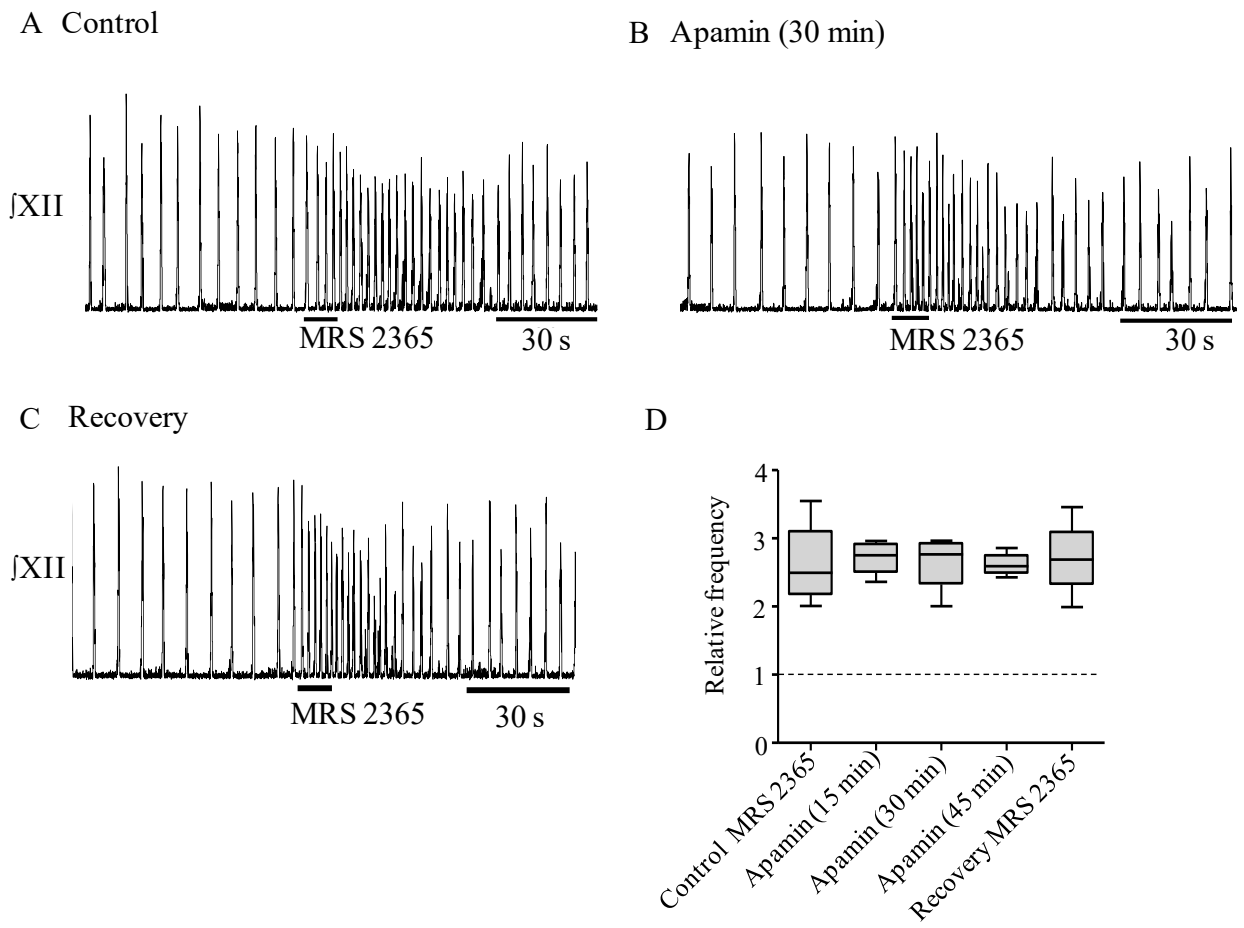

Figure 10. Apamin has no effect on the MRS 2365-evoked frequency increase. A, Representative traces of ∫XII showing the frequency increase evoked by MRS 2365 (100 µM, 10 Sec) in control (A) and after 30 min of Apamine (3 µM, B). C. Recovery injection D. Box plot of group data showing that Apamine does not affect the MRS 2365 response (n=4, $p<0.05$, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). All data are plotted relative to baseline levels.

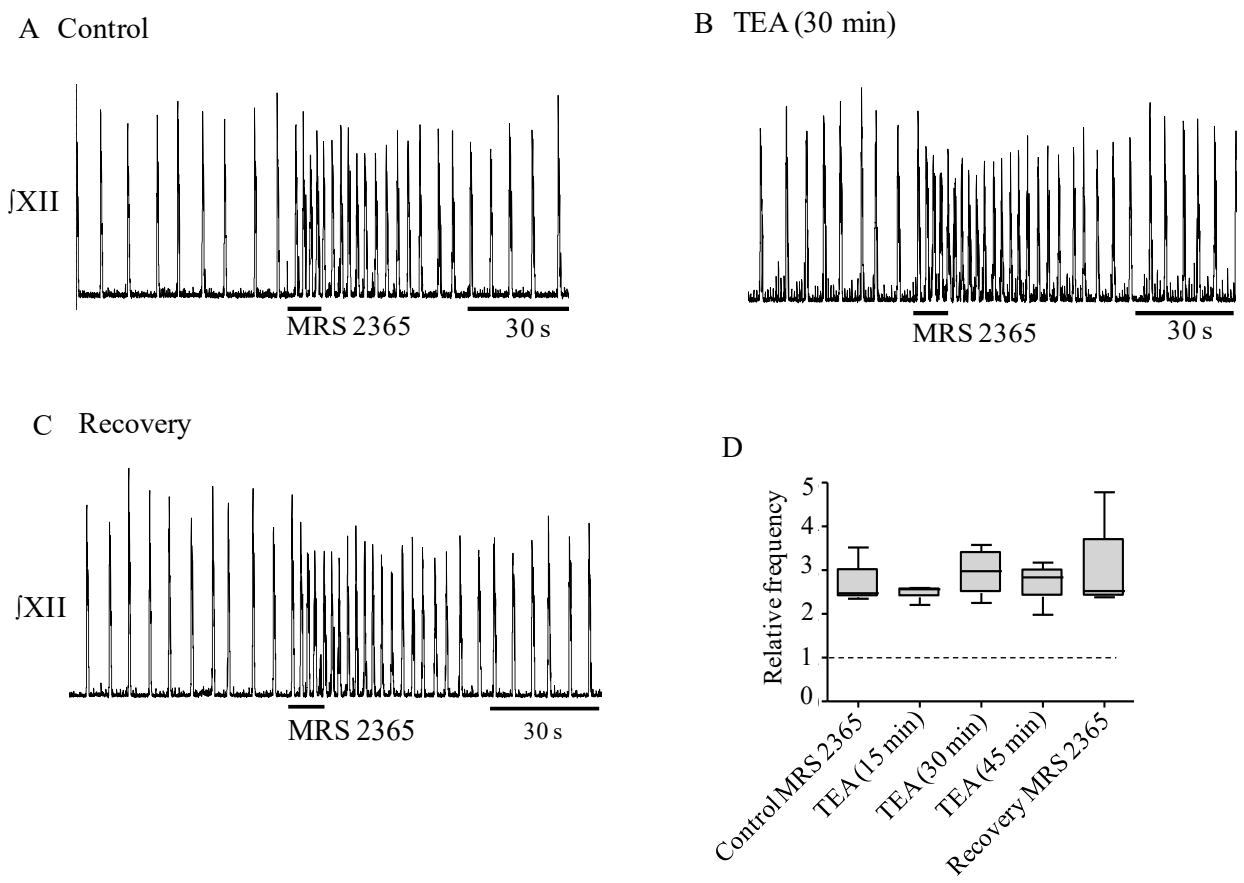

Figure 11. TEA has minimal effect on MRS 2365 evoked frequency. A, Representative traces of ∫XII showing the increase in response to $P2Y_1$ agonist MRS 2365 (100 μM, 10 Sec). B, after a 30 minute application of TEA (1mM) locally to the preBötC. C. Recovery injection D, Comparison of group data reveals a statistically non-significant potentiating of the response at 30 and 45 min of TEA injection. (n=4, $p<0.05$, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). All data are plotted rel. to baseline levels.

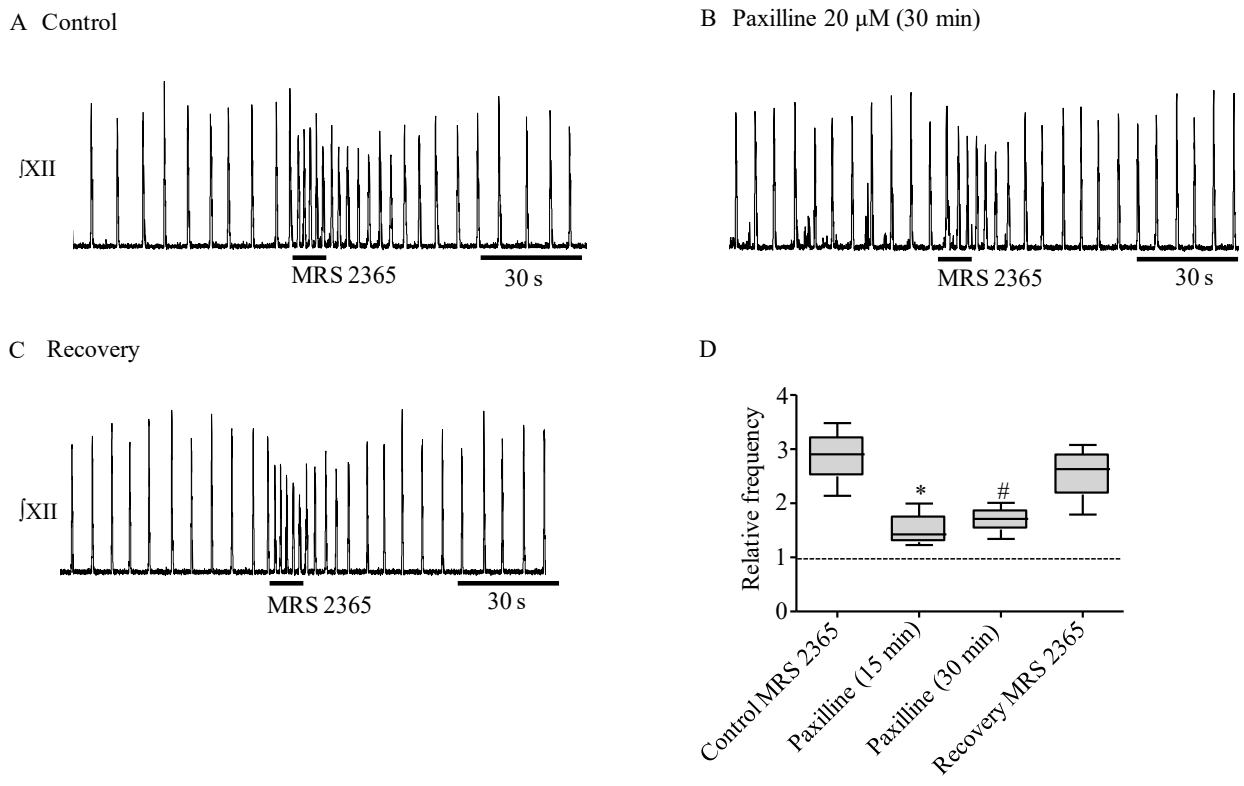

Figure 12. Paxilline at 20 µM attenuated MRS 2365-evoked frequency increase. Representative traces of ∫XII nerve showing the frequency increase evoked by the $P2Y_1$ agonist MRS 2365 (100 µM, 10 Sec) in control (A) and after 30 minute application of paxilline (20 µM) locally to the preBötC (B). C. Recovery injection D. Comparison of group data reveals a significant reduction in the MRS 2365-evoked response after 15 and 30 minutes of paxilline ($n=3$, $p<0.05$, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). All data are plotted relative to baseline levels.

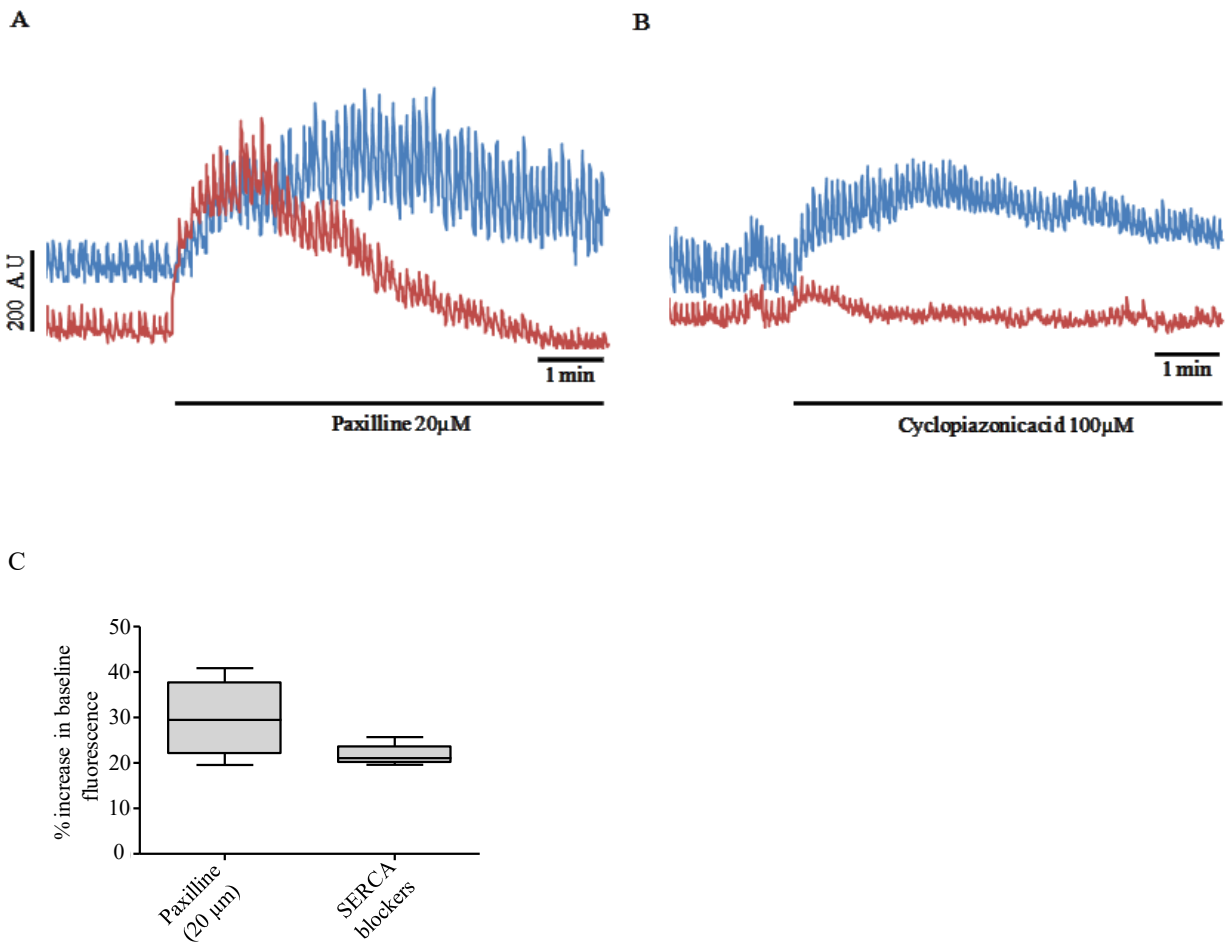

Figure 13. Paxilline at 20 µM and SERCA blockers produce similar increases in baseline Ca^{2+}_i fluorescence of inspiratory neurons. Fluorescence intensity measurements from two inspiratory neurons showing the increase in the baseline fluorescence (Ca^{2+}_i) evoked by local application of paxilline (20 µM) (A) B. Same neurons showing an increase in baseline fluorescence (Ca^{2+}_i) evoked by local application of CPA (100 µM) C, Group data from 4 inspiratory cells from 2 slices showing average effects on baseline Ca^{2+} fluorescence evoked by paxilline and SERCA blockers respectively ($p = 0.26$, paired t test)

Paxilline (10 μM) increased baseline fluorescence by 2.6 ± 1.6% while Thapsigargin over the same neurons caused a significant, 11-fold greater increase in Ca^{2+} fluorescence that was 29 ± 11% greater than baseline (n=4 from 2 slices p=0.0194, paired t test). These experiments suggest that at 10μM paxilline has minimal effect on SERCA.

Paxilline at 10 μM had no significant effect on $Ca^{2+}{}_i$. To ensure that thapsigargin was at a concentration where off-target actions on SERCA were not a concern, I repeated the MRS 2365 experiments using paxilline at a concentration 10-fold lower than that at which the it had no measurable effect on intracellular Ca^{2+}. Paxilline at 1.0 μm reduced the MRS 2365-evoked frequency increase from a peak of 2.45 ± 0.31 fold in control to 1.79 ± 0.45 at 15 min ($p > 0.05$) and 1.62 ± 0.25 after 30 min (n = 6, significant p=0.04, repeated measures ANOVA, Bonferroni's multiple comparison test). These experiments suggest that BK channels are a downstream effector of $P2Y_1$ receptors. The absolute baseline frequency values of control and in 15 and 30 min of paxilline (1 μM) are 12.6 ± 0.6, 13.2 ± 0.6 and 11.4 ± 1.2 BPM respectively, where as peak absolute frequency value are 31.2 ± 4.2, 21 ± 3 and 18 ± 1.8 BPM respectively

Paxilline blocks both Type I and Type II BK channels. To distinguish the relative contributions of these two subtypes, I next assessed the effects of a Type I selective blocker, Iberiotoxin, on the MRS 2365-evoked frequency increase. The response of a single preparation and group data are shown in Fig. 16. In control, MRS 2365 injections into the preBötC evoked a 2.24 ± 0.1 fold frequency increase while in presence of Iberiotoxin for 15, 30 and 45 min, MRS 2365 evoked 2.58 ± 0.26, 2.90 ± 0.54 and 2.74 ± 0.34 fold increases, respectively (n = 4, $p>0.05$, repeated measures ANOVA, Bonferroni's multiple comparison test). The absolute baseline frequency values of control and in 15, 30 and 45 min of Iberiotoxin are 12 ± 1.8, 12.6 ± 0.6, 12.6 ± 0.6 and 13.2 ± 0.6 BPM respectively, where as peak frequency value are 27 ± 1.2, 32.4 ± 3.6, 37.2 ± 6.6 and 36 ± 4.8 BPM respectively

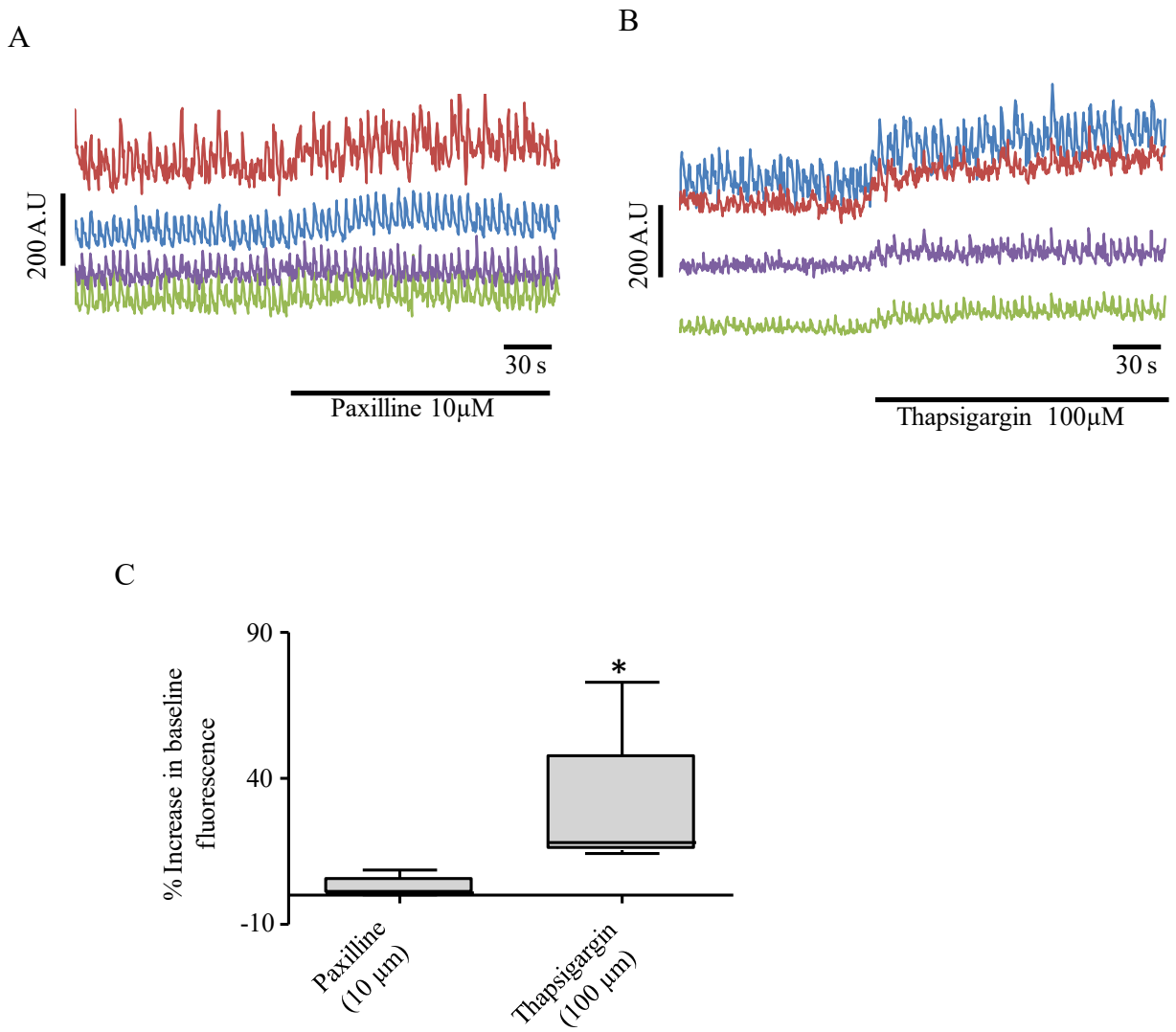

Figure 14. Paxilline at 10 µM has minimal effect on the baseline Ca^{2+} fluorescence of inspiratory neurons. Baseline Ca^{2+} fluorescence measurements of four preBötC inspiratory neurons in control and during local application of paxilline (10 µM) into the preBötC (A) and thapsigargin (100 µM, B). C. Group date taken from 5 inspiratory cells from 2 slices showing a 2.5% and 29% increase in baseline Ca^{2+} with paxilline and Thapsigargin respectively ($p = 0.0194$, paired t test).

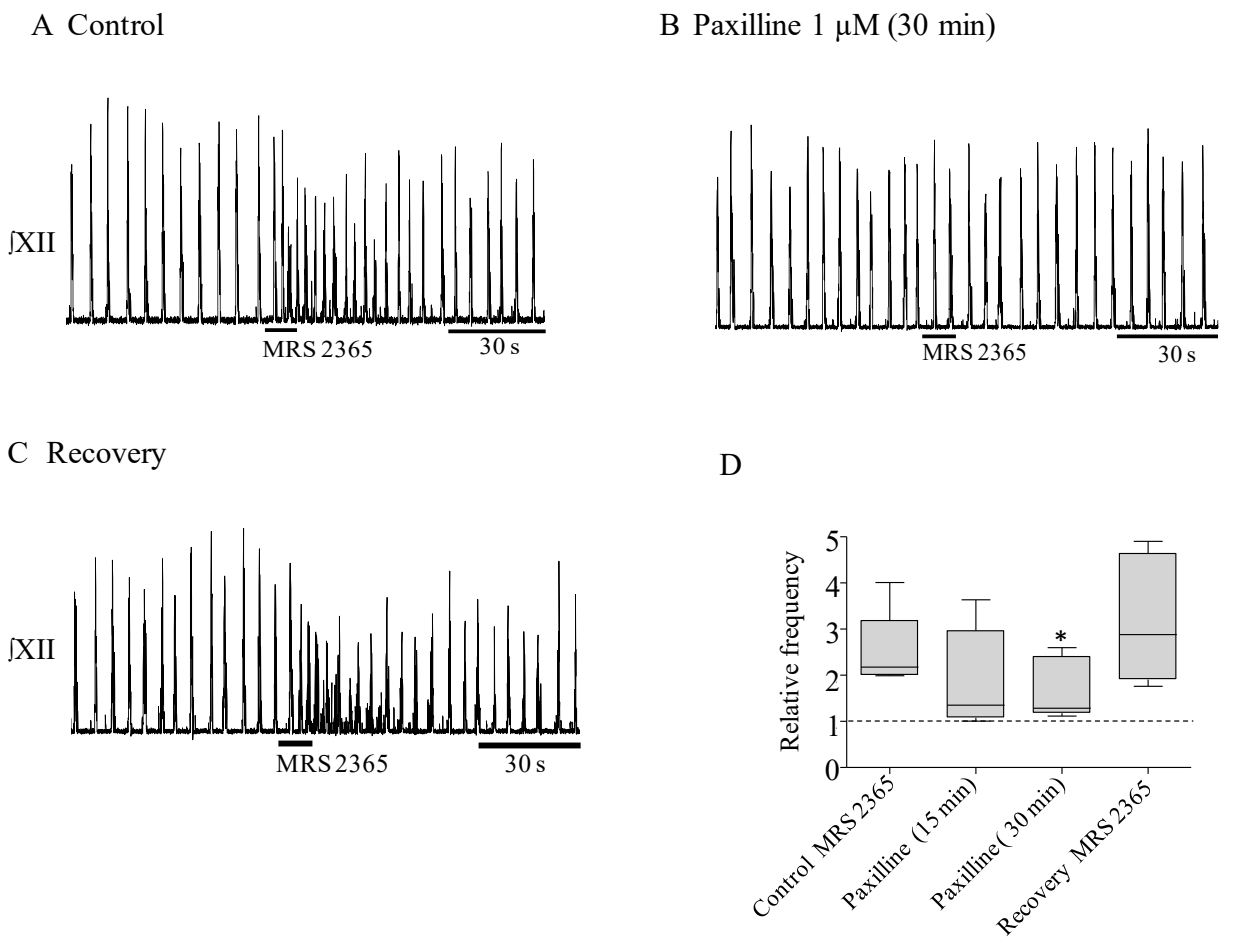

Figure 15. Paxilline at 1 µM attenuated MRS 2365-evoked frequency increase. Representative traces of ∫XII nerve activity showing the frequency increase evoked by local application of MRS 2365 (100 µM, 10 sec) in control (A) and after 30 minute application of paxilline (1 µM) locally to the preBötC (B). C. Recovery injection D. Group data showing the effects of MRS 2365 on frequency after 15 and 30 min of locally applied paxilline. * indicates a significant difference from control (n = 6 p = 0.04, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). All data are plotted relative to baseline levels.

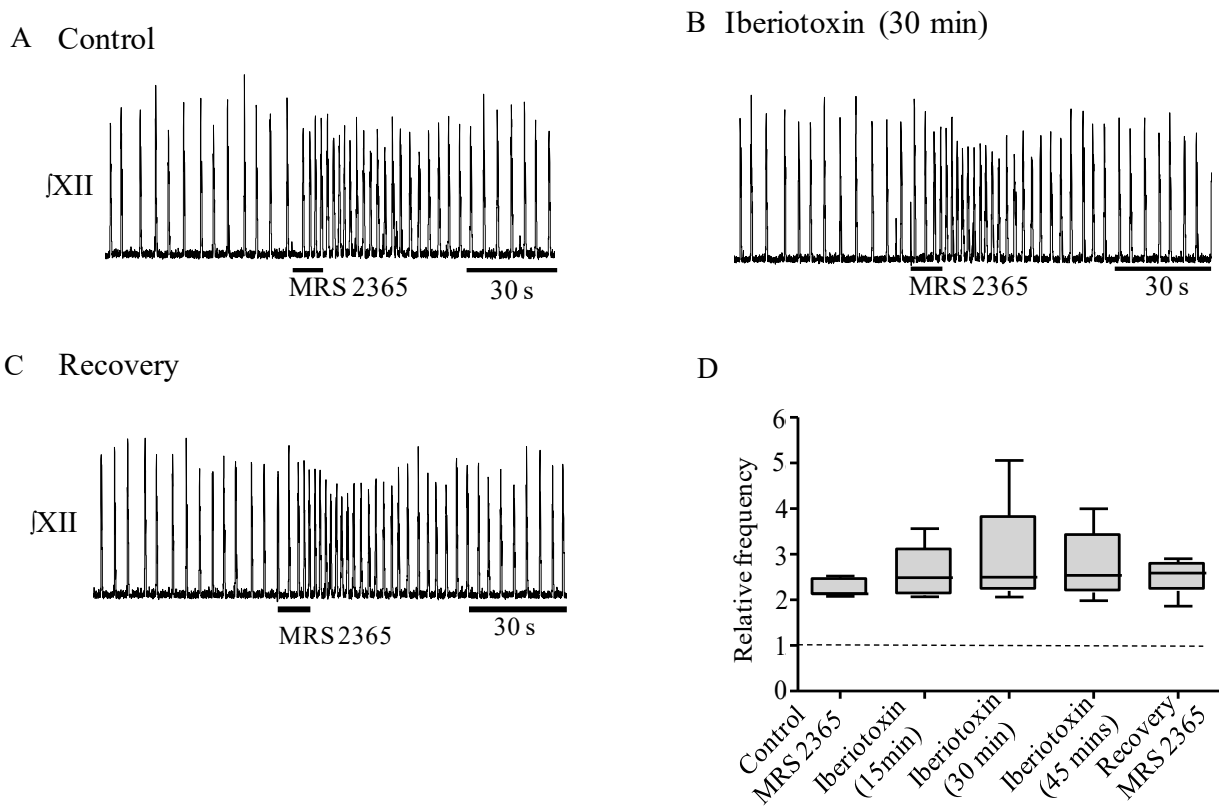

Figure 16. Iberiotoxin had no significant effect on MRS 2365 evoked frequency increase. Representative traces of ∫XII nerve activity showing the increase in frequency evoked by MRS 2365 (100 µM, 10 Sec) in control (A) and after 30 min application of Iberiotoxin (25 µM) locally to the preBötC (B). C. Recovery injection D. Box plot of group data showing no effect of Iberiotoxin on the MRS 2365-evokde frequency response ($n = 5$, $p < 0.05$, repeated measures ANOVA, Bonferroni's Multiple Comparison Test). All data are plotted relative to baseline levels.

5.0 Discussion

5.1. Signaling pathways mediating the effects of $P2Y_1$ receptor activation on Ca^{2+}_i and preBötC frequency

ATP released in the preBötC during hypoxia attenuates the secondary hypoxic respiratory depression primarily by acting on $P2Y_1$ receptors (Rajani et al., 2015). The molecular mechanisms underlying the $P2Y_1$ receptor mediated excitation of the inspiratory network was unclear. The main objective of my thesis was to dissect the molecular mechanisms underlying $P2Y_1$ receptor-mediated excitation of preBötC inspiratory network. I first demonstrated that inspiratory neurons in the preBötC show an increase in Ca^{2+}_i following $P2Y_1$ receptor activation. However, as predicted for a heterogeneous neuronal network containing excitatory and inhibitory neurons, I also demonstrated that inspiratory neurons are differentially sensitive to $P2Y_1$ receptor-mediated increases in intracellular Ca^{2+} that are derived, at least in part, from thapsigargin and CPA-sensitive intracellular Ca^{2+} stores. Finally, I established that Type II BK channels are one downstream target of $P2Y_1$ receptors that contribute to the overall increase in frequency evoked by ATP in the preBötC.

5.2 $P2Y_1$ receptor activation increases Ca^{2+}_i of preBötC inspiratory neurons

Many neuromodulators, such as amines and neuropeptides and neurotransmitters modulate rhythm via GPCR signaling systems. These neuromodulatory inputs originate from numerous areas distributed throughout the central nervous system and can either excite or inhibit respiratory activity, depending on the receptor they activate.

Endogenous GABA via $GABA_B$ receptors, opioids from periacqueductal gray via μ opioid receptors (Mellen et al. 2003) and norepinephrine from the A5 cell group in the ventrolateral pons via α_2 receptors (Hilaire, Monteau, and Errchidi 1989) all depress respiratory frequency. Treatment of animals with pertussis toxin the day prior to slice preparation and in vitro experimentation attenuated the respiratory depression caused by activation of these receptors, suggesting they are coupled through the $G_{i/o}$ signaling pathway (Johnson et al. 2006).

On the other hand acetylcholine via M_3 receptors (Shao and Feldman 2000, 2005), norepinephrine from locus ceruleus via α_1 receptors (Viemari and Ramirez 2006), histamine from tuberomammillary nucleus via H_1 receptors (Dutschmann et al. 2003), serotonin from raphe magnus via 5-HT_2 receptors

(Peña and Ramirez 2002; Ptak et al. 2009). ATP (Huxtable et al. 2009, 2010; Lorier et al. 2007) released from glia of the ventrolateral medulla (Angelova et al. 2015) via $P2Y_1$ receptors, substance P from dorsal raphe via NK_1 receptors (Gray et al. 1999; Pena 2004), cholecystokinin from periaquedutal gray, nucleus tractus solitarius and raphe magnus via CCK_1 receptors (Ellenberger and Smith 1999) and TRH from raphe magnus (Greer, al-Zubaidy, and Carter 1996) all increase respiratory frequency. Analysis of recombinant receptors in a host of expression systems all support that these receptors act via the $G_{\alpha q}$ GPCR signaling pathway. However, there is little direct evidence that these neuromodulators activate the $G_{\alpha q}$ pathway specifically in preBötC neurons.

$P2Y_1$ receptors are also presumed to act via $G_{\alpha q}$. Recombinant $P2Y_1$ receptor studies (Von Kugelgen and Wetter 2000) and $P2Y_1$ receptor signaling studies in various brain regions (Abbracchio et al. 2006) support this hypothesis. Conventionally, activation of the $G_{\alpha q}$ signaling pathway dissociates the heterotrimeric G protein into αq and βγ subunits. The αq subunit activates phospholipase C, which subsequently hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 promotes release of Ca^{2+} from intracellular stores by binding to IP_3 receptors on the endoplasmic reticulum. Ca^{2+} and DAG activate PKC to mediate cellular and ultimately network effects (Berridge 2002).

$P2Y_1$ receptor excitation of the preBötC inspiratory network also appears to be mediated, at least in part, via the $G_{\alpha q}$ signaling pathway. First, activation of $P2Y_1$ receptors via local application of MRS 2365 caused an increase in $Ca^{2+}{}_i$ in inspiratory neurons that was sensitive to the SERCA-blockers thapsigargin and CPA. In addition the network effects of $P2Y_1$ receptor activation were attenuated by chelation of intracellular Ca^{2+} (with BAPTA AM), inhibition of IP_3 receptors (with 2-APB) and PKC (with chelerythrine chloride) (Rajani et al. 2015), which are all part of the $G_{\alpha q}$ signaling pathway.

Despite these potent actions of $P2Y_1$ receptor agonists on the preBötC inspiratory neurons and network frequency and the data supporting that $P2Y_1$ receptors signal through $G_{\alpha q}$ activation to cause increases $Ca^{2+}{}_i$, I cannot conclude unequivocally that $P2Y_1$ receptors are coupled to $G_{\alpha q}$ in preBötC. $P2Y_1$ receptors can also act via the $G_{\alpha i}$ signaling pathway to increase $Ca^{2+}{}_i$. In CFTR-expressing Chinese hamster ovary cells, $P2Y_1$ receptor-mediated Ca^{2+} increases are sensitive to pertussis toxin (Marcet et al. 2004). In rat sympathetic neurons pertussis toxin abolishes closing of GIRK channels mediated by $P2Y_1$ receptors, further suggesting $G_{\alpha i}$ coupling (Erb and Weisman 2012). $G_{\alpha i}$ can

induce increases in Ca^{2+}_i through the subunits of $G_{i\beta\gamma}$ protein that can activate PLC and trigger release of Ca^{2+} from various signaling pathways (Bugrim 1999; Smrcka and Sternweis 1993). $G_{\alpha s}$ can also increase Ca^{2+}_i through an IP_3-dependent pathway. $G_{\alpha s}$ activation then increases PKA via cAMP. PKA-mediated phosphorylation of IP_3 receptors alters sensitivity to IP_3 (Tovey et al. 2008). Alternatively, cAMP-triggered Epac, exchange proteins activated by cAMP can activate PLC via β2-adrenoceptors expressed in HEK cells producing IP_3 triggered release of Ca^{2+} from Endoplasmic reticulum (Schmidt et al. 2001). $P2Y_1$ receptors can also form heteromeric complexes with adenosine (A_1) receptors. These hybrid receptors respond to ADPβS to activate both $G_{\alpha i}$ and $G_{\alpha q}$ (Yoshioka et al. 2001). Thus, while data strongly support a role for $G_{\alpha q}$ signaling in the $P2Y_1$ receptor-mediated potentiation of inspiratory frequency, additional experiments are required to confirm this conclusion.

5.3 PreBötC inspiratory neurons are differentially sensitive to $P2Y_1$ receptor activation

The preBötC is a heterogeneous neuronal network. In addition to inspiratory and a smaller number of expiratory neurons, the preBötC contains a small population of post inspiratory neurons (Lieske et al. 2000; Smith et al. 1991). Within the inspiratory neuron group, there are pacemakers and non-pacemaker neurons. Not all inspiratory preBötC neurons or even pacemaker neurons are excitatory; some are inhibitory (Morgado-Valle, Baca and Feldman 2010; Winter et al. 2009). The high sensitivity of the network to $P2Y_1$ receptor activation suggested to me that preBötC neurons are differentially sensitive to $P2Y_1$ receptor activation. More specifically, we predict that a population of neurons important in rhythm generation, or in providing direct excitatory drive to rhythm generating neurons, shows greater sensitivity to $P2Y_1$ receptor activation than other neurons. Certainly equivalent activation of excitatory and inhibitory mechanisms is less likely to result in powerful excitation than selective activation of excitatory neurons. Based on magnitude of Ca^{2+} fluorescence increases evoked by MRS 2365, we found two distinct classes of neurons; 60 % of neurons (12/20) showed strong increases in Ca^{2+} fluorescence while 8/20 responded very weakly. Importantly, experiments in which neurons did not respond to MRS 2365 were only included in the analysis if there were other neurons in the slice that responded to MRS 2365; i.e., we had a positive control to exclude false negative responses. These data are consistent with our hypothesis that the high ATP sensitivity reflects differential sensitivity of inspiratory preBötC neurons. What remains is to

determine the phenotype of the MRS 2365-sensitive and insensitive neurons; i.e., are the sensitive neurons exclusively excitatory?

It is also interesting to note that when slices had sensitive inspiratory neurons, all inspiratory neurons were sensitive. When the slice contained insensitive inspiratory neurons, all inspiratory neurons were insensitive. Importantly, non-inspiratory cells adjacent to non-responders did respond to MRS 2365. Thus, I am confident that this negative response is meaningful. One possible explanation is that slices with insensitive neurons were cut more rostrally than those with sensitive neurons, which were cut at the rostral surface of the preBötC. This possibility is consistent with the observation that the inspiratory rhythm is most sensitive to activation of $P2Y_1$ receptors in the preBötC (Lorier et al. 2007). However, I did not document the slice boundaries in the different experiments. Thus, additional experiments are required to address whether inspiratory neurons rostral to preBötC are less sensitive to $P2Y_1$ receptor activation than those within the preBötC.

5.4 Ca^{2+} released from intracellular stores contributes to $P2Y_1$ receptor-mediated increase in respiratory frequency

Ca^{2+} is major second messenger that regulates a variety of intracellular signaling pathways and processes. Ca^{2+} is generally taken up and stored within the endoplasmic reticulum (ER) in most of the cell types (Berridge 2002) and released into the cytosol in response to messengers such as IP_3 (Berridge 1998). The released Ca^{2+} ions have many different functions depending on the type and location of the cell. SERCAs are an important family of ATPases that are responsible for maintaining high levels of Ca^{2+} in the ER. SERCA transfers Ca^{2+} from the cytosol of the cell to the lumen of ER at the expense of ATP hydrolysis. Blockade of SERCA activity will eventually result in depleting of ER Ca^{2+} (Berridge 1998). Here I showed that both the increase in intracellular Ca^{2+} in inspiratory preBötC neurons and network frequency increase evoked by $P2Y_1$ receptor activation were sensitive to SERCA blockade.

However, neither thapsigargin nor CPA completely blocked the MRS 2365-evoked, $P2Y_1$ receptor-mediated Ca^{2+} and frequency increases. This incomplete block may reflect that additional signaling pathways contribute to the actions of $P2Y_1$ receptors or that the thapsigargin/CPA pathway, the presumptive $G_{\alpha q}$-IP_3-increased $Ca^{2+}{}_i$ pathway, was not completely blocked. When bath applied at 100 μM, SERCA blockers almost completely abolished the MRS2365-evoked increase in

intracellular Ca^{2+}. In some of the imaging experiments this concentration also blocked the frequency effect (see Fig. 3D).

However, in the experiments that examine the mechanisms underlying the frequency effects of $P2Y_1$ receptor activation, thapsigargin was locally applied at 100 μM into the preBötC. Under these conditions the thapsigargin-mediated inhibition was smaller than during bath application. This is not surprising. During local injection, the actual drug (thapsigargin) concentration decreases exponentially from the point source at the injection pipette tip. Indeed previous works comparing the effects of bath- vs. locally applied drugs suggest that to produce similar actions, the locally applied concentration needs to be ~10-fold greater than when bath applied. (Liu et al. 1990)

These data suggest that the actions of thapsigargin and CPA are dose-dependent, but even when bath-applied the SERCA blockers did not completely block the effects of $P2Y_1$ receptor activation on frequency. The insensitive component of the MRS 2365 effect could reflect: 1) alternate second messenger signaling pathways; 2) presence of SERCA pumps that are insensitive to Thapsigargin (Liang and Sze 1998)or CPA; or, (3) that it may not be possible to complete deplete internal Ca^{2+} stores (Treiman, Caspersen, and Christensen 1998).

As discussed previously, evidence also exists that $P2Y_1$ receptors can couple to $G_{\alpha i}$. In CFTR-expressing Chinese hamster ovary cells, $P2Y_1$ receptor mediated Ca^{2+} increases are sensitive to pertussis toxin (Marcet et al. 2004). In rat sympathetic neurons pertussis toxin abolishes closing of GIRK channels mediated by the $P2Y_1$ receptor, further suggesting $G_{\alpha i}$ coupling (Erb and Weisman 2012).

$P2Y_1$ receptor activation also causes excitation in various brain regions. For example in posterior hypothalamus activation of $P2Y_1$ receptor causes excitation (Sergeeva et al. 2006). Even though $P2Y_1$ receptor are excitatory in nature it is not always true that activation leads to network excitation for example activation of $P2Y_1$ receptor exerts excitatory action of inhibitory interneurons, which ultimately causes inhibition of the hippocampal CA3 neurons (Kawamura et al. 2004).

5.5 Minimal contribution of GIRK, SK and voltage gated K^+ channels to $P2Y_1R$ mediated network excitation.

Having demonstrated that the $P2Y_1$ receptor-mediated increase in preBötC frequency is produced in part by activation of a signaling cascade the triggers Ca^{2+} release from intracellular stores, my objective was to define the downstream ion channel mediating this excitation. I screened a number of channels based on the fact that they are modulated either by $P2Y_1$ receptor or $G_{\alpha q}$ signaling pathway and they may influence inspiratory rhythm (Rajani et al. 2016).

5.5.1 GIRK channels

GIRK channels are a family of inward-rectifier potassium channels, activated by $G_{\beta\gamma}$ component of GPCRs that cause hyperpolarization of neurons when activated. Four subunits of GIRK channel are identified in mammals (GIRK1-GIRK 4) (Dascal et al. 1993; Ferrer et al. 1995; Krapivinsky et al. 1995; Krapivinsky et al. 1995). GIRK1 to GIRK3 are expressed more commonly in the brain (Lüscher and Slesinger 2010). The GIRK 2 subunit is expressed in the preBötC where it is proposed to mediate the opioid depression of breathing (Montandon, Liu, and Horner 2016). Consistent with this, breathing rate and genioglossus muscle amplitude decreased when ML297, a specific GIRK channel activator, was microperfused into the preBötC of anesthetized adult male rats (Montandon et al. 2016).

GIRK channels are inhibited by $BaCl_2$ so I tested the effects of this divalent cation on the MRS 2365-evoked frequency increase. $BaCl_2$ on its own affected baseline inspiratory rhythm and evoked a substantial increase in background tonic activity that sometimes made it difficult to discriminate individual inspiratory bursts. These effects clearly indicated that $BaCl_2$ was diffusing into the slice, but even with the perturbations of rhythm, it was evident that $BaCl_2$ did not block the ability of MRS 2365 to increase frequency, strongly suggesting that GIRK channels are not the ion channel that underlies the $P2Y_1$ receptor-evoked frequency increase.

5.5.2 Voltage gated K^+ channels (VGKC)

VGKCs are activated during depolarization and selectively permeable to K^+ ions. Eight VGKC were identified in mammals (Coetzee WA, et. al., 1999). Currents carried by VGKC can be broadly classified into noninactivating delayed rectifier-type (K_{DR}) and transient A-type (I_A) currents (Johnston et. al., 1995, Mathie et. al., 1995). TEA preferentially blocks IK_{DR} over I_A (Lien et al., 2002; Melnick et al., 2004).

Many K^+ channel species may contribute to delayed rectifier K^+ currents, including members of the voltage-dependent K_v1, K_v2, and K_v3 gene families and others, (Wei et. al., 1996, Hille et. al., 2001, Salkoff et. al., 1992). The specific combination of delayed rectifier candidates expressed in the preBötC is not known. Effect of specific blockade of IK_{DR} on rhythm generation and modulation are also unknown. In rat (P_0-P_4) brainstem-spinal cord preparation TEA decreased frequency (Onimaru et al., 2003).

A role for VGKCs in mediating the excitatory actions of $P2Y_1$ receptors on preBötC rhythm, however, appears unlikely. Local application of TEA (1mM) caused an increase tonic discharge recorded from the XII nerve but the MRS 2365-evoked frequency was not significantly affected by TEA.

5.5.3 SK Channels

SK channels are a family of Ca^{2+} activated K^+ channels. Unlike BK channels, these channels are activated solely by raise in the intracellular Ca^{2+}. No evidence exists that these channels are involved in rhythm generation but can modulate rhythm, depending on species and perhaps preparation.

Apamin had minimal effect on the MRS 2365-evoked frequency increase, suggesting SK channels may not be a downstream effector channel of $P2Y_1$ receptors. The only caveat here is that because Apamin did not affect baseline rhythm I cannot be 100% confident that it diffused into the slice and blocked SK channels. I also did not perform a positive control to demonstrate that Apamin had washed in. However, Apamin has been used multiple times in similar situations (Zavala-Tecuapetla et al. 2008) where it readily diffused into tissue and antagonized SK. Thus, while I cannot completely exclude the possibility that Apamin had not washed in, data suggest that SK channels do not mediate the actions of $P2Y_1$ receptor activation on preBötC rhythm.

5.6 Type II BK channels may be the downstream effector channel of $P2Y_1$ receptor

BK channels are large conductance K^+ channels that contribute to the fast afterhyperpolarization phase of most cells.

Similar to SK channels, BK channels are not involved in rhythm generation but appear to contribute to the $P2Y_1$ receptor-mediated increase in inspiratory frequency. Paxilline at 20 μM significantly attenuated the MRS 2365-evoked frequency increase. However, Ca^{2+} imaging data indicated that at

this concentration paxilline also increased intracellular Ca^{2+} concentration. Thus, it is not possible to attribute this antagonism to the actions of paxilline at BK channels. The inhibition may have been due to inhibition of SERCA as previously reported at high concentrations (Bilmen et al. 2002). Imaging experiments also showed that 10 μM paxilline did not affect intracellular Ca^{2+}, suggesting minimal inhibition of SERCA at 10 μM. However, to be sure that any actions of paxilline were not due to inhibition of SERCA, we used a 10-fold lower concentration of paxilline (1 μM) to test whether BK channels contribute to the $P2Y_1$ receptor-mediated increase in inspiratory frequency.

Paxilline, a blocker of Type I and II BK channels, was re-tested at a concentration of 1 μM at the network level where it significantly attenuated the frequency increase evoked by MRS 2365 in the preBötC, suggesting that BK channels may be a downstream effector channel of $P2Y_1$ receptors. Interestingly, Iberiotoxin, which blocks Type I BK channels with very low affinity for Type II BK channels (Wang, Jaffe, and Brenner 2014), had no effect on the MRS 2365-evoked frequency increase. Thus, it is most likely the Type II BK channels through which MRS 2365 acts to increase frequency. The MRS 2365-evoked frequency effect was not completely blocked by paxilline. Following factors may contribute to this. First, BK channel blockade by paxilline is inversely proportional to the open probability of channels. Intracellular Ca^{2+} increases open probability of BK channels, thereby decreasing the ability of Paxilline to block channels (Sanchez and McManus 1996). As I demonstrated that $P2Y_1$ receptor activation causes an increase in intracellular Ca^{2+} and responders are more sensitive to $P2Y_1R$ activation, it is possible that in slices having more responders maximum block by paxilline may not be achieved. Secondly, it is also possible that BK channels are not the only ion channel on which $P2Y_1$ receptors act to increase frequency. As discussed earlier, $P2Y_1$ receptors modulate a wide variety of channels. It is completely possible that the $P2Y_1$ receptor-mediated excitation of the preBötC network results from the activation of BK and other channels.

Above mentioned two factors may all have contributed to the variability in the magnitude of the paxilline-mediated inhibition of the MRS 2365 frequency increase which ranged between preparations from no inhibition to a maximum of almost 70%.

6.0 Summary

The main objective of this thesis was to advance understanding of the molecular mechanisms by which $P2Y_1$ receptors activation increases ventilation. The main contributions were the demonstrations that: i) $P2Y_1$ receptor activation in preBötC inspiratory neurons causes release of Ca^{2+} from intracellular stores; ii) the $P2Y_1$ receptor-evoked Ca^{2+} increase contributes to network excitation; iii) as predicted of a network comprising excitatory and inhibitory preBötC inspiratory neurons, not all preBötC inspiratory neurons are excited by $P2Y_1$ receptor activation; and iv). Activation of BK channels contributes to the $P2Y_1$ receptor-mediated excitation of inspiratory rhythm.

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