PhD Thesis

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ATP and mechanisms of central CO_2 chemosensitivity

by

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Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any other award. Where other sources of information have been used, they have been acknowledged

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Summary

ATP release from the surface of the ventro-lateral medulla (VLM) is integral to the hypercapnic response *in vivo* and can be seen *in vitro*. By employing horizontal slices of the ventral medulla containing the ventral chemosensitive nuclei, I have developed a model that consistently evokes hypercapnia-induced ATP release *in vitro*. Using this preparation I have studied CO_2-triggered ATP release by means of microelectrode biosensors. I conclude that it is the change in PCO_2 itself, and not associated pH changes that accompany it, that is directly responsible for eliciting ATP release from the surface of the VLM. In addition ATP release from this region may have a role in the response to hypocapnia as well as hypercapnia. Using pharmacological agents I have demonstrated that gating of connexin hemichannels mediates ATP release. The dorso-ventral distribution of Cx26 ascertained via quantitative PCR and immunofluorescence makes this hemichannel the most likely candidate. Dye loading the cells responsible for ATP release with carboxyfluorescein, which co-localised with Cx26, revealed these cells reside in the pia mater and sub-pial astrocytes. Application of gap-junction antagonists, with selectivity towards connexin 26, greatly reduced ATP release in response to elevated CO_2 *in vitro* and *in vivo* and reduced the tone of ATP at the VLM surface. Moreover, by loading Cx26 expressing HeLa cells with ATP, I was able to recapitulate the entire *in vivo* response. Therefore I propose that ATP is released from sub-pial astrocytes and leptomeningeal cells through connexin 26 hemichannels in response to alterations in PCO_2. Here Cx26 performs a dual role, as both the chemosensory transducer and the conduit for ATP release.

Abbreviations

µM - Micromole

5,7-DHT - 5,7-dihydroxytryptamine

5-HT - Serotonin

8-OH DPAT - 8-hydroxy-2-(di-n-propylamino) tetralin

Å - Angstroms

ACh - Acetylcholine

aCSF - Artificial cerebrospinal fluid

ADP - Adenosine diphosphate)

Ag/AgCl – Silver silver chloride

AMP - Adenosine mono-phosphate

ASIC - Acid sensing ion channel

ATP - Adenosine triphosphate

AVP - Arginine vasopressin

AZ - Acetazolamide

BSA - Bovine serum albumin

CA - Carbonic anhydrase

Ca^{2+} - Calcium ions

$CaCl_2$ – Calcium chloride

CBF - Carboxyfluorescein

CCHS - Congenital hypoventilation syndrome

cDNA - Complementary deoxyribose nucleic acid

CI - Chemosensitivity index

Cl^- - Chloride ions

CO_2 - Carbon dioxide

Co^{2+} - Cobalt ions

$CsCl_2$ - Caesium chloride

Cx - Connexin

Da - Daltons

DAB - Di-amino benzene

DIDS - 4,4'-diisothiocyano-2,2'-stillbene-disulfonic acid

DMEM - Dulbecco's modified eagle's medium

ECF - Extracellular fluid

FITC - Fluorescein isothiocyanate

GABA - Gamma aminobutyric acid

GFAP - Glial fibrillary acidic protein

H_2O - Water

H_2O_2 – Hydrogen peroxide

HA - Hypercapnic acidosis

HCl - Hydrochloric acid

HCO_3^- - Bicarbonate ions

HEK - Human embryonic kidney

HeLa cells – Ovarian carcinoma cells from Henrietta Lacks

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HR - Heart rate

HVC – Hypercapnic ventilatory response

IA - Isocapnic acidosis

I_{can} - Ca^{2+}-activated non-specific cation current

IH - Isohydric hypercapnia

I_{NaP} - Persistent sodium current

IP3 - Inositol triphosphate

K^+ - Potassium ions

KCl – Potassium chloride

KCN - Potassium Cyanide

kDa - Kilodaltons

KOH - Potassium Hydroxide

LC - Locus coeruleus

M – Mole

MAP2 - Microtubule associated protein

MGL - Marginal glial layer

$MgSO_4$ – Magnesium sulphate

mM - Millimole

mmHg – Millimetres of mercury

mRNA - Messenger ribose nucleic acid

mV – Millivolt

NA - Nucleus ambiguus

NaCl – Sodium chloride

NaCN - Sodium cyanide

NaH_2PO_4 – Sodium dihydrogen orthophosphate

NBC - Sodium bicarbonate cotransporter

NH_4^+ - Ammonium ion

NHE - Na^+/H^+ exchangers

NK1R - Neurokinin 1 receptor

NPPB - 5-nitro-2-(3-phenylpropylamino)benzoic acid

NREM - Non-rapid eye movement

NTS - Nucleus tractus solitarius

O_2 - Oxygen

P1 - Purinergic receptor type 1

P2 - Purinergic receptor type 2

P2X - Purinergic receptor type 2, sub type X

P2Y - Purinergic receptor type 2, sub type Y

pA - Picoamp

pA.s – Picoamps x seconds

PBN - Parabrachial nucleus

PBS - Phosphate buffer solution

PCO_2 - Partial pressure of carbon dioxide

PCR - Polymerase chain reaction

PFA - Paraformaldehyde

pFRG - Parafacial respiratory group

pH - The cologarithm of the activity of dissolved hydrogen ions (H+).

pH_e - Extracellular pH

pH_i – Intracellular pH

PNA - Phrenic nerve activity

PND - Phrenic nerve discharge

PNMT - Phenylethanolamine N-methyltransferase

PO_2 - Partial pressure of oxygen

post-I - Post-inspiratory

PPADS - pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate

pre-I - Pre-inspiratory neurons

pS - Picosiemens

PVN - Paraventricular nucleus

REM - Rapid eye movement

RM - Raphé magnus

RO - Raphé obscurus

RP - Raphé pallidus

RTN - Retrotrapezoid nucleus

Rubisco - Ribulose-1,5-bisphosphate carboxylase oxygenase

RVLM - Rostral ventrolateral medulla

sAC - Soluble adenylate cyclase

SIDS - Sudden infant death syndrome

SON - Supraoptic nuclei

SP-SAP - Substance P conjugated to saporin

TASK - Tandem-pore acid sensing potassium channels

$TEACl_2$ - Tetraethylammonium chloride

TM - Transmembrane

TMN - Hypothalamic tuberomamillary nucleus

TNP-ATP - 2',3'-O-(2,4,6-Trinitrophenyl) adenosine 5'-triphosphate

TRH - Tyrosine hydroxylase

TRPM4 - Transient receptor potential cation channel, subfamily M, member 4

TTX - Tetrodotoxin

VRAC's - Volume regulated anion channels

VRG - Ventral respiratory group

Chapter 1: Introduction

1.1 Respiratory regulation

Generation and control of respiratory rhythm is one of the most important functions performed by the brain. This rhythm is generated by pacemaker cells, whose activity can be modulated to meet metabolic demands. The vital regulation of this function is controlled by the medulla oblongata, which is situated rostral to the spinal cord and ventral to the cerebellum at the base of the skull. There are several nuclei within this brain region which communicate, control and modulate respiratory output (Berne and Levy, 1998). The respiratory cycle is incessant, and perturbations in its control can have profound effects; dysregulation of this system often leads to the cessation of life. The two most widely studied diseases of the autonomic control of respiration are congenital central hypoventilation syndrome (CCHS) and sudden infant death syndrome (SIDS).

1.1.1 Congenital central hypoventilation syndrome (CCHS)

CCHS was first described in 1970, and is predominantly defined by cardiorespiratory dysregulation (Weese-Mayer et al., 2008). It is characterised by an intact respiratory system and neural network but with an absence of chemoreception, leading to hypoventilation and apnoeas particularly during sleep (Gallego and Dauger, 2008). It is associated with over 20 genetic mutations, however it is now accepted that the major cause of this disease is abnormalities in the homeobox gene Phox2b (Weese-Mayer et al., 2008, Gallego and Dauger, 2008). Phox2b is expressed in many respiratory and chemosensory areas; the area postrema, nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus, the dorsomedial portion of the spinal trigeminal tract, intermediate reticular nucleus, nucleus ambiguus (NA), retrotrapezoid nucleus (RTN), parabrachial nucleus (PBN), the carotid bodies (Kang et al., 2007) and the locus coeruleus (LC) (Gallego and Dauger, 2008). There are also implications that Phox2b mutations may also lead to abnormalities in the hypothalamus, though it is not expressed there. CCHS patients often present with thermoregulatory control deficits, a function performed by this brain region (Gallego and Dauger, 2008). It is required for the development of most neural cell types in the central and peripheral nervous system and thus a heterozygotic Phox2b knockout

phenotype, where one allele of the gene is replaced the human mutation which carries a 7 polyalanine repeat, is embryonic lethal (Gallego and Dauger, 2008).

Phox2b co-localises with VGlut-2 in the RTN, though cited by the authors as a neuronal marker it is not exclusive to this cell type. Thus there may be a role for Phox2b in non-neuronal cells as well. Despite its expression in many areas, Phox2b mutations cause a 77% loss of RTN/pFRG (parafacial respiratory group) neurons with an apparently otherwise intact respiratory network (Dubreuil et al., 2008, Guyenet, 2008). This loss causes gasping or reduced and haphazard breathing eventually leading to death shortly after birth (Dubreuil et al., 2008). This led to the hypothesis that at birth breathing and chemoreception is entirely dependent on RTN neurons (Guyenet, 2008). However it cannot be ruled out that the RTN is the major site of integration for all peripheral and medullary chemoreceptors and therefore its loss removes all chemoreception by indirect as well as direct means (Guyenet, 2008).

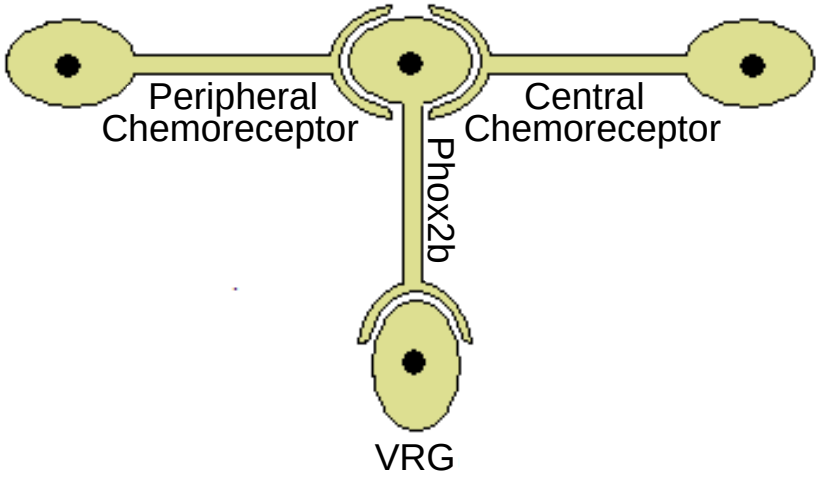

Figure 1: Hypothesised location of Phox2b in the chemoreflex. The Phox2b positive neurons serve as an integrator for all central and peripheral chemosensitive pathways. Any removal of this neuronal subset will stop chemosensory neurons from innervating the ventral respiratory group (VRG).

The prediction that CCHS patients have lesions between the central and peripheral carbon dioxide (CO_2) integrator and respiratory rhythm generator is not new (Shea, 1997). Interestingly CCHS may allow some compensation (Guyenet, 2008), as patients maintain their partial pressure of carbon dioxide (PCO_2) within normal parameters during wakefulness although with much greater variation than controls (Shea, 1997). Importantly the mouse model of the human disease does not show cardiovascular abnormalities like their human counterparts (Gallego and Dauger, 2008). This may be due to humans having additional mutations in genes such as ret1

or due to the nature of the mutation (Gallego and Dauger, 2008). Humans have a polyalanine repeat whilst mice are null mutants, thus human proteins may still bind to their targets without inducing their intracellular pathway, effectively antagonising them which cannot happen in the mouse model (Gallego and Dauger, 2008). The addition of this polyalanine repeat may also lead to incorrect trafficking of intracellular proteins, which could have downstream effects. In either case the neurons may appear morphologically normal, but the network may not be intact. In other words neurons in other brain regions may appear normal but Phox2b mutations cause a loss or gain of function causing them to act abnormally. If this were true then it would appear that only the RTN is affected, whilst in truth the loss of Phox2b would have much further reaching effects. This would appear a more logical conclusion as the chemosensitivity of RTN neurons alone cannot account for the full hypercapnic ventilatory response.

1.1.2 Sudden infant death syndrome (SIDS)

Sudden infant death syndrome (SIDS) is characterised by a perverse chemosensitive response during sleep, which results in apnoea. It has been linked to the dysregulation of the serotonergic system (Richerson, 2004) particularly mutations in the serotonin transporter protein (Weese-Mayer et al., 2008). Interestingly as with CCHS patients many SIDS patients present with mutations in Phox2b, these mutations lead to abnormal differentiation of serotonergic neurons (Weese-Mayer et al., 2008). Therefore Phox2b mutations do not with the exception of the loss of RTN neurons have an otherwise intact respiratory network as has been previously stated (Dubreuil et al., 2008, Guyenet, 2008). Therefore the Phox2b containing neuronal pathway may be a common pathway altered in all sleep apnoea related disorders (Weese-Mayer et al., 2008).

SIDS like CCHS has been causally related to the loss of many chemosensitive and respiratory nuclei, particularly the medullary raphé (see section 1.4.3), the pre-Bötzinger complex and the arcuate nucleus. The pre-Bötzinger complex is the respiratory rhythm central pattern generator in rats (Smith et al., 1991) and has been identified in humans (Lavezzi and Matturri, 2008). Thus any mutations leading to a loss of, or abnormal function in, these neurons will result in apnoea. In one study abnormalities in the pre-Bötzinger complex were identified in 41% of sudden death patients (Lavezzi and Matturri, 2008).

There are several hypothesises as to which chemosensitive areas in the VLM of lower order mammals (see section 1.3) relate to their human counterparts (figure 2), though all agree that the human arcuate nucleus is a key central chemosensitive site. Some believe that there is no homologue to the rostral chemosensitive sites, area M and the RTN (Filiano et al., 1990), whereas others believe that it is the arcuate nucleus (Mulkey et al., 2004, Okada et al., 2001). If there is no rostral homologue the arcuate nucleus may be the caudal chemosensitive area, area L, and the raphé complex of cats and rats; as the cells of these regions are identical in description and cover similar areas (Filiano et al., 1990). By contrast those who have implicated the arcuate nucleus as the rostral homologue implicate the nucleus conterminalis as the human homologue of the cat and rat area L (Okada et al., 2001). There is an area S homologue, which is also opposed to the sub-pial space. Also both humans and lower order mammals have raphé pallidi (Filiano et al., 1990). There is also a thickened marginal glial layer (MGL) in the human which is comparable to that of lower order mammal (Filiano et al., 1990) as they cover similar areas upon 3D reconstruction.

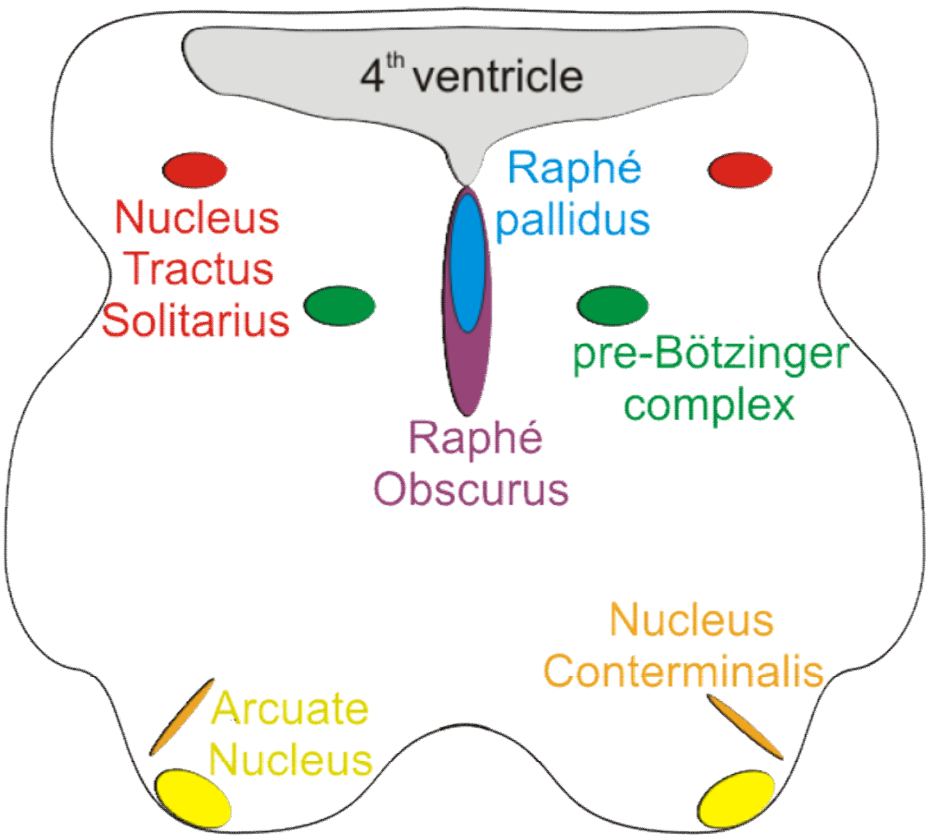

Figure 2: Human chemosensitive and respiratory nuclei.

In any case arcuate nucleus abnormalities are commonly seen in SIDS patients (Lavezzi and Matturri, 2008), these include low neural density, volume and number of neurons (Matturri et al., 2002, , Biondo et al., 2003). Since these areas in humans as well as the human pre-Bötzinger complex homologue (Lavezzi and Matturri, 2008), have all been implicated in the pathology of sudden infant death syndrome (Matturri et al., 2002, , Biondo et al., 2003, Lavezzi and Matturri, 2008), any progress made in chemoreception and respiration in lower order mammals could advance our understanding of human diseases.

1.2 Chemosensitivity

Chemoreception is the body's ability to detect specific chemical changes. In terms of respiration chemoreception is the ability of the brain to detect changes in blood gases (oxygen and carbon dioxide) and to alter breathing to keep their levels within tightly controlled parameters. The O_2 sensitive chemoreceptors are external to the brain in the carotid bodies. CO_2/pH sensitive cells are also in the carotid bodies (Peers and Buckler, 1995), but major sites are also within the brain (Feldman et al., 2003). Of the two gases it appears that CO_2 provides the most powerful drive to breath (Haldane and Priestley, 1905). Hypercapnia from re-breathing expired air causes similar increases in respiratory rate to exercise and does not affect physiology. In contrast hypoxia (low O_2) causes cyanosis (blueing of the lips and fingertips etc.), which is not seen during physical exertion. As athletes do not become cyanotic before their breathing rate increases, this was interpreted to mean that CO_2 is the primary drive to breathe (Haldane and Priestley, 1905). In support of this, it has been shown that mean arterial PCO_2 ($PaCO_2$) does not change with exercise (Forster et al., 1986). In conjunction PCO_2 in arterial blood is not different well below, above or at sea level, until extreme altitudes (>2500ft) are reached or prolonged periods of time are spent at altitude (Haldane and Priestley, 1905, Catron et al., 2006). In contrast PO_2 varies dramatically during short term exposure to moderate altitude (Haldane and Priestley, 1905). Therefore PCO_2 is tightly regulated even at the expense of PO_2, as it is at rest (Haldane and Priestley, 1905). In fact an increase in arterial PCO_2 by as little as 1mmHg leads to a 20-30% increase in ventilation (Feldman et al., 2003) and increasing inspired CO_2 to 4% increases ventilation (177%) (Haldane and Priestley, 1905). As CO_2 levels rise, depth of breathing increases as does total ventilation

(Haldane and Priestley, 1905). In contrast inspired O_2 must fall to 13% before changes in respiration occur (Haldane and Priestley, 1905).

The central CO_2 chemoreceptors, which are responsible for detecting changing levels of CO_2, are situated close to the respiratory centres and provide a powerful driving force for respiration (Thomas et al., 1999). Moreover it is changes in the partial pressure of carbon dioxide (PCO_2) of arterial blood that provides this powerful drive for respiratory rhythm (Haldane and Priestley, 1905), and causes the changes in ventilation that serves to alter clearance of CO_2 via the lungs (Berne and Levy 1998). This is also true during exercise where increased ventilation is proportional to increased CO_2 produced by the work, so increased breathing serves to clear the extra CO_2 and keep blood levels constant (Haldane and Priestley, 1905). Therefore there must be an underlying homeostatic process that serves to detect levels of systemic CO_2 and changes respiratory frequency, in order to return levels of CO_2 to the norm. The fact that CCHS patients are able to manage their PCO_2 during wakefulness has led to the hypothesis that chemoreception is only functional during sleep. However there are many more factors that contribute to respiratory control during wakefulness as compared to sleep, it may be the contribution of these additional factors that allows them this compensation (see section 1.8)

Once in the extracellular fluid (ECF) CO_2 is converted to bicarbonate ions and protons. Although this process occurs it does so at a very slow rate. This can however be dramatically increased by carbonic anhydrase, which catalyses the reaction (figure 3). Thus there are three possible signalling molecules that could act as chemosensory signals.

$$CO_2 + H_2O \underset{CA}{\longleftrightarrow} HCO_3^- + H^+$$

Figure 3: The conversion of carbon dioxide to hydrogen ions under the influence of carbonic anhydrase (CA). Possible chemosensory signals are highlighted in black.

In order to be classified as a chemosensitive neuron certain characteristics must be displayed. Neurons must respond to CO_2 or pH intrinsically, project to the respiratory centres and once stimulated must lead to alterations in ventilation (Putnam et al., 2004, Guyenet et al., 2005b, Richerson, 2004). A neuron that responds intrinsically to CO_2 must contain a protein that directly binds CO_2, causing a change in conformation, leading to an alteration in the neurons excitability. It must be remembered that neurons inhibited by CO_2 are as important as those excited by it

(Putnam et al., 2004). The anatomical connections between the pre-Bötzinger complex and chemosensitive neurons means that NK1R (neurokinin 1 receptor: a marker of pacemaker cells in this region) ablation in the pre-Bötzinger complex leads to the loss of the hypercapnic and hypoxic responses (Feldman et al., 2003). There are several areas that have been implicated in chemoreception and they will be discussed here.

1.3 The Ventrolateral medulla

The surface of the ventro-lateral medulla has a denser capillary network than the surrounding tissue (Göbel et al., 1990). The penetrating vessels of the medulla are surrounded by a perivascular space (Okada et al., 2001), and lead to elongated and labryrinthic invaginations of the sub-arachnoid space which form cisternae at the surface of the medulla (Okada et al., 2001). This leads to a higher blood flow than is seen in the surrounding tissue which is followed by higher glucose utilisation in adult rats (Göbel et al., 1990). This allows for greater blood gas sampling by the cells of the medulla, making the arterial branches perfusing the rostral ventrolateral medulla (RVLM) the most important in chemoreception (Okada et al., 2001). Importantly there is an intimate relationship between CO_2 excitable cells and surface and penetrating vessels in the ventral medulla, the basal lamina of these vessels have neuronal axon terminals and somata abutted to them (Okada et al., 2001). These cells often displayed a concave shape and a surface indentation is associated with fine vessels (Okada et al., 2002).

1.3.1 Chemosensitive sites of the VLM surface

The importance of the medulla in chemosensory signalling has long been known and the first major site that responds to inspired CO_2 was discovered on the VLM surface 45 years ago (Mitchell et al., 1963). Increasing the PCO_2 or hydrogen ion (H^+) concentration at a discrete locus on the surface of the VLM dramatically increased breathing. This area of the rostral VLM was designated area M after its founder (Mitchell et al., 1963). Interestingly the effect of changes in H^+ alone on respiration were slower than the effects of changing CO_2/H^+ simultaneously (Mitchell et al., 1963). In conjunction application of acetylcholine (ACh) or nicotine (which acts at nicotinic ACh receptors) to the sub-arachnoid space around 8^{th} and 9^{th} cranial nerve rootlets increased tidal volume and frequency (Mitchell et al., 1963).

Application of sodium cyanide (NaCN) and lobeline (an AChR antagonist) to area M depressed respiration (Mitchell et al., 1963). In conjunction cooling of this area leads to a pronounced apnoea in both adult and neonate goats (Forster et al., 1997) and adult cats (Mitchell et al., 1963). Repeated applications of ACh agonists led to a desensitisation of the response and cauterising it diminished it dramatically (Mitchell et al., 1963). Procaine (a local anaesthetic) depressed tidal volume and did not penetrate more than 0.35mm into the medulla (Mitchell et al., 1963).

They concluded that the neurons or their dendrites creating this response must be close to the surface since hydrogen and bicarbonate ions diffuse slowly through neural tissue and poorly penetrate the cellular membrane if at all (Mitchell et al., 1963). The neurons of area M thought to participate in this response are located dorsal to the ventral surface and consist of thin unmyelinated dendrites running parallel to the pial surface (Okada et al., 2001). The conclusions of these experiments were that the VLM contributes approximately 40% of the total hypercapnic ventilatory response (Mitchell et al., 1963).

The discovery of this chemosensitive area on the surface of the ventral medulla led to investigations to locate other areas that may participate in the hypercapnic ventilatory response. A second series of experiments by the same group identified a separate area at a more caudal location, designated as area L (Loeschcke et al., 1970). This area was located in and around the hypoglossal nerve rootlets, and electrical stimulation of this area caused an increase in respiratory rate (Loeschcke et al., 1970). However cooling of this area in goats had an effect only in neonates (Forster et al., 1997). The neurons of area L are located between 80-200μm from the surface of the medulla (Okada et al., 2001). Acetylcholinergic cells of this region importantly have close contact with penetrating vessels, ideally locating them to sense changes in blood chemistry (Okada et al., 2001). A third intermediate area was located between area M and L, designated area S. This region though apparently not chemosensitive itself contains neurons of passage vital to the chemosensitive response (Loeschcke, 1982). The neurons of area S are located 30-50μm from the ventral surface which were more dense than area L (Okada et al., 2001).

The stimulation or inhibition of breathing by the rostral (area M), intermediate (area S) and caudal (area L) chemosensitive loci, may have been due to a direct effect on the respiratory central pattern generator. To determine this, a series of ventral surface cooling experiments were conducted. Cooling of any area was never able to

eliminate breathing in goats (Forster et al., 1997). Therefore neurons near the VLM surface are not essential to sustain breathing in the awake goat (Forster et al., 1997). However it did have a profound effect on the response to hypercapnia and hypoxia (Forster et al., 1997). Greater effects of cooling were seen in the response to hypercapnia than hypoxia and during exercise; thus the effects of cooling are due to the inactivation of CO_2 sensitive neurons (Forster et al., 1997). As respiration was reduced across all parameters tested, it appears that chemosensitive neurons of the VLM provide a facilitatory effect on more dorsal areas independent of chemoreception (Forster et al., 1997). Although the experiments described so far show that sites on the VLM surface do in fact contribute to the ventilatory response to hypercapnia, they do not distinguish whether these are intrinsic responses or due to the integration of the peripheral chemoreceptors. Cooling of the ventral surface was without effect on peripheral chemosensory stimulation; thus the VLM does not integrate peripheral chemoreceptors (Forster et al., 1997).

1.3.2 Cell types involved in CO_2 chemosensation

The medulla has a thickened marginal glial layer, which extends from the surface of the medulla to a depth of 150μm. The MGL of the rostral VLM contains numerous neurons, although only few neurons are seen in the MGL of many other brainstem regions (Okada et al., 2001). Many c-Fos studies have been undertaken to identify chemosensitive neurons of the VLM. c-Fos is a proto-oncogene that becomes active when neurons fire, and thus is a marker of neuronal excitability. There are however some limitations to its use and data collected by these studies must be carefully interpreted. In order to increase levels of c-Fos to a detectable level, neurons must be activated for a sustained period of time; typically animals are put into CO_2 rich environments (>9%) for at least 1 hour. This is a very unphysiological stimulus and could lead to the activation of neurons that do not typically respond to hypercapnia. It must also be noted that not all neurons express c-Fos and so may be misinterpreted as non-chemosensitive. Conversely, c-Fos is expressed in all neurons that express it and are active, whether they participate in the response to a given stimulus or not; thus some neurons will be misconstrued as chemosensitive when they are not. Finally c-Fos studies are unable to distinguish between neurons that are intrinsically sensitive to a given stimulus (primary order neurons) and those stimulated by synaptic transmission (second order neurons).

As one would expect c-Fos studies have shown CO_2-induced positive cells in the ventral medulla (Belegu et al., 1999, Sato et al., 1992, Wickström et al., 1999). Most c-Fos positive cells lie predominantly within a few hundred micrometers of the ventral surface, but some are seen at deeper levels when 21% CO_2 was inhaled for 1 hour (Belegu et al., 1999) or 10% for 3 hours (Okada et al., 2002). In contrast exposure to lower CO_2 levels, 13-15% for 1 hour, c-Fos immunoreactivity in deeper structures was not different to controls (Sato et al., 1992). Neurons embedded within the MGL are typically of small diameter and surround fine vessels, whereas those seen in deeper structures have a larger diameter (Okada et al., 2001, Okada et al., 2002). These c-Fos studies have shown that 67-75% of c-Fos positive cells reside within the first 50μm of the surface of the VLM (Sato et al., 1992, Okada et al., 2002), which constitutes approximately 29% of all superficial cells (Okada et al., 2002). Interestingly c-Fos expression in small diameter neurons within the MGL is unaffected by the blockade of synaptic activity with either low calcium/high magnesium or Tetrodotoxin (TTX) (Okada et al., 2002). On the other hand larger diameter neurons showed almost no c-Fos reactivity in either of the synaptic block solutions (Okada et al., 2001). The topology of c-Fos staining in the VLM closely resembles that of areas M, L and S (Sato et al., 1992). Intriguingly shortly after birth the VLM is the only location in which c-Fos can be induced by hypercapnia (Wickström et al., 1999). In conjunction injections of HCl, creating a fall in pH of 0.2pH units (a chemosensory stimulus in the absence of CO_2), a similar drop to that seen in arterial pH during hypercapnia, increased c-Fos staining in all surface regions of the medulla (Douglas et al., 2001).

1.3.3 The marginal glial layer of the VLM may contribute to CO_2 chemosensory transduction

It is important to remember that although neurons are present at the surface of the VLM, the primary cell type that resides here are astrocytes. Therefore one cannot discount their participation in the hypercapnic ventilatory response. In fact it has been speculated (Okada et al., 2001, Filiano et al., 1990) that the primary chemoreceptor cells may be a kind of neuroglia which process hypercapnia and release chemical messengers. Thus the significance of glia near the medullary surface in central chemoreception needs to be further studied.

The first indication that glia may participate in the adaptive changes in breathing to alteration in PCO_2 was that presumed glia cells within the MGL depolarise in response to hypercapnia (Fukuda et al 1978, Ritucci et al 2005). In one study this was shown to be a direct interaction with H^+ as they depolarise in response to changes in pH_e without concomitant changes in PCO_2 (Fukuda et al., 1978). However this study did not distinguish whether this is a direct effect of H^+ on these cells, or a secondary effect of H^+ acting on neurons of this region, which in turn influence these cells. Nevertheless the reason for this depolarisation and the exact role they play is a yet an unanswered question.

Importantly perfusion of the rostral VLM with fluorocitrate, a glial toxin, reduced the hypercapnic ventilatory response by 10% in anaesthetised rats (Erlichman et al., 1998). However these studies are by no means conclusive, fluorocitrate only inhibits the tricarboxylic acid cycle but does not affect the ribose shunt pathway. Thus under these experiments it may still be possible for glia to produce adenosine triphosphate (ATP) and may leave other intracellular processes intact. Importantly the animals were not treated with isocitrate, which could bypass the effects of fluorocitrate on astrocytes and thus recover the animals to act as an important control. In addition glia are closely associated with blood vessels in the medulla (Bradley et al., 2002). It is therefore possible that this association exists so they may sample the PCO_2 content of arterial blood. In addition a small proportion of SIDS patients present with a low GFAP (glial fibrillary acidic protein; a marker of glia) density (Biondo et al., 2003); thus loss of glia in humans may lead to sleep apnoeas in a subset of patients. Therefore though their involvement has not been conclusively proven, it cannot be ruled out.

1.3.4 Evolutionary origins of chemosensitive sites on the surface of the VLM

CO_2 chemosensitivity of the VLM surface may have arisen from our amphibian ancestors. There are discrete nuclei on the surface of the medulla of frogs adjacent to cranial nerve (CN) 5 and CN 10 which are chemosensitive (Tayor et al., 2003). Although the Frog chemosensitive sites are not homologous to the previously described mammalian sites, they share many important similarities (Tayor et al., 2003). Unilateral injections of CO_2 rich solutions into each chemosensitive site causes an increase in ventilation (Tayor et al., 2003). Lesions of either the CN 5 or CN 10 regions removes the hypercapnic ventilatory response and these sites are within

100µM of the surface (Tayor et al., 2003). Intriguingly lesions of the caudal site but not the rostral site reduced resting ventilation, showing that some chemosensitive sites in the bull frog also provide facilitatory drive to the respiratory rhythm generator (Tayor et al., 2003).

1.4 Chemosensitive neurons of the VLM

Although the VLM is undoubtedly important it does not consist of a homogenous pool of neurons and is comprised of several chemosensitive nuclei.

1.4.1 Primary and secondary chemosensitive neurons

Not all primary chemosensitive neurons feed directly into the respiratory rhythm generator (figure 4). For instance glomus cells of the carotid body stimulate the carotid sinus nerve (Spyer et al., 2003), which synapse in the nucleus tractus solitarius, which innervates the retrotrapezoid nucleus (Moreira et al., 2007). These neurons excite the pre-Bötzinger complex (Mulkey et al., 2004) which project to motor neurons that control respiratory muscles (Duffin et al., 2000).

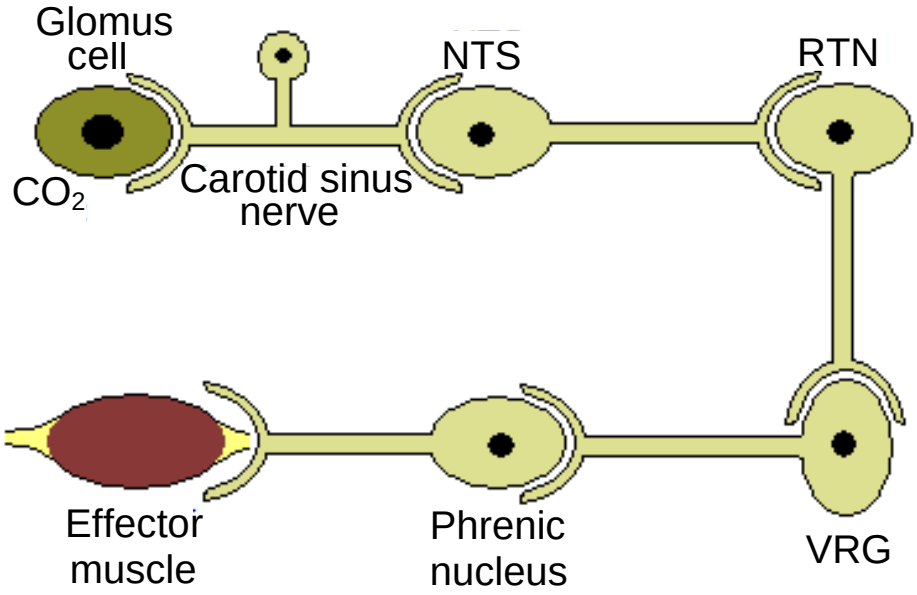

Figure 4: The peripheral chemosensory pathway from the glomus cells of the carotid body to the diaphragm.

In this case the glomus cell is the primary chemosensor as it is responsible for detecting fluctuations in PCO_2. The other neurons involved are known as second order chemosensory neurons, as they are important in conveying the message of the primary chemosensor. Importantly the loss of any of the constituent cells of this pathway will result in a loss of the response.

Thus it is important that, when performing experiments, we distinguish between whether we are affecting first or second order chemosensory cells. If experiments are performed which remove cells that have an identified chemosensory transducer then we can be sure we have removed a primary chemosensor. Alternatively cells can be identified as primary chemosensors by using a cocktail of antagonists for all known neuro- and gliotransmitters, though this has the risk that an unknown transmitter may play a role. Several types of lesioning studies can be performed to disrupt a reflex arc. By lesioning different aspects of the arc we can evaluate its involvement in the physiological reflex. However these studies must be interpreted carefully.

Electrolytic lesions and cauterisation aimed at destroying the cells of a chemosensitive nucleus, could cause a reduction in the ventilatory response to hypercapnia. However electrolytic lesions do not just destroy the somata of neurons in a specific region, they will also destroy neurons of the passage. If the axons of neurons that pass through an area are destroyed it may remove the reflex, but this will appear to be due to the removal of the somata in this area.

Genetic lesioning, such as the removal of genes in the form of knockout mice will remove a genetic component from a cell type of the reflex arc. This can be in the form of a neurotransmitter such as serotonin which will remove serotonergic signalling and thus test the participation of serotonergic neurons in a reflex. Alternatively it could remove a chemosensory transducer, the removal of this molecule could remove a specific subset of neurons or many neurons in the pathway. However even if serotonergic signalling is removed, the pathway may still remain intact but employ a different transmitter such as acetylcholine. Hence it would appear that this neurotransmitter does not participate in the response providing a false negative. Alternatively animals with a constitutive lesion from conception may show developmental compensation for this loss, thus creating the illusion this gene is not important.

Finally there is pharmacological lesioning. The use of pharmacological agents to antagonise a receptor removes the influence of a neurotransmitter and thus can provide evidence for its involvement in the reflex. However a pharmacological agent may have non-specific effects; thus it will affect neurons in a way which is not intended. Therefore it may also provide a false positive.

1.4.2 Respiratory neurons

Respiratory (increased CO_2/H^+) and metabolic acidosis (increased H^+ at constant PCO_2) increase frequency in neonatal rat 'en bloc' preparations (Kawai et al., 2006). Though the responses to metabolic and respiratory acidosis may cause similar changes in ventilation, they are mediated by different channels (Kawai et al., 2006).

A rise of CO_2 from 2-8% increases phrenic nerve discharge (PND) by 70% (Kawai et al., 1996), due to a reduced burst length and expiratory interval in mice (Kawai et al., 1996, Infante et al., 2003), whereas burst amplitude stays unchanged in the rat (Kawai et al., 2006). Alterations in PND are a result of the effect that moderate shifts in pH or PCO_2 have on membrane excitability (Kawai et al., 1996). Hypercapnic (respiratory) acidosis depolarises inspiratory neurons resulting in their discharging, whereas hypocapnic alkalosis has the opposite effect (Kawai et al., 2006).

When solutions are switched from hypocapnic alkalosis to hypercapnic acidosis pre-inspiratory (pre-I) neurons increased firing during all phases of their bursting (Kawai et al., 2006). In contrast to inspiratory related neurons post-inspiratory (post-I) and expiratory neurons hyperpolarised in response to hypercapnia and decreased discharge (Kawai et al., 1996). In the presence of TTX or in calcium free solutions to stop synaptic activity, both post-I and expiratory neurons retained their hyperpolarising response but in the absence of discharge (Kawai et al., 1996), whereas inspiratory neurons depolarised (Kawai et al., 1996).. As well as bursting neurons that respond to hypercapnia there are tonically firing neurons that are sensitive to it, these neurons were either excited by CO_2 or depressed by it. Tonically excited neurons depolarised in response to hypercapnia and increased their discharge, these neurons also depolarised in TTX and low calcium (Kawai et al., 1996). Tonically depressed neurons hyperpolarised and decreased discharge in response to increased PCO_2, and maintained their change in membrane polarity in synaptic block (Kawai et al., 1996). Thus during hypercapnia there is reduced firing of expiratory neurons and an increase in firing in inspiratory related neurons. The maintenance of membrane potential changes in synaptic block has been thought to show that respiratory neurons are intrinsically chemosensitive (Kawai et al., 2006).

The cell bodies of respiratory neurons reside between 90-400μm from the surface with dendrites projecting to within 50μm of the VLM surface regardless of cell body depth (Kawai et al., 2006). Cell body depth was independent of response time and not deep enough to elicit the fast responses seen with hypercapnia; hence it

is the dendrites that are likely to be chemosensitive (Kawai et al., 1996). In conjunction neurons that lack dendrites but whose cell bodies are at the same depth as hypercapnia modulated respiratory neurons are insensitive to CO_2 (Kawai et al., 1996).

As well as neurons of the Bötzinger and pre-Bötzinger complex, pre-I neurons are intrinsically and synaptically chemosensitive (Kawai et al., 2006). Excitation of these neurons by hypercapnia increased both the frequency and amplitude of drive potential (Kawai et al., 2006). As hypercapnic (respiratory) acidosis caused greater responses than isocapnic (metabolic) acidosis and isohydric hypercapnia (increased CO_2, at a constant pH) it appears that CO_2 itself has an effect (Kawai et al., 2006). Though it must be stated that the respiratory neurons of the adult *in vivo* have been shown to be unresponsive to H^+ (Ribas-Salgueiro et al., 2003). Even if they are not chemosensitive, they must at least be second order neurons, but which transmitters affect them?

1.4.3 Medullary raphé neurons

The medullary raphé neurons are serotonergic neurons that either have their cell bodies in the marginal glia layer or if their cell bodies are in deeper structures and have dendrites that run parallel to the surface (Ribas-Salgueiro et al., 2005). In conjunction with their close proximity to the VLM surface serotonergic neurons are closely associated with arteries. These arteries perfuse the medulla before the rest of the brain, thus enabling serotonergc neurons to sense blood gases before they are affected by brain metabolism (Richerson, 2004). Phenylethanolamine N-methyltransferase (PNMT), a marker of adrenergic cells also co-localises with markers of serotoninergic cells. These PMNT positive cells were often in direct apposition to the basal lamina of small capillaries, intraparenchymal blood vessels and neighbouring astrocytic processes (Okada et al., 2001). Other studies have also shown that neurons expressing serotonergic markers are tightly apposed to both surface and penetrating large arteries of the VLM (Bradley et al., 2002, Severson et al., 2003), and that only glial end feet separate these neurons from large blood vessels (Bradley et al., 2002).

These cells have been shown to be highly sensitive to fluctuations in pH_i *in vitro* (Ribas-Salgueiro et al., 2005, Wang et al., 2002) and to some extent *in vivo* (Mulkey et al., 2004, Ribas-Salgueiro et al., 2003, Veasey et al., 1995). Their chemosensitivity has been shown to vary with age, during the first two postnatal

weeks there was an increase in responsive neurons (3-18%) *in vivo* (Wang and Richerson, 1999), and an increase in the strength of the response in both neurons that are inhibited and excited by hypercapnia in cultured raphé neurons (Wang and Richerson, 1999). After the second postnatal week, the response remains unchanged with 22% (Veasey et al., 1995) to 27% of cells in midline raphé being excited by hypercapnia (Okada et al., 2002). An increase in inspired CO_2 increases ventilation by increasing inspiratory burst amplitude in conscious cats (Veasey et al., 1995). In conjunction exercise which increases arterial CO_2 also increases activity in raphé neurons of cats *in vivo* (Veasey et al., 1995). The increase in firing of these neurons will lead to increase in respiration, which will reduce $PaCO_2$ back to resting levels. Raphé neurons are also either highly or mildly responsive to isocapnic acidosis, and displayed an increased firing frequency of burst patterning in anaesthetised rats (Ribas-Salgueiro et al., 2003).

It appears that the serotonin 1a receptor (5-$HT1_a$) is an important receptor in these neurons. A 5-HT_{1a} receptor antagonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH DPAT) reduced firing in raphé neurons when injected intravenously (IV) or subcutaneously (Veasey et al., 1995). Blockade of 5-HT using methysergide (a 5-$HT_{1/2}$ receptor antagonist) not only reduces respiratory rate but also reduces c-Fos staining in these areas, both *in vivo* and *in vitro* (Bodineau et al., 2004).

1.4.3.1 Alterations of pH_i is the chemosensory stimuli of raphé neurons

Raphé neurons *in vitro* responded equally to all forms of chemosensory stimuli, isohydric hypercapnia (IH), hypercapnic acidosis (HA) and isocapnic acidosis (IA) (Wang et al., 2002). However the increase in firing did differ between these chemosensory stimuli so that IH>IA>HA (Wang et al., 2002). These responses were unaffected by application of the carbonic anhydrase inhibitor acetazolamide (AZ), leaving the conclusion that it is in fact alterations in pH_i that are primarily responsible for the change in firing rate (Wang et al., 2002). It has also been shown that their intrinsic chemosensitivity to pH_e comes from the presence of TASK (tandem-pore acid sensing channels) 1 and 3 channels (Mulkey et al., 2007b). These channels are highly sensitive to certain anaesthetics such as halothane (Mulkey et al., 2007b), which may provide a reason for their low CO_2 sensitivity in anaesthetised animals *in vivo* (Mulkey et al., 2004). The fact that TASK knockouts show no deficiency in their CO_2 response has left doubt over whether serotonergic neurons are functional

chemosensors (Mulkey et al., 2007b). However since these mice have lacked these channels since birth it is possible that they have compensated for this somehow.

Although their intrinsic chemosensitivity has been called into question, it is still certain that they participate in the response to hypercapnia; as animals seem to be unable to compensate for the loss of serotonergic neurons (Li and Nattie, 2008b). Therefore it is highly likely the TASK channels are not the primary chemosensory transducer for serotonergic neurons. Thus serotonergic neurons appear to be second order neurons, responding to the release of a transmitter from primary chemoreceptors. The remaining questions therefore are which transmitter is influencing these neurons and why are they so closely associated with blood vessels?

Their close association with blood vessels has always been assumed to be to increase their ability to detect changes in arterial PCO_2. Importantly serotonergic neurons are not directly apposed to blood vessels, instead they are actually associated with glial end feet that surround the artery (Bradley et al., 2002). Thus it may be that it is their association with glia that is important and the proximity of these neurons to arteries is due to the necessity of glial contact with arteries. In other words, glia are in close contact with arteries, as they are responsible for sensing arterial PCO_2, they then release a transmitter to signal alterations in arterial PCO_2 which is detected by serotonergic neurons. Therefore serotonergic neurons are closely associated with glial end feet around blood vessels, so that they may detect this signal.

1.4.3.2 Anatomical connections of the raphé nuclei

Anterograde tracers injected into the caudal VLM, looking for efferent projections revealed connections with: the rostral VLM, lateral reticular formation and the nucleus tractus solitarus of the medulla; the parabrachial nucleus, locus coeruleus and the A5 region of the pons; the motorneurons of the cranial nerves in the cat (Stocker et al., 1997). Once stimulated the medullary raphé exert their influence over the hypercapnic ventilatory response by innervating respiratory and other chemosensory centres (Ribas-Salgueiro et al., 2005, Mulkey et al., 2007a).

1.4.3.3 Serotonergic neurons influence over the drive to breathe

Serotonergic neurons are a source of tonic drive for respiration (Richerson, 2004, Hodges et al., 2008). Inhibition of the raphé obscurus inhibits the persistent sodium current (I_{NaP}) in the pre-Bötzinger complex (Pace et al., 2007). I_{NaP} currents provide a depolarising current when pacemaker neurons are hyperpolarised stabilising

their Vm (Tryba and Ramirez, 2004). This keeps the pacemakers Vm within a range where bi-stable properties can be expressed (e.g., regenerative plateau formation), which is vitally important to their function (Tryba and Ramirez, 2004). Thus raphé neurons project to the pre-Bötzinger complex and increase excitability (Pace et al., 2007). A second source of tonic input is derived from the raphé magnus (RM) (Li et al., 2006). Inhibition of the medullary raphé in a location that include raphé pallidus (RP) and raphé obscurus (RO) causes hypoventilation (Li et al., 2006), thus they provide a facilitatory drive to respiration. However they are not essential for rhythm generation (Hodges et al., 2008). Thus any excitation or inhibition here will lead to alterations in respiratory rate. The exact nature of their participation is unclear as there are differential reports as to how these neurons influence breathing. Serotonin transporter knock out mice exhibit higher breathing rates than wild types of both sexes (Li and Nattie, 2008b), however ablation of medullary raphé with 5,7-dihydroxytryptamine (5,7-DHT) did not affect basal respiration (Penatti et al., 2006).

1.4.3.4 Serotonergic neurons influence over chemoreception

They may also influence adaptive breathing to alterations in CO_2, through the release of substance P, TRH (tyrosine hydroxylase) and serotonin which are co-expressed in raphé neurons, and known to excite chemosensory neurons (Mulkey et al., 2007a, Richerson, 2004). The absence of serotonergic neurons by genetic (cre-lox) re-combination causing the removal of the homeobox gene *Lmx1b* only in serotonergic neurons leads to a reduced response to CO_2 challenges (~50%) (Hodges et al., 2008). These mice show a developmental compensation with greater emphasis on peripheral receptors; importantly the hypercapnic ventilatory response could be rescued by intracerebroventricular injections of 5-HT (Hodges et al., 2008). The fact that the hypercapnic ventilatory response could be rescued by the addition of the neurotransmitter released by the cells that were removed indicates that the rest of the respiratory network is intact in these animals and that this is not due to a non-specific effect of the removal of the gene.

Serotonin transporter knockout mice have increased 5-HT in their extracellular fluid (ECF), which leads to a desensitisation of their receptors, and a decrease in their respiratory response to CO_2 (Li and Nattie, 2008b): approximately 48-68% in males and 15-38% in females (Li and Nattie, 2008b). Though in pigs it has been shown that lesions of 5-HT neurons with 8-OH DPAHT leads only to a decrease in the CO_2

response in males and only during NREM (non-rapid eye movement) sleep (Penatti et al., 2006) as there are sex differences in the ability to compensate for medullary lesions (Penatti et al., 2006). The differences between these studies may also be because CO_2 sensitivity does not change during post-natal life in pigs as it does with rats. The idea that chemosensitivity differs between these two species is further supported by the fact that aberrations in serotonergic neurons do not affect hypoxia in rodents (Hodges et al., 2008, Li and Nattie, 2008b), but do affect hypoxic responses during NREM in piglets (Penatti et al., 2006).

1.4.3.5 Effects of different raphé nuclei on respiration

Although serotonergic neurons are usually grouped together it must be said that the raphé consist of three nuclei; RM, RP and the RO, which all contain serotonergic neurons in adult rats (Connelly et al., 1989). It is important to note that these areas provide differential responses on respiration when electrically stimulated; the RM is associated with inhibition of respiratory drive, whilst the RP provides a facilitatory role and the RO causes both inhibitory and excitatory responses (Cao et al., 2006). Stimulation of the RM depressed the inspiratory phase and ceased respiratory movements at the end of the expiratory phase, thus slowing respiration (Cao et al., 2006); it should also be noted that this area inhibits the response to hypoxia (Penatti et al., 2006). Thus the RM could provide a GABAergic (gamma aminobutyric acid) input into the respiratory system and the RP a serotonergic one (Cao et al., 2006). c-Fos studies have revealed that during hypercapnia greater activity is seen in the caudal nuclei of the raphé, in the RP and RO, the raphé neurons known to facilitate respiration (Belegu et al., 1999). This mediation may arise from raphé spinal projections (Cao et al., 2006, Weston et al., 2004) in conjunction with those to other medullary nuclei (Cao et al., 2006).

It must also be noted that as well as differential responses between nuclei, there are differential responses between neurons in the same locale, such that some are inhibited by hypercapnia and others excited by it: chemosensory inhibited cells act oppositely to chemosensory activated cells (Wang et al., 2002). Although the strength of inhibition on breathing varies dramatically, depending on the location within the medullary raphé (Li et al., 2006). These neurons were also stimulated by all forms of chemosensory stimuli so that the magnitude of inhibition was HA=IH>IA (Wang et al., 2002).

1.5 The retrotrapezoid nucleus

The RTN is located ventral to the facial (VII) nucleus and contains glutamatergic Phox2b positive neurons (Mulkey et al., 2004, Kang et al., 2007) which do not contain serotonin (Connelly et al., 1989). The neurons are located at 130-230μm from the ventral surface, with a few cell bodies located in the MGL (Mulkey et al., 2004). Their dendrites project ventrally with extensive secondary dendrites located within the MGL (Mulkey et al., 2004). Since 67% of c-Fos positive cells lie within the first 200μm of the ventral surface and 60% c-Fos positive cells lie rostral to the midline (Sato et al., 1992), a vast proportion of the cells stained in this study are RTN neurons. This location enables the dendrites to sample CSF in close proximity to the basilar artery, and thus sense CO_2 of the blood before it is affected by cellular metabolism (similar to the medullary raphé). These cells are exquisitely sensitive to CO_2 when levels are above 4% *in vivo* (Mulkey et al., 2004, Hewitt et al., 2004) and *in vitro* (when it leads to hypercapnic acidosis) (Mulkey et al., 2004), and from injections of AZ (resulting in decreased pH) directly into this nuclei (Hewitt et al., 2004). Though their pH sensitivity *in vitro* is half of that *in vivo*, as the temperature difference between these preparations significantly alters their pH sensitivity (Guyenet et al., 2005a). This discrepancy may also be due to the large pH changes used in the *in vitro* preparation (~0.5 pH units) which could not be achieved *in vivo* (~0.15 pH units see section 1.9.4). Their CO_2 sensitivity is preserved in the presence of blockers of excitatory transmission both in the RTN and the pre-Bötzinger complex; and it has been shown that the central pattern generator (CPG) activity during hypercapnia is secondary to RTN firing (Mulkey et al., 2004). Thus during hypercapnia increased firing in RTN neurons causes increased discharge in pre-Bötzinger neurons.

1.5.1 Intrinsic chemosensitive properties of RTN neurons

Their intrinsic pH sensitivity has been shown to be linked to a background K^+ current (Mulkey et al., 2004), it appears that hypercapnia blocks outward rectifying potassium channels (figure 5(i)) (Wellner-Kienitz et al., 1998). Unlike serotonergic neurons this effect is not dependent on TASK channels (Mulkey et al., 2007a). The RTN contains over 2000 glutamatergic neurons that are activated by acidification via reductions in potassium conductance (Guyenet, 2008). Although they are intrinsically chemosensitive, it has not been ruled out that respiratory acidosis could release an

unknown substance from neighbouring glia or blood vessels and this substance could activate receptors located on the RTN neurons causing the modulation of their potassium conductance (Guyenet et al., 2005b). Although these authors have ruled out serotonergic and purinergic modulation of their pH sensitivity in neonates (Mulkey et al., 2004, Mulkey et al., 2007a), this has not been done in adult preparations or *in vivo*; thus their modulation by these transmitters in the mature hypercapnic response cannot be ruled out. However although serotonin does not affect the intrinsic pH sensitivity of these neurons, it is not without effect; serotonin and pH are additive showing different mechanisms in RTN neurons (Mulkey et al., 2007a). Therefore it has been hypothesised that serotonin could bring RTN neurons close to their firing threshold, magnifying their response to pH changes and thus increasing central chemosensitive reflex gain (Mulkey et al., 2007a). Since at least one other transmitter affects these neurons, it may be a yet overlooked transmitter that performs a similar role.

1.5.2 Influences on RTN neurons

The RTN provides the major drive to breathe, as it is the major site of CO_2 sensing and CO_2 is the major drive to breathe, but it also comes under the influence of many feed forward inputs and reflexes (Guyenet, 2008). Activity of RTN neurons is unregulated by descending inputs from the hypothalamus involved in respiration (Guyenet, 2008). The RTN receives convergent inputs from lung mechanoreceptors, peripheral chemoreceptors and cardiopulmonary C-fibre afferents which first synapse in the NTS (Moreira et al., 2007, Takakura et al., 2006). Though peripheral denervation does not affect their response to hypercapnia (Mulkey et al., 2004). It also receives direct inputs from the NTS (a known chemosensitive site), particularly glutamatergic neurons of the commissural NTS as activity here causes spiking in the RTN (Takakura et al., 2006, Bodineau et al., 2000). It also receives inputs from the ventrolateral NTS but whether these are chemo- or baro- receptors was not elucidated (Bodineau et al., 2000), however it is most likely that that these are slow adapting pulmonary stretch receptors (Moreira et al., 2007).

The RTN also receives inputs from the medullary raphé and injections of 5-HT, TRH and substance P (neurotransmitters released by serotonergic neurons) all increased PND and single cell firing rate of neurons in the RTN (Mulkey et al., 2007a). There is an implication that the serotonergic neurons of the raphé obscurus provide a tonic drive to the RTN (Li et al., 2006) as it does for the pre-Bötzinger

complex (see section 1.4.3.3). Inhibition of serotonergic neurons in the caudal medullary raphé markedly potentiates the effect of inhibition in the RTN (Li et al., 2006). Inhibition of these areas causes a hypoventilation and a substantial reduction in CO_2 response (Li et al., 2006). The extensive afferent projections to the RTN has led to the proposal that the RTN could be a chemosensory integrator (Takakura et al., 2006), as other respiratory reflexes probably modify respiration by altering chemoreceptor drive (Moreira et al., 2007). However some believe that this is not the final common pathway (Guyenet, 2008), as cooling the VLM in the vicinity of the RTN did not affect the response to carotid nerve stimulation (Forster et al., 1997). The fact that the RTN receives inputs from peripheral CO_2 sensors and is itself pH sensitive may explain why lesions here reset the accepted arterial CO_2 to above normal levels and may explain why it is so important in the drive to breathe (Nattie, 2006). Thus this region appears to be both a primary and secondary chemosensory nucleus.

1.5.3 Connections between the RTN and the VRG

Glutamatergic neurons of the VLM (RTN) project ipsilaterally to the VRG and pontine respiratory group (PRG) (Mulkey et al., 2004, Connelly et al., 1989), pre-Bötzinger complex and Bötzinger complex (Weston et al., 2004) but not the spinal cord (Weston et al., 2004, Kang et al., 2007). The latency in the response from stimulation of these areas shows the projections to be unmyelinated neurons. RTN neurons are poised to provide a tonic excitatory drive to the CPG (Guyenet et al., 2005b, Nattie, 2000), as well as chemosensory information (Nattie, 2000). Cooling of the VLM, which affects the RTN, has shown that the RTN has a facilitatory function on breathing (Forster et al., 1997). However there are discrepancies between the effects of lesions on room air breathing, having either no effect (Akilesh et al., 1997) or causing hypoventilation (Li et al., 2006). A hypoventilation would appear more plausible due to the fact the RTN provides a tonic drive to the pre-Bötzinger complex (Li et al., 2006). The RTN neurons receive inhibitory inputs from the CPG and, when blocked, the intensity of firing in the RTN rises with PCO_2 until saturated (Guyenet et al., 2005a). In conjunction, after CPG blockade their firing becomes tonic (Guyenet et al., 2005a).

Although the RTN is involved in the hypercapnic ventilatory response, these neurons are not activated until CO_2 levels increase to 5%. At low levels of inspired

CO_2 below the RTN activation point, frequency changes occur indicating that more than just this nucleus is involved (Li and Nattie, 2002).

Nucleus	pH_e	pH_i	CO_2	HCO_3^-	TTX/ low Ca^{2+}	Excitatory block
RTN	+	+		?	+	+
NTS	+					+
Raphé	+	+		?		
pre-Bötzinger complex	?	?			+	+

Table 1: Summary of features of areas involved in the hypercapnic adaptive ventilatory response. + represent what is known, ? represent what is hypothesised and blank squares represent what is unknown.

1.6 Distributed versus specialised theory of chemosensitivity

At present there are two theories as to how the medulla oblongata detects changes in arterial CO_2; the specialised chemosensitivity theory, which states that there are a few dedicated cells which monitor pH and send information to the respiratory centres: and the distributed chemosensitivity theory, which states that all brainstem neurons are chemosensitive and it is a summation of the effects on the medulla as a whole that causes the change in CPG output (Guyenet et al., 2005b, Guyenet, 2008). Although many brainstem neurons are pH sensitive, some neurons (i.e. RTN) are dramatically more chemosensitive (Guyenet et al., 2005b), though their chemosensitivity is determined using extremely unphysiological changes in extracellular pH. TASK channels confer intrinsic pH-sensitivity to most neurons (Mulkey et al., 2007b) and TASK knock outs show no alterations in the hypercapnic ventilatory response, thus the intrinsic chemosensitivity of most medullary neurons is not required for the chemoreceptive response (Mulkey et al., 2007b). In conjunction after excitatory block, the RTN remains chemosensitive, but the CPG does not (inferring that it is not intrinsically chemosensitive) (Guyenet et al., 2005b). Since the pH sensitivity of the majority of medullary neurons is not necessary for the full hypercapnic ventilatory response, and there are apparent intrinsically sensitive neurons in discrete nuclei which are essential for adaptive breathing to hypercapnia, chemosensitivity is most likely conveyed by specialised groups of neurons within the brain.

1.7 The peripheral nervous system

In the 1930s a series of experiments in anaesthetised dogs, led to the search for the role of the carotid body in respiratory control (Heymans and Bouckaert, 1930). Though it was known that respiration could be altered by the peripheral nervous system, this was the first time it had been shown to be due to activation of the carotid body (Heymans and Bouckaert, 1930). They observed that by removing the carotid body they were able to remove respiratory related apnoeas to intravenous adrenaline and that increasing pressure within the carotid body increased breathing (Heymans and Bouckaert, 1930). Glomus cells are chemosensors of the carotid body and are the principle peripheral chemoreceptors (Peers and Buckler, 1995). Heeringa et al 1979 estimate their input to be 20-50% of the total hypercapnic ventilatory response (Nattie, 2000).

In these cells a rise in intracellular Ca^{2+} leads to neurosecretion (Peers and Buckler, 1995). During hypoxia this is due to the inhibition of K^+ channels hypoxia (Peers and Buckler, 1995), whereas for CO_2 transduction it is through alterations in pH_i (Peers and Buckler, 1995).

1.8 Complexity of chemosensitivity

Though chemosensitivity is determined by specialised groups of neurons, these neurons appear to be present in multiple nuclei throughout the brain (Mulkey et al., 2004, Filosa et al., 2002, Wang et al., 2002, Nattie and Li, 2002a). What is the reason for such complexity and the necessity of multiple sites? It has been hypothesised that one set of chemosensors could be the primary set, but other areas compensate for its loss (Richerson, 2005). Although redundancy is likely to occur in such an important function as breathing, this is not likely to be the only reason.

Conditions placed on the respiratory system are constantly changing, increased CO_2 during exercise will be accompanied by lactic acid formation, whereas re-breathing expired air will not. Thus as conditions change, the influence of individual chemoreceptors changes, which would increase the adaptability/flexibility of the system (Richerson, 2005). In support of this there are many physiological reflexes associated with hypercapnia and different areas perform different functions (Nattie, 2000). There are many stimuli that cause chemosensitive cells to discharge; in neurons this is primarily pH_e (as in the RTN) and pH_i (as in the raphé). Thus different

regions sense different signals and the summation of these signals induces the appropriate response to the appropriate physiological conditions (Nattie, 2000, Su et al., 2007). There may also be some temporal specificity, in which some areas respond to acute and others to chronic conditions (Nattie, 2000). The response to hypercapnia is also not universal at all sites, they respond to different chemicals (i.e. pH, CO_2 etc) and do so at different levels (i.e. <5% or >5% CO_2) and thus may represent functional subdivisions, so some may mediate small changes and others more pathophysiological conditions.

Whatever the reason it is apparent that multiple systems are required (Su et al., 2007, Putnam et al., 2004, Li and Nattie, 2008a). The robust nature of the response to CO_2 depends on additive or greater interactions of inputs from multiple locations (Nattie, 2000) and some tonic baseline input is required from all chemoreceptors to allow full expression of the systemic response (Nattie, 2000). It is sure that the sensitivity of neuronal processing of hypercapnia is enhanced if all of the neurons in the pathway are chemosensitive (Su et al., 2007). This may provide a reason for the widespread chemosensitivity in the medullary neurons conferred by TASK channels, and why these channels, though present are not essential. Finally many sites involved in CO_2 detection provide stability in a closed loop (Li and Nattie, 2008a), and allow for corrections of regional imbalances of blood flow and metabolism, and provide redundancy and malleability (Li and Nattie, 2008a).

1.8.1 Complexity of chemosensitivity and arousal state

During wakefulness respiratory control is determined by behavioural and arousal related influences and their integration with chemoreceptive feedback (Shea, 1997). The integration of descending voluntary and involuntary and rhythmic and non-rhythmic respiratory drives which act on both the respiratory rhythm generator and the spinal motorneurons, determine our basal ventilatory rate (Shea, 1997). When we are in rapid eye movement (REM) sleep however there is a loss of voluntary inputs and a change in chemoreflexes so that the complexity (a measure of the inputs to a physiological signal) of respiratory pattern decreases (Dragomir et al., 2008). This change in complexity during arousal states may explain why CCHS patients show little perturbation in CO_2 regulation whilst awake. If given no physiological stimulants (i.e. air re-breathing, exercise etc), CCHS shows little qualitative difference in respiratory adaptation to the altered behavioural conditions (Shea, 1997). Patients

might learn behavioural cues which replace intrinsic responses (Shea, 1997), for instance they may increase breathing during exercise because they have learnt to do so and not through involuntary reflexive control.

This reduction in complexity may be due to enhancement of the GABAergic system during REM sleep, and muscimol (a $GABA_A$ agonist) injections reduce the complexity of breathing during NREM as well as REM sleep (Dragomir et al., 2008). This reduction in complexity during REM sleep will partly be due to independence of breathing on VLM chemoreception (Forster et al., 1997). During NREM sleep breathing can be made more dependent on VLM chemoreception if animals are peripherally denervated (Forster et al., 1997): Thus following carotid body denervation, cooling of the ventral surface during NREM and wakefulness led to apnoeas, whereas breathing in intact animals recovered (Ohtake et al., 1996).

Further support for this comes from studies in conscious rats where the RTN and raphé were remarkably immunoreactive for c-Fos after hypercapnia when compared to other arousal states (Okada et al., 2002). Though other studies have suggested that RTN and not the raphé are important in the conscious animal (Nattie, 2001). A study in humans has seen that the sensitivity to changes in end tidal (expired) CO_2 were higher during wakefulness (Trinder et al., 2006). Interestingly the increase in ventilation at arousal is not due to a shift from sleeping chemoreception to waking; it is due to an increase in the behavioural and arousal related inputs to the system (Trinder et al., 2006). Although GABAergic enhancement explains the alterations during REM sleep it does not however explain the alterations in chemosensitivity during NREM sleep. Studies into non-respiratory (cortical) related chemoreception, from the olfactory system and trigeminal reflex, may provide some insights. Cortical chemosensory inputs also alter during sleep stages, for instance olfactory responses are lost during NREM and REM sleep whereas trigeminal responses are lost only during REM sleep (Stuck et al., 2006). However cortical event related potentials are still seen during sleep, thus chemosensory stimuli are processed during sleep, but are processed differently (Stuck et al., 2006).

1.8.2 Anaesthesia and chemosensitivity

There is also a dramatic difference in the hypercapnic ventilatory response during anaesthesia and either wakefulness and sleep states. Contrary to what one would expect conscious rats exhibited more restricted c-Fos than anaesthetised ones

(Okada et al., 2002). This is due to the unequal suppression of chemosensitive nuclei, such that nuclei whose activities are low during consciousness can exert greater influence during anaesthesia. Also, as in conscious animals, chemoreception during anaesthesia is more dependent on structures of the VLM, such as the RTN, lateral reticular nucleus, RP and MGL (Okada et al., 2002). Under anaesthesia the influence of the NTS, which normally contributes 20-30% of the total hypercapnic ventilatory response in both sleep and wakefulness (Nattie and Li, 2002a), increases to 39% (Nattie, 2001) and the input of medullary raphé doubles (Nattie, 2001). Though these measurements provide a useful guide as to the contribution of these sites one must remember that if a site is acidified, hyperventilation can occur and cause an alkalisation at other sites that may counteract the effects of the original stimulus and so they can only be used as guidelines (Li and Nattie, 2002).

1.8.3 RTN and arousal state

Under anaesthesia the RTN plays a massive role in the CO_2 response and the drive to breathe (Nattie, 2006). Anaesthesia differs from sleep, it depresses other chemoreceptive sites more than the RTN thus increasing its influence on the total response (Li et al., 1999). Lesions here create a 39% decrease in the response to CO_2 during wakefulness and 52% reduction during anaesthesia, but do not affect room air breathing (Li and Nattie, 2002). In conjunction the tonic facilitatory role of the RTN alters with anaesthesia, cooling of area M where many of the RTN dendritic projections are found, shows differences between wakefulness and sleep and anaesthesia; during wakefulness and sleep there was a decrease in respiration which recovered, but in anaesthetised animals there was persistent apnoea (Forster et al., 1997, Ohtake et al., 1996). The facilitatory role of the RTN may also differ between sleep and wakefulness. Bilateral injections into the RTN of substance P conjugated to saporin, a ribosome-inactivating protein, (SP-SAP; which resulted in cell death in this nuclei) reduced induced hypoventilation during wake but not sleep (Nattie and Li, 2002b), whereas inhibition of the RTN causes hypoventilation during wakefulness and NREM sleep (Li et al., 2006, Nattie and Li, 2002b).

The chemosensitive response of these neurons does not seem dependent on arousal state. CO_2 dialysis (25%: which causes tissue pH changes equivalent to 7% inspired CO_2) into the RTN causes a change in the amplitude of PND by 20% and accounts for 25% of the total response in anaesthetised animals and an increase in

tidal volume of 17-24% during wakefulness (Li et al., 1999). However the relative contribution of this area does not seem to be affected in NREM sleep; 24% during wakefulness and 27% during NREM sleep (Li et al., 2006). In conjunction both uni- and bi- lateral injections of SP-SAP reduced the CO_2 response by 51% during wakefulness and 52% during NREM sleep. One study seems to contradict this: in awake rats 50-100% CO_2 dialysis increased ventilation by 10-30% during wakefulness but not when asleep, though this may be due to failure to distinguish between NREM and REM sleep (Li et al., 1999).

1.8.4 Arousal state and serotonergic neurons

Hypercapnia induced changes in breathing decrease when 8-OH DPAT lesions are applied to the RM and RP, due to tidal volume changes during sleep and wakefulness (Taylor et al., 2005). Though reductions in their CO_2 response was higher during wakefulness (22%) than sleep (10%) at lower concentrations of 8-OH DPAT, interestingly at high concentrations the responses were not different (Taylor et al., 2005). A similar result was seen *in vivo* though the number of raphé neurons that increased firing in response to hypercapnia remained constant (22%) the magnitude being much lower during sleep (Veasey et al., 1995). Thus hypoventilation and reduced chemoreception during sleep may be due to a loss of chemosensitivity of raphé neurons during sleep (Richerson, 2005). Interestingly inhibition of the medullary raphé causes hypoventilation during wakefulness and NREM sleep (Li et al., 2006, Nattie and Li, 2002b). 8-OH DPAT also altered room air breathing only during wakefulness implying a tonic drive to breathe exists only during wakefulness (Taylor et al., 2005).

Due to the drastic changes in the influence of different chemosensitive areas with arousal state, experiments need to be performed to determine exactly which areas are and are not active during each stage of the sleep-wake cycle. This may be achieved using established methods for obtaining functional connections in the respiratory network (Duffin et al., 2000).

1.9 pH regulation

1.9.1 pH regulation and chemosensitive neurons

In early postnatal life neurons from non-chemosensitive areas such as the hypoglossal (XII) nucleus show pH_i recovery from acidification caused by hypercapnic acidosis (Nottingham et al., 2001), whereas neurons from chemosensitive

areas such as the RTN do not (Nottingham et al., 2001, Ritucci, 2005 #194, Ritucci et al., 2005). The fall in pH_i associated with hypercapnic acidosis in younger animals was significantly greater in the RTN compared to the XII nucleus (Nottingham et al., 2001). The pH_i changes in acute tissue slices from younger animals exposed to high CO_2 was more profound than older animals in the RTN (Nottingham et al., 2001). Thus the RTN had a lower internal buffering power than the XII nucleus, but for both nuclei internal buffering increased with age (Nottingham et al., 2001). This leads to similar profiles in pH_i regulation in both nuclei whether they are chemosensitive or not after P11 (Nottingham et al., 2001).

RTN neurons respond to hypercapnic acidosis (fall in pH_e) in a similar way to isohydric hypercapnia (constant pH_e) (Ritucci et al., 2005). However animals of all ages were able to regulate their pH_i in isohydric hypercapnia (no pH_e change) (Nottingham et al., 2001, Ritucci et al., 2005). This tells us two things, firstly changes of pH_i are more significant than changes of pH_e in chemosensory signalling in RTN neurons (Ritucci et al., 2005, Chesler, 2003). Secondly decreases in pH_e has an inhibitory effect on recovery, and must inhibit transporters responsible for rectification of pH_i in response to an acid load (Nottingham et al., 2001) and indicates Na^+/H^+ exchangers (NHE transporters). To further support this, amiloride, a known inhibitor of NHE transporters (Paris and Pouyssegur, 1983) reduces pH_i recovery in the RTN (Nottingham et al., 2001). Whereas DIDS (4,4'-diisothiocyano-2,2'-stillbene-disulfonic acid) a bicarbonate transporter inhibitor was without effect (Nottingham et al., 2001). This indicates that brain stem neurons use NHE antiporters (figure 5(ii)) that are more readily inhibited by pH_e in chemosensitive neurons than non-chemosensitive neurons, implying chemosensitive and non-chemosensitive cells express different isoforms (Chesler, 2003). Nevertheless it must be noted at this point that amiloride has many non-specific effects and so one must be careful when attributing its effects solely to blockade of NHE transporters.

The RTN is exposed to a continuous acid load offset by extrusion of H^+ by NHE (Nottingham et al., 2001). Since chemosensitive neurons can regulate pH_i if pH_e is constant, NHE in the medulla allows pH_i recovery in IH but not HA (Putnam, 2001). Importantly 90% of RTN neurons have a maintained fall in pH_i, but only 50% respond to hypercapnic acidosis, thus maintained pH_i is not a marker of chemosensitive neurons (Ritucci et al., 2005). Therefore chemosensitive neurons must have a fall in pH_i and a molecular transducer sensitive to it (Ritucci et al., 2005).

These experiments also do not distinguish where the pH_i regulation takes place and have left a need for dendritic pH_i recording (Putnam, 2001). Since pH_i recovers it has been hypothesised that increases in intracellular HCO_3^- may play a role (as it does in glomus cells) as it will increase in IH (Ritucci et al., 2005). In addition the Cl^-/HCO_3^- exchanger is present in the VLM and is also inhibited by pH_e, whereas it is not in non-chemosensitive cells or the NTS (Chesler, 2003).

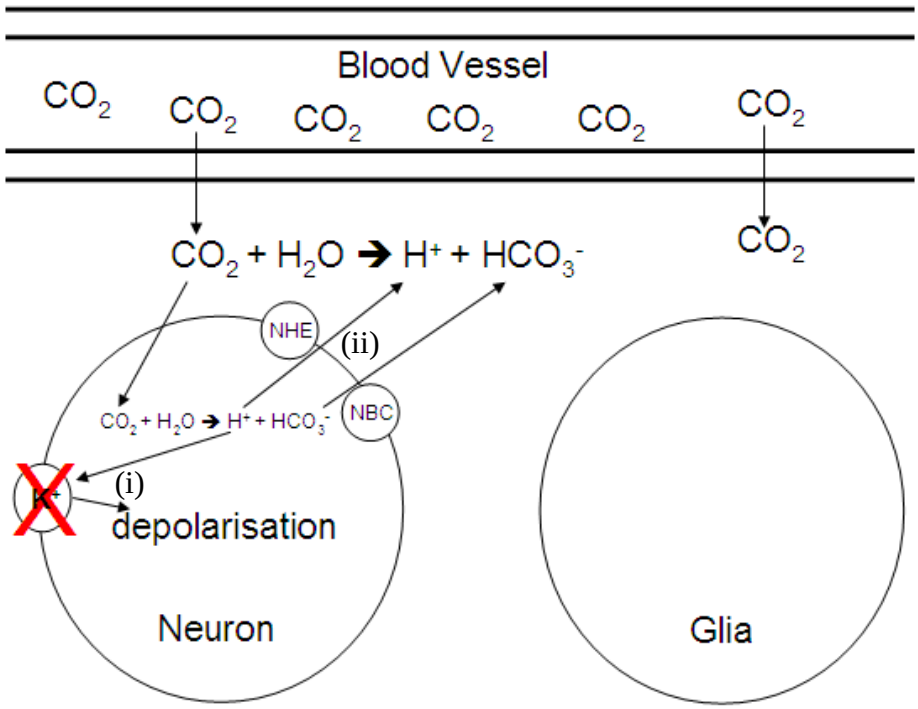

Figure 5: pH regulation of neurons in chemosensitive areas of the medulla. Carbonic anhydrase converts CO_2 to H^+ in the extracellular fluid and neuron.

1.9.2 pH regulation and glia

Glial mechanisms of pH_i regulation include NHE (for acid extrusion: figure 6 (i)) (Dietmar and Rose, 1996, Chesler, 2003); sodium driven and non-sodium driven Cl^-/ HCO_3^- exchanger for (for HCO_3^- extrusion) (Chesler, 2003, Dietmar and Rose, 1996); sodium bicarbonate cotransporter (NBC: for HCO_3^- extrusion: figure 6(ii)) at resting membrane potential are especially prevalent in glial cells (Chesler, 2003).

In cortical glia, 20mM NH_4^+ caused a biphasic intracellular pH change (basic-acidic), which glia were able to recover from (Faff et al., 1996). Removal of sodium from the perfusate caused an intracellular acidification, implying a loss of pH_i regulation through NHE (Faff et al., 1996). This was confirmed by blockade of pH_i recovery by amiloride (Faff et al., 1996), though again non-specific effects of this drug cannot be excluded. In conjunction, pH_i recovery was blocked by bicarbonate

and sodium free solution (Faff et al., 1996). However the removal of bicarbonate alone was insufficient to inhibit pH_i recovery (Faff et al., 1996). This shows that cortical glia express sodium dependent bicarbonate transporters, both Cl^-/HCO_3^- exchanger and NBC transports (Faff et al., 1996).

The resting pH_i of glia in the medulla in the NTS and RTN is 7.3 (Erlichman et al., 2003). The glia of both regions acidify upon removal of bicarbonate ions from the milieu (Erlichman et al., 2003); however their mechanisms of pH regulation are different. Glia of the RTN are alkalised by increased K^+ in the perfusate (figure 6(iii)), whereas this has no effect in the NTS (Erlichman et al., 2003). This increase in intracellular pH was blocked by DIDS (Erlichman et al., 2003), however this is by no means a specific inhibitor of NBC transporters. The removal of chloride ions from the aCSF caused a decrease in the intracellular pH of glia in both nuclei, however the fall in pH_i in the RTN was greater than that of glia cells in the NTS (Erlichman et al., 2003). Thus like cortical glia, medullary glia in both regions have Cl^-/HCO_3^- exchangers, NHE and NBC transporters (Erlichman et al., 2003, Chesler, 2003) (Ritucci et al., 2005). However the activity of these transporters differs between nuclei (Erlichman et al., 2003).

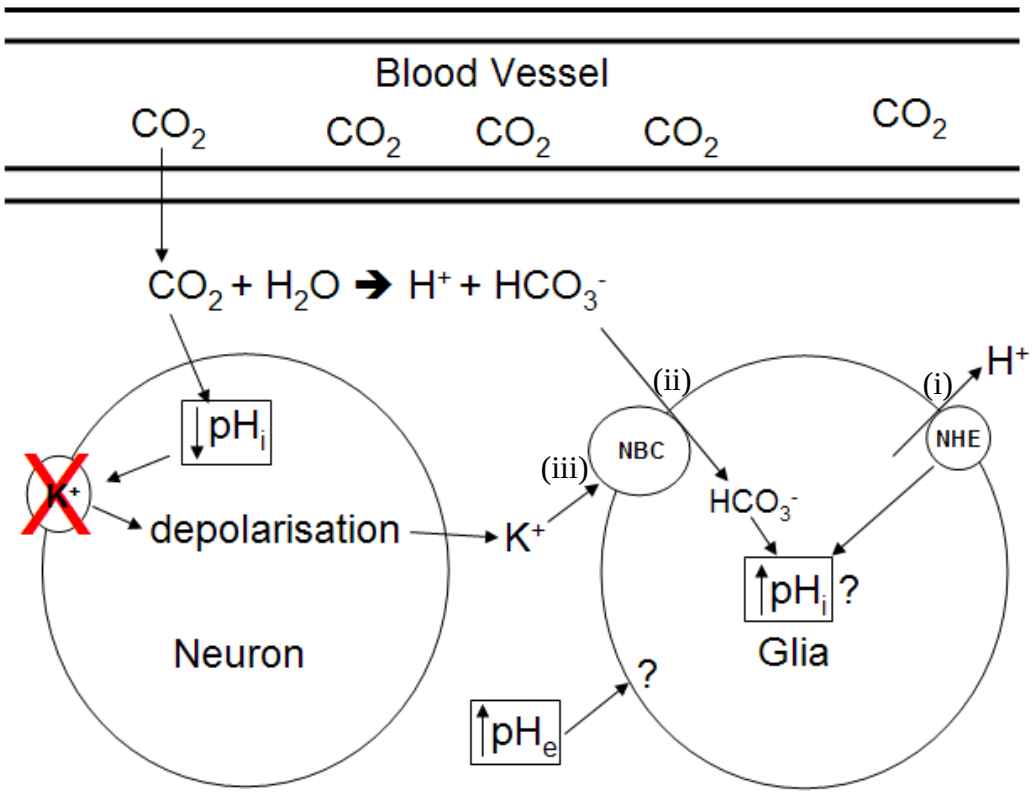

Figure 6: pH regulation of medullary astrocytes and interactions with neurons. Question mark denotes an unexplained role of the mechanism.

1.9.3 pH regulation in the medulla

Hypercapnia initiates neuronal firing, due to alterations in pH_i. The extrusion of HCO_3^- from these neurons causes an extracellular alkalisation (figure 7(i)). Neuronal activity increases extracellular K^+ concentration, which cause glial cells to depolarise (Chesler, 2003), as is seen in hypercapnic conditions (Ritucci et al., 2005). This depolarization causes a 4mV shift in membrane potential (Ritucci et al., 2005), which brings it to the reversal potential of NBC transporters (Dietmar and Rose, 1996, Chesler, 2003). Once this membrane potential is reached, glial cells begin to take up bicarbonate ions from the ECF (figure 7(ii)) (Dietmar and Rose, 1996), this acts to reduce the alkalisation caused by neuronal pH regulation (Erlichman et al., 2003). Thus during hypercapnia, glial cells show intracellular alkalisation (figure 7(iii)) (Erlichman et al., 2003, Chesler, 2003, Dietmar and Rose, 1996). This increase in glial pH_i can be blocked by DIDS**,** implying that it is due to the NBC (Erlichman et al., 2003). Thus a biphasic response to neuronal firing should be seen, first an alkalisation (neuronal pH regulation), followed by an acidic one (glia pH regulation). Interestingly this biphasic response is commonly seen upon neuronal firing (Chesler, 2003, Dietmar and Rose, 1996).

There are many other factors that will contribute to the pH of the ECF. The increased neuronal firing will cause the release and re-uptake of neurotransmitters, which require proton motive forces (figure 7(iv)). Such neurotransmitters include glutamate (Chesler, 2003, Dietmar and Rose, 1996), ACh, aspartate and 5-HT (Dietmar and Rose, 1996), many of which are released in the VLM during hypercapnic stimulation. In conjunction with this, the activation of GABA (gamma amino butyric acid) receptors allows passage of HCO_3^- out of cells, again causing an alkaline shift (Chesler, 2003, Dietmar and Rose, 1996). Thus neuronal firing acts to alkalise the ECF by two different mechanisms, which supersede the small effect of glial HCO_3^- uptake. In fact neuronal firing causes alkaline shifts of the ECF by 0.1-0.2 pH units (Chesler, 2003). This appears to make perfect sense as an alkalisation of the ECF increases synaptic activity in terms of excitatory transmitters such as glutamate (figure 7(v)) and suppresses inhibitory transmitters such as GABA (Dietmar and Rose, 1996). Thus during hypercapnia synaptic transmission in the vicinity of chemosensitive neurons is enhanced. Interestingly an alkaline pH in the ECF surrounding chemosensitive neurons was proposed by Kalia and Ransom 25 years ago (Ballanyi et al., 1999).

This poses several very interesting questions. Although a decrease in pH_i and increase in pH_e would aid the chemosensitive response (by triggering neuronal firing and enhancing synaptic transmission, respectively) in terms of neurons, how does this environment effect glia (figure 7(vi))? If they are unable to counteract the effects of chemosensitive neurons on pH_e, why do they express NBC transporters and remove bicarbonate from the extracellular space? The answer to this seemingly paradoxical question is that it is the regulation of glia pH_i, and not pH_e that is important. If this is the case then why is an increase in glial pH_i so important? The answer to this question remains unknown, but implies that glia have an unknown role in chemosensitivity that surpasses their participation in pH_e regulation (figure 7(vii)).

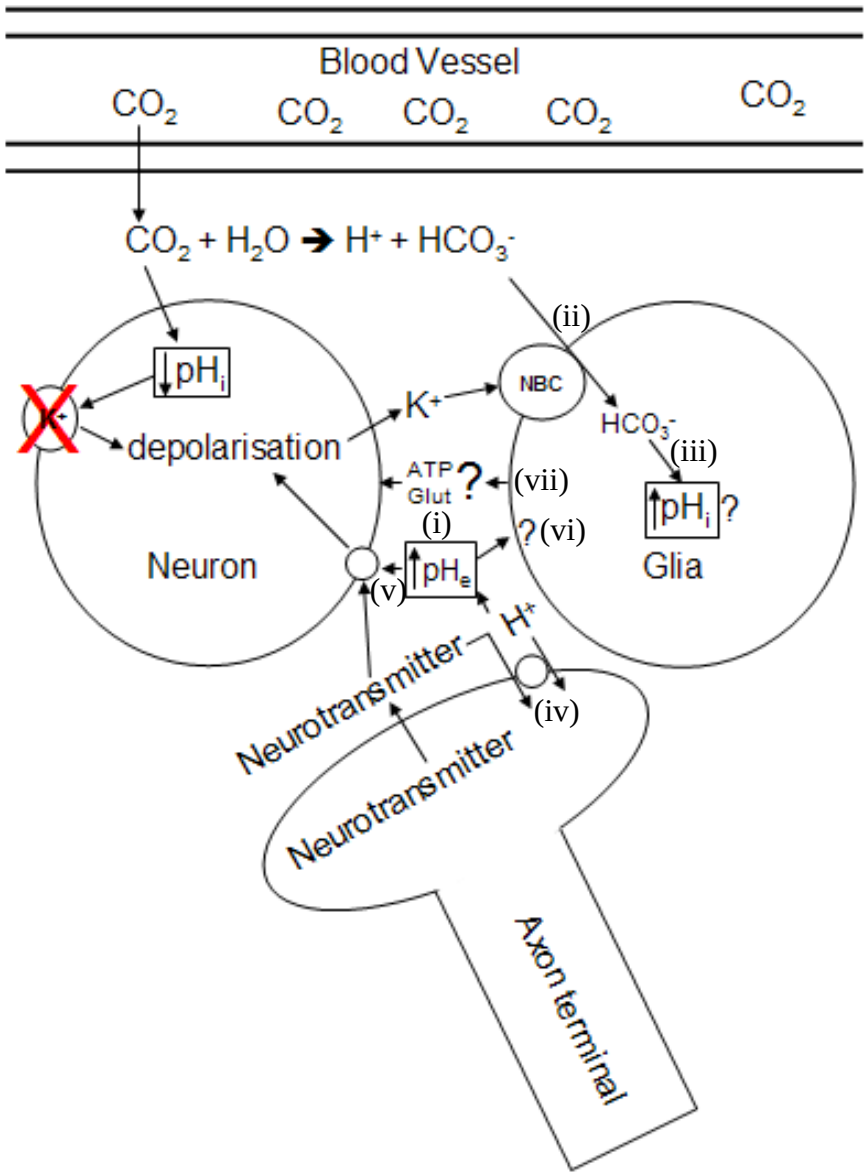

Figure 7: pH regulation in the ventral surface of the medulla. Though the mechanisms of pH regulation are understood in terms of functions in chemosensitive neurons, many questions remain unanswered about the effect of pH changes on glial cells, denoted by question marks.

1.9.4 Recapitulating the in vivo environment

The second important question then is what stimulus *in vitro* would more appropriately replicate this environment? Hypercapnic acidosis would appear to be unsuitable. The constant perfusion of aCSF (artificial cerebrospinal fluid) would act to wash away any consequent compensatory pH changes in the ECF surrounding neurons and glia in the tissue. In conjunction the tissue would be bathed in an acidic solution, creating an extracellular environment opposite to that which has been hypothesised *in vivo*. Though this would stimulate chemosensory neurons through alterations of their pH_i it would inhibit synaptic transmission through its actions on pH_e. Second to this, hypercapnic acidosis actually causes a profound and sustained decrease in glial pH_i (Ritucci et al., 2005), again contrary to *in vivo* conditions. Isohydric hypercapnia on the other hand would not induce such profound acidification of the internal pH of glia, but would still provide the initial fall in pH required to stimulate chemosensitive neurons. However this stimulus also does not provide an alkaline condition in the ECF, for the same reason as stated for hypercapnic acidosis. So what would more accurately recapitulate the *in vivo* environment? Firstly we know that an increase in CO_2 is required to alter the pH_i of chemosensitive neurons, however it would need to be present in an alkaline solution. In this way increased CO_2 would reduce the pH_i of chemosensitive neurons, but would maintain the extracellular alkalosis. In conjunction an aCSF with this composition would cause an increase in both the pH surrounding glia as well as their cytosol.

The importance of the role of constant perfusion of *in vitro* preparations is best shown with experiments using carbonic anhydrase inhibitors. In humans *in vivo* experiments, injections of acetazolamide (AZ) increase the CO_2 response (Vovk et al., 2000). It is thought to do this by blunting the peripheral response (through blockade of carbonic anhydrase), and enhancing central chemosensitivity (through lowered pH_e) (Vovk et al., 2000). In adult cats *in vivo* methazolamide (a blocker of carbonic anhydrase) and acetazolamide injected into the pre-Bötzinger area increased both the amplitude and frequency of phrenic nerve discharge (Solomon et al., 2000). However bath applied AZ is without effect on the CO_2 response of *in vitro* preparations (Necakov et al., 2002). This was determined to be because the CO_2 concentration of the perfusate determines CO_2 concentration in tissue, rendering the extracellular carbonic anhydrase useless. In contrast *in vivo* the CO_2 concentration is determined by the action of carbonic anhydrase in the tissue and thus alterations are seen (Necakov

et al., 2002). Of course it may be that the carbonic anhydrase is washed out of *in vitro* preparations but is present *in vivo.* In either case, these results show that we must be careful when designing *in vitro* models to make sure that *in vivo* conditions are met, an alkaline extracellular pH with intracellular acidification.

In my opinion this is one of the biggest problems facing the study of chemosensitivity at present. All the studies discussed to this present point have been undertaken using different models of *in vitro* hypercapnia. This makes comparisons between studies difficult and causes contradictions between studies of the same chemosensitive nuclei. One of the first and most obvious steps that needs to be undertaken is the measurement of extracellular and intracellular pH *in vivo*. Once this has been done as it has for other brain regions then *in vitro* models can be designed that replicate this environment. At present many studies, such as those of RTN neurons, utilise a decrease of up to 0.5 pH units, a value that can never be obtained under normal conditions *in vivo*. These studies have gone on to claim that under these conditions the chemosensitivity index (CI: the amount by which a neuron increases firing under hypercapnic conditions compared to controls) makes them prime candidates for central chemosensitivity and that they could contribute almost the entire chemosensitive response. If more modest shifts, closer to those that could be seen *in vivo* are used then the CI of these neurons is nowhere near what it must be to make these claims.

1.9.5 Implications of glia involvement in central CO_2 chemosensitivity

Fluorocitrate is an inhibitor of the tricarboxylic acid cycle in glial cells. This produces highly toxic effects and leads to glial dysfunction. It has been reported that injection of fluorocitrate into the RTN of conscious adult rats caused an increase in ventilation and heightens the response to CO_2, due to increased extracellular acidification (Holleran et al., 2001). This finding would seem to imply that glia play no part in the response to chemosensitivity. However in this series of experiments 50% of the animals went into respiratory distress (Holleran et al., 2001), for obvious reasons these experiments were discontinued. This does however provide an intriguing question, why did fluorocitrate have such opposite effects on the response to hypercapnia? There are a few possibilities as to why this occurred. One hypothesis would be that the location of the injection sites were different between these two sets of animals, and therefore the site of glia dysfunction is important. A second idea is

that the animals which survived had a lower diffusional spread of the toxin and thus it is the amount of glia that are affected which is important. In another series of experiments, injections of fluorocitrate into the RTN of conscious rats again increased breathing due to tissue acidification (Erlichman et al., 1998). However in this series of experiments fluorocitrate caused a reduced maximal response to inspired CO_2 (Erlichman et al., 1998). Since the location of the injections were identical between these two experiments one would assume it is the volume of the area covered by the fluorocitrate that is important, though this cannot be elucidate. However intraperitoneal injections of methoxysulphate another glial toxin into neonatal rats, which should affect a much larger proportion of glia than site directed injections, resulted in reduced levels of basal ventilation and a reduction in their CO_2 response (Young et al., 2005). As stated previously these experiments do not provide definitive proof of glial involvement, merely they imply the possible involvement of this cell type.

1.10 Maturation of the chemosensory response

Medullary chemosensitivity is present throughout foetal development (Eugenin et al., 2006). In early stage foetuses embryonic day 13-16 (E13-16) this is due to an increase in burst amplitude of the phrenic nerve with no change in frequency (Eugenin et al., 2006). However at E17-18, this changes to an increase in frequency that is accompanied by a reduction in amplitude (Eugenin et al., 2006). Surprisingly chemosensitivity exists before respiratory rhythm is seen, thus it can influence ventilation as soon as the respiratory pacemaker begin firing (Eugenin et al., 2006).

In neonatal preparation hypercapnia stimulates ventilation in general, but does not elicit metabolic response that may modify the demand for ventilation (Putnam et al., 2005). Neonates like early foetuses increase ventilation by an increase in tidal volume (Studen et al., 2001), though this changes to an increase in frequency after P3 (Wickström et al., 2002). Therefore like foetal preparations chemosensitivity varies with age (Putnam et al., 2005). It is at its highest between P1-5, then wanes to reach a nadir at P8 at which point it begins to rise until the adult response is reached by P21 (Putnam et al., 2005, Studen et al., 2001). Thus, like autonomic control of other systems chemosensitivity appears to show a tri-phasic response (Putnam et al., 2005). The decrease in respiratory response corresponds to a reduction in c-Fos staining

which occurs after 6 days (Wickström et al., 1999). The increase in respiration from the nadir is coincident with astrocytic maturation which occurs between P8-P12 (Putnam et al., 2005, Ritucci et al., 2005, Wickström et al., 2002, Davis et al., 2006), again implicating some form of glia involvement in the hypercapnic ventilatory response. Another series of c-Fos studies have shown that the RTN is more active in early postnatal life than adulthood (though this true of chemosensation as a whole) (Belegu et al., 1999). This may account for such high sensitivity in early post-natal life. Some of these changes may be due to the fact that chemoreceptors are closer to the surface in neonates than in adults (Forster et al., 1997). Thus during early postnatal life CO_2 sensitivity decreases as cell bodies of chemosensory nuclei move further away from the surface where they were able to detect fluctuation in blood chemistry more easily. Their ability to compensate for this after P8 may be due to the migration of dendrites to the surface to re-establish their association with blood vessels. Once adult chemosensitivity is established it appears to remain constant throughout life, at least in humans (Browne et al., 2003).

1.11 ATP as a transmitter

The first evidence that adenosine and related compounds could affect excitable cells came in 1929, where the perfusate from minced bull hearts caused a bradycardia in dogs and heart block in guinea pigs when directly applied to the muscle (Drury and Szent-Györgyi, 1929). The active compound was purified and shown to contain adenine, a pentose sugar and phosphoric acid with an empirical formula of $C_{10}H_{14}O_7N_4P\text{-}H_2O$ (Drury and Szent-Györgyi, 1929), thus indicating either adenosine or adenosine mono-phosphate (AMP). Applications of these compounds replicated the effects of the whole heart perfusate in guinea pigs and dogs and caused a bradycardia in rabbits (Drury and Szent-Györgyi, 1929).

The first evidence for ATP as a peripheral transmitter did not come however until 1970. Guinea pig intestines were stimulated electrically and the perfusates applied to the intestine of other guinea pigs (Burnstock et al., 1970). Since only the perfusates of stimulated guinea pig intestines induced any response, it implies that something must be released upon stimulation. To investigate this further the perfusates were dried and purified (Burnstock et al., 1970). The minced bull heart perfusates contained a high quantity of purines (Burnstock et al., 1970). Applications

of adenosine replicated the effects of the endogenously released ligand in frogs and turkeys as well as rodents (Burnstock et al., 1970). However it could not be determined whether the release of adenosine was from the nerve terminals or the surrounding tissue. In order to study the release from the nerves alone, Auerbach's plexuses were removed from turkey gizzards, stimulated and the resultant perfusate was dried and purified (Burnstock et al., 1970). This resulted in the identification of ATP and ADP (adenosine diphosphate) as the active compounds. These were much more potent inhibitors of gut motility than adenosine (Burnstock et al., 1970). When the ATP was perfused through the vasculature of the stomach of toads, the ATP was hydrolysed to adenosine (Burnstock et al., 1970). As ATP had differential effects on segments of ileum, there was a proposal of two purinergic receptors for ATP (Burnstock et al., 1970), thus creating the basis of the three aspects of ATP signalling we know today, where ATP, its metabolite adenosine and ectonucleotidases (which are responsible for the hydrolysis of ATP to adenosine) can all have influences over the same reflex.

It was not until 1992 however that the first evidence for ATP as a central neurotransmitter appeared (Edwards et al., 1992). It was noticed that fast ligand gated synaptic currents in the medial habenula were not blocked by a cocktail of antagonists to all neurotransmitters known at the time (Edwards et al., 1992). These currents were however sensitive to suramin (a purinergic antagonist) and desensitised by α,β methyl-ATP (a non-hydrolysable analogue) (Edwards et al., 1992). The currents evoked by ATP were still present in excised patches showing the presence of a fast ligand gated ion channel sensitive to ATP in the post-synaptic membranes of these neurons (Edwards et al., 1992). The final and most important step in proving the existence of purinergic signalling was the cloning of the purinergic receptors.

1.12 Purinergic receptors

In 1978 it was first established that there are two types of purinergic receptors, P1 receptors modulate the response to adenosine, whilst P2 receptors are responsible for conveying the effects of ATP (Burnstock and Kennedy, 1985). Subsequently P2 receptors have been subdivided into two distinct classes, ionotropic P2X and metabotropic P2Y (Burnstock and Kennedy, 1985).

1.12.1 P2X receptors

There are seven types of P2X receptors, which are non-selective cation channels gated by ATP (Torres et al., 1999). ATP is a strong agonist whereas ADP is a weak agonist for P2X but AMP (adenosine monophosphate) and adenosine have no effect (Buell and Rassendren, 1998). Though there are many agonists and antagonists for P2X receptors, the specificity of these agonists and antagonists are limited, leading to problems with pharmacological identification (Lambrecht, 2000).

Though they do not discriminate between monovalent cations the permeability of the channels reduces with increasing atomic number (Torres et al., 1999), though permeability to low atomic number cations can be equal (Evans et al., 1996). In addition they are permeable to divalent cations (Torres et al., 1999, Ma et al., 2006) and modulated by others (Ma et al., 2006, Evans et al., 1996). Their increase in permeability to these ions upon binding of ATP is not through dilatation of the pore[1] (Ma et al., 2006), thus it must be due to the unplugging of the gate. The ion conduction pathway is predominantly formed on the second transmembrane (TM2) helix, though it cannot be ruled out that TM1 may play a role (Li et al., 2008). It is the external half of the TM2 helix that contains the gate, which is thought to be hydrophobic (Li et al., 2008). This domain may also convey the selectivity of the channel to ionic permeability (Li et al., 2008). These channels have pore sizes and permeabilities similar to those of 5-HT and nACh channels (Evans et al., 1996) and share structural feature with ASIC (acid sensing ion channel) channels (Li et al., 2008). These channels like ASIC channels are highly sensitive to pH, probably conveyed by the areas that are structurally similar to ASIC. P2X 1, 3 and 4 currents are attenuated by acidification, whereas alkalisation is without effect (Stoop et al., 1997). In contrast $P2X_2$ currents are potentiated with acidification (Stoop et al., 1997). Though they are highly sensitive to large changes in pH, their responses to changes within physiological range are small (Stoop et al., 1997). The effects of pH on these channels are not exerted through changes in pore size, it changes the affinity of the binding site for its agonist (Stoop et al., 1997).

1.12.2 Co-assembly of P2X channels

These receptors, with the exception of $P2X_6$, have been shown to form homo-ogliomeric assemblies in HEK (human embryonic kidney) cells (Torres et al., 1999).

[1] Dilatation may occur with prolonged exposure to ATP or other ligands

In conjunction they are also capable of forming hetero-oligomeric channels (figure 8). $P2X_7$ is structurally and functionally different to other P2X receptors and this is why it only appears to form homomeric channels (Buell and Rassendren, 1998). It is the longest P2X protein, possibly from a P2X gene fusing with a pore forming gene, giving it an extra codon (Buell and Rassendren, 1998). Unlike other P2X channels which are responsible for signal transduction, $P2X_7$ channels are part of the apoptotic pathway (Buell and Rassendren, 1998).

Heteromeric assembly of sub-types that share similar pharmacological properties, such as $P2X_6$ and $P2X_4$ which are insensitive to pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) and suramin, will create a channel with the same properties as the individual sub-types (Collo et al., 1996). This form of heteromeric channel formation appears to be a common occurrence as $P2X_4$ and $P2X_6$ share a similar distribution (Collo et al., 1996). When subtypes with different properties co-assemble, under different conditions one sub-type dominates the other. This is can be shown by the pH sensitivity of these channels (Stoop et al., 1997). $P2X_3$ currents are attenuated by acidification but $P2X_{2/3}$ currents are increased with acidification, showing that the $P2X_2$ receptor dominates the channel (Stoop et al., 1997). In some cases however this can cause the formation of channels with new properties. $P2X_{1/2}$ channels show biphasic modulation to acidification, the first phase resembles that of $P2X_1$ and second $P2X_2$ when expressed in Xenopus oocytes (Brown et al., 2001). In conjunction alkaline solutions also increased agonist potency of this hetero-oligomer, a property not seen for either of the homomeric channels (Brown et al., 2001) and acidification potentiated the $P2X_1$ like currents, where it usually attenuates them (Brown et al., 2001). Purinergic receptors may also modulate the actions of other ion channels, i.e. $P2X_5$ can mediate ASIC currents in a calcium independent manner (Spelta et al., 2004).

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
$P2X_1$	+	+	+	−	+	+	−
$P2X_2$		+	+	−	+	+	−
$P2X_3$			+	−	+	−	−
$P2X_4$				+	+	+	−
$P2X_5$					+	+	−
$P2X_6$						−	−
$P2X_7$							+

Figure 8: Summary of homomeric and heteromeric assembly of P2X receptors. Taken from Torres et al *J Biol Chem* 274(10), 6653-6659 (1999).

1.12.3 The $P2Y_1$ receptor

$P2Y_1$ receptors are activated by ADP and ATP but are insensitive to AMP and adenosine (Ralevic and Burnstock, 1998). $P2Y_1$ receptors are G-protein coupled receptors (GPCR) and thus mediate slower responses than P2X receptors (Ralevic and Burnstock, 1998). They are usually found coupled to G_q, G_{11} and G_i proteins (Ralevic and Burnstock, 1998). There are two known pathways that these receptors modulate (Ralevic and Burnstock, 1998). The first is the inositol triphosphate (IP_3) pathway, which it initiates by activation of phospholipase C, leading to increased calcium mobilisation within cells (Ralevic and Burnstock, 1998). The second is the inhibition of the adenylate cyclase pathway (Ralevic and Burnstock, 1998). In addition P2Y, like P2X receptors, can also modulate other channels (Wirkner et al., 2003).

1.13 Purine receptors in VLM

ATP binding has been seen in the medulla (Fong et al., 2002), and many purinergic receptors are expressed there. The medulla (of the marmoset and rat) has widespread staining for all P2X ionotropic receptors, though the distribution of each varies (Yao et al., 2000, Thomas et al., 2001). In the rat $P2X_{1, 2, 5, 6}$ were seen throughout the VLM (Thomas et al., 2001), whereas $P2X_3$ and $P2X_4$ are located only in more caudal and medial portions (Thomas et al., 2001). $P2X_6$ is the most commonly observed receptor in the VLM and immunoreactivity was very strong throughout the entire rostro-caudal extent and was the most heavily expressed P2X receptor on the VLM surface (Thomas et al., 2001). $P2X_{2, 3, 4, 6}$ are primarily located on dendrites with dense staining in the fibre matrix (Yao et al., 2000). Interestingly the $P2X_2$ channel the only channel enhanced by acidification and $P2X_5$ which can modulate acid sensing channels were highly expressed and showed localised staining (Kanjhan et al., 1999, Yao et al., 2000). Interestingly heavy staining of P2X immunoreactivity was seen at the pial surface (Thomas et al., 2001). It must be noted here that there are differential reports to the expression of $P2X_1$ (Yao et al., 2000, Thomas et al., 2001), this has been attributed to the use of different $P2X_1$ antibodies, some of which have been shown to react with tissue from $P2X_1$ knockout mice (Ashour et al., 2006). In conjunction many nuclei in the medulla have been shown to contain $P2Y_1$ receptors (Kanjhan et al., 1999). The expression of many purinergic receptors and widespread ATP binding in the medulla lead to a series of experiments to define its role there.

1.13.1 ATP in the VLM

Ten years ago the first evidence for the role of ATP in chemosensation began to arise. In peripherally denervated rats bilateral injections of suramin into the retrofacial area raised the threshold level of CO_2 required to initiate the hypercapnic ventilatory response (Thomas et al., 1999). In conjunction it also diminished the increased frequency and amplitude of ventilatory response seen with increased inspired CO_2 in adult rats (Thomas et al., 1999). Extracellular recordings showed that suramin and PPADS primarily exert their effects on neurons that pace inspiratory rhythm, such that they reduce or abolish the excitatory effect of CO_2 on inspiratory neurons in the Bötzinger or pre-Bötzinger complexes of the VRG (Thomas and Spyer, 2000) and pre-I neurons of the pFRG (Thomas and Spyer, 2000, Kawai et al., 2006).

In conjunction the direct application of ATP to inspiratory related areas excites them (Thomas and Spyer, 2000). In contrast to its effect on inspiratory related areas of the VRG, PPADS and suramin were without effect on expiratory related areas, post-inspiratory and expiratory neurons of the VRG (Thomas and Spyer, 2000). However ATP did excite them (Thomas and Spyer, 2000), causing a reduction in phrenic nerve activity (Thomas et al., 2001), but only when they are in their active phase, (Gourine et al., 2003). In addition α,β-methyl ATP replicated this response (Thomas et al., 2001).

The location of purinergic receptors in numerous chemosensitive and respiratory nuclei and the effects of ATP on respiratory neurons led to the hypothesis that ATP may play a role in respiratory control and chemosensitivity. Evidence has begun to imply that ATP is released tonically at the VLM surface (Spyer et al., 2003). Therefore two hypotheses emerged: firstly that ATP may be released in response to hypercapnia or secondly pH changes in the VLM associated with hypercapnia may increase the efficacy of $P2X_2$ thus leading to an increased influence of tonic ATP release, though it may be a mixture of both. Importantly $P2X_2$ receptor knock out mice do not show perturbations in their response to inspired CO_2 (Rong et al., 2003), and ATP is released at the VLM surface (Gourine et al., 2005a), leading to the conclusion that it is release of ATP from the ventral surface and not a change in sensitivity of purinergic receptors for their ligand (Spyer et al., 2003).

Intriguingly ATP released from the ventral surface was confined to the most superficial layer and overlies the classical chemosensitive areas first described in the cat almost fifty years ago (Gourine et al., 2005a). ATP release was shown to be

released immediately after end tidal CO_2 begins to increase and precedes any adaptive breathing (Gourine et al., 2005a). Applications of the purinergic receptor antagonists, PPADS and TNP-ATP (2',3'-O-(2,4,6-Trinitrophenyl) adenosine 5'-triphosphate), not only reduced the hypercapnic ventilatory response but also the threshold level of CO_2 required to initiate the response *in vivo* (Gourine et al., 2005a). In conjunction applications of ATP to the ventral surface replicated the ventilatory response to hypercapnia (Gourine et al., 2005a). However P2X receptor blockade did not completely ablate the response, indicating a role for P2Y receptors (Gourine et al., 2005a). Interestingly P2Y receptor agonists also increase respiratory rate, showing their involvement (Gourine et al., 2005a). Since residual increases in respiratory rate remain after blockade of purinergic receptors, there must be other transmitters involved (Spyer and Thomas, 2000). Since ATP causes excitation of breathing, only neurons responsible for increasing inspiratory rate that have ATP receptors are involved in mediating respiratiory changes induced by this transmitter (Spyer and Thomas, 2000).

1.13.2 ATP and hypoxia

As well as its role in modulating the ventilatory response to hypercapnia ATP release in the medulla is also involved in the response to hypoxia. During hypoxia ATP is released from deeper structures than hypercapnia, approximately 400-800µm from the ventral surface (Gourine et al., 2005b). There is also a delay in the release of ATP upon hypoxic stimulation, it is not released until after hypoxia induced augmentation of respiratory activity (Gourine et al., 2005b), thus it is not responsible for initiating increased respiratory drive. Application of PPADS during hypoxia augmented hypoxic ventilatory depression, thus ATP acts to oppose reductions in breathing seen in the second phase of the hypoxic ventilatory response (Gourine et al., 2005b). Peripherally denervated animals showed no increase in respiration seen during the first phase of the hypoxic ventilatory response (Gourine et al., 2005b). However they still exhibit hypoxic ventilatory depression and the associated ATP release that counteracts it, though at much lower levels (Gourine et al., 2005b). Thus ATP is vital to the central response to both hypoxia and hypercapnia.

1.13.3 $P2X_2$ and chemosensation

Mice deficient in $P2X_2$ and $P2X_{2/3}$ receptors have normal responses to mild hypoxia, but perverse responses to severe hypoxia (Rong et al., 2003). At 7.5% O_2

profound respiratory depression was observed in these mice but not in $P2X_3$ knockouts or wild types (Rong et al., 2003). Carotid body afferents exhibit ongoing activity under normal conditions, and are silenced when levels of O_2 reach 50% (Rong et al., 2003). Applications of PPADS to the carotid body of wild type mice blocks background activity and hypoxia induced increases in sinus nerve activity (Rong et al., 2003), whereas applications of ATP or other purinergic agonists increased firing, in carotid body afferents (Rong et al., 2003). This basal level of firing is reduced in $P2X_2$ double and $P2X_{2/3}$ knockouts, but not when $P2X_3$ is knocked out alone (Rong et al., 2003). In conjunction ATP applications to the carotid body of $P2X_2$ and $P2X_{2/3}$ knockouts cause only modest rises or no increase in respiratory rate (Rong et al., 2003). Thus the response to peripheral O_2 chemoreception is mediated by $P2X_2$ containing receptors, which are located on afferent terminals surrounding glomus cells (Rong et al., 2003).

$P2X_2$ and $P2X_3$ messenger ribose nucleic acid (mRNA) is also found on petrosal neurons (Prasad et al., 2001, He et al., 2006), where hypoxia causes a reversible sub-threshold depolarisation in type 1 cells, which are the site of chemosensory transduction (Prasad et al., 2001). Applications of ATP stimulates calcium waves in these cells, which can be blocked by PPADS and suramin (He et al., 2006). The reduction of calcium waves in the cells upon application of these antagonists reduces firing in post-synaptic petrosal neurons (Prasad et al., 2001) and removes the ventilatory response to chronic hypoxia (He et al., 2006). Thus ATP is a common mediator of the peripheral and central chemosensitive responses (Spyer et al., 2003). Though ATP is undoubtedly involved in these processes the cells types and mechanisms involved have not yet been elucidated.

$P2X_{2,\ 3,\ 2/3}$ knockout mice however show absolutely no alterations in their response to hypercapnia compared to their wild type counterparts (Rong et al., 2003). This was concluded to mean that these receptors do not play a role in the response to hypercapnia. However ATP also acts as a co-transmitter during hypercapnia in carotid bodies (Prasad et al., 2001). Since these cells express purinergic receptors and ATP is released here it would be strange if this hypothesis were true. Although animals seem unable to compensate for the loss of this receptor (Finger, 2005, Rong et al., 2003), they are able to compensate for the loss of carotid inputs (Pan et al., 1998). Therefore it may be that there is a re-organisation of the chemosensory pathway, to adjust for the loss of this receptor's contribution in the carotid body. A second note is that the

involvement of $P2X_2$ was selected on the basis that it is positively modulated by decreasing pH, which is thought to occur at the VLM surface. However as discussed earlier it may be that this region alkalises during hypercapnia, if this is the case then the $P2X_2$ receptor is the least likely P2X receptor to be involved in this response. The lack of alterations to inspired CO_2 in mice lacking this receptor may reflect this. In conjunction if an alkalisation were to occur as predicted, it would actually serve to increase the affinity of all P2X (excluding $P2X_2$) to ATP. Thus it may be that release of ATP works with an increased sensitivity of purinergic receptors at the VLM surface during hypercapnia.

1.14 ATP and respiration in neonates

There are hotspots in acute medullary tissue slices from neonates that are excited by ATP. These hotspots correspond closely to the region from which the strongest respiratory field potentials are recorded and incorporates the pre-Bötzinger complex (Lorier et al., 2007). The response to ATP is dose dependent in that 1mM ATP increased respiratory frequency, but 10mM ceased activity (Lorier et al., 2004). ATP's actions on the pre-Bötzinger complex increases the frequency of bursting but is without effect on burst amplitude in neonates (Lorier et al., 2004). It evokes currents in inspiratory neurons of pre-Bötzinger complex (Lorier et al., 2008) by opening cation channels and inhibiting a K^+ current (Lorier et al., 2008). Applications of ionotropic purinergic receptor antagonists into this area did not counter the effect of exogenous ATP and pH was without effect, implying P2Y involvement (Lorier et al., 2007). The response to exogenous ATP was biphasic, and the secondary slowing was dependent on ATP hydrolysis (Lorier et al., 2007). Suramin reduced basal respiration, and Cu^{2+} (an allosteric modulator) increased resting frequency (Lorier et al., 2007) implying $P2X_2$ involvement in this region (Lorier et al., 2004). That PPADS alone did not affect bursting but does reduce the response to exogenous ATP (Lorier et al., 2004) indicates that tonic release of ATP is not seen at this developmental stage. Interestingly P2Y antagonists that do not affect P2X receptor induce larger current changes than ATP itself (Lorier et al., 2008). As for adult rats ATP evokes currents in expiratory neurons and non-respiratory neurons via P2X and P2Y receptors in the neonate (Lorier et al., 2008). Intriguingly, PPADS did not affect the CO_2 response in neonates as it does in adults (Lorier et al., 2004), implying that ATP signalling during hypercapnia occurs as chemosensitivity matures.

1.14.1 ATP and respiration in adults

Bilateral injections of suramin into the pre-Bötzinger and Bötzinger complexes reduced basal phrenic nerve activity and baseline firing of inspiratory neurons that reside there (Thomas et al., 1999, Thomas and Spyer, 2000, Gourine et al., 2003), implying tonic release of ATP in this region (Spyer et al., 2003), though surprisingly no tone of ATP from the ventral surface of the medulla was seen upon removal of ATP biosensors (Gourine et al., 2005a). Interestingly unilateral injections of suramin had no effect on basal respiration, but did block the response to ATP (Thomas et al., 2001). Intriguingly adenosine antagonists augmented the effect of hypercapnia on these neurons (Thomas and Spyer, 2000), indicating that the effects of ATP post-hypercapnia are self limiting.

As stated earlier inspiratory neurons in pre-Bötzinger complex express P2Y receptors. Transfection of this receptor alone into astrocytoma cell lines is sufficient to cause intracellular calcium mobilisation (Fam et al., 2000) and dorsal spinal cord astrocytes raise intracellular Ca^{2+} upon P2Y receptor activation (Fam et al., 2000). Thus P2Y receptors could potentiate the Ca^{2+}-activated non-specific cation current (I_{can}) through propagating calcium waves in inspiratory related neurons, in a similar manner to metabotropic glutamate receptors (Funk et al., 2008). Thus during hypercapnia an increase in ATP levels will cause greater activation of P2Y and increased intracellular calcium, and thus greater activation of TRPM4 (Transient receptor potential cation channel, subfamily M, member 4), leading to an influx of sodium and an increase in the firing rate.

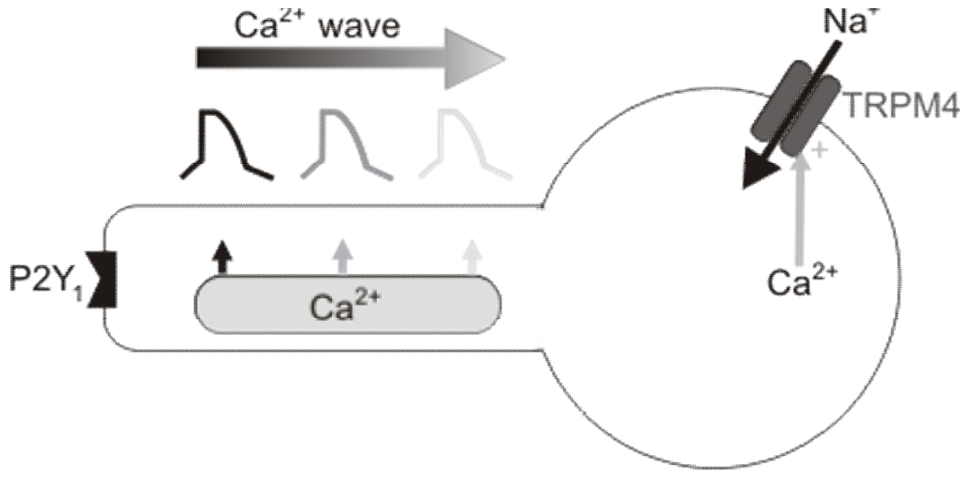

Figure 9: Possible participation of P2Y receptors in the modulation of putative pacemakers in the pre-Bötzinger complex. P2Y activation leads to the induction of calcium waves, which activate TRPM4 channel leading to sodium influx and depolarisation. Figure adapted from Del Negro and Hayes *J Physiol* 586, 2245-2246 (2008).

1.15 ATP and Chemosensory neurons of the VLM

The distribution of serotonergic neurons of the medullary raphé (Bradley et al., 2002) closely resembles that of sites that release ATP under hypercapnic conditions (Gourine et al., 2005a) (figure 10).

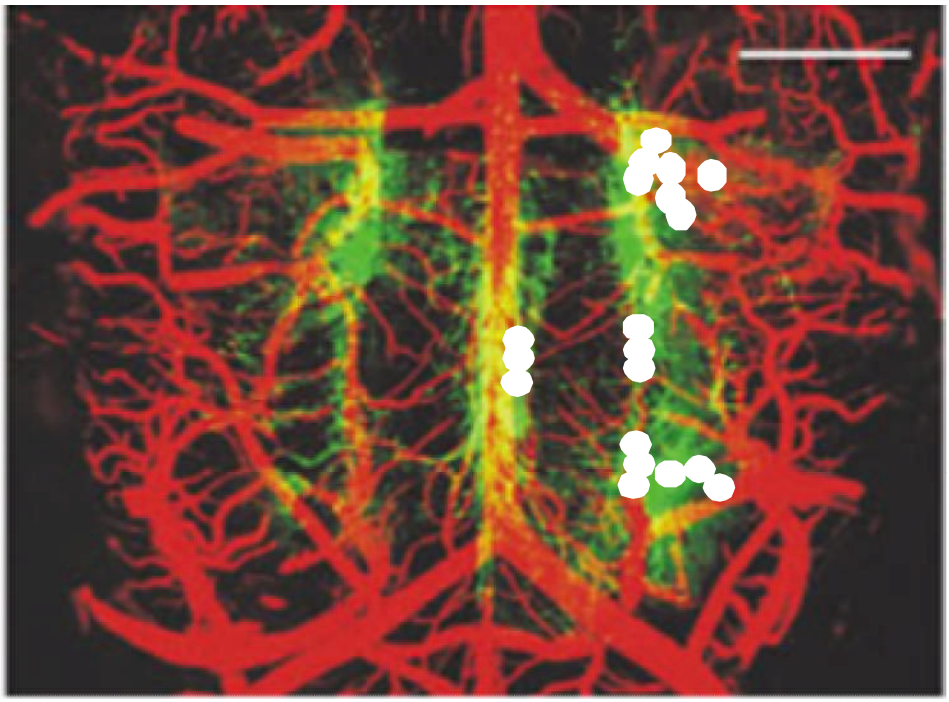

Figure 10: Association between serotonergic neurons of the VLM surface (green) with ATP release sites at the VLM surface (white circles). Picture adapted from Bradley et al *Nature Neuroscience* 5, 401 - 402 (2002) and Gourine et al *Nature* 436, 108-111 (2005a).

Injections of ATP into the RM caused a marked inhibition of respiration (Cao and Song, 2007), similar to electrical stimulation of the same area (Cao et al., 2006). In contrast, injections of ATP into the RP caused facilitation in a dose dependent manner (Cao and Song, 2007), similar to electrical stimulation (Cao et al., 2006). The response of ATP injected into the RM and RP could be blocked by ATP antagonists, however it did not affect basal respiration (Cao and Song, 2007), implying that ATP release needs to be stimulated before it affects these regions. This is not surprising, as although these neurons contribute to the hypercapnic ventilatory response, they are not known to tonically influence respiration. Interestingly the highest sensitivity of raphé to ATP is located in the caudal VLM (Ribas-Salgueiro et al., 2003), in an area that would incorporate the RO. This area in contrast to the other raphé nuclei does have a tonic effect on the respiratory drive. Therefore if tonic release of ATP is seen at the ventral surface, as it is in the vicinity of the pre-Bötzinger complex, then ATP

may also affect basal respiration through its influence here. Interestingly the chemosensitivity of raphé neurons has been shown to be age dependent, as is ATP release from the ventral surface, leaving the intriguing possibility that ATP release and chemosensitivity may develop together. This could mean either that serotonergic neurons are responsible for ATP release, or that ATP may be responsible for the chemosensitivity of serotonergic neurons.

The RTN also lies beneath the most rostral site of ATP release. Neurons that reside here are directly excited by ATP through the activation of P2Y receptors (Mulkey et al., 2006). ATP also inhibits these neurons indirectly from the activation of P2X receptors on inhibitory neurons, though whether these are GABAergic or glycinergic is unknown (Mulkey et al., 2006). Though PPADS was without effect on the baseline firing of these neurons, these experiments were performed on neonatal rats, at an age where ATP release is not seen. However since these neurons are modulated by ATP and express purinergic receptors, one would expect them to respond to ATP released in this vicinity.

1.16 Methods of ATP detection

In order to study the mechanisms by which ATP is released in depth, one must use reduced preparations or transfection of proteins thought to act as conduits into naïve cells. One must then show that the preparations are able to release ATP. There are three main methods of ATP detection employed today.

The luciferase/luciferin reaction: - Luciferin is a pigment of the common fire fly, which reacts with oxygen under the influence of luciferase in an ATP dependent manner. Luciferin is converted to oxyluciferin and energy, which is released in the form of light (figure 11). The ATP dependence of the reaction is due to the formation of an intermediate protein luciferyl adenylate during the conversion pathway (Dukhovich et al., 1996).

Figure 11: Conversion of Luciferin to oxyluciferin under the influence of a luciferase enzyme.

By stimulating cells in the presence of luciferase and luciferin, light will be emitted in the vicinity of ATP release. However this method is quite difficult to get to work and generally it provides only qualitative data.

The second way of detecting ATP release is by the use of ATP sensor cells. Here a cell line is transfected with the $P2Y_1$ receptor, which when activated induces calcium waves in these cells. The cells are placed over regions of interest and loaded with a calcium sensitive dye. Thus when the cells responsible for ATP release are activated calcium waves are detected in the reporter cell. This can provide a very sensitive detection mechanism to very low concentrations of ATP and can map discrete areas of ATP release. However again this method is only qualitative.

The final method of detection is the use of ATP microelectrode biosensors. The sensors contain a platinum wire encapsulated by a porous glass coating. The glass coating traps enzymes which convert glycerol to H_2O_2, an electroactive compound, against the platinum wire. The H_2O_2 reacts with the platinum wire causing a change in current which can be recorded. This method provides a more quantitative measure of ATP release, as it an ATP dependent process. However these sensors are quite large in diameter ~50 μm and thus cannot be used to detect ATP within a synapse. In addition the platinum wire can be affected by small electroactive compounds. The use of null sensors and a di-amino benzene (DAB) screening layer minimise these effects, but they require some training in the interpretation of the results.

1.17 Mechanisms of ATP release

There are several forms of ATP release described in the literature that are of both glial and neuronal origins. There is classical synaptic vesicular release from terminal boutons of neurons in the medial habenula (Edwards et al., 1992). Here ATP is stored in vesicles in post-synaptic neurons. During neuronal firing calcium entry causes vesicle docking and priming readying them for release. Upon the next bout of calcium entry, these vesicles fuse with the membrane and cause exocytosis of ATP into the synaptic cleft. It then acts on purinergic receptors in the medial habenula causing them to fire and eliciting a response.

Recently this form of vesicle docking and neurotransmitter release has been shown to occur in the dorsal root ganglion (DRG) of the spinal cord. Here ATP is also transported in vesicles ready for release in a similar fashion to that which is seen at the synapse. However, in contrast to being released from the terminal bouton, ATP is

released quantally from the somata of neurons in the DRG through the endo- and exo-cytosis pathways (Zhang et al., 2007a). In contrast to stimulating neurons in this region, ATP released here stimulates satellite glia, through stimulation of $P2X_7$ receptors (Zhang et al., 2007a).

In contrast, glial derived ATP release takes many forms. In hippocampal slices, glia release ATP via SNARE-dependent vesicular release in the same manner as neurons of the medial habenula (Pascual et al., 2005). Here ATP is hydrolysed to adenosine which affects synaptic plasticity leading to long term facilitation and increasing basal transmission (Pascual et al., 2005). Further evidence supporting this form of release from astrocytes came when astrocytes were shown to contain ATP loaded vesicles which undergo spontaneous release (Striedinger et al., 2007). By disrupting proteins that are responsible for the fusion of vesicles with astrocytic cell membranes, ATP release from these astrocytes was abolished (Striedinger et al., 2007). Spontaneous vesicular release of ATP in response to calcium waves is seen in astrocyte progenitors and aids neural progenitor migration (Striedinger et al., 2007). The final conclusive evidence was provided in the form of an inducible knockout of SNARE protein in GFAP positive glia, which can be used to remove this form of signalling.

Hippocampal astrocytes also store glutamate and ATP in secretory granules in lysosomatal vesicles (Zhang et al., 2007b). The lysosomatal vesicles can undergo both full and partial fusion with the glial cell membranes in this area (Zhang et al., 2007b). Partial fusion occurs spontaneously through a ‘kiss and run’ mechanism resulting in only a small amount of transmitter release (Zhang et al., 2007b). In contrast full fusion is only seen under hypoxic conditions and occurs through a similar mechanism to exocytotic release (Zhang et al., 2007b). In addition oxygen-glucose deprivation, the *in vitro* model of ischeamia, induces ATP release through maxi-anion channels in primary cortical cell cultures from neonatal mice (Liu et al., 2008). These mechanisms are unlikely to be the primary source of ATP at the surface of the medulla during hypercapnia, as ATP is not released there in response to hypoxic stimulation. However this may provide the molecular mechanism for ATP release at depths of 400-800μm from the surface during hypoxia.

In cow aortas, hypotonic stress induces release of ATP through volume regulated anion channels (VRAC’s) (Hisadome et al., 2002). In these cells, as in the bladder, hypotonic solutions lead to cellular swelling causing the activation of the

channel. Upon channel opening ATP release acts in a paracrine fashion to neighbouring cells to induce a co-ordinated response.

Finally a common mechanism of ATP release in many systems is through gap-junction hemichannels. Calcium waves in astrocytes were thought to be propagated by electrical coupling through gap junctions. Calcium waves in cortical astrocytes however were seen to pass over areas devoid of cells; thus there must be an alternative pathway that can perform this function (Cotrina et al., 1998, Hassinger et al., 1996). The inhibition of these waves by purinergic antagonists suggest the paracrine release of ATP fulfilled this role (Cotrina et al., 1998, Pearson et al., 2005, Cotrina et al., 2000). This was confirmed when glial cells in the RPE, C6 glioma cells and cultured astrocytes were shown to release ATP (Pearson et al., 2005, Stoutt et al., 2002), which acted to propagate the calcium wave (Pearson et al., 2005, Stoutt et al., 2002, Peuchen et al., 1996), through activation of P2Y receptors (Peuchen et al., 1996, Cotrina et al., 1998). Interestingly connexins and purinergic receptors form transcriptomes (Iacobas et al., 2007), meaning these genes are co-ordinately expressed. This may be because the expression of both of these proteins in a single glial cell is required for calcium waves.

If one can identify the mechanism of ATP release induced by hypercapnia at the ventral surface of the VLM, it will aid in the identification of the cell types responsible for it. The first step in determining which mechanism is employed here is to test the dependence of it on extracellular calcium, as this can distinguish between SNARE and non-SNARE dependent mechanisms. This can also determine whether ATP is released from primary chemosensory cells or is secondary to firing of neurons, as can the use of TTX. Once the pool of possible targets has been reduced, pharmacology can then be employed to distinguish which of the remaining mechanisms is utilised.

Chapter 2: ATP is released from the VLM in response to changes in PCO_2, and may influence the response to both hypo- and hypercapnia in rats

2.1 Abstract

Despite the discovery of the ventral chemosensitive areas of the medulla oblongata, the cell types and mechanisms involved in CO_2 chemosensory transduction have remained elusive. Recently, ATP release from these areas has been demonstrated to be a vital component of the CO_2 chemosensory reflex. By cutting horizontal slices of the ventral medulla that contain the ventral chemosensitive nuclei, I studied CO_2-evoked ATP release by means of microelectrode biosensors. I have developed a model that reliably recapitulates hypercapnia-induced ATP release *in vitro*, by mimicking a key characteristic of the *in vivo* stimulus: an increase in PCO_2 that is accompanied only by modest changes in extracellular pH. By systematically altering pH, HCO_3^- and CO_2 levels, I have excluded extracellular acidification as the stimulus for ATP release. To test the possible involvement of intracellular pH, I modified it both directly with propionate and ammonium chloride and indirectly by blocking carbonic anhydrase. ATP release was not evoked or modified by these treatments. I conclude that it is the change in PCO_2 itself that is directly responsible for eliciting ATP release from the surface of the VLM.

2.2 Introduction

Respiration is a ceaseless activity essential for life that begins *in utero* and continues until death. Without this vital process it would be impossible for large multicellular organisms to exist, and even relatively brief pauses can lead to profound effects on major organs. To provide adequate ventilation dependent on activity and environmental conditions, respiration is subject to a variety of mechano- and chemosensory feedback mechanisms. The partial pressure of oxygen (PO_2) in arterial blood is sensed by the peripheral nervous system. In contrast the PCO_2 of blood is predominantly detected centrally (Feldman et al., 2003). Of the two blood gases, carbon dioxide provides the most powerful drive to breathe (Haldane and Priestley, 1905), and is therefore more tightly controlled. Sites within the central nervous

system responsible for detecting these changes are known as chemoreceptors, and are positioned so that they are able to efficiently detect fluctuations in blood gases and influence the central respiratory pattern generator to evoke adaptive changes in breathing.

Breakthroughs in biosensor technology have led to new experimental approaches that have enhanced the way we conduct scientific research. Their invention has made conducting previously impossible investigations possible, particularly in the field of chemosensitivity (Gourine et al., 2005a, Gourine, 2005, Spyer et al., 2003). Since the effects of this homeostatic mechanism could be measured only through changes in respiratory output, mechanisms of adult chemosensitivity have until now been studied only *in vivo* or in a semi-intact working heart brainstem preparation. The complexity of both the preparation and the system responsible for chemoreception means that identification of the molecular mechanisms of chemosensory transduction are extremely difficult without a reduced preparation. Although rhythmic activity can be studied *in vitro*, it is limited to neonatal preparations (Paton et al., 2002). Whilst these preparations have been undeniably invaluable, chemosensory mechanisms of the neonate differ to the adult (Putnam et al., 2005, Wickström et al., 2002, Davis et al., 2006). They are thus poor models, ineffective for the study of the adult response. Using microelectrode biosensors, I have created a new *in vitro* model for studying the classical chemosensitive areas of the VLM surface of the adult rat, in isolation from the rest of the system: by utilising ATP release as a measure of chemosensory output, I am no longer reliant on respiratory activity. This has allowed for the first time, in depth studies into these regions.

The surface of the medulla has multiple sites for chemoreception, the first of these discovered was found on the ventrolateral medullary surface (here on in called the classical chemosensitive areas) (Loeschcke et al., 1970, Mitchell et al., 1963, Loeschcke, 1982). Not only do these areas respond to chemosensory stimuli, cooling of them decreases the ventilatory response to hypercapnia (increased CO_2) (Forster et al., 1997, Ohtake et al., 1996). Though they were discovered almost fifty years ago, the cell types and mechanisms involved have remained enigmatic, until recently. ATP is released from sites that correspond to the classical chemosensitive areas (Gourine et al., 2005a). Moreover, applications of exogenous ATP replicate the response to

increased CO_2 and applications of its antagonist PPADS to the site of ATP release reduces the ventilatory response to hypercapnia (Gourine et al., 2005a).

To develop understanding of chemosensory transduction further, I thought it pertinent to characterise the nature of the CO_2 stimulus that evokes ATP release (Wang et al., 2002, Mulkey et al., 2004). Changes in PCO_2 could act directly or indirectly either through extracellular or intracellular pH or through changes in HCO_3^- . A common consensus is that CO_2 dependent acidification acts on pH sensitive channels. For example pH dependent closure of K^+ channels has been proposed as the primary focus of chemosensory transduction in chemosensitive neurons (Duprat et al 1997. In particular TASK1 channels (TWIK-related acid sensing potassium channel 1) were favoured in this role. However $TASK^{-/-}$ mice do not have perturbations in their hypercapnic response (Mulkey et al., 2007b). Nevertheless other pH sensitive channels remain, such as further TASK channels or acid sensing ion channels (ASICs). By understanding the nature of the CO_2-evoked ATP release I hope to gain new insight into the transduction mechanisms themselves.

2.3 Methods

Once in the artificial cerebrospinal fluid (aCSF) carbon dioxide reacts with water and is in equilibrium with bicarbonate and hydrogen ions under the influence of carbonic anhydrase. Although this will occur spontaneously, this process is greatly accelerated by this enzyme. Thus, the response to hypercapnia could be mediated by any of these molecules excluding water (figure 1).

$$CO_2 + H_2O \underset{CA}{\longleftrightarrow} HCO_3^- + H^+$$

Figure 1: The conversion of CO_2 to H^+ and HCO_3^- is catalysed by carbonic anhydrase (CA).

I therefore systematically altered all three of the possible stimulatory molecules (figure 2a). To recapitulate the *in vivo* environment more accurately, I kept pH changes to a minimum. A solution containing 80mM HCO_3^- ions was bubbled with 9, 12 or 15% CO_2 creating a pH change of +0.15, 0.0 or -0.15 pH units respectively. For a 50mM HCO_3^- solution, CO_2 levels of 6, 9 and 12% gave the same pH changes that were seen in the 80mM HCO_3^- solution (figure 2a). The PCO_2 of all isohydric and control solutions was measured on a Siemens RapidLab 248 blood gas analyser (figure 2b).

a

HCO_3^- / pH	50 mM	80mM
	PCO_2 (mmHg)	
7.65	41	62
7.5	55	71
7.35	68	92

b

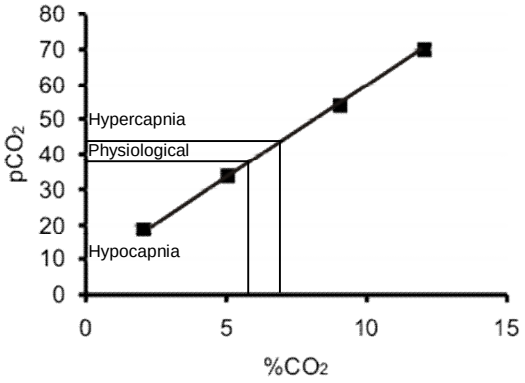

Figure 2: Constitution of solutions and the calculation of PCO_2. a) The relationship between PCO_2, HCO_3^- and extracellular pH of solutions. b) The relationship between the percentage and partial pressure of CO_2 at pH 7.5.

2.3.1 Tissue preparation

Experiments were performed on adult Sprague-Dawley rats (4-6 weeks). The medulla oblongata was removed in its entirety from the animal with no spinal cord and a ventral tissue slice was sectioned at 400μm. The medulla was dissected out in cold (approximately 4-6ºC) aCSF (NaCl 124mM, $NaHCO_3$ 26mM, NaH_2PO_4 1.25mM, KCl 3mM, $MgSO_4$ 11mM, $CaCl_2$ 1mM and D-glucose 10mM, equilibrated with 95%O_2, 5 % CO_2). During the sectioning care was taken to ensure that no blood found its way onto the medullary surface, for successful recording of ATP release. It was also essential that the pia mater, the innermost membrane surrounding the brain, remained intact throughout the preparation. Once removed the preparation was placed into a perfusion chamber containing standard aCSF (NaCl 124mM, $NaHCO_3$ 26mM, NaH_2PO_4 1.25mM, KCl 3mM, $MgSO_4$ 1mM, $CaCl_2$ 1mM and D-glucose 10mM equilibrated with 95%O_2, 5 % CO_2) at 33ºC at a flow rate of approximately 5ml.min^{-1}.

2.3.2 Sensor placement

Once in the perfusion chamber, two 0.5mm biosensors were placed on the medullary slice in a variety of locations according to medullary landmarks (figure 3). At all sensor positions the tissue was treated with a standard stimulus of 80mM HCO_3^- solution bubbled with 91% O_2, 9% CO_2 (pH 7.65), as this elicited the largest amount of ATP release (figure 10) and therefore would give me the greatest chance to observe it and any changes that may occur.

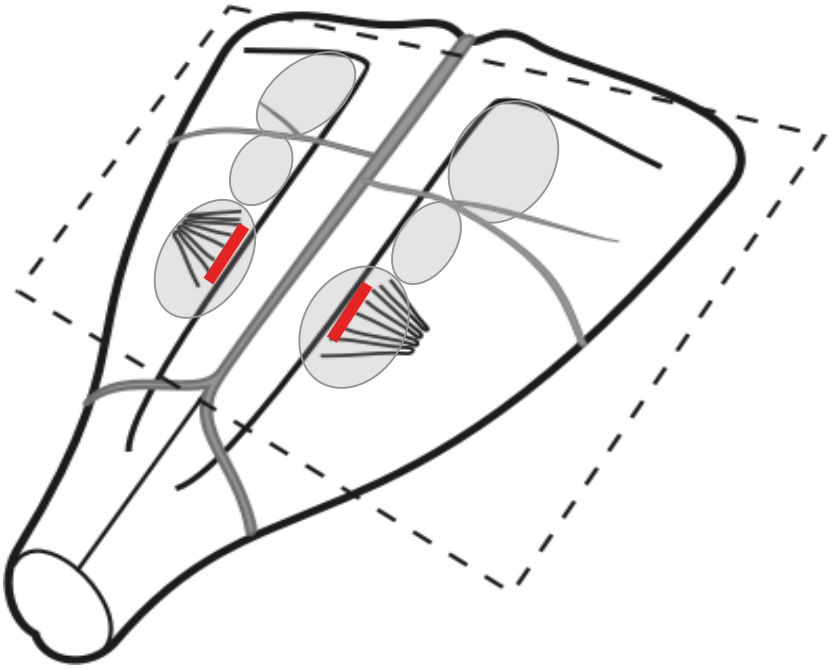

Figure 3: Placement of ATP microelectrode biosensors on the ventral surface of the medulla. Grey circles represent the classical chemosensitive areas. Red lines represent the placement of 0.5mm biosensors.

2.3.3 ATP Biosensors

The biosensors require glycerol as a substrate, to produce the electro-active molecule hydrogen peroxide. As this is an ATP dependent process, hydrogen peroxide is formed in proportion to ATP. Being electro-active, hydrogen peroxide can be oxidised at a platinum wire, polarized to 500 mV relative to Ag/AgCl (silver/silver chloride). The reduction of the platinum generates an electrical recording (figure 4). 2mM glycerol was therefore present in all solutions to enable biosensor operation (figure 4).

Null sensors are identical to ATP biosensors with a polymer layer devoid of enzymes. They react to any non-specific electro-active compounds or electrical disturbances in the perfusion chamber. This activity can then be subtracted from that of the ATP biosensor, thus providing more accurate recordings (figure 5).

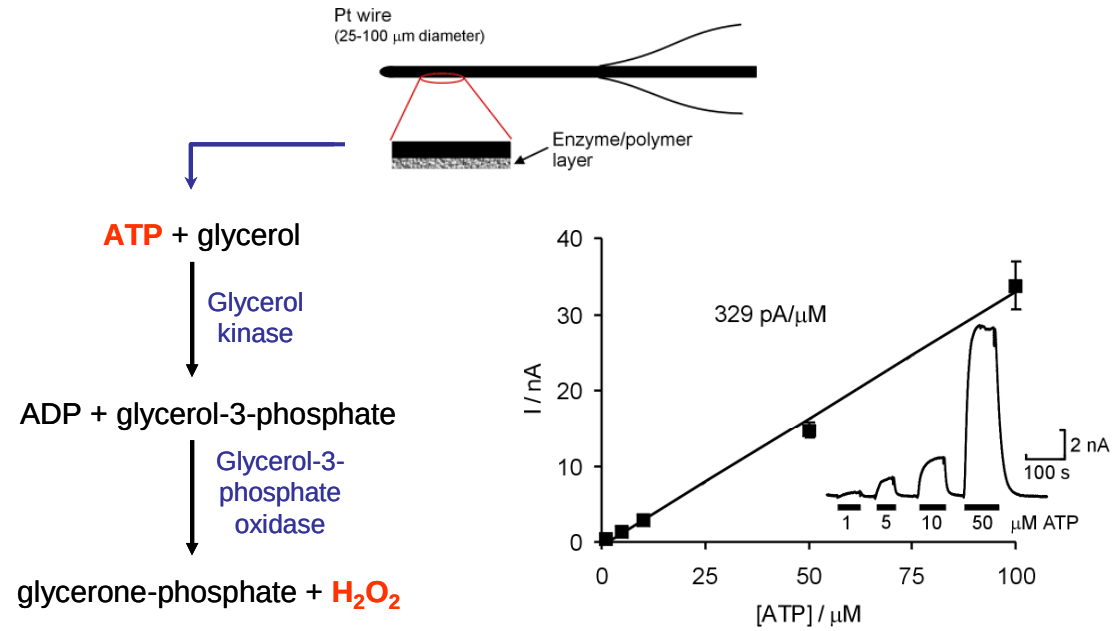

Figure 412: Enzymatic cascade of the ATP biosensor. Note that glycerol is essential for biosensor operation. (Picture supplied by Sarissa biomedical).

Sensors were calibrated with 10μM ATP before and after each experiment. By comparing the total current change evoked by a known concentration of ATP I can calculate the amount of ATP released during each experiment (figure 5).

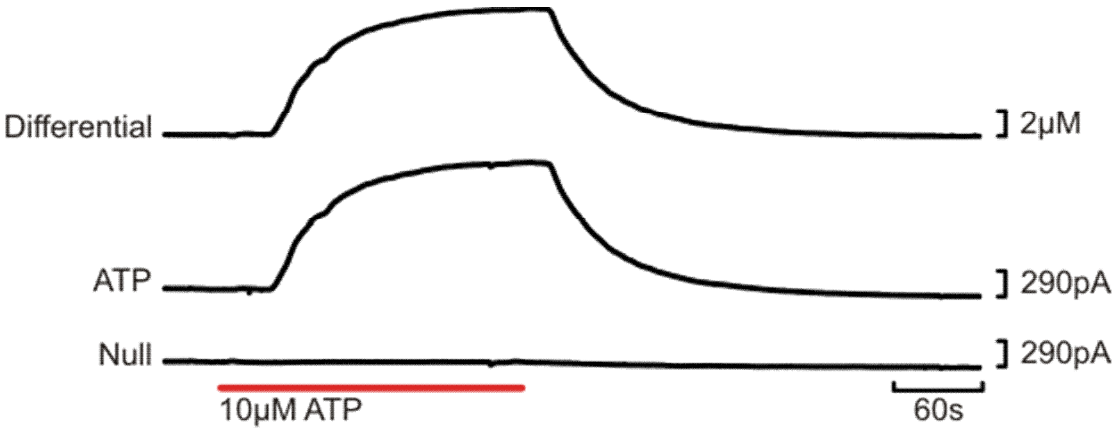

Figure 5: Calibration tracers of null and ATP biosensors. Top: Trace made from the subtraction of the null signal from the ATP signalling. Middle: Raw trace from the ATP biosensor. Bottom: Raw trace from the null biosensor during calibration with 10μM ATP.

2.3.4 Hypercapnic acidosis

The tissue slice was left for 15 minutes to equilibrate (pH 7.5), before undergoing a 3 minute treatment in the hypercapnic solution (pH 7.1 or 7.2). The tissue underwent a further two 3 minute hypercapnic washes at 15 minute intervals. After the final hypercapnic stimulus, the tissue was left for 10 minutes before the biosensors were removed and calibrated. The hypercapnic acidotic solution is the same composition as the control solution (26mM HCO_3^- and 124mM NaCl), bubbled with 9% CO_2, 91%O_2 causing an approximate pH change of -0.4 pH units from pH 7.5 to 7.1.

2.3.5 Isohydric Hypercapnia

The tissue was left to equilibrate (pH 7.5) for 30 minutes, after which it underwent a 5 minute treatment with an isohydric solution, either an isohydric hypercapnic solution containing 80mM HCO_3^- and 70mM NaCl (altered to keep sodium levels and osmolarity constant), or an isohydric solution containing 50mM HCO_3^- and 100mM NaCl. The tissue was then washed in the standard aCSF for a further 45 minutes (to allow for recovery), the sensors removed (to allow measurement of the tone) and calibrated.

2.3.6 Isohydric Hypocapnia

Preparation of the tissue and placement of the sensors was identical to that for isohydric hypercapnia in the caudal location (figure 3). Tissue was left to equilibrate for 30 minutes, then went into a 5 minute treatment with isohydric hypocapnic solution (10mM HCO_3^- and 140mM NaCl) and bubbled with 98% O_2, 2% CO_2 (pH 7.5), before a 45 minute wash in standard aCSF.

2.3.7 Intracellular pH changes.

After the initial recovery period, 80mM HCO_3^- aCSF saturated with 91% O_2, 9% CO_2 was applied for 5 minutes, followed by a 30 minute wash to assess responsiveness of the tissue to CO_2. The intracellular pH modifiers, propionic acid (Fisher) 5mM or NH_4Cl 20mM (Sigma), were then applied for a period of 5 minutes in standard aCSF followed by a 30 minute wash in standard aCSF before the sensors were removed and calibrated.

Alternatively, after an assessment of tissue responsiveness, acetazolamide 500 µM (Sigma), to test for the participation of carbonic anhydrase, was applied for a period of 20 minutes in standard aCSF to allow it to take affect. Subsequently the

tissue underwent a second application of a hypercapnic 80mM HCO_3^- aCSF containing acetazolamide, followed by a 30 minute wash in standard aCSF containing the drug. The tissue was then washed for a further 20 minutes in standard aCSF before the sensors were removed and calibrated.

2.4 Results

2.4.1 Hypercapnic acidosis

I began my experiments by utilising the classical model of chemosensation, hypercapnic acidosis (figure 6). Hypercapnic acidosis, an increase in PCO_2 associated with a fall in pH, is known to cause ATP release at the VLM surface (Gourine et al., 2005). Although this stimulus provided me with ATP release (3.2 ± 0.2µM, n=12), it was not reliable from preparation to preparation. Furthermore I was unable to evoke multiple episodes of ATP release, essential for assessing its mechanisms. The reason for the labile nature of ATP release was unknown. However I decided to seek better models for studying hypercapnia *in vitro*.

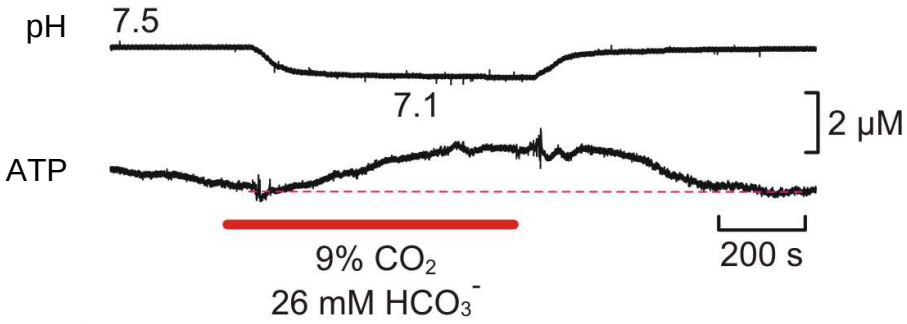

Figure 6: Hypercapnic acidosis evokes ATP release from the ventral surface of the medulla. Note that ATP is released almost immediately upon hypercapnic stimulation. Sensor signal is net ATP release (null sensor subtracted from the ATP trace)

2.4.2 Physiological changes of PCO_2/pH cause ATP release

To provide a more physiological stimulus I utilised a modified isohydric hypercapnic solution containing 80mM HCO_3^-, 9% CO_2, pH 7.65. This solution has a PCO_2 of 62 mmHg equivalent to a moderate episode of hypercapnia *in vivo*. This model evoked multiple bouts of release (2.2 ± 0.3µM, n =13) over an extended period of time from the caudal chemosensitive site (figure 7a and e). In conjunction the second episode evoked sufficient ATP for further investigation (1.5 ± 0.2µM, n=13) (figure 7a). At this site ATP release can diffuse at least 400µm into the medulla (figure 7b) at a concentration high enough to activate purinergic receptors (2.3 ± 0.1µM, n =5) (figure 7e). However ATP release is not present in deeper structures

(0.0 ± 0.3 µM, n=4) with similar sensor placement (figure 7c and e). As seen in a previous study (Gourine et al., 2005a) ATP release was also observed in the rostral chemosensitive site (0.8 ± 0.1µM, n =4) (figure 7d and e). ATP release was not seen outside of the classical chemosensitive areas (i.e. close to the lateral cut surface; data not shown) therefore this mechanism is site specific. It is limited to the most superficial layers of the VLM and overlies all chemosensitive regions that reside there.

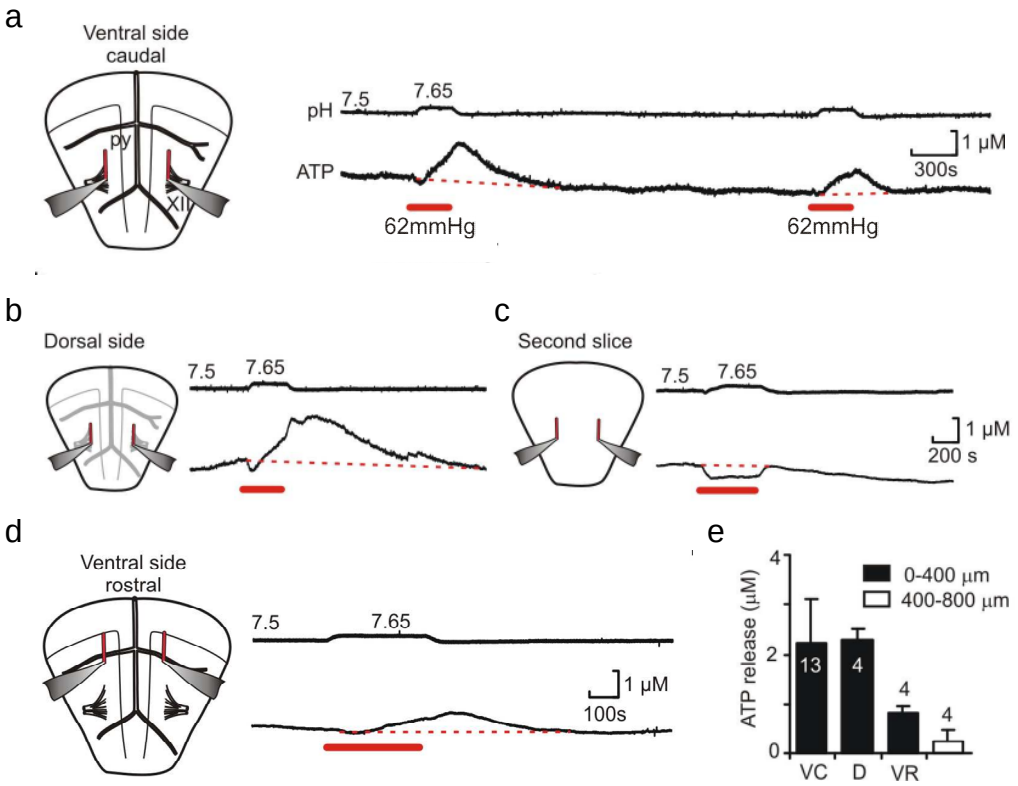

Figure 7: CO_2-evoked ATP release from the ventral surface of the medulla. a) Multiple episodes from the caudal site. b) ATP release can be detected from the dorsal surface of the ventral most slice (0-400µm) tissue. c) The second tissue slice is devoid of ATP release (400-800µm) (the fall below baseline seen here is likely due to some residual pH sensitivity of the biosensors which does not effect the sensors equally). d) ATP release from the rostral site, equivalent position to the RTN. e) Summary of ATP release from different sites of the medulla.

A basal concentration of ATP, a tone, was measured by removing the sensors from the ventral surface of the medulla and measuring the resulting fall in the sensor signal (3.2 ± 0.9µM, n=13) (figure 8). This concentration of ATP is sufficient to cause sustained excitation of purinergic receptors, in the vicinity of the ventral surface. This tone was not present in the second slice (0.1 ± 0.3µM, n = 4). This, the first description of tonic release of ATP at the ventral surface of the medulla implies that ATP may provide a continuing source of drive to the chemosensory and respiratory nuclei that reside there.

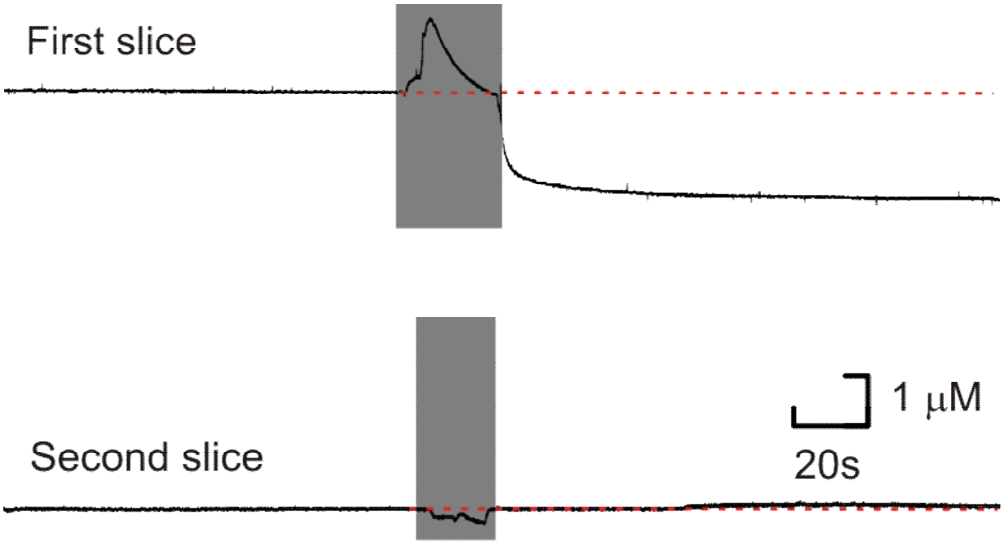

Figure 8: ATP tone on the ventral surface of the medulla. Shaded area designates where the sensors were removed. The tone is measured as the difference between the current of the differential recording after the sensor is removed from the slice compared to the current when the sensors are in contact with the tissue dotted line.

2.4.3 Systematic variation of PCO_2, pH and HCO_3^-

To determine the key features of the CO_2 stimulus I systematically varied PCO_2, pH and HCO_3^-. The modified isohydric hypercapnic models elicited ATP release in all combinations tested (figure 9 and 10a).

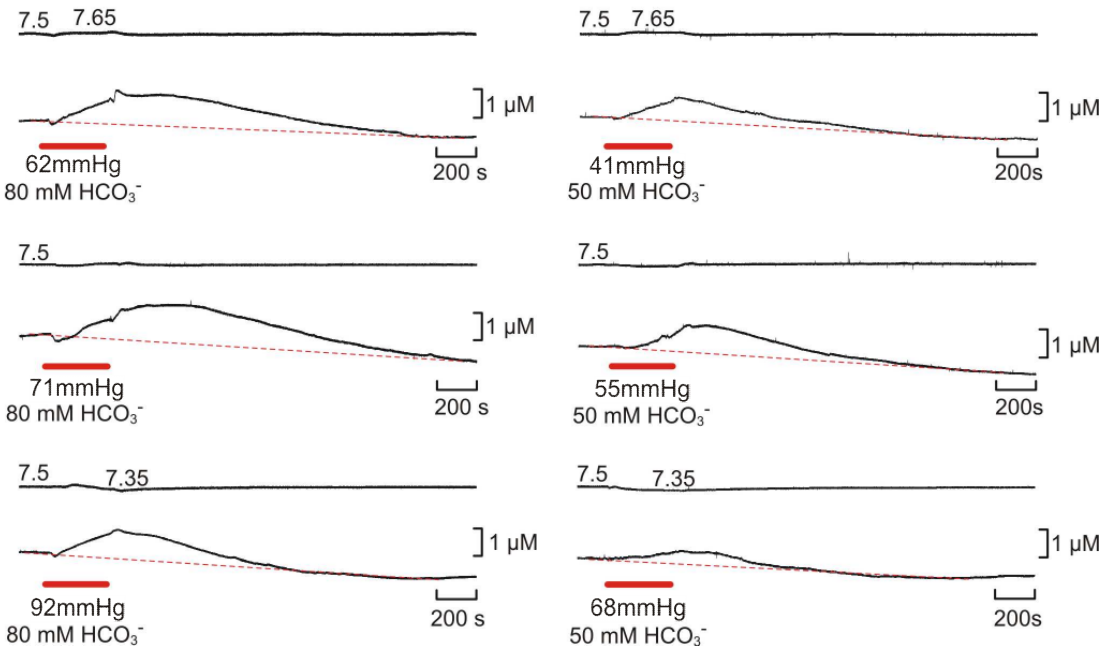

Figure 9: Effects of different models of hypercapnia on ATP release from the ventral surface of the medulla. Note that peak ATP release decreases with extracellular acidification, and increases at the same pH with increasing concentrations of CO_2.

ATP release is governed by interactions between PCO_2 and pH, with PCO_2 having an excitatory effect and pH having an inhibitory effect (figure 10); thus for any given pH ATP release increases with increases in PCO_2. In fact the positive effect of PCO_2 appears to be able to overcome the inhibitory effect of pH if the concentration is high enough (figure 10). Therefore I have excluded extracellular pH as a possible stimulus. The solutions bubbled with 9% CO_2 provided similar PCO_2 shifts (80mM, 9%: 34-62 mmHg and 50mM, 9% CO_2 34-54.5 mmHg) that are seen during *in vivo* experiments (40-60 mmHg). Therefore these models appear to provide a much better replication of *in vivo* conditions and will enable the further study of this mechanism.

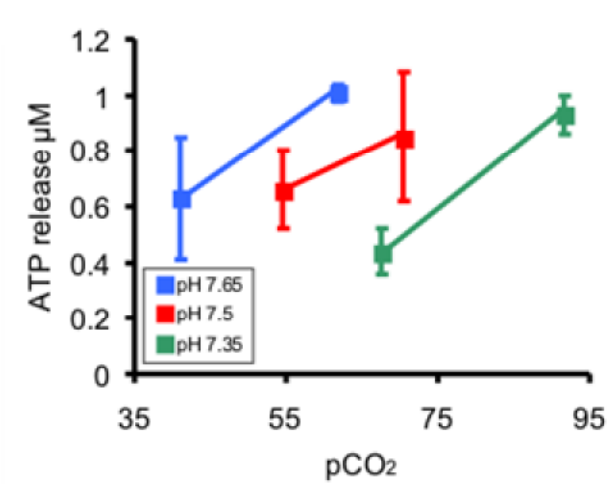

Figure 10: Effects of different models of hypercapnia on ATP release from the ventral surface of the medulla. a) Systematic changes in HCO_3^-, pH and CO_2. Note that increasing PCO_2 can overcome the inhibitory effect of extracellular acidification.

2.4.4 Isohydric hypocapnia

The discovery of the ATP tone led me to postulate that a decrease in PCO_2 may act to reduce ATP release and thus any tonic influence it may have. Isohydric hypocapnia (PCO_2 19mmHg) had the opposite effect to isohydric hypercapnia, and significantly reduced basal ATP levels at the surface of the medulla (-0.6 ± 0.1µM, n=6; figure 11). Interestingly this is similar to that of tissue treated with a similar increase in PCO_2 (55mmHg) at the same pH (0.7 ± 0.1µM, n=4). Note also that the duration of the response to hypo- and hyper- capnia shared the same relationship (low PCO_2: 1988 ± 327 secs, n = 6. High PCO_2: 1779 ± 181 secs, n = 4).

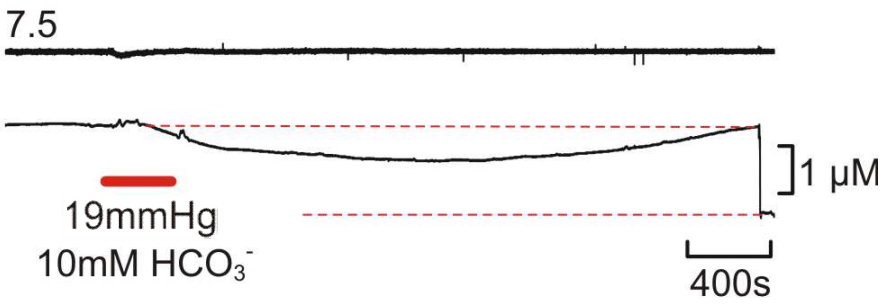

Figure 11: Reduction in ATP tone on the ventral surface of the medulla caused by isohydric hypocapnia. Note the sustained fall in ATP tone caused by a fall in PCO_2.

2.4.5 ATP release at the VLM highly correlates to PCO_2 concentrations

The relationship between the percentage and partial pressure of CO_2 was linear (figure 2b), whereas the relationship between ATP release and PCO_2 at pH 7.5 exhibited a trend towards saturation at high levels of PCO_2 (figure 12), with the steepest part of the curve within physiological range. Since pH is not a contributing factor in this comparison, there must be a physiological reason for the plateau, for example the rate at which the mechanism of ATP release can work within the cell.

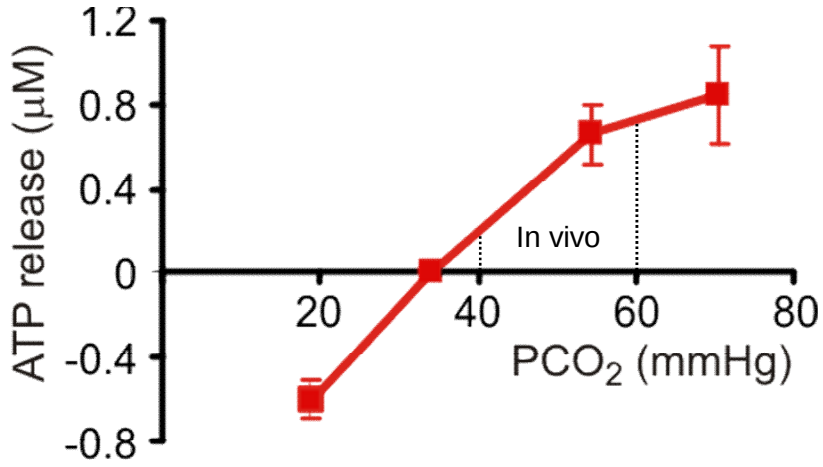

Figure 12: The effect of increasing PCO_2 at pH 7.5. Note that there is an almost linear response between ATP release and PCO_2 over a clinically relevant range of PCO_2.

2.4.6 Intracellular pH modifiers

A change in PCO_2 can potentially alter intracellular pH. Therefore to test whether this might evoke ATP release I used propionate and ammonium chloride to decrease or increase intracellular pH respectively.

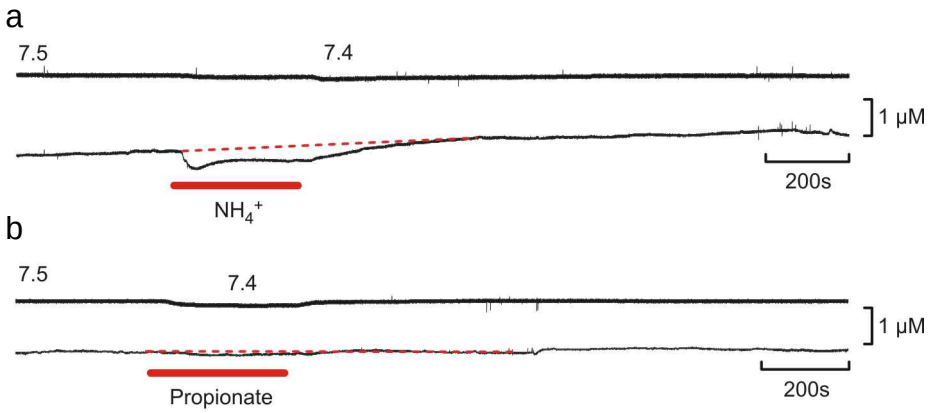

Figure 13: Effects of intracellular pH changes on ATP release. a) 20mM Ammonium chloride (reduction below baseline here is due to a subtraction difference created by differential pH sensitivities of the biosensors b) 5mM Propionic acid.

Modification of intracellular pH was without effect. Neither propionate, which causes intracellular acidification (Dulla et al., 2005), nor ammonium chloride (Trapp et al, 1996) which causes intracellular alkalisation were sufficient to elicit a response (0.0 ± 0.0µM, n=3 and 0.0 ± 0.1µM, n=5, respectively) (figure 13). This suggests that alterations of intracellular pH without accompanying changes in PCO_2 are not sufficient as a cellular mechanism to cause ATP release.

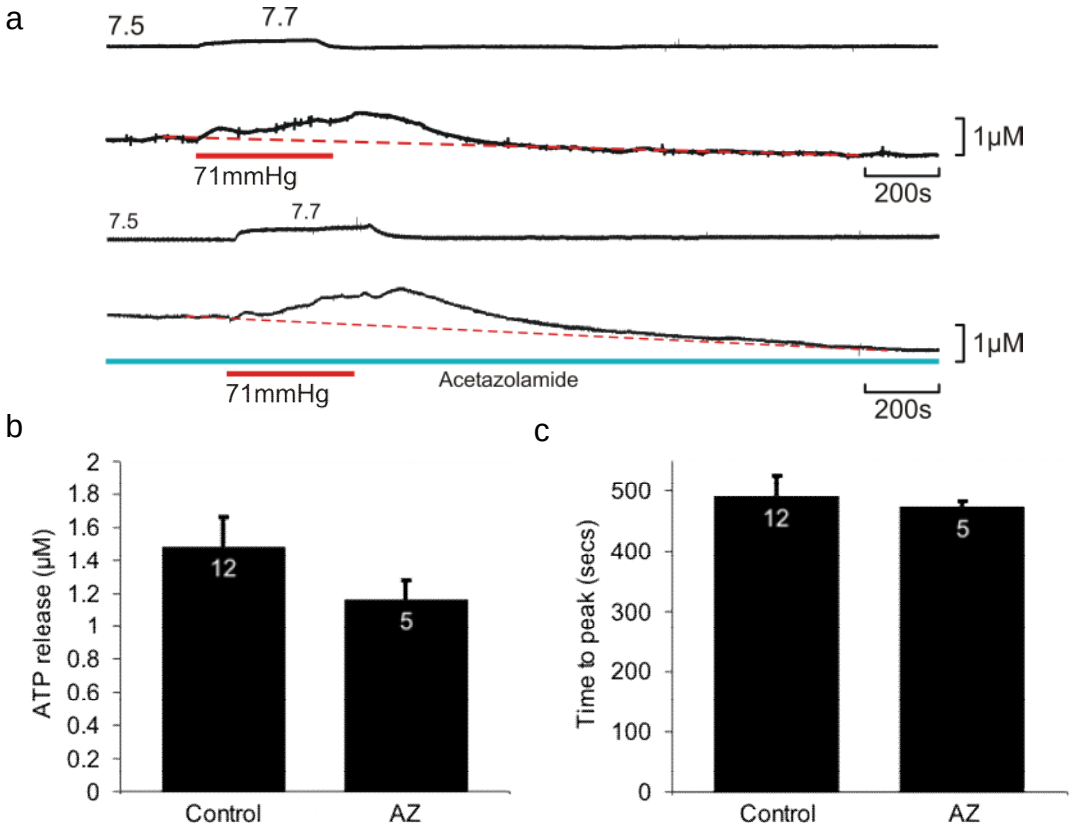

Figure 14: The effects of carbonic anhydrase blockade on ATP release a) profile of ATP release b) Peak ATP release c) Time taken for ATP release to reach its peak.

To test the possible involvement of carbonic anhydrase I used acetazolamide, a blocker of this enzyme. Acetazolamide did not reduce the response to isohydric hypercapnia compared to interleaved controls (figure 14a and c). It was also without effect on the time taken for ATP release to reach its peak (figure 14b and c). Therefore hypercapnia-triggered ATP release does not depend on carbonic anhydrase, thus the mechanism is likely to be directly modulated by CO_2.

2.5 Discussion

2.5.1 ATP release from the ventral surface

I began by replicating the *in vitro* experiments performed by Gourine et al 2005, and evoked ATP release. However the inability of this preparation to elicit repeated episodes led me to search for alternative methods of hypercapnic stimulation. My search for a more appropriate stimulus, led me to use quasi-isohydric solutions similar to those used in previous investigations into chemosensitive stimuli (Filosa and Putnam, 2003, Richerson, 2004, Wang et al., 2002, Ritucci et al., 2005, Otsuguro et al., 2006). Use of thee stimuli led to reliable and repeatable ATP release on the VLM surface (figure 7, 9 and 10).

2.5.2 ATP release in the ventral medulla

I examined the location of ATP release from the VLM (figure 7). My objective was to test if this more physiological stimulus would evoke ATP release from other classical chemosensitive regions. I found that it did but at lower levels compared to the caudal region (figure 7e). I next clarified that ATP release was confined to the ventral surface, as it was for Gourine *et al* 2005. Since ATP release was not seen on the surface of the second slice, from cells directly adjacent to those of the dorsal surface of the first tissue slice, I concluded that the ATP must be released at the ventral surface, but is capable of penetrating at least 400µm dorsally.

Interestingly the pre-Bötzinger complex is located 150-400µm from the ventral surface (Kuwana et al., 2006) and sends dendritic projections to it (Kawai et al., 1996). The pre-Bötzinger complex is the kernel for respiratory rhythm generation (Smith et al., 1991), and has not only been shown to contain purinergic receptors, but to be modulated by ATP (Lorier et al., 2004, Lorier et al., 2007). Therefore at these concentrations and this level of penetration, ATP would be placed to affect both the somatatic and dendritic P2Y receptors and thus directly affect respiratory rate.

Serotonergic neurons of the medullary raphé, whose cell bodies and dendrites reside within 150µm of the VLM surface (Ribas-Salgueiro et al., 2005, Richerson, 2004) are also modulated by ATP and applications of ATP cause a similar response to electrical stimulation of these nuclei (Cao et al., 2006, Cao and Song, 2007). Since serotonergic neurons provide a tonic drive to the pre-Bötzinger complex, which stabilise the membrane potential there (Pace et al., 2007), as well as sending chemosensory information to the respiratory rhythm generator (Richerson, 2004, Ribas-Salgueiro et al., 2005, Hodges et al., 2008, Bodineau et al., 2004, Li and Nattie, 2008b), ATP signalling is again poised to influence both respiratory rate and the chemosensitive response.

The retrotrapezoid nucleus (RTN) is situated directly under the rostral chemosensitive site, with its cell bodies and dendrites located at the ventral surface of the medulla (Mulkey et al., 2004). The RTN provides tonic excitatory drive to the pre-Bötzinger complex and appears to contain intrinsically chemosensitive neurons (Mulkey et al., 2004, Li et al., 2006, Weston et al., 2004, Nattie, 2000). The RTN is of major importance to chemosensory drive; moreover it has been shown to contain both P2Y and P2X receptors. The chemosensitive neurons are directly excited by exogenous ATP acting through $P2Y_1R$ (Mulkey et al., 2006). At this site too, ATP can again alter both the respiratory and chemosensory drive.

2.5.3 Characterisation of the stimulus

Under hypercapnic conditions *in vivo* the blood pH changes by approximately 0.15 units (Gozal et al., 1995), a value I have also observed (see chapter 3). It is however unknown if the same pH change occurs in the extracellular fluid; it has been hypothesised that pH may actually become alkaline (Ballanyi et al., 1999) as proton transporters on chemosensitive neurons remove them from the extracellular space. It is also probable that the actions of such proton transporters in conjunction with bicarbonate ion transporters means that no pH change would be seen.

Interestingly ATP release varied as a consequence of PCO_2, and not extracellular pH (which had an inhibitory effect) or HCO_3^- levels (figure 9). The primary stimulus for the response to hypercapnia must therefore be dependent on PCO_2. However this data does not discriminate whether it is a direct action of CO_2 or consequent changes in intracellular pH. It does however remove the possibility of the

involvement of acid sensing ion channels, as they are activated by extracellular pH and are not affected by PCO_2.

To test the possible involvement of intracellular pH (pH_i) changes, I used pharmacological agents that can change pH_i, without affecting extracellular pH. These agents were not sufficient to elicit ATP release (figure 13). To further investigate the effects of pH_i on ATP release, I antagonised the effects of carbonic anhydrase to alter the effect of CO_2 on changes in pH_i. Attenuating the actions of this enzyme slows the conversion of CO_2 to HCO_3^- and H^+; thus changes in pH_i are protracted and should lead to either alterations in the amount of ATP released or increase the time taken for ATP release to reach its peak. I saw no significant change in either the peak of ATP release or the time taken for ATP release to reach its peak (figure 14). Thus I excluded changes in pH_i and HCO_3^- as possible stimuli for evoking the release of ATP, leaving only changes in PCO_2. Isohydric hypercapnia causes much smaller changes to pH_i when compared to hypercapnic acidosis (Ritucci et al, 2005), which indicates it is of lesser importance in this preparation.

2.5.4 Implications for chemoreception

This is the first time that chemosensitivity has been shown to derive directly from changes in PCO_2. This provides an interesting insight into chemosensation as a whole, and it would appear that different chemosensitive sites respond to different signalling molecules. RTN neurons can be excited by changes in extracellular pH (pH_e) (Mulkey et al., 2004), while serotonergic neurons respond to pH_i (Wang et al., 2002), by contrast ATP releasing cells respond to PCO_2 (this study). This may be a contributing reason as to why there are so many chemosensitive sites, each senses different aspects of the same signal. These signals may then converge, if all sites are stimulated then a large response is seen, but if only a few are stimulated a smaller response is seen. It may also provide a mechanism by which adaptation to chronic disease may be seen. For instance in terms of metabolic acidosis, pH_e remains constantly low and above the threshold for activation of breathing. Under these conditions this pathway may effectively desensitise leaving other remaining aspects of the stimulus (PCO_2, pH_i etc) as being important.

2.5.5 Reductions in ATP release at the VLM during hypocapnia may modulate breathing

Another exciting finding of this current study is that there appears to be tonic release of ATP from the chemosensory sites (figure 8). Thus during eucapnia ATP may provide a tonic drive to breathe. More interestingly, it would also allow purinergic signalling to underlie the response to hypocapnia. Indeed when I reduced PCO_2, I found this caused a reduction in ATP tone at the ventral surface in the caudal chemosensitive area (figure 10 and 11). During hypocapnia the respiratory rate decreases, thus slowing the excretion of CO_2 from the lungs and bringing it back into normal parameters. This reduction in tone could remove the tonic excitatory influence of ATP from the respiratory and chemosensory systems and thus leads to a slowing in breathing. Physiologically hypocapnia is more commonly observed than hypercapnia e.g. during excursions to high altitude, thus this form of respiratory control, by reductions in ATP release at the VLM surface, is likely too play a more prominent role.

2.6 Summary

To conclude, I have discovered a new physiological stimulus for eliciting ATP release from the classical chemosensitive areas. ATP is released from the classical chemosensitive areas in response to changes in PCO_2, and is confined to the ventral surface of the medulla. It can however reach nuclei important in chemosensitivity and respiration and may influence the response to both hypo- and hypercapnia.

Chapter 3: ATP is released through connexin 26 hemichannels on the leptomeningeal cells and on sub-pial astrocytes at the VLM surface in response to hypercapnia

3.1 Abstract

ATP release from the ventro-lateral medullary (VLM) surface is a key component in the hypercapnic response *in vivo* and can be seen *in vitro* (Gourine et al 2005). In the previous chapter I demonstrated that ATP is released as a consequence of changes in PCO_2 rather than pH and may have a role in the response to hypocapnia as well as hypercapnia (chapter 2). Using our new, more physiological stimulus I have now studied the mechanisms and cell types involved in this process. I have demonstrated that ATP release is independent of extracellular Ca^{2+} and have evidence that gating of connexin hemichannels mediates ATP release. Quantitative PCR and immunofluorescence show that connexin 26 is highly expressed at the VLM surface. Application of gap-junction antagonists, with selectivity towards connexin 26, greatly reduced ATP release in response to elevated CO_2 *in vitro* and *in vivo* and reduced the tone of ATP at the VLM surface. Dye loading studies have shown that that the cells responsible for releasing ATP are of glial origin and reside within the first 150μm of the VLM surface and a second group of cells in the leptomeninges. Therefore I propose that ATP is released from sub-pial astrocytes and leptomeningeal cells through connexin 26 hemichannels in response to alterations in PCO_2.

3.2 Introduction

ATP is known to be released from specific sites on the surface of the ventral medulla (Gourine et al., 2005a). Though the chemosensitivity of these sites was discovered nearly fifty years ago (Loeschcke et al., 1970, Mitchell et al., 1963, Loeschcke, 1982), the cell types and mechanisms involved in this process remain unknown. The previous chapter shows that changes in PCO_2 directly, and not through its actions on either extracellular or intracellular pH, was the determining factor (chapter 2). Importantly, although extracellular pH was removed as a possible

stimulus, it was found to be a contributing factor to the amount of ATP seen in response to hypercapnia. A fall in pH_e reduced the amount of ATP seen at the ventral surface, for equivalent levels of PCO_2 (chapter 2). These discoveries have provided some insight as to what may be occurring in this process, although a mechanism that is PCO_2 sensitive and reduced by decreases in pH_e has not yet been described.

There are known mechanisms of ATP release elsewhere in the brain and body. ATP can be released through synaptic vesicular neuronal exocytosis, such as in adapting pulmonary stretch receptor afferents seen in the NTS (Gourine et al., 2008). In addition neuronal vesicular release can be seen extra-synaptically, such as in the dorsal root ganglia (Zhang et al., 2007a). Cultured astrocytes are also capable of exocytotic release of neurotransmitters via lysosomatal fusion (Zhang et al., 2007b). All of these processes have been shown to be exquisitely sensitive to extracellular calcium for many of their processes, therefore its removal should inhibit these mechanisms.

There are also non-exocytotic mechanisms of release such as gap junction hemichannels. ATP release has been shown by connexins (Kang et al., 2008) in the cochlear (Zhao et al., 2005) and retina (Pearson et al., 2005) and pannexins in Xenopus oocytes (Bao et al., 2004) and an implied role in the taste buds (Romanov et al., 2007, Huang et al., 2007). Hemichannels have been shown to open in response to lowered extracellular calcium; thus, if mediated by hemichannels, ATP release should be independent of this stimulus. Finally, volume regulated anion channels (VRACs): these maxi-anion channels are the route for ATP release in aortic endothelial cells (Hisadome et al., 2002). VRACs are highly dependent on intracellular calcium (Takano et al., 2005, Lang et al., 1998), yet the dependence on extracellular calcium for the activation of this ion channel family is largely unknown. Since these mechanisms show differential response to altering calcium ions, alterations in extracellular calcium could provide insights into the release mechanism.

One implication from the previous chapter is the inhibitory effect of decreasing pH_e. Importantly this discovery removes the involvement of acid sensing channels. TASK channels (TWIK-related acid sensing potassium channels) and ASIC (acid sensing ion channels) are activated by decreasing extracellular pH (Duprat et al., 1997, Waldermann et al., 1997). In contrast, gap junctions and hemichannels close with decreasing extracellular pH, which suggests that it may be either connexin or pannexin mediated.

It is also unknown whether ATP release is of glial or neuronal origin. Since it is confined to the most superficial structures of the ventral medulla (Gourine et al., 2005a), one must look at the cell types that reside there. The VLM surface has a thickened marginal glial layer, which is to say that the first 150μm of the ventral medulla is enriched in glia with very few neuronal cell bodies seen (Sato et al., 1992, Okada et al., 2001). An important discovery in terms of this hypothesis was that silent cells within the MGL depolarise in response to hypercapnia, indicating a yet unknown role for glia in the hypercapnic response (Fukuda et al., 1978, Ritucci et al., 2005). Okada *et al* (2001) speculated that glia may be the primary chemoreceptors, and that neurons involved in chemosensitivity may be acting in response to transmitters released from this cell type.

The MGL of the medullary surface differs from the MGL throughout the rest of the brain, in that, whilst they are few, it does contain neuronal cell bodies. Although many neurons send dendritic projections to the ventral surface, there are few studies that report axonal projections there. However since the concentration of ATP release seen in the dorsal surface of a cut tissue slice is not different to that seen on the ventral surface, it may be released from deeper structures at levels between 150-400μm. The neurons at this level and those that reside in the MGL do receive afferent inputs and it is possible that the neurotransmitter released by these afferent inputs is ATP. Possible neuronal sources of ATP are the medullary raphé (Richerson, 2004) and the RTN (Mulkey et al., 2004) which reside in the VLM or commissural NTS which sends projections to every aspect of the VLM surface (Takakura et al., 2006, Moreira et al., 2007, Bodineau et al., 2000). The ventral slice preparation removes the possibility of release from more dorsal structures such as the NTS. It may also be plausible that it released from a yet undiscovered set of interneurons residing in this region.

In this study I aim to identify the mechanism of ATP release at the ventral surface of the medulla, and in doing so try to elucidate molecular markers for the cell types involved. Using these molecular markers I hope to identify the location and types of cells that are involved in this response.

3.3 Methods

3.3.1 In vitro tissue preparation

Tissue was extracted from 4-6 week old Sprague Dawley rats as described in Chapter 2. Biosensors were placed and calibrated in a similar manner to that described in the previous chapter. The composition of solutions used also remained unaltered.

3.3.2 Application of pharmacological agents

All chemicals and compounds are from Sigma, unless otherwise indicated. 2 mM glycerol was added to all solutions to enable operation of the ATP biosensor. After the initial recovery period, 80mM HCO_3^- aCSF (NaCl 70mM, $NaHCO_3$ 80mM, NaH_2PO_4 1.25mM, KCl 3mM, $MgSO_4$ 11mM, $CaCl_2$ 1mM and D-glucose 10mM) saturated with 91%O_2, 9% CO_2 was applied for five minutes followed by a 30 minute wash period in standard aCSF (to test for tissue responsiveness). The pharmacological agent (carbenoxolone 10 µM, carbenoxolone 100 µM, Co^{2+} 500 µM, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) 200 µM, proadifen 200 µM or probenecid 1 mM) was then applied for a period of 20 minutes in standard aCSF to allow it to take affect. A second application of a hypercapnic 80mM HCO_3^- aCSF containing the appropriate drug was applied followed by a thirty-minute wash in standard aCSF containing the drug. The tissue was then washed for a further twenty minutes in standard aCSF before the sensors were removed (to allow measurement of the tone; see chapter 2) and the sensors calibrated.

3.3.3 Effects of extracellular Ca^{2+}

After the initial recovery period, 80 mM HCO_3^- aCSF saturated with 9% CO_2 was applied for five minutes (to assess tissue responsiveness) followed by a 30 minute wash period. The Ca^{2+}-free aCSF (standard aCSF in which $CaCl_2$ was substituted by $MgCl_2$ with the addition of 1 mM EGTA: ethylene glycol tetraacetic acid) was then applied for 20 minutes before a second application of a Ca^{2+}-free 80 mM HCO_3^- aCSF saturated with 91%O_2, 9% CO_2 (80 mM HCO_3^- aCSF in which $CaCl_2$ was substituted by $MgCl_2$ with addition of 1 mM EGTA). This was followed by a 30 minute wash in Ca^{2+}-free aCSF. The tissue was then washed for a further twenty minutes in standard aCSF before the sensors were removed (to allow measurement of the tone) and then calibrated.

3.3.4 Effects of Cx26 blockers on the response to zero calcium

After the initial recovery period of 15 minutes, Ca^{2+}-free aCSF was applied for 10 minutes. The solution was switched to Ca^{2+}-free aCSF containing a Cx26 blocker (carbenoxolone 100 µM, Co^{2+} 500 µM, NPPB 200 µM or Proadifen 200 µM) was applied for five minutes, followed by a 30 minute wash in Ca^{2+}-free aCSF. The tissue was then washed for a further twenty minutes in standard aCSF before the sensors were removed (to allow measurement of the tone) and calibrated.

The effects of the all drug applications were compared to the second episode of ATP release in time-matched controls.

3.3.5 In vivo recordings

Experiments were performed on 16 adult (age 10-12 weeks) male anaesthetised and artificially ventilated Sprague-Dawley rats and carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. The ventral surface of the medulla was exposed as described previously (Gourine et al., 2005a). Activity of the phrenic nerve was recorded as an indicator of respiratory activity. End-tidal levels of CO_2 were monitored on-line and kept at designated level by altering respiratory volume and frequency. ATP and null sensors (Sarissa Biomedical Ltd) were placed in a direct contact with the designated areas of the ventral surface of the medulla oblongata. Hypercapnia was then induced by titrating CO_2 into the respiratory mixture for a period of 3-5 min.

The rats were anaesthetised with urethane (1.6 g/kg, I.P.). Adequate anaesthesia was ensured by maintaining stable levels of arterial blood pressure, heart and central respiratory rate. The femoral artery and vein were cannulated for measurement of arterial blood pressure and administration of anaesthetic, respectively. The trachea was cannulated and the animal was ventilated with a mixture of 50% oxygen and 50% nitrogen (unless otherwise required by the protocol) using a positive pressure ventilator (Harvard rodent ventilator, model 683) with a tidal volume of ~2 ml and a ventilator frequency similar to spontaneous frequency (~60 strokes/min). The animal was then injected with gallamine triethiodide (Flaxedil™, 10 mg/kg, I.V.; then 1-2 mg/kg/h, I.V.) and was placed in a stereotaxic frame. The ventral surface of the medulla oblongata (VLM) was exposed as described previously (Gourine et al 2005).

Activity of the phrenic nerve was recorded as an indicator of central

respiratory drive. The signal was amplified (x 20,000), filtered (500-1500 Hz) and rectified and smoothed (τ = 50 ms). Partial pressures of O_2 and CO_2 as well as pH of the arterial blood were measured every 1-2 h. End-tidal levels of CO_2 were monitored on-line using a fast-response CO_2 analyser (model Capstar-100, CI Inc., Ardmore, PA, USA) and kept at a designated level by altering tidal volume and respiratory frequency. In all the experiments the partial pressure of oxygen (PO_2) in the arterial blood was kept at >100 mmHg to ensure minimal drive from the peripheral chemoreceptors. The body temperature was maintained with a servo-controlled heating pad at 37.0 ± 0.2°C.

ATP and null sensors were connected to a MicroC potentiostat (WPI, Sarasota, Florida, USA) or Duostat Me200+ (Sycopel international Ltd) and held on a stereotaxic micromanipulator. The sensors were aligned with the pyramidal tracts and major blood vessels running on the VLM and then were placed in a direct contact with the VLM 0.1-0.5 mm lateral from the pyramidal tracts. The sensitive part of the sensor was ~0.5 mm in length placed over a significant portion of the VLM chemosensitive areas just rostral from the XII nerve roots. The identical placement of the ATP sensors and null sensors was achieved by aligning them to the pyramidal tracts, landmark blood vessels and by means of the vernier scale of the manipulator. Once the sensors were placed, the exposed area of the brain and both sensors were covered with an excessive volume of modified Bulmer's buffer (100 mM NaCl, 10 mM phosphate buffer, 1 mM $MgCl_2$ and 2 mM glycerol) and a period of 20-30 min was allowed until a steady baseline was obtained.

3.3.6 Quantitative PCR

300µm slices were cut along the ventral-dorsal axis of the rat medulla and RNA extracted using the RNeasy kit (Qiagen). Reverse transcription (by standard methods) was performed and the resultant complementary DNA (cDNA) was used in subsequent QPCR analysis using an Applied Biosystems Real Time 7000. Rat β-actin, Connexin 26, 32, 36, 43 and Pannexin 2 primers were designed using the primer design package (Primer Express) supplied with the Applied Biosystems Real Time 7000 and were synthesised by Invitrogen. Each QPCR reaction was performed in triplicate and the experiment was performed 3 independent times (3 rats).

Primer sequences were: β -actin forward (F) aggccaaccgtgaaaagatg and reverse (R) gcctggatggctacgtacatg; Cx26 (F) gggagaggatgaggcaacct and (R) aatgtttgcccgggagatg; Cx32 (F) ccaacacggtggactgctt and (R) aggcggcgagcataaaga; Cx36 (F) cctacggagaagacggtctttc and (R) aggcggcgagcataaaga; Cx43 (F) tttccccgacgacaacca and (R) tggctaatggctggagttcat; and pannexin 2 (F) gctggtcaccctggtcttca and (R) cagtagccacgggcgtaca.

3.3.7 Immunocytochemistry

400 µm thick slices containing the ventral surface of medulla oblongata were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer pH 7.4 for 45 mins at room temperature. They were then placed in 30% sucrose in 0.1 M phosphate buffer solution (PBS), pH 7.4 and left overnight at 4^{o}C. The fixed tissue was then embedded in Cryo-M-Bed (Bright Instruments) and re-sectioned into 20µm transverse sections. All primary and secondary antibodies were made up in immunosolution (0.1M phosphate buffer, 0.1% BSA (bovine serum albumin), 0.1% sodium azide, 0.4% Triton X-100, pH 7.4). Slices were blocked overnight at 4^{o}C in 10% horse serum. Chick anti-GFAP (Chemicon) and mouse anti-Connexin 26 (Invitrogen) were applied at 1:500 and 1:200 respectively and incubated overnight at 4^{o}C. The slices were then washed in 0.1 M phosphate buffer and were incubated with appropriate secondary antibodies for 2 hrs (Alexafluor 488 goat anti-mouse for Connexin 26 and β-galactosidase and Alexafluor 594 goat anti-chick for GFAP, both obtained from Invitrogen. After further washing the slices were dried and mounted in Vectashield (Vector Labs) and visualised under an SP2 Leica confocal microsope. Some sections (in this case 60µm thick) were processed as free-floating sections. In these instances sections were also stained for MAP2 (1:500, Sigma). Slices were finally mounted in Vectashield DAPI (Vector Labs).

3.3.8 Immunohistochemistry of LacZ reporter

I received whole medullas from heterozygote Cx26$^{+/LacZ}$ transgenic mice (a gift from Klaus Willecke), where a LacZ reporter gene had been inserted in place of one copy of the Cx26 gene. The medullas were sectioned on a cryostat at 60µm intervals as described in the previous section. Free floating tissue sections were incubated in 500µl of 15% X-Gal buffer (0.075g X-Gal, Melford Labs, in 500µl DMF), 50µl X-Gal buffer solution (0.165g potassium ferricyanide and 0.211g

potassium ferrocyanide in 50ml PBS) and 1µM magnesium sulphate. The tissue was left overnight at 37ºC to develop and washed with PBS. Sections were then dried and mounted in Vectashield (Vector Labs) and visualised under an Olympus light microscope. All reagents are from Sigma unless otherwise stated.

3.3.9 Dye loading of medullary slice

After preparing the slice as above and an initial recovery period, 80mM HCO_3^- aCSF saturated with 9% CO_2 containing either carboxyfluorescein (CBF, 100µM) or its fixable form fluorescein isothiocyanate (FITC, 100µM) was applied for 5 minutes, followed by a wash of 30 minutes in standard aCSF. Controls underwent a five-minute application of the fluorescent compound in standard aCSF followed by a fifteen-minute wash. To wash out the dye previously loaded into the tissue, a five minute application of 80mM HCO_3^- aCSF the saturated with 9% CO_2 was given in the absence of the dye, followed by a 10 minute wash in standard aCSF. These experiments were performed on the ventral medullary slice from 3 rats – each replicate gave the same result. Carboxyfluorescein was chosen as it has a similar size and charge as dyes that connexin 26 is permeable to (Zhao 2005, Webber et al 2004).

3.3.10 Data analysis

All measurements of ATP release are expressed as mean ± sem. Statistical comparisons were made with a one-way ANOVA, the Kruskal-Wallis test or unpaired sample t-tests where appropriate. All comparisons are two-sided. Analysis of cell labelling to create pixel intensity histograms was performed with Image J software.

3.4 Results

3.4.1 ATP is released through hemichannels

Different mechanisms of ATP release are either sensitive or insensitive to the concentration of extracellular calcium. Therefore to reduce the pool of possible mechanisms I examined whether CO_2-evoked ATP release requires extracellular Ca^{2+}. The application of the calcium free aCSF by itself induced a profound release of ATP (4.0 ± 0.6 µM, n=4, figure 1a). Interestingly, an increase in PCO_2 was still able to evoke release of ATP on top of this elevated baseline (2.0 ± 0.5 µM, n=4) not significantly different control conditions (2.2 ± 0.9 µM, n = 13, unpaired t-test, P = 0.18, figure 1a). CO_2-evoked ATP release is therefore independent of extracellular Ca^{2+}, removing neuronal or glial exocytosis as possible mechanisms.

That lowered extracellular Ca^{2+} could evoke ATP release, suggested gap junction hemichannels as conduits for ATP release, as these will gate open in the absence of extracellular Ca^{2+} (Müller et al., 2002, Peracchia and Peracchia, 2004). In conjunction with this it has also been widely reported that gap junction hemichannels are inhibited by large decreases in pH (Trexlar et al., 1999, Peracchia and Peracchia, 2004), as is ATP release in response to increased PCO_2 (chapter 2). Carbenoxolone, the broad-spectrum gap junction hemichannel blocker, inhibited ATP release triggered by a calcium free solution (Fig 1b) as well as that evoked by CO_2 (Fig 1c and d), supporting the involvement of hemichannels. Carbenoxolone also reduced tonic levels of ATP present at the ventral surface, suggesting that this tone arises from spontaneous gating of gap junction hemichannels (1.4 ± 0.5 µM, n=9; Kruskal-Wallis H=5836, p<0.001, compared to control; figure 1).

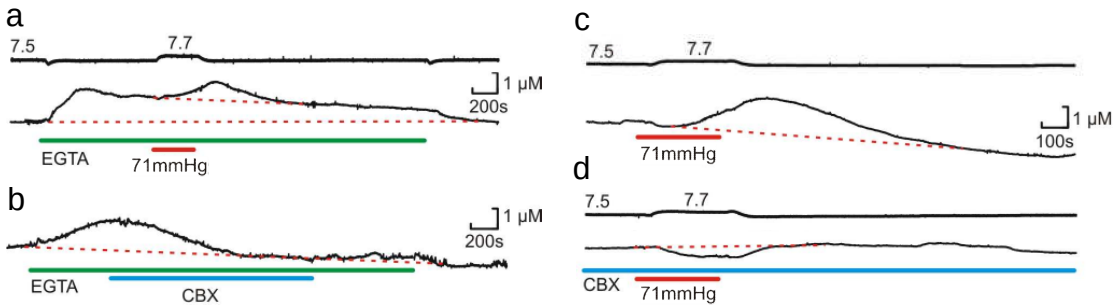

Figure 1: ATP release is hemichannel mediated. a) Effect of Calcium free aCSF (Containing 1mM EGTA and divalents balanced with $MgCl_2$) on ATP release at the ventral surface of the medulla. b) Effect of 100µM carbenoxolone on EGTA (0 $CaCl_2$ 2mM $MgCl_2$) evoked ATP release. c) Control ATP release. d) Effect of 100µM carbenoxolone on hypercapnia induced ATP release (negative shifts are due to subtraction artefacts). Note that 100µM carbenoxolone blocks ATP in response to the removal of calcium as well as increased CO_2.

3.4.2 Connexin 26 hemichannels are expressed at the VLM surface

There are many different types of gap junction protein that can form functional hemichannels including connexins and pannexins. To establish which gap junction proteins may be present at the surface of the VLM, I ran a cold PCR screen of different connexin and pannexin sub-types. I found that only Cx26, 32, 36, 43 and Pan 1 and 2 were present in this brain region.

Using quantitative PCR, I determined the expression of connexin and pannexin mRNA at different levels along the ventro-dorsal axis of the medulla oblongata. Whilst connexins 32, 36, and 43 and pannexin 2 were roughly uniformly distributed throughout the medulla, connexin 26 (Cx26) was enriched near the ventral surface as opposed to more dorsally located areas of the brainstem (Figure 2). I

confirmed these data by examining immunocytochemical localization of Cx26 protein. Cx26 immunoreactivity was confined to the very ventral margin of the parenchyma at the medullary surface and the pia mater (Fig 4) as reported by others (Solomon et al 2001). Thus Cx26 is localised to the VLM, the area that ATP release is confined to, making it the most likely candidate for involvement in this mechanism.

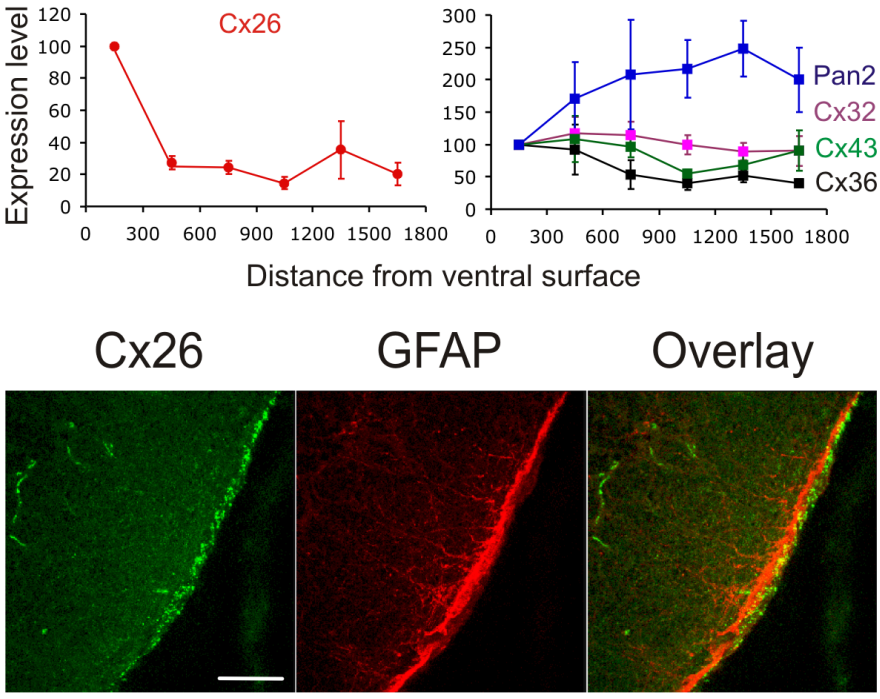

Figure 2: Connexin 26 is localised to the ventral surface of the medulla. Upper panels: Expression levels of Connexin and pannexin mRNA through the dorso-ventral axis of the medulla. Lower panels: Location of Cx26 protein at the VLM surface and its correlation with GFAP. Note that Cx26 appears to be expressed at the end feet of glial cells. Scale bar represents 40µm

3.4.3 Connexin 26 hemichannels are expressed the leptomeninges and capillaries

The cellular expression of the Cx26 protein is controversial. The creation of a LacZ reporter mouse has been at the centre of this debate, since inconsistencies have been reported between the expression of Cx26 in these mice as compared to immunocytochemical data. In order to try and resolve some these discrepancies and to provide unbiased documentation, I thought it appropriate to also investigate the expression of Cx26 in these mice. β-galactosidase positive nuclei were densest in the leptomeninges, particularly in the region that relates to the highest levels of ATP release, both *in vitro* and *in vivo* (figure 3). LacZ staining was also observed in the cells adjacent to penetrating vessels of the medulla and was particularly prevalent in areas suspected to respond to hypercapnia evoked ATP release (see chapter 1), such

as the medullary raphé complex (figure 3). This indicates that ATP may be released directly from the leptomeninges and cells lining blood vessels.

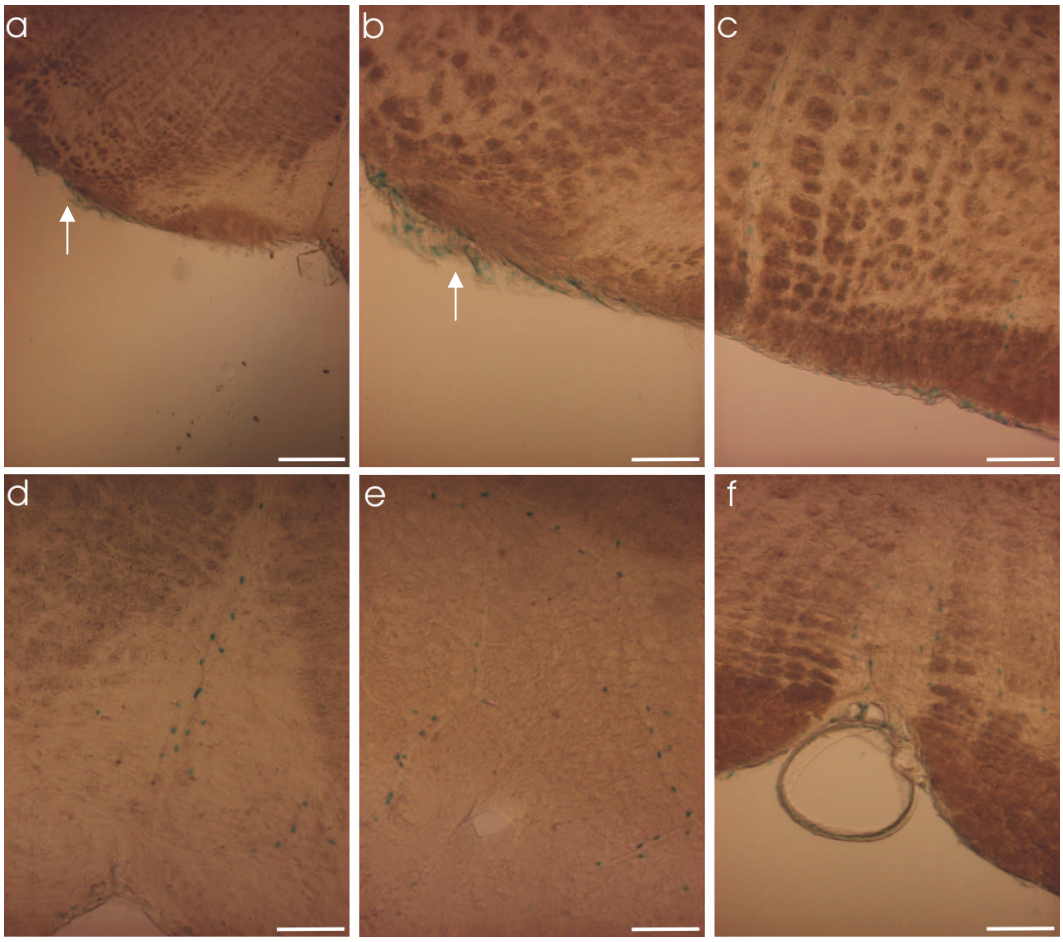

Figure 3: β-galatosidase immunoreactivity in Cx26$^{+/LacZ}$ reporter mice. a) low magnification view of the ventral medulla showing Cx26 expression in the leptomeninges (scale bar 300μm). B and c) Higher power magnification of a) (white arrows indicate biosensor placement). d, e and f) Localisation of Cx26 on penetrating vessels of the medulla. Scale bars represent 100μm unless otherwise stated.

3.4.4 Connexin 26 positive nuclei are enveloped by a GFAP cytoskeleton

Under the light microscope the expression of Cx26 in the LacZ reporter mice was localised in the pia. However, extensive co-localisation of Cx26, GFAP and FITC in the glial limitans was observed on the confocal microscope I therefore wanted to identify if any β-galactosidase immunoreactivity from Cx26$^{+/LacZ}$ mice co-localised with GFAP in the glial limitans. As under the light microscope β-galactosidase immunoreactivity was seen throughout the leptomeninges and the surface and penetrating vessels (figure 4). However at the surface of the ventral medulla there was co-localisation of β-galactosidase and GFAP, showing that the most superficial astrocytes of the medulla express Cx26, thus confirming the immuncytochemistry

data in the previous section. At higher magnification it was apparent that β-galactosidase positive nuclei were enveloped by GFAP (figure 4). Interestingly no co-localisation was observed in the blood vessels or the pia indicating that more than one cell type may be involved in the release of ATP, namely sub-pial astrocytes and pericytes.

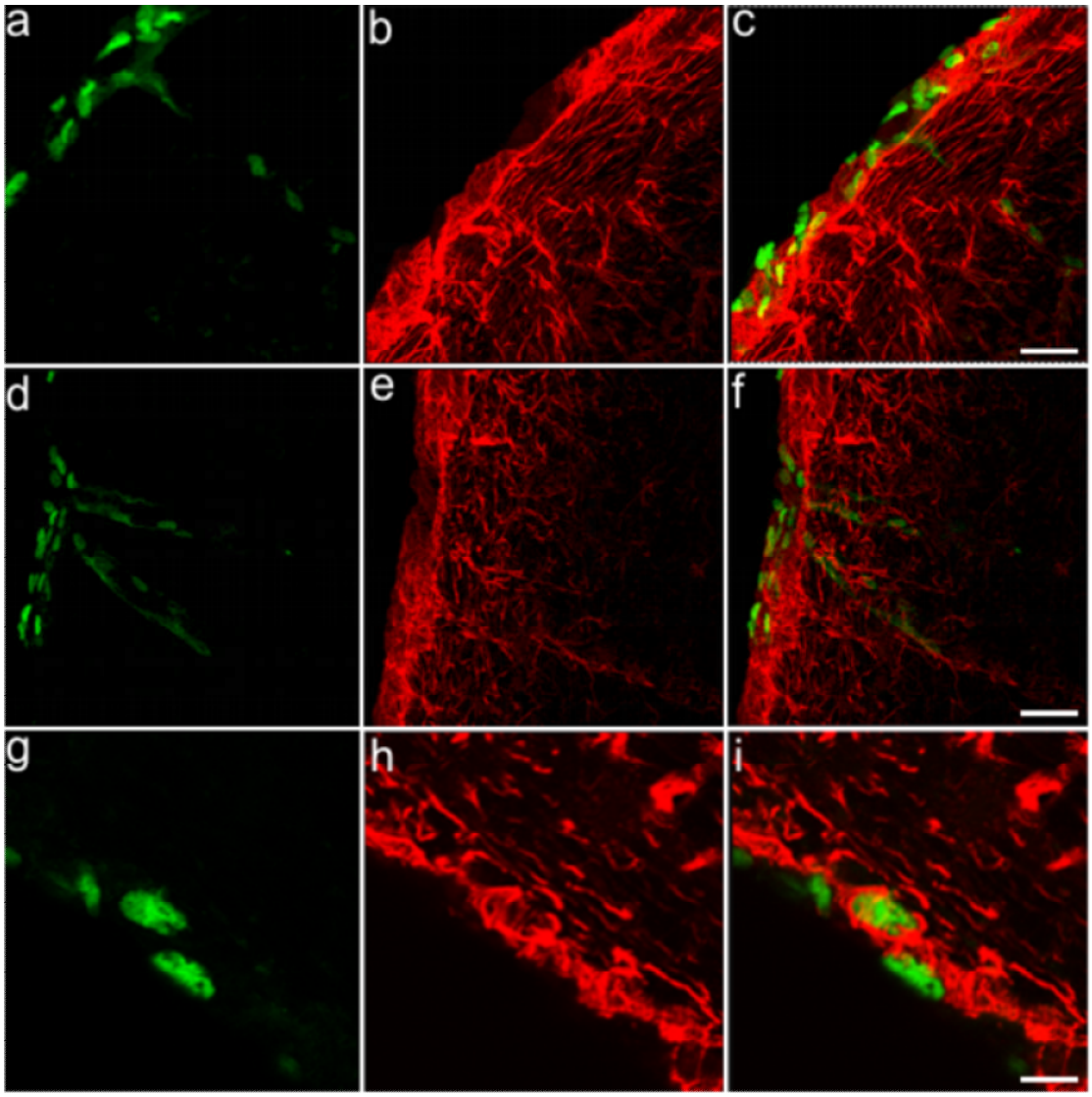

Figure 4: β-galactosidase (a marker of Cx26) positive nuclei are enveloped by GFAP. a, and d) β-galactosidase staining of the medullary surface, g) at higher magnification. b, and e) GFAP staining, h) at higher magnification. c and f) overlay i) at higher magnification. Scale bar for a, b and c represents 30μm; d, e and f represents 40μm; g, h and I represents 10μm. Note that β-galactosidase is located at the surface of the medulla and is associated with the vasculature. Co-localisation of GFAP and β-galactosidase appears as yellow in pictures c and f, in addition i shows that GFAP cytoskeleton encases β-galactosidase stained nuclei.

3.4.5 Blocking Connexin 26 hemichannels reduces ATP release in vitro

The localized expression of Cx26, an anion-selective hemichannel capable of conducting ATP (Zhao, 2005), in structures located within the ventral medullary chemosensitive regions, suggests the involvement of this particular channel in the release of ATP in response to CO_2. I tested this hypothesis by applying inhibitors more selective for Cx26 hemichannels (Co^{2+}, proadifen and NPPB) (Zhao, 2005, Ripps et al., 2004). All three compounds greatly reduced the amount of ATP released from the ventral surface of the medulla in response to a CO_2 challenge (one way anova, P=0.004, t-tests P<0.05 in all cases, Fig 5a-d).

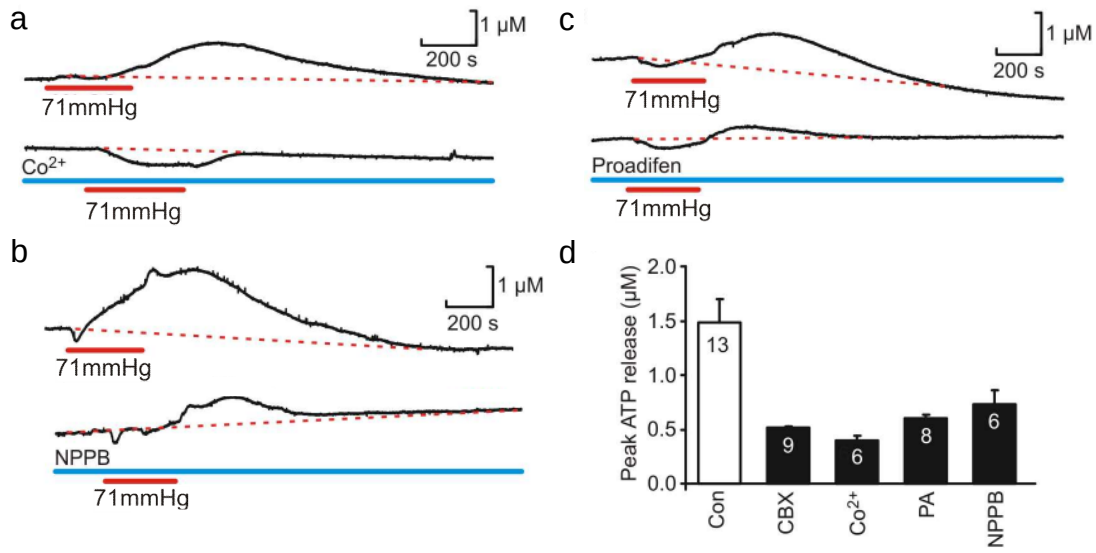

Figure 5: Inhibition of ATP release by connexin 26 selective hemichannel blockers. A) Cobalt 500µM B) NPPB 200µM C) Proadifen 200µM D) Graph of peak ATP release for all hemichannel blockers and controls calculated as the difference between the red dotted line and the peak of the black line. Negative shifts are due to subtraction artefacts.

3.4.6 Blocking Connexin 26 Hemichannels reduces ATP release in response to EGTA and reduces tonic levels of ATP at the VLM surface

To confirm the results obtained with carbenoxolone, I assessed the Cx26 selective blockers against all forms of ATP release seen at the VLM surface. Co^{2+}, proadifen and NPPB also reduced the ATP tone present at the ventral surface, suggesting that it is the Cx26 hemichannel that is spontaneously gating to give rise to tonic levels of ATP seen in this region (respectively to 0.6 ± 0.3 µM, n=6; 2.0 ± 0.6 µM, n=8; and 1.6 ± 0.4 µM, n=6; Kruskal-Wallis H=5836, p<0.001, compared to control; Fig 6).

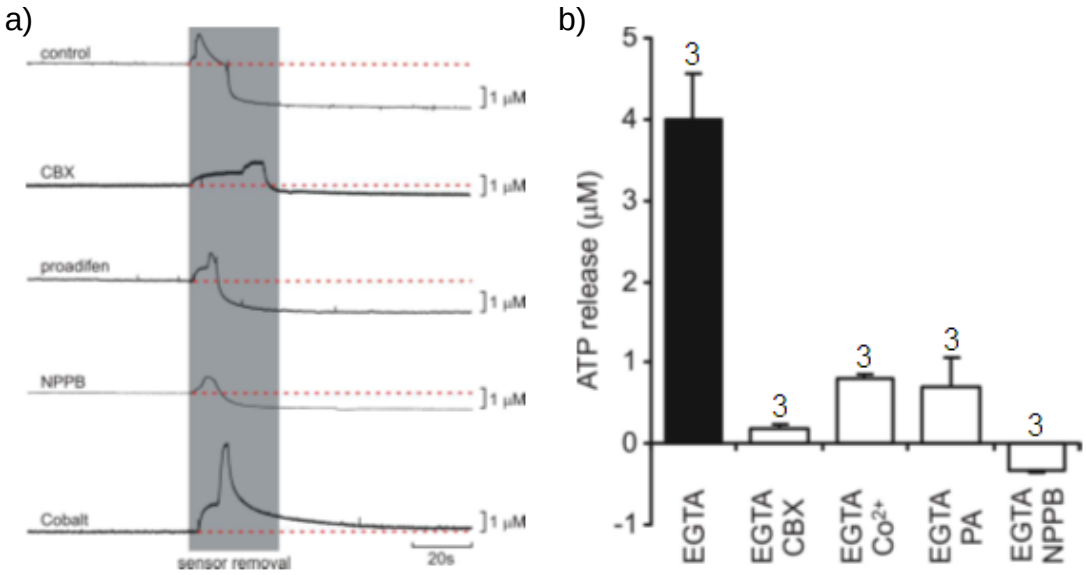

Figure 6: Inhibition of tonic and reduced calcium evoked ATP release by connexin 26 selective hemichannel blockers A) Reduction of tonic ATP levels by Connexin 26 selective hemichannel blockers, grey box shows where sensors were removed B) Graph of peak ATP release in response to removal of extracellular calcium for all hemichannel blockers and controls.

3.4.7 Blocking Pannexin 1 hemichannels does not affect ATP release in response to hypercapnia

As pannexin 1 hemichannels have been reported as conduits for ATP release (Romanov et al., 2007, Huang et al., 2007, Bao et al., 2004), I tested two agents at concentrations selective for these channels, (Silverman et al., 2008): carbenoxolone (10 µM); and probenecid (1mM). However these manipulations had no effect on CO_2-evoked ATP release ruling out a significant role for pannexin 1 hemichannels in CO_2 chemosensory transduction at the ventral medullary surface (Figure 7)

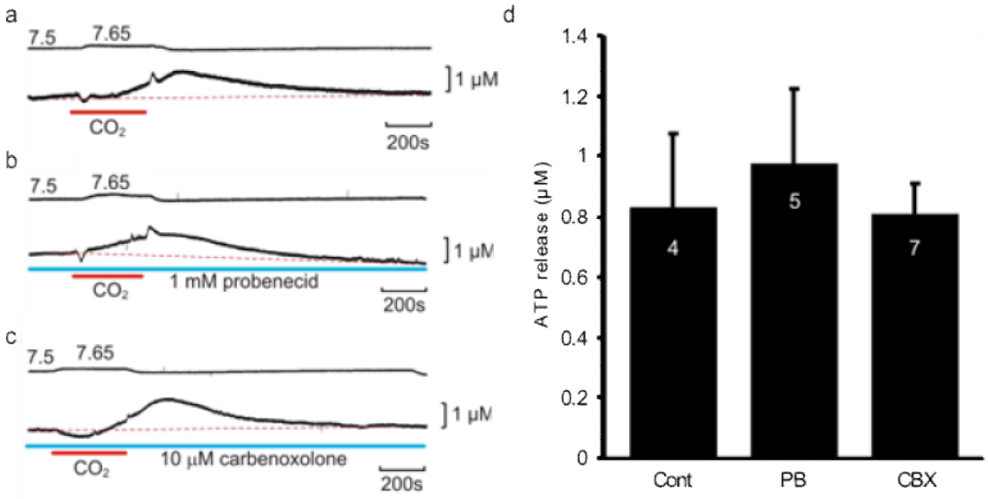

Figure 7: Effect of pannexin 1 blockade on ATP release. A) probenecid 1mM B) Carbenoxolone 10µM C) Control D) Bar graph for comparisons between controls and blockers calculated as the difference between the red dotted line and the peak of ATP release.

3.4.8 Blocking Connexin 26 Hemichannels reduces ATP release in vivo

To test whether ATP release through Cx26 hemichannels contributes to the increases in breathing seen in response to elevated CO_2 *in vivo*, I made recordings from the ventral medullary surface of anaesthetized and artificially ventilated adult rats. Application of either NPPB or proadifen to the surface of the medulla greatly reduced CO_2-evoked ATP release and significantly reduced the adaptive increases in respiratory activity (Fig 8e-g). This reduction of the CO_2-induced respiratory response following inhibition of Cx26 at the ventral surface of the medulla was similar to that caused by ATP receptor antagonists (Gourine et al., 2005a) and is similar to the contribution of the ventral surface chemosensors to the total adaptive ventilatory response to CO_2 (Nattie, 2001). Interestingly carbenoxolone perfusion into the RTN, a chemosensitive site that may be a possible target for ATP, reduces the hypercapnic ventilatory response by a similar value (17%) in adult rats (Hewitt et al., 2004). These *in vivo* data strongly suggest that ATP released via Cx26 hemichannels mediates the adaptive increase in ventilation following an increase in inspired CO_2.

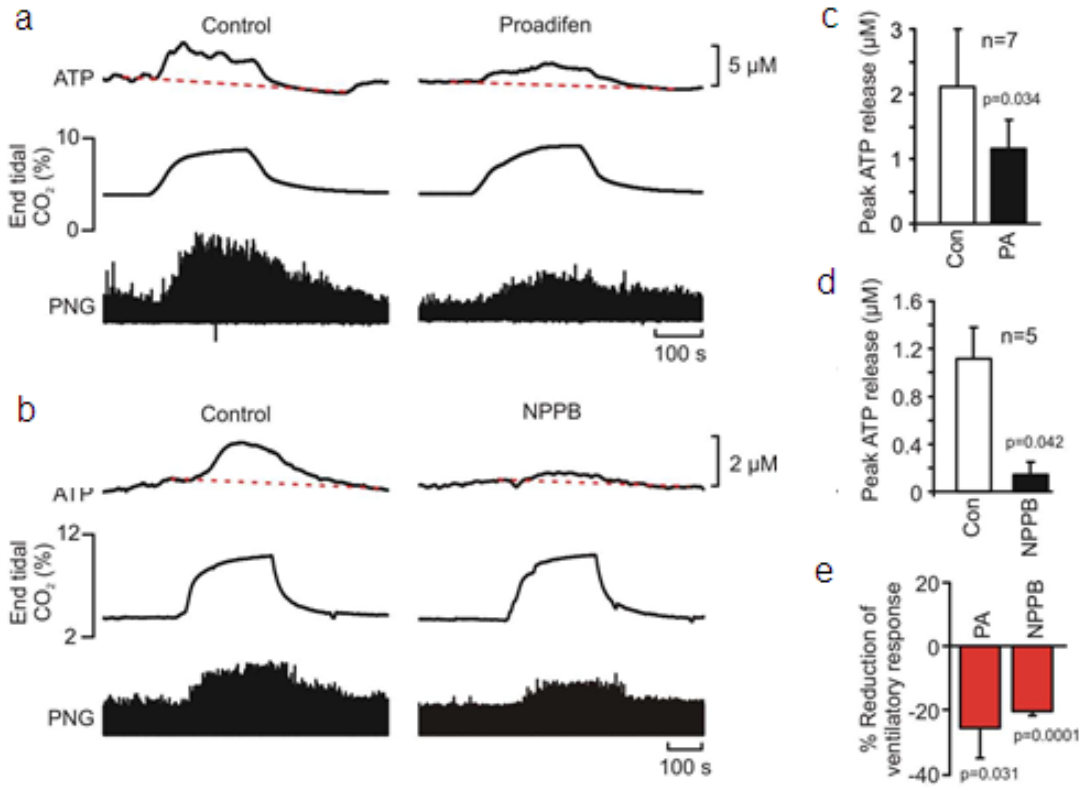

Figure 8: Effect of connexin 26 blockade on ATP release and respiration in vivo. a) 200µM proadifen b) 200µM NPPB c) Bar graph for comparisons between controls and proadifen e) Bar graph for comparisons between controls and NPPB e) effect of hemichannel blockade on the total ventilatory response

3.4.9 Identifying CO_2 sensitive cells

When Cx26 hemichannels open it should be possible to load cells that express it with a low molecular weight fluorescent anionic dye. Since this hemichannel opens in response to CO_2, the dye should pass down a concentration gradient and fill the CO_2-sensitive cells of the medulla if it is present in the milieu during the stimulus. Brief exposure of the isolated ventral medullary slice to carboxyfluorescein caused modest dye loading over the entire ventral surface of the medulla including the parenchyma and vasculature (Fig 9a). This again suggests the spontaneous gating of hemichannels under control conditions (95% O_2, 5% CO_2), and is consistent with our observations of a Cx26-dependent ATP tone.

However, elevated levels of PCO_2 (5 minutes of 9% CO_2, 80 mM HCO_3^-) induced a significant increase in carboxyfluorescein loading throughout the ventral medullary surface (including the pia and surface vessels: Fig 9a). I was able to subsequently reduce dye loading to below control levels by exposing the slice to a PCO_2 of 62mmHg (5 minutes of 9% CO_2, 80mM HCO_3^-) in a standard aCSF (Fig 9a). Re-opening of the channel with an increase in PCO_2 in the absence of the dye allows evacuation of the carboxyfluorescein, as it passes down the concentration gradient in the opposite direction.

To identify the sensor cells, I used immunocytochemical labelling of transverse sections of medullary slices. Isolated tissue slices were first loaded with FITC and then, to confirm that cell loading was a consequence of the opening of Cx26 hemichannels, were counterstained for this protein. To identify the cellular origin of ATP release, I also counterstained for GFAP (Fig 9b and c) and microtubule associated protein 2 (figure 9e). To show that FITC loading was through Cx26, I also counter stained for this protein (figure 9d). This revealed that the CO_2-dependent FITC loading was colocalized with Cx26 and GFAP immunoreactivity. Conversely, there was virtually no colocalization with MAP2 – a specific neuronal marker, even though MAP2 positive neuronal processes were closely juxtaposed to the FITC labelled cells. Thus CO_2 is sensed by astrocytes and leptomeninges at the ventral surface of the medulla oblongata

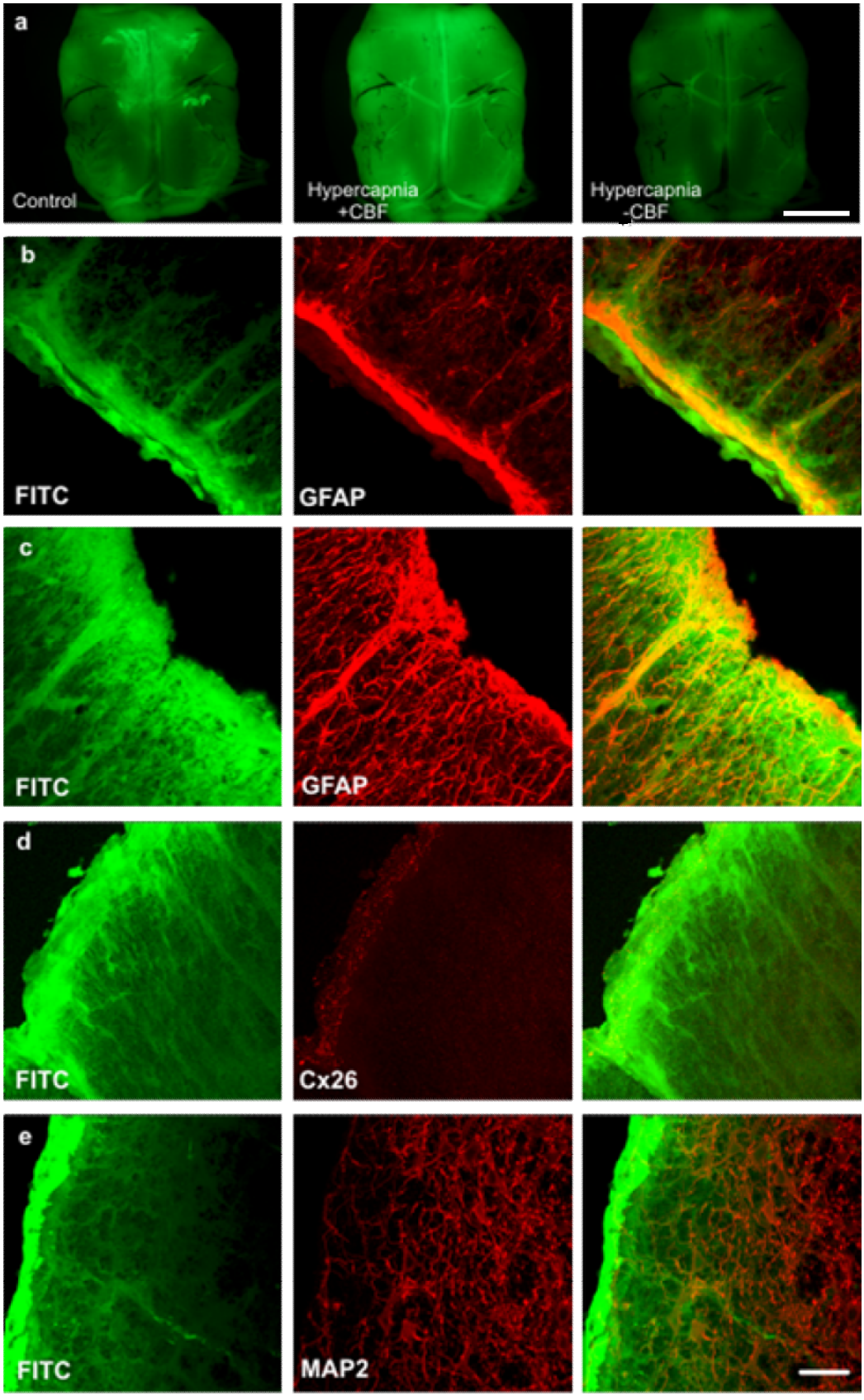

Figure 9: Alterations in PCO_2 allow low molecular weight dyes diffuse into sub-pial astrocytes through C26 hemichannels. a) Carboxyfluorescein in tissue slice. b and c) FITC localised with GFAP d and e) FITC and Cx26 Note the dye loading associated with the vasculature and leptomeninges of the medulla in a). Scale bars represent - top: 5mm for a) and bottom: 40µm for b) - e).

3.5 Discussion

3.5.1 ATP release is hemichannel mediated

The profile of ATP release seen upon the removal of extracellular calcium has been observed in the retina, where ATP is released through connexin 43 (Pearson et al., 2005). The relationship between extracellular calcium ion concentration and ATP release may explain why the removal of calcium from the VLM surface potentiates the response to hypercapnia, whereas addition attenuates it, in adult cats (Berkenbosch and Adan, 1974, Berkenbosch et al., 1976). In conjunction a fall in pH_e will close hemichannels, which explains the inhibitory effect of decreases in pH_e on ATP release. The increase in pore opening caused by alkaline pH in addition to that caused by increasing PCO_2 reveals why our 80mM HCO_3^- hypercapnic solutions provides us with the greatest amount of ATP. In support of these implications, applications of the broad spectrum hemichannel blocker carbenoxolone significantly reduced ATP released in response to changes in PCO_2.

Carbenoxolone also significantly reduced the ATP released initiated by the removal of extracellular calcium. The additive effect of removing extracellular calcium and increasing PCO_2 on ATP release indicates their modes of action are through different binding sites. A third final discovery from this series of experiments was the reduction of ATP tone seen at the surface of the ventral medulla caused by carbenoxolone. This implies that the tonic levels of ATP at the VLM surface are created by the spontaneous gating of gap junction hemichannels. As there is a basal level of carbon dioxide in the blood (PCO2 ~40mmHg) and this leads to a basal PCO_2 at the VLM surface, this causes the gating of the hemichannel and thus ATP release. When PCO_2 increases its effect on hemichannel gating increases and thus greater levels of ATP are seen; when PCO_2 decreases its effects lessen and reduces ATP levels at the ventral surface, thus ATP could have affects during both hypo- and hypercapnia as seen in chapter 2. This provides a new role for gap junction proteins in the ventilatory system, as previously their role was thought to be confined to modulating rhythm generation and synchronising inspiratory motor output (Solomon et al., 2003).

3.5.2 Connexin 26 is enriched at the VLM surface

Although many connexin and pannexin sub-types are found at the ventral surface, only connexin 26 mRNA was enriched in the ventral slice compared to more

dorsal tissue (300-1800μm from the surface), with the others showing a more linear and ubiquitous expression. Since ATP release is confined to VLM surface and most hemichannel proteins are not I would expect to see ATP elsewhere if it were due to any connexin except connexin 26.

The localisation of connexin 26 has come under scrutiny. Immunological studies show connexin 26 is exclusively expressed in sub-pial astrocytes and sub-ependymal layers (Mercier and Hatton, 2001). However, studies using genetic coupling of β-galactosidase to the connexin 26 promoter to create a reporter allele show that it is limited to the meninges only (Filippov et al., 2003). The discrepancy between these two investigations has called the specificity of the Cx26 antibody into question. However staining was confined to the very superficial cells, and were cross reactivity occurring, one would expect a more ubiquitous staining profile in keeping with the expression of other connexin sub-types. The major source of cross reactivity is thought to be Cx30; importantly we did not find this protein present with cold PCR. This connexin has been described only in one study, which reported it to be highly expressed in the facial nucleus and superior olive (Condorelli et al 2002). Staining of these regions was never seen with the Cx26 antibody I used; therefore I assume that no cross reactivity has occurred. In addition the $Cx26^{+/LacZ}$ reporter mice still contain the neomycin selection gene; importantly this is a powerful promoter and may affect not only the expression of the reporter but may also affect the control of genes that surround it. However the reporter mice used in this study had the selection gene removed, so that this is not a factor. As only one of the Cx26 alleles has been replaced by the reporter allele, some Cx26 positive cells will not express β-galactosidase. Thus there are draw backs to either technique. In addition the reporter mice show only nuclear staining and not where the protein is expressed, in contrast the antibody stain shows the location of the protein, but not which cells are expressing it. Therefore I employed both techniques in conjunction with real-time PCR to try and overcome these problems.

The co-locations of Cx26 and glial fibrillary acidic protein (GFAP) suggest that Cx26 is expressed in the end feet of sub pial astrocytes in direct contact with the pia mater. This would place these hemichannels in direct contact with our biosensors. In addition the $Cx26^{+/LacZ}$ mice also showed Cx26 positive nuclei at the surface of the medulla, though not all of it was encased by GFAP. Thus there appears to be a second cell type that expresses this protein, which is not of glial origin. There was also dense

β-galactosidase staining of the pia mater and cells surrounding blood vessels, which may be of the same cellular origin. This also provides an insight into the delicacy of the dissection that leads to our preparation. If Cx26 is in direct contact with the pia mater and is highly expressed in the pia mater itself, then any disruption to this meningeal layer removes a vast amount of and causes damage to this protein and thus no ATP release is seen.

3.5.3 Connexin 26 is the conduit for ATP release in vitro

Pharmacological identification of gap junction hemichannels is not yet achievable, due to a lack of specific blockers. Though all hemichannel blockers universally affect all subtypes, they do so differentially. Therefore I identified several compounds that are more selective for connexin 26 over other hemichannels (Ripps et al., 2004). I found that Co^{2+}, NPPB and proadifen all significantly reduced the amount of ATP released in response to changes in PCO_2 and EGTA and also reduced tonic levels of ATP. The actions of pharmacological blockade combined with the anatomical location of connexin 26, demonstrates that all three mechanisms are not just mediated through hemichannels but specifically connexin 26 *in vitro*.

Pannexin 1 is one of the only hemichannels that can be distinguished pharmacologically. Pannexins, particularly pannexin 1, can act as pathway for ATP release. Therefore to remove this as a possible source of ATP release under hypercapnic conditions I applied carbenoxolone at concentrations effective enough to inhibit pannexin 1 but not connexin hemichannels, and probenecid a selective blocker of Pan 1. I found that applications of either pannexin 1 hemichannel blocker reduced ATP release at the VLM surface, leaving connexin hemichannels as the only possible source.

3.5.4 Connexin 26 is the conduit for ATP release in vivo

Now I had identified a conduit by which ATP may pass from the intracellular to extracellular space in vitro, I wanted to confirm this observation *in vivo*. This would not only verify the mechanism of ATP release but also that our model of ATP release at the VLV surface was indeed valid. Significant reductions in ATP release at the VLM surface by applications of NPPB and Proadifen were accompanied by significant reductions in the hypercapnic ventilatory response. This result show that ATP release at the VLM surface of intact anaesthetised animals is also mediated through Cx26, and also confirmed the importance of purinergic signalling in the

hypercapnic ventilatory response. Moreover the reduction in the increase in ventilation was identical to the contribution of; the VLM surface, purinergic receptors and gap junctions to the total response. The combination of these results shows that ATP release through gap junction hemichannels at the VLM surface is the sole contributor to participation of this region in the total response.

3.3.5 CO_2 responsive cells can be dye loaded

Connexin hemichannels have been shown to be selective for the charge and size of a molecule. Connexin 26 for instance is anion selective and allows the uptake of Alexa 350 (MW 350) and Alexa 488 (MW: 570) but not Alexa 594 (MW: 760) (Weber et al., 2004). Importantly, ATP is a negatively charged molecule with a molecular weight of 507.2 (Stenesh, 1998). Dye loading studies are heavily utilised for the study of gap junctions and hemichannels: for instance hemichannel expression can be identified in cells of the cochlear, based upon dye selectivity (Zhao, 2005).

I hypothesised that it would be possible to dye load cells involved in the release of ATP in the VLM, and that opening the channel by increasing PCO_2 should allow passage of the dye down the diffusion gradient into the cell. Since connexin 26 hemichannels have shown a preference for dyes such as Alexafluor 350 (Zhao, 2005, Weber et al., 2004), I chose a dye of similar charge and weight, carboxyfluorescein (CBF; MW 376). I found that applications of CBF in control aCSF caused modest dye loading in accordance with the ATP tone seen; this shows that Cx26 hemichannels are either in a sub-conductance state or exhibit spontaneous gating under control conditions. A second application of CBF in the presence of elevated CO_2 caused a significant increase in dye loading at the medulla surface, indicating an increase pore opening of the hemichannel in response to changes in PCO_2 allows more dye in and therefore ATP out. I found that it was possible to evacuate CBF from the cell by again opening the channel with increased PCO_2 in the absence of the dye, this time the direction of the diffusion gradient would be the same as for ATP allowing it to escape. This discovery not only provided me with further evidence of my working hypothesis but also gave me proof of principle in terms of my ability to dye load the cells involved in this response.

3.5.6 ATP is released from Cx26 positive sub-pial astrocytes

The counterstaining of dye loaded medullas with cellular identifiers enabled me to determine the origin of ATP release at the cellular level and their relationship

with connexin 26 hemichannels. I found that FITC labelling was confined to the first 150µm of the VLM, an area that extends from the surface to the boundary of the MGL. In concordance with this I found a high correlation between dye loading and GFAP, the densest levels of FITC matched the densest levels of GFAP, after the initial 150µm the tissue was devoid of both FITC and GFAP staining. Where FITC penetrates deeper into the medulla it appears to do so down glia projections, especially around blood vessels. Using both methods of Cx26 detection, there was shown to be an association between GFAP and Cx26 expression. Conversely, the correlation between MAP2 and FITC staining was almost mutually exclusive, whereas FITC staining ceased at 150µm, MAP2 staining began. There was evidence of some MAP2 staining within the MGL in agreement with reports of neurons residing here. However, the MAP 2 staining seemed to exist between FITC staining with no overlap seen. Thus I can exclude neuronal involvement in this process.

3.6 Summary

ATP is released from sub-pial astrocytes through connexin 26 hemichannels. Blockade of this release mechanism causes reduction in breathing that is equal to blocking the effects of this signalling molecule at its receptors or to the destruction of the cells responsible for releasing it.

Chapter 4: Connexin 26 is the molecular transducer for CO_2 chemosensitivity

4. 1 Abstract

In previous chapters I have shown that connexin hemichannels are responsible for the release of ATP at the surface of the medulla in response to hypercapnia. These channels are constitutively active and are responsible for tonic ATP release at the surface of the ventral medulla. The dorso-ventral distribution of Cx26 along with the profile of pharmacological blockers makes this hemichannel the most likely candidate. Its expression in the pia and sub-pial astrocytes stained with carboxyfluorescein in a hypercapnic solution also supports this hypothesis. However the molecular transducer for detecting fluctuations in PCO_2 has yet to be elucidated. In this chapter I show that blockade of CO_2 or HCO_3^- sensitive second messenger systems is ineffective at reducing ATP release. In addition I tested the CO_2 sensitivity of five different connexins, and show that they exhibit different responses. Of the connexins tested only hemichannels consisting of β-connexins opened in response to elevated CO_2. Moreover, I show that the expression of Cx26 protein alone in HeLa cells is sufficient to confer CO_2 sensitivity and ATP release. By loading these transfected cells with ATP I was able to recapitulate the entire *in vivo* response with a single protein. Thus I propose a dual role for Cx26, as both the chemosensory transducer and the conduit for ATP release, in the hypercapnic ventilatory response.

4.2 Introduction

The discovery of ATP release from the classical chemosensitive areas of the ventral surface of the medulla, has led to a series of investigations to characterise the stimulus and elucidate the mechanisms and cell types involved. It is dependent on changes of PCO_2, which may lead to its involvement in the response to hypocapnia as well as hypercapnia (chapter 2). Blockade of Cx26 hemichannels decreased the tonic levels of ATP and reduced hypercapnia-induced ATP release both *in vitro* and *in vivo*. Moreover, this reduction in ATP release weakens the hypercapnic ventilatory response (HVC). The decrease in the HVC is equivalent to the contribution of the VLM surface to the overall response (Nattie, 2001), the blockade of purinergic receptors and is similar to the participation of all gap junction hemichannels in this

region (Hewitt et al., 2004). The discovery that Cx26 mediated ATP release led to the identification of the cellular source of ATP as sub-pial astrocytes (Mercier and Hatton, 2001) and more likely the leptomeninges (Filippov et al., 2003). This identified for the first time a primary role for astrocytes and leptomeninges in the hypercapnic response. Although, the conduit for ATP release from glial cells is known to be Cx26 (chapter 3), the CO_2 transducer has yet to be identified.

Previous work has suggested that CO_2 is not detected directly but instead via consequent in pH, either intracellularly (pH_i) (Wang et al., 2002, Filosa et al., 2002) or extracellularly (pH_e) (Mulkey et al., 2004). The general consensus is that changes in either pH_i or pH_e act on acid sensing cation ion channels on these neurons. Acid sensing ion channels (ASIC) are proton sensitive cation channels (Shimokawa et al., 2005). They are activated by rapid extracellular acidification (Shimokawa et al., 2005), which acts on the extracellular face of the protein (Waldermann et al., 1997). Upon activation they allow an influx of cations (Shimokawa et al., 2005), usually sodium (Waldermann et al., 1997). However they usually require large alterations in pH not within physiological range. TASK (tandem-pore acid sensing potassium channels) 1 and 3 channels confer intrinsic pH sensitivity to many medullary neurons (Mulkey et al., 2007b). Nevertheless TASK knockout mice show no deficiency in their ventilatory response to CO_2 (Mulkey et al., 2007b). Thus they are not now thought to be the transducer of CO_2 chemoreception. Inward rectifying potassium channels are sensitive to alterations in pH within the physiological range (Pineda and Aghajanian, 1997), and have been shown to convey chemosensitivity to neurons in the locus coeruleus (Pineda and Aghajanian, 1997). In conjunction cells in this region have reduced responses to hypercapnia acidosis, when an array of potassium channels or L-type calcium channels are inhibited by tetraethylammonium (TEA) or nifedipine respectively (Filosa and Putnam, 2003). However the blockade of other channels in these studies may have affected the excitability of these neurons and thus provided a false positive or an exaggerated response. In this chapter I demonstrate that Cx26 is sufficient for CO_2 sensing. Expression of this protein alone in HeLa cells conveys chemosensitivity to them. Its high sensitivity to alterations in PCO_2 in excised patches shows that it is an intrinsic chemosensor. Moreover expression of this protein to HeLa calls can completely recapitulate ATP release at the VLM surface in response to hypercapnia. Thus Cx26 appears to be a new chemosensor.

The involvement of Cx26 in this response has provided the first insight into the mechanism which may underlie ATP release at the VLM surface. Exploiting this as an initial starting point, I began to unravel the mystery that surrounds the mechanism underlying this phenomenon. I have already shown that one carbon dioxide and bicarbonate sensitive second messenger, carbonic anhydrase, is not involved in this process (chapter 2) and here I eliminate the other, adenylate cyclase. More importantly the expression of a single protein, Cx26, is all that is necessary to transfer CO_2 sensitivity to a non-chemosensitive cell line. In this chapter I show that the CO_2 binding domain resides on the extracellular face of the protein. In conjunction I also provide evidence for the first time that this protein is indeed capable of allowing passage of ATP through it. Therefore I propose that Cx26 is not only the conduit by which ATP is released from sub-pial astrocytes but is also a transducing molecule for identifying changes in extracellular PCO_2.

4.3 Methods

4.3.1 In vitro recordings

4-6 week old Sprague-Dawley rats were sacrificed by an overdose of isofluorane, the brain stem was rapidly dissected free under chilled aCSF, mounted on a block and a 400 µm thick slice comprising the ventral surface of the medulla was cut. This slice was then placed in the recording chamber maintained at 33ºC. ATP and null biosensors were obtained from Sarissa Biomedical Ltd. They were placed on the ventral surface of the medullary slice in equivalent positions either side of the midline. I report the difference between the ATP and null biosensor recordings to give an accurate measure of ATP release.

After the initial recovery period, 80mM HCO_3^- aCSF saturated with 9% CO_2 was applied for five minutes followed by a 30 minute wash period in standard aCSF. This enabled me to test the viability of the preparation. The pharmacological agent SQ22536 (Tocris; 100 µM) was then applied for a period of 20 minutes in standard aCSF to allow it to take effect. A second application of a hypercapnic 80mM HCO_3^- aCSF containing the drug was applied followed by a thirty-minute wash in standard aCSF containing the drug. The tissue was then washed for a further twenty minutes in standard aCSF before the sensors were removed (to allow measurement of the tone) and the sensors calibrated. Since there is a decrease in ATP release between applications of hypercapnic solutions to the same tissue slice, ATP release in the

presence of the inhibitor was compared to interleaved controls.

After the recovery period control tissue slices were exposed to 5 minute application of 80mM HCO_3^- aCSF saturated with 9% CO_2. The tissue was then washed with control solution for fifty minutes before a second 5 minute application of the hypercapnic solution. The tissue was then washed for a second time before the sensors were removed and calibrated.

4.3.2 Cell culture

I maintained WT and Cx26 expressing HeLa cells in Dulbecco's Modified Eagle's Medium (DMEM) with the following supplements: 1mM glutamine (Melford Labs), 10% foetal calf serum (Invitrogen) and penicillin/streptomycin (Sigma) at 10U/ml and 10µg respectively. In addition, the Cx26 cells were under selective pressure with puromycin (Sigma) at 1µg/ml. All cells were grown at 37^{o}C in a humidified 95% O_2/5% CO_2 incubator. For patch clamp recordings the cells were plated out in 6 well plates at 2 x 10^6 cells per well for the Cx26 and 1 x 10^6 cells per well for the WT HeLa cells.

4.3.3 Dye loading Cells

Sub-confluent Wild type (WT), Cx26, Cx30, Cx32, Cx36 and Cx43 expressing cells (on coverslips) were loaded with CBF (200µM), and experiments were repeated at least 3 times. After an initial recovery period, 80mM HCO_3^- aCSF saturated with 9% or 12% CO_2 containing carboxyfluorescein (CBF, 200µM) was applied for 5 minutes, followed by a wash of 10 minutes in standard aCSF containing CBF, then a 20 minute wash in standard aCSF. Controls underwent a 15 minute application of the fluorescent compound in standard aCSF followed by a 20 minute wash. Each replicate gave the same result. Carboxyfluorescein was chosen as it has a similar size (MW 376) and charge (anionic) as dyes that Cx26 is permeable to (Zhao 2005, Webber et al 2004). 40 cells were selected at random for each cell line, condition and pixel intensity measured using an image analysis programme (image J); on each fluorescent picture a region of interest was drawn around a single cell and analysed. The median pixel intensities were taken and ranked in a cumulative probability distribution; the distributions were then analysed using a Mann-Whitney U test.

4.3.4 Patch clamp recordings

Coverslips containing non-confluent cells were placed into a perfusion chamber at 28°C in sterile filtered standard aCSF. Standard patch clamp techniques were used to pull outside out or inside out isolated membrane patches or for whole cell recordings. Although intracellular acidification has been shown to close hemichannels, a recent report has shown that this is dependent on the type of buffer used e.g. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Therefore I employed an intracellular solution that contained what has been termed "a good buffer". Whole cell patch pipettes contained an intracellular solution (K-Gluconate 120 mM, $CsCl_2$ 10 mM, TEACl 10 mM, EGTA 10 mM, ATP 3 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, sterile filtered, pH adjusted to 7.2 with KOH); Outside out patch pipettes contained an intracellular solution (K-Aspartate 120 mM, $CsCl_2$ 10 mM, TEACl 10 mM, EGTA 10 mM, ATP 6 mM, $MgCl_2$ 4 mM, $CaCl_2$ 1 mM, sterile filtered, pH adjusted to 7.2 with KOH) inside out patch pipettes contained standard aCSF. Isolated patches of the outside out configuration were held at -40mV and inside out patches were hold at +40mV. After a reasonable baseline had been recorded, patches were exposed to isohydric aCSF solutions saturated with variable amounts of CO_2 for 1 minute, followed by a wash period. Integrals of isolated patch recordings were taken and compared by the Friedman test.

4.3.5 ATP release through Cx26 transfected cell lines

The cells (both Cx26-expressing and wild type) were exposed to 10 mM ATP in the presence of 80 mM HCO_3^- aCSF saturated with 9% CO_2 (to open the Cx26 channels and load them with exogenous ATP). They were then washed for 30 minutes in standard aCSF before being exposed to the hypercapnic stimulus to test for release. Preloading with ATP was essential to see CO_2-evoked ATP release from Cx26-expressing HeLa cells.

4.4 Results

4.4.1 SQ22536

The gating of connexin hemichannels in response to increases in PCO_2 could be due to the activation of a CO_2 sensitive second messenger and subsequent phosphorylation of the channel by protein kinases. A soluble adenylate cyclase (sAC) is a conserved CO_2/HCO_3^- sensor that is expressed in the brain (Zippin et al., 2001,

Wuttke et al., 2001, Chen et al., 2000). To test for the involvement of this enzyme, I used a general adenylate cyclase inhibitor SQ22536.

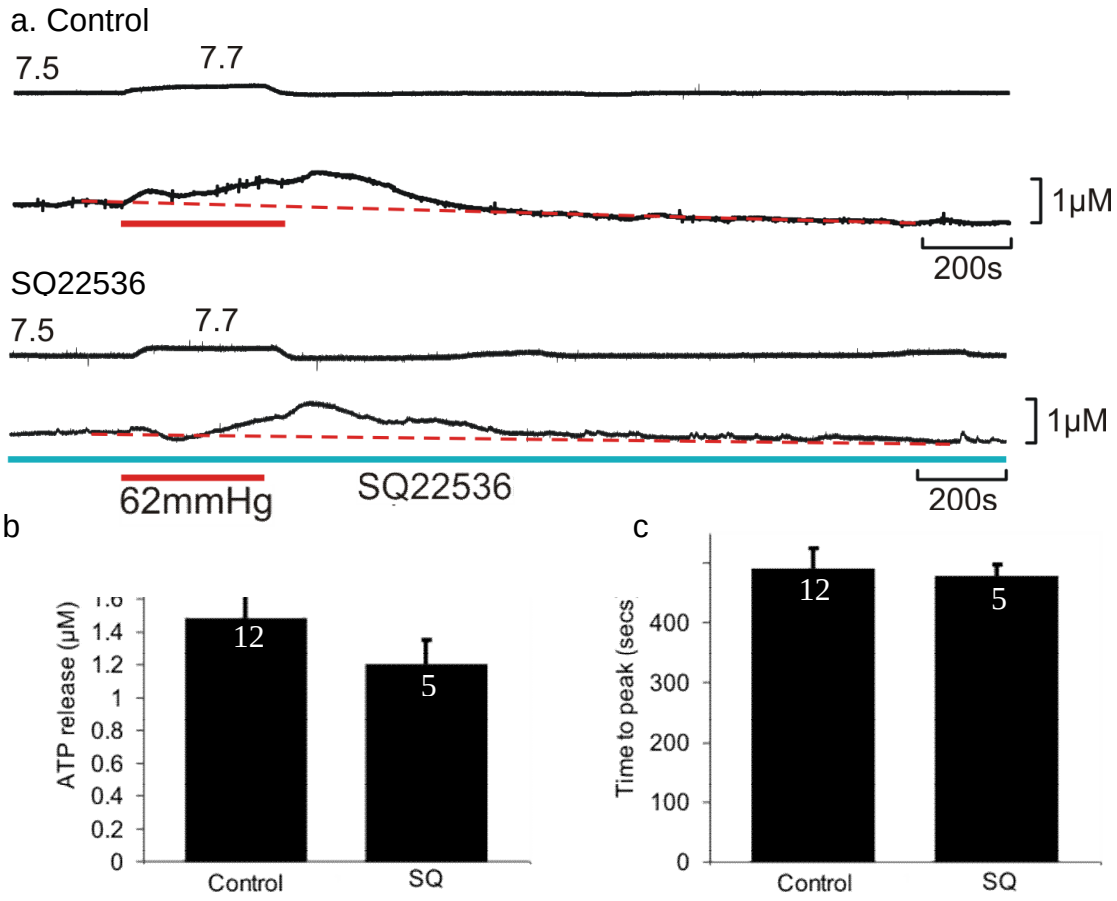

Figure 1: CO_2-dependent ATP release is not dependent on an adenylate cyclase. a) Profile of ATP release in standard aCSF (9% CO_2, 80mM HCO_3^-; red bar) and in the presence of adenylate cyclase inhibitor. b) Effect of SQ22536 on peak ATP release. c) Effect of SQ22536 on the timing of ATP release.

SQ22536 did not reduce the response to isohydric hypercapnia compared to interleaved controls (figure 1a and c). SQ22536 was also without effect on the time taken for ATP release to reach its peak. The gating of Cx26 in response to hypercapnia does not therefore depend on sAC, the production of cAMP and the subsequent phosphorylation of the channel. Therefore a direct interaction between CO_2 and this protein seems likely.

4.4.2 Expression of Cx26 in HeLa cells allows dye loading

If there is a direct interaction between CO_2 and Cx26, then transfection of Cx26 should be necessary and sufficient to confer chemosensitivity. To test whether

expression of Cx26 is sufficient to impart CO_2 sensitivity, I expressed Cx26 in HeLa cells. Dye loading was apparent in transfected HeLa cells, but absent in wild type controls. The fluorescence profile of the wild type cells contains staining in one cell (figure 2). Membrane disruption caused by cell death is presumed to have allowed the passage of dye into the cell body. On the other hand, all Cx26 positive cells loaded with dye, as can be seen by comparison with bright field illumination. This implies that expression of Cx26 protein alone confers chemosensitivity to cells.

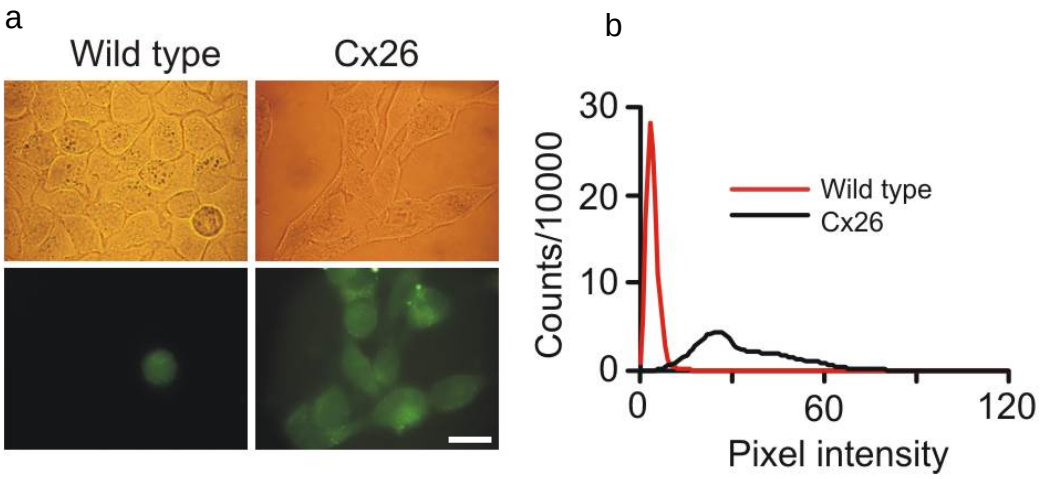

Figure 2: Cx26 transfected HeLa cells can be loaded with low molecular weight dyes at 9% CO_2. a) Cell loading with Carboxyfluorescein in 9% CO_2/ 80mM HCO_3^- aCSF: Upper panels bright field illumination; Lower panels 488 fluorescence. b) Histogram of pixel intensity obtained over the entire field of epifluorescence. Note that the wild type curve shows a lot of pixels at low intensity (~5) and that the Cx26 curve shows pixels at high, albeit different, intensities (~10-60), thus the greater range. Scale bar represents 20µm.

Although expression of Cx26 confers CO_2 sensitivity in this cell line, whether the dye loading is a direct effect of Cx26 or an up regulation of another protein is not certain. Thus to show that it is a direct consequence of Cx26 expression, I repeated the protocol in the presence of a hemichannel blocker proadifen. Dye loading of Cx26 positive cells was blocked by proadifen (figure 3). This demonstrates that it is the presence of Cx26 hemichannels themselves and not some contingent change in the HeLa cells that is responsible for the CO_2-dependent loading. Cell intensity for carboxyfluorescein dye loading was significantly reduced, though not completely removed. However proadifen at this dose does not completely block CO_2-induced ATP release.

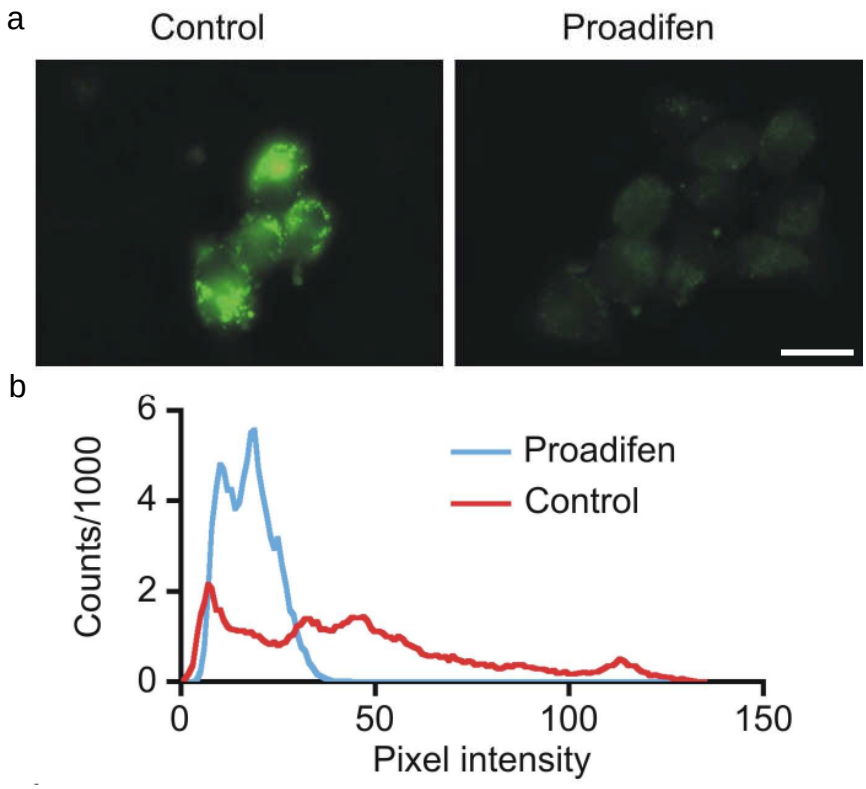

Figure 3: Fluorescent loading of transfect HeLa cells can be blocked by a Cx26 hemichannel blocker. a) Upper panels 488 fluorescence of Cx26 transfected HeLa cells in the presence of carboxyfluorescein and 9% CO_2/80mM HCO_3^- note that loading is blocked by proadifen. b) Histogram of pixel intensity obtained over the entire field of epifluorescence. Note that the wild type curve shows a lot of pixels at low intensities, albeit different, (~10-25) and that the Cx26 curve shows pixels at high, albeit different, intensities (~10-140), thus the greater range. Scale bar represents 20µm.

4.4.3 PCO_2 has differential effects on various members of the hemichannel protein family

Although I have shown that expression of Cx26 confers CO_2 sensitivity in HeLa cells (figure 2 and 3), it may be that expression of any connexin hemichannel could perform this function. To test this hypothesis I obtained HeLa cells that expressed all the relevant medullary connexins and tested their response to CO_2 with dye loading. Connexin hemichannels have several open and closed states; typically connexin hemichannels can exhibit spontaneous gating. Therefore cells exposed to standard aCSF containing CBF showed background staining (figure 4 and 6).

Comparisons between median differences in cell loading between control solutions and experimental solutions showed differential effects of hypercapnia on connexin hemichannels. Cx26 and Cx32 showed increased staining, whereas Cx30 and Cx36 showed no change and Cx43 showed a decrease in staining. This shows that CO_2 sensitivity is conveyed only by specific connexin hemichannels. Wild type cells exposed to hypercapnic solutions in the presence of CBF did not show any fluorescent loading (data not shown).

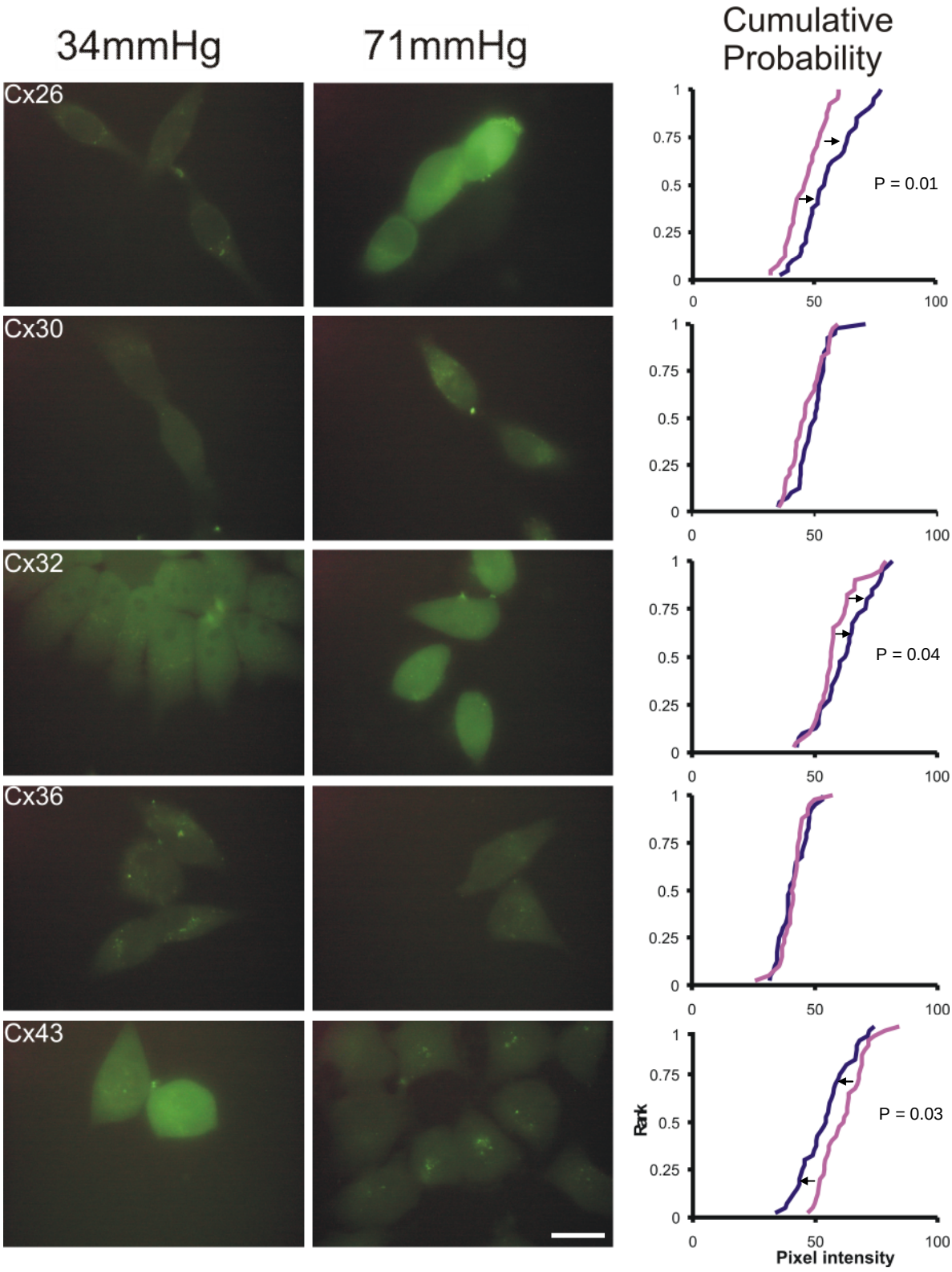

Figure 4: Dye loading of connexin expressing HeLa cells shows that CO_2 has varying effects on different connexin hemichannels. Column 1 is the carboxyfluorescein loading in standard aCSF, due to constituent open states of gap junction hemichannels. Column 2 shows carboxyfluorescein staining in the presence of 12%CO_2/80mM HCO_3^- aCSF. Column 3 shows the cumulative probability of control loading (pink line) and loading with the stimulus (blue line). Each row represents a different connexin expressed in HeLa cells. Scale bar represents 20µm.

4.4.4 Increasing CO_2 alters the conductance of connexin expressing HeLa cells

I made whole cell patch clamp recordings from the HeLa cell lines expressing various connexins to examine the gating and sensitivity of hemichannels in response to CO_2 challenges. I found that conductance of HeLa cells containing Cx26, 30 and 32 hemichannels increased with increasing PCO_2. In contrast those which possess Cx43 hemichannels exhibited reduced conductance when exposed to hypercapnic solutions. Cx36 transfected HeLa cells on the other hand were unaffected by alterations in PCO_2.

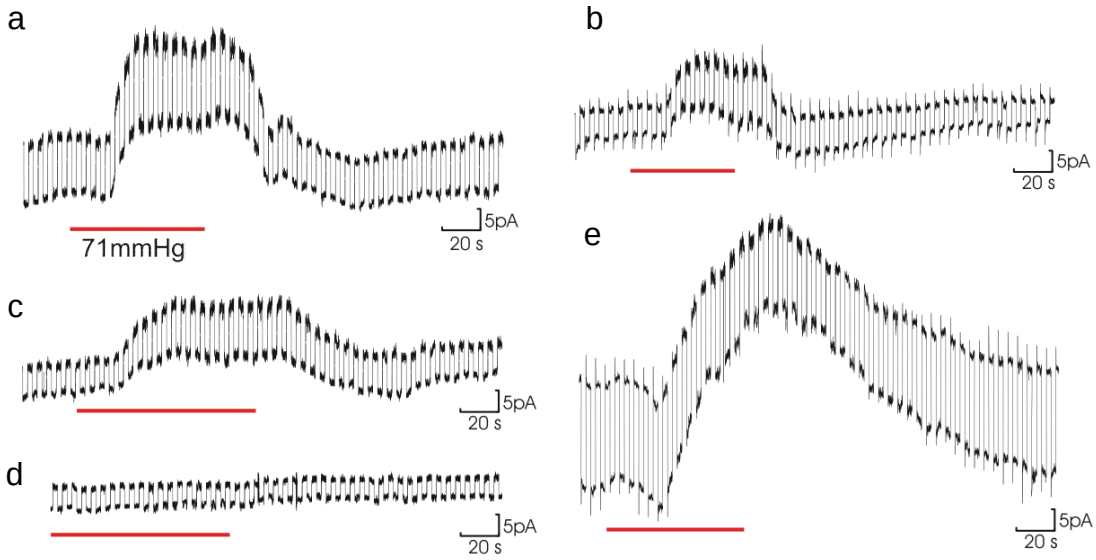

Figure 5: CO_2 (12% in 80mM HCO_3^- aCSF) alters the conductance of hemichannels expressed in HeLa cells. Whole cell patch clamp recordings taken from HeLa cells expressing a) Cx26. b) Cx30. c) Cx32. d) Cx36. e) Cx43. Conductance is measured as the change in current caused by a 10mV step, divided by the change in voltage.

Interestingly the pattern of conductance change of each hemichannel was very similar to the change in fluorescence (figure 5 and 6). However at 95% confidence, only Cx26, Cx30 and Cx32 were significantly different to zero (figure 5 and 6). The lack of significance seen with Cx43 is due to the large standard deviation (figure 5 and 6).

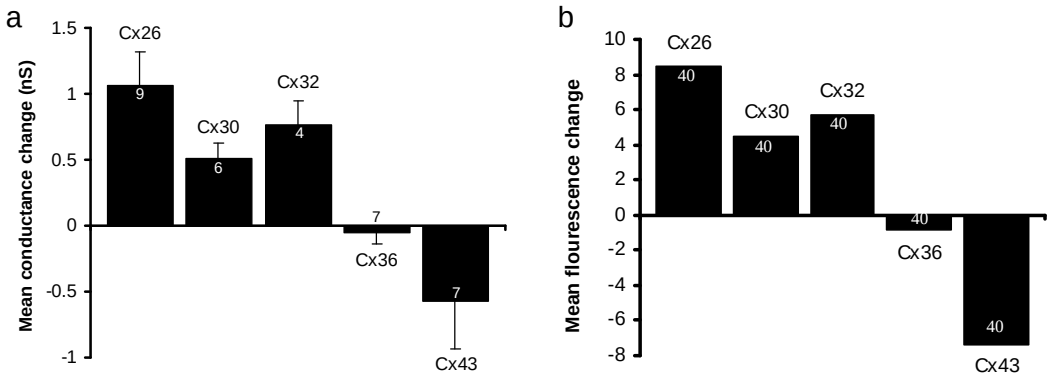

Figure 6: Alteration in dye loading of HeLa cells are caused by a change in hemichannel conductance induced by 12% CO_2 80mM HCO_3^- alters hemichannel conductance and in a similar way. a) Mean conductance change from whole cell patch clamp data. b) Median fluorescence change in dye loaded HeLa cells.

4.4.5 The connexin 26 hemichannel exhibit graded sensitivity to PCO_2

To test whether Cx26 is sensitive to changes in PCO_2 over the likely physiological range, I examined whole cell conductance changes to graded levels of PCO_2. I found that Cx26 hemichannels are exquisitely sensitive to changes in PCO_2. Compared to a resting PCO_2 34mmHg hypercapnic solutions (PCO_2 55 and 71mmHg) dramatically increased the whole cell conductance. Thus CO_2 acts to unblock the gate of the hemichannel and it spends more time in an open state, which would allow the passage of more ATP into the ECF. Interestingly decreasing PCO_2 to 19mmHg caused a decrease in whole cell conductance (figure 7) compared to control levels (PCO_2 34mmHg).

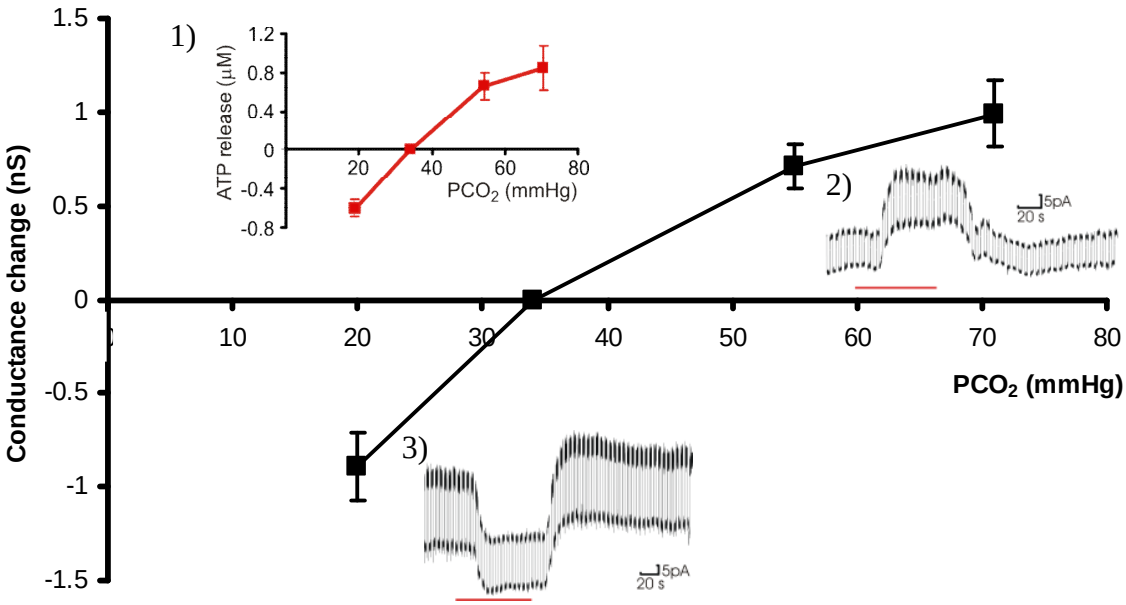

Figure 7: Effect of changes in CO_2 on Cx26 hemichannel conductance. Insert 1) Graph to show the effect of CO_2 on ATP release from the VLM surface. Insert 2) Whole cell patch clamp recording Cx26 expressing cell line during application of 12% CO_2/80mM HCO_3^- aCSF. Insert 3) Whole cell patch clamp recording Cx26 expressing cell line during application of 2% CO_2/10mM HCO_3^- aCSF.

Thus this causes the channel gate to close and would restrict the flow of ATP out of the cell. This relationship between Cx26 channel and gating and PCO_2 is almost identical to that for PCO_2 and ATP release. Therefore it is highly probable that this mechanism is working at the surface of the VLM during hypercapnia and hypocapnia.

4.4.6 The connexin 32 hemichannel exhibit graded sensitivity to PCO_2

To test whether Cx32 is also sensitive to changes in PCO_2 over the likely physiological range I examined whole cell conductance changes to graded levels of PCO_2. I found that Cx32 hemichannels are also sensitive to increases in PCO_2. Compared to a resting PCO_2 (34mmHg) hypercapnic solutions (PCO_2 55 and 71mmHg) increased the whole cell conductance. However in contrast to Cx26, decreasing PCO_2 to 19mmHg did not affect whole cell conductance (figure 8) compared to control levels (PCO_2 34mmHg). As the relationship between Cx32 channel gating and PCO_2 differs to that for PCO_2 and ATP release it is highly unlikely that Cx32 is the conduit by which ATP is released at the surface of the medulla. During hypercapnia there seems to be a rightward shift in the sensitivity of this channel to PCO_2 compared to Cx26. Therefore it is highly probable that this mechanism is not working at the surface of the VLM (Mulkey et al., 2007b).

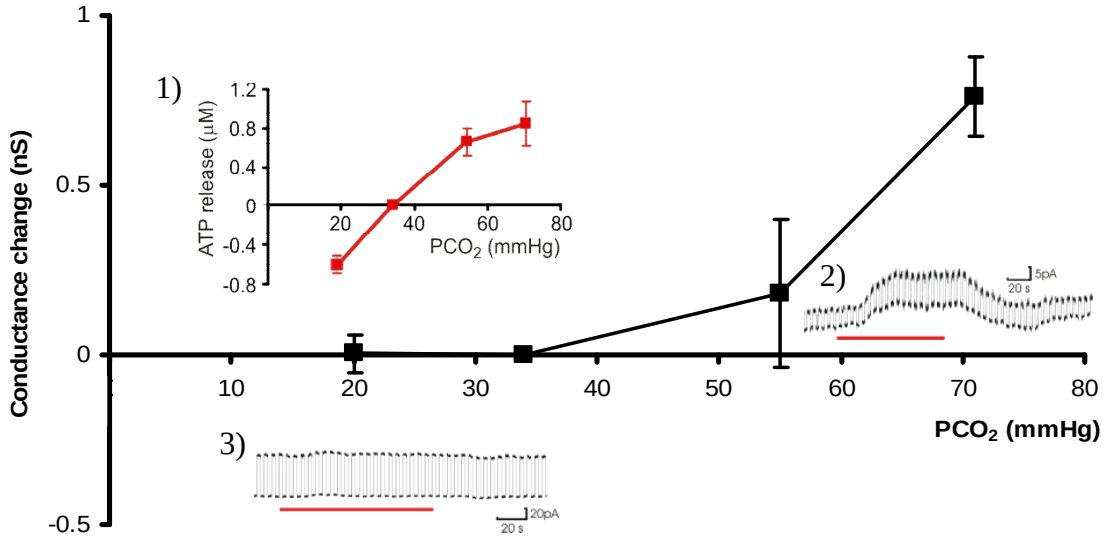

Figure 8: Effect of changes in CO_2 on Cx32 hemichannel conductance. Insert 1) Graph to show the effect of CO_2 on ATP release from the VLM surface. Insert 2) Whole cell patch clamp recording Cx26 expressing cell line during application of 12% CO_2/80mM HCO_3^- aCSF. Insert 3) Whole cell patch clamp recording Cx26 expressing cell line during application of 2% CO_2/10mM HCO_3^- aCSF.

4.4.7 Excised patches from HeLa cells transfected with Cx26 respond to alterations of PCO_2

My data shows that expression of Cx26 is sufficient to endow HeLa cells with "physiological" sensitivity to PCO_2. This strongly suggests that Cx26 is directly CO_2 sensitive. However this sensitivity could involve interactions with unknown proteins.

Therefore I made patch clamp recordings from isolated membrane patches. As this removes the protein from the intracellular components of the cell, any change seen will be a direct interaction between CO_2 and the channel. Moreover if the timing of the response of either inside out or outside out configuration may give me a starting point to try and distinguish where CO_2 may be binding. Analysis of patch clamp studies showed that outside out patches of Cx26 positive HeLa cells showed a large conductance of 130pS (the gradient of the current-voltage relationship; figure 11). At negative holding potentials, outside out patches showed a large inward current and a significant increase (~6 fold) in the time the channel spent in the open state (increase of 846000 pA.s, n = 8; figure 9 and 10) during the application of hypercapnic solutions compared to wash periods. In addition applications of hypocapnic solutions channels spent a decreased (~8 fold) amount of time in the open state (reduction of 328000 pA.s, n = 7; Friedman test, $P>0.05$; figure 9 and 10). Wild type controls did not show any significant change between control and hypercapnic states (Friedman test, $P<0.05$), as no channel gating was seen in these isolate patches no control for hypocapnic solutions could be taken. Preliminary data from isolated inside out patches showed a similar trend in current change with ~10 fold increase in the open state during hypercapnia compared to control periods (n = 2; figure 10 and 11) and ~3fold decrease during hypocapnia (n=1). The time taken for the increased or decreased gating to occur was shorter in inside out patches than in outside patches, implying that the CO_2 binding domain lies closer to the intracellular portion of the channel and may reside in the C-terminus.

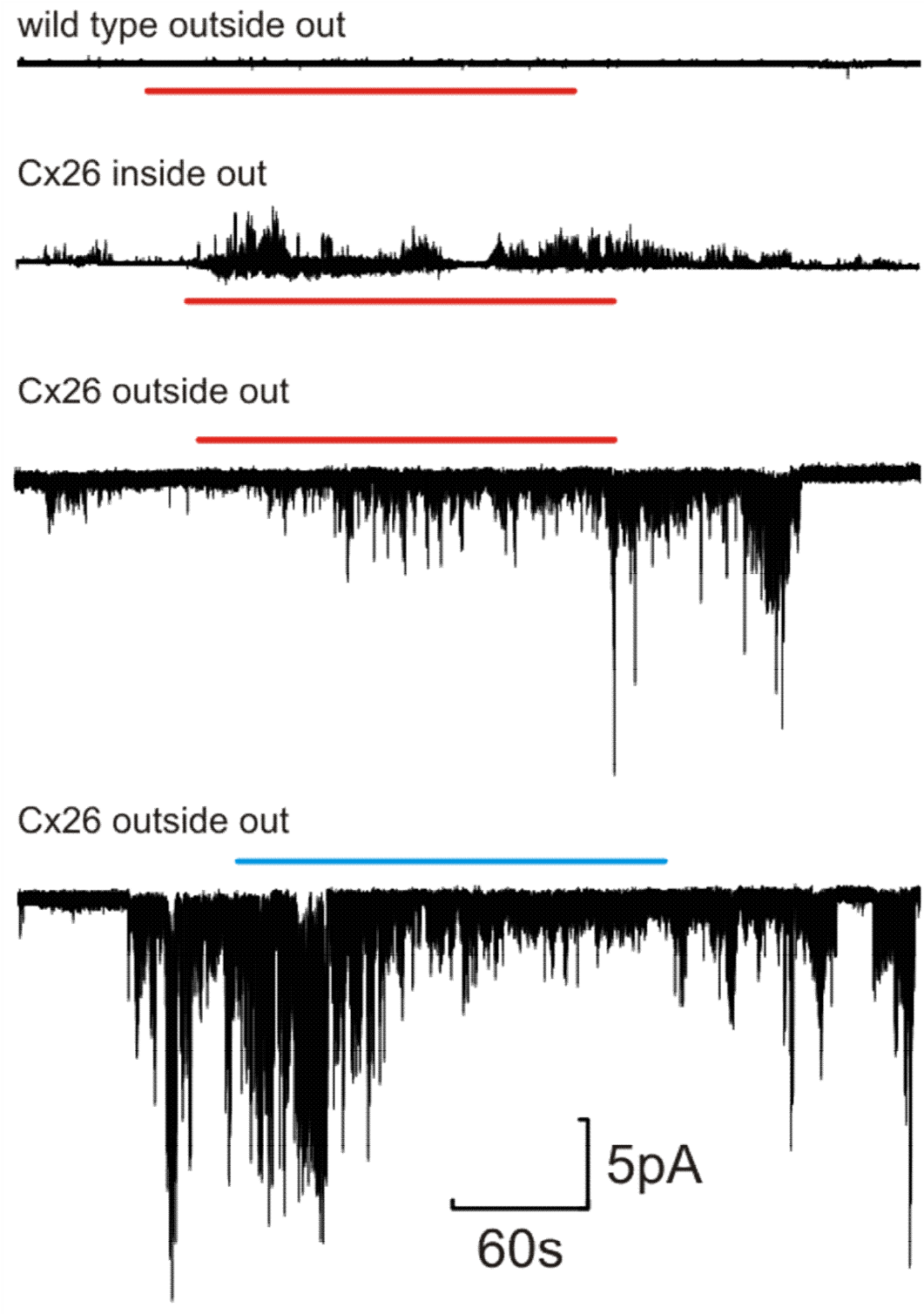

Figure 9: Isolated patches containing Cx26 are responsive to changes in PCO_2. Red line represent applications of hypercapnic solution (71mmHg), blue lines indicate application of hypocapnic solution (19mmHg).

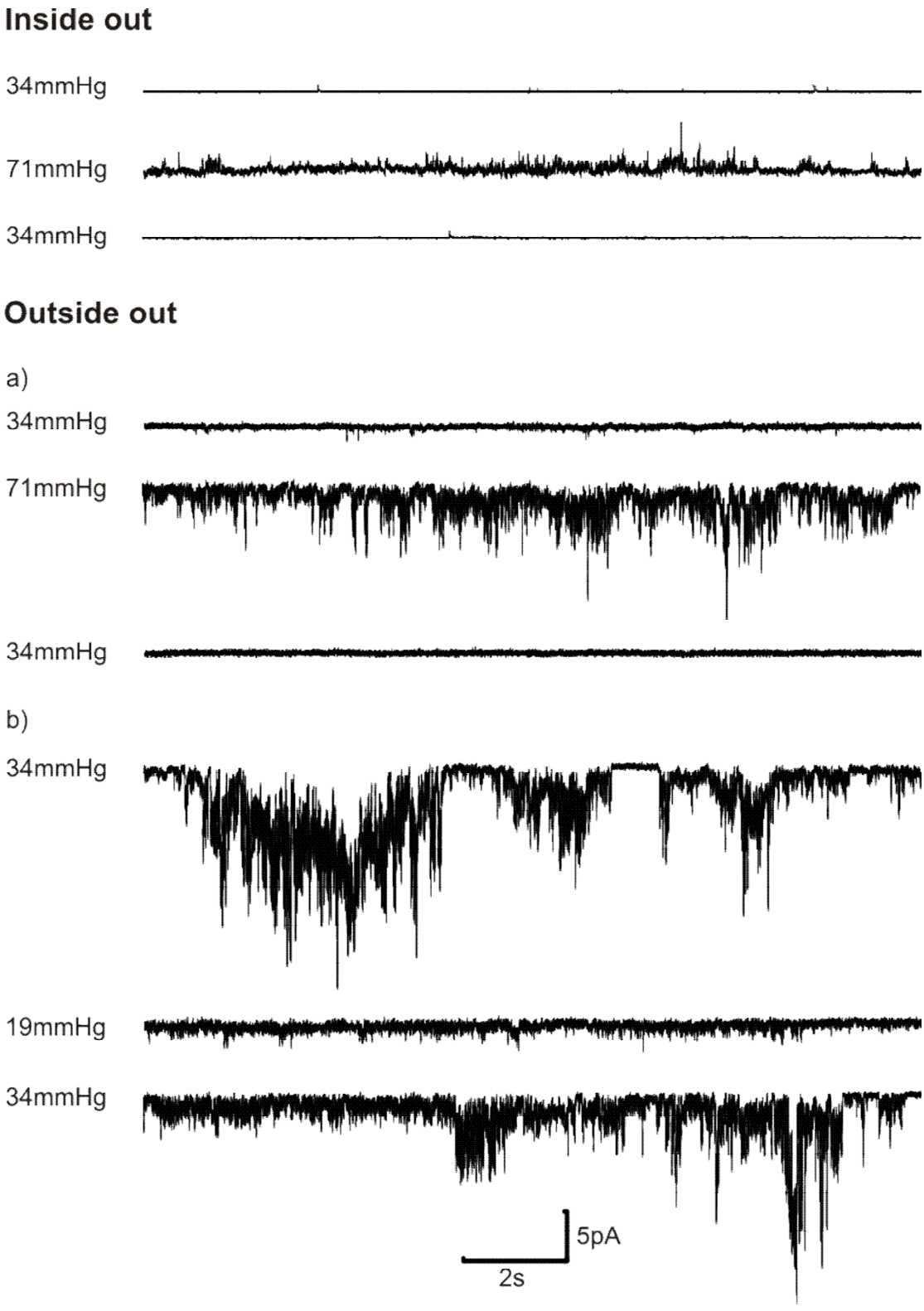

Figure 10: Enlarged channel activity from excised patches containing Cx26. a) Excised patch that exhibited low spontaneous gating. b) excised patch that exhibited a lot of spontaneous gating.

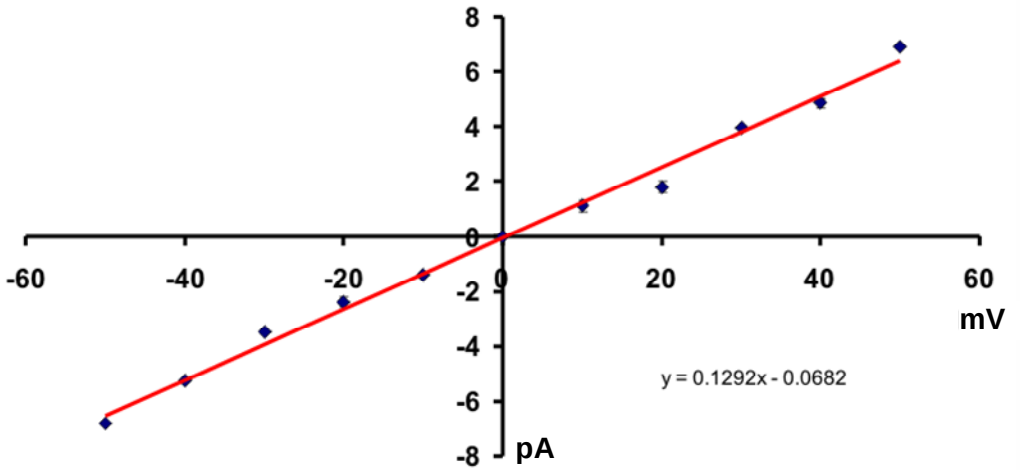

Figure 11: The current voltage relationship of the Cx26 hemichannel reveals a conductance of 130pS. Conductance of the channel was calculated from the gradient of the line.

4.4.8 Cx26 hemichannels are capable of allowing passage of ATP into the extracellular space

I have shown that CO_2 directly gates Cx26 hemichannels and causes ATP release from the VLM surface where Cx26 is heavily expressed. However whether Cx26 is capable of releasing ATP and whether it is able to do so in response to CO_2 is still unknown. To test this I placed ATP biosensors over sub-confluent Cx26 expressing HeLa cells and applied a hypercapnic solution. In pre-loaded Cx26-expressing HeLa cells ATP release was detected with biosensors (0.3 ± 0.04 µM, n=5: figure 10). ATP release was not evident from wild type cells (0.03 ± 0.2 µM, n=6, p=0.005) that had undergone the same treatment (figure 12).

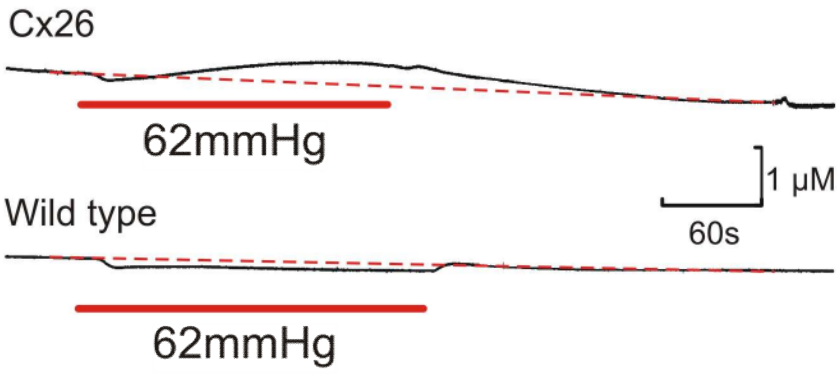

Figure 12: Cx26 transfected cell lines are capable of releasing ATP. Cells were preloaded by opening hemichannels with a quasi-isohydric stimulus (91% O_2, 9% CO_2 and 80mM HCO_3^-) in the presence of 10mM ATP.

4.4.9 CO_2 sensitivity may be more common than first thought

Excised patches of the outside out configuration (using the gluconate based whole cell intracellular patch solution) revealed a low conductance channel (~11pS) in both Cx26 transfected and wild type HeLa cells. This channel exhibit spontaneous gating at 34mmHg CO_2 and increased in frequency with the application of a solution equilibrated to 71mmHg CO_2 and decreased in frequency with the application of 19mmHg CO_2 containing solution (figure 12). This channel had a reversal potential of -50mV, which led to the choice of a -40mV holding potential for the outside out excised patches and the use of a different intracellular solution for the testing of isolated patches containing Cx26.

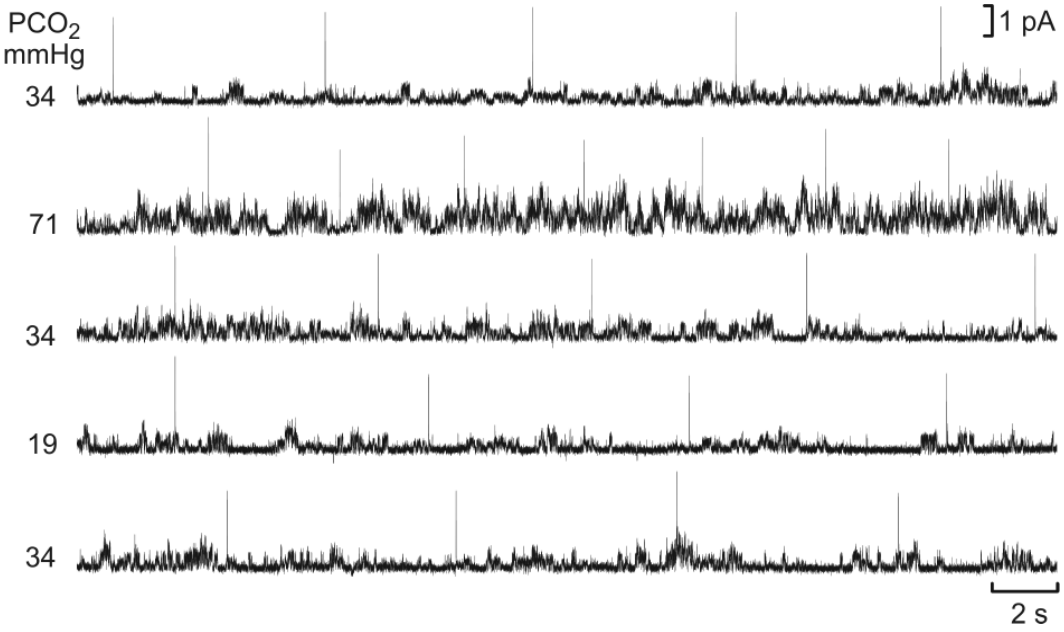

Figure 13: HeLa cells contain an endogenous CO_2-senisitive channel. Isolated patches were held at +10mV.

4.5 Discussion

4.5.1 CO_2/HCO_3^- sensitive second messengers do not contribute to ATP release

In previous investigations I had shown that ATP at the VLM surface is mediated by Cx26 hemichannels in response to changes in PCO_2. I have removed conversion of CO_2 to HCO_3^- and H^+ by blockade of carbonic anhydrase previously (chapter 1). I therefore investigated possible HCO_3^- sensitive second messengers (Chen et al., 2000) that may be responsible for converting increases in PCO_2 to increased gating of Cx26 hemichannels indirectly. Adenylate cyclase activates many

intracellular pathways and some connexin hemichannels are modulated by the adenylate cyclase/cAMP pathway (Duncan and Fletcher, 2002, Saez et al., 1990). In conjunction adenylate cyclase is sensitive to CO_2/HCO_3^- (Zippin et al., 2001, Wuttke et al., 2001, Chen et al., 2000); thus CO_2 activation of this enzyme may lead to an intracellular cascade that causes the release of ATP. I found that SQ22536 inhibition was without effect on any of the parameters recorded. This removed the only known second messenger system that I know of that could be involved in this mechanism, leaving the most plausible explanation that Cx26 is directly regulated by CO_2.

4.5.2 Cx26 is sufficient for CO_2 sensitivity

I obtained HeLa cells transfected with Cx26 protein (a gift from Klaus Wilecke). Knowing from previous investigations that carboxyfluorescein was able to penetrate cells via Cx26, I employed it in the present study. To test the direct CO_2-dependent modulation of Cx26 (Chapter 3) I applied carboxyfluorescein in the presence of my CO_2 stimulus (5 minutes 9% CO_2 80mM HCO_3^-, fig 5a). Cx26 positive cells readily loaded with the dye, compared to their wild type counterparts. Changes of PCO_2 also caused comparable changes in the conductance of these Cx26 expressing HeLa cells. In fact this increase in whole cell conductance closely resembles changes in ATP caused by altered CO_2 levels in chapter 2. The combination of these data sets shows that as PCO_2 increases the channel conductance, and therefore the Cx26 hemichannel spends more time in its open state, leading to an increase in ATP release at the VLM surface; when PCO_2 decreases the opposite is true. As dye loading was not seen in wild types which lack Cx26, carboxyfluorescein must have entered the Cx26 expressing cells through this hemichannel. To confirm that this loading was in fact as a direct consequence of transfection with Cx26, I repeated the experiments in the presence of the Cx26 selective inhibitor proadifen. Proadifen, profoundly reduced dye loading in response to alterations of PCO_2, confirming that Cx26 is responsible for dye loading and gating of the Cx26 hemichannels is directly sensitive to the levels of CO_2.

4.5.3 Expression of Connexin hemichannels leads to differential responses in CO_2 sensitivity and dye loading.

Although I have obtained blockers more selective to Cx26 than other gap junction hemichannels proteins, these compounds are not without effect on other members of this protein family. Therefore I used a dye loading and patch clamp

screen to test the CO_2 sensitivities of all the prime candidates for ATP release at the ventral surface of the medulla. I had predicted that these proteins would either be positively gated by CO_2 or be insensitive to it. The results of this experiment show that in fact some hemichannels are closed by this stimulus. Cx43 is known to be the least selective hemichannel in terms of both size and charge of molecules it allows to either enter or leave a cell (Weber et al., 2004). Upon increasing CO_2, the channel gate closes (figure 4), the increased time the channel spends in its closed state restricts the flow of low molecular weight dyes into the cell and thus reduces loading (figure 4 and 6). A reduction of dye loading implies that fewer molecules would be able to move in the opposite direction, thus under hypercapnic conditions less ATP would be seen; since ATP release increases I can exclude any involvement of this hemichannel in this process. Likewise Cx36 is seemingly insensitive to CO_2, it cannot therefore participate in the hypercapnia induced release of ATP (figure 4 and 6). This leaves only three possible hemichannels, Cx26, Cx30 and Cx32; interestingly these proteins are the most closely related according to phylogenetic studies, whereas Cx36 and Cx43 are found at greater distance on the phylogenetic tree (Manthey et al., 1999: figure 14). This relationship between phylogenetics and CO_2 sensitivity implicates that CO_2 sensitivity evolved to serve a specific function and has been selected for ever since. Though Cx30 is positively modulated by CO_2, from the cold PCR experiments (data not shown), we know that Cx30 is not present in the whole medulla, and therefore can also be ruled out as the molecular transducer.

This leaves us with only two principal candidates, Cx26 and Cx32. The patch clamp and fluorescence data shows that Cx26 undergoes larger conductance changes at 12% CO_2 than its cousin. More importantly, Cx32 expressing HeLa cells did not show a reduction in conductance when PCO_2 was lowered to 19mmHg, thus it cannot be responsible for the tonic levels of ATP seen at the VLM surface. Nevertheless during extremely pathological hypercapnia it may contribute to ATP release. The rightward shift in CO_2-sensitivity means that once Cx26 is saturated with CO_2 and thus releasing ATP at full capacity, Cx32 may then begin to open and thus allowing higher levels of ATP release to be achieved. Interestingly Cx32 may also be heavily expressed in the pre-Bötzinger complex (Solomon et al 2001), which is present in my second slice. Therefore it is possible that during hypercapnia, CO_2 may act upon Cx32 to increase electrical coupling and therefore increase respiratory rate. Since Cx26 and

Cx32 are both expressed at the VLM surface and are able to co-assemble, they could be both involved in this response as homo- and hetero- meric channels.

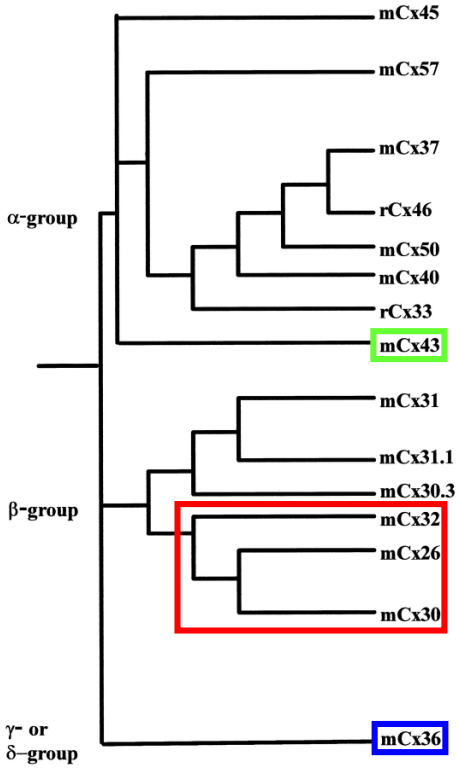

Figure 14: Phylogeny tree of connexin hemichannels. Red box denotes positively modulated CO_2 sensitive connexin hemichannels. Green box denotes negatively modulated CO_2 sensitive channel. Blue box denotes CO_2 insensitive channels. Picture adapted from Manthey et al J. Biol. Chem., Vol. 274, Issue 21, 14716-14723.

4.5.4 The CO_2 binding domain lies on the intracellular face of the Cx26 protein

Once I had determined the CO_2 dependent gating of Cx26, I began to study this using patch clamp techniques. This enabled me to study the protein in isolation from any intracellular components of chemosensitive cells that may be specialised to this response. Interestingly, my hypercapnic stimulus initiated large transient inward currents at negative membrane potentials in Cx26 positive patches excised in an outside out orientation. Importantly, this type of response was not seen in equivalent patches from wild type HeLa cells. Thus CO_2-dependent gating occurs when the protein is removed from any intracellular influence and is studied in isolation. In conjunction, current changes evoked by increases in CO_2 in patches of an inside out configuration showed similar, albeit faster responses. This may indicate that the effect of carbon dioxide is mediated by a binding domain accessible from either face of the Cx26 hemichannel protein, but appears to be located on the intracellular side of the

channel. Interestingly, carbon dioxide binding domains have never been described before in connexin hemichannels.

Surprisingly, many investigations have looked into the relationship between hemichannel gating and carbon dioxide. The chemical gate of gap junction hemichannels is commonly referred to as the CO_2 gate, though it also interacts with calcium ions and protons. However these studies employ unusually high levels of CO_2, between 30 and 100% (Trexlar et al., 1999, Peracchia et al., 1999, Peracchia et al., 2003, Peracchia, 2004, Peracchia and Peracchia, 2004). At these levels carbon dioxide causes massive extracellular and intracellular acidification. It is the interaction between this acidification and the closure of hemichannels that is being studied. Importantly many of these studies use "bad buffers" for studying hemichannels, i.e. HEPES (Bevans and Harris, 1999, Tao and Harris, 2004). These buffers contain taurine analogues (sulphonated amines), which when protonated block hemichannel pores. Therefore the closure of the hemichannel is due to interactions between the protonated buffer and the channel, and not protonation of the channel itself. The levels employed in this study are much closer to physiological levels, and have uncovered a unique relationship.

4.4.5 ATP can be released from cells transfected with Cx26 protein

Once I had determined that Cx26 is sensitive to CO_2 and that this sensitivity showed a close relationship to that of CO_2-triggered ATP release I wanted to confirm that this channel was able to act as a conduit for ATP release. I began by testing this hypothesis on untreated HeLa cells transfected with Cx26 protein, using ATP microelectrode biosensors. Surprisingly I found that no ATP release could be detected. I hypothesised that this may be due to the cellular concentration of ATP of HeLa cells. Therefore I loaded the HeLa cells with ATP using my stimulus as a method of gating the channel to allow influx of this molecule down the concentration gradient. I found that pre-loaded cells released significant levels of ATP (figure 11). It has been hard to reconcile the fact that Cx26 is expressed over the entire surface of the ventral medulla, yet ATP release is localised to discrete areas. This disparity can be resolved by this finding; in conjunction with Cx26 expression the concentration of ATP content of the cells involved may be elevated. This means that either pial cells or sub-pial astrocytes must either have an extraordinary high rate of ATP production or a specific sub-cellular organisation; such that mitochondria are situated close to the hemichannel or

they are compartmentalised so that the ATP can be stored beneath the hemichannel at high concentrations. Alternatively it may be that either the level of ectonucleotidases, enzymes responsible for the degradation of ATP, may be lower in chemosensitive areas. Thus ATP levels will be higher in the ECF of chemosensitive areas.

4.5.6 Implications for chemosensitivity

Intrinsic chemosensitivity is currently defined as the maintenance of a chemosensitive response in the absence of synaptic transmission, methods to achieve this include the removal of extracellular calcium or the use of a cocktail of antagonists for most neurotransmitters. Using this definition many neurons of the respiratory network and medulla oblongata as a whole have a small degree of intrinsic chemosensitivity (Kawai et al., 1996, Mulkey et al., 2004, Richerson, 2004, Nattie, 2000, Nattie and Li, 2002a). Importantly removing extracellular calcium will not inhibit hemichannel mediated ATP release. In conjunction the synaptic blocker cocktails used do not block gap junctions or contain purinergic antagonists. In light of the evidence presented here I believe that intrinsic chemosensitivity should be defined as the maintenance of a chemosensitive response in the absence of synaptic transmission (including purinergic antagonists) and gap junction hemichannel blockade. I also believe that in order to truly identify a chemosensitive cell one must demonstrate that it possesses the relevant molecular transducer.

Until recently the search for a chemosensory transducing molecule has focused on tandem pore potassium channels such as TASK channels (TWIK-related acid sensing potassium channel), particularly TASK 1. TASK 1 channels exhibit pH sensitivity over the physiological range, making it an apparently attractive chemosensory target. Although this molecule does convey intrinsic pH sensitivity to many neurons, TASK 1 knock out mice show no perturbations in the hypercapnic ventilatory response (Mulkey et al., 2007b). Thus the search for an acid sensing cation permeable channel continues. With many chemosensitive nuclei located close to the surface of the brain, containing purinergic receptors and sensitive to ATP, may be Cx26 is what we have all been looking for Cx26 acts as the chemosensor and purinergic receptors confers chemosensitivity to the neuron.

Chapter 5 Discussion

5.1 Implications for chemoreception

I have uncovered what appears to be the first incidence of a chemosensory mechanism that directly detects alterations in PCO_2 instead of the proxy of pH, as its primary source of detection. Generally it is fluctuations in pH that are primarily responsible for stimulation of chemosensory neurons. The RTN is directly stimulated by extracellular pH (pH_e) (Mulkey et al., 2004) in both bicarbonate and HEPES buffered saline. The raphé neurons are stimulated by an alteration in intracellular pH associated with all models of respiratory and metabolic acidosis (Wang et al., 2002). Interestingly fluctuations in bicarbonate ions acting at adenylate cyclase have also been implicated in both the RTN (Ritucci et al., 2005) and glomus cells of the carotid body (Summers et al., 2002). Thus with the inclusion of CO_2 acting directly on ATP releasing cells in the superficial medulla, all possible forms of chemosensory transduction are utilised in the response to hypercapnia as has been predicted from previous investigations (Harada et al., 1985). Thus Gray's multiple factor theory seems to apply, though with a slight modification. Gray stated that pH, CO_2 and O_2 all exert individual effects that contribute additively to give the full chemosensory response (Putnam et al., 2004, Loeschcke, 1982). I shall reiterate a modified form of Gray's proposition that pH, CO_2 and HCO_3^- all exert independent effects on separate chemosensitive nuclei and the net ventilation under any given condition is the sum of the partial effects of each of the separate agents through their actions on separate chemosensitive nuclei, whether this be a diction of increased CO_2 (or pH) or a decrease in PCO_2 (or pH).

5.1.1 Importance of the detection of multiple factors

In terms of complexity of the system as a whole this appears logical. The recognition of different molecules in different areas would allow the brain to compute the exact condition of the entire system. During metabolic acidosis areas that respond to changes in either pH_i or pH_e would be stimulated, but areas responsible for detecting HCO_3^- and CO_2 fluctuations would not. During respiratory acidosis all areas would be excited. During exercise there is an increase in H^+ due to an increase in anaerobic respiration, however bicarbonate levels reduce as it buffers lactate, thus only H^+-sensitive sites stimulate respiration, with bicarbonate dampening it slightly. Thus it allows not only great flexibility of the system, but the implementation of the

appropriate response to a given physiological environment as was discussed earlier (Nattie, 2000, Su et al., 2007). It will also allow great adaptation of the system, during chronic disease there may be prolonged periods when one of these chemosensory triggers remains constitutively high, such as H^+ concentration during metabolic acidosis. During such chronic disease there is often a resetting of the acceptable level of CO_2 in the blood. By providing a system that monitors multiple facets of the same chemical reaction (i.e. conversion of CO_2), the respiratory system can still adapt to changes in inspired or metabolic CO_2 even when one of the signaling molecules of this reflex is already saturated.

The fact that I saw tonic ATP at the surface of the VLM, in conjunction with this multiple factor and multiple site theory would also seem to show the robust nature of the response to CO_2 does indeed depend on additive or greater, interactions of inputs from multiple locations (Nattie, 2000) and some tonic baseline input is required from all chemoreceptors to allow full expression of the systemic response (Nattie, 2000).

5.2 ATP release is hemichannel mediated

There are several possible mechanisms of ATP release defined in the introduction. The removal of extracellular calcium was without effect on ATP release, eliminating synaptic release from neurons as a plausible mechanism. The removal of extracellular Ca^{2+} also caused ATP release in the absence of CO_2 alterations. ATP release on removal of extracellular Ca^{2+} has been observed for gap junction hemichannels in the retinal pigment epithelia, where ATP is released through connexin 43 (Cx43) hemichannels (Pearson et al 2005). The additive affect of removing extracellular calcium and increasing PCO_2 on ATP release indicates their modes of action are through different binding sites or mechanisms. It also removes most sources of neuronal mechanisms of transmitter release, which was the first suggestion that the primary source of ATP release is of glial origin.

Though the effects of low calcium suggest the involvement of gap junction hemichannels, it is by no means conclusive proof. Thus I next employed the use of carbenoxolone. Carbenoxolone is a known uncoupler of gap junctions and an inhibitor of hemichannel conductance. The first noticeable and expected result is that carbenoxolone blocked the release of ATP in response to lowered Ca^{2+}, showing that it is able to block hemichannel mediated release of ATP at the ventral medullary

surface. Carbenoxolone significantly inhibited hypercapnia activated ATP release at the VLM surface, supporting its involvement here. Interestingly it also significantly reduced tonic levels of ATP seen at the VLM surface. It appears that hemichannels spontaneously gate at the ventral medulla in response to basal levels of CO_2, upon hypercapnic stimulation there is an increase in the gating of connexin hemichannels leading to an increase in ATP concentration at the VLM surface.

5.3 Connexin 26 (Cx26) mRNA is enriched at the VLM surface

Although my evidence suggests that ATP release from the VLM surface is mediated by connexin hemichannels, there is a vast array of connexin hemichannels capable of releasing ATP. I had to then determine which connexin hemichannel was acting as the conduit in the superficial medulla. Reports had shown that connexins 26, 30, 32, 36 and 43 are the most abundantly expressed in mammalian brains (Solomon and Dean, 2002). Therefore I ran a cold PCR screen for these proteins and confirmed that, with the exception of connexin 30 (Cx30), all were present in the medulla. Interestingly the VLM expresses the highest levels of 26, as compared to the rest of the brainstem (Solomon and Dean, 2002) a result confirmed here. I also found high levels of both pannexin 1 and 2 in this brain region. To narrow down the possible candidates I ran real time PCR to look for the enrichment of message at the VLM surface, as I was trying to match regional specificity of ATP release with the expression levels of a specific connexin. Of the many connexin and pannexin sub-types expressed in the medulla, only Cx26 showed site specificity to the ventral surface. The other connexins and both pannexins showed a more or less uniform distribution throughout the ventro-dorsal extent of the medulla. Therefore one would expect to see ATP release elsewhere in the medulla, if other connexins were responsible for the passage of ATP into the extracellular space. It is therefore more than reasonable to conclude that Cx26 is the most probable conduit for ATP release and this conclusion warranted further investigation.

5.3.1 Cx26 protein is confined to the most superficial layer of the VLM

The detection of mRNA using real time PCR only allowed localisation of Cx26 to within 400μm of the ventral surface. To identify if there was a further localisation within this limited boundary, I employed the use of a Cx26 antibody and a transgenic mouse with a lacZ reporter allele, where a β-galactosidase sequence is inserted into the open reading frame of one copy of the gene. The reason for this dual

approach was the discrepancy in the expression of Cx26 by these two methods. LacZ reporter mice show that Cx26 is expressed only in the meninges surrounding the brain (Fillipov et al 2003), whereas immunofluorescent studies had shown it to be in the underlying glia and the cells lining the ventricles, the sub-ependymal layer (Mercier and Hatton 2001). I found exactly the same differences in this study; however confocal microscopy showed GFAP staining enveloping Cx26 positive nuclei in $Cx26^{+/LacZ}$ mice. With the Cx26 antibody, staining the superficial cells of the medulla and the lacZ mice showing staining in the pia and blood vessels. The staining of all penetrating vessels supplying chemosensory nuclei especially those supplying the serotonergic raphé neurons is a very intriguing finding (the importance of the raphé complex will be discussed later). The highly localised expression of Cx26 to the pia means that absolute levels are extremely low, thus a 5 fold decrease as is seen in the real time PCR experiments would only represent very low amounts of Cx26 throughout the rest of the dorso-ventral axis. Thus the presence of Cx26 on the penetrating vessels could account for all of the Cx26 expression seen in the subsequent slice in the real time PCR. If this is the case then Cx26 is expressed only where cells exposed to the highest fluctuations of PCO_2 are seen and thus the detecting mechanism will be most sensitive.

One possible reason for the discrepancy between these two results is the cross reactivity of the Cx26 antibody. Since antibody staining was confined only to the most superficial of cells this is unlikely, coupled to the fact that the connexin reported as being responsible for cross reactions of this antibody is not present in the medulla. A much more likely explanation is that these two forms of staining detect Cx26 in different cellular locations. The β-galactosidase transcript is switched on whenever the promoter of the Cx26 allele that it is attached to is activated. The transcript is only expressed in the nuclei of these cells, whereas the Cx26 antibody binds to the protein when expressed in the cell membrane. Were a glial cell anchored to the pia responsible for the production of Cx26, it would not be distinguishable under light microscopy from the pia itself. If glia cells were anchored strongly to the pia and only a single layer of glia cells produced Cx26, then it is conceivable that they are removed with the pia creating a false negative in the PCR results of Filippov *et al* 2003. A second though highly unlikely possibility is that Cx26 mRNA is made in pial cells then transported to sub-pial astrocytes where it is translated and expressed. Though highly unlikely this would not be without its merits, this would allow the expression

of Cx26 in homomeric channels only, as sub-pial astrocytes manufacture many different connexin sub-types. Thus in either case the lacZ mouse would show staining that is either of, or appears to be of, pial origin, whilst the antibody will show astrocytic staining. Whatever the case it would suggest that the removal of the pia removes Cx26 hemichannels. Therefore in any adult *in vitro* or *in situ* preparation experimenters must make sure not to disturb the meningeal layer, as is required in this preparation. Otherwise this arm of the chemosensitive response will not be present and will lead to results unrepresentative of the *in vivo* response.

5.4 All Cx26 positive cells dye load

The application of our stimulus must unblock the gate of the connexin hemichannels to a degree that would allow a molecule of at least 509Da to pass through it, in order to allow the passage of ATP into the extracellular space. Connexin hemichannels are non-directional channels: thus if the channel will allow the diffusion of molecules down the diffusion gradient into the extracellular space, it will allow the passage of dyes down the diffusion gradient into the intracellular space. Dye loading studies of this type are heavily utilised for the study of gap junctions and hemi-channels, for instance hemichannel expression can be identified in cells of the cochlear, based upon dye selectivity (Zhao 2005). As Cx26 is most permeable to anionic low molecular weight dyes such as Alexa 350 (Weber et al., 2004, Zhao, 2005) I used a fluorescent dye of similar molecular weight (CBF; MW 376) and charge.

I firstly exposed the medulla to CBF in the absence of any hypercapnic stimulus. The tonic release of ATP suggests that the channel is spontaneously gating and thus some background staining was inevitable. Alternatively many hemichannels of some species including human Cx26 are found in a partially open sub-conductance state and this could have led to background staining and tonic ATP release (González et al., 2006, Ripps et al., 2004). Any further increase in staining from this baseline level was therefore representative of dye uptake due to hypercapnia triggered opening of hemichannels. This is also important as many cells express hemichannels, which are also constitutively open or spontaneously gate, thus there will be background activity due to non-Cx26 containing hemichannels. There was an increase in dye loading over the entire surface of the medulla upon activation of the hemichannels that reside there, with increased CO_2. Interestingly coronal sections of the medullas loaded in this manner, showing this to be confined only to the most superficial cells

and cells around blood vessels. This loading was confined only to areas that expresses Cx26 and was absent from tissue that does not express Cx26. This was only true for Cx26 and no other connexin.

5.4.1 Dye loading is confined to sub-pial astrocytes, the pia mater and blood vessels

Using this method I aimed to answer the question of whether sub-pial astrocytes are involved in this process and whether they express Cx26. Using this method I loaded cells with FITC and then counterstained them with markers of cellular identity and Cx26 antibodies. GFAP was used as a marker of glial cells and microtubule associated protein 2 (MAP2) was used as a specific neuronal marker.

FITC labelling was confined to the MGL, the first 150µm, of the ventral medulla and was extensively co-localised with GFAP. The densest staining of FITC overlaps with the densest staining observed for GFAP. In addition FITC staining penetrated into the medulla in cells that surround blood vessels, which would allow for the excitation of deeper structures. Conversely dye loading and MAP2 staining was mutually exclusive, though a correlation was seen. MAP2 staining was seen in close apposition to dye loading, implying a close association between neurons and ATP releasing cells with in the MGL. Therefore when CO_2 levels increase it is detected by Cx26 positive glia, which release ATP possibley directly onto neuronal processes within the MGL.

5.4.2 Cx26 is expressed in sub-pial astrocytes and possibly pericytes

The co-localisations of Cx26 and GFAP at the light microscopy level suggest that Cx26 is expressed in the end feet of sub pial astrocytes in direct contact with the pia mater and therefore the blood vessels that reside there. In conjunction β-galactosidase stained nuclei that were not enveloped in GFAP appear in a similar distribution to pericytes, though no co-localisation was seen with NG2 (a marker of pericytes). Thus these cells, whatever they may be, which are associated with blood vessels and the pia (which contains blood vessels) may contribute to hypercapnia-triggered ATP release. Surprisingly using both antibody staining and lacZ reporter mice I saw Cx26 staining throughout the rostro-caudal and medio-lateral extent of medulla. The lateral staining where ATP release sites are not observed indicates that Cx26 alone is not sufficient for ATP release. This may mean that an intracellular intermediary is needed or specific internal environments are required. However the expression of Cx26 over the entire surface of the medulla including all ATP release

sites and its restriction to the ventral surface like ATP means that ATP is released only in areas where Cx26 is expressed and not from areas that are devoid of it. Therefore this shows that there is a high correlation with Cx26, though not all Cx26 positive cells release ATP. This cannot be said of other connexins, such as connexin 32 (Cx32) which is heavily expressed in the pre-Bötzinger complex (Solomon et al., 2001), an area devoid of ATP release.

5.4.3 The expression of connexin sub-types is cell specific

Connexin expression is cell type specific and this may also lead to some clues to the hemichannel involved in releasing ATP. More than 90% of astrocytes in the central nervous system express Cx43 (Rash et al., 2001, Dermietzel et al., 1991). As relatively only a handful of astrocytes release ATP in response to alterations of PCO_2 and in a site specific manner, this would seem to imply that Cx43 is not involved in this process, though it does not directly exclude it. Cx30 is astrocyte specific and is never seen in neurons or oligodendrocytes (Rash et al., 2001); however its lack of expression in the medulla excludes it as a possibility. Connexin 36 (Cx36) is purely a neuronal connexin (Rash et al., 2001) and thus can be excluded. Cx32 in a mature brain is found only in neurons (Solomon et al., 2001) and oligodendrocytes (Rash et al., 2001), which do not exhibit GFAP staining (Kashima et al., 1993). In fact Cx32 is never found in association with GFAP in the dentate gyrus (Melanson-Drapeau et al., 2003) or in Schwann cells (the oligodendrocytes of the peripheral nervous system) (Nicholson et al., 2001) or the medulla (Solomon et al., 2001).

In the peripheral nervous system there is an inverse regulation of GFAP and Cx32, such that GFAP is up regulated in the absence of Cx32 and almost undetectable when it is expressed in Schwann cells (Nicholson et al., 2001). Thus it is highly unlikely that the cells dye loaded in these experiments were due to the opening of Cx32 gap junction positive hemichannels, due to the co-localisation of dye loading with GFAP. Cx26 on the other hand is located only in astrocytes (Rash et al., 2001, Mercier and Hatton, 2001) or pial cells (Filippov et al., 2003, Rash et al., 2001) though they are expressed in neural progenitors (Rash et al., 2001), its localisation with neurons in animals of this age is extremely limited (Solomon et al., 2001). In contrast it is up regulated in glial cells with age. Thus no Cx26 is found in animals younger than P7, an age where no ATP is seen in vitro (Huckstepp, id Bihi and Dale; unpublished observation). However is it present in adults in large quantities (Solomon

et al., 2001), at an age where ATP release occurs (this study). Interestingly the development of Cx26 expression is coincident with the chemosensitivity of ATP sensitive raphé neurons. Neurons of the raphé complex are not chemosensitive until P12 (Wang and Richerson, 1999) and Cx26 expression in astrocytes of the ventral medulla begins around P11 (Solomon et al., 2001).

5.4.4 ATP release requires additional environmental conditions

Since dye loading was not limited to ATP releasing sites, it is not due to the localisation of an intracellular intermediatory that gates Cx26 hemichannels. If it were, I would have expected localised increases in CBF and FITC loading that would correlate only with ATP releasing areas of the VLM, which there were not. Instead this implies that all Cx26 hemichannels open under hypercapnic environments. That is not to say that an intermediate isn't involved in the gating of Cx26 hemichannels just that if there is it is expressed in all Cx26 positive cells. Thus it must be an internal environment unrelated to Cx26 expression that governs whether ATP is released. That dye loading and evacuation were observed during hypercapnia suggests that if a diffusion gradient exists, molecules can exit superficial cells through Cx26 hemichannels. This poses an interesting question, is it then in fact that ATP release sites are governed by whether a diffusion gradient for ATP exists or not? If this were true then only areas where a diffusion gradient exists would release ATP, and this would explain the discrepancy between global dye loading and localised ATP release.

Initial experiments to test whether Cx26 positive HeLa cells are capable of releasing ATP were unsuccessful. If as hypothesized it is the diffusion gradient of ATP between the cell and the ECF, increasing the cellular content of ATP should enable transfected HeLa cells to release ATP. I chose to load the cells, by applying our stimulus with ATP present in the milieu. I selected this method for three main reasons. Firstly it would take a very long time to individually inject each cell, and this provides a method for loading all HeLa cells simultaneously in a rapid fashion. Secondly it would provide proof of the principle of my proposed mechanism twice in one experiment, for the ATP to get in and out of the cell it would require CO_2-activation of the hemichannel. Finally, this is a non-invasive method, and thus I thought it could not be said that the ATP released was an artifact caused by the method with which I loaded them.

This method provided very reproducible results, and ATP was seen in all experiments where pre-loading took place. This suggests that in order to see CO_2-activated ATP release two things must be present, firstly a hemichannel comprised of a connexin (or connexins) that is positively modulated by CO_2 and secondly a gradient for ATP to diffuse down. I have already proposed three methods by which the second may be attained in Cx26 positive cells they are: Cx26 positive cells must either have an extraordinary high rate of ATP production or a specific cellular organisation, such that mitochondria are situated close to the hemichannel or they are compartmentalised so that the ATP can be stored beneath the hemichannel at high concentrations. Further investigations may provide evidence for which of these occurs.

5.5 Cx26 is sufficient for CO_2 sensitivity

To further study the intrinsic properties of this membrane channel, and other proteins of this family which are expressed in the medulla I obtained HeLa cells transfected with Cx26, 30, 32, 36 and 43. HeLa cells are almost completely devoid of any endogenous connexins, thus making them ideal for studies such as this. Since I had already shown that permeation of these channels in response to CO_2 occurs, I employed the use of dye loading in this study. I began by confirming that what I had seen *in vitro* tissue slices was in fact due to movement of the dye through connexin hemichannels. As there was mounting evidence for the participation of Cx26, I began with HeLa cells expressing this protein. Cx26 transfected cells loaded readily with carboxyfluorescein in the presence of 9% CO_2 in an elevated bicarbonate solution. In contrast staining was never seen in wild type HeLa cells. Thus it appears that Cx26 expression alone is sufficient to convey CO_2-sensitivity to a cell. To confirm this and to make sure that the insertion of the gene had not caused the upregulation of another unrelated membrane pore, I repeated the experiments in the presence of a gap junction hemichannel inhibitor, proadifen. The presence of proadifen in the perfusate significantly reduced the amount of dye that entered these cells, confirming that it was not due to a non-specific side effect. To further confirm this I used whole cell patch clamp to study any conductance changes of this channel in response to this stimulus. In accordance with the dye loading studies I found an increase in the membrane conductance in these cells indicating the opening of a large channel. As this membrane change was not observed in wild types, which have all of the same

membrane channels as the transfected cells with the exception of Cx26, I concluded that it must be this channel which is opening.

HeLa cells are an immortal cancerous cell line of ovarian origin. One would expect that these cells are not usually programmed to be chemosensitive, and that their expression of intracellular proteins and enzymes would be different to that of superficial medullary glia cells. It would not therefore be unfair to assume that connexin hemichannels in these cells would not be regulated in the same way as in the medulla. However it may be that another chemosensory transducer in these cells may be interacting with the Cx26 hemichannel causing it to gate in response to CO_2 where usually it would not. Thus I repeated the experiments in cell lines expressing other connexin hemichannels. This served two purposes: firstly it could rule out any non-specific of hemichannel transfection unless of course all of these hemichannels react in the same way; secondly it would allow us to determine if all hemichannels are sensitive to CO_2 and if so in what manner. For this series of experiments however I used the higher concentration of CO_2 (12%), as my whole cell data had indicated it provided greater conductance changes.

5.5.1 Connexin hemichannels are regulated differentially by CO_2

Connexin hemichannels are usually flickering between sub-conductance states and are not usually fully closed (or open) (Bukauskas and Peracchia, 1997, Ripps et al., 2004). Thus I had predicted to see non-specific background loading as I did for my *in vitro* tissue slice preparation previously. Therefore to control for this I used the same protocol in the presence and absence of changes in PCO_2. This also would allow me to detect reductions in conductance in response to hypercapnia as well as increases.

The first striking observation was that the background staining for Cx43 is actually as high and in some cases higher than loading of cells stimulated by CO_2. This may be due to many reasons: Cx43 transfected channels may express more hemichannels than other transfect cell lines; Cx43 may spend more time in open states than other connexin hemichannels or it is more permeable to the dye than other transfected cell lines. My patch clamp data shows it has the second largest resting conductance after Cx26, in addition Cx43 is one of the most permeable hemichannels (Weber et al., 2004). Thus it appears to be a combination of the two. Interestingly Cx43 hemichannels close in response to increased PCO_2, with large decreases in conductance and dye loading intensity. Physiologically this is a vital function. Cx43 is

the gap junction responsible for electrical coupling of excitable cells in the heart (Duncan and Fletcher, 2002). During arrhythmia gap junctions uncouple in order to stop the spread of damage in cardiac tissue (Cascio et al., 2005). During pathophysiological conditions in myocardial tissue (i.e. myocardial infarction), PCO_2 increases, this would cause Cx43 hemichannels to close and thus would act to preserve non-affected tissue from damage and/or over excitation. Cx43 channel closure in response to elevated PCO_2 is inconsistent with their involvement in ATP release at the ventral medullary surface, and thus we can conclude that they are not involved.

Cx36 the only existing gamma connexin was insensitive to alteration in PCO_2 by either method used. Interestingly this connexin is derived from the original gene duplication of the common precursor. Therefore it may be the 'purest' connexin in terms of structure and function and may imply that CO_2 sensitivity has involved from an 'inert' precursor. Importantly a lack of CO_2 sensitivity implies that it is not a non-specific reaction between an intracellular component of HeLa cells and hemichannels in general. Thus with the exception of Cx26 we have excluded all other connexins for one reason or another. Thus it is most likely that Cx26 is the conduit of ATP release, to quote Sir Arthur Conan-Doyle, "Once you eliminate the impossible, whatever remains, no matter how improbable, must be the truth"[2].

All three β-connexins however were sensitive to hypercapnia - this is not surprising as Cx30 arose from the gene duplication of Cx26 (Dahl et al., 1996) - and all three are highly related. The lack of Cx30 in the medulla means this will no longer be discussed here. Both Cx26 and Cx32 were shown to be significantly different in both experiments. Of the two connexins Cx32 was seen to be the least sensitive to CO_2, by approximately 30% in both series of experiments. In addition the conductance of Cx32 did not decrease with lowered PCO_2 levels; thus there is no correlation between ATP release at the VLM surface and Cx32 hemichannel conductance at the same level of PCO_2. One would expect that the release mechanism would be through the most sensitive hemichannel to the stimulus, in this case Cx26. Though it may be possible that ATP is released through both hemichannels or a heteromeric channel comprised of both connexins. For all of the reasons stated earlier (particularly the lack of Cx32 in GFAP positive cells) and in light of this new finding,

[2] From Sherlock Holmes a Scandal in Bohemia (a form of *modus tollendo ponens*/disjunctive syllogism)

Occam's razor/*lex parsimoniae*[3], would suggest that it is through Cx26. There was also an interesting correlation between ATP release and Cx26 hemichannel conductance. A reduction of PCO_2 by 60% causes a reduction in ATP release by 32% and Cx26 conductance by 28%, whereas increasing PCO_2 by 62% causes a 41% increase in ATP and a 44% increase in Cx26 conductance.

5.6 Properties of Cx26

The hemichannels mediating ATP release have been shown to contain all of the properties to designate them as Cx26. In conjunction to the location and cell type specificity, they share many other properties. Cx26 hemichannels are found to be constitutively open (Ripps et al., 2004), in line with tonic ATP release; it has been shown to be blocked by all the inhibitors used here (Ripps et al., 2004, Zhao, 2005). They are closed by extracellular calcium (Ripps et al., 2004). I have excluded all other medullary connexins with the exception of Cx26, and have shown that at every stage Cx26 appears to be the most likely candidate.

5.7 Structure of hemichannels

Gap junctions comprise of two hexameric connexons or pannexons each made of 6 sub-units individually called connexins or pannexins. Homomeric hexamers comprise a single type of connexin protein (Evans and Martin, 2002). Alternatively they may be heteromeric, where they comprise two different connexin proteins (figure 1) (Evans and Martin, 2002). In terms of gap junction they may be homotypic where both connexons are identical (whether they are homo- or heteromeric: figure 1). Otherwise they are heterotypic where each hemichannel is different, again regardless of whether the individual hexamers are homo- or heteromeric (figure 1).

[3] *entia non sunt multiplicanda praeter necessitatem:* all things being equal the simplist explanation is the best

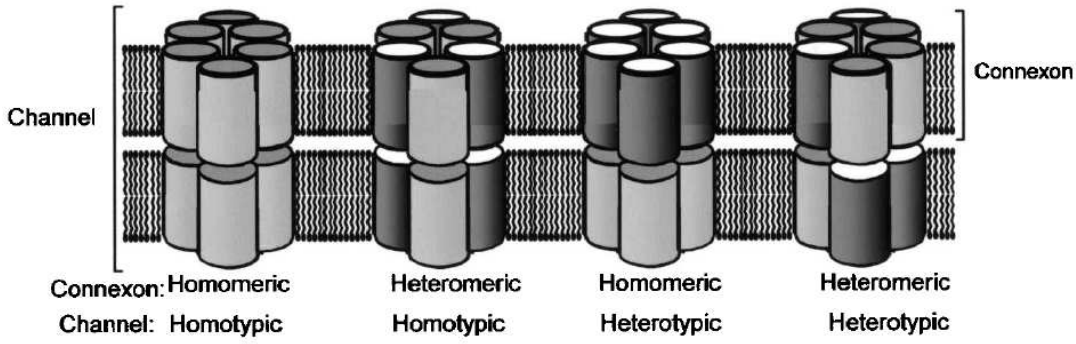

Figure 1: Arrangement of connexin proteins in gap junctions. Figure taken from Evans and Martin, Molecular membrane biology 19; 121-136 (2002)

Each connexin is named after its molecular weight (i.e. Cx26 is 26kDa). They consist of four transmembrane regions, two extracellular loops and one intracellular loop. The carboxy- and amino- terminals are located intracellularly (Spray et al., 2006). There are 20 connexins that have been identified, separated into three subfamilies. Type 1 connexins are known as α-connexins and contain connexins above 32kDa in size (connexins 33, 37, 40, 43, 45, 46, 50, 57) (Rozental et al., 2000). Type 2 connexins are known as β-connexins and are comprised of connexins lower than 33 kDa (26, 30, 30.3 31 31.1 32) (Rozental et al., 2000). There is only one known type 3 or γ-connexin that is Cx36 (Rozental et al., 2000). The major difference between connexin proteins is found in the carboxy tail, and this forms the major contribution to the difference in their molecular weight (Evans and Martin, 2002). There are also three pannexins, named pannexin 1, 2 and 3, whose structure, though very similar to connexins, contain significant differences (figure 2).

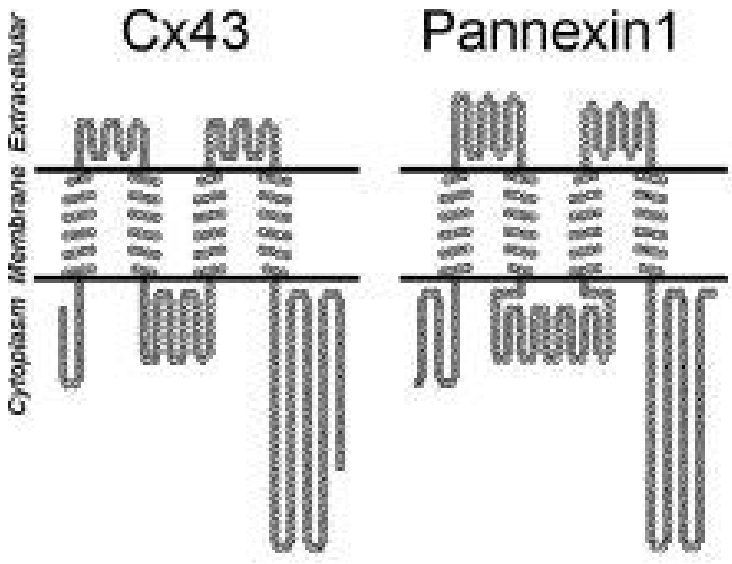

Figure 2: Two dimensional structure of connexin and pannexin proteins in cell membranes.

Hemichannels were once thought to only be an intermediatory, found in the membrane whilst they laterally diffuse into gap junction plaques (Spray et al., 2006). Now they are known to connect the cytoplasm of cells to the extracellular space (Spray et al., 2006). Connexins are known to contain at least two gates (Peracchia et al., 2003, Peracchia et al., 1999, González et al., 2006), a voltage gate that opens or closes with alterations in membrane potential thus increasing or decreasing contact between excitable cells during times of activation. The second gate known as the chemical gate is sensitive to pH_i and will be discussed later. Interestingly both gates can influence each other (Peracchia et al., 2003, Peracchia et al., 1999, González et al., 2006).

5.7.1 Structure of Cx26

Cx26 has a positive cytoplasmic face, which creates an environment that concentrates negatively charged molecules such as ATP at the entrance to the pore (Maeda et al., 2009). Conversely the pore of the channel is negatively charged, this will accelerate the movement of the negatively charged molecules through the channel through electrostatic repulsion (Maeda et al., 2009). Hence the amino acid side chains that line the pore and its entrance create an anion selective channel, perfect for conducting molecules such as ATP (Maeda et al., 2009). The channel itself is funneled having a pore diameter of 40Å at the cytoplasmic entrance and 25Å at the extracellular side (Maeda et al., 2009). However just before the extracellular cavity the channel narrows to 14Å (Maeda et al., 2009) creating the size selectivity for molecules with a molecular weight of <750.

5.7.2 Gating mechanisms of gap junctions

There are currently three gating mechanisms proposed for gating of connexin hemichannels; a rotational gate that assumes that a conformational change in the connexin causes the rotation of all six subunits which forms a pore (Peracchia, 2004); a ball and chain gating mechanisms, which assumes that the amino acid terminal of the connexin forms a hinge containing a ball like structure on the end, a conformational change in the hinge causes the ball to be situated either inside or outside of the pore (Evans and Martin, 2002, Peracchia, 2004); and a cork and bottle mechanism, this requires the binding of an intracellular molecule to the intracellular face of the connexin to form a plug, the binding sites are altered to determine whether

binding can or cannot take place (Peracchia, 2004). It is now universally accepted that the rotational gate model is not used by connexins.

Thus connexins either use a ball and chain or bottle or cork gating mechanism in response to decreased pH_i, the form of gating used appears to differ between connexins. Cx43 has an extremely long carboxy- tail; upon acidification this tail binds to the second half of its intracellular loop thus blocking the entrance to the channel pore and this is known as the chemical gate (Duffy et al., 2002). The massively reduced length of the β-connexins means that they are unable to form these complexes with their intracellular loop. Thus many low molecular weight connexins have calmodulin binding sites on their C-termini, Cx26 for instance has a single calmodulin binding site whereas Cx30 has two (Blödow et al., 2003). Surprisingly Cx50 which has the second longest carboxyl tail of all connexins also uses this form of gating mechanism (Peracchia and Peracchia, 2004). Thus it appears that either connexins use a ball and chain or a cork and bottle where calmodulin provides the plug.

5.8 The CO_2 binding domain may lie in the carboxy-terminus of the Cx26 protein

So far all of the experimental evidence from this study and that in the literature point to the regulation of ATP through the direct gating of Cx26 hemichannels. However it may still be that an intracellular component participates in this response. Therefore I used excised membrane patch clamp techniques to study the intrinsic properties of the channel. An excised patch contains a small piece of membrane containing the channel and almost no cytosol. In patches of an outside out orientation, increases in PCO_2 induced large transient outward currents at negative membrane potentials. These currents were absent from patches of the same configuration from wild type cells. The lack of cytosolic content and absence of any response in patches lacking the channel shows that Cx26 is indeed intrinsically sensitive to CO_2. In contrast patches of an inside out orientation showed large outward currents at positive holding potentials. The faster response of the channel to CO_2 in the inside out configuration indicates that the binding domain may lie closer to the intracellular face.

As Cx26 is situated close to blood vessels and is readily accessible to CO_2 in either orientation, this would place the CO_2 sensor directly on contact with the CO_2 passing through a blood vessel into the ECF of the superficial medulla. This would enable it to detect changes in PCO_2 almost immediately. In terms of a chemosensory mechanism this is almost as perfect as it can be. Almost instantly upon CO_2 diffusing

through the blood vessel wall it will reach Cx26 hemichannels, these will activate releasing ATP without having to wait for any intracellular cascade. That this is one of the simplest mechanisms ever described, the transducer and effector are one and the same molecule and it all occurs rapidly, is what makes this such an interesting proposition.

5.8.1 Gating of Cx26 hemichannels

Cx26 is thought to be comprised of two uncorrelated structures separated by a hinge (Purnick et al., 2000), where the N-terminus of the protein is flexible (Purnick et al., 2000). The first ten amino acids are integral to maintaining a central pore density (Oshima et al., 2008) and thus they constitute a plug (Oshima et al., 2008, Purnick et al., 2000, Young and Peracchia, 2004). Thus it is predicted that this channel operates via a ball and chain mechanism, with the first ten residues lining the pore of the channel.

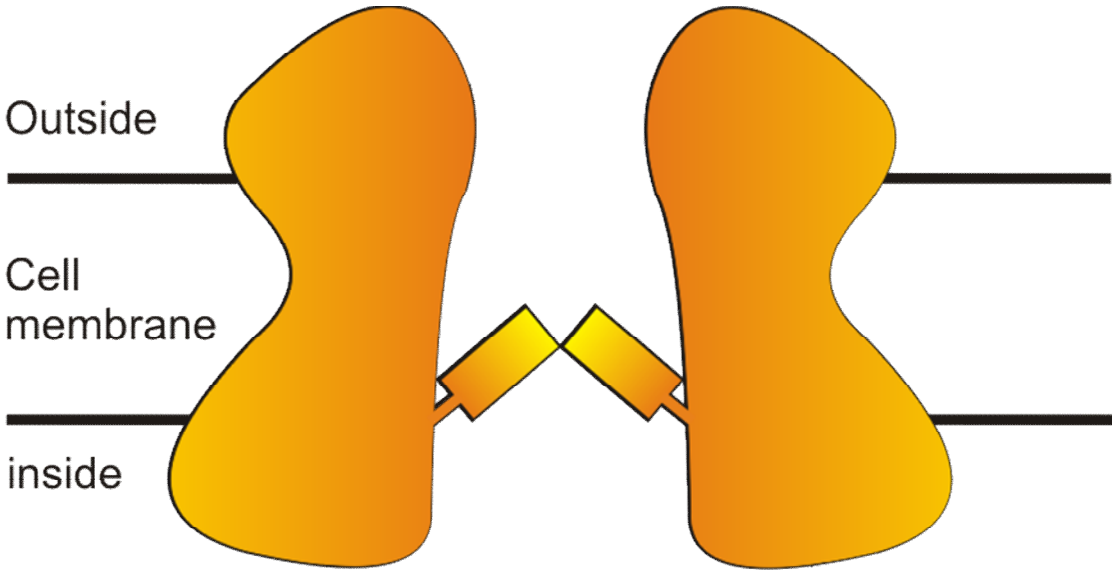

Figure 3: Gating mechanism of Cx26. The channels is gated from the intracellular compartment via a ball made up of at least the first ten residues of the N-terminus.

5.9 Possible CO_2 binding domains on connexin proteins

Cx26 has the same structure as all other connexins with the shortest carboxy tail in the mammalian connexin family. It has 4 transmembrane domains, 2 extracellular loops and an intracellular loop and is similar to Cx32 (Zhang and Nicholson, 1994).

Carbon dioxide (CO_2) is a small molecule and thus it is unlikely that a complex binding domain will exist on the connexin protein, for this molecule to interact with. CO_2 is known to interact with at least one protein, haemoglobin (Hb), thus it is likely that CO_2 will interact in a similar manner with Cx26 as with Hb. In red blood cells, CO_2 binds to the uncharged terminal amino groups to form carbamino compounds (-NH-CO_2H) on the α and β chains (Kilmartin and Rossi-Bernardi, 1971). Alternatively, a second formation of carbamino compounds occurs in rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase). Here carbamino compounds are formed between the free amine of a Lysine and the carboxy side chains of Glutamate and Aspartate, this compound is stabilised by free magnesium ions acting as a coordination ion (Taylor and Andersson, 1997). That increased magnesium in the intracellular patch solution was required for Cx26 gating in the presence of CO_2 seems to imply that this form of coordinate compound is formed in Cx26 and is responsible for the gating of this protein. Therefore I have looked for basic residues on the proposed ball of the Cx26 protein and acid residues that they might bind to. My hypothesis is that once CO_2 reaches the gate it will bind to the amino groups forming carbamino compounds. Once bound to the free amine it will be able to form co-ordinate compounds with amino acids lining the pore (figure 4).

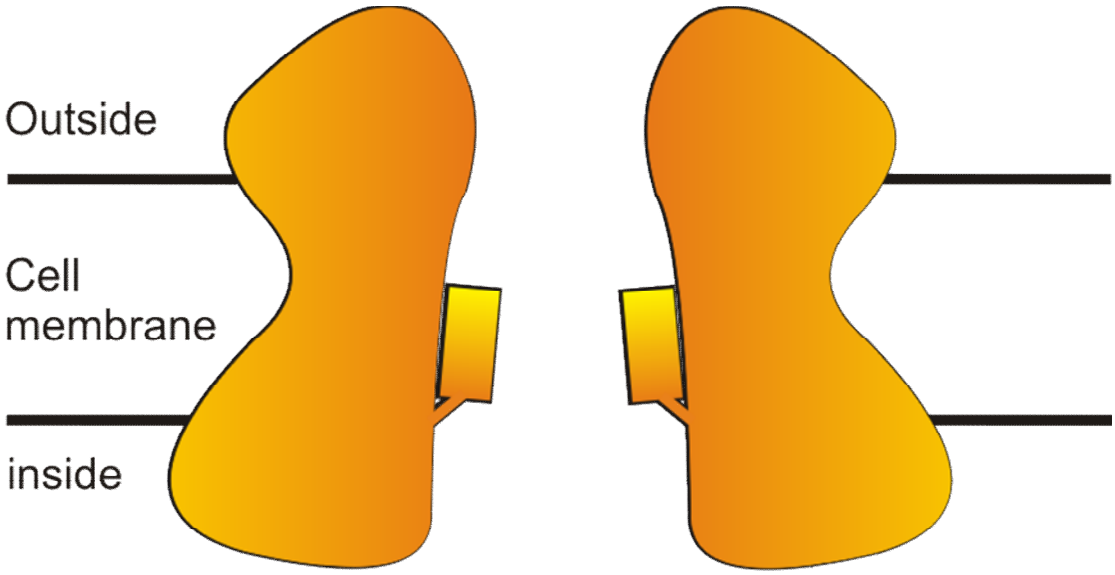

Figure 4: Possible CO_2 gating mechanism of Cx26. CO_2 binds to uncharged amines on the plug of the Cx26 N-terminus forming carbamino compounds and neutralising basic resides. Neutralisation causing a loss of attraction between acidic and basic side chains, hence causing the plug to move out of the pore.

Using sequence alignment (ClustalW) of all five connexins (figure 5) for which I have classified the CO_2 sensitivity of, I have identified possible sites of action for CO_2. I have done this by looking for basic residues in the sequence. Once aligned and coloured for acidity I have looked for regions that have a conserved basic sequence between CO_2 activated connexins, which are not seen in hemichannels that do not gate in response to CO_2. In doing so I found two regions of interest, fascinatingly in these regions on Cx36 and Cx43, contain no basic residues. Thus I believe that it is these regions which are responsible for reacting with CO_2 in all CO_2 sensitive connexins.

It can be seen from the sequence alignments of the N-terminus of the protein that Cx26, 30 and 32 contain a KH…..K or RH…..R motif not seen in Cx36 or Cx43 thus providing regions that could be responsible for intracellular gating. A second region CO_2 may bind to is the intracellular loop which shows an acidic motif in Cx26, 30 and 32 (EE.D..E, E….ED and D….EE, respectively) a motif not seen in the other connexins. Unfortunately the 3.5Å structure does not resolve this region. The difference between the basic and acidic side chains of these connexins may cause differential binding of the plug and thus explain the differences in CO_2 sensitivity.

As we can see from the sequence alignment the highly basic region found in all three CO_2-positively gated connexins, but not Cx43 or Cx36 lies on the proposed ball. The acidity of the amino acids of this region falls in line with the patch clamp and dye loading data. Interestingly based upon the model of amino acid structure related to Cx26 we can see that there does not appear to be any acidic in this region. The most likely binding domain for the ball is found on the intracellular loop, which is unexpected. If however the intracellular loop more closely resembled that of Cx32, which it may do as no antibody reactivity was seen in this area, then what would appear to be the intracellular loop now becomes the beginning of the transmembrane domain 3. This would allow the binding of the hypothesised ball to the internal face of the pore (see figure 6). We can see that the intra-pore binding domain is highly acidic and thus would easily bond to the highly basic n-terminus. Both of these regions are highly variable on Cx43 and thus it may act oppositely.

It must also be noted that there are amphoteric amino acids, which may act as acids or bases at different pH's. They may not directly interact with carbon dioxide, but they are closely associated with acidic and basic amino acids. This close proximity means that they may influence the interactions between CO_2 and the amino

acids that surround them. Therefore I have highlighted them in these regions specified below.

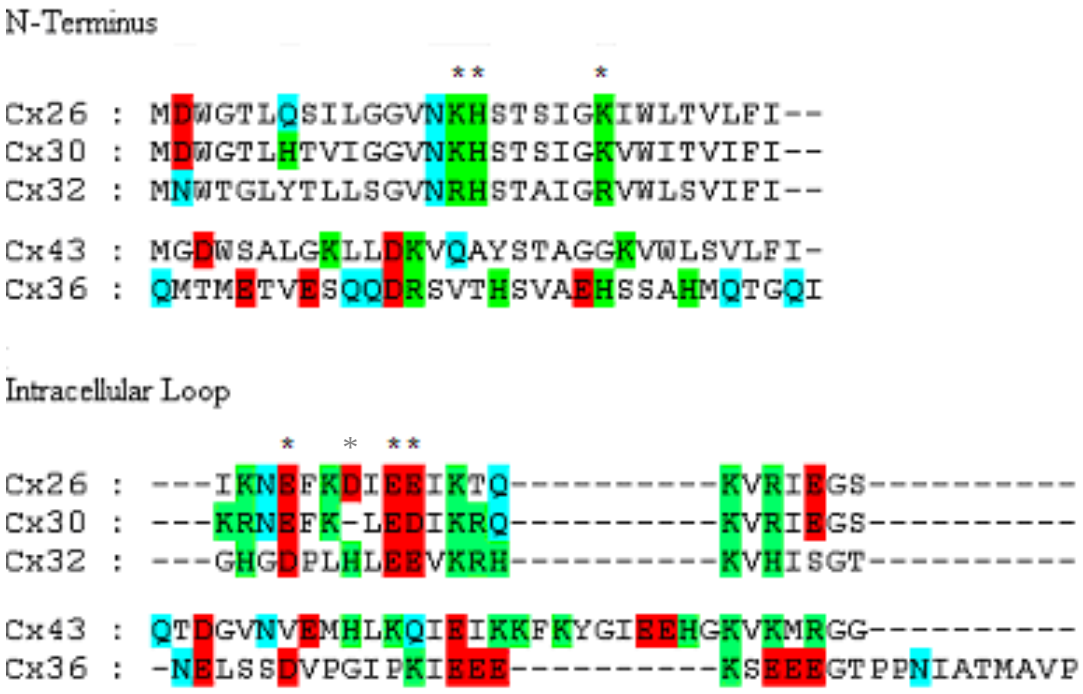

Figure 5: Sequence alignments for 5 connexins showing differences in acidic (coloured red), basic (coloured green) and amphoteric (blue) side chains. Areas of interest are highlighted with asterisks.

The ease of access to the intracellular loop in inside out patches to CO_2 may explain why gating occurs faster in this configuration compared to outside patches, where CO_2 has to diffuse across the membrane before it can form a carbamino compound. Alternatively CO_2 may diffuse into the cell neutralising the basic amino acids leaving them unable to bind to acidic residues and thus the ball is repelled from the pore. In contrast it has already been shown that acidic conditions cause the binding of the carboxy terminal to an unspecified domain on the intracellular face of the Cx43 protein (Duffy et al., 2002). May be this is one of the two regions I have specified here or may be an interaction between all three. Thus there are a few possibilities as to how this mechanism may occur, but it is likely to contain the regions I have described here.

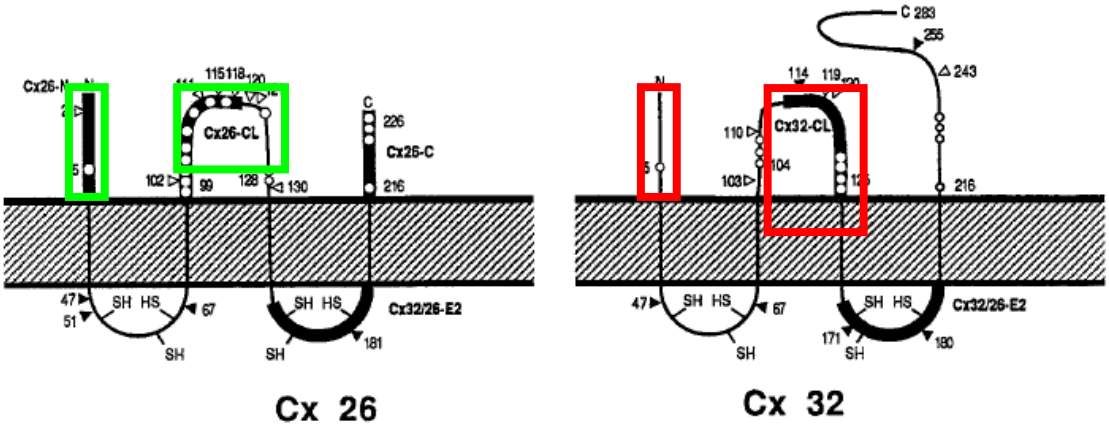

Figure 6: Location of possible CO_2 reactive domains on connexin hemichannels. Left Cx26. Right Cx32. Red/green boxes show differences between CO_2-activated and deactivated connexins, blue boxes show conserved regions. Picture adapted from Zhang and Nicholoson J Membrane Biol 139 15-29 (1994).

5.10 Cx26 is the conduit for ATP release in vitro

So far all the indications point towards the involvement of Cx26 in the release of ATP in response to hypercapnia with evidence against the involvement of other connexins. It is located in the right place, its expression levels coincide with the development of ATP signaling, its association with surface and penetrating blood vessels, its co-localization with dye loaded cells and its co-localization with GFAP and ATP release is not seen outside of the areas that express this channel. I next sought to see if I could provide evidence of its participation pharmacologically. This however provides problems. Pharmacological identification of gap junction hemichannels is not yet achievable, due to a lack of specific blockers. Though all hemichannel blockers universally affect all subtypes, they do so differentially. I therefore identified compounds that are more selective for Cx26 than other hemichannels cobalt, NPPB and proadifen (Ripps et al 2004), though cobalt and NPPB have been shown to affect other hemichannels (Kang et al., 2008, Cotrina et al., 1998). Cobalt is generally used at much higher levels (2mM) (Huettner et al., 2006) than this study (500µm) and does not affect all hemichannels (Huettner et al., 2006). Proadifen on the other hand has been shown only to affect Cx26 (Zhao, 2005). Nevertheless the removal of all of the relevant connexins means that even without specificity the effect of these blockers is highly likely their effects are on ATP are through their actions on Cx26.

All three gap junction blockers significantly reduced all forms of ATP release. The blockade of ATP release in response to removal of extracellular calcium shows

that they block hemichannels. The reduction in CO_2-activated ATP release by these selective blockers shows that it is highly likely that Cx26 acts as the conduit for release *in vitro*. In conjunction the loss in tonic release of ATP also implies that this release is through the same mechanism as that in response to hypercapnia and not parallel pathways.

In contrast to the lack of specificity of connexin hemichannel blockers, pannexin 1 can be pharmacologically distinguished. Since it has been shown to release ATP I thought it pertinent to test its involvement here. Both low concentrations of carbenoxolone and probenecid were without effect on ATP release in response to changes in PCO_2 and tonic levels of ATP release. Thus I have excluded this as the mechanism underlying ATP release in the superficial medulla.

5.10.1 Cx26 is the conduit for ATP release in vivo

Although I had characterised the release sites of ATP *in vitro* and matched them to those seen *in vivo* and tried to create an environment that more accurately recapitulates *in vivo* conditions. I could not be certain that what I had seen was not an artefact of the extraction method or stimulus that I provided. In conjunction although *in vitro* methods are phenomenal for building models and gathering data, I believe they must be validated by replication *in vivo*. Therefore I applied proadifen and NPPB to an *in vivo* preparation of the adult rat. I had selected these blockers for two reasons, firstly the effects of carbenoxolone applied to the VLM surface had been previously described, and cobalt at this concentration has a significant effect on calcium channels. Secondly proadifen has a tendency to precipitate out of the solution if it is not dissolved in and kept in specific conditions. ATP release during hypercapnia was significantly reduced by these blockers and in conjunction so was adaptive breathing.

The reduction in the hypercapnic ventilatory response caused by these blockers was similar to that caused by: ATP receptor antagonists (Gourine et al 2005), all gap junction hemichannels in the superficial medulla (Hewitt et al 2004) and the contribution of the ventral surface chemosensors to the total adaptive ventilatory response to CO_2 (Nattie 2001). Thus confirming not only that Cx26 mediates the release of ATP, but also that it is the primary gap junction protein involved in chemosensory transduction. In addition it implies that Cx26 contributes the major chemosensory drive in the superficial medulla.

5.10.2 Divalent ion concentrations effect respiration and the response to hypercapnia

In adult cats *in vivo* intracisternal perfusion of aCSF containing a dye causes staining only in the most superficial layer of the entire medullary surface (Berkenbosch and Adan, 1974, Berkenbosch et al., 1976). Thus aCSF perfused in this way causes drug delivery or a change in environment of the MGL presumably without effect on any other medullary nuclei. Artificial CSF containing varying concentrations of Ca^{2+} was perfused in this manner and caused changes not only in the hypercapnic response but also resting ventilation. Increasing calcium concentrations, reduced resting ventilation whilst decreasing them increased ventilation (Berkenbosch and Adan, 1974, Berkenbosch et al., 1976). Although these authors had no mechanistic explanation of their findings, I can however reinterpret them in light of my data. The alterations of calcium used in this investigation are sufficient to cause an increase or decrease in gap junction hemichannel gating. The closure of hemichannels at the VLM surface would result in a loss of tonic ATP; thus its continual facilitatory drive is removed and a reduction in ventilation is seen. Therefore addition of calcium which closes hemichannels at the VLM surface has a similar effect on respiration as application of purinergic antagonists in a similar area. In conjunction a decrease in calcium, which would open hemichannels, causes an increase in resting ventilation, due to an increase in facilitatory drive. In addition the removal of calcium caused a potentiation of the hypercapnic ventilatory response, whereas increases in calcium ions attenuated it (Berkenbosch and Adan, 1974, Berkenbosch et al., 1976). It appears that potentiation or attenuation of hemichannel conductance in the superficial layers of the medulla results in similar effects in the ventilation in response to increased CO_2. Importantly the response to hypoxia, which is not controlled by, or does not cause release of, ATP at the ventral surface of the medulla (Gourine et al., 2005b), is unaffected by alterations of Ca^{2+} in this region (Berkenbosch and Adan, 1974).

Using the same technique, the effect of magnesium ion concentrations was assessed in the same manner (Berkenbosch et al., 1976). Alterations of magnesium concentrations caused identical effects on respiration and chemosensitivity as those of calcium, however to a lesser extent (Berkenbosch et al., 1976). Interestingly the effect of magnesium on hemichannel conductance are the same as that of calcium ions; however calcium has a much higher binding affinity and so is a more potent inactivator (Ebihara et al., 2003). Thus the effects of divalent ion concentration on respiration can possibly be explained through hemichannel mediated ATP release in

the adult response. To further support this I could investigate how calcium concentrations influence the CO_2-sensitivity of Cx26 hemichannels.

Similar responses to these divalent cations were also reported in the neonate, which is surprising as ATP release is not seen in rats of this age. This can be reconciled as it appears that the mechanisms responsible for these alterations are much more complex. The effect of low calcium in the neonate has been attributed to membrane stabilisation and the augmentation of calcium-dependent potassium channels (Kuwana et al., 1998), which can contribute to neuronal chemosensitivity from before birth (Wellner-Kienitz et al., 1998). Unfortunately the effect of divalent cation concentration cannot be directly compared as calcium concentrations were altered throughout the entire medulla of the neonatal preparation; hence it will affect many processes and not just hemichannels.

5.10.3 Cx26 has an alternative pathway for membrane trafficking

Cells use a different trafficking mechanism for Cx26 to every other connexin. Connexins as a family are manufactured and formed into hemichannels in the golgi apparatus and transported to the membrane via the endoplasmic reticulum. If a cell produces only one connexin isoform in a given period of time it will form homomeric hemichannels, if it produces more than one they will be constructed into heteromeric channels in the ER. The treatment of cells expressing Cx26, 32 and 43 with brefeldin A, a golgi toxin, leads to the loss of Cx43 and 32 but does not affect Cx26 (Evans and Martin, 2002, Gemel et al., 2004, Martin et al., 2001). In contrast treatment of cells with nocodazole, a cytoskeleton inhibitor of microtubule polymerisation, reduced Cx26 without effect on Cx32 or 43 (Martin et al., 2001). Thus it appears important for cells to ensure that Cx26 is expressed as a homomeric unit. Importantly the pathway for the manufacture and trafficking of Cx26 homomeric channels is a much faster route than that for Cx32 and Cx43 (Evans and Martin, 2002).

Since Cx26 and 32 can form heteromeric channels it would also appear that Cx26 can if necessary use both routes. Thus there are two conclusions that can be drawn from these results; Cx26 must be inserted into the membrane rapidly and as a homomeric channel. This begs the question, why is this hemichannel so important? Firstly I believe that it must be to some extent its sensitivity to CO_2. Though this is a vital role in the response to hypercapnia, there are also indications that this may be useful in other physiological situations (these will be discussed later). Interestingly

Cx26 has only been shown to localise with other β-connexins, in one study with an array of connexins it co-localised only with Cx32 (Elfgang et al., 1995). In addition it only co-assembles with one other connexin, Cx30 (Locke et al,. 2006). Thus Cx26 only seems to co-assemble with other CO_2-activated connexins when forming gap junctions and possibly therefore hemichannels. Therefore even when using the normal connexin trafficking route there appears to be a need to conserve CO_2-activated hemichannels even if only in the heteromeric form.

5.11 Implications for chemosensitivity

Intrinsic chemosensitivity is currently defined as the maintenance of a chemosensitive response in the absence of synaptic transmission, these methods include the removal of extracellular calcium or the use of a cocktail of antagonists for most neurotransmitters. Using this definition many neurons of the respiratory network and medulla oblongata as a whole have a small degree of intrinsic chemosensitivity (Kawai et al., 1996, Mulkey et al., 2004, Richerson, 2004, Nattie, 2000, Nattie and Li, 2002a). However removing extracellular calcium will not inhibit hemichannel mediated ATP release. Furthermore the synaptic blocker cocktails used do not block gap junctions (Putnam et al., 2004) or contain purinergic antagonists. In light of the evidence presented here I believe that intrinsic chemosensitivity should be defined as the maintenance of a chemosensitive response in the absence of synaptic transmission (including purinergic antagonists) and gap junction hemichannel blockade. This however does not account for the unknown and thus contains the same flaws as the previous definition. Therefore I believe that in order to truly identify a chemosensitive cell, one must identify a molecular transducer present in the cell that remains chemosensitive in isolated patches, such as in this study; once identified, blockade or removal of this channel *in vivo* must result in a reduced ventilatory response to hypercapnia.

Until recently the search for a chemosensory transducing molecule has focused on tandem pore acid sensing potassium such as TASK channels (TWIK-related acid sensing potassium channel), particularly TASK 1. Although this molecule does convey intrinsic pH chemosensitivity to many neurons, TASK 1 knock out mice show no perturbations in the hypercapnic ventilatory response (Mulkey et al., 2007b). Thus the search for an acid sensing cation permeable channel continues. Importantly ionotropic purinergic receptors are non-selective cation channels. With many

chemosensitive nuclei located close to the surface of the brain, containing purinergic receptors and being sensitive to ATP, may be Cx26 is what we have all been looking for; Cx26 acts as the chemosensor and purinergic receptors convey chemosensitivity to the neuron.

5.12 ATP and pH regulation

Now that I have discussed the properties of Cx26, its location and its involvement in ATP release, I would like to discuss how pH regulation may effect ATP release and how ATP release may affect pH regulation (for a reminder of pH regulation in the medulla please see chapter 1.9).

When CO_2 diffuses out of blood vessels it will cause the release of ATP through Cx26 from sub-pial astrocytes (figure 7(i)). This will activate P2Y receptors on the dendrites of surrounding neurons leading to their subsequent depolarisation (figure 7(ii)). In a similar time frame CO_2 will cause intracellular acidification in chemosensitive neurons again leading to their depolarisation (figure 8(i)). The effects of one should work to amplify the other, thus leading to a heightened response. The removal of bicarbonate from the cytosol of the neuron acts to alkalise the extracellular space (figure 8(ii)). The alkalisation of the extracellular space will aid the transport of ATP through Cx26 hemichannels (figure 8(iii)): this will act to amplify the sensitivity of these cells to CO_2. The increased release of ATP will thus provide a positive feed back loop with the neuron.

As the neuron fires, extracellular potassium levels increase causing the depolarisation of the sub-pial astrocytes and the subsequent reversal of NBC transporters (figure 9(i)). This will cause an influx of HCO_3^- ions causing intracellular alkalisation (figure 9(ii)), which should a taurine analogue be present, unblocks the pore of the channel and leads to enhanced ATP signalling (figure (9iii)). Thus the increased firing of the neuron will provide a positive feedback loop with the sub-pial astrocytes.

In conjunction the release of other neurotransmitters in the vicinity of the superficial medulla will also increase pH_e thus creating an environment that enhances not only their own affinity for their receptor but the affinity of ATP to its receptors (figure 10(i) and (ii)). Once ventilation has corrected PCO_2 levels in the blood ATP signalling decreases, by this point pH_i recovery mechanisms present in the neurons have brought its pH_i back to normal (as is seen in with isohydric hypercapnia: figure

10(iii)). Thus with no intracellular or extracellular signalling it ceases firing. This would reduce extracellular potassium and thus reverse the NBC transporter, so that it returns glia pH_i back to normal levels and thus the system returns back to its starting point. Therefore it creates a system where each component works to increase the efficiency of all the other components in the system. In conjunction the system communicates with each other via positive feedback to provide a well coordinated response.

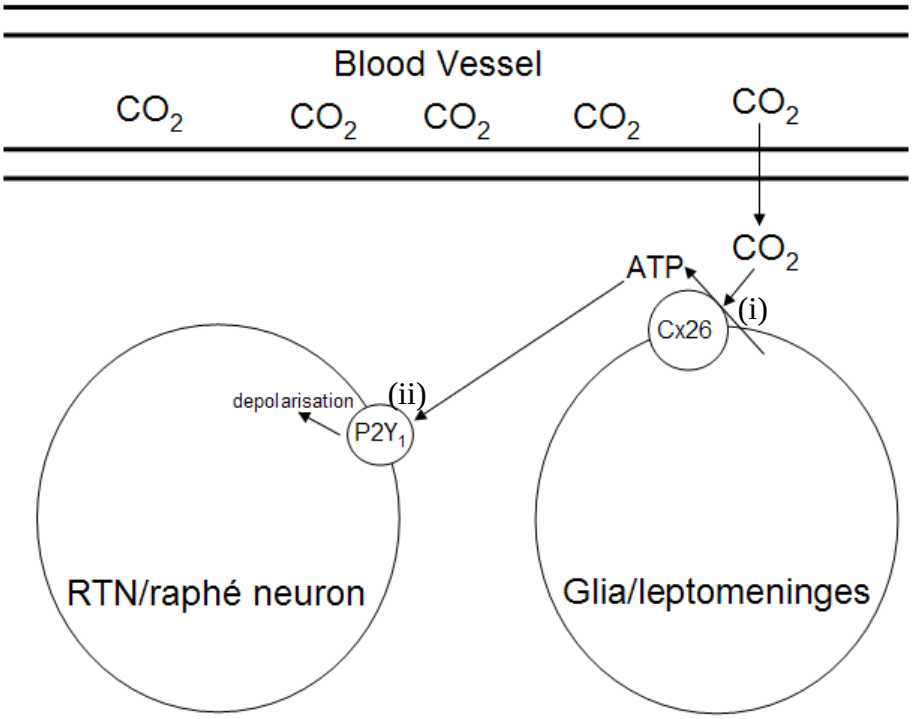

Figure 7: The effect of CO_2 entering the ECF of the superficial medulla on the Cx26 positive cells that reside there. As Cx26 positive cells are closer to the surface and the mechanism of ATP release occurs extracellularly, they will respond first.

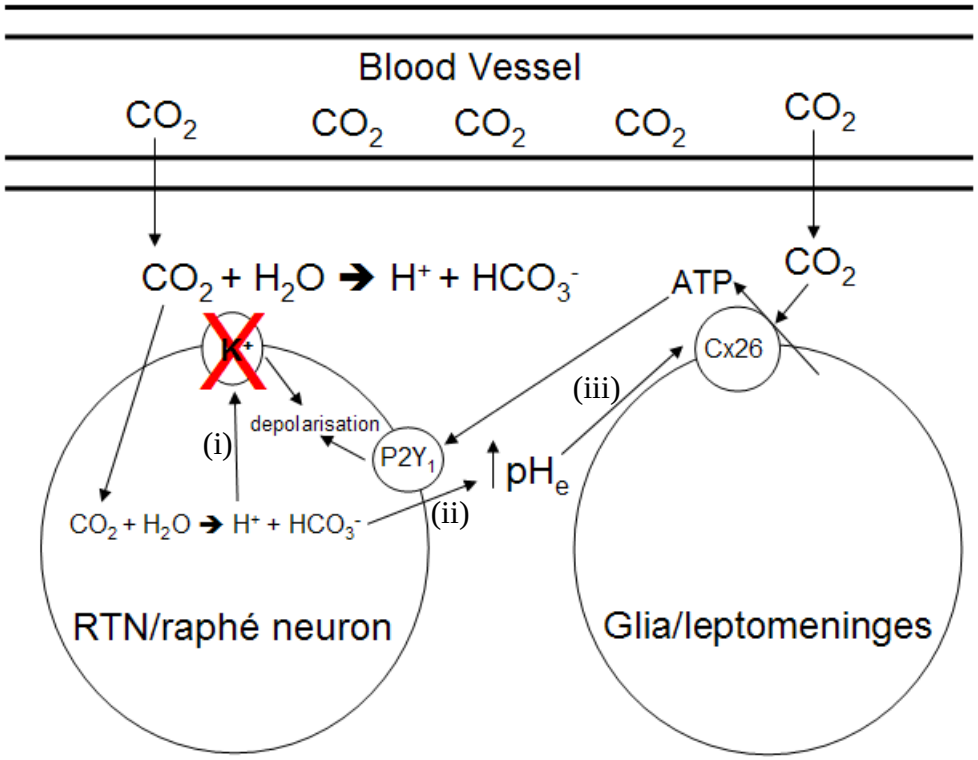

Figure 8: The effect of CO_2 entering the ECF of the superficial medulla on neurons of the medulla and the subsequent amplification of ATP release through Cx26 hemichannels.

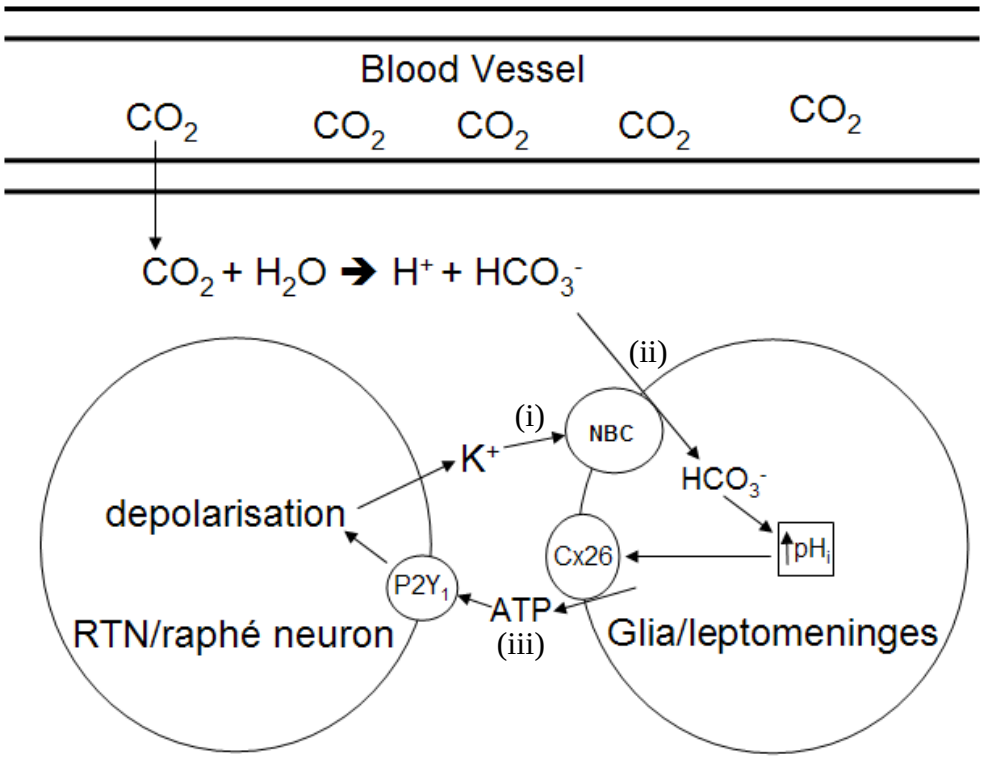

Figure 9: Feedback loop created from the neuronal depolarisation causing the reversal of NBC transporters on glia.

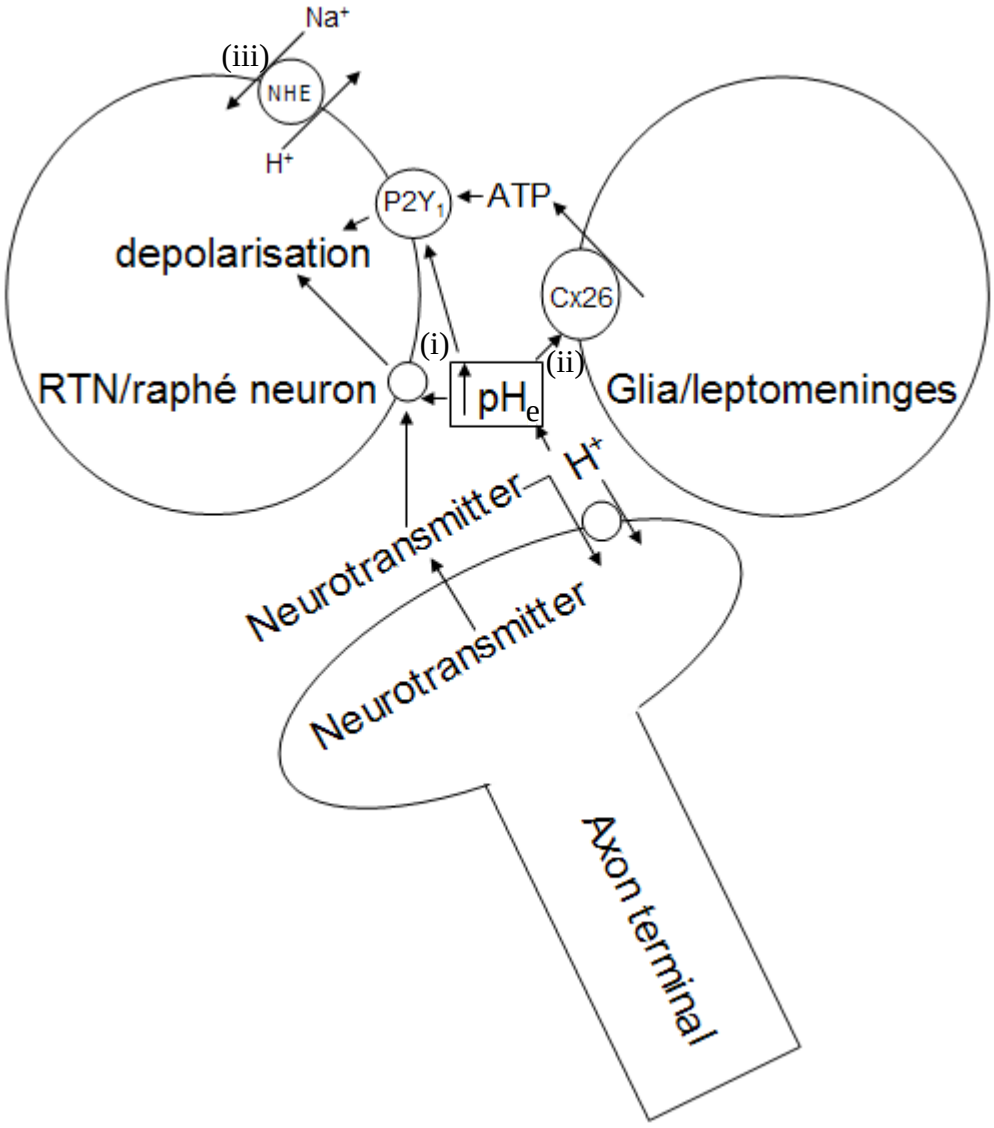

Figure 10: Increased transmission caused by alteration in extracellular pH, caused by the release of neurotransmission.

In terms of glia this would account for: the localisation of NBC transporters on glial cells, which stand no chance of altering pH_e in the face of neuronal firing; the need for glial cells to depolarise in response to hypercapnia, by the exact amount required to reverse this transporter; the need for glial to increase their pH_i during hypercapnia. In conjunction it also explains why neurons continue to fire during hypercapnia once pH_i returns to control levels; once the system begins, it is the release of ATP from glia that propagates the system and not the pH_i of the neurons.

During metabolic or hypercapnic acidosis, even in the face of increased PCO_2 ATP signalling through Cx26 will not occur, due to the effects of extracellular

acidification on the channel. In this situation pH_e and/or concomitant changes in pH_i will provide the driving force for neuronal activation, with no ATP driving the system, were pH_i to recover the neuron would cease to fire. Therefore the system continues until pH levels return to normal upon which pH_i will recover and the system will cease.

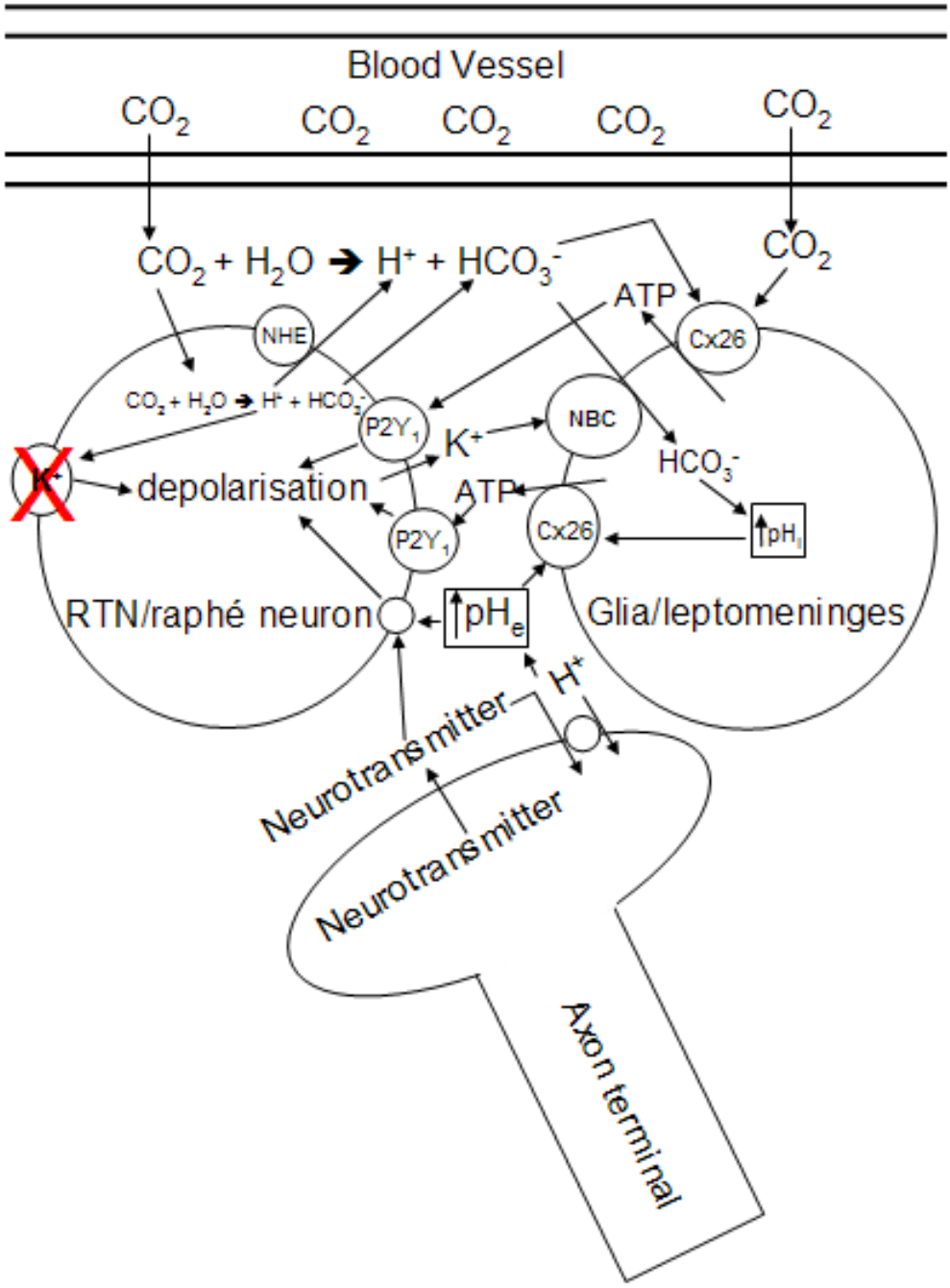

Figure 11: Overview of chemosensory transduction at the VLM surface

This explains the need for pH regulation during hypercapnic acidosis and isohydric hypercapnia. Using the pH regulatory mechanisms which occur in these cells combined with how pH_e and pH_i act on different chemosensory transducers the system becomes flexible and adaptable to different physiological requirements. In some instances the system works in tandem with each other and in others independently, thus creating a spectrum of responses from the same neuronal pathways.

5.13 Evidence for Cx26 mediated ATP release as a CO_2 sensor for the entire brain

Cx26 expression is seen in: radial glia of mice (Bittman and LoTurco, 1999); sub-ependymal and sub-pial astrocytes (Mercier and Hatton, 2001); the meninges (Filippov et al., 2003); blood vessels; and ATP receptor binding is seen in the NTS, caudal VLM, RVLM, inferior olive, the XII nucleus, lateral reticular nucleus, gracile nucleus and cuneate nucleus in the medulla alone (Fong et al., 2002). I predict that this mechanism must be working elsewhere in the brain. To begin with I identified the systemic responses to hypercapnia. During hypercapnia there is an increase in breathing rate, blood pressure, arousal/vigilance and anxiety/fear and a decrease in heart rate and micturition. I therefore identified regions of the brain that are responsible for causing these effects and then looked for evidence that they were: activated by CO_2, contain purinergic receptors/ are responsive to ATP and are located in close vicinity to Cx26. From this I have identified areas where this mechanism may be occurring. I will begin by describing targets of ATP at the VLM surface; I will extend this to the medulla, then to the brain and finally possible targets in other physiological systems.

5.13.1 Serotonergic raphé neurons

Serotonergic neurons of the medullary raphé, have cell bodies and dendrites that reside within 150µm of the VLM surface (Ribas-Salgueiro et al., 2005, Richerson, 2004), directly against Cx26 positive cells of the medullary surface. Cx26 is also on penetrating vessels of the medulla particularly at the midline, a pattern shown by radial glia (Mori et al., 1990). The processes of these radial glia terminate at the somata of raphé neurons in rabbits and cats (Mori et al., 1990). Other studies have also shown that neurons expressing serotonergic markers are tightly apposed to large arteries of the VLM, both surface and penetrating (Bradley et al., 2002, Severson et al., 2003), and that only glial end feet separate these neurons from large blood vessels

(Bradley et al., 2002). The close association between these neurons and Cx26 positive cells implies that this relationship may be advantageous. Raphé neurons are important in both the drive to breathe and adaptations to hypercapnia (Richerson, 2004, Ribas-Salgueiro et al., 2005, Hodges et al., 2008, Bodineau et al., 2004, Li and Nattie, 2008b). These neurons are only mildly responsive in neonates whereas they are highly sensitive to hypercapnia in adults where ATP release is observed (Wang and Richerson, 1999). Interestingly this pattern of chemosensitivity closely matches the expression of Cx26 protein in astrocytes (Solomon et al., 2001). As these neurons have been shown to be responsive to ATP (Cao and Song, 2007), it would be surprising if the development of their chemosensitivity was not due the development of CO_2-activated ATP release. In conjunction the multiple functions performed by other raphé neurons outside of the medulla, such as increased arousal, means that this mechanism acting through serotonergic neurons could be far reaching. Thus I propose that in the ventral medullary raphé neurons are the primary target for ATP release.

5.13.2 The pre-Bötzinger complex

The pre-Bötzinger complex sends projections to within 50µm of the MGL (Kawai et al 1996) and have cell bodies within 150-400µm of the ventral surface (Kuwana et al., 2006). Since the pre-Bötzinger complex is the site of respiratory rhythm generation any effect here will directly alter breathing (Smith et al., 1991). This area contains purinergic receptors and is modulated by ATP (Lorier et al., 2004, Lorier et al., 2007, Thomas et al., 2001). There is also evidence that the tonic release of ATP provides continual excitatory drive to this nucleus (Thomas et al., 1999, Thomas and Spyer, 2000, Gourine et al., 2003, Spyer et al., 2003). The proposed mechanism of action for ATP in this brainstem region is shown in Chapter 1 and will not be discussed here any further.

Interestingly the CO_2-activation of hemichannels may play a subsidiary role in the pre-Bötzinger complex; in this case one that does not involve Cx26. The sensitivity of Cx32 to CO_2 and its location in the pre-Bötzinger complex may not be coincidental. Though not commented on at the time, it has already been seen to show that the conductance of Cx32 in the presence of CO_2 increases before it decreases in the presence of high CO_2 (Young and Peracchia, 2004); these increases could coincide with low concentrations of CO_2 before the solution fully equilibrates. When PCO_2 in the vicinity of this nucleus increases it may increase the functional conductance

between cells connected by Cx32 gap junctions. This would serve to increase electrical coupling and thus would increase the synchronicity of the firing patterns in this area. Thus during hypercapnia it should serve to increase respiratory rate.

5.13.4 The nucleus tractus solitarus

The nucleus tractus solitarus (NTS) is a known site of chemosensitivity and acts as the relay for the peripheral nervous system (Nattie and Li, 2002a) and receives input from the pre-Bötzinger complex (Kalia et al., 1979). It is situated superficially in the dorsal medulla (Okada et al., 2001) and surrounds the 4th ventricle, an area where we have seen staining Cx26 expression in mice expressing LacZ reporter allele (data not shown). Breathing either 21% or 10-15% CO_2 for 1 hour increases c-Fos immunoreactivity in this region (Sato et al., 1992, Belegu et al., 1999). Unilateral lesions of this region in humans causes reduced responses to hypercapnia (Morrel et al., 1999, Morrel et al., 2001). During wakefulness they have normal breathing (Morrel et al., 2001); however during sleep they display irregular breathing (Morrel et al., 1999, Morrel et al., 2001). This is probably due to the reliance of the response on peripheral chemoreception during this arousal state. Conversely focal perfusion of 25% of CO_2 into the NTS in rats during wakefulness and sleep, increases respiration during wakefulness and NREM sleep but not REM sleep.

The NTS contains many sub-nuclei which project to different regions of the brainstem (Loewy and Burton, 1978). Anatomical tracer studies show that the NTS projects to the lateral reticular nucleus (which contains the respiratory centres) (Otake et al., 1992, Ruggiero et al., 2000) and the dorsal raphé (Ruggiero et al., 2000), the raphé pallidus and obscurus (Zec and Kinney, 2003, Loewy and Burton, 1978), dorsal motor vagal complex (Loewy and Burton, 1978, Otake et al., 1992, Zec and Kinney, 2003), the retrotrapezoid nucleus (Otake et al., 1992), the nucleus ambiguus (Zec and Kinney, 2003, Loewy and Burton, 1978), the retrofacial nucleus (in an area that contains the pFRG) (Loewy and Burton, 1978), the A5 region of the pons, (Otake et al., 1992) and the parabrachial nucleus (Loewy and Burton, 1978), the pressor regions of the RVLM (Zec and Kinney, 2003, Loewy and Burton, 1978) and to spinal motor neurons (Loewy and Burton, 1978). Thus it is poised to influence not only respiration and chemosensitivity, but also cardiovascular control.

The NTS expresses all known P2X receptors (Yao et al., 2000) and responds to ATP. ATP is released rhythmically in the NTS from pulmonary stretch receptor

afferents during lung inflation and is phased locked with tracheal pressure (Gourine et al., 2008). Here it excites pump cells and its actions are mediated through co-transmission with glutamate, in an additive manner (Gourine et al., 2008). It has been postulated that glutamate release triggers ATP release which feeds forward to amplify glutamate transmission (Braga et al., 2008). In conscious animals ATP and glutamate antagonism is required to block both pressor and bradycardiac but not tachypnoeic responses to peripheral stimulation with KCN (Braga et al., 2007), which has been shown to stimulate all unmyelinated axons in the carotid body and not just hypoxia responsive cells (Alcayaga et al., 1999). Injections of ATP into the commissural NTS evoke bradycardia and tachypnoea, although with hypotension (Antunes et al., 2005, Braga et al., 2008). In the intermediate NTS ATP injections reduce phrenic nerve activity (PNA) and heart rate (HR) (Antunes et al., 2005). In conjunction the NTS is under the influence of adenosine caused by the hydrolysis of extracellular ATP release (Spyer et al., 1997). At low doses, adenosine injections cause an increase in blood pressure and a bradycardia (De Paul and Machado, 2001). Therefore ATP release is likely to affect the respiratory and cardiovascular response to hypercapnia, through the actions of ATP and adenosine (figure 12), either directly or through the enhancement of glutamatergic transmission.

The NTS changes postnatally and action potentials in the NTS are highest in the NTS between the ages of P11-14 (Vincent and Tell, 1997). Interestingly the same time as Cx26 expression appears, as P2X receptors can desensitise to ATP, it may be that after a few days from the onset of tonic ATP release the NTS lowers its sensitivity. Cobalt an inhibitor of Cx26 hemichannels stops spiking activity in adult rats (Vincent and Tell, 1997), though this may be through its actions on calcium channels. Injections of carbenoxolone into the NTS reduce basal respiration and adaptive changes in breathing associated with hypercapnia (Parisian et al., 2004). Interestingly a cocktail of antagonists that did not include purinergic antagonists reduced ventilation, which returned upon wash out (Parisian et al., 2004), however synaptic block with cobalt, which would also inhibit Cx26, affected respiration for days after the injection (Parisian et al., 2004). Intracisternal perfusion of low calcium or high magnesium solutions that would effect only the surface of the dorsal and ventral medulla reduced basal respiration and the hypercapnic ventilatory response (Berkenbosch et al., 1976, Berkenbosch and Adan, 1974), though this may be due to the effects on the VLM alone. Thus in the NTS there is at least circumstantial

evidence that hemichannel mediated ATP release may play a role. Fascinatingly, ATP release was not observed from the dorsal surface of the medulla (Gourine et al., 2005a), in this preparation the dorsal surface was accessed through the removal of the cerebellum; which would disrupt the pia mater in this region.

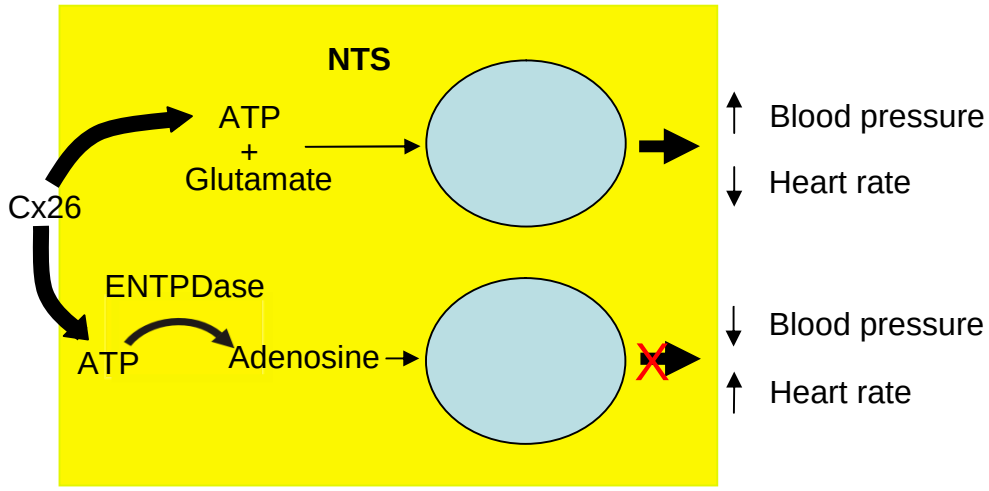

Figure 12: The actions of ATP and adenosine in the NTS on cardiovascular regulation.

5.13.5 The C1 region of the rostral ventrolateral medulla (RVLM)

The RVLM is an important nucleus in blood pressure regulation and can do so in response to hypercapnia (Moreira et al., 2006). It was first hypothesised that the RTN was the source of drive to these neurons (Moreira et al., 2006). However RTN neurons are glutamatergic and glutamate is not responsible for this excitation (Moreira et al., 2006). Thus it was proposed that the C1 region of the RVLM was either intrinsically chemosensitive or that the signal comes from another chemosensitive site that use alternative transmitters (Moreira et al., 2006). This may be from the input from the PVN (paraventricular nucleus; see below) or through Cx26 mediated ATP release. The pressor regions are located close to the surface near the sites I have demonstrated to release ATP. The pressor response areas of the RVLM express both P2X & Y (Ralevic et al., 1999), as well as adenosine receptors (Spyer et al., 1997). Applications of ATP into the VLM in regions known to influence blood pressure and increases the pressor response associated with hypercapnia (Thomas et al., 1999). Injections of ATP into this region in the absence of hypercapnia cause pressor responses, and surprisingly a decrease in respiration (Thomas et al., 2001). Interestingly the RVLM receives tonic ATP input, which I suggest is from Cx26 mediated release as the descending input from the PVN are not tonically active (Spyer

et al., 1997). Interestingly P2 receptors could modulate directly and independently the respiratory and cardiovascular pathways via actions in the VLM (Spyer and Thomas, 2000). The RVLM is also sensitive to adenosine, which is produced by hydrolysis of ATP and adenosine antagonists can influence the pressor response elicited by hypothalamic stimulation (Spyer et al., 1997) .

5.13.6 The locus coeruleus (LC)

The LC in the pons supplies tonic chemosensory input (Infante et al., 2003). It is located at the ponto-medulla border directly underneath the 4th ventricle, placing it in direct contact with Cx26 expressing cells. Stimulation of the LC causes dopamine and noradrenaline release in the cortex (Devoto et al., 2005) which has been linked to arousal and increased vigilance (Devoto et al., 2005). It has also been shown to send projections to the medial habenula (Yao-Hua, and Ku., 2002), and thus has been linked to fear and anxiety (Li and Ku, 2002, Gorman et al., 2000, Devoto et al., 2005). At rest the LC exhibits oscillatory behaviour (Oyamada et al., 1998, Filosa et al., 2002), due to phase locking with respiration through glutamatergic and adrenergic projections (Oyamada et al., 1998). Upon hypercapnic stimulation LC neurons lose respiratory related phasic behaviour (Oyamada et al., 1998). These neurons are driven by alterations in pH_i and retain chemosensitivity during synaptic blockade (Filosa et al., 2002).

Although unlike many of the nuclei mentioned here they do not show maturational changes (Filosa et al., 2002), they do express P2X and P2Y (Yao et al., 2000, Nieber et al., 1997, Sansum et al., 1998) receptors which co-localise with catecholaminergic neurons (Yao et al., 2000). Thus ATP is likely to stimulate these neurons leading to the release of noradrenaline and/or dopamine elsewhere in the brain. ATP and ADP causes depolarisation and purinergic antagonists further reduce LC currents already bathed in a cocktail of synaptic blockers (Nieber et al., 1997). Interestingly the minimum amount of ATP release required to stimulate these neurons is 3µM (Nieber et al., 1997) which is the same amount seen in *in vivo* preparations (Gourine et al., 2005a). Suramin depresses LC neurons in the absence of agonists implying tonic regulation through ATP (Nieber et al., 1997). Interestingly the profile of CO_2 sensitivity of these neurons (figure 13) in the rat is similar in profile to that of ATP release or conductance changes in Cx26 in response to CO_2 (see chapter 2 and 3)

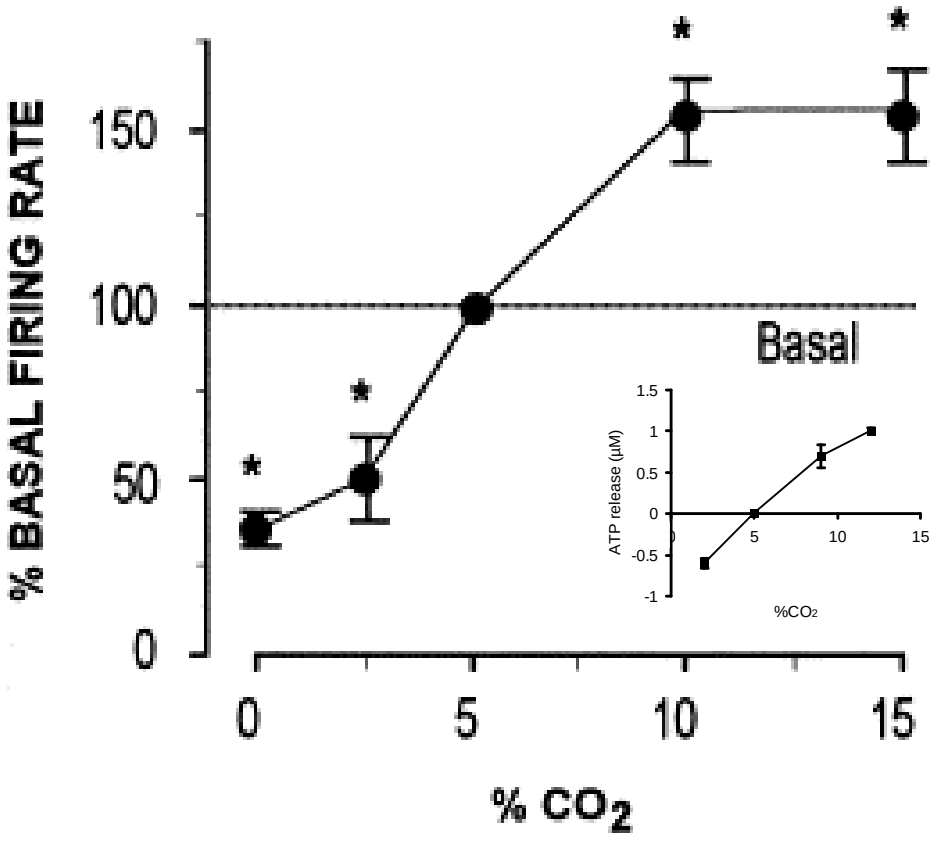

Figure 13: CO_2 dose response curve for neurons in the locus coeruleus. Inset) ATP release in response to alterations of $\%CO_2$ through Cx26 hemichannels at the VLM surface. Note the similarity of the profile to that of ATP release and Cx26 conductance in response to alterations to CO_2 seen in chapter 1. Picture taken from Pineda *Neuroscience* 77, 723-743 (1997)

5.13.7 ATP and hypothalamus

The hypothalamic paraventricular nucleus (PVN) is thought to be the master controller of blood pressure regulation. Stimulation of the PVN excites the pressor areas of the rostral ventrolateral (RVLM) pressor areas via the release of glutamate (Coote et al., 1998), leading to an increase in blood pressure. The PVN also projects to the phrenic nucleus and the spinal cord (Yeh et al., 1997). Neurons projecting to the phrenic nucleus express c-Fos during hypercapnia and thus are activated during it (Kc et al., 2002, Berqin et al., 2000). Injections of L-glutamate into the PVN also cause an increase in breathing frequency and amplitude and increased blood pressure (Yeh et al., 1997). Thus the PVN is poised to influence blood pressure and respiration through intermediate nuclei and directly through motor neurons. The PVN is excited by ATP and has been shown to contain a heterogeneous collection of P2X receptors (Whitlock et al., 2001). It is located next to the 3rd ventricle (Cham et al., 2005). Interestingly the ependymal layers of the 3rd ventricle express Cx26 (Mercier and Hatton, 2001).

In the adult cat hypercapnia reduces spontaneous bladder contractions (Barlett Jr and Knuth, 2003). ATP injections into the PVN and supraoptic nuclei (SON) also reduces urine outflow (Mori et al., 1992). It does so by the release of arginine vasopressin (AVP) (Mori et al., 1992, Mori et al., 1994), which act on V2 receptors (Nakamura et al., 2003). c-Fos studies show that AVP secreting cells in the SON and PVN are excited by hypercapnia (Kc et al., 2002). Thus during hypercapnia the PVN and SON release AVP into the blood in response to ATP and causes antidiuresis via V2 receptors (Mori et al., 1992, Mori et al., 1994). The antagonism of AVP during eucapnia had no effect on bladder contraction, only when ATP was injected into the SON (Mori et al., 1994). Thus there must be a release of ATP in the SON, most probably during hypercapnia. Injections of ATP did not effect blood pressure or phrenic nerve discharge (Mori et al., 1994), however it was injected in nanomolar concentrations and may not have been high enough to stimulate neurons of these pathways.

Figure 14: Cx26 is located near hypothalamic nuclei that influence physiological responses to hypercapnia. Coronal sections of the midbrain showing the location of; A) The tuberomamillary nucleus. B) Cx26 expression Mercier and Hatton *J Comp Neuro* 431, 88-104 (2001). C) The PVN (blue) and Son (green).

Thus the PVN and SON contain purinergic receptors and are excited by ATP and hypercapnia and stimulation of this area causes responses associated with the systemic response to hypercapnia. Therefore the hypothalamic PVN and SON are possible sites where this mechanism is working.

5.18.8 ATP and tuberomamillary nucleus

The hypothalamic tuberomamillary nucleus (TMN) is located in the posterior hypothalamus near the ventral surface and it is involved in alertness / vigilance and arousal from sleep. Within the TMN there is an excitatory tone of ATP, which acts through P2Y 1 and 4 (Sergeeva et al., 2006). Applications of ATP excite all TMN neurons (Sergeeva et al., 2006), and enhances glutamatergic and GABAergic

transmission from glia through P2X (Sergeeva et al., 2006). This nucleus is excited by hypercapnia and expresses c-Fos after exposure to CO_2 (Johnson et al., 2005). Therefore the involvement of Cx26 hemichannels release in both the anterior and posterior hypothalamus may take place. In fact preliminary studies utilising dye loading and biosensors have implicate that this mechanism may be at work in this brain region.

5.13.9 ATP and habenula

The medial habenula is situated in the thalamus close to the floor of the lateral ventricles and is responsible for increasing fear / anxiety and learned helplessness and depression (Shumakea et al., 2003, Pobbe and Zangrossi Jr, 2008). It is located in an area where ependymal cells are stained for Cx26 (Mercier and Hatton, 2001). We know that ATP acts as a fast neurotransmitter (Edwards et al., 1992). However P2Y receptors in this region cause long term facilitation of glutamatergic currents (Price et al., 2003). This facilitation was only seen when concentration of ATP were low (<10μM) and approximately equal to what has been seen in this investigation when the tone and released ATP are added (~7μM). This facilitation is due to the action of P2Y receptors on pre-synaptic terminals. The lateral habenula receives inputs from the LC (Yao-Hua, and Ku., 2002), which is excited during hypercapnia. If these neurons are glutamatergic and if ATP release were to be released via Cx26 hemichannels in this region it may act to cause long term facilitation of neurons originating in the LC and potentiate the response to fear.

5.13.10 ATP and vascular control

Cx26 is expressed heavily in the vascular endothelia, and these cells are responsive to ATP. ATP increases leg blood flow and vascular conductance (Calbert et al., 2006). Injections of ATP into the human leg cause vasodilatation (Calbert et al., 2006), probably through the release of nitrous oxide (Bao et al., 2004). ATP causes dilation of cerebral arteries in low concentrations and constriction in high concentrations (Shuba and Vladimirova, 1980) through its actions on P2X and P2Y receptors (Clifford, 2008, Burnstock et al., 1970). P2X receptor activation causes constriction and P2Y dilation (Clifford, 2008). In the retina this has been linked to the release of ATP from glia cells (Metea and Newman, 2006). Calcium waves in the glia cells surrounding blood vessels, always preceded dilation of blood vessels (Metea and Newman, 2006). Release of caged intracellular calcium in glia, to mimic endogenous

calcium waves also caused vaso- constriction and dilation (Metea and Newman, 2006). These vasomotor responses are inhibited by the antagonism of ATP (Metea and Newman, 2006).

Thus an increase in CO_2 in the blood vessels will activate Cx26 hemichannels, either on glia cells or from the endothelial cells themselves. This will lead to ATP release which acts on the endothelial cells causing vasodilatation. During hypercapnia this will lead to increased blood supply in the ventral medulla, which would increase CO_2 to the medulla, a distinctively favourable physiological reflex. However this may not be its only application. During increased neuronal activity the metabolic production of CO_2 increases, this would act to open Cx26 hemichannels and lead to ATP release in the local vicinity. This would cause vasodilatation of the blood vessels supplying this region, increasing delivery of oxygen to this region. If an area of the brain becomes inactive, metabolic CO_2 decreases leading to a reduction in tonic ATP levels. Thus the blood vessels in this region would constrict. Therefore CO_2-activated ATP release through Cx26 hemichannels would cause regional changes in blood flow that match the metabolic demand of discrete areas of the brain.

5.13.11 The placenta

The possible involvement of ATP release in vascular control may explain why $Cx26^{-/-}$ transgenic mutations are embryonic lethal. This mechanism may also play a vital role *in utero*. Cx26 is heavily expressed in embryos and the placenta of mice. Homozygous deletion of Cx26 is embryonic lethal (Filippov et al., 2003), yet it does not cause any morphological changes of either the placenta or the embryo, though both appeared smaller than their wild type counterparts. Could Cx26 mediated ATP release provide the answer. In a developing embryo the metabolic demands are high, thus during periods growth or any enhanced metabolic activity an increase in PCO_2 will occur. This would raise the level of PCO_2 in the placenta. This increased PCO_2 will open Cx26 hemichannels that reside there, causing ATP release. This will act directly on foetal vasculature and may build up in the intravillous space where it could diffuse to the maternal blood supply. This could lead to vasodilatation in the placenta and thus an increase in blood flow on both the maternal and foetal sides. This increase in blood flow serves two purposes; firstly it would increase the supply of oxygen and essential nutrients to the foetus, essential for its continued growth. Secondly it will cause the removal of any waste products that may be toxic to the foetus. In mice

lacking Cx26, this will not occur; this would lead to anoxia (due to increased foetal respiration and lack of O_2 delivery) and a build up of toxic waste products in the foetus, eventually leading to death. In heterozygous mice with a decreased but not absent Cx26 mediated ATP control of vasculature, this would occur at a slower rate and would lead to a decreased birth weight. Alternatively, it may be the opening of Cx26 hemichannels alone without any involvement from ATP. Thus CO_2 opens the hemichannel allowing the passage of glucose from the intravillous space into the foetal blood supply. Fascinatingly foetal mice with homozygous deletions of Cx26 but not heterozygous or wild type foetus from the same litter, showed a 60% decrease in glucose up take from maternal blood. This was originally suggested to mean that Cx26 aids in the uptake of glucose directly. I suggest that it is due to a lack of delivery of glucose to / in the placenta, which is the cause of decreased uptake. This may or may not work in conjunction with a loss of Cx26 mediated glucose uptake. Thus Cx26 mediated ATP release appears to be functionally active in other physiological system as well as the physiological chemosensory reflex.

5.14 Summary

I have created a new *in vitro* preparation for the study of chemosensitivity in the VLM surface, and proposed a new model for recapitulating *in vivo* conditions of hypercapnia more accurately. I have identified a new intrinsically chemosensitive target for chemosensory transduction, Cx26. This is the first record of connexin hemichannel gating in response to a physiological stimulus, and may provide the long sort after true physiological function of hemichannels, such as reduced coupling in the heart or increased transmission in the pre-Bötzinger complex. I have shown a primary role for glia in a sensory reflex, which may also provide a better understanding of acid base regulation in these cells. Finally I have produced a novel mechanism by which the brain may detect changes in CO_2, which may be one of the fastest and simplest cellular pathways ever described.

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