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PhD Thesis

School of Life Sciences

University of Warwick

On the modulation of connexin 26 by CO_2

by

Louise Meigh

April 2014

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

- All patch clamp experiments and analysis were performed by Professor Nicholas Dale.
- Elastic network modelling was performed by Dr. Martin Cann and Dr. Thomas Rodgers of Durham University.
- A subset of experiments in Chapter 4 were performed by Sophie Greenhalgh, an undergraduate student under my supervision.
- A subset of experiments in Chapter 6 were performed by Daniel Cook, an undergraduate student under my supervision

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Summary

The mechanism through which changes in PCO_2 in the blood are detected is much disputed. Although many believe the stimulus for CO_2 detection to be the associated increase in H^+, increasing evidence supports a role for direct CO_2 detection. In a recent development, Huckstepp et al demonstrated that connexin 26 hemichannels open in response to elevated CO_2 in the absence of a pH change. This model however remained incomplete, with the mechanism of CO_2 interaction with the channel being unknown.

In this work I have employed site directed mutagenesis and dye loading studies to identify the CO_2 binding site of connexin 26. This was found to be lysine 125, with binding through the formation of a carbamate bond. Mutational and modelling studies support an intersubunit salt bridge between the subsequent negative charge and the positive arginine 104 as the mechanisms of channel opening.

Using this model for connexin 26 channel opening I was also able to manipulate the channel to respond to novel stimuli. These new mutants act both to support our mechanism for channel opening and to provide tools for further research. The production of connexin 26 channels lacking the key residues of interest also provides the basis of a novel method for producing animal models lacking CO_2-sensitvity for further research in vivo.

The findings of this work confirm that connexin 26 interacts with CO_2 through direct binding. Connexin 26 therefore represents the first established CO_2 sensor in the chemosensitive areas of the brain and strongly supports the idea that CO_2 itself participates in monitoring PCO_2 levels. As this model requires no accessory proteins, this work also offers the intriguing possibility that CO_2-sensitvity may be an important function of this protein in other tissues and supports the idea of connexin proteins as novel ligand gated channels.

Abbreviations

µM- micro mole

µm-micro meter

Å-angstrom

aCSF –artificial cerebrospinal fluid

Ask1-apoptosis signal-regulated kinase 1

ATP-adenosine triphosphate

BSO-L-Buthionine sulphoximine

Ca^{2+}- Calcium ion

$CaCl_2$-calcium chloride

CALHM1-calcium homeostasis modulator 1

CCHS- congenital central hypoventilation syndrome

CNO- clozapine-N-Oxide

CNS- central nervous system

CO_2- carbon dioxide

Cx- connexin

DMEM- Dulbecco's modified eagle medium

DNA- deoxyribonucleic acid

EGTA- ethylene glycol tetraacetic acid

ENM-elastic network modelling

ER- endoplasmic reticulum

ERGIC- ER-Golgi intermediate compartment

FCS- fetal calf serum

GFAP-glial fibrillary protein

GFP- green fluorescent protein

GPCR- G protein coupled receptor

GSH-glutathione

GSSG-glutathione disulphide

H^+- proton

H_2O-water

HCl-hydrochloric acid

HCO_3^- - bicarbonate

K^+ -potassium ion

Kcal- kilocalorie

KCl- potassium chloride

KDa-kilodalton

KO-knock out

L -litre

LC-locus coeruleus

Mg^{2+}-magnesium ion

$MgSO_4$- magnesium sulphate

mmHg- millimetres of mercury

Mol-mole

MTSES-2-sulfonatoethyl methanethiosulfonate sodium salt

mV- millivolts

Na^+-sodium ion

NAC- N-acetyl cysteine

NaCl - sodium chloride

NAD^+-nicotinamide adenine dinucleotide

$NaHCO_3$- sodium bicarbonate

$NaHPO_4$- sodium phosphate

$NaNO_3$- sodium nitrite

nM- nano mole

NO-nitric oxide

NO_2-nitrogen dioxide

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

O_2- oxygen

PCO_2- partial pressure of CO_2

PCR- Polymerase Chain reaction

ROI- region of interest

ROS-reactive oxygen species

RTN-retro trapezoid nucleus

SIDS- sudden infant death syndrome

VMS- ventral surface of the medulla

WT- wild type

Chapter 1 – Introduction

1.1 Introduction to breathing

1.1.1 Elevated CO_2 causes the drive to breathe

The production of ATP from glucose is a defining feature of all animals. This process uses oxygen and therefore maintaining a continuous supply of oxygen is key in maintaining life. This reaction also produces CO_2 as a waste project which needs to be excreted. Acquiring oxygen and removing CO_2 are both achieved by breathing. The importance of oxygen might mean that that lack of oxygen (hypoxia) would be the driving force for breathing. However, in reality a large portion of the drive to breathe is determined by CO_2 levels (Praud *et al.*, 1997). In blood and other bodily fluids CO_2 reacts with H_2O to produce HCO_3^- and H^+ via a reaction catalysed by carbonic anhydrase (Figure 1).

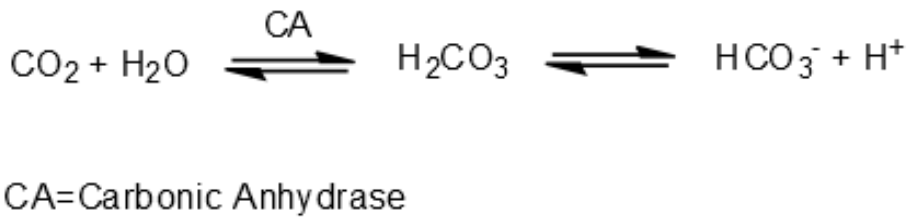

Figure 1 Reaction of CO_2 with water

As a consequence, an increase in the concentration of CO_2 in the blood (PCO_2) produces a corresponding increase in H^+ and therefore a decrease in blood pH. As important biological processes often rely on pH sensitive enzymatic reactions, a relatively small change in pH can have dramatic consequences, with a decrease in only 0.1µM being fatal (Richerson, 2004). Human beings produce approximately

300L of CO_2 a day (Snyder, 2006) and therefore constant monitoring and excretion of CO_2 is required. Increases of systemic PCO_2 are detected by what are known as chemosensitive cells. These are cells which respond directly to changes in CO_2 without the requirement for input or signals from other cells. For these cells to be important they must be able to evoke adaptive physiological changes in response to increases in PCO_2. This occurs largely through making breathing deeper (increasing tidal volume) and/or faster (increasing respiratory frequency). Both of these act to escalate the removal of CO_2 from the body. Chemosensitive cells involved in respiration are largely found in the medulla oblongata, although they can also found peripherally such as in the carotid body (Feldman *et al.*, 2003; Zhang & Nurse, 2004). The medulla oblongata lies in the hind brain and is known to be the centre of many autonomic processes such as control of digestion and heart rate.

1.1.2 Defects in CO_2 chemosensitivity

Defects in these CO_2 chemosensitive cells have been shown to have dramatic consequences. Congenital central hypoventilation syndrome (CCHS), also known as Ondine's curse, encompasses a subset of breathing difficulties in patients which present without damage to the lungs, chest or central nervous system (Mellins *et al.*, 1970). In adult patients with CCHS breathing continues normally during wakefulness but the drive to breathe is lost during sleep leading to suffocation if the person is not artificially ventilated. Other symptoms of this condition include headaches and lethargy but these can vary between suffers. One symptom always present is a decreased blood pH (Mellins *et al.*, 1970). As increases in PCO_2 lead to a drop in blood pH it appears that defects in the CO_2 chemosensitivity are

responsible for this condition. One curious aspect of CCHS is the maintenance of the drive to breath while sufferers are awake. This suggests that while awake the CO_2 chemosensory drive plays a less important role, possibly because activities during waking hours enable the excretion of CO_2 without the need for the chemosensitive cells to be involved. A study by Gozal et al appears to support this hypothesis (Gozal & Simakajornboon, 2000). In this work, the legs of CCHS patients were moved while they slept simulating the effects of normal activity during the day. These individuals showed enhanced ventilation during sleep as evidenced by an increase in respiratory frequency and improved gas exchange (Gozal & Simakajornboon, 2000). This is thought to result from activation of mechanoreceptors in the legs which may be responsible for promoting breathing during exercise (McCloskey & Mitchell, 1972).

The inability to detect CO_2 levels correctly has also been implicated in some cases of sudden infant death syndrome (SIDS). Although SIDS can be used as a generic diagnosis for unexplained death in young children, true cases are characterised by spots of blood on the lungs, thymus and heart (Beckwith, 1988). This damage is thought to be caused by increased negative pressure due to breathing difficulties, specifically a missed inspiration before swallowing which leads to windpipe closure and consequential suffocation (Beckwith, 1988). Incidences of SIDS deaths have been found to correlate to environments likely to be high in CO_2, for example high pollution areas where the pollution leads to condensation of CO_2 around ground level and high temperatures where sweating can cause CO_2 to pool around the sleeping infant (Corbyn, 1993). If the infant is unable to disperse the pooled CO_2

from around its nose and mouth, either by restriction of movement by heavy bedding or by a physiological inability to detect increases in PCO_2, the child will suffocate (Corbyn, 1993). A study of 15 infants being investigated for sleep apnea discovered 4 of these infants lacked a CO_2 chemosensory response (Folgering & Boon, 1986). Of these 4 infants, 2 died of SIDS and one required a ventilator to prevent death during sleep. The 2 deceased infants were found to have damage or bleeding in the medulla, known to be important in central CO_2 sensitivity (Folgering & Boon, 1986). These results indicate that defects in CO_2 response are responsible for at least a subset of SIDS deaths. Also of interest was the fourth child who later developed a functioning CO_2 chemosensitive response (Folgering & Boon, 1986). This suggests multiple CO_2 detection mechanisms are involved in central chemosensitivity, the importance of which may vary during development. Several studies have confirmed this hypothesis, with most animals showing a reduction in CO_2 chemosensitivity in the first week of life with activation and response to CO_2 increasing to adult levels in the third week (Wickstrom *et al.*, 2002; Dwinell *et al.*, 2011; Holley *et al.*, 2012).

1.2 Investigating CO_2 chemosensitivity

1.2.1 pH and CO_2 chemosensitivity

Having demonstrated the importance of the CO_2 chemosensory mechanisms it is now important to investigate how changes in PCO_2 are detected in chemosensitive cells. As shown in Figure 1 an increase in PCO_2 also leads to an increase in H^+ and in HCO_3^-. Cells detecting a change in PCO_2 could therefore be responding to CO_2 as a direct stimulus, an elevation in bicarbonate levels or a decrease in pH. In 1911 Winterstein hypothesised that CO_2 was detected through the same receptor as O_2

(Loeschcke, 1982). For this to be true the receptor must respond to a common stimulus, which he identified as H^+. In 1956 this same author conceded that oxygen and CO_2 were detected by different systems (Loeschcke, 1982). However the idea that H^+ must act as the sole stimulus has remained and is still considered by many to be the key to CO_2 chemosensitivity.

There are many examples of data supporting H^+ as the main, or in some cases only, stimulus of CO_2 sensitivity (Jarolimek *et al.*, 1990; Solomon *et al.*, 2000; Wang *et al.*, 2002). For example, Loeschcke et al demonstrated that perfusion of constant CO_2 with decreasing pH caused an increase in ventilation in the anaesthetized cat (Loeschcke, 1982). Increases in PCO_2 in the absence of a change in pH was shown to have no effect (Loeschcke, 1982). Despite the author's criticisms of other work which did not measure stimulus directly at the chemosensitive cells, measurements in this experiment were taken from the cerebrospinal fluid (Loeschcke, 1982) which has been shown to not represent the pH of the extracellular fluid (Eldridge *et al.*, 1985). The author also admits that due to the carbonic anhydrase reaction, alterations in either CO_2 or pH can affect the other, especially when applied some distance from the chemosensory receptor (Loeschcke, 1982). H^+ has been demonstrated to act as a stimulus to increased PCO_2 in various regions of the medulla, suggesting that it has a role in chemosensitivity (Fukuda & Honda, 1976; Wellner-Kienitz *et al.*, 1998; Putnam *et al.*, 2004). The mechanism through which this can occur is unknown but a large number of the candidate receptors are K^+ channels (Wu *et al.*, 2004; Yamamoto *et al.*, 2008; Gestreau *et al.*, 2010). In this model decreases in intracellular pH lead to closure of K^+ channels which results in

membrane depolarisation and action potential firing with consequent release of neurotransmitters (Filosa *et al.*, 2002). Alteration of internal pH may involve acid transporters such as the Na^+/H^+ exchanger (Wiemann *et al.*, 1999).

1.2.2 CO_2 as a direct stimulus

As early as 1936, a role for direct sensing of CO_2 in chemosensitivity was being identified (Loeschcke, 1982). These data, along with Gray's multiple factor theory which suggests that all three stimulants (H^+, CO_2 and HCO_3^-) may cause the drive to breathe, were however largely dismissed (Gray, 1946). This was due to the criticism that their work did not measure the stimuli at the site of the chemoreceptor and therefore that their results could be explained by an undetected local pH change (Loeschcke, 1982; Eldridge *et al.*, 1985). Other studies were similarly discounted. For example, Borison et al demonstrated that infusion of acid produces a much smaller change in ventilation than respiratory acidosis (a pH change caused by CO_2) (Borison *et al.*, 1977). This work however applied the acid intravenously and therefore the amount of acid reaching the central chemoreceptors was not known.

To aim to rectify this Eldridge et al compared phrenic nerve activity in paralysed cats as a result of either respiratory acidosis (arising from CO_2) or metabolic acidosis (application of HCl) while measuring the pH of the extracellular fluid (Eldridge *et al.*, 1985). Eldridge demonstrated that application of acid alone results in phrenic nerve responses half that of those produced from application of CO_2. Again this supports an independent role for CO_2 in chemosensitivity with it being responsible for 50% of the respiratory effect (Eldridge *et al.*, 1985). This same result was also obtained by Teppema et al (Teppema *et al.*, 1983).

Work by Schuitmaker aimed to quantify the effects of PCO_2 and H^+ in ventilatory responses, as well as the relative importance of central and periphery chemoreceptors (Schuitmaker *et al.*, 1987). This was achieved by comparing the effects of respiratory and metabolic acidosis at both of these sites. The carotid body (periphery chemoreceptor) was shown to contribute approximately 20% of the ventilation response, with pH as the main stimulus (Schuitmaker *et al.*, 1987). In contrast the central chemoreceptors were responsible for the majority of the PCO_2 chemosensory response (80%) with only approximately 30% of this being accounted for by changes in H^+ (Schuitmaker *et al.*, 1987). This suggests that 70% of the central chemosensory response may be due to changes in CO_2 directly.

To further dissect the role of CO_2 in chemosensitivity, H. Shams investigated the deferential effects of pH and CO_2 in the anaesthetized cat (Shams, 1985). By measuring pH at the surface of the medulla in response to inhaled CO_2, he was able to infuse sufficient HCO_3^- into the blood to prevent a change in pH at the surface of the medulla. By monitoring respiration in these animals he was able to see an increase in respiratory frequency and tidal volume in response to PCO_2 at a constant pH (Shams, 1985)(Figure 2). Although significant these changes in respiration were not as large as those evoked by PCO_2 when pH was allowed to vary (Shams, 1985). These results suggest pH and CO_2 can both act as a stimulus for elevated PCO_2 and that they act cumulatively to evoke a larger respiratory response to hypercapnia.

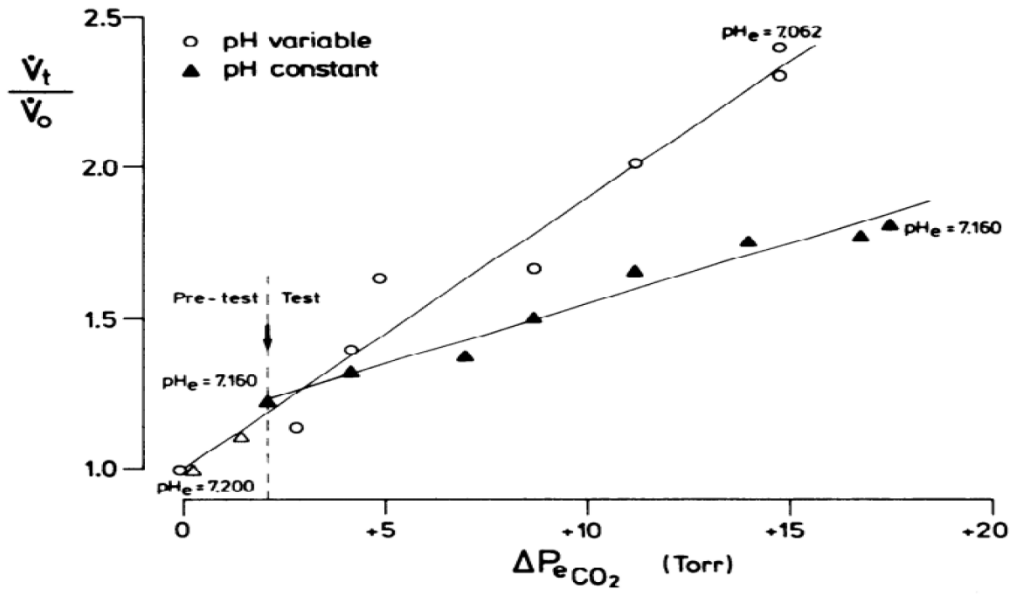

Figure 2 pH and CO_2 contribute to central chemosensitivity Reproduced from (Shams, 1985). In anesthetized cats increases in PCO_2 at constant pH evoke an increase in respiration. This implies both H^+ and CO_2 are stimuli from PCO_2 chemosensitivity. Y axis is a measure of respiration while the X axis shows increasing PCO_2 concentrations.

A direct role for CO_2 was also identified by Harada et al, this time in an in vitro preparation consisting of the brain stem, spinal cord and phrenic nerve (Harada *et al.*, 1985). In the intact animal the phrenic nerve signals to the diaphragm and so activity in this nerve can be used as a proxy of breathing. Decreases in pH, in the absence of a change in PCO_2, were shown to increase both respiratory frequency and tidal volume. In contrast, elevated PCO_2 at constant pH lead to an increase in tidal volume only (Harada *et al.*, 1985). This same distinction between the effects of CO_2 and H^+ was documented by other authors (Gray, 1946). The response to CO_2 was also shown to be transient in nature, with prolonged CO_2 administration leading to acclimatisation. The pH response did not show this behaviour (Harada *et al.*, 1985). This data demonstrates pH and CO_2 represent two independent stimuli with different effects on the respiratory system.

1.2.3 The problem of intracellular pH

Despite these findings, the scientific community remains largely unconvinced of the possibility of CO_2 as a direct signalling molecule. While previous opinion was that a change in extracellular pH resulted in the chemosensitive response, a shift in opinion has now occurred which highlights the importance of internal pH (Ritucci *et al.*, 2005). The ability of CO_2 to pass through membranes and, through the actions of carbonic anhydrase, decrease intracellular pH was demonstrated by Jacobs in 1920 (Boron, 2010). As a result responses to CO_2 could be viewed as reflecting the sensing of intracellular pH changes rather than as a direct effect of CO_2. This idea appeared to be supported by work in snail neurons which demonstrated that in these cells increases in PCO_2 do lead to a decrease in internal pH (Thomas, 1976). Hareda et al addressed this situation in their 1985 paper where they acknowledged the transient nature of the CO_2 response mimicked the transient nature intracellular pH response (Thomas, 1976; Harada *et al.*, 1985). However, as the respiratory consequences of CO_2 and pH were distinct (effecting tidal volume only vs. tidal volume and respiratory frequency), they supported them as two separate stimuli. As intracellular pH was not measured in the examples above, the authors can do little to dispel this assumption. However, the statement that CO_2 can affect intracellular pH in all cells may in itself be flawed. This is based on Overtons rule, which states that gases can freely diffuse across membranes (Boron, 2010). This basic assumption may however be untrue as at least one group of membranes has demonstrated no permeability to CO_2 (Boron, 2010). This property may depend on the number of membrane associated proteins which both reduce the surface area

of available lipid to diffuse through and may interact with the gases non-specifically reducing their permeability (Boron, 2010). Recent studies have taken this further indicating that membranes with a no specialised gas channels show very little CO_2 permeability, as do membranes which contain a normal amount of cholesterol (Itel *et al.*, 2012). This suggests CO_2 could only freely diffuse across membranes if there were suitable channels present or if the cholesterol level was unusually low, e.g. as in mitochondrial membranes (Itel *et al.*, 2012). Also as the reaction of CO_2 with H_2O requires catalysis by carbonic anhydrase, an internal pH change could only be produced if this enzyme were present within the cells. As such internal pH is unlikely to explain the CO_2 responsiveness of all cells.

Recent work by Huckstepp et al suggests PCO_2 modulation can occur in the absence of a change in intracellular or extracellular pH and independently of bicarbonate (Huckstepp *et al.*, 2010b). In these experiments the CO_2 sensor is thought to be connexin 26 (Huckstepp *et al.*, 2010b; Huckstepp *et al.*, 2010a). This protein forms hemichannels in the membrane which can be gated by a stimulus. With elevated PCO_2, these channels were shown to open allowing the movement of dye into, or ATP out of the cells (Huckstepp *et al.*, 2010b; Huckstepp *et al.*, 2010a). The potential effects of internal pH were assessed by physically altering pH using NH_4Cl and by inhibition of carbonic anhydrase within the cells themselves (Huckstepp *et al.*, 2010b). CO_2-dependent ATP release was unaffected by these manipulations. In addition, CO_2 dependent channel opening was also observed during patch clamp studies, even in isolated patches of both the inside-out or outside-out configuration. In the isolated patches, the intracellular cytosolic contents are lost

and pH on both sides of the membrane can be controlled by the experimenter (Huckstepp *et al.*, 2010a). These results strongly imply that internal pH changes are not always the stimulus for CO_2 chemosensitivity and that in some cells CO_2 acts as its own stimulus. These results represented a great boon for the field of pH independent CO_2 chemosensitivity. Nevertheless key unanswered questions remain, such as how CO_2 interacts with these proteins.

1.3 Gap junction proteins

1.3.1 Pannexin and CALHM1 channels

Gap junctions are pores that form between adjoining cells allowing the transfer of ions, metabolites and signalling molecules. In mammalian biology, gap junctions are formed by connexin or pannexin proteins and are produced by the docking of hemichannels on opposing cells. Pannexin proteins could also be considered a gap junction protein, although the ability of hemichannels of this protein to form gap junctions is disputed (Bruzzone *et al.*, 2003; Locovei *et al.*, 2006). The hemichannels themselves are hexamers of the connexin or pannexin protein. Connexins and pannexins proteins display very similar tertiary structure but have very little sequence similarity (Abascal & Zardoya, 2013) and are actually the result of convergent evolution which may indicate the importance of these channels to life. Pannexin proteins are related to the invertebrate gap junction proteins the innexins which perform the role of connexin proteins in these animals (Phelan & Starich, 2001). Pannexin proteins are known to be important in the CNS, taste buds and carotid body (Bruzzone *et al.*, 2003; Huang *et al.*, 2007; Zhang *et al.*, 2012). Another protein that forms channels similar to gap junctions is Calcium Homeostasis

Modulator 1 (CALHM1). Like connexin or pannexin hemichannels, channels of CALHM1 are hexamers with a large central hole allowing non specific transport (Siebert *et al.*, 2013). Unlike the other proteins, however CALHM1 does not form gap junctions but instead exists as isolated channels (Siebert *et al.*, 2013). CALHM1 channels are known to be involved in sweet, bitter and umami tastes where they are thought to release ATP after voltage dependant channel opening (Taruno *et al.*, 2013).

1.3.2 Introduction to connexin proteins

Of particular interest in this work are the connexin proteins, due to the finding by Huckstepp et al that connexin 26 (Cx26) may be directly modulated by CO_2. The connexins are a family of proteins of over 20 members. The family can be further divided into several sub families based on sequence similarities (Figure 3).

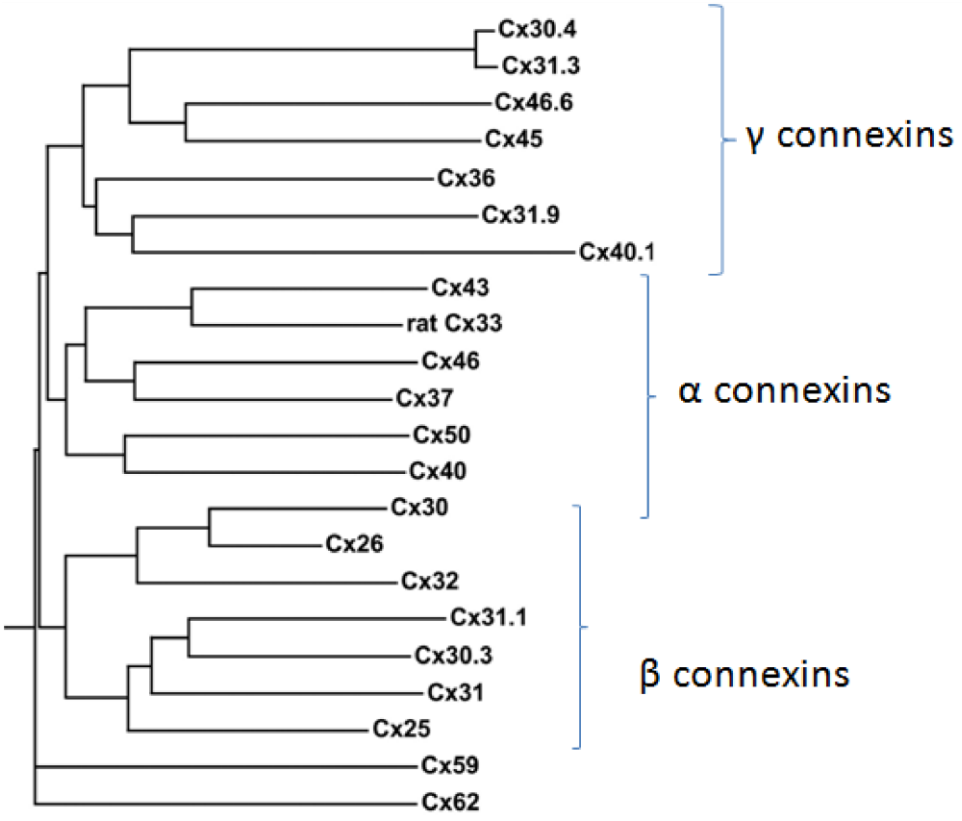

Figure 3. Phylogenetic tree of human connexin proteins Reproduced from (Saez *et al.*, 2003)

As previously stated connexin channels are found in their hexameric form, known as a hemichannel or connexon. Hemichannels in the membrane of opposing cells unite to form a gap junction (Figure 4). These interactions between cells allow the transfer of signalling molecules or metabolites up to 1KDa in size and although there appears to be some preference for positively or negatively charged molecules depending on the channel, size exclusion appears to be the strongest form of channel specificity.

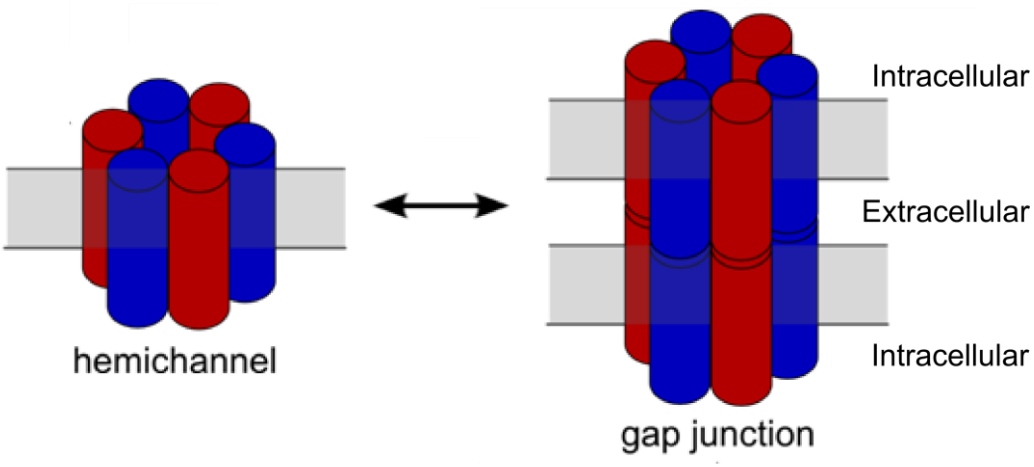

Figure 4 Hemichannel and gap junction structure

1.3.3 Tissue specific connexin expression

Connexin channels are present in all tissues of the body however their expression occurs in a tissue specific manner. The importance of connexin proteins is evidenced by the number of diseases caused by their mutation. For example connexins 26 is expressed in the inner ear and mutations in this protein are responsible for 10-50% of non-syndromic sensorineural hearing loss cases (hearing loss not resulting from problems or deformities in the ear canal) (Petersen & Willems, 2006). These forms of deafness are those that are not associated with any other disease phenotype. Mutations of this gene take two forms: a premature stop codon or a missense mutation (Petersen & Willems, 2006). Premature stop codons lead to a lack of gap junctions and are associated with profound hearing loss (Petersen & Willems, 2006). In contrast, missense mutations produce a less severe form of deafness which may be due to reduced permeability of the gap junctions to signalling molecules such as IP3 (Beltramello *et al.*, 2005). Rather than electrical coupling, gap junctions in the organ of Corti are thought to control potassium

homeostasis (Schulte & Adams, 1989; Wang *et al.*, 2009). Cx26 deafness mutations are also associated with skin disorders as Cx26 is also expressed in the proliferating cell layers. Skin diseases associated with Cx26 manifest themselves as hyper-proliferation of the skin cells (Petersen & Willems, 2006). Connexin 26 has been shown to be unique amongst connexin proteins by slowing the growth of HeLa cells which may implicate that normal Cx26 expression has an anti-cancer role that involves preventing proliferation of cells (Mesnil *et al.*, 1995). If this link is correct we would expect this proliferation control to be lost in the mutants associated with Cx26 skin diseases.

Another important disease linked to defects in connexin proteins is X-linked Charcot-Marie-Tooth disease which encompasses a group of diseases characterised by nerve degradation. Cx32 is expressed in the myelin sheath and appears to form gap junctions between the Schwann cells and the plasma membrane of the axon speeding transmission of metabolites (Zhao *et al.*, 1999). Mutations in Cx32 are linked with this disease and result in either the prevention of gap junction formation or over activation of connexin hemichannels which leads to the loss of precious metabolites (Abrams *et al.*, 2001; Liang *et al.*, 2005).

Connexins 46 and 50 are expressed in the lens, with mutations in these connexins leading to cataracts. Some mutations produce proteins that are unable to form functional gap junctions due to lack of trafficking to the membrane and retention of protein in the ER (DeRosa *et al.*, 2007). Gap Junctions are required for transport of metabolites between cells and as a consequence these mutations disrupt the homeostatic balance of lens cells destroying its clarity. Another mutation of Cx50,

G46V was shown display normal gap junction trafficking but also enhanced hemichannel activity (Minogue *et al.*, 2009). Due to the low Ca^{2+} environment of the lens, cells expression of these mutants display increased hemichannel opening and will die from loss of metabolites.

Connexins are known to be important in innumerable other examples including Cx26 and 32 in the liver (Nicholson *et al.*, 1987), Cx40 in blood vessels and the heart (de Wit *et al.*, 2000) and Cx31 in placenta (Plum *et al.*, 2001). The specific diseases associated with connexin mutations indicate the important roles of different connexin isoforms in the different tissues. The differences in connexin properties are thought the arise at least in part from the differences in length of the C-terminal tail which can affect the tertiary structure and therefore post-translational modification such as phosphorylation (Lampe & Lau, 2004). The tissue specific expression of connexins may also indicate a safety net, where by multiple connexins with similar properties are expressed in the same tissue to compensate for potential defects. It appears from these studies that each connexin serves an independent role and that the assumption that these proteins act purely as passive pores is over simplistic.

1.3.4 Connexin protein structure and gating

All connexin proteins have the same generic structure. This consists of 4 transmembrane domains linked by flexible linker loops. The N- and C-terminus lie on the intracellular side of the membrane (Figure 5). Formation of hemichannel from their subunits is known to occur in the ER or Golgi with assembled connexins being trafficked to the membrane (George *et al.*, 1999). Intracellular stores of

connexons are also present to increase the rate of connexin turnover in the cells (George *et al.*, 1999).

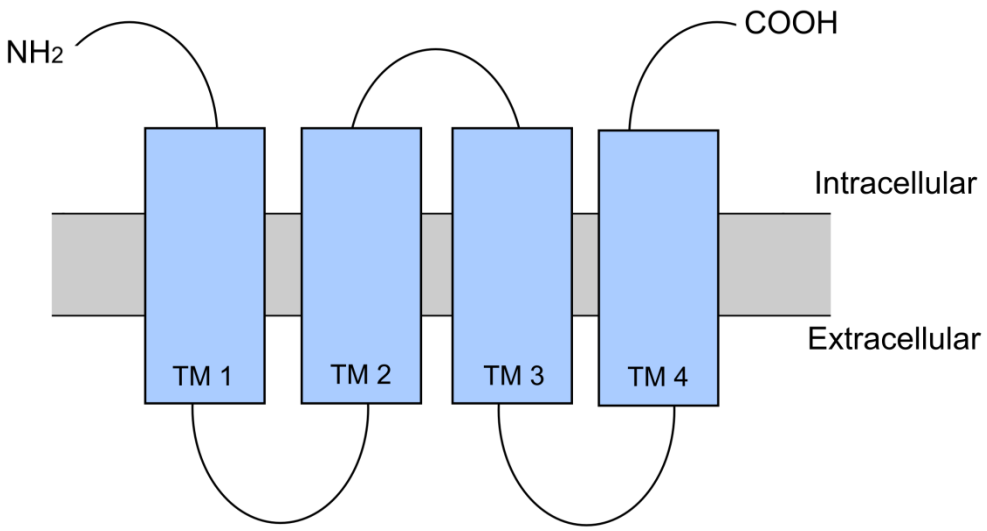

Figure 5 Structure of the connexin protein TM= transmembrane domain

The mechanism of connexin gating is still a matter of conjecture, with three possible mechanisms suggested. One possible mechanism is the rotational model likened to the closing of a camera iris (Muller *et al.*, 2002). In this mechanism rotation of the connexin subunits at the intracellular side acts to reduce the diameter of the connexin pore (Muller *et al.*, 2002). Ca^{2+} dependent closure of Cx26 has been demonstrated to employ this mechanism as shown in AFM studies (Muller *et al.*, 2002).

Connexin channel closure could also occur via physical blockade of the channel, either by a separate molecule or by part of the connexin protein itself. One suggested mechanism for the Ca^{2+}-dependent closure of connexin 32 is the occlusion of the connexin pore by the Ca^{2+} ions themselves, preventing gap junction

signalling and effectively closing the channel (Gomez-Hernandez *et al.*, 2003). Binding of Ca^{2+} was found to occur at a ring of 12 aspartate residues which line the inside the channel (Gomez-Hernandez *et al.*, 2003). Interestingly the mutation of aspartate 178 to a tyrosine is a known mutation of X-linked Charcot-Marie-Tooth disease which leads to a loss of Ca^{2+} sensitivity and disordered gap junction opening (Gomez-Hernandez *et al.*, 2003). Blockade of the channel also appears to be involved in pH gating mechanisms. Connexin channels are known to close in low pH however recent evidence suggests that rather than directly sensing H^{+}, connexin channels may be sensitive to protonated aminosulfonates (Locke *et al.*, 2011). In Cx26, the protonated aminosulfonate taurine was shown to close the channel by interacting with the C-terminal tail causing it to lose its interaction with an intracellular loop. As a result the C-terminus is free to physically block the channel (Locke *et al.*, 2011).

The final hypothesised mechanism for connexin gated involves the N-terminus of the protein forming a plug within the channel. The crystal structure of Cx26 reveals that the N-terminus of the protein lines the surface of the pore, narrowing it into a funnel (Maeda *et al.*, 2009). This is confirmed by electron density studies which reveal a density in the pore which is lost in truncated N-terminus mutants (Oshima *et al.*, 2007, 2008). It is believed that this mechanism may be important in voltage sensing of connexin channels whereby changes in potential disrupt intersubunit, electrostatic interactions between the N-terminus and transmembrane region leading to the N-terminus being released and blocking the channel (Maeda *et al.*, 2009).

As there is strong evidence for each of these binding mechanisms it may be possible that they reflect different gating properties directed against different stimuli. They may also be different mechanisms by which the same stimuli act to block different connexins e.g. the different mechanism for Ca^{2+} occlusion in Cx26 and 32 (Muller *et al.*, 2002; Gomez-Hernandez *et al.*, 2003).

1.4 Connexin Hemichannels

1.4.1 Introduction of connexin hemichannels

Although the roles of gap junctions are well known, the function of isolated hemichannels are less well studied. As transport through these channels is passive, open hemichannels can still allow the release of signalling molecules into the extracellular space. This mechanism offers a calcium independent route in which cells could communicate with cells outside of their immediate proximity. This was initially quite a controversial idea with many arguing that hemichannel signalling would not be possible as the channel opening would kill cells by non-specific efflux of their intracellular contents. Despite this, hemichannel signalling has been observed. One example of this is Stout's finding that Cx43 hemichannels in astrocytes are capable of releasing ATP (Stout *et al.*, 2002). One criticism with this result could be that the mechanical stimulation used to evoke ATP does not reflect a physiological stimulus and instead the ATP release could reflect a pathological process. These doubts were dispelled with Pearson et al 2005 paper which investigated ATP through Cx43 hemichannels in the retina (Pearson *et al.*, 2005).

They found that ATP release occurred spontaneously due to changes in intracellular Ca^{2+} and had a role in the development of the retina, with ATP signalling leading to cell proliferation (Pearson *et al.*, 2005). ATP release through hemichannels, composed of Pannexin-1, has also been shown to be important in the taste buds where it transmits information from the primary sensory cells to the afferent neurons (Huang *et al.*, 2007). Unlike other hemichannel forming proteins Pannexin-1 is specifically enriched in the taste buds and ATP release prevented by pannexin specific blockers (Huang *et al.*, 2007).

1.4.2 Hemichannel ATP release in chemosensitivity

A role for ATP release from hemichannels has also been implicated in CO_2 chemosensitivity with Gourine et al demonstrating that the ATP receptor antagonist PPADS reduced the increase in phrenic nerve activity associated with hypercapnia (Gourine *et al.*, 2010). As demonstrated in the pannexin-1 example, the opening of connexin channels provides a route through which ATP can be released and signalling can be initiated. Connexin channels are known to respond to a number of stimuli with channel closure (Locke *et al.*, 2011; Zonta *et al.*, 2012); however few examples are known to cause the opening of connexin channels. One of these stimuli is CO_2 which has been shown to cause channel opening (Huckstepp *et al.*, 2010b; Huckstepp *et al.*, 2010a). This is of particular interest to us as connexin proteins therefore have the potential to act as a conduit for PCO_2 detection in respiration.

The ability to open in response to elevated PCO_2 appears to be a unique property of the β subfamily of connexins, with connexins 26, 30 and 32 being shown to open in

response to CO_2 (Huckstepp *et al.*, 2010a). In contrast the γ connexin Cx36 demonstrated no CO_2-sensitvity, while the α connexin Cx43 closed in response to elevated PCO_2 (Huckstepp *et al.*, 2010a). Of the β connexins tested, Cx26 appears to be most suited for CO_2 chemosensitivity, with its sensitivity being centred around 40mmHg, the physiological level of CO_2 (Huckstepp *et al.*, 2010a). Cx26 is therefore able to respond to small changes away from normal PCO_2 despite this high base level.

Connexin 26 is known to be expressed in areas known to be important in CO_2 chemosensitivity and the control of respiration, namely the ventral surface of the medulla oblongata (VMS) (Solomon *et al.*, 2001; Mulkey *et al.*, 2004; Huckstepp *et al.*, 2010b). In the VMS Cx26 is present only within 300 µm of the ventral surface and around penetrating blood vessels (Huckstepp *et al.*, 2010b). These areas are best suited for measuring systemic PCO_2 without interference from local metabolism. In contrast, other connexin proteins found in the medulla (Cx32,36 and 43) are found to be uniformly distributed throughout this area and not to favour expression at the surface (Huckstepp *et al.*, 2010b).

The VMS is also implicated in the release ATP (Gourine *et al.*, 2005b; Gourine *et al.*, 2010; Huckstepp *et al.*, 2010b) and is known to exhibit a higher than normal basal tone of ATP (Huckstepp *et al.*, 2010b). These higher resting levels indicate reduced breakdown and elevated production of ATP, supporting a role of this signalling molecule in this area (Huckstepp *et al.*, 2010b). The presence of a CO_2 sensitive connexin and elevated ATP production in a known chemosensitive site therefore strongly indicate that ATP release from these channels may be involved in the

hypercapnic response. This is further supported by the very similar characteristics of CO_2 dependent channel opening and ATP release as demonstrate by Huckstepp et al (Huckstepp *et al.*, 2010b) (Figure 6).

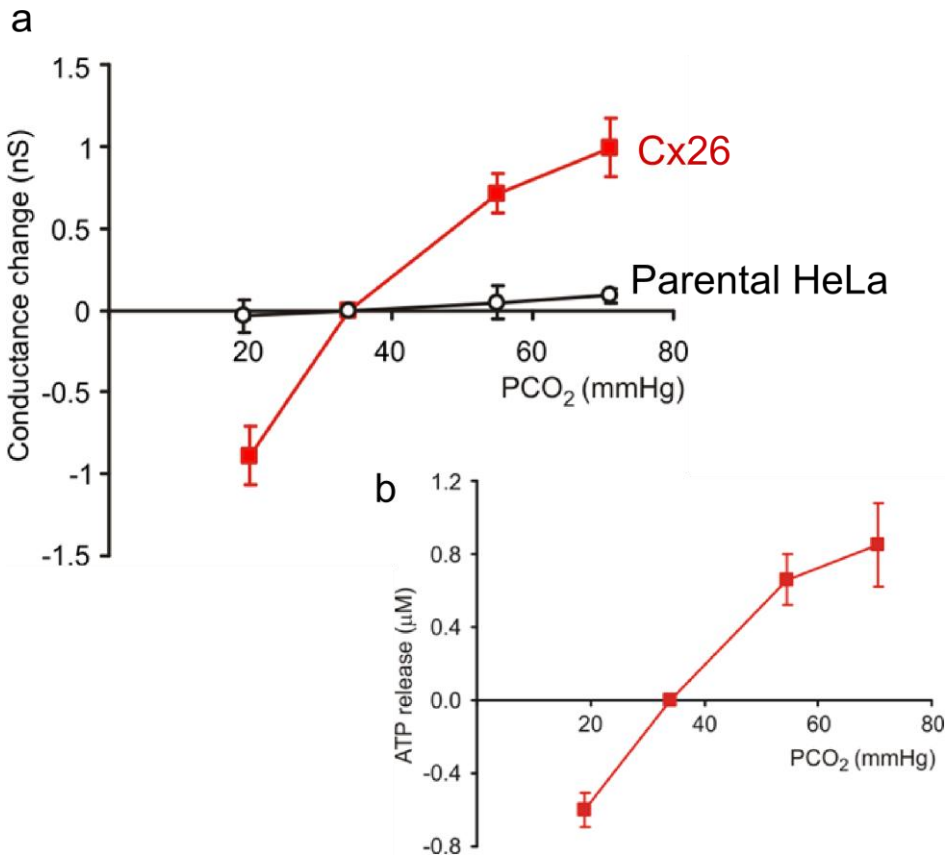

Figure 6 ATP is released from connexin hemichannels. Reproduced from (Huckstepp *et al.*, 2010b) a) Patch clamp data for Cx26 cells and parental HeLa cells reveals only cells expressing connexins display CO_2 dependent channel opening. b) ATP release, as detected by ATP biosensors, displays the same pattern as channel opening.

The role of connexin channels in the ventilatory response to hypercapnia has also been directly demonstrated through work by Gourine et al (Gourine *et al.*, 2005b). In these experiments carbenoxolone, a connexin channel blocker, reduced the

elevated phrenic nerve activity usually produced in response to hypercapnia (Gourine *et al.*, 2005b). This response was also reduced by the ATP antagonist PPADS confirming that ATP release through connexin channels is likely to be the mechanism involved (Gourine *et al.*, 2005b). The ability of connexin channels to release ATP has been demonstrated, both in vivo and in vitro, by Huckstepp et al and independently verified by Wenker et al (Huckstepp *et al.*, 2010b; Huckstepp *et al.*, 2010a; Wenker *et al.*, 2012). In these experiments ATP release has been shown to be independent of Ca^{2+} concentration, discounting vesicle release, but shown to be sensitive to carbenoxolone (Huckstepp *et al.*, 2010b; Wenker *et al.*, 2012). The hemichannels involved were deemed to be connexin proteins due to a blockade of ATP release at high carbenoxolone levels (100µM, blocks connexin channels) but not lower concentrations (10µM, blocks only pannexin channels) (Huckstepp *et al.*, 2010b). The dependence of activity on cobalt suggested a role for connexin hemichannels rather than gap junctions between cells (Huckstepp *et al.*, 2010b). Agents known to cause a reduction in the ATP response, such as proadifen or NPPB were shown to reduce CO_2-dependent ATP release from Cx26 and to reduce the ventilatory response to hypercapnia (Huckstepp *et al.*, 2010b). As both ATP and connexin hemichannel blockade reduced ventilation by a similar amount it follows that ATP released through hemichannels is important in the ventilatory response in the RTN (Gourine *et al.*, 2005b).

Taken together these results suggest that CO_2 interacts with connexin hemichannels, specifically Cx26, in the VMS causing them to open. The unique localisation of Cx26 results in systemic PCO_2 levels being detected, through

proximity to the surface and blood vessels, despite the local metabolism of the brain. Opening of connexin hemichannels in the astrocytes and leptomeninges allows the release of ATP in to the extracellular fluid. This provides a route for the stimulation of neural networks and as a result breathing is altered to adapt to an elevation in CO_2. Despite this largely complete model there remain gaps in the current knowledge. Primary amongst these is a description of how CO_2 interacts with Cx26. Previous work by Huckstepp et al demonstrate that the PCO_2 dependent channel opening of Cx26 was independent to changes in external pH or bicarbonate ions (Huckstepp *et al.*, 2010b). Internal pH was also largely discounted due to an apparent independence to changes in internal pH through buffering experiments, an independence to carbonic anhydrase and the maintenance of response in inside out and outside out patch clamp (Huckstepp *et al.*, 2010b; Huckstepp *et al.*, 2010a). It is worth noting that Wenker et al have previously supported stimulus detection through Kir4.1-Kir 5.1 potassium channels resulting in the opening of connexin channels and subsequent ATP release (Wenker *et al.*, 2010). This implies a change in internal pH is responsible for PCO_2 detection. In order to fully discount a role for internal pH in direct CO_2 detection in Cx26, the mechanism of CO_2 interaction with this system must be identified. By identifying a CO_2 binding it may also be possible to describe the mechanism by which channel opening can occur and consequently produce a complete model for direct CO_2 detection by Cx26.

1.5 Summary

In the healthy individual, increases in PCO_2 result in elevated ventilation. These physiological responses act to aid the excretion of the gas from the blood and consequently maintain a suitable blood pH. An increase in H^+ has been considered the stimulus for this response despite contradictory evidence supporting a role for direct CO_2 detection. Experiments by Huckstepp et al have identified Cx26 as a possible transducer for direct CO_2 detection as this gap junction protein appears to open in response to elevated CO_2 providing a route for ATP release. Although compelling evidence, this study did not identify a mechanism for CO_2 binding and therefore the change in internal pH thought to accompany elevated CO_2 has not been completely discounted as a potential stimulus. In the absence of this result the concept for direct CO_2 detection remains incomplete.

1.6 Experimental aims

This thesis aims to address three key questions involving the CO_2-sensitvity of Cx26, namely:

i. Does CO_2 directly interact with this channel to cause it to open?

ii. If so, what is the nature of this interaction?

iii. How does this interaction cause the conformational change required for CO_2 dependant channel opening?

Chapter 2 – CO_2 chemosensitivity is restricted to Cx26, 30 and 32, a subset of the β connexins.

2.1 Abstract

A subset of connexin proteins has previously been shown to open in response to elevated CO_2. The previous study demonstrated CO_2-sensitivity to be restricted to the β family of connexin proteins and to be independent of changes in pH or bicarbonate. Here we aim to determine whether the subset of β connexin tested comprise all of the CO_2-sensitive connexins or whether the closely related connexin 31 can also open in response to increases in CO_2. Using a dye loading assay we have demonstrated that connexin 31 is not CO_2-sensitive. From this work it can be concluded that CO_2-sensitivity is a specific property of one branch of the β connexin phylogenetic tree. This suggests a unique structural feature present in these connexins allows interaction with CO_2.

2.2 Introduction

2.2.1 CO_2 chemosensitivity is a key physiological mechanism

In the blood and bodily fluids, CO_2 reacts with water in a reaction partially catalysed by carbonic Anhydrase (Figure 1). As H^+ ions are a product of this reaction, an increase in the concentration of CO_2 in the blood quickly leads to a decrease in blood pH. Changes in blood H^+ as small as 0.1μM can be fatal due to the pH sensitive nature of enzymes and the biological processes they perform (Richerson, 2004). As such, monitoring and reducing CO_2 levels (partial pressure of CO_2, PCO_2) is a key physiological process. When abnormally high PCO_2 levels are detected there are several ways in which the body acts to reverse this, including increased

breathing and vasodilatation to remove excess CO_2 through the lungs and panic and anxiety responses, that encourage the subject to leave areas with high CO_2 atmospheres (Loeschcke, 1982; Fathi *et al.*, 2011). Despite the relatively well understood consequences of elevated CO_2, the molecular mechanisms involved in the actual detection of CO_2 remain less conclusive. Several candidate receptors have been suggested, which are thought to detect CO_2 or some proxy of CO_2 based on the carbonic anhydrase reaction.

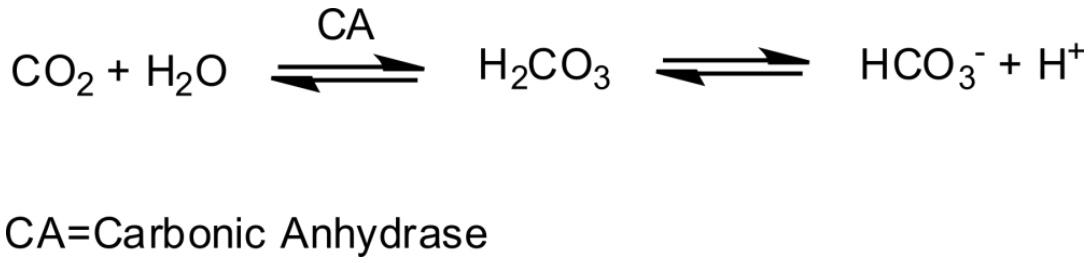

Figure 1 CO_2 reacts in blood to produce changes in pH levels

2.2.2 pH as the sole proxy for CO_2 detection

The most popular hypotheses for CO_2 detection assume the pH change resulting from the reaction of CO_2 with water, to be the stimulus for the physiological changes observed. Several works have demonstrated that changes in pH are capable of bringing about responses similar to that of inhaled CO_2, with the pivotal experiment demonstrating that acidic perfusion into the fourth ventricle could mimic changes in ventilation in the anaesthetized cat (Loeschcke, 1982). The specific transducer molecule for pH dependant CO_2 detection is however yet to be

elucidated, with several candidate molecules being suggested such as Kir, TASK and ASIC channels (Casamassima *et al.*, 2003; Jasti *et al.*, 2007; Mulkey *et al.*, 2007; Yamamoto *et al.*, 2008; Gestreau *et al.*, 2010; Trapp *et al.*, 2011). Thus far no one receptor is able to fully explain the chemosensitive response. For example, inwardly rectifying potassium (Kir) channels have been suggested to have a role in CO_2 chemosensitivity. These channels allow the movement of the K^+ ions into the cells, reducing membrane potential towards resting potential and suppressing neuronal firing (Hibino *et al.*, 2010). It is suggested that the decrease in pH associated with increases in PCO_2 leads to an inhibition of Kir channels and a consequent increase in depolarisation and neuronal activity (Wu *et al.*, 2004). It was demonstrated through in situ hybridisation that a subset of Kir channels, specifically Kir 4.1 and 5.1 heterodimers, are highly expressed in many known CO_2 chemosensitive regions such as the Locus coeruleus (LC) and ventral surface of the medulla oblongata (Wu *et al.*, 2004). In support of this idea, selective deletion of Kir 5.1 in the LC of mice, lead to a reduced response to hypercapnia in isolated cultures (D'Adamo *et al.*, 2011). However, other studies demonstrate no respiratory phenotype in Kir 5.1 knockout rodents and little co-localisation of Kir 4.1 and 5.1 in the medulla oblongata through immunohistochemisitry studies (Yamamoto *et al.*, 2008; Trapp *et al.*, 2011). This suggests the Kir channels may have some role in CO_2 chemosensitivity of the LC neurons but not in all known CO_2 chemosensitive sites. This study also demonstrates that the known CO_2-sensitivity of the LC neurons may be redundant in the whole animal response, with other chemosensitive areas compensating for the LC and producing no respiratory phenotype. No pH sensitive modulator thus far has been shown to account for all CO_2 chemosensitivity. This

suggests a complex system of CO_2 chemosensitivity utilising many CO_2/H^+ detecting molecules and comprising several chemosensitive areas of the brain.

2.2.3 CO_2 as a direct stimulus

The suggestion that CO_2 itself could also have a role in CO_2 chemosensitivity is deemed controversial, with most groups believing pH alone acts as the proxy for elevated PCO_2. Evidence against this point of view has however been mounting since 1946 when Grey et al identified that respiratory acidosis leads to bigger changes in respiration parameters, than the corresponding pH change due to metabolic acidosis alone (Gray, 1946). Increased responses were also seen to respiratory acidosis when monitoring phrenic nerve activity directly (Eldridge *et al.*, 1985). This implies a secondary stimulus must act in respiratory acidosis, that isn't present when metabolic acidosis alters pH alone, and that these stimuli act cumulatively. This idea was further confirmed by Shams et al who, by removing the pH change associated with increased PCO_2, by using simultaneous bicarbonate administration, demonstrated that CO_2 can act as a stimulus for increased phrenic nerve activity independently of pH (Shams, 1985). As with the less controversial pH detection mechanism, the molecular mechanisms involved with direct CO_2 detection are yet to be elucidated.

2.2.4 Connexins hemichannels in CO_2 chemosensitivity

Huckstepp et al have presented Connexin 26 as a candidate receptor for direct CO_2 modulation (Huckstepp *et al.*, 2010b; Huckstepp *et al.*, 2010a). This protein, which forms hemichannels at the cell surface has been found to be highly localised to

important CO_2 chemosensitive areas (Huckstepp *et al.*, 2010b). Hemichannels are known for their ability to be involved in cell signalling, as these gated channels can allow the passage of molecules up to 1KDa in size out of cells (Simpson *et al.*, 1977). Cx26 itself has been demonstrated to be capable of releasing ATP for paracrine signalling; a mechanism through which CO_2 interaction could bring about the physiological consequences such as altered breathing etc (Huckstepp *et al.*, 2010b). The ability of Cx26 , and other β connexins, to open in responses to elevated CO_2 has been demonstrated via dye loading and patch clamp experiments in both rat medullary slices and stably transfected HeLa cells (Huckstepp *et al.*, 2010a).

The β subfamily of the connexin phylogenetic tree has been demonstrated to be unique in the connexin family by responding to increased in CO_2 with channel opening. The δ connexin, Cx36 and the α connexin, Cx43 did not demonstrate channel opening with elevated CO_2 (Huckstepp *et al.*, 2010a). The β branch of the connexin family tree could however be considered 2 sub-branches. The smaller branch contains the three β connexins previously demonstrated to be CO_2-sensitive, namely Cx26, 30 and 32 (Huckstepp *et al.*, 2010a). The second branch contains all other β connexins which are yet to be investigated. In order to investigate the frequency of CO_2 chemosensitivity within the β connexins, the most appropriate connexin will be selected using the phylogenetic tree and accessed for CO_2-sensitivity through dye loading methods.

2.3 Methods

2.3.1 Constructing the connexin phylogenetic tree

A β connexin phylogenetic tree was constructed using phylogeny (Dereeper *et al.*, 2008). The following connexins were included in the tree: Cx26 (accession number NP_001004099), Cx30 (NP_445840), Cx32 (NP_058947), Cx25 (NP_940970), Cx30.3 (NP_446436), Cx31.1 (NP_062114.1) and Cx31 (NP_062113). In line with experiments performed by (Huckstepp *et al.*, 2010a), subsequent experiments will involve connexin proteins from Rattus norvegicus, therefore sequences from this species have been used for the comparison. The exception to this is Cx25 which does not have an available protein sequence in Rattus norvegicus. To our knowledge there remains no identified homologue to Cx25 in rat or mouse (Willecke *et al.*, 2002). Therefore the Homo sapiens sequence was used for this comparison.

2.3.2 HeLa cell culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma), 10% FCS (Biosera), 1:1000 pen/strep and supplemented with 3mM $CaCl_2$. Cells were grown in a humidified 5% CO_2 incubator at 37 degrees.

2.3.3 Transient connexin transfection

Cx31 (accession number NM_019240.1) was synthesised by Genscript USA and subcloned into the pCAG-GS vector

For transient transfections, cells were plated in 6-well plates at 5 x 10^4 cells per well Following the GeneJuice® transfection reagent (Merck-Millipore) user protocol,

cells were transfected with 1µg of the appropriate DNA. Experiments were performed when the connexin proteins had reached the cell membrane. This was found to be approximately 3 days for Cx31.

2.3.4 Experimental solutions

Control aCSF: 124mM NaCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 3mM KCl, 10mM D-glucose, 1mM $MgSO_4$, 1mM $CaCl_2$.

The solution was saturated with 95% O_2/5% CO_2 in order to provide the cells with oxygen throughout experimentation. This solution has a final pH of 7.5 and a PCO_2 of 35mmHg.

50mM HCO_3^- Isohydric Hypercapnic aCSF: 100mM NaCl, 50mM $NaHCO_3$, 1.25mM NaH_2PO_4, 3mM KCl, 10mM D-glucose, 1mM $MgSO_4$, 1mM $CaCl_2$.

This solution was saturated with ~9% CO_2 to match the final pH to that of the control aCSF, namely pH 7.5. This removes any potential effects of changes in extracellular pH. The final PCO_2 of this solution is 55mmHg.

Zero Ca^{2+} aCSF: 124mM NaCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 3mM KCl, 10mM D-glucose, 2mM $MgSO_4$,1mm $MgCl_2$ 1mM EGTA.

As with the control aCSF experiments, this solution is with 95% O_2/5% CO_2 and has a final pH of 7.5.

2.3.5 Dye loading assay

Coverslips plated with a sub confluent level of Cx31 expressing HeLa cells were placed in a small flow chamber for dye loading analysis.

The dye loading assay, previously described by (Huckstepp *et al.*, 2010a), involves exposing the cells to carboxyfluorescein dye. Carboxyfluorescein is a membrane impermeable dye of molecular mass 380 Da. Consequently carboxyfluorescein can only enter HeLa cells when connexin channels expressed in the membrane are opened to some stimulus. When the stimulus is removed, the connexin channels close trapping the dye inside the cells. This dye can then be visualised using epifluorescence microscopy which excites the dye using light at a wavelength of 470nm. As a result the carboxyfluorescein dye emits light at 525nm which can be detected and recorded. Here we investigate the affect of the increased CO_2 on the gating properties of connexin proteins. The connexin proteins used for transient transfection in this experiment express a mCherry tag on their C-terminus which can be visualised at 587nm.

During control experiments cells were exposed to control aCSF containing 200µM carboxyfluorescein for 15 minutes. The cells were then washed with control aCSF for 30 minutes to remove external carboxyfluorescein.

For CO_2 investigations, cells were exposed to isohydric hypercapnic solution containing 200µM carboxyfluorescein for 10 minutes, followed by 5 minutes of control aCSF with 200µM carboxyfluorescein. This second step assures any loaded dye is not washed away during channel closure. As with the control experiments,

these cells were again washed with control aCSF for 30 minutes to remove any extracellular dye which could produce false results.

A lack of loading with increased CO_2 could occur due to non sensitivity of the connexin channels to CO_2. However, it could also occur if the connexin channels expressed were not in the membrane or were not functioning to allow the transport of dye. To attempt to distinguish between these two possibilities, a positive control was performed. Low extracellular calcium is known to result in connexin channel opening (Muller *et al.*, 2002). We therefore exposed the cells to our "Zero Ca^{2+} aCSF solution" which is not supplemented with Ca^{2+}, unlike other solutions, and contains 1mM EGTA for Ca^{2+} chelation. Cells for this experiment were exposed to Zero Ca^{2+} aCSF containing 200µM carboxyfluorescein for 10 minutes, followed by Control aCSF containing 200µM for 5 minutes. As with the CO_2 experiments this ensured loaded dye was not washed away before channel closure. The cells were finally washed with control aCSF for 30 minutes. The dye loading protocol can be seen in Figure 2.

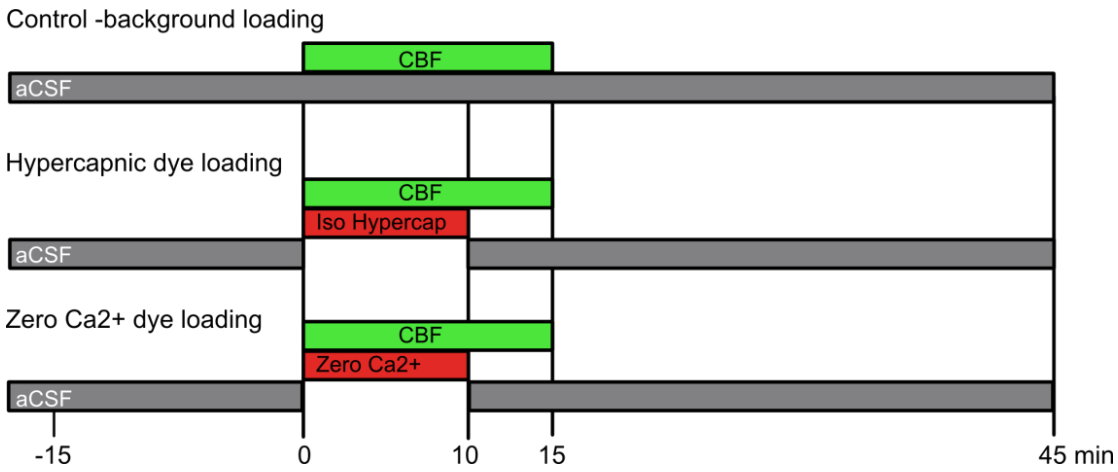

Figure 2 Graphical representation of dye loading protocol, detailing control, hypercapnic CO_2 and zero Ca^{2+} experiments

Dye loading was also used to investigate the effect of pharmacological blockers on transfected HeLa cells. Control and hypercapnic solutions and dye loading were performed as above. For hemichannel blocker experiments, the cells were pre-incubated with the blocker of interest for 15 minutes. Presence of the blocker was maintained during the dye loading portion of a hypercapnic experiment. The hemichannel blockers investigated were 100µM carbenoxolone, 1mM probenecid or 20µM ruthenium red. A graphical representation of the dyeloading can be seen in Figure 3.

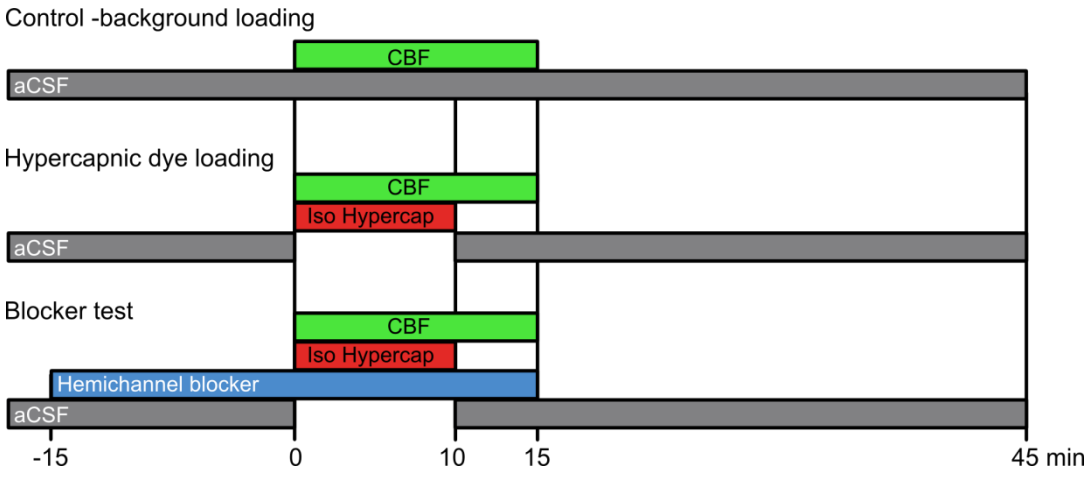

Figure 3 Graphical representation of hemichannel blocker dye loading. Hemichannel blockers tested were carbenoxolone, probenecid and ruthenium red.

2.3.6 Data capture and analysis

In all experiments the cells were then imaged using epifluorescence (Scientifica Slice Scope, Cairn Research OptiLED illumination, 60x water Olympus immersion objective, NA 1.0, Hamamatsu ImageEM EMCCD camera, Metafluor software).

Experiments were repeated at least 5 times, with each repeat performed on cells resulting from a different transient transfection.

Images produced during all experiments were analysed using image J. Background brightness can often differ between experiments and this can prevent proper comparison of results. To resolve this, background brightness was measured and subtracted from the entire image for each individual image. The resulting images reflected only the brightness of the cells themselves. By drawing a region of interest (ROI) around individual cells the mean pixel intensity for these individual cells was calculated. During each repetition this was repeated for at least 40 cells for each condition and the mean pixel intensities plotted as cumulative probability distributions. Median differences in CO_2 response were compared for Cx31 and Cx26 using the Mann Whitney test significance test.

2.3.7 Cx26 adaptations

As a comparison to Cx31, mCherry-tagged Cx26 connexin channels were also investigated by dye loading. This connexin channel was previously shown to be CO_2-sensitive by this assay. Cx26 (accession number NM_001004099.1) was synthesised by Genscript USA and subcloned into a pCAG-GS.

2.4 Results

2.4.1 Cx31 could provide a comparison to Cx26

Previous work by Huckstepp et al established connexin 26 to be CO_2-sensitive. In order to understand the mechanism involved it is important to establish the specificity of this response within the β connexins. To identify the best connexin to investigate CO_2-sensitivity within this subgroup a phylogenetic tree of the Rattus

norvegicus β connexin sequences was constructed using phylogeny (Dereeper *et al.*, 2008)(Figure 4).

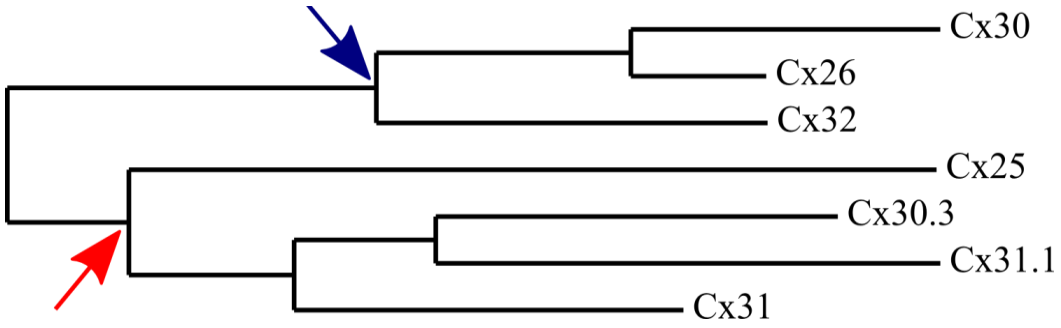

Figure 4 β connexin phylogenetic tree. Constructed at Phylogeny. Two distinct sub branches can be seen. One contains Cx32, 26 and 30, which have previously been shown to be CO_2-sensitive. The second group are yet to be tested for CO_2-sensitivity. Blue arrow indicates the common ancestor of connexins 30, 32 and 32. The red arrow indicates the common ancestor of connexins 31, 31.1, 30.3 and 25

The phylogenetic tree constructed was shown to be in agreement with those previously constructed for connexins (Bruzzone, 2001; Saez *et al.*, 2003). The connexins previously tested for CO_2-sensitivity were shown to form one sub-branch on the tree, which derived from a common ancestor not shared by the second larger group. In order to determine whether CO_2 dependent channel opening is a property of all β connexins, a candidate connexin from the second branch must be selected for dye loading analysis.

We have identified Cx31 as the best candidate for further experimentation. Although Cx25 is closest connexin to the CO_2 sensitive group, this protein is not commonly expressed. In contrast, Cx31 which is also closely related to Cx26 is widely expressed and associated with genetic disease. This connexin, unlike Cx25, also has a rat homologue allowing direct comparison with previous Cx26 experiments (Willecke *et al.*, 2002). The use of Cx31 therefore fulfils our

requirements for investigating Cx26 sensitivity while potentially providing its own intrinsic merit by investigating the possibility of CO_2-sensitvity in the role of Cx31 in the inner ear and skin (Xia *et al.*, 1998; Gottfried *et al.*, 2002). Cx31 has also been previously used for fluorescent tagged experiments (Liu *et al.*, 2009; Oh *et al.*, 2013). As we intend to express our connexin with a c-terminal mCherry tag, prior use of Cx31 indicates the protein will be correctly trafficked to the membrane and form functional channels.

Finally knowing the CO_2-sensitivity of Cx31 allows us to determine the CO_2 chemosensitivity of the other connexins in this group. If Cx31 were to be non CO_2-sensitive there are 2 potential ways in which this situation could be achieved: the subgroup of Cx32, 30 and 26 gained CO_2-sensitivity from their shared common ancestor or a common ancestor of Cx31, not shared by Cx26, 30 and 32, lost CO_2-sensitivity. In both of these situations, the CO_2-sensitivity of Connexins 31.1 and 30.3 will be the same as Cx31, as they would share the hypothesized common ancestor. Following the criteria described above Cx31 was chosen to be used to determine whether CO_2 chemosensitivity is a property of all β connexins or just the sub-branch previously tested.

2.4.2 Cx31 is non CO_2-sensitive

The CO_2-sensitvity of Cx31 was investigated via dye loading and compared to the previously known sensitivity of Cx26. In line with previous experiments, transiently transfected connexin 26 HeLa cells showed increased dye loading when exposed to elevated CO_2 in the presence of carboxyfluorescein (Figure 5). Conversely Connexin

31 expressing HeLa cells did not show increased dye loading under the same conditions (Figure 5). This suggests unlike the previous β connexins tested, Cx31 is not sensitive to changes in CO_2.

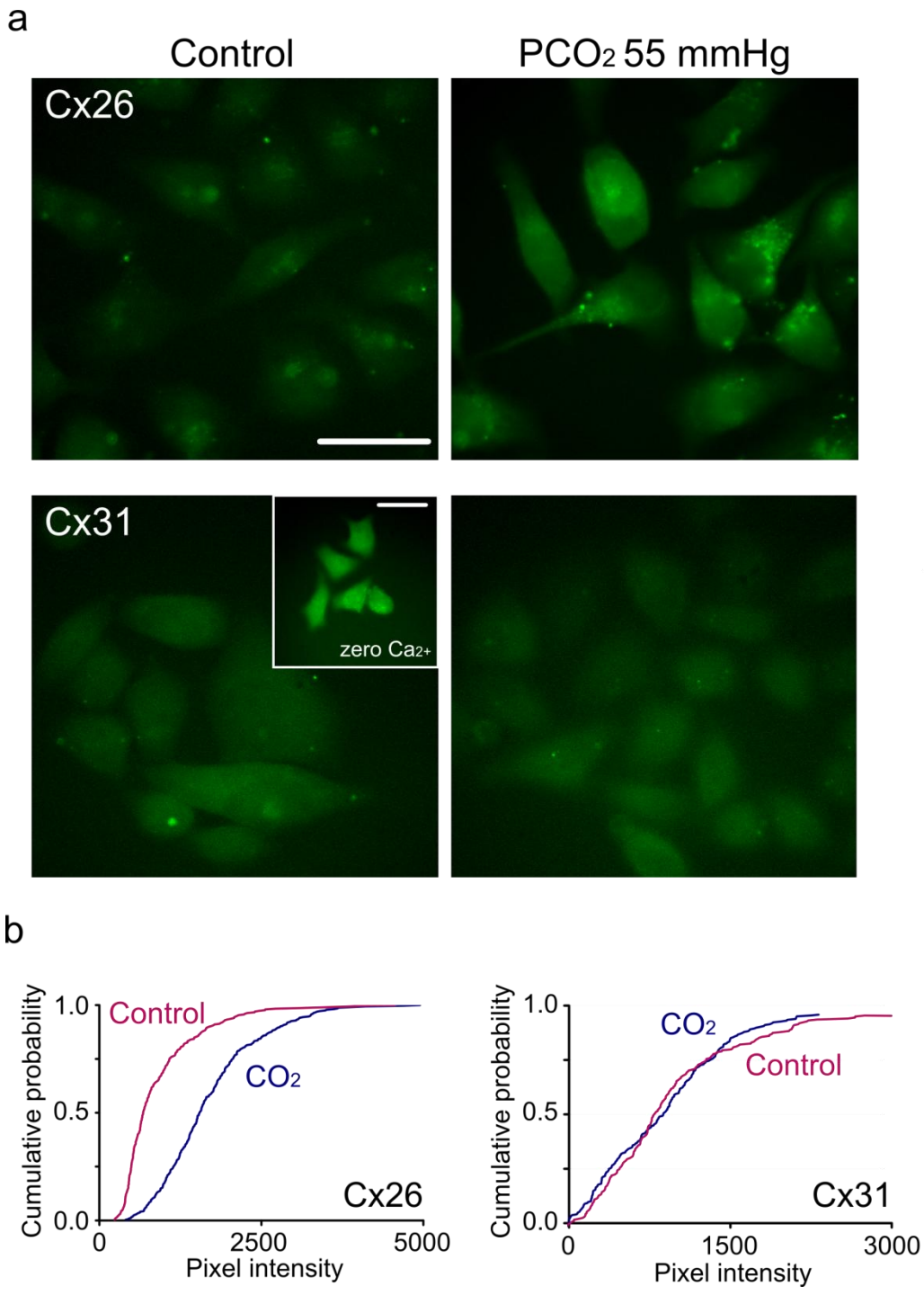

Figure 5 Cx31 is not sensitive to CO_2. a) Dye loading images for Cx26 and Cx31 at control and elevated CO_2 levels. All scale bars represent 50 µm. Cx31 inset shows zero Ca^{2+} positive control. b) Cumulative probability graphs of all cells measured in the 5 repeats.

In order to confirm the validity of this result a positive control was also tested. Connexin channels are known to open in response to low Ca^{2+} levels (Muller *et al.*, 2002). Although the method by which this occurs is not confirmed, it is believed that calcium ions can act to block the channel, an effect which is lost when calcium ions are removed (Ebihara & Steiner, 1993; Zonta *et al.*, 2012). The positive control was achieved by the exclusion of supplementary Ca^{2+} and by the addition of EGTA. Dye loading was observed for Cx31 using this "Zero Ca^{2+}" positive control. This confirms the connexin channels are present in the membrane and are functioning correctly and thus confirms their non-sensitivity to CO_2 (Figure 5).

2.4.3 Parental HeLa cells contain no endogenous gap junction proteins

The Zero Ca^{2+} positive control was also used to establish that the parental HeLa cells were free from endogenous connexins. Zero Ca^{2+} experiments were performed for parental HeLa cells and demonstrated no dye loading. In contrast, HeLa cells transfected with Cx31 or Cx26 revealed increased dye loading when extracellular Ca^{2+} was removed (Figure 6). Background control loading was also lower in the parental HeLa cells. This also supports an absence of endogenous connexins, as the spontaneous opening of connexin channels leads to a small increase in dye loading in transfected cells.

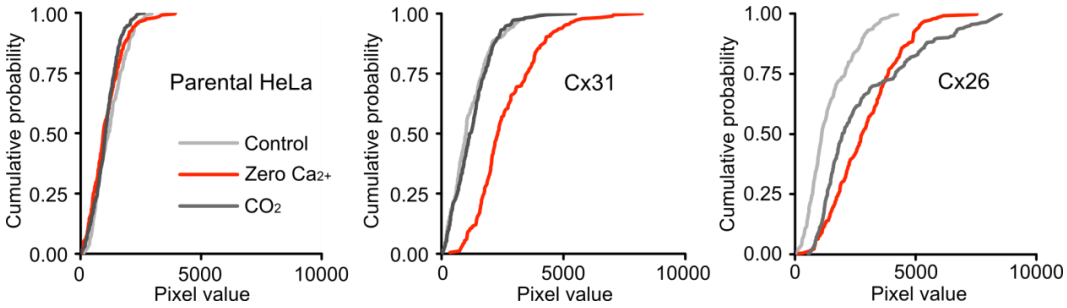

Figure 6 Parental HeLa cells contain no endogenous connexins as demonstrated though Zero Ca^{2+} dye loading. Cumulative probability distributions for all cells over 5 repeats.

The lack of CO_2-sensitivity in the parental HeLa cells indicates the increased dye loading observed in the Cx26-expressing cells accurately reflects the CO_2-sensitive nature of this protein. To further support this, various pharmacological blockade experiments were performed to exclude the role of other hemichannels. The increases in dye loading seen with elevated CO_2 were prevented by the application of 100µM carbenoxolone to the cells. At the concentrations used, this drug blocks channel opening in both connexin and pannexin channels (Huckstepp *et al.*, 2010b). Conversely, CO_2 dependent dye loading was not prevented by application of 1mM probenecid or 20 µM ruthenium red (Figure 7). These drugs block pannexins-1 and CALHM1 hemichannels respectively (Bruzzone *et al.*, 2005; Silverman *et al.*, 2008; Taruno *et al.*, 2013). These results indicate that Cx26 is responsible for the elevated dye loading seen and confirms the earlier study which concluded Cx26 to be CO_2-sensitive (Huckstepp *et al.*, 2010a).

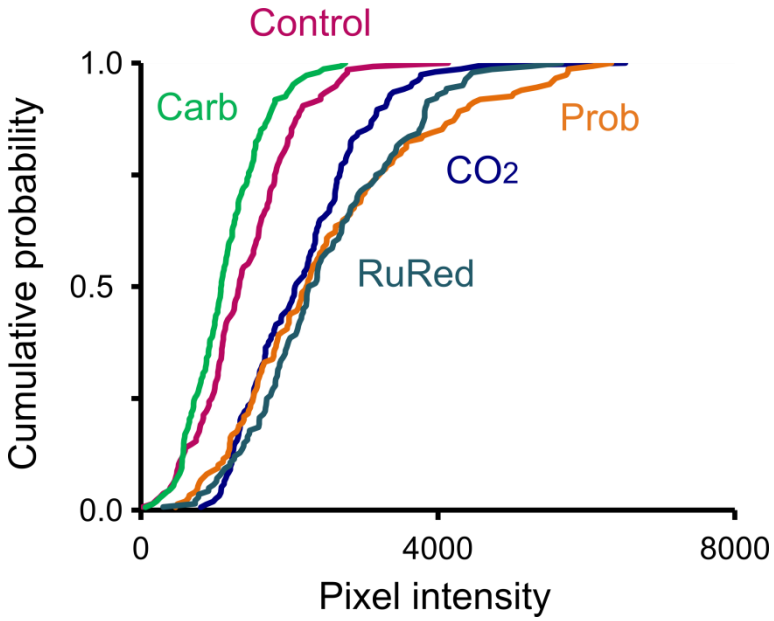

Figure 7 Carbenoxolone blocks Cx26 CO_2 response. Cumulative probability distribution showing the blockade of CO_2 dependent dyeloading by carbenoxolone. No effect is seen with selective pannexin or CALHM1 blockers.

2.4.4 Connexin 31 is correctly expressed in HeLa cells

The connexin genes used for these experiments were fusion proteins expressing a mCherry fluorescent tag at their C-terminus as it was hoped this would provide information of the synthesis and trafficking of the protein. Fluorescent tags have previously been used at this location and have demonstrated normal expression and trafficking to the membrane (Laird *et al.*, 2001; del Corsso *et al.*, 2006). As carboxyfluorescein fluoresces at green wavelengths, a red tag was chosen. Of the red tags available mCherry is considered the most promising due to its increased photo stability and reduced dimerisation compared to natural red fluorescent proteins (Campbell *et al.*, 2002; Shaner *et al.*, 2005). Strong mCherry signals were seen for both Cx31 and Cx26, demonstrating the expression of these proteins in the

HeLa cells (Figure 8). This confirms that our results for Cx31 reflect a true negative response and do not result from lack of protein expression.

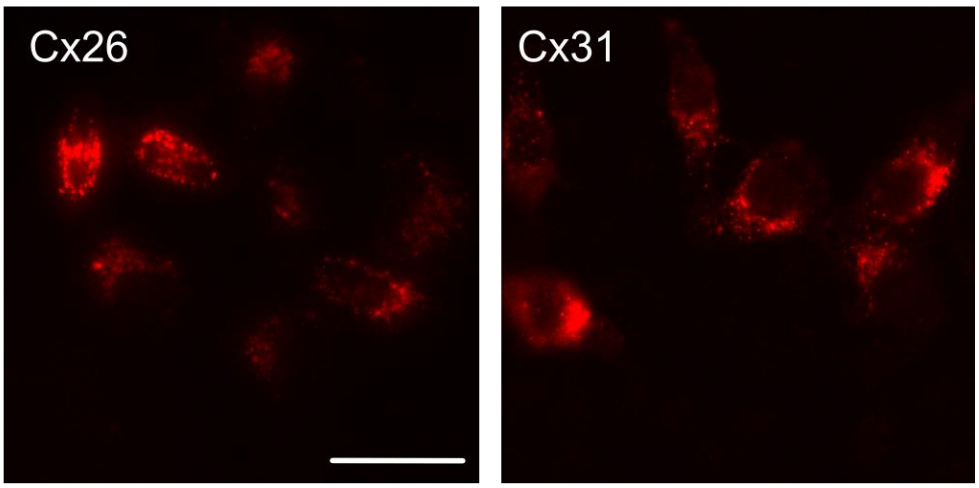

Figure 8 Connexin proteins are correctly expressed in HeLa cells as demonstrated through mCherry signal**.** mCherry visualised by epifluorescence at 587nm . Scale bar 50 µm.

2.5 Discussion

2.5.1 CO_2 dependent channel opening is specific to Connexins 26, 30 and 32

Huckstepp et al demonstrated the β sub-group to be unique amongst connexin proteins by experiencing channel opening in response to elevated CO_2. Here we determined Cx31, a β connexin closely related to those previously tested, to be non CO_2-sensitive. There are two possible explanations for the differences in CO_2 chemosensitivity observed in these experiments: Cx31 could be unique amongst β connexins in being insensitive to CO_2; or the subgroup of which Cx31 is a member is entirely non CO_2-sensitive. We consider the latter to be more likely as it is simpler to envisage a change in CO_2-sensitivity involving a common ancestral gene from

which the other related β connexins are derived, rather than the presence or absence of CO_2-sensitivity varying gene by gene. The appropriateness of this assumption will be investigated in later work in this thesis. However accepting this assumption to be true for the moment, it implies that the smaller group, comprising connexins 26, 30 and 32, is unique amongst β connexins by demonstrating CO_2-dependent channel opening.

There are two possible ways in which this difference in CO_2 chemosensitivity could be achieved. Firstly, the common ancestor of *all* β connexins could have been CO_2-sensitive. At some point, the sequence or binding moiety responsible would have been lost in the last common ancestor of Cx31, Cx31.1 and Cx30.3. Alternatively, the common ancestor of all β connexins was insensitive to CO_2 and a random mutation in the last common ancestor of Cx26, 30 and 32 lead to a gain of this function only in these connexins. As members of the α and δ connexins were previously shown to be non CO_2-sensitive (Huckstepp *et al.*, 2010a) we favour a gain of CO_2-sensitivity in the Cx26 branch as the more likely mechanism.

2.5.2 Physiological relevance

We have demonstrated that CO_2 dependant channel opening is a specific property of 3 β connexins, Cx26, 30 and 32. The CO_2-sensitivity of these proteins could therefore be an important mechanism in any tissue in which they are located. Of particular interest to us is Cx26 which has previously been identified to be highly localised to areas of known CO_2-sensitivity, specifically those involved in changes to respiration in response to elevated CO_2 (Huckstepp *et al.*, 2010b). The role of Cx26 in CO_2-sensitivity is further supported by experiments which determined Cx26 to be

capable of releasing ATP in response to elevated CO_2, a signalling molecule known to be important in CO_2 dependant changes in respiration (Gourine *et al.*, 2005b).

Cx31 was selected for use in these experiments based on its structurally similarity to the previously tested Cx26. Investigation of this protein may however have its own intrinsic merit. Cx31 is highly expressed in several tissues and its misexpression is known to result in a number of genetic diseases. Like Cx26, Cx31 is expressed in the organ of Corti in the ear (Xia *et al.*, 1998; Xia *et al.*, 2000). These two gap junctions appear to be responsible for K^+ recycling in distinct locations in the cochlea but defects in both can result in deafness (Kikuchi *et al.*, 1995; Xia *et al.*, 1998). Cx31 is also expressed in the keratinocytes in the skin. Mutations that prevent the formation of gap junctions between keratinocytes through defective trafficking to the membrane lead to Erythrokeratodermia variabilis (Gottfried *et al.*, 2002). This skin disorder leads to patches of thickened skin suggesting a role for Cx31 gap junctions in cell signalling to control the proliferation of skin cells.

As CO_2-sensitvity has been discounted in Cx31 we can now determine that this stimulus is not relevant in these pathologies. Further experimentation also provides us with the possibility to gain a greater understanding of the functionality of the Cx31 hemichannel through its comparison to Cx26.

CO_2-sensitivity has previously been demonstrated in HeLa cells, stably transfected with Cx26. This result was confirmed in this work, with transiently transfected HeLa cells. The demonstration of CO_2-sensitivity in this non native system suggests direct CO_2 binding to be responsible for channel opening. Direct binding of CO_2 to Cx26 requires a binding site. Having identified β connexins that are both CO_2-sensitive

and non-sensitive, we now have the basis for a sequence comparison that may allow us to identify a binding motif or residue that is present only in the CO_2-sensitive connexins.

Chapter 3- Cx31 can be conferred with CO_2-sensitivity by the inclusion of a carbamylation motif

3.1 Abstract

Carbamate bond formation occurs through the interaction between CO_2 and an amine group. This interaction is biologically important in several contexts including CO_2 binding to haemoglobin, carbon fixation by RuBisCo and antibiotic resistance by β lactamase. After identifying Cx31 to be non sensitive to CO_2, we compared this sequence to that of known CO_2-sensitive connexins, Cx26, 32 and 30. Based on the hypothesis that CO_2 may bind through a carbamate bond, we specifically searched for conserved lysines absent in Cx31. This identified a conserved motif in Cx26, and specifically lysine 125, as a potential CO_2 binding site. Mutational studies to include this conserved region in Cx31 produced a CO_2-sensitive mutant as determined through dye loading and patch clamp assays. These experiments determined the conserved domain containing lysine 125 as sufficient to confer CO_2-sensitivity to connexin proteins and suggest this residue may represent a direct binding site for CO_2.

3.2 Introduction

3.2.1 CO_2 interactions

In the previous chapter (Chapter 2) we demonstrated that CO_2 dependent channel opening is a specific property of a small group of β connexins. CO_2 could interact with these proteins, either directly by binding to the connexin or indirectly through an accessory protein.

One possibility for an accessory protein in CO_2 detection could be a G-protein coupled receptor, with members of this family known to interact with CO_2 in insects. Drosophila measure CO_2 levels to identify stressed individuals, allowing them to exercise avoidance behaviour (Suh *et al.*, 2004). CO_2 detection in these animals is known to be conducted through a specialised set of odour receptors known to express two 7 membrane G-protein coupled receptors (GPCRs) (Jones *et al.*, 2007). Selective loss of these G proteins removes CO_2 chemosensitive behaviour, while leaving the odour pathway intact (Yao & Carlson, 2010). Homologues of these GPCRs also appear to be responsible for CO_2 detection in mosquitoes, which they use to locate human victims (Robertson & Kent, 2009). The role of G proteins in CO_2 chemosensitivity is not restricted to insects, with a similar response recently being demonstrated in the mouse. Like the drosophila, mice have been shown to have specialised olfactory cells which are believed to be involved in CO_2 avoidance behaviour (Meyer *et al.*, 2000). In Drosophila this response has been demonstrated to be independent of pH (Badre *et al.*, 2005). Huckstepp et al demonstrated that the CO_2-sensitivity of Cx26 was also pH independent (Huckstepp *et al.*, 2010b) and thus GPCRs could potentially have a role in this mechanism. In this hypothetical model, activation of GPCRs would lead to a signalling cascade resulting in connexin channel opening and subsequent ATP release. It has however also been demonstrated that the specialised cells expressing GPCRs produce elevated levels of carbonic anhydrase, with the CO_2 avoidance behaviour lost in carbonic anhydrase mutants (Meyer *et al.*, 2000; Hu *et al.*, 2007). Taken together these findings indicate that bicarbonate, rather than CO_2, is the stimulus of GPCR activation. As Cx26 channel opening has been shown to

be independent of changes in bicarbonate levels (Huckstepp *et al.*, 2010a), and we would have to hypothesize that HeLa cells coincidentally expressed this hypothetical CO_2-sensitive GPCR, we think it unlikely that GPCRs could be involved in the response reported in the previous chapter.

In systems that have been demonstrated to be apparently pH independent, a reliance of carbonic anhydrase is still often observed (Brearley *et al.*, 1997; Boijink *et al.*, 2010; Hu *et al.*, 2010). This suggests in complicated systems of CO_2 detection, pH or HCO_3^- are often the stimulus rather than direct CO_2 binding. As detection of CO_2 by Cx26 has shown to be independent of pH and HCO_3^- (Huckstepp *et al.*, 2010b) this suggests that the sensitivity reported here does not fall into any previously established system of CO_2 signalling. A plausible hypothesis is that CO_2 binds directly to Cx26, and that no other signalling cascades or proteins are required.

Previous work by Huckstepp et al (Huckstepp *et al.*, 2010a) also makes the idea that CO_2-dependent opening of Cx26 requires an accessory protein less plausible. In this study inside-out and outside-out patch clamp experiments were performed. In both of these methods a small section of membrane is isolated to enable single channel conductance recordings and CO_2 chemosensitivity was demonstrated for Cx26 channels under these conditions. For accessory proteins to be relevant in CO_2 dependent channel opening they would need to be isolated in the same membrane patch and be capable of exerting their response at the membrane, in the absence of the intracellular media. While this is not impossible, the hypothesis of an accessory protein being involved seems considerably more complex and therefore less

attractive than a direct interaction between Cx26 and CO_2. Nevertheless to settle the issue we need to understand in detail the mechanism and nature of this hypothesized interaction.

3.2.2 CO_2 binding through carbamate formation

One way in which CO_2 can bind directly to proteins is through a carbamylation reaction. These reactions are present in several biological systems. For example, in haemoglobin carbamylation reduces oxygen binding affinity allowing O_2 to be released at the tissues (Kilmartin & Rossi-Bernadi, 1971). In bacteria, the cell wall production catalysed by β lactamase is dependent on carbamylation on both subunits of the enzyme (Golemi *et al.*, 2001). Carbamate formation is even important in plant processes, with the enzyme Ribulose bisphosphate carboxylase requiring carbamylation to perform its role in carbon fixation during photosynthesis (Lorimer, 1979). There are several indications that the interaction here could be due to carbamylation. Firstly, we would expect CO_2 binding to be a specific interaction and as such we might expect a strong covalent bond to occur. However, it must also be labile, as responses to increased CO_2 must desist when CO_2 levels return to normal. These properties match the carbamate bond exactly, which is both covalent and reversible.

Secondly, the equilibrium of the carbamate reaction means decreases in pH lead to reduced carbamate formation, as increases in H^+ concentration push the reaction towards free CO_2 (Figure 1). Data supporting these dynamics were observed in previous experiments into the CO_2 sensitivity of Cx26, which demonstrated that

under alkaline conditions smaller changes in CO_2 were required to produce Cx26 channel opening (Huckstepp *et al.*, 2010b).

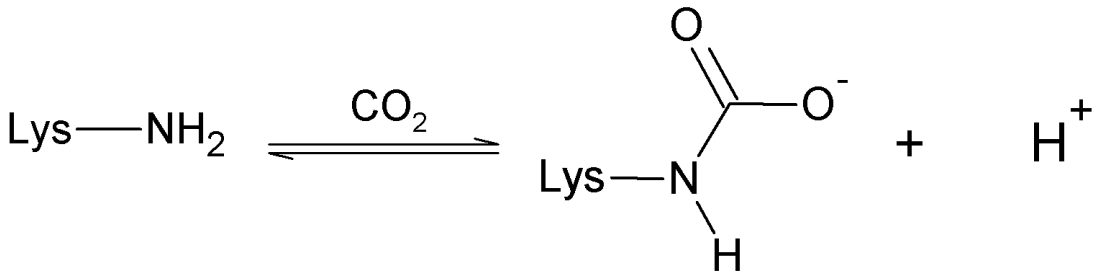

Figure 1 Carbamate formation reaction. The reversible nature of the bond means increased H^+ impedes carbamate formation.

One important property of the carbamate bond is that it requires stabilisation due to the negative charge produced upon CO_2 binding. Previous experiments have revealed a dependence of CO_2 sensitivity in Cx26 on Mg^{2+} (unpublished study). This dependence could suggest a role in carbamate stabilisation. Another possibility is that positively charged residues could stabilise a carbamate bond. As connexins exist as hexamers in the membrane there could be a multitude of residues that could act to neutralise the carbamate's charge.

Carbamate bonds in proteins form between CO_2 and the ε-amino group of lysine. In order for carbamylation to be physiologically important in CO_2 sensitivity, we would expect the binding site to be present only in the CO_2-sensitive connexins. We therefore aimed to identify a lysine present specifically in the CO_2-sensitive connexins. Cx26 contains 16 lysines that could potentially act as a CO_2 binding site if the carbamate hypothesis is correct. The candidate lysine must also be present in the other connexins shown to be CO_2-sensitive, connexins 30 and 32 and absent in

CO_2-insensitive connexins. In the previous chapter we demonstrated that Cx31 is non CO_2-sensitive and as such this will be used in the sequence comparison for binding site assignment.

Here we aim to identify a potential CO_2 binding site using sequence comparison of the above mentioned connexins. Upon identification of a candidate binding site, the contribution of this residue to CO_2 sensitivity can be analysed via mutational studies.

3.3 Methods

3.3.1 Sequence comparison

Amino acid sequences of various connexins were tested for sequence similarity. The connexins tested were: Cx26 (accession number NP_001004099), Cx30 (NP_445840), Cx32 (NP_058947), Cx31 (NP_062113), Cx30.3 (NP_446436), Cx25 (NP_940970) and Cx31.1 (NP_062114.1).

Cx26 experiments have been performed using the sequence of Cx26 from rat. For these finding to be important in human chemosensitivity it is important to also compare the rat and human sequences for conserved lysines. The accession numbers for the comparison are as follows: Homo sapiens Cx26 (NP_003995.2), Rattus norvegicus Cx26 (NP_001004099), Mus musculus Cx26 (NP_032151.1), Felis catus Cx26 (XP_003980299), Orcinus orca Cx26 (XP_004281848), Tursiops truncatus Cx26 (XP_004310707) and Heterocephalus glaber Cx26 (XP_004895537).

3.3.2 HeLa cell culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma), 10% FCS (Biosera), 1:1000 pen/strep. As with the experiments in the previous chapter (Chapter 2) they were supplemented with 3mM $CaCl_2$ and grown in a humidified 5% CO_2 incubator at 37 degrees.

DNA mutations were performed by Genscript USA.

Cells were plated onto coverslips in 6 well plates at a concentration of 5 x 10^4 cells per well. Using genejuice transfection reagent cells were transiently transfected with 1µg DNA.

3.3.3 Dye loading

For the dye loading assay, cells were analysed after 3 days. Analysis was performed using the solutions and protocol outlined in Chapter 2. Briefly, cells were exposed to either control or elevated CO_2 aCSF containing 200µM carboxyfluorescein for 10 minutes. Next, cells were exposed to control aCSF containing 200µM carboxyfluorescein for 5 minutes. A wash with control aCSF for 30 minutes ensures all carboxyfluorescein is removed from the outside of the cells. As carboxyfluorescein is membrane impermeable, dye can only enter the cells if the connexin channels open in respond to increased CO_2. If the mCx31 mutant was non-CO_2 sensitive, as was Cx31, we would expect no increase in dyeloading with the elevated CO_2 solution. The pH of the increased CO_2 solution is matched to the control aCSF to ensure no effect of pH on these experiments.

3.3.4 Data Capture and analysis

Cells were imaged by epifluorescence as described in Chapter 2.

Background subtracted median pixel intensities were obtained for approximately 40 cells in 5 repeats. These data were used to produce cumulative probability distributions. Median differences in pixel intensities between control aCSF and Hypercapnic aCSF were used to access CO_2-sensitivity through comparison to Cx31 using Mann Whitney Statistical analysis. Cx31 and the 2 mutants, mCx31 and mCx31 K-R, were also analysed for statistical difference using the Kruskal-Wallis statistical test.

3.3.5 Whole cell patch clamp

All patch clamp experiments were performed by Professor Nicholas Dale.

For patch clamp experiments, HeLa cells were plated at a subconfluent level of approximately 1×10^4 cells per well. As with the dyeloading experiments, cells were analysed from 3 days post transient transfection with the gene of interest.

Patch clamp recordings were performed used standard patch clamp techniques. Coverslips containing cells were perfused with standard aCSF following the recipe outlined in the previous chapter (Chapter 2) to ensure the cells remained healthy and oxygenated during the experimentation. A glass electrode is used to make a seal with the outside of a HeLa cell of interest. The inside of this electrode is filled with solution to mimic the intracellular content. For these experiments the

intracellular fluid in the patch pipette consisted of 120mM K-gluconate, 10mM CsCl, 10Mm TEACl,10mM EGTA, 3mM ATP, 1Mm $MgCl_2$, 1mM $CaCl_2$. This solution was sterile filtered and pH adjusted to 7.2 with KOH. As whole cell patch clamp was being performed, extra suction was applied to the cell until the cell membrane was ruptured, leaving the patch electrode plugging the hole in the cell. Unlike other patch clamp methods which measure channel conductance in small membrane fragments, this method allowed us to investigate the channel opening properties of a large number of channels. Electrical currents in the cells are subsequently detected through a platinum wire in contact with the fluid within the patch pipette. Maintaining a constant voltage in the experiment allows changes in current to be assessed. In these experiments this was achieved using a holding potential of -40 mV using regular steps of 5 s to -50 mV to assess whole-cell conductance. Changes in whole cell conductance can be interpreted as changes in channel opening and as such the channel opening in response to changes in CO_2 levels can be observed.

3.4 Results

3.4.1 A motif containing Lysine 125 is conserved only in CO_2-sensitive connexins

It has been previously shown that a subset of β connexins respond to increases in CO_2 with channel opening (Huckstepp *et al.*, 2010a). In order for this specificity to be possible, the sensitive connexins must contain a CO_2 binding moiety that is lacking in non CO_2-sensitive connexins. Based on the hypothesis that carbamate formation may be important in CO_2 binding, sequence comparison was used

identify a conserved lysine residue in the CO_2-sensitive connexins 26, 30 and 32. The potential lysine should also be absent in Cx31, known to be non CO_2-sensitive. Connexin 26 contains 16 lysine residues that could potentially act as CO_2 binding sites. Of those lysines conserved in the CO_2 sensitive connexins, only 2 were absent in Cx31. One of these, Lysine 168 was found to be conserved in other β connexins not yet tested for CO_2 sensitivity, namely connexin 25 (Figure 2).

```
Cx26         1 MDWGTLQSILGGVNKHSTSIGKIWLTVLFIFRIMILVVAAKEVWGDEQADFVCNTLQPGCKNVCYDHYFPISHIRLWALQ  80
Cx30         1 MDWGTLHTVIGGVNKHSTSIGKVWITVIFIFRVMILVVAAQEVWGDEQEDFVCNTLQPGCKNVCYDHFFPVSHIRLWALQ  80
Cx32         1 MNWTGLYTLLSGVNRHSTAIGRVWLSVIFIFRIMVLVVAAESVWGDEKSSFICNTLQPGCNSVCYDHFFPISHVRLWSLQ  80
Cx31         1 MDWKKLQDLLSGVNQYSTAFGRIWLSVVFVFRVLVYVVAAERVWGDEQKDFDCNTRQPGCTNVCYDNFFPISNIRLWALQ  80
Cx25         1 MSWMFLRDLLSGVNKYSTGTGWIWLAVVFVFRLLVYMVAAEHVWKDEQKEFECNSRQPGCKNVCFDDFFPISQVRLWALQ  80

Cx26        81 LIMVSTPALLVAMHVAYRRHEKKRKFMKGEIKNEFKDIEEIKTQKVRIEGSLWWTYTTSIFFRVIFEAVFMYVFYIMYNG  160
Cx30        81 LTFVSTSALLVAMHVAYYRHETARKFIRGEKRNEFKDLEDIKRQKVRIEGSLWWTYTSSIFFRIIFEAAFMYVFYFLYNG  160
Cx32        81 LILVSTPALLVAMHVAHQQHIEKKMLRLEGH-GDPLHLEEVKRHKVHISGTLWWTYVISVVFRLLFEAVFMYVFYLLYPG  159
Cx31        81 LIFVTCPSMLVILHVAYREERERKHRQKHGEHCAKLYSHPGKKH-----GGLWWTYLFSLIFKLIIELVFLYVLHTLWHG  155
Cx25        81 LIMVSTPSLLVVLHVAYHEGREKRHRKKLYV------------SPGTMDGGLWYAYLISLIVKTGFEIGFLVLFYKLYDG  148

Cx26       161 FFMQRLVKC-NAWPCPNTVDCFISRPTEKTVFTVFMISVSGICILLNITELCYLFIRYCSG------------KSKR---  224
Cx30       161 YHLPWVLKC-GIDPCPNLVDCFISRPTEKTVFTVFMISASVICMLLNVAELCYLLLKLCFR------------RSKR-TQ  226
Cx32       160 YAMVRLVKC-EAFPCPNTVDCFVSRPTEKTVFTVFMLAASGICIILNVAEVVYLIIRACAR------------RAQRRSN  226
Cx31       156 FTMPRLVQCASVVPCPNTVDCYIARPTEKKVFTYFMVGASAVCIILTICEICYLIFHRIMRGLSKDKSTKSISSPKSSSR  235
Cx25       149 FSVPYLIKC-DLKPCPNTVDCFISKPTEKTIFILFLVITSCLCIVLNFIELSFLVLKCFIK----------CCLQKYLKK  217

Cx26       225  ----------------------------------------PV---------------  226
Cx30       227  AQRNHP----NHALKESKQNEMNELISD---SGQNAITSFPS---------------  261
Cx32       227  PPSRKGSGFGHRLSPEYKQNEINKLLSEQDGSLKDILRRSPGTGAGLAEKSDRCSAC  283
Cx31       236  ASTCRC----HHKLLESGDLEAV----PADDKLQASAPSLTPI--------------  270
Cx25       218  PQVLSV-------------------------------------------------  223
```

Figure 2 Sequence comparison of β connexins reveals 2 potential CO_2 binding sites. Comparison reveals lysine 168 (blue) and lysine 125 (red) to be conserved in the CO_2-sensitivity Cx26, 30 and 32 and absent in non CO_2-sensitive Cx31. Cx31.1 and Cx30.3, hypothesized to be non CO_2-sensitive, were also compared (not shown) and also lacked these 2 residues.

In the previous chapter we discussed how the lack of CO_2 dependent channel opening in δ and α connexins demonstrated by Huckstepp et al makes it unlikely that Cx31, or the common ancestor it shares with its sub-branch, lost CO_2-sensitivity (Huckstepp *et al.*, 2010a). Instead we hypothesise that a common ancestor of Cx26,

30 and 32 gained this property. If this is correct Cx25 would also lack CO_2-sensitivity and thus the reappearance of lysine 168 in this connexin discounts it from being an important CO_2 binding site (Figure 3).

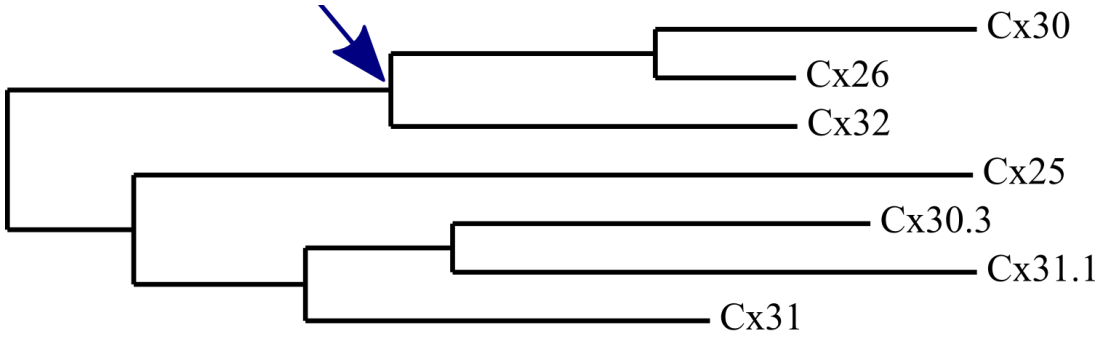

Figure 3 Cx25 does not share a common ancestor with Cx26 that Cx31 does not. As CO_2-sensitivity is thought to have been gained in the smaller subgroup, Cx25 is non CO_2-sensitivity and so Lys168 is not the CO_2 binding site. Arrow points to hypothesized common ancestor that gained CO_2-sensitivity.

The second lysine matching our prior criteria was Lysine 125. This residue is present only in the CO_2 sensitive connexins, Cx26, 30 and 32, being absent in the non sensitive Cx31 and also all connexins evolving from it. Lysine 125 is also of interest as it forms part of a short sequence the entirety of which is absent from non CO_2-sensitive connexins but largely conserved in the CO_2-sensitive connexins. This conserved domain was also found to be conserved not only in mice but also in all mammals thus far investigated, including humans (Figure 4). This is an interesting finding as it suggests a role for this residue not only in our experiments using rat connexins but also in human CO_2 chemosensitivity. The importance of the conservation of these residues in other species will be discussed further in the discussion.

```
Human Cx26            81   LIFVSTPALLVAMHVAYRRHEKKRKFIKGEIKSEFKDIEEIKTQKVRIEGSLWWTYTSSIFFRVIFEAAFMYVFYVMYDG  160
Mouse Cx26            81   LIMVSTPALLVAMHVAYRRHEKKRKFMKGEIKNEFKDIEEIKTQKVRIEGSLWWTYTTSIFFRVIFEAVFMYVFYIMYNG  160
Rat   Cx26            81   LIMVSTPALLVAMHVAYRRHEKKRKFMKGEIKNEFKDIEEIKTQKVRIEGSLWWTYTTSIFFRVIFEAVFMYVFYIMYNG  160
Killer whale Cx26     81   LIFVSTPALLVAMHVAHYRHEKKRKFMKGEIKSEYKDIEEIKSQKVRIEGSLWWTYTGSIFFRVIFEAAFMYVFYVMYDG  160
Naked mole rat Cx26   81   LIFVSTPALLVAMHVAYRRHEKKRKFIKGEMKSEFKDIEEIKSEKVRIEGSLWWTYTSSVFFRVIFEGAFMYVFYIMYNG  160
Dolphin Cx26          81   LIFVSTPALLVAMHVAHYRHEKKRKFMKGEIKSEYKDIEEIKSQKVRIEGSLWWTYTGSIFFRVIFEAAFMYVFYVMYDG  160
Cat Cx26              81   LIFVSTPALLVAMHVAYRRHEKKRKFIKGEIKNEFKDIEEIKSQKVRIEGSLWWTYTSSIFFRVIFEAVFMYVFYIMYDG  160
```

Figure 4 Lysine 125 is conserved in human Cx26. Sequence comparison demonstrates lysine 125 and conserved sequence is conserved in various species including human.

3.4.2 CO_2 binding at Lysine 125 could allow carbamate stabilisation by Arginine 104 on the neighbouring subunit

In order to determine whether this conserved sequence has any functionality in CO_2 sensitivity, the protein was investigated using the existing crystal structure (Maeda *et al.*, 2009). This revealed the conserved sequence to form part of an α helix which orientates Lysine 125 towards Arginine 104 on the neighbouring subunit at a distance of 6.5 Å (Maeda *et al.*, 2009) (Figure 5). We hypothesised that carbamylation of Lysine 125 allows salt bridge formation with Arginine 104. This interaction across the subunit interfaces could provide the conformational changes required for channel opening upon CO_2 binding.

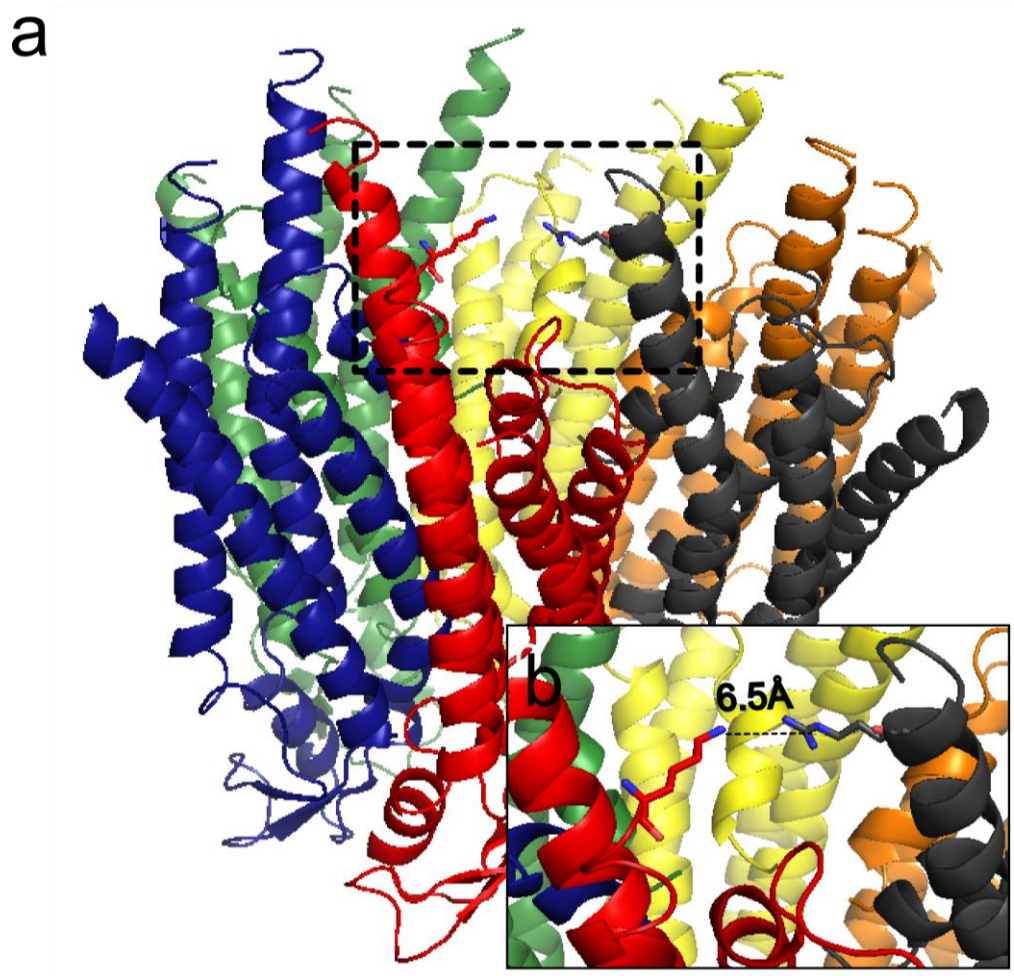

Figure 5 Analysis of Lysine 125 using the protein data bank file (2zw3) of Connexin 26. a) Connexin 26 hexamer, dotted line highlights Lysine 125 on the red subunit. b) Magnification shows conserved domain (red α helix) orientates lysine 125 towards Arginine 104 on neighbouring subunit (grey). The distance between these 2 residues is 6.5A allowing a salt bridge to form after carbamylation.

3.4.3 CO_2-sensitivity is conferred to Cx31 by the inclusion of the conserved sequence containing Lysine 125

To investigate the importance of this conserved region in CO_2 sensitivity, a mutated version of Cx31 was produced. This new protein, mCx31, has the amino acid sequence of Cx31 but with the insertion of 5, and the substitution of 2, conserved amino acids from Cx26 (Figure 6) This mutant contains not only the hypothesised

CO_2 binding site, lysine 125, but the entirety of the sequence conserved in the CO_2-sensitive connexins. This sequence is thought to orientate bound CO_2 towards Arginine 104 in Cx26 and was consequently included to ensure correct orientation in this mutant.

Figure 6 Sequence comparison of Cx31 and mCx31 demonstrating mutation performed. mCx31 differs from Cx31 by the insertion of the sequence TQKVRIE which replaces KH in the original sequence. K is lysine 125 the suspected binding site for CO_2.

mCx31 was investigated for CO_2 chemosensitivity via the dye loading assay. When CO_2 levels were increased to 9% CO_2, increased dye loading was observed in mCx31 expressing HeLa cells (Figure 7). This dyeloading was shown to be significantly different ($p=0.008$) to Cx31 loading and indicates CO_2-sensitivity has been produced in this mutant. This demonstrates the conserved domain containing Lysine 125 to be sufficient for CO_2 dependent opening of connexin channels. In Cx31 residue 104 is a lysine and as such the positive charge required for salt bridge formation is maintained.

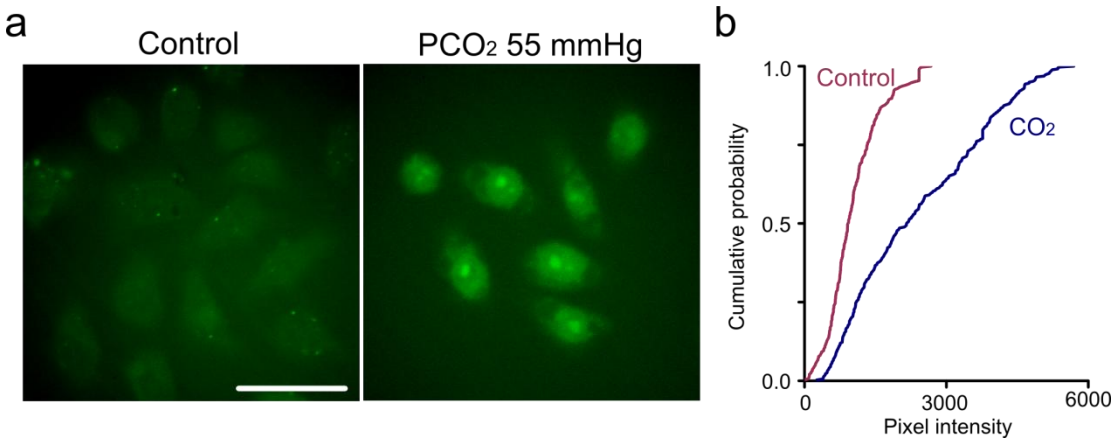

Figure 7 mCx31 is sensitive to CO_2 as analysed by dye loading assay. a) Dye loading images for mCx31 at control and elevated CO_2 levels. mCx31 shows increased dyeloading with elevated CO_2 indicating channel opening. Scale bar 50 µm b) Cumulative probability results for all cells measured during 5 repeats. mCx31 shows a shift towards higher pixel intensities with increased CO_2.

3.4.4 Channel opening in mCx31 is confirmed by whole cell patch clamp analysis

Although the dye loading assay has been previously established, it is not a commonly used assay. In order to assess the validity of the assay and to confirm the result produced, CO_2 chemosensitivity of mCx31 was also assessed by whole cell patch clamp. When CO_2 levels were increased to 55mmHg, mCx31 cells demonstrated a change in whole cell conductance indicative of channel opening (Figure 8). Cx31 expressing HeLa cells were also investigated by whole cell patch clamp studies and no changes in channel conductance were observed with hypercapnic solutions (Figure 8). Previous work by Huckstepp et al showed patch clamp was a suitable assay for demonstrating channel opening in HeLa cells expressing Cx26 (Huckstepp *et al.*, 2010a). For the Cx31 patch clamp experiments a positive control was not possible. This was due to an absence in Ca^{2+} leading to destruction of the seal between patch pipette and HeLa cell of interest. As such the experiment ended before any changes in conductance could be observed.

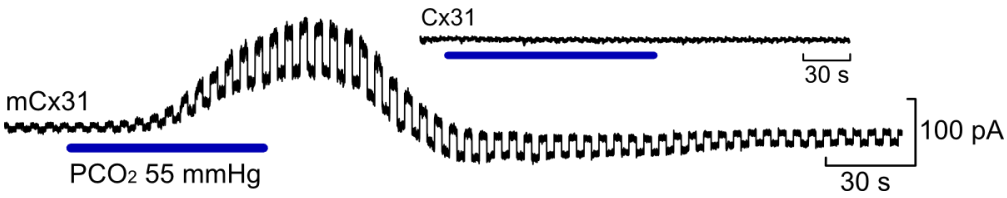

Figure 8 mCx31 is sensitive to CO_2 as demonstrated by patch clamp analysis. Patch clamp results for mCx31 demonstrate channel opening with increases in CO_2. Cx31 shows no conductance change with changes in CO_2 suggesting no CO_2-sensitivity.

3.4.5 CO_2-sensitivity in mCx31 is lost with the removal of Lysine 125

In order to confirm that Lysine 125 is the CO_2 binding site in the conserved region of Connexin 26, mCx31 K125R was produced. This mutant has the same sequence as mCx31 but with lysine 125 replaced with an arginine (Figure 9).

```
mCx31      91 VILHVAYREE RERKHRQKHG EHCAKLYSHP GKTQKVRIEG GLWWTYLFSL IFKLIIELVF 150
mCx31 K125R 91 VILHVAYREE RERKHRQKHG EHCAKLYSHP GKTQRVRIEG GLWWTYLFSL IFKLIIELVF 150
```

Figure 9 Sequence comparison of mCx31 and mCx31 K125R. Replacement of Lys125 with an arginine will prevent carbamylation at this location.

Our hypothesis states that CO_2-sensitivity was conferred to mCx31 from Lysine 125 and not the surrounding residues. If this is correct mCx31 K125R should be non CO_2-sensitive. Arginine residues have a pKa of 12.5 and as a result at physiological pH the amine group will exist in the form of NH_3^+. Lysine residues in contrast have a lower pKa of 10.5. The amine group of this amino acid is therefore less basic and as such more readily loses a proton to exist in the NH_2 form, if present in a restricted hydration space within the protein. As carbamate formation requires the free

electrons on the nitrogen of NH_2, lysines can undergo carbamylation. These electrons form a bond with hydrogen in NH_3^+ and as such arginine residues cannot.

Dye loading analysis of mCx31 K-R revealed no increased dye loading with increases in CO_2 (p=0.028 compared to mCx31) (Figure 10). CO_2-sensitivity of mCx31, and therefore by extension Cx26, depends on lysine 125 which represents a direct CO_2 binding site.

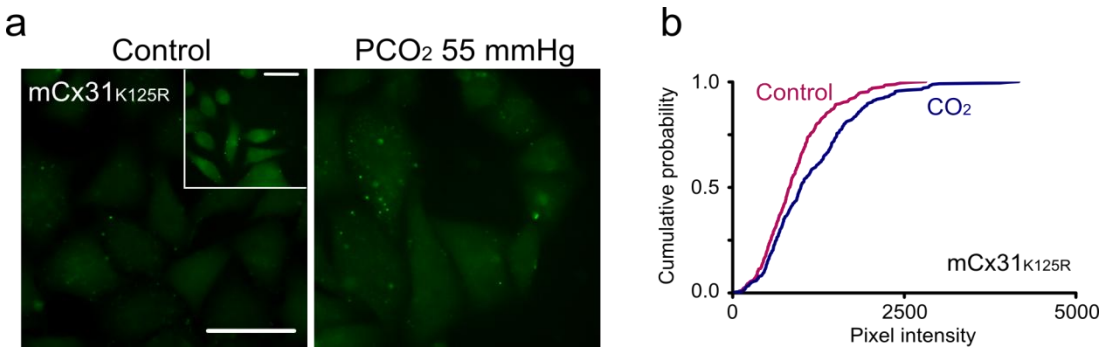

Figure 10 mCx31 K125R is non CO_2-sensitive. a) Dye loading analysis of mCx31 K-R. Inset shows Zero Ca^{2+} loading. All scale bars 50µm. b) Cumulative probability distribution of all cells from 5 repeats.

The Kruskal-Wallis test was used to compare median change in pixel intensity for control and 55mmHg CO_2 for the 3 Cx31 variants: Cx31, mCx31 and mCx31 K-R. This revealed the responses to come from different populations, indicating the response to CO_2 by mCx31 is statistically different to that of Cx31 and mCx31 K-R (p=0.049) (Figure 11).

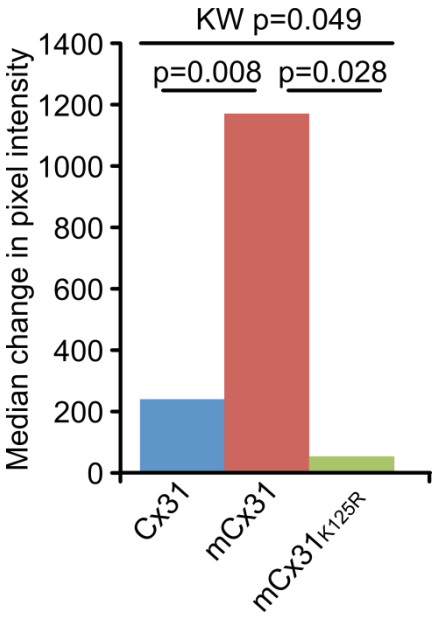

Figure 11 Comparison of the CO_2-sensitvity of mCx31, mCx31 K125R and WT Cx31. Both Mann-Whitney U and Kruskal-Wallis analysis confirms a gain in CO_2-sensitivity in mCx31 mutant

The findings of this chapter were published in the paper "CO_2 directly modulates connexin 26 by formation of carbamate bridges between subunits" (Meigh *et al.*, 2013).

3.5 Discussion

3.5.1 CO_2-sensitive connexins have a conserved motif around Lysine 125

Beginning with the hypothesis that carbamate formation may be important in CO_2 binding, we have identified lysine 125 as being a potential CO_2 binding site, being present only in CO_2-sensitive connexins. By examining the crystal structure of Cx26 it became evident that the conserved sequence forms part of an α helix, that orients Lysine 125 towards Arginine 104 on the neighbouring subunit. The importance of this residue was confirmed when the conservative substitution of this residue by arginine was shown to manipulate CO_2-sensitivity in a Cx31 mutant.

The identification of Lysine 125 as a conserved residue supports carbamylation as the method of CO_2 binding in CO_2 chemosensitivity. As previously described, carbamate interaction require a number of conditions to form. As carbamate bonds produce a permanent negative charge, they require stabilisation through charge neutralisation. We hypothesised that Mg^{2+} could provide this stabilisation based on the previous dependence on Mg^{2+} seen in Cx26 chemosensitivity (Unpublished work). However, analysis of the crystal structure reveals another possibility. The conserved region present around Lysine 125 orientates this residue towards Arginine 104 on the neighbouring subunit with a distance of only 6.5Å. An interaction between these two residues would not only neutralise charged residues but also provide a mechanism through which conformational change could be produced. As connexin 26 is a symmetrical channel with 6 subunits there would be 6 potential CO_2 binding sites and thus 6 intersubunit interactions. This is a way in which the small interaction of CO_2 binding could produce the large conformational change required for channel opening. The importance of this carbamate bridge in CO_2-sensitivity will be investigated by further experimentation. Interestingly if carbamate stabilisation is occurring through an intersubunit salt bridge, Mg^{2+} is not required in this context. Previous work suggested CO_2-sensitivity to be dependent on Mg^{2+} however its role is yet to be elucidated.

An interesting consequence of the identification of Lysine 125 as the potential site of carbamylation is that this residue is on the intracellular side of the membrane. Therefore CO_2 must travel through the membrane to bind at this site. CO_2 can diffuse through some membranes but must be passively trafficked through others,

depending on the fluidity of the membrane concerned. Aquaporin channels, which are involved in H_2O transportation, are known to also traffic CO_2 through non permeable membranes (Boron, 2010). Passive diffusion or aquaporin trafficking are both possibilities in the Cx26 system.

3.5.2 Speculations on the evolution of CO_2-sensitivity

The ability to open in response to CO_2 has been shown to be a unique property of Cx26, 32 and 30 and consequently we hypothesize that this group gained this functionality through random mutation. We have determined that CO_2-sensitivity results from the presence of the conserved region surrounding lysine 125 and as such we would expect the pivotal point in the evolution of CO_2-sensitvity would be the insertion of these residues, probably through a duplication error in the ancestral gene. Interestingly a positively charged residue (K or R) is present at residue 104 in both CO_2-sensitive and non sensitive connexins. This suggests this residue existed prior to the duplication error and through this serendipitous event CO_2-sensitvity in connexin proteins was produced.

All mammals investigated thus far appear to maintain this conserved region. This indicates it to be of paramount importance physiologically and to have appeared early in evolutionary history. Killer Whale, Dolphin and Naked mole rat were amongst the mammals tested for these residues of interest (Figure 4). These animals are of particular interest as due to their habitat constituting a high CO_2 environment. Naked mole rats live in underground colonies of up to 300 animals. The cramped quarters coupled with the poor ventilation leads to CO_2 levels higher than at the surface. Killer whales and dolphins can only breathe when they are at

the surface and therefore must hold their breath for long periods of time leading to an increase in blood CO_2. In both of the environments, CO_2-sensitivty of the animals must be reduced to prevent CO_2 evoked responses at what for these animals is "normal" CO_2. Interestingly, these animals maintain the conserved domain and arginine 104 and consequently this is not the route of the differences in CO_2-sensitivity seen. Several subtle differences in the sequence of Cx26 can be seen in these animals. These may reflect changes to the tertiary structure of the protein, affecting the ease with which CO_2 can bind and therefore the sensitivity of the species to changes in CO_2.

In this chapter we have demonstrated that CO_2-sensitivity can be conferred on the non sensitive Cx31 by the presence of Lysine 125. To investigate the physiological relevance of this finding it is important to determine the role of these residues in Cx26. Analysis of the crystal structure of Cx26 also revealed a possible role of Arginine 104 in changing channel conformation. Further experimentation will investigate the roles of arginine 104 and lysine 125 in Cx26 will aim to investigate the importance of this interaction in this natively CO_2-sensitive protein.

Chapter 4- CO_2-sensitivity in Cx26 occurs via intersubunit salt bridge formation

4.1 Abstract

In the previous chapter I demonstrated that the presence of a lysine residue at position 125 of Cx31 is sufficient to confer CO_2 sensitivity to this previously non-sensitive connexin. We hypothesize that carbamate formation at this location leads to channel opening via a salt bridge interaction with Arginine 104. In order to relate this discovery to physiologically relevant CO_2 chemosensitivity, it is important to demonstrate these residues also have a role in the connexin proteins normally present in chemosensitive regions of the brain. Using mutational studies we demonstrated that the removal of lysine 125 or arginine 104 in connexin 26 lead to a loss of CO_2-dependent channel opening. We also performed mutational studies to mimic a salt bridge interaction between these two residues. These results strongly support our hypothesis that carbamate formation at lysine 125 and the subsequent formation of a salt bridge to arginine 104 on a neighbouring subunit is capable of producing channel opening. Elastic Network Modelling studies performed indicate that a salt bridge interaction between these residues restricts channel movement biasing the channel towards an open state.

4.2 Introduction

4.2.1 Cx26 in Chemosensitivity

Connexin 26, a gap junction protein, has been suggested to have a potential role in CO_2 chemosensitivity due to the presence of hemichannels in the medulla oblongata. Immunohistochemistry studies reveal this connexin to be highly

localised to the first 300µm of tissue (Huckstepp *et al.*, 2010b). Further evidence supporting a role for Connexin 26 in CO_2 chemosensitivity is this hemichannel's ability to open in response to elevated CO_2. This has been demonstrated both in medullary slices and cultured HeLa cells and was capable of releasing ATP, a signalling molecule known to be an important in the CO_2 chemosensitive response (Huckstepp *et al.*, 2010b; Huckstepp *et al.*, 2010a). Using our knowledge of the CO_2 chemosensitivity of Cx26 and the non-sensitivity of Cx31 (Chapter 2), we were able to identify both a potential binding domain for CO_2 and a mechanism through which channel opening could occur (Chapter 3). Carbamate bond formation at lysine 125 results in a negative charge being located in close proximity to the positively charged arginine 104, on a neighbouring subunit. A salt bridge between these two residues could hypothetically restrict channel movement. This could cause the channel to remain in its open state when CO_2 was bound resulting in the CO_2 dependent channel opening we observe.

4.2.2 Experimental strategies to test the carbamylation hypothesis

Loss of function mutations

In order to test the mechanism hypothesised we must first establish the importance of both of these residues in Cx26. Replacement of lysine 125 with an arginine residue prevents CO_2 binding as carbamate formation requires the amine group to be in the NH_2 form. Lysine residues have a pKa of 10.5 and these residues are capable of proton donation at physiological pH when located in areas of restricted hydration. Arginine residues however have a pKa of 12.5 making them highly basic. As a result they are poor proton donors and with remain in their protonated form

at physiological pH regardless of the environment they are in. Arginine consequently cannot act as a CO_2 binding site. The production of a Cx26 K125R mutant therefore allows us to test the hypothesis that lysine 125 represents the CO_2 binding site in Cx26.

We have also identified arginine 104 as being important in salt bridge formation and therefore channel opening. This interaction relies on the positive charge associated with the arginine residue. Replacement of arginine 104 with an alanine residue would prevent this interaction. Loss of CO_2 chemosensitivity in a Cx26 R104A mutant would thus produce the first direct evidence for the formation of a salt bridge as being important for the detection of CO_2.

Gain of function mutations

Testing the carbamylation hypothesis with a gain of function strategy could be achieved by mimicking the salt bridge that would form on carbamylation, by introducing appropriately charged residues. In order to mimic this hypothetical bridge experimentally, we can place a permanent negative charge at position 125, e.g. by the mutation K125E. The glutamate at this position could be regarded as an analogue of the carbamylated lysine. We would predict that the mutated channel would be constitutively open but insensitive to CO_2.

Alternatively we could put a negative charge at position 104 e.g. by the mutation R104E. Under control conditions this would effectively reverse the carbamate bridge, with the positive residue now being present at position 125. Once again we would predict that the mutated channel would be constitutively open but

insensitive to CO_2. Furthermore constitutive channel opening in this mutant channel, as indicated through dye loading experiments, would suggest that an electrostatic interaction between these two residues is responsible and sufficient for the CO_2 chemosensitivity of Cx26.

4.2.3 Elastic Network Modelling to test the carbamylation hypothesis

Mutational studies are not the only method that can be used to test the carbamylation hypothesis, computational models can also be employed. Previously, the molecular modelling of proteins has been a complicated process requiring the model to include calculations of the deformities in bond angles, lengths and rotation as well as changes in repulsion effecting electrostatic interactions. As a result, modelling protein molecules often took a prohibitive amount of computational power. This was changed in 1996 when Tirion adapted the concept of Elastic Networking models to work with proteins (Tirion, 1996). In these models bond angles and lengths are considered to be constant due to the limited flexibility that the covalent bonds allow. Instead the rotations of the bonds alone are used to model appropriate protein movements. In these simulations, carbons of the protein backbone represent the nodes of the model. These are connected by springs to represent the bonds between them. An appropriate cut-off distance is chosen to allow springs to represent not only covalent bonds but electrostatic interactions. All springs are equivalent and do not take into consideration specific bond properties which result in the simplicity of this model (Tirion, 1996; Rodgers *et al.*, 2013a) Once a model has been produced for a protein of interest the

molecular motion of the protein can be investigated. Movements requiring the lowest energy are likely to be accurate for a protein. These models can also be used to compare protein movements under different conditions. We hypothesize that salt bridge formation is responsible for channel opening. By mimicking a bond the salt bridge bond and comparing it to the unbound state, we can determine how this effects the movement of the channel.

4.3 Methods

Experiments pertaining to Cx26 K125E and Cx26 R104E were performed in conjunction with Sophie Greenhalgh, then an undergraduate at the University of Warwick, who worked under my direct supervision.

4.3.1 Mutant connexin production

Cx26 K-R was synthesised by Genscript USA and subcloned into a pCAG–GS vector.

Cx26 K-E, Cx26 R-E and Cx26 R-A mutants were produced in the lab using the Agilent Quikchange protocol. The pCAG-GS vector containing Cx26 was shown to be unsusceptible to mutation through the quikchange method. To allow mutation Cx26 was first subcloned into a PUC 19 vector. Once successful subcloning was confirmed by sequencing, site directed mutagenesis was performed to achieve the desired mutation.

The primers for mutation are as follows: Cx26 K-E forward 5’ AAG AGA TCA AAA CCC AGG AGG TCC GTA TCG AAG GG 3’ reverse 5’ CCT TCA TGA ACT TCT CTT TCT

TTT CGT GTC TCC GGT AAG CC 3', Cx26 R-E forward 5'GGC CTA CCG GAG ACA CGA AAA GAA AGA GAA GTT CAT GAA GG 3'reverse 5' CCT TCA TGA ACT TCT CTT TCT TTT CGT GTC TCC GGT AAG CC 3', Cx26 R-A forward 5' GGC CTA CCG GAG ACA CGA AAA GAA AGC GAA GTT CAT GAA GG 3' reverse 5' CCT TCA TGA ACT TCG CTT TCT TTT CGT GTC TCC GGT AGG CC 3'.

In keeping with the quikchange manual, the PCR reaction was performed over 16 cycles to achieve a single amino acid change. The extension time was selected as 6 minutes which allowed for the advised 1 minute per Kb of DNA with the vector, mCherry and gene accounting for 5.1 Kb. The PCR reaction was then digested for 1h at 37 degrees with 1 µl Dpn1. This enzyme digests methylated DNA and thus removes the template, leaving only the newly synthesised, mutated DNA. Agarose gel electrophoresis confirmed that synthesised DNA was present and therefore that the PCR reaction had been successful. Heat shock transfection into Top 10 E.Coli cells and subsequent DNA amplification and DNA purification through Fermentas miniprep kit allowed the mutation to be confirmed through sequencing. The mutated connexin were finally subcloned into a pCAG-GS vector for use in dyeloading analysis.

4.3.2 HeLa cell culture

Cells were cultured as detailed in the previous chapters (Chapter 2). Cells were plated onto coverslips in 6 well plates at a concentration of 5 x 10^4 cells per well. Using genejuice transfection reagent cells were transiently transfected with 1µg of the gene of interest.

4.3.3 Dye loading

For mutants Cx26 K125R and Cx26 R104A dyeloading was performed as outlined in Chapter 2, performing control, CO_2 and zero Ca^{2+} experiments. For mutants Cx26 K125E and Cx26 R104E no zero Ca^{2+} was performed as it was expected that these mutants would be constitutively open. Instead the sensitivity of dye loading to carbenoxolone was examined. As carbenoxolone is a connexin channel blocker, we used this to test whether any constitutive dye loading was due to the misexpressed mutant Cx26 as opposed to some other endogenous pathway. During carbenoxolone experiments, cells were exposed to control aCSF containing 200µM carboxyfluorescein and 100µM Carbenoxolone for 10 minutes followed by 5 minutes of control aCSF with 200 µM carboxyfluorescein. The cells were then washed with control aCSF for 30 minutes to remove external carboxyfluorescein (Figure 1).

Control and CO_2 experiments were performed as described in Chapter 2.

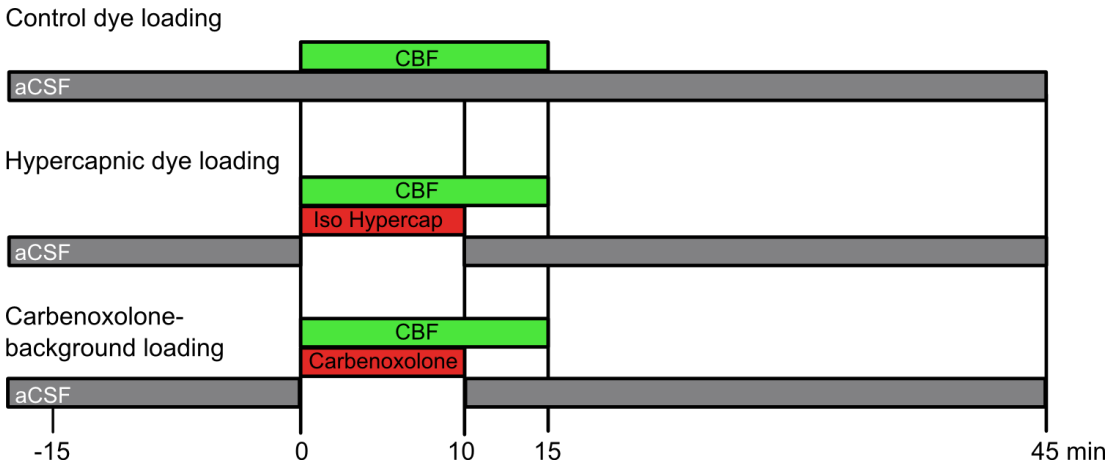

Figure 1 Graphical representation of dyeloading protocol, detailing control, hypercapnic and carbenoxolone experiments

4.3.4 Data capture and analysis

Cells were imaged using epifluorescence as descrbed in chapter 2. Experiments were repeated at least 5 times, with each repeat being performed on cells from a seperate transient tranfection.

Images were analysed through Image J. Background adjusted mean pixel intensities were used to produce cumulative probability distributions as described previously (Chapter 2). Mutants Cx26 K-R and Cx26 R-A were compaired to WT Cx26 and each other through the Mann Whitney and Kruskal Wallace statistical significance tests by comparing median differences in CO_2 responses.

Mutants Cx26 K-E and Cx26 R-E were analysed differently with dyeloading responses compared for Control, CO_2 and Carbenoxolone responses for each mutant separately. We compared the three conditions (control CO_2 and carbenoxolone) with the Kruskal-Wallis ANOVA and performed pairwise comparisons with the Mann Whitney U- test.

4.3.5 Elastic Network modelling

These simulations were performed by Martin Cann and Thomas Rodgers at Durham University using the information provided by us as to the location of the carbamate bridges.

Elastic network model simulations were produced using the protein data bank file 2ZW3. In this model the protein is simplified by representing it as protein backbone carbons (Cα) connected by Hookean springs (Tirion, 1996; Rodgers *et al.*, 2013a). All bonds were represented as an equivalent spring with a spring constant of 1kcal mol^{-1} $Å^{-2}$. The cut-off radius for spring formation was 8 Å. This cut-off radius allows non covalent interactions to also be included in the model. For the CO_2-bound protein an additional spring was included between residues K125 and R104 on the neighbouring subunit to mimic the intersubunit bond(Rodgers *et al.*, 2013b) (Figure 2).

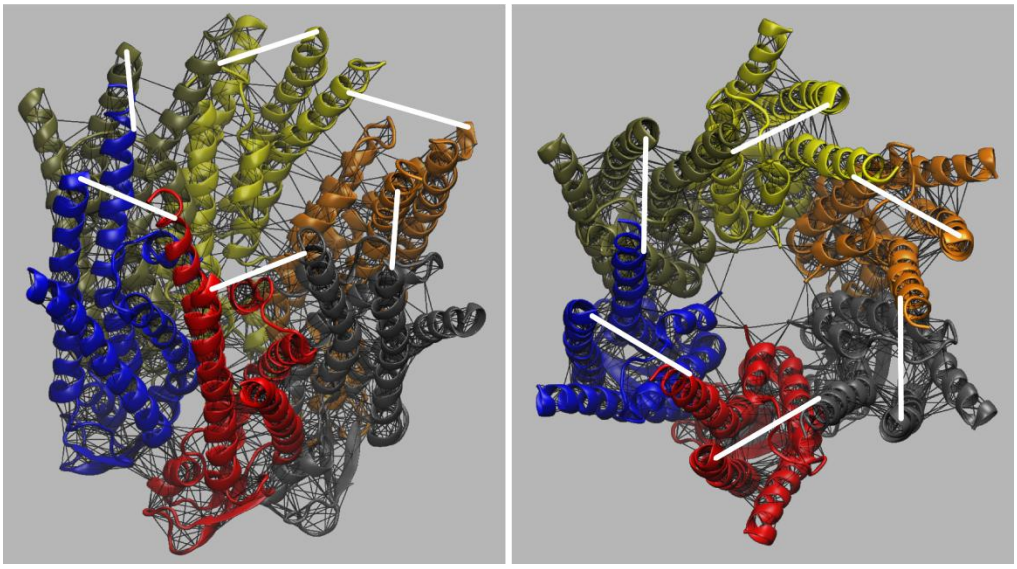

Figure 2 Modelling of Cx26 hemichannel. Modelled from the PDB file with balls representing α carbons, connected by springs. White lines represent additional springs inserted to model the carbamate salt bridge hypothesis

4.4 Results

4.4.1 Cx26 loses CO_2-sensitivity with the loss of lysine 125

As with the previous chapters, CO_2-sensitivity of the synthesised mutants was measured using the dye loading assay. Previous experiments have demonstrated that the presence of lysine 125 in Cx31 is sufficient to confer CO_2-sensitivity to this previously non CO_2-sensitive connexin. In order to confirm these results and to test their validity in the physiological CO_2 chemosensitive response, these experiments were repeated in Cx26, the connexin family member of interest. The Cx26 K125R mutant differs from Cx26 in a single amino acid substitution, Lysine 125 to an arginine residue (Figure 3). As arginine residues cannot form carbamate bonds, the native CO_2-sensitivity of Cx26 would be lost if the hypothesised mechanism is correct.

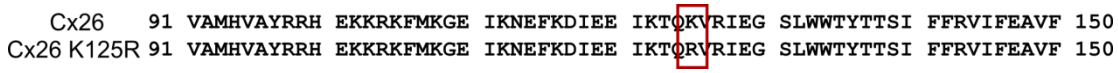

Figure 3 Sequence comparison of Cx26 and Cx26 K125R demonstrating mutation performed. Cx26 K125R differs from Cx26 by the amino acid substitution of lysine 125 to arginine. This mutation prevents carbamate formation at residue 125.

Dye loading analysis of Cx26 K125R demonstrated no increases in dye uptake in response to an increase to 9% CO_2 ($p=0.004$ compared to WT Cx26) (Figure 4). To ensure connexin channels were functioning correctly a positive control was performed by exposing the HeLa cells to carboxyfluorescein in the absence of calcium. This confirmed the presence of functional hemichannels through increased dye loading (Figure 4). The CO_2-sensitivity of Cx26 therefore requires a lysine residue at position 125. The dependence of CO_2-sensitivity on the presence of this residue suggests direct binding and supports the carbamate hypothesis for CO_2 dependent channel opening.

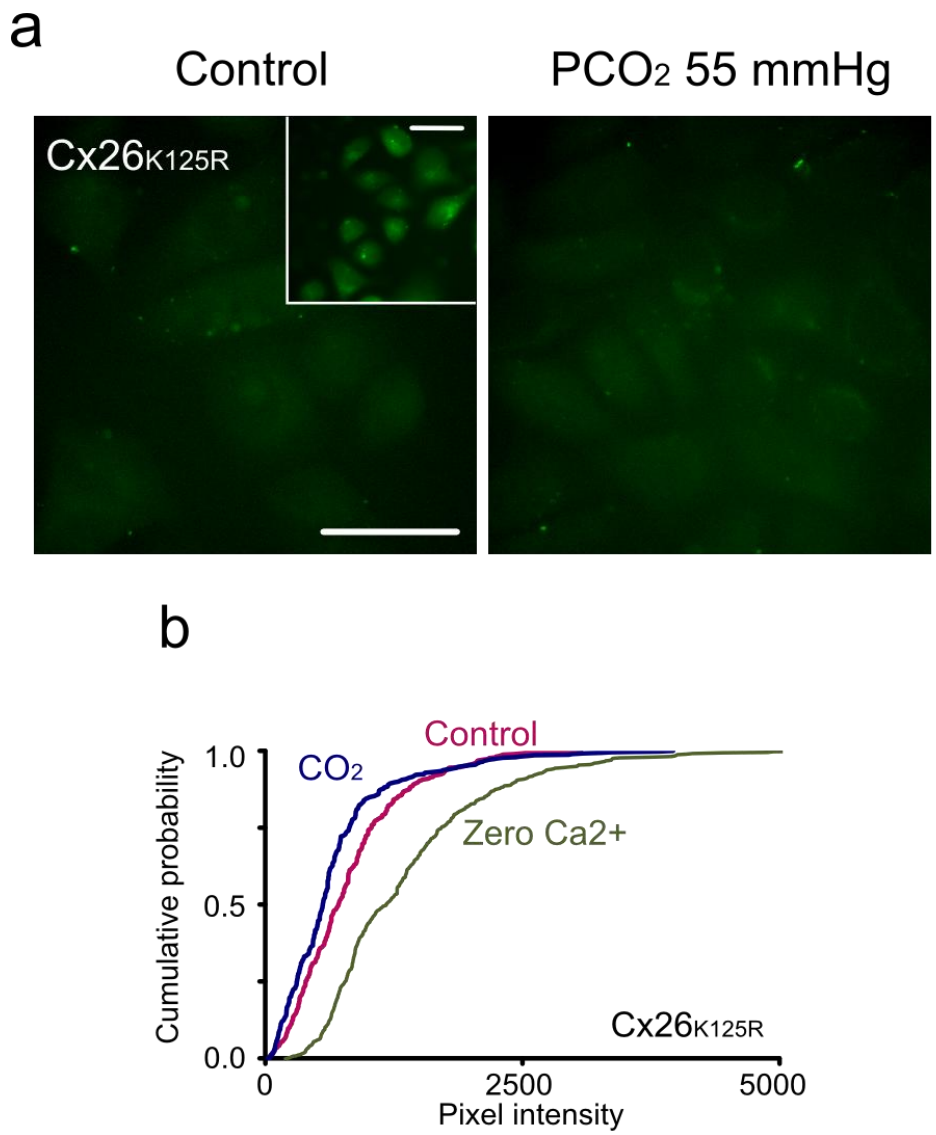

Figure 4 Cx26 K125R is not sensitive to CO_2 when detected by dye loading assay**.** a) Dye loading images for Cx26 K125R. All scale bars represent 50µm. Inset shows zero Ca^{2+} positive control. b) Cumulative probability graphs of all cells measured in the 5 repeats.

4.4.2 Arginine 104 is also important for Cx26 CO_2-sensitivity

Previous results demonstrate that the interaction of CO_2 with lysine 125 appears to produce CO_2 dependent channel opening in connexin proteins. As a conformational change is required for channel opening, it follows that the carbamate produced must interact with other parts of the channel in order to produce the required change in conformation. We suggest a salt bridge between the negative charge at position 125 and the positive charge at arginine 104 on the neighbouring subunit could provide the required conformational change. Electrostatic interactions require a distance of less than 4Å to take effect (Kumar & Nussinov, 2002). Residues 125 and 104 on neighbouring subunits are separated by a distance of 6.5Å with the carbamate bond projecting approximately 2.9 Å into this space (C-O 1.43Å, C-N 1.47Å) (Allen *et al.*, 1987). Although these distances could vary due to charge effects on bond length and angle, the charged residues are separated by approximately 3.6Å making a salt bridge interaction possible. In order to determine whether an intersubunit salt bridge is responsible for channel conformational change, the Cx26 R-A mutant was produced (Figure 5). Unlike Cx26 K125R, this mutant maintains a lysine at position 125 and therefore has the potential to bind CO_2. This mutant varies from previous mutants by the replacement of arginine 104 with an alanine residue. This substitution removes the positive charge that would be required for the electrostatic interaction between subunits.

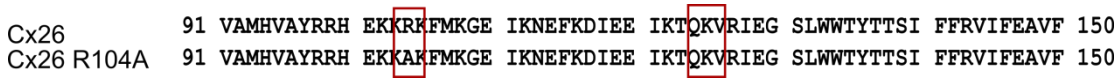

Figure 5 Sequence comparison of Cx26 and Cx26 R104A demonstrating mutation performed. These sequences differ due to an amino acids substitution at arginine 104 to alanine. This removes the other side of the salt bridge hypothesised to be involved in CO_2 chemosensitivity.

Dye loading analysis of this mutant revealed a loss of CO_2-sensitivity ($p=0.016$ compared to WT Cx26) (Figure 6). As with previous mutants, proper channel functionality and location in the membrane were confirmed using a "zero Ca^{2+}" positive control. These experiments confirm that a positive charge at position 104 is required for CO_2-sensitivity in Cx26 and are therefore in agreement with the salt bridge hypothesis.

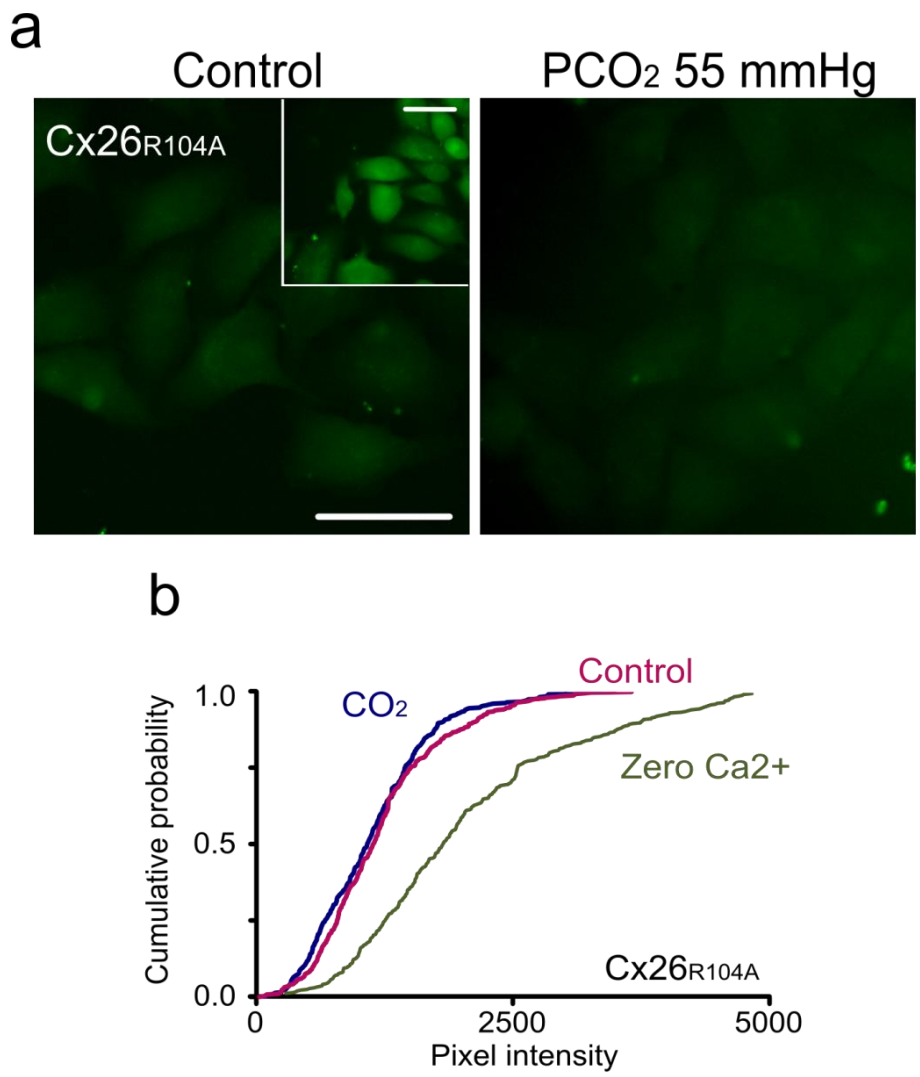

Figure 6 Cx26 R104A is not sensitive to CO_2 as measured by dye loading assay. a) Dye loading images for Cx26 R-A. All scale bars represent 50 µm. Inset shows zero Ca^{2+} positive control. b) Cumulative probability distribution of all cells measured over the 5 repeats.

The three Cx26 variants, WT Cx26, Cx26 K125R and Cx26 R104A, were investigated by the Kruskal-Wallis ANOVA and pairwise testing with the Mann-Whitney U test. Changes in median pixel intensity in response to elevated CO_2 were compared. It was found that these mutants belonged to separate populations (p=0.01) revealing CO_2-sensitivity to be lost in the Cx26 K125R and Cx26 R104A mutants (Figure 7).

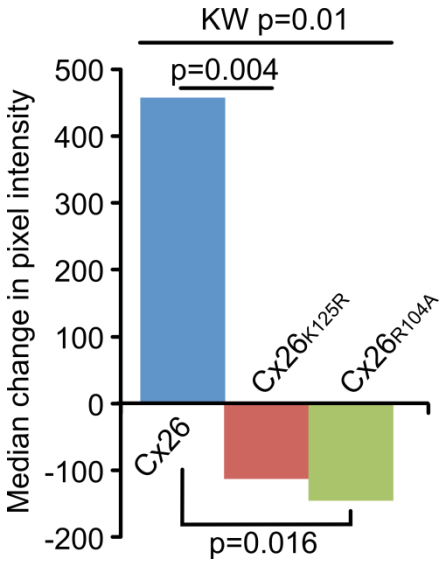

Figure 7 Comparison of the CO_2-sensitivity of Cx26 K125R, Cx26 R104A and WT Cx26. Both Mann-Whitney U and Kruskal-Wallis support a loss of CO_2-sensitvitity in the mutants compared with WT Cx26.

4.4.3 Connexin 26 channel opening can be induced by a negative charge at position 125

We have demonstrated that residues lysine 125 and arginine 104 of Cx26 are required for CO_2 chemosensitivity. We suggest that CO_2 binding at lysine 125 is the key to inducing the conformational change required for CO_2-sensitivity, through salt bridge formation to residue arginine 104 on the neighbouring subunit. This salt bridge can occur due to the negative charge produced when CO_2 binds to the lysine side chain producing a carbamate. In order to investigate the importance of this interaction, a mutated connexin was produced to mimic the negative charge produced upon CO_2 binding. Cx26 K125E differs from Cx26 by having a glutamate residue at position 125 (Figure 8). As glutamate has a negatively charge side chain that protrudes into the cavity in which CO_2 would bind, it could potentially act to replicate the effect of a carbamate group at this position (Figure 9).

```
Cx26       91 VAMHVAYRRH EKKRKFMKGE IKNEFKDIEE IKTQKVRIEG SLWWTYTTSI FFRVIFEAVF 150
Cx26 K125E 91 VAMHVAYRRH EKKRKFMKGE IKNEFKDIEE IKTQEVRIEG SLWWTYTTSI FFRVIFEAVF 150
```

Figure 8 Sequence comparison of Cx26 and Cx26 K125E demonstrating mutation performed. Glutamate at position 125 mimics the binding of CO_2 to lysine 125 to determine whether this interaction is important in channel opening.

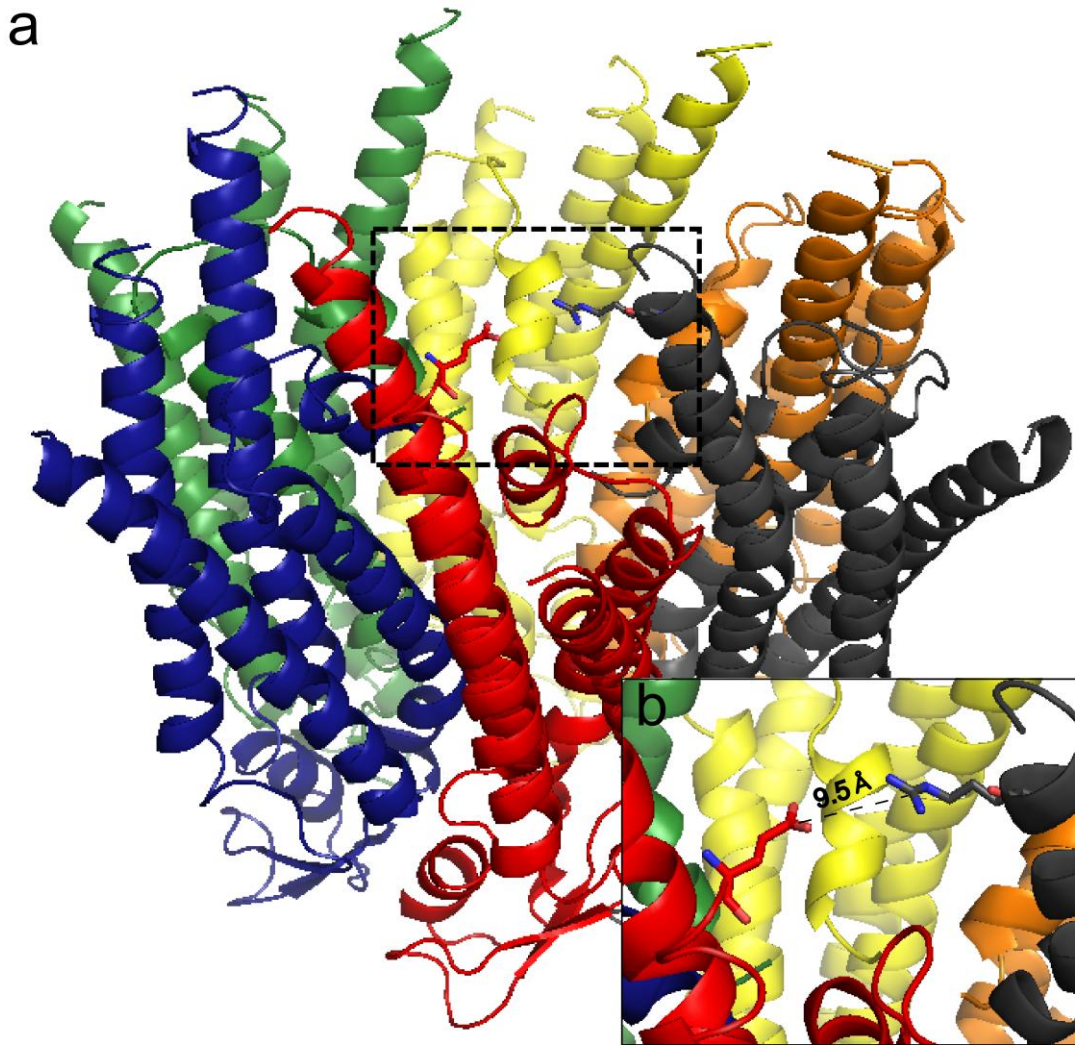

Figure 9 Structure of Cx26 K125E. a) Cx26 hexamer with residues of interest highlighted. b) Glutamate 125 (red) and Arginine 104 (grey) are 9.5Å apart in new mutant.

We found that HeLa cells expressing Cx26 K125E exhibited a high degree of constitutive dye loading in the absence of a CO_2 stimulus (Figure 10). Furthermore,

the mutated channel did not exhibit any further dye loading above this constitutive background when exposed to a hypercapnic stimulus (Figure 10).

In order to confirm that the high level of dye loading under control conditions was due to constitutive opening of Cx26 K125E, a new control experiment was designed. We tested whether the connexin blocker, carbenoxolone, to block the mutant channels. This method was chosen as carbenoxolone had previously been demonstrated to block ATP release via Cx26 (Rozental *et al.*, 2001; Huckstepp *et al.*, 2010b) and my results from Chapter 2 demonstrated that carbenoxolone was effective at blocking CO_2-dependent dye loading in WT Cx26-expressing HeLa cells. The presence of 100µM carbenoxolone greatly reduced dye loading into the Cx26 K125E-expressing HeLa cells compared to dye loading under control conditions ($p=0.004$) (Figure 10). This demonstrates that the Cx26 K-E mutant the connexin hemichannels are constitutively open. There was no statistically significant difference between the dye loading of WT Cx26-expressing HeLa cells in the control and carbenoxolone conditions ($p=0.274$). This indicates that WT Cx26 hemichannels (unlike those of Cx26 K125E) are mostly closed under control conditions in normal aCSF. These results indicate a permanent negative charge at lysine 125 locks the channel in an open state and suggests carbamate formation at this location could cause the conformational changes required for CO_2 dependent channel opening.

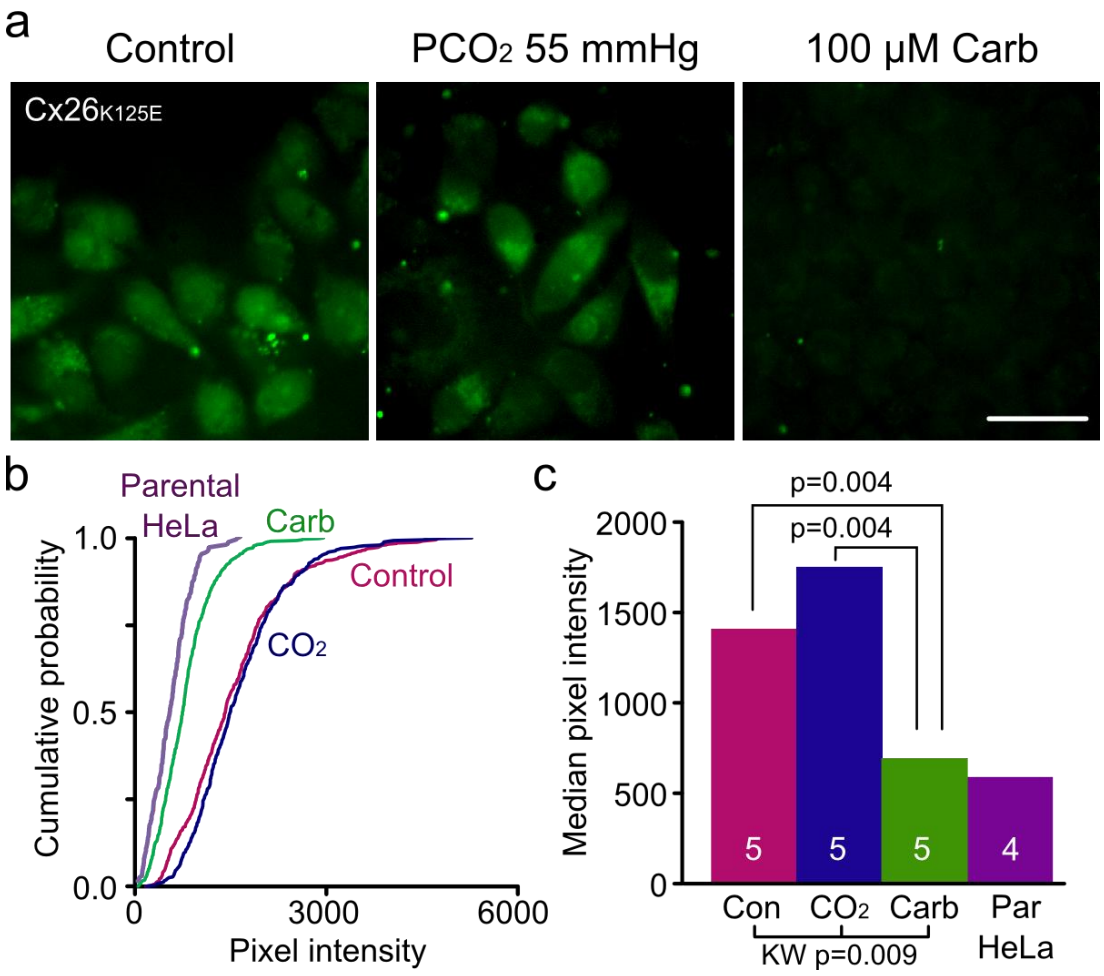

Figure 10 Cx26 K125E is constitutively open as shown by dye loading assay. a) Dye loading images for Cx26 K-E for control, elevated CO_2 and connexin blocker conditions. Scale bar represents 50µm b) Cumulative probability distribution for all cells measured over 5 repeats. Also present are parental HeLa cell data. c) Median pixel intensity data for control, CO_2, Carbenoxolone and parental HeLa conditions, displaying Mann-Whitney and Kruskal-Wallace statistical test data.

4.4.4 A salt bridge in either direction is capable of channel opening

The Cx26 K125E mutant has demonstrated that an intersubunit interaction between the positively charged arginine 104 and a negative carbamate group at lysine 125 is capable of inducing channel opening. If this interaction were solely responsible for the conformational change observed, it should be possible to reverse the interaction and still observe channel opening. In order to demonstrate this, we made the mutant Cx26 R104E. In this mutant lysine 125 remains present

but arginine 104 is now replaced with a glutamate residue (Figure 11). The glutamate residue introduces a negative charge at position 104. In the absence of CO_2, lysine 125 could be positively charged and form a salt bridge with the E104 of the neighbouring subunit. This potentially allows a reversal of the salt bridge normally produced in the CO_2-sensitive connexins.

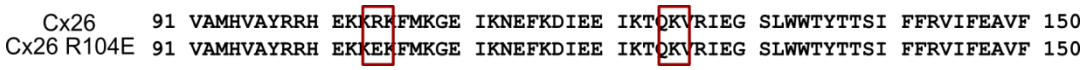

Figure 11 Sequence comparison of Cx26 and Cx26 R104E demonstrating mutation performed. Glutamate at position 104 mimics a salt bridge between residues 104 and 125 to determine whether this interaction can cause channel opening.

We found a high degree of constitutive dye loading under control conditions in aCSF that was blocked by carbenoxolone (p=0.004) (Figure 12). As with the previous mutation K125E, this result supports the idea that a salt bridge interaction between residues 104 and 125 on neighbouring subunits results in a constitutively open channel. As channel opening can be observed with a salt bridge made in either direction, these data support the interaction between residues 104 and 125 on adjacent subunits as the key to inducing conformational change.

In the presence of increased CO_2, no change in dye loading, was observed in the Cx26 R104E mutant (p=0.579 c.f. control loading) (Figure 12). As lysine 125 is present in this mutant, CO_2 could in theory bind to this residue. This would remove the positive charge required to maintain the channel in the constitutively open mutant and might be expected to reduce dye loading. However, no change with hypercapnia was observed, suggesting that CO_2 cannot bind at this position.

The loss of CO_2-sensitivity may arise for two related reasons. Firstly, in this mutant there is no longer a positive charge at residue 104 to stabilise the negatively-charged carbamate, in fact there is now a negative charge. This makes it less likely that CO_2 would bind to lysine 125. Secondly, channel opening in Cx26 R104E requires that K125 be positively charged to allow intersubunit salt bridge formation. It is also possible that the presence of R104E may alter the chemical environment to favour the protonated form of the K125 side chain. Because carbamate bonds cannot form at protonated amine groups, this is a further reason why Cx26 R104E no longer interacts with CO_2.

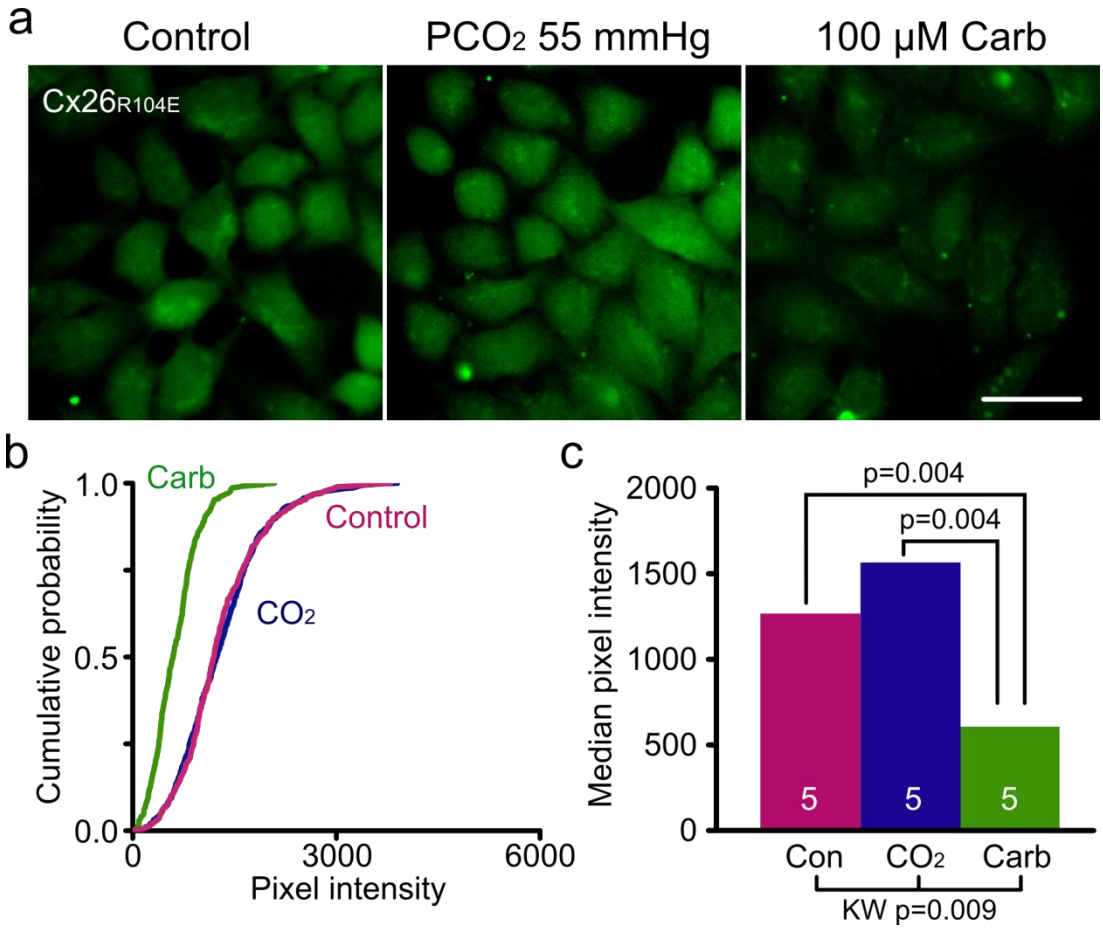

Figure 12 Cx26 R104E is constitutively open as shown by the dye loading assay. a) Dye loading images of Cx26 R-E for control, elevated CO_2 and connexin blocker conditions. Scale bar represents 50µm b) Cumulative probability distribution for all cells measured over 5 repeats. c) Mean pixel intensity data for control, CO_2 and Carbenoxolone, displaying Mann-Whitney and Kruskal-Wallace statistical test data.

4.4.5 Elastic Network Modelling studies indicate channel opening with salt bridge between residues 125 and 104 on neighbouring subunits

We hypothesize that salt bridge formation between lysine 125 and arginine 104 induces channel opening. To provide support for the hypothesis that an electrostatic interaction between these residues is capable of producing a conformational change, collaborators at the University of Durham produced Elastic Network models for Connexin 26. These models use the X-ray crystallography

structure of connexin 26 to model the protein as a series of alpha carbons with the bonds between them modelled as springs. The motion of the entire protein in the EMN is split into a number of modes which describe movements within that protein. The modes of movement were compared for Cx26 and Cx26 with CO_2 bound, replicated by an extra spring joining lysine 125 and arginine 104 on the neighbouring subunit.

In the unbound model the lowest frequency mode was found to represent full opening and closure of the channel through occlusion of the channel pore by the α helices. This mode accounted for 40% of total protein movement. In the CO_2 bound model, this mode had dropped to account for only 2% of global protein movement. This means in the CO_2 bound state the channel is restricted and can no longer become fully occluded (Figure 13).

These results demonstrate that the hypothesized salt bridge between arginine 104 and lysine 125 is a plausible mechanism by which to bring about conformational changes in Cx26. The ENM indicates this is due to restricting channel movement upon CO_2 biding which causes the channel to become locked in an open state.

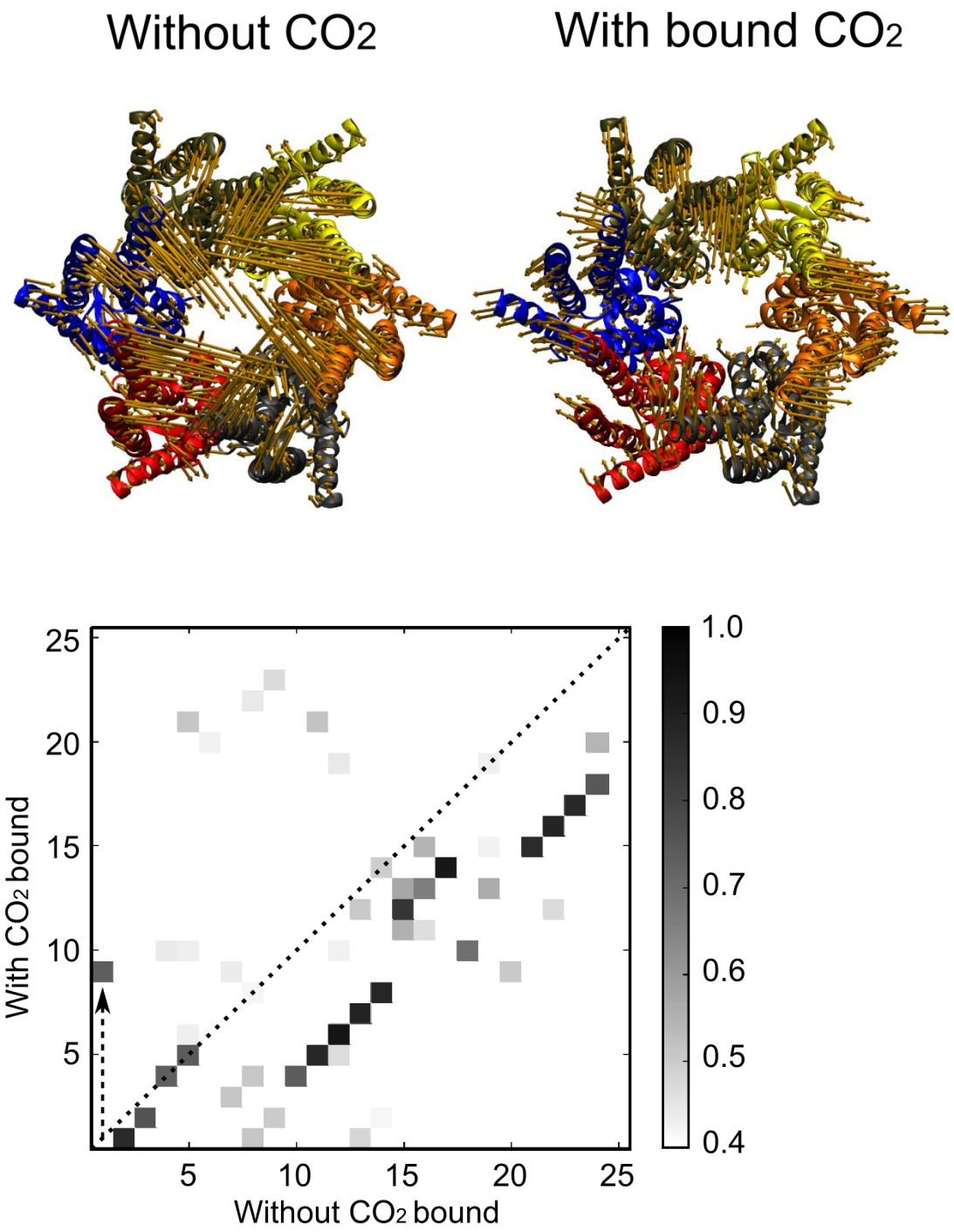

Figure 12 Bound CO_2 changes the mode of Cx26, biasing it towards an open state. Without CO_2 bound channel closure is mode one. With CO_2 bound this changes to mode 9, indicating CO_2 binding prevents channel closing. Constructed from elastic network modelling.

The findings of this chapter have been published as part of the paper "CO_2 directly modulates connexin 26 by formation of carbamate bridges between subunits" (Meigh *et al.*, 2013).

4.5 Discussion

4.5.1 Lysine 125 is required for CO_2 sensitivity in Cx26

In the previous chapter (Chapter 3) we demonstrated that the presence of lysine 125 conferred CO_2 chemosensitivity to the previously non-sensitive Cx31. For physiological CO_2-sensing, Cx26 is the connexin of interest as it is found in areas of the medulla oblongata which are known to be involved in the chemosensory control of respiration (Feldman *et al.*, 2003). In order to confirm that our previous findings could also be applied to Cx26, mutation studies were performed to remove lysine 125 from this protein. We demonstrated that the replacement of this lysine residue with an arginine prevented the CO_2 dependent dye loading of Cx26 expressing HeLa cells as documented in this and previous studies. This supports our hypothesis that lysine 125 provides a direct binding site for CO_2. As connexin 26 is known to be located in the medulla oblongata, a known site of CO_2 sensitivity, its ability to directly bind CO_2 provides a mechanism through which pH-independent CO_2 chemosensitivity can occur.

4.5.2 Arginine 104 is also required for CO_2 chemosensitivity

In order for CO_2 binding to produce channel opening, this small action must be capable of producing large conformational changes. In the previous chapter we identified arginine 104 as having a potential role in this process due to its close proximity to the site of carbamate formation. Carbamates have a negative charge

and require interaction with positive charges to be stabilised. As residue 104 of the neighbouring subunit is less than 4Å from the carbamate, a salt bridge is possible. In this chapter we determined that arginine 104 is important in CO_2-dependent channel opening of Cx26. Mutation of arginine 104 to remove the positive charge prevents channel opening probably due to a loss of an electrostatic interaction between the residues of interest. In order to confirm this, experiments to mimic a salt bridge at the hypothesised sites were tested.

4.5.3 Engineering a permanent salt bridge between lysine 125 and arginine 104 opens connexin channels

Mutational studies to replace lysine 125 with a glutamate residue produced 2 interesting findings. Firstly, mimicking a salt bridge interaction between these residues led to a constitutively open channel as seen by elevated dye loading during control conditions. This confirms our hypothesis that salt bridge formation between these residues can induce a conformational change. Secondly, no additional dye loading was seen in the Cx26 K125E mutant under elevated CO_2 conditions. This further supports the hypothesis that lysine 125 is a unique CO_2 binding site in Cx26 channel opening as its loss removes CO_2 sensitivity.

4.5.4 Elastic network modelling confirms a salt bridge interaction can induce a conformational change

Theoretical modelling was also used to confirm our salt bridge results. Hypothetical bonds between the residues of interest were shown to change channel dynamics, biasing the channel towards the open state.

Through experimental and theoretical studies we have provided a full mechanism to explain the opening of Cx26 channels by CO_2. This highlights the importance of 2 residues both of which are required for channel opening. Using this information it should be possible to produce Cx26 channels which lack CO_2 sensitivity while retaining other functionality. This offers an intriguing new way of producing knock out organisms that will be discussed in the next chapter.

4.5.5 The environment around Lysine 125 makes it a specific binding site

Our results indicate that Lysine 125 is the unique CO_2 binding site in Connexin 26, as removal of this residue removes CO_2 sensitivity despite the presence of 15 other lysines in the protein. This suggests the environment around lysine 125 is able to support carbamate formation while that of the other lysine residues do not. One potential reason for this is the presence of arginine 104. As previously stated the maintenance of carbamate bonds requires the negative charge to be neutralised. In our model this is provided by the positively charged side chain of arginine 104. CO_2 binding at other lysine residues may therefore occur but the lack of available positive charges for neutralisation would prevent them from persisting. Another possibility is that CO_2 only binds to lysine 125 and not to any other lysine residues. This could be explained by this lysine being present in a unique environment, favouring CO_2 binding that would not usually occur elsewhere. Earlier in this chapter we explained that CO_2 binding to lysine residues requires the amine group to be in the form of NH_2 and used this for our justification for the K125R mutation of Cx26. Arginine has a pKa of 12.5 and therefore will exist in the protonated NH_3^+ form in any environment. Lysine is less basic, with a pKa of 10.5, and is therefore

more likely to exist as NH_2 but only when present in an area of restricted hydration. Lysine 125 could therefore be unique among the lysine residues in connexin 26 in that it is an environment that allows it to exist in a non-protonated form. One way in which this could be achieved would be for the residue to be protected from the environment by being within the hydrophobic membrane lipids. The crystal structure of Cx26 does not support this, placing lysine 125 outside the plane of the membrane (Maeda *et al.*, 2009). Restricted hydration could also be achieved if the residue was found in a pocket within the protein surrounded by hydrophobic residues. The cytoplasmic loops, which connect the transmembrane domains, and the C terminal tail remain unresolved in the Cx26 crystal structure (Maeda *et al.*, 2009). This offers an interesting possibility as the positioning of these residues could provide the restricted area required. The basis of the special environment required for carbamate formation is yet to be explained, requiring further study and the resolution of these residues in an improved crystal structure.

4.5.6 Flexibility of Cx26 and gating models

Salt bridge interactions are known to occur when oppositely charged residues are located less than 4Å apart (Kumar & Nussinov, 2002). In the Cx26 K125E mutant produced in this chapter the distance between glutamate 125 and arginine 104 is 9.5Å and yet constitutive channel opening is evident. If our hypothesis that channel opening occurs due to a salt bridge interaction between these residues is correct, the connexin channel must be sufficiently flexible to allow these residues the move close enough to interact. There are 2 hypotheses for how hemichannel

opening/closure can occur, which may be dependent on the stimulus affecting the channel. Connexin channels could close through physical blockade of the channel either by a separate molecule or by the c-terminal tail of the connexin monomers (Oh *et al.*, 2004; Locke *et al.*, 2011; Zonta *et al.*, 2012). Channel opening/closure could also be caused by rotation of the channel hexamers (Muller *et al.*, 2002). This could provide a route by which the residues of interest could be moved within the distances required for interaction. In this mechanism however, the residues would only be close enough to interact if the channel was present in the open state. Permanent opening of connexin channels in the absence of a stimulus would lead to cell death, as the passive movement of molecules out of the cell would lead to loss of important metabolites including ATP and NAD^+ and disruption of ion balance across the membrane(Bruzzone *et al.*, 2001; Zhao, 2005; Huckstepp *et al.*, 2010b). Temporary connexin channel opening has however been observed which can occur in the absence of any stimulus. This random flickering of the channel can explain how the functionalised residues can be bought close enough to interact and to lock the channel into an open state.

4.5.7 Cx26 as a CO_2 receptor

The assignment of lysine 125 as a CO_2 binding site identifies Cx26 as a CO_2 receptor. The response of Cx26 to CO_2 could be equated to other ligand gated receptors with direct ligand binding leading to a conformational change and subsequent channel opening. Upon opening of ligand gated channels, charged ions such as Na^+ or K^+ can move in or out of the cell leading to depolarisation and signalling. These channels are highly specific for the ions they can transport. In comparison to other

ligand gated channels, connexin channels have a large central pore which will passively allow the transport of molecules up to 1KDa in size (Simpson *et al.*, 1977). Connexin 26 has been demonstrated to favour the transport of positivity charged molecules however this is not restrictive as the negatively charged molecule ATP is also transported (Huckstepp *et al.*, 2010b; Kanaporis *et al.*, 2011). This low level of specificity, compared to other ligand gated channels, results in connexin channels being capable of releasing a number of different signalling molecules in response to elevated CO_2. ATP released from hemichannels can act as a signalling molecule through its ability to bind P2X and P2Y purinergic receptors on the surface of cells. P2Y receptors are GPCRs and as such ATP binding is capable of initiating intracellular signalling. This occurs through increased production of inositol 1,4,5-trisphosphate (IP3) which leads to the release of Ca^{2+} from intracellular stores (Streb *et al.*, 1983; Bezprozvanny & Ehrlich, 1993; Svichar *et al.*, 1997). The propagation of elevated Ca^{2+} between cells, known as calcium waves, is an important part of the signalling cascade with release of ATP from hemichannels being indicated as the source in various tissues (Stout *et al.*, 2002; Anselmi *et al.*, 2008). P2X receptors are ligand gated ion channels with ATP binding leading to cell depolarisation as a result of ion flow (Abbracchio & Burnstock, 1994). As connexin channels transmit signalling molecules through large non specific pores, bi-directional transport is also possible. Elevated intracellular Ca^{2+}, and consequently Ca^{2+} signalling, can also be induced by the influx of Ca^{2+} from outside of the cell. ATP is known to be capable of causing Ca^{2+} influx into cells and hemichannels have been shown to permeable to Ca^{2+} in this direction (Barry & Cheek, 1994; Fiori *et al.*, 2012). The CO_2 dependent opening of connexin hemichannels, and the subsequent

release of ATP, therefore provides several routes for the initiation of signalling cascades both through paracrine signalling and through altered Ca^{2+} levels in the detection cell itself. Connexin hemichannels have also been implicated in the transport in other signalling molecules including NAD^{+}, glutamate and glutathione (Bruzzone *et al.*, 2001; Stridh *et al.*, 2008; Jiang *et al.*, 2011).

Through demonstrating the ability of Cx26 to directly bind CO_2 we present this protein as a novel CO_2 receptor. This has interesting implications as Cx26 is expressed in tissues other than the medulla, such as the ear, liver and uterus (Nicholson *et al.*, 1987; Grummer *et al.*, 1994; Kikuchi *et al.*, 1995). A role for CO_2 modulation in these tissues becomes a distinct possibility when Cx26 is capable of initiating signalling cascades in the response to elevated CO_2. Further implications for Cx26 as a CO_2 receptor will be discussed in Chapter 7.

Chapter 5- Dominant negative strategy for selectively disrupting CO_2-sensitivity of native Cx26

5.1 Abstract

In this work we have demonstrated that the CO_2-sensitivity of Cx26 requires carbamate formation at lysine 125 and subsequent salt bridge formation to arginine 104 on a neighbouring subunit. This interaction restricts channel movement, biasing it towards an open state. As both residues are required for this interaction, it follows that homomeric or heteromeric hemichannels containing mutant Cx26 subunits which lack both of these residues would have diminished CO_2-sensitivity. Heteromeric connexons were produced by transiently transfecting WT Cx26 expressing HeLa cells with a Cx26 K125R R104A dominant negative mutant (dnCx26). Dye loading revealed the native CO_2-sensitivity of these cells was completely abolished 6 days post-transfection. In comparison stably transfected Cx26 cells that were not transfected with dnCx26 maintained their CO_2-sensitivity throughout the 7 day monitoring period. The ability of this mutant to remove CO_2 chemosensitivity in previously CO_2-sensitive cells offers a promising new method for producing animal models, which would express Cx26 but would lack this mechanism of CO_2 dependent channel opening. This could be of great use not only in observing the effects on CO_2-sensitivity in central chemosensitive cells but also in peripheral tissues containing Cx26 where the effect of CO_2 is unknown.

5.2 Introduction

5.2.1 Cx26 could act as a CO_2 detector in peripheral tissues

Connexin 26 hemichannels have been found to be highly localised to the first 300μM of the surface of the medulla oblongata, an area known to be important in CO_2 chemosensitivity (Bou-Flores & Berger, 2001; Wickstrom *et al.*, 2002; Gourine *et al.*, 2005b; Huckstepp *et al.*, 2010b). As Cx26 is known to exhibit CO_2 dependent channel opening, a role for this channel in the CO_2 chemosensitive response is highly probable. In order to determine the importance of Cx26 in this system, animals lacking the Cx26 channel opening must be tested for their response to elevated levels of CO_2. We may also wish to test for the importance of CO_2 dependent channel opening of Cx26 in other tissues. For example Cx26 is expressed in the endothelium of the uterus. Expression was shown to be low in non-pregnant rats, becoming highly expressed around the time of embryo implantation (Grummer *et al.*, 1994). As with all living things, blastocytes are also known to express CO_2. Many species are capable of delayed implantation whereby blastocytes do not immediately implant in the uterus wall and in the mouse, these blastocytes are known to produce less CO_2 than those implanting immediately (Torbit & Weitlauf, 1975). CO_2 levels could therefore be a determining factor in blastocyte implantation, with Cx26 acting as the detection moiety. Direct CO_2 monitoring could also be important in the lungs. CO_2 has been shown to affect the contraction and relaxation of the lungs in response to hypercapnia (Emery *et al.*, 2007). It is thought that this increases the amount of air that can reach the alveoli, thus aiding excretion of excess CO_2. The way in which CO_2 interacts with the lung is

unknown. Cx26 offers a possible explanation of this, being expressed in the lungs throughout life (Carson *et al.*, 1998).

5.2.2 CO_2-sensitivity can be investigated using Knock out Animals

The simplest way to determine the importance of connexin 26 in these systems would be to investigate the physiological differences in animals that do not express Cx26 in any of their tissues. This however is not possible as systemic knock out of Cx26 is lethal to embryos. These KO animals demonstrate severely reduced uptake of glucose and other nutrients from the mothers blood and appear to die of starvation at day 11 of development (Gabriel *et al.*, 1998). Cx26 also appears to have a role in neuronal migration in the developing nervous system which would also be lethal to the embryos (Valiente *et al.*, 2011).

Developments in investigating the role of Cx26 CO_2-sensitivity have been made using selective Cx26 knockout animals via the cre lox system. In this system mice expressing a ”floxed” Cx26 locus (the coding sequence flanked by loxP sites) (Pfeifer *et al.*, 2001) are crossed with mice expressing a cre recombinase only in specific cell types. The cre recombinase will excise the DNA sequence between the two loxP sites thereby giving cell-specific deletion of the Cx26 gene. The specificity of Cx26 deletion therefore comes down to the specificity of expression of the cre recombinase. In mice exhibiting Cx26 deletion specifically in the medulla oblongata, whole body plethysmography demonstrated a reduction in increased tidal volume response to increased CO_2, compared with WT animals (N. Dale, G. Koentges, X. Zhang, J. Zhang unpublished study). For the experiment described above, Cre expression was driven by the wnt-1 or GFAP promoters providing activation

respectively in neural crest derived cells and GFAP-expressing cells. This process provides a sophisticated method of achieving gene knockouts in specific cell types. However, this selection method is unavailable for rat and therefore selective deletion of Cx26, and consequently its role in CO_2 chemosensitivity, cannot be investigated in these animals. Another disadvantage to this system is the customisation required for each cell type. For each tissue investigated a line of specific knockout mice must be designed and breed. Although this process is the current norm, it remains a costly and time consuming process. Finally, as connexin hemichannels are an important part of the signalling cascade, complete deletion of these proteins could potentially lead to large changes in cell physiology. Although in the current system deletion occurs in selective tissues there remains the possibility of cell effects unrelated to CO_2 affecting the experimental results. For these reasons deletion of Cx26, even selectively in tissues and specific cells, may give results that are capable of more than one interpretation.

5.2.3 A more precise way to investigate CO_2-sensitivity by using Cx26 K125R R104A

In this thesis I have demonstrated that the mechanism for the CO_2 dependent channel opening of Cx26 relies on salt bridge formation between a carbamate formed at lysine 125 and the positive charge at arginine 104 on the neighbouring subunit. Cx26 mutated to remove these residues individually lack CO_2-sensitivity (Chapter 4). As channel opening involves an intersubunit interaction, subunits of a double mutant that lacks both residues should be unable to interact with either neighbouring subunit. It should therefore produce hemichannels insensitive to CO_2 when expressed both on its own or when forming heteromeric channels with WT

Cx26 (Figure 1). We therefore refer to this subunit as a dominant negative Cx26 (dnCx26). If this is correct the introduction of this mutant into tissues which endogenously express Cx26 would provide an exquisitely specific way of removing CO_2-sensitivity from the Cx26 hemichannels while maintaining Cx26 expression and the presumed other functions of these hemichannels and gap junctions.

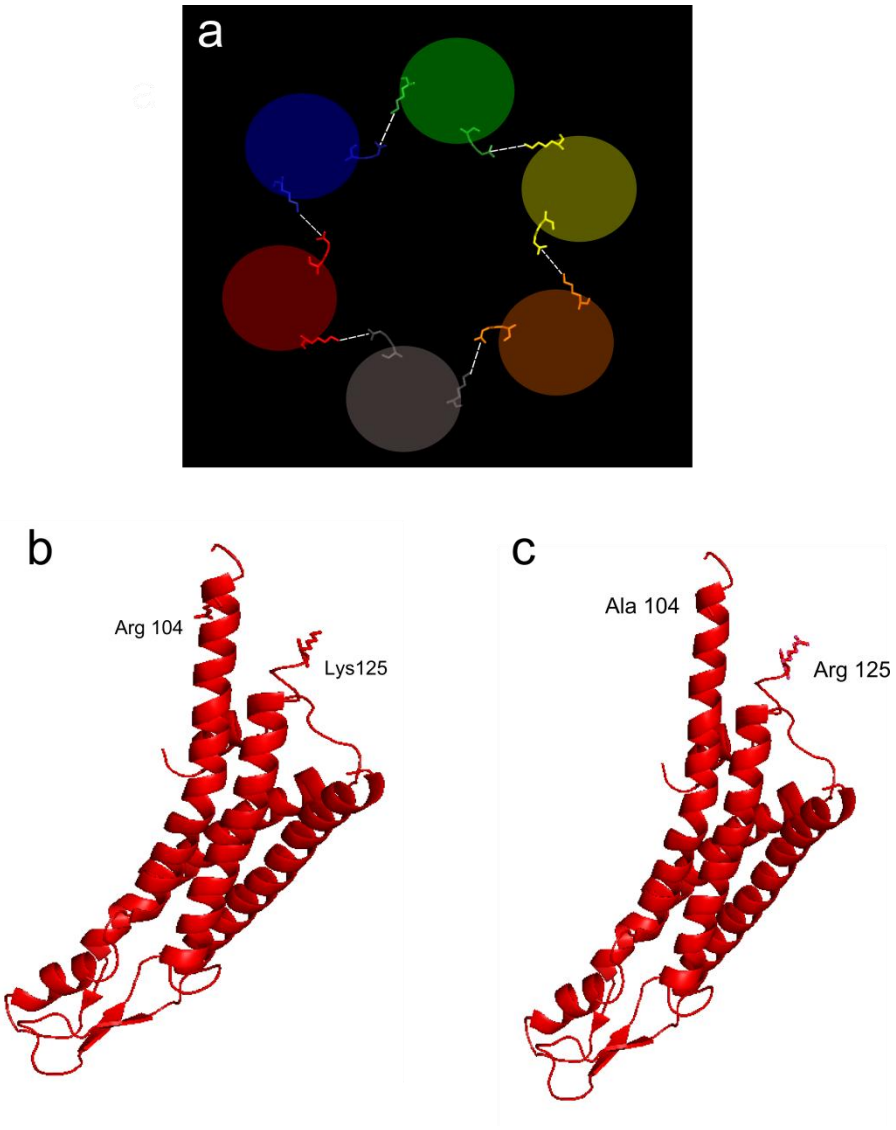

Figure 1 Cx26 can be mutated to prevent it from interacting with its neighbours. a) Residues 104 and lysine 125 shown for each subunit. The salt bridge interaction between them is shown with dotted lines. b) Native Cx26 subunit showing the residues of interest. c) The dnCx26 subunit with arginine 104 mutated to alanine removing the required charge for salt bridge formation and lysine 125 replaced with an arginine to prevent CO_2 binding.

This putative dominant negative strategy depends on how connexins are assembled into connexons. As with other proteins, synthesis of connexin proteins occurs in the endoplasmic reticulum. The trafficking of these proteins to the cell surface is however disputed. Trafficking of connexins 32 and 43 has been shown to be sensitive to Brefeldin A indicating a reliance on the Golgi apparatus. In contrast Cx26 was shown to be insensitive to this drug, instead appearing to use a microtubule dependent method of trafficking to the membrane (Martin *et al.*, 2001). This result has however recently been disputed with Brefeldin A sensitivity demonstrated for Cx26 trafficking (Thomas *et al.*, 2005). Nevertheless a novel method of Cx26 trafficking is supported by the faster speed with which Cx26 reaches the membrane compared to other connexin proteins(George *et al.*, 1999). Cx26 has also been shown to have distinct intracellular stores from Cx32 and 43. Connexin proteins in these stores are largely found in the hexameric, hemichannel form and only this form of connexin is trafficked to the membrane (George *et al.*, 1999). Oligmerisation of connexin proteins appears to begin in the ER, progressing during the secretory pathway and being completed in the Golgi apparatus for Cx32 and at the ER-Golgi intermediate compartment (ERGIC) for Cx26 (Zhang *et al.*, 1996; George *et al.*, 1999). Cx26 hemichannels are known to have a turnover of approximately 5 hours, with cell surface hemichannels being replaced from a pool of connexins in the ERGIC (Fallon & Goodenough, 1981; George *et al.*, 1999).

As oligomerisation of connexin proteins occurs after synthesis, it follows that the co-expression of non-endogenous dnCx26 subunits and endogenous WT Cx26 subunits could result in a population of heterogeneous hemichannels containing

both connexins. We could also expect homomeric channels containing either WT or dnCx26 subunits. Homomeric dnCx26 hemichannels should lack CO_2-sensitivity, while the sensitivity of the heterogeneous channels will depend on the number of salt bridge interactions that are required to induce conformational change in Cx26.

The number of CO_2 molecules required to cause channel opening is currently unknown. This however could be of paramount importance in determining the success of this double mutant strategy. For example, if 6 bound CO_2 molecules are required to induce the conformational change needed for channel opening, the incorporation of 1 mutant connexin hemichannel would prevent CO_2-dependent channel opening. If however 3 interactions would induce sufficient conformational change, CO_2-sensitivity would have a 40% probability of being maintained with the inclusion of 2 mutant subunits but would be lost with the introduction of 3 or more. One possible way to further investigate this is to re-examine the dose-response relation of Cx26 for CO_2 as recorded in Cx26-expressing HeLa cells by Huckstepp et al (2010). This relationship can be described by the Hill equation:

$$I = (PCO_2/K)^h/\{1+ (PCO_2/K)^h\}$$

Where K is a constant that represents the affinity of binding and h the Hill coefficient. The Hill coefficient for the dose response relation of a receptor can be interpreted as giving an indication of how many ligand molecules need to bind to effect opening. For a typical GPCR the Hill coefficient is usually between 1 and 2

We have replotted the conductance change of Cx26 channels versus PCO_2 and fitted the Hill equation to it (Huckstepp *et al.*, 2010a). The Hill coefficient capable

of adequately fitting the experimental data can be either 5 or 6 suggesting that either 5 or 6 CO_2 molecules are needed to achieve opening (Figure 2). As there are known to be 6 subunits in Cx26, a harmonious idea is that there are likely 6 ligands required for hemichannel opening, with one CO_2 binding to each Lysine 125 in the protein. If all 6 salt bridges must be formed to cause channel opening then the introduction of even 1 mutant subunit into Cx26 should destroy CO_2-sensitivity. This suggests that this method of abolishing CO_2-sensitivity is likely to be effective.

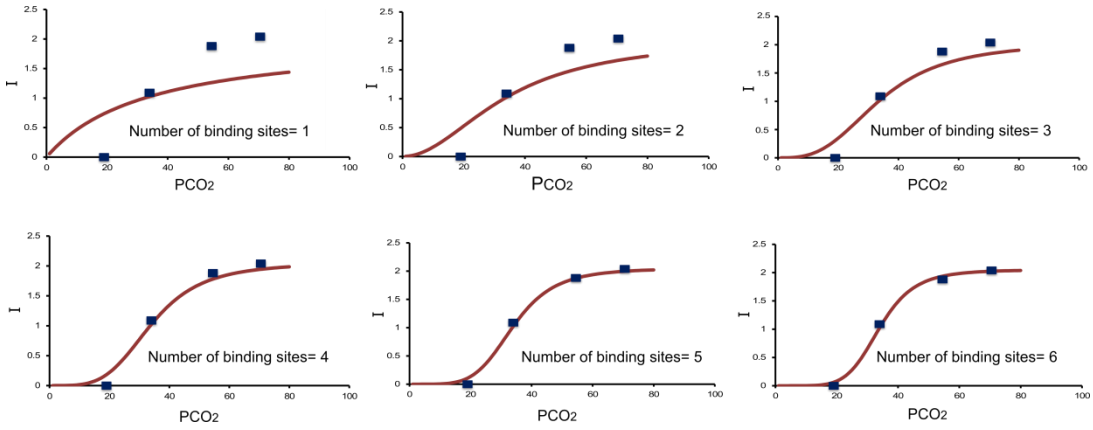

Figure 2 The binding of 6 ligands in the Hill equation mimics the dose-dependence of Cx26 CO_2-sensitivity. Patch clamp results of changes in cell conductivity with increases in CO_2 can be seen in blue. The relationship between free ligand concentration and binding affinity can replicate this sigmoidal relationship when the protein has 5 or 6 binding sites (red). In contrast 1 to 4 binding sites do not replicate the dynamics of Cx26.

Targeted expression of mutant Cx26 could be achieved through cell specific viral infection using promoters directed towards the cell of interest. This method of producing CO_2-insensitive animals would address many of the concerns with the current method of transgenesis. Firstly, CO_2-sensitivity of Cx26 could be lost from any animal or any tissue by delivery of the mutant connexin gene. The responses of rat or any other species could therefore be investigated using this method.

Secondly as the viral promoter would determine mutant Cx26 expression, the breeding of specific animal lines would not be required, making this a less time consuming process. This method also offers the possibility of investigating the CO_2 responses of the same individual before and after loss of Cx26 CO_2-sensitivity thus providing a clear indication of effect. Finally, this method would prevent CO_2 dependent channel opening of Cx26 while nevertheless maintaining hemichannel expression and thus its other functions. Normal cell signalling would consequently not be effected leading to clearer and more accurate results concerning the importance of CO_2-sensitivity.

In order to determine the feasibility of this approach, experiments were performed in HeLa cells using the dnCx26 subunit. This allowed us to determine whether this mutant was CO_2-sensitive and also whether it was capable of removing CO_2-sensitivity in cells expressing WT (CO_2-sensitive) Cx26.

5.3 Methods

5.3.1 Mutant connexin production

Mutant Cx26 R104A K125R (dnCx26) DNA was produced by Quikchange site directed mutagenesis using Cx26 K125R as the template DNA. PCR conditions for the Quikchange protocol were as described previously using primer sequences as follows: Cx26 R104A forward 5'GGC CTA CCG GAG ACA CGA AAA GAA AGC GAA GTT CAT GAA GG 3', reverse 5'CCT TCA TGA ACT TCG CTT TCT TTT CGT GTC TCC GGT AGG CC 3'.

5.3.2 HeLa cell culture

HeLa cells stably expressing Cx26 were maintained in DMEM supplemented with 1:1000 pen/strep and 3mM $CaCl_2$. Cells were grown in a 5% CO_2 humidified incubator. Loss of CO_2 sensitivity was expected to be a gradual process due to the time required for the double mutant Cx26 protein to move to the cell membrane and become incorporated into the connexons. Therefore cells were plated onto coverslips at densities that would allow dye loading to be followed over a 5 day time scale beginning at 3 days post transfection. To ensure cells were at an appropriate confluency for experimentation, cells were plated at 3 different densities: 5×10^4, 1×10^4 and 5×10^3 cells per well.

These Cx26 expressing HeLa cells were transiently transfected with dnCx26 using the Genejuice transfection agent protocol. Cx26 expressing cells were also plated as above but with no transfection. These cells provided a control to ensure that CO_2 sensitivity would be otherwise maintained when not transfected with the dnCx26. dnCx26 was also investigated by transient transfection into parental HeLa cells. This allowed us to confirm whether, in agreement with previous experiments, this mutant lacked CO_2 sensitivity. For these experiments cells were plated at 5×10^4 cells and investigated 3 days post transfection to allow time for the protein to be trafficked to the cell membrane.

5.3.3 Dye loading assay

Dye loading was performed as in the previous chapters, with control, CO_2 and zero Ca^{2+} conditions being tested over a 5 day period, beginning at 3 day post-

transfection. Dye loading was performed for stably transfected Cx26 expressing HeLa cells and stably transfected Cx26 HeLa cells transiently transfected with dnCx26.

Dye loading was also performed for transient transfection of WT parental HeLa cells with the dnCx26. Again control, hypercapnic CO_2 and zero Ca^{2+} conditions were analysed.

5.3.4 Data capture and analysis

Cells were imaged using the epifluroscent microscope mentioned previously (Chapter 2). Median pixel intensities were collected for at least 40 cells for 5 repetitions and used to produce cumulative probability distributions. Statistical significance was determined using Mann Whitney statistical tests.

5.3.5 Detecting mCherry

mCherry signals were obtained as outlined in Chapter 2. Median pixel intensity at 8 points along each cell were collected and the mean of these over all cells were used to produce an approximation of mCherry expression. This allows us to compare to location of mCherry, and therefore connexin trafficking in the various mutants (Figure 3).

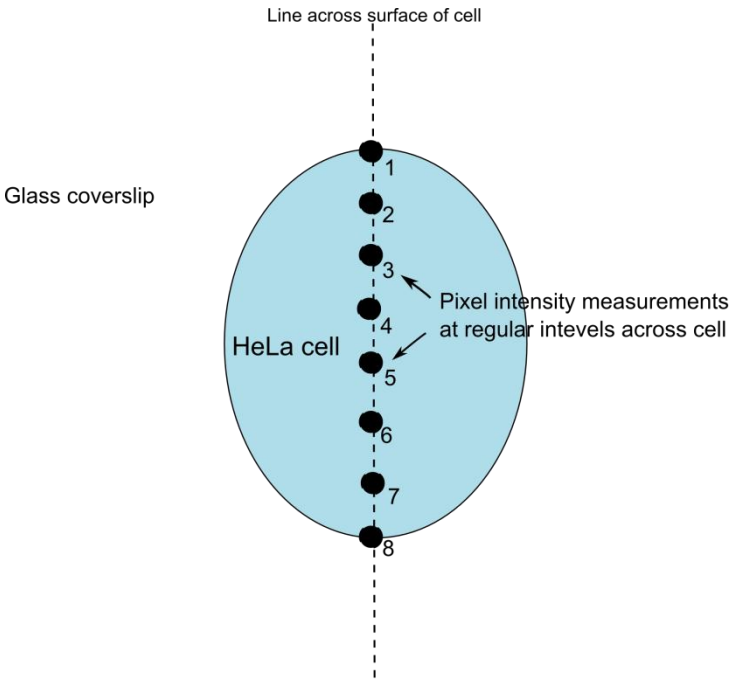

Figure 3 Diagrammatic representation of protocol for mCherry localisation measurements

5.4 Results

5.4.1 dnCx26 forms hemichannels insensitive to CO_2

As the dnCx26 mutant lacks lysine 125 and arginine 104, the key residues in carbamate bridge formation, we would expect dnCx26 hemichannels to lack CO_2-sensitivity. In support of our previous experiments, dye loading assays performed on WT HeLa cells transiently transfected with these double mutant connexins displayed no CO_2-sensitivity ($p=0.028$ versus WT Cx26). Channels were shown to be functioning correctly otherwise, as determined by zero Ca^{2+} conditions described previously (Figure 4).

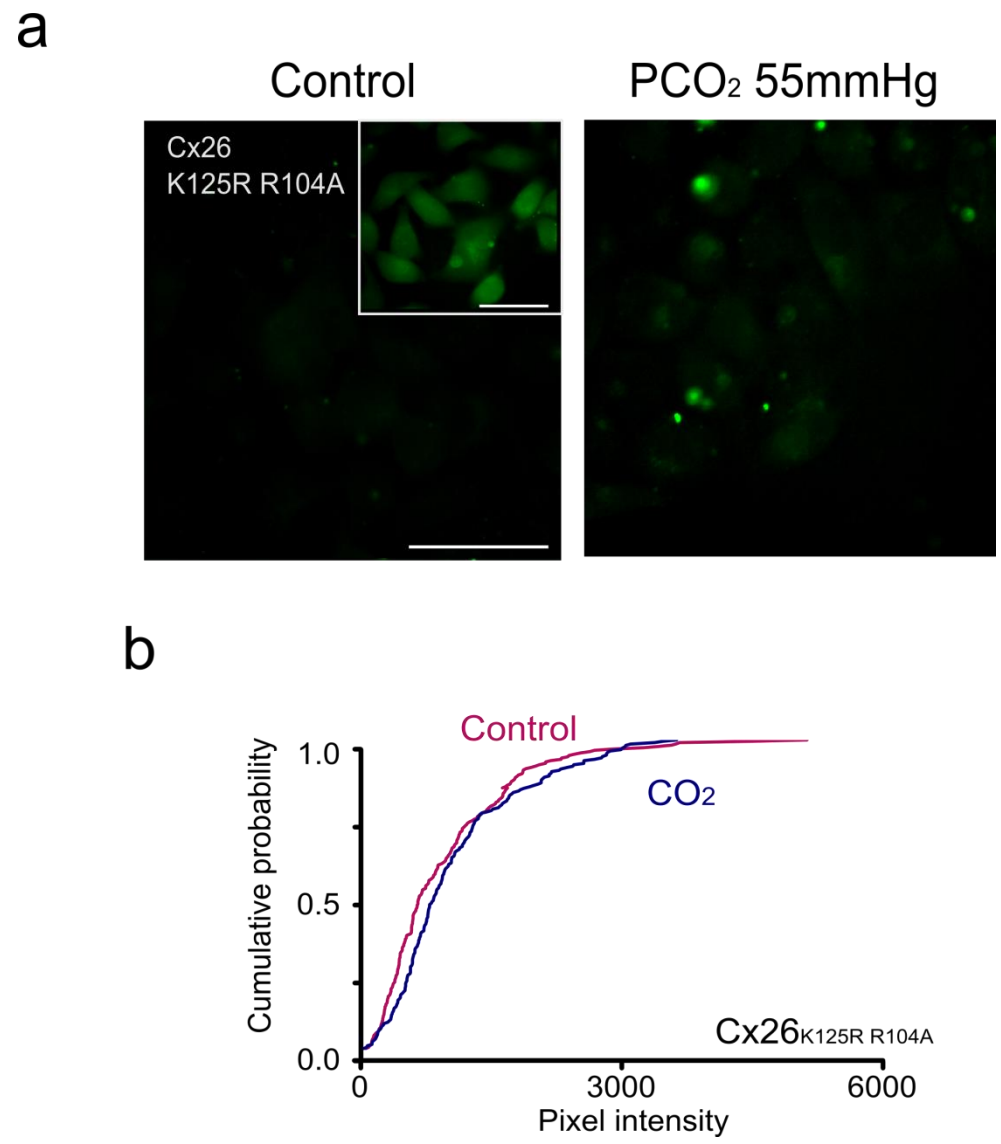

Figure 4 dnCx26 is non-CO_2 sensitive. a) Dye loading assay images for control and elevated CO_2 conditions for dnCx26. Zero Ca^{2+} response is seen as inset. All scale bars are 50 µm.b) Cumulative probability distribution for dnCx26 reveals no CO_2 sensitivity. Distribution shows all data for n=5 experiments.

5.4.2 dnCx26 can destroy CO_2-sensitivity of Cx26 expressing cells

To test whether the dnCx26 subunit does indeed work in a dominant-negative fashion, we transfected this subunit into HeLa cells stably expressing WT Cx26. We then monitored their ability to open in response to CO_2 over a 5 day period, beginning at 3 days post-transfection. This extended time frame was used on the assumption that loss of CO_2-sensitivity would not be immediate but would only

occur after connexin synthesis, co-assembly and trafficking to the membrane. HeLa cells stably expressing WT Cx26, but not transfected with dnCx26, were also monitored over the same period to confirm that CO_2-sensitivity would be maintained over this period.

The CO_2-sensitivity of WT Cx26-expressing HeLa cells was maintained throughout the 5 experimental days with the peak sensitivity to CO_2 evident at day 3 (Figure 5). This increase in sensitivity may reflect an elevated amount of connexin synthesis due to the HeLa cells having settled into their new environment. At day 4, CO_2 sensitivity begins to show a modest decline. This pattern therefore reflects the normal evolution of CO_2 sensitivity of Cx26-expressing HeLa cells in culture.

Cells transfected with dnCx26 also demonstrated a small increase in CO_2 sensitivity over experimental days 1 and 2. However, instead of reaching its peak at day 3, the CO_2 sensitivity of the transfected cells began to decline and became significantly different to that of untransfected cells by day 4 (Figure 5). Indeed by experimental day 4 the cells transfected with dnCx26 exhibited no sensitivity to CO_2. Nevertheless, the positive control of testing the effect of zero Ca^{2+} on these cells revealed increased dye loading throughout the 5 day experimental period (Figure 5). This confirms that Cx26 hemichannels continue to be expressed and functional throughout the experimental period. They must therefore lack CO_2 sensitivity on account of the coassembly with dnCx26 subunits.

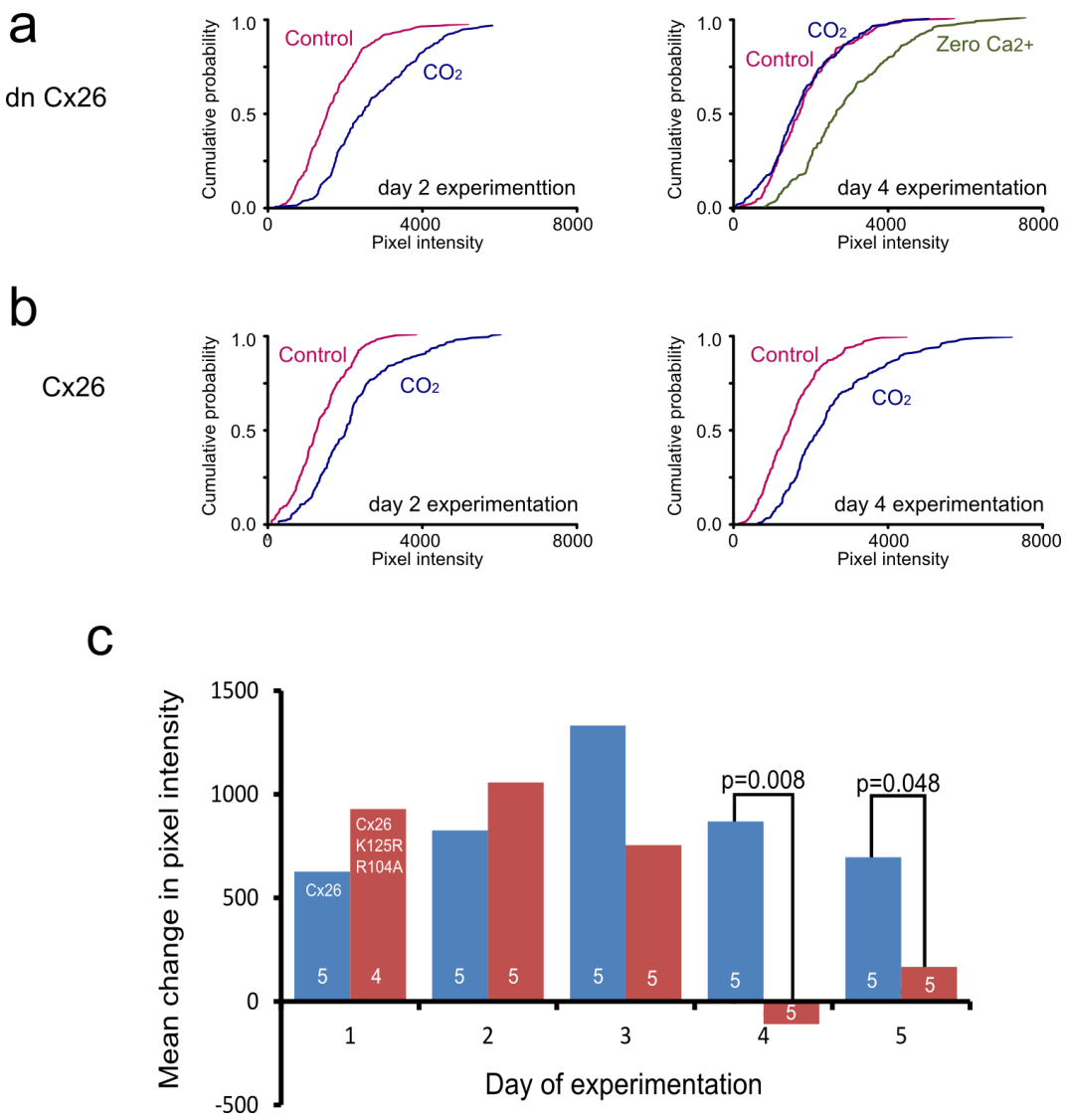

Figure 5 dnCx26 destroys CO_2-sensitivity in WT Cx26 expressing cells. a) Cumulative probability distributions demonstrating loss of CO_2-sensitivity in dnCx26 transfected cells at 6 days post transfection. b) In contrast WT Cx26 cells (not transfected with dnCx26) maintain CO_2-sensitivity during experimentation. c) Mean change in pixel intensity in response to hypercapnia for dnCx26-transfected and non-transfected Cx26 expressing HeLa cells over the 5 day experiment period. P values calculated using Mann-Whitney test for statistical significance.

5.4.3 dnCx26 mCherry suggests trafficking does not occur immediately after transfection

The dnCx26 protein is a fusion protein with mCherry. Consequently the expression of this protein can be visualised using epifluorescence. In Chapter 2 we presented mCherry expression patterns for connexin hemichannels at the cell surface. This expression pattern was shown to be similar to the expression pattern of dnCx26 at

day 5 experimentation (Figure 6). mCherry in these cells is visualised as isolated puncta surrounding the cell. In contrast, at the second day of experimentation connexin expression is more diffuse with the mCherry signal largely present in the cell interior. The Cx26 stably expressed in these cells is not a fusion protein and consequently all mCherry signal emanates from the mutant, transiently transfected protein. The visualisation of mCherry indicates that mutant protein is synthesised in Cx26 cells before loss of CO_2 sensitivity is seen but that this protein is not trafficked to the membrane. In order to attempt to quantify this change in mCherry, pixel intensity was recorded at 8 points along the cross-section of the cells. The mean of the values at each of these points can be seen in Figure 6. At 4 days-post-transfection a peak in pixel intensity is visible at point 5 representing the middle of the transfected cells. In contrast, at 7 days post-transfection peaks are present at points 2 and 7 indicating mCherry at the cell surface. Although this represents a crude method of mCherry quantification, this result supports the visual assessment that mCherry is not present in the membrane at 4 days post-transfection. This suggests protein trafficking to the membrane is the rate limiting step in removal of CO_2 sensitivity in these cells.

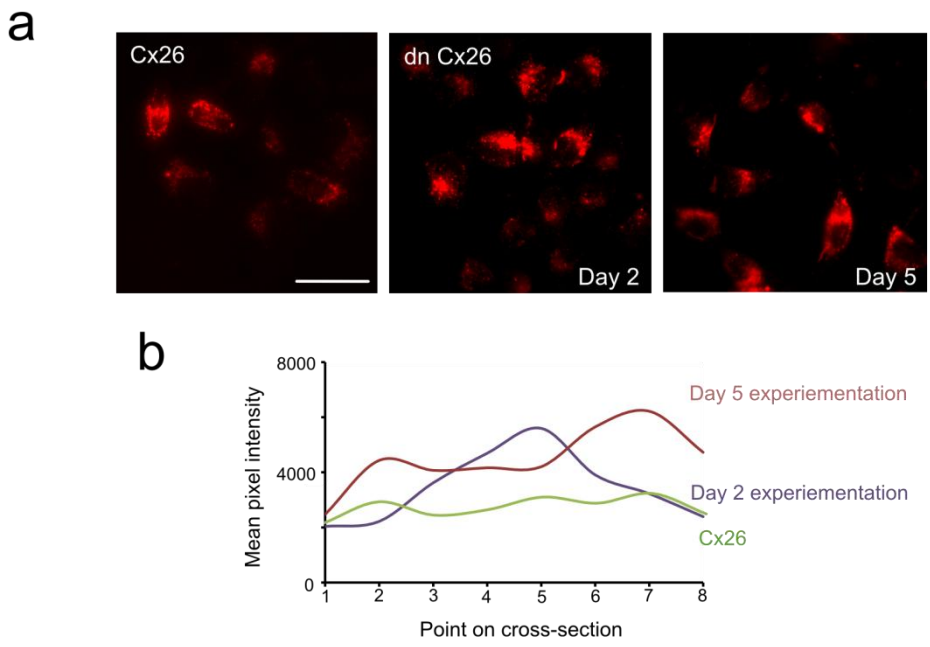

Figure 6 dnCx26 is not in the membrane at 4 days post-transfection. a) mCherry images comparing an example mCherry signal of a protein in the membrane(Cx26) to mutant protein. At day 2 experimentation mCherry signal is diffuse and concentrated on the interior of cell. At day 5 experimentation mCherry is visible in the cell. b) Mean pixel intensities at 8 points along the cross-section of dnCx26-expressing cells stably expressing Cx26. At day 2 experimentation (13 cells) mCherry is present in the middle of the cells where as at 5 days experimentation (9 cells) it is present near the cell surface. mCherry is also seen at the surface in WT Cx26 expressing cells (8 cells).

5.5 Discussion

5.5.1 dnCx26 removes native CO_2 sensitivity

We have demonstrated that the CO_2 chemosensitivity of cells natively expressing Cx26 can be removed by the co-expression of a mutant connexin lacking lysine 125 and arginine 104. This mutant was independently shown to be non CO_2-sensitive confirming our previous finding that these residues are involved in CO_2 dependent channel opening of Cx26. Dye loading analysis of the natively sensitive cells in the absence of transient transfection revealed these cells maintained CO_2-sensitivity over a 5 day period. This demonstrates that homogenous channels of Cx26

maintain their CO_2-sensitivity over this period and therefore the the loss of CO_2-sensitivity must result from the production of heteromeric hemichannels that contain both Cx26 and dnCx26 subunits. This result, which tests a prediction arising from our previous work gives very strong support to the carbamylation model of CO_2-dependent opening of Cx26.

CO_2 dependent channel opening requires an interaction between subunits and therefore the presence of 3 mutant connexins in a hemichannel could completely destroy CO_2-sensitivity by the removal of all 6 intersubunit interactions. Depending of the organisation of the connexins, a hemichannel containing 3 mutant channels could also maintain 1 or 2 interactions (if the dnCx26 subunits were adjacent to each other). The incorporation of 2 mutant connexins would maintain the potential for 2 or 3 bonds depending on the arrangement, while a hemichannel containing one dnCx26 subunit would still maintain the potential for 4 bonds. The composition of the heteromers produced is unknown, as is the minimum number of bonds required for channel opening. Using the Hill equation, as described in the introduction to this chapter, suggests the need for 5 or 6 intersubunit carbamate bridges to open Cx26. If this is correct, the introduction of just one mutant subunit would be sufficient to destroy channel sensitivity. This could be further explored with elastic network modelling which could indicate the number of intersubunit interactions required for channel opening. As dye loading demonstrates these heteromers are not CO_2-sensitive, this information could be used to speculate on the composition of the channel.

5.5.2 Speculations on connexin trafficking

Loss of CO_2-sensitivity with co-transfection was not immediate, occurring approximately 6 days post transfection. Connexin turnover however is known to occur much quicker than this, with the half life of Cx26 shown to be 5 hours in mouse hepatocytes (Fallon & Goodenough, 1981). Assuming similar dynamics in HeLa cells, we would expect less than 1% of the connexins present in the membrane at time of transient transfection to be present 40 hours later. Incorporation of the mutant connexin into the membrane was however not evident for a further 4 days. This suggests that connexin 26 turnover may occur from a reserve of connexin monomers that move to the cell surface as they are required. For the dnCx26 subunit tested here, and all mutants tested previously, expression was observed from approximately 3 days post-transfection in WT HeLa, compared to 6 days when expressed in Cx26 cells. This suggests that a cell with no Cx26 reserves (i.e. parental HeLa Cells) can synthesise connexin hemichannels and transport them the cell surface in 3 days. When connexins are already being expressed, as in the Cx26 HeLa cells, 3 extra days are required to move a sufficient number of connexins to the surface to see an effect. This further supports the idea of connexin protein reserves, which must be diminished before the mutant protein can be trafficked to the cell surface.

The aim of this experiment was to determine whether native CO_2-sensitivity could be removed from cells by the expression of Cx26 lacking the 2 residues of interest. The results of the dye loading analysis reveal that CO_2-sensitivity could be removed from HeLa cells stably expressing Cx26. As a consequence the delivery of mutant

connexin to CO_2-sensitive cells in vivo could provide a method of producing animals lacking CO_2 dependent Cx26 channel opening while leaving Cx26 intact. This method should avoid the widespread disruption caused by gap junction deletion and allows the test of various tissues by the design of a tissue specific promoter rather than breading a knock out animal. The success of the dnCx26 subunit in destroying the CO_2-sensitivity of Cx26-expressing HeLa cells warrants further work to produce viral expression systems that can be used to control expression in specific cell types in animal studies.

Chapter 6 - Using knowledge of the residues of interest Cx26 can be adapted to respond to other stimuli

6.1 Abstract

Our previous mutational studies have demonstrated that the CO_2 sensitivity exhibited by Cx26 is due to a salt bridge interaction between adjacent subunits of the connexin channel. Any adaptations to the connexin channel maintaining or replicating a bridge at this location should therefore allow the channel to be opened to other, non native stimuli. Through mutational experiments we produced Cx26 K125C and Cx26 K125C R104C mutants. Dye loading analysis of these mutants showed them to be sensitive to changes in NO_2 and redox potential respectively. By understanding the molecular mechanisms involved in CO_2 detection by Cx26 we are now able to produce a series of molecular tools allowing us to detect and monitor a variety of molecular stimuli.

6.2 Introduction

6.2.1 NO/NO_2 sensitivity

In the previous chapters of this work we have deciphered the mechanism by which elevated CO_2 leads to channel opening in connexin 26. We determined that CO_2 binding relies on covalent bond formation to lysine 125 and that channel opening occurs through an electrostatic interaction between subunits, resulting in restriction of channel movement. By understanding that channel opening relies on these 2 interactions it should be possible to engineer the channel to be sensitive to new stimuli while at the same time confirming our findings about the mechanism of CO_2 sensitivity.

NO is an important biological signalling molecule having been implicated in a myriad of signalling pathways including roles in synaptic plasticity, blood flow and gastric acid secretion to name but a few (Bohme *et al.*, 1991; Esplugues *et al.*, 1996; Wilderman & Armstead, 1997). NO can react with O_2 to produce $NO_2^{\cdot}$. As $NO_2^{\cdot}$ is a free radical, it has a free electron which it can use to oxidise cysteine residues. This results in a free electron on the cysteine residue which can subsequently bind NO or NO_2 (Forman *et al.*, 2004)(Figure 1).

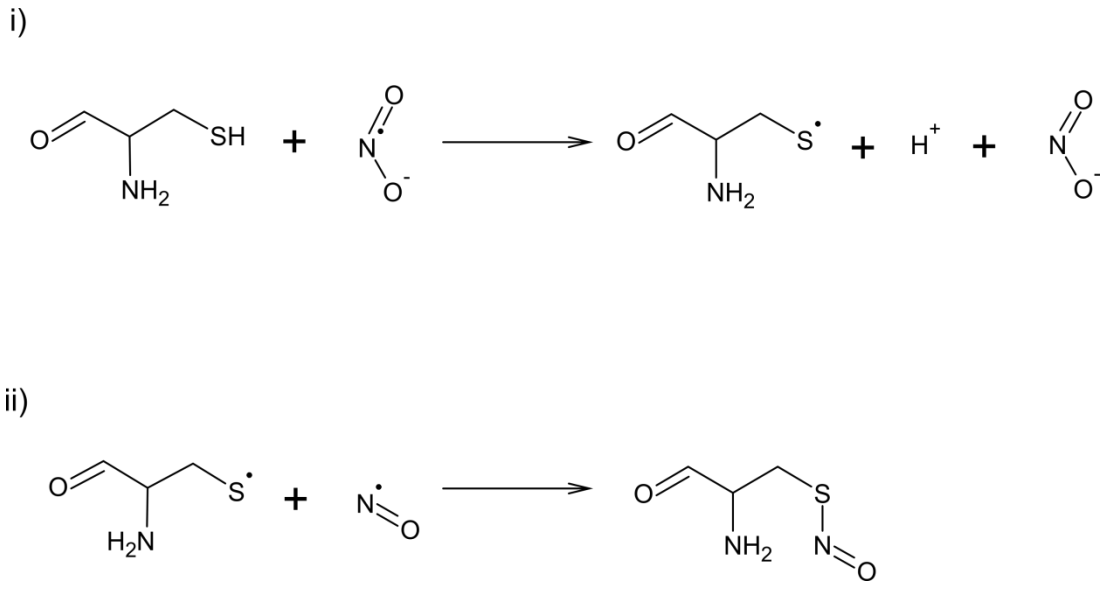

Figure 1 NO/NO_2 can bind to cysteine residues. Activation of cysteine residues by NO_2 (i) allows subsequent binding by NO (ii).

The activation of this reaction by NO_2 is of particular interest to us due the similarities between this molecule and CO_2. Although the molecules are different in shape, due to the free electron of nitrogen, these molecules are of similar size. The similarities of the structure of these molecules suggest residue 125 should be accessible to NO_2, as it is to CO_2. As we know that NO_2/NO can bind cysteine

residues, replacement of lysine 125 with a cysteine should make this residue a NO_2 binding site. Upon binding, NO_2 receives a negative charge and as such the intersubunit electrostatic interaction seen with CO_2 binding should be maintained in this system. As a result NO_2 binding should also result in channel opening. Cx26 adapted in the way described above should become a NO/NO_2 detector, as Cx26 is a CO_2 detecting molecule. As this molecule is designed based on our prior hypothesis of the mechanism of Cx26, successful NO_2 binding and channel opening would provide further support for our mechanism of CO_2 dependent channel opening in Cx26.

6.2.2 Redox sensitivity

We could also modify the channel to replace the native carbamate bridge between subunits with a disulphide bond. This would occur between cysteine residues at positions 125 and 104 and would have the potential of making the mutant Cx26 sensitive to redox states. Redox sensitivity is known to be important in biological processes. Reactive oxygen species, such as H_2O_2, often break down into molecules with an unpaired electron. These free radical molecules are highly reactive and can cause damage to DNA and oxidation of amino acid side chains. Although largely damaging, ROS can also act as signalling molecules, usually to activate protective pathways against the oxidative effect. The reaction of ROS with the SH of the cysteine side chain leads to the formation of sulphenic acid (-SOH). If this residue is close to another cysteine residue a disulphide bond can form. As this covalent interaction moves cysteine residues to a new position and then locks them into place with a covalent bond, disulphide bond formation often leads to dramatic

conformational change compared with the reduced state (Ryu, 2012). This can lead to changes in enzyme function or signalling cascades. One way in which changes in enzymatic function can be altered is by direct inactivation of an active site cysteine as occurs in various phosphatases (Caselli *et al.*, 1998). Another less obvious way in which redox sensitivity can alter activity is seen in the kinase Ask1. Under physiological conditions this enzyme is bound to thioredoxin, leading to its ubiquitination and degradation. Oxidation of thioredoxin however leads to its release and activation (Saitoh *et al.*, 1998; Liu & Min, 2002). Disulphide bond formation has the potential to provide a good analogue for the intersubunit interaction with channel opening now resulting from an oxidative environment rather than elevated CO_2.

6. 3 Methods

Experimental procedures in this chapter were performed in conjunction with Daniel Cook, an undergraduate at the University of Warwick, who worked under my supervision.

6.3.1 Mutant connexin production

Cx26 K125C and Cx26 K125C R104C mutants were produced as outlined in the previous chapters (Chapter 4). Briefly Cx26 Puc19 was used for mutation via the Quikchange protocol. The primers used were as follows: Cx26 K125C forward 5' CGA AGA GAT CAA AAC CCA GTG CGT CCG TAT CGA AGG GTC CC 3' reverse 5' GGG ACC CTT CGA TAC GGA CGC ACT GGG TTT TGA TCT CTT CG 3', R104C for Cx26 K125C R104 forward 5' CCG GAG ACA CGA AAA GAA ATG CAA GTT CAT GAA GGG AGA G 3'

reverse 5' CTC TCC CTT CAT GAA CTT GCA TTT CTT TTC GTG TCT CCG G 3'. The DNA produced was processed using DPN1 digest and bacterial transformation. DNA mutation was confirmed by genomic sequencing.

6.3.2 HeLa cell culture

HeLa cells were cultured as detailed in the previous chapters (Chapter 2). Briefly cells were cultured in DMEM, 10% FCS, 3mM $CaCl_2$. For experimentation cells were plated onto coverslips at a density of 5 x 10^4 cells per well. Transient transfections were performed using the genejuice protocol.

6.3.3 Dye loading for NO_2 sensitivity

Control aCSF and Zero Ca^{2+} recipes are as outlined in Chapter 2.

<u>NO_2 aCSF</u>: 124mM NaCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 3mM KCl, 10mM D-glucose, 1mM $MgSO_4$, 1mM $CaCl_2$, 100µM $NaNO_3$

Dye loading analysis was performed as described in previous chapters. Here, however we aim to determine the sensitivity of the mutant to NO_2 not CO_2. Coverslips plated with Hela cells transiently transfected with Cx26 K125C were exposed to aCSF containing 200µM carboxyfluorescein and 100µM Sodium Nitrite for 10 minutes. This was followed by aCSF with 200µM carboxyfluorescein for 5 minutes. A 30 minutes wash with aCSF ensures that all dye is removed from the outside of the cells to allow imaging. Control and zero Ca^{2+} aCSF dye loading

experiments were performed as previously described (Chapter 2). Dye loading was also performed with WT Cx26 to allow comparison of the results (Figure 2).

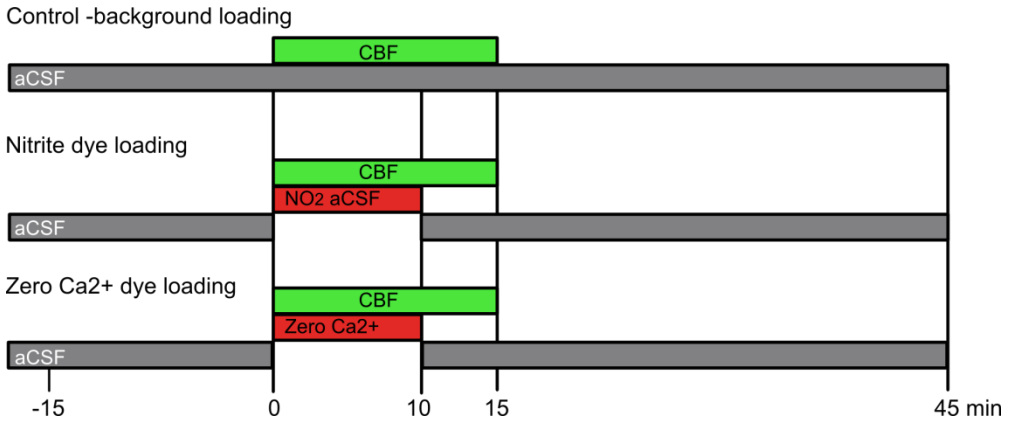

Figure 2 Graphical representation of dye loading experiment.

6.3.4 Dye loading for Redox sensitivity

Reduced aCSF: 124mM NaCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 3mM KCl, 10mM D-glucose, 1mM $MgSO_4$, 1mM $CaCl_2$, 4 mM N-Acetyl-L-Cysteine.

Adaptor aCSF: 124mM NaCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 3mM KCl, 10mM D-glucose, 1mM $MgSO_4$, 1mM $CaCl_2$, 1mM L-Buthionine-Sulfoximine, 600µM 2-sulfonatoethyl methanethiosulfonate sodium salt (MTSES).

Oxidised aCSF: 124mM NaCl, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4, 3mM KCl, 10mM D-glucose, 1mM $MgSO_4$, 1mM $CaCl_2$, 1mM L-Buthionine-Sulfoximine.

These solutions were saturated with 95% O_2/5% CO_2 in order to provide the cells with oxygen throughout experimentation.

For the Cx26 K125C R104A mutant, dye loading was used to test the redox sensitivity of this new connexin channel. Zero Ca^{2+} experiments were performed as described in previous chapters (Chapter 2). However, an additional 5 minutes aCSF wash was included before the carboxyfluorescein dye loading step.

The redox state of the cell was manipulated by altering the levels of glutathione within the cell. To promote a reduced redox state, 4mM N-acetyl cysteine was included in the "reduced" aCSF to enhance the synthesis of glutathione (Miners *et al.*, 1984). To induce an oxidized redox state, 1mM buthionine sulfoximine was included in the "oxidized" aCSF to inhibit the synthesis of glutathione (Griffith & Meister, 1979).

If disulphide bond formation between C125 and C104 is possible under control conditions in normal aCSF, the connexin channel could remain open during the wash period. This could potentially lead to a loss of dye from the cells before imaging could take place. However, previous experiments with the constitutively open Cx26 K125E mutants suggest dye loading would still be visible under these conditions (Chapter 4). To reduce the likelihood of this happening, we performed the manipulations by always going from the reduced state, to the oxidized state. We then washed the carboxyfluorescein away with reduced aCSF to promote channel closure during the wash period. By comparing this result to the normal aCSF control we can also determine the native state of the cysteine residues introduced into this system i.e. do they exist as cysteine residues or as a disulphide bond during normal conditions. A no-stimulus control equivalent to the aCSF

control experiments described in previous chapters was also given. In this case the cells were exposed to 200µM carboxyfluorescein for 15 minutes in reduced aCSF followed by a 30 minute reduced aCSF wash. The reduced aCSF was introduced 5 minutes before the 15 minute carboxyfluorescein step.

To test for redox dependent hemichannel opening the cells were exposed to oxidized aCSF with the aim of inducing formation of a disulphide bridge between C125 and C104. Cells were first exposed to reduced aCSF for 5 minutes. Cells were then exposed to oxidized aCSF plus 200µM carboxyfluorescein for 10 minutes. Reduced aCSF with 200µM carboxyfluorescein was then applied for 5 minutes before washout of the carboxyfluorescein with reduced aCSF for 30 minutes. .

The final experiment concerned the addition of an adaptor molecule to the oxidation protocol. This was done to address the possibility that C125 and C104 might be too far apart to form a disulphide bridge. The inclusion of the MTSES adaptor will enable the cysteine residues to form a bridge over a greater distance if normal flexibility of the channel could not bring the two residues sufficiently close (Figure 3). Cells were exposed to 600µM MTSES for 5 minutes. This step is the reason for the additional 5 minutes included in the other protocols. After this pre-incubation step the dye loading protocol followed that of the oxidation experiment.

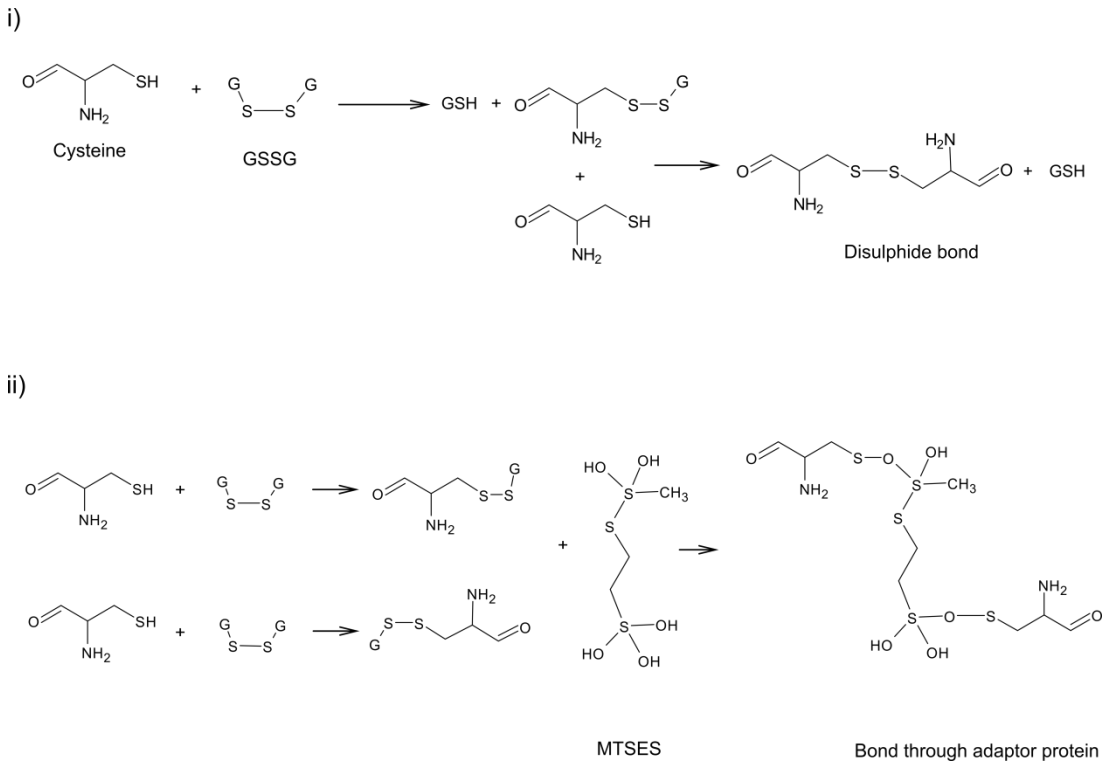

Figure 3 MTSES can be used to replicate disulphide bonds when the distance between cysteine residues would be prohibitive. Disulphide bonds can form between cysteine residues (i). If the cysteine residues are too far apart in space, MTSES can be used to bridge the gap (ii).

A summary of these dyeloading experiments can be seen below (Figure 4)

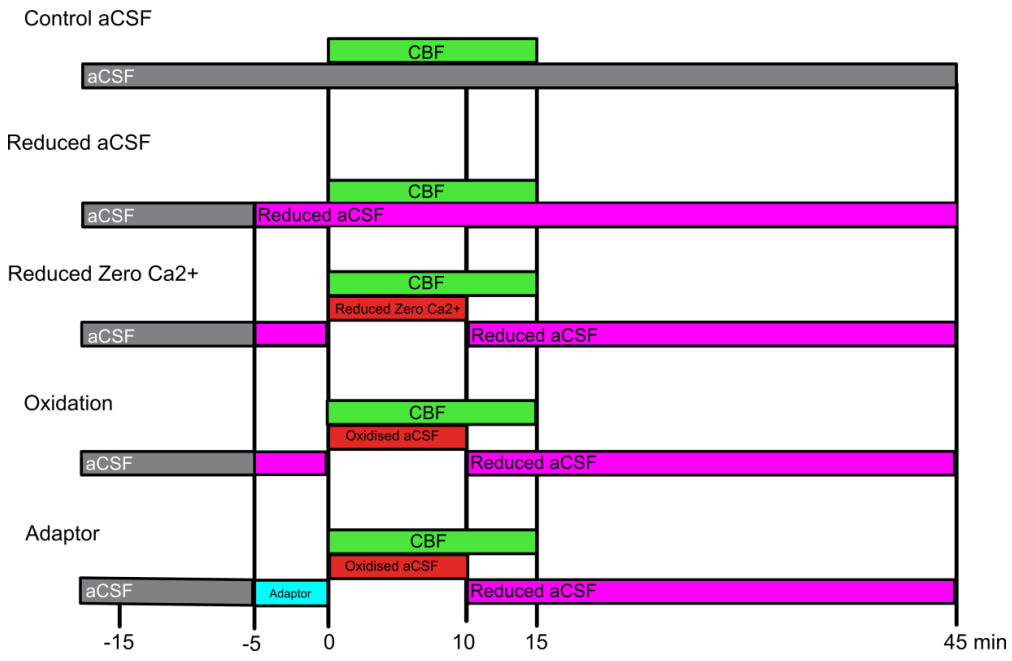

Figure 7 Graphical representation of dye loading protocol for redox experiments.

As with the other experiments, images were analysed using image J and data presented in a cumulative probability distribution. Experiments were performed n=5 with each experiment being performed on cells resulting from a separate transient transfection. Approximately 40 cells were sampled under each condition for each repeat. Conditions were compared for the mutants using the Mann-Whitney test for significance.

6.3.5 Whole cell patch clamp

All patch clamp experiments were performed by Professor Nicholas Dale.

Hela cells were plated at a subconfluent level of approximately 1 x 10^4 cells per well and analysed from 3 days post transient transfection with the gene of interest.

Patch clamp recordings were performed used standard patch clamp techniques as described in Chapter 3. Channel conductance was observed upon application of 1mM BSO or 3mM NAC. WT Cx26 was also tested for redox sensitivity.

6.4 Results

6.4.1 NO_2 sensitivity can be conferred to Cx26 by inclusion of a cysteine residue at position 125

Our previous results demonstrate that an intersubunit bridge between residues 125 and 104 induces hemichannel opening. We reasoned that we could replace the native carbamate bridge with a SNO bridge, by including a cysteine residue at position 125 in the mutation Cx26 K125C (Figures 5, 6). Nitrosylation of the cysteine

residue would create a SNO moiety (Figure 1) that would be sufficiently polar to form a bridge with R104 in the neighbouring subunit and thus open the channel.

```
Cx26       91 VAMHVAYRRH EKKRKFMKGE IKNEFKDIEE IKTQKVRIEG SLWWTYTTSI FFRVIFEAVF 150
Cx26 K125C 91 VAMHVAYRRH EKKRKFMKGE IKNEFKDIEE IKTQCVRIEG SLWWTYTTSI FFRVIFEAVF 150
```

Figure 5 Sequence comparison of Cx26 and Cx26 K125C. This mutation has a cysteine at position 125. It is hoped this will convert Cx26 to be NO_2 sensitive.

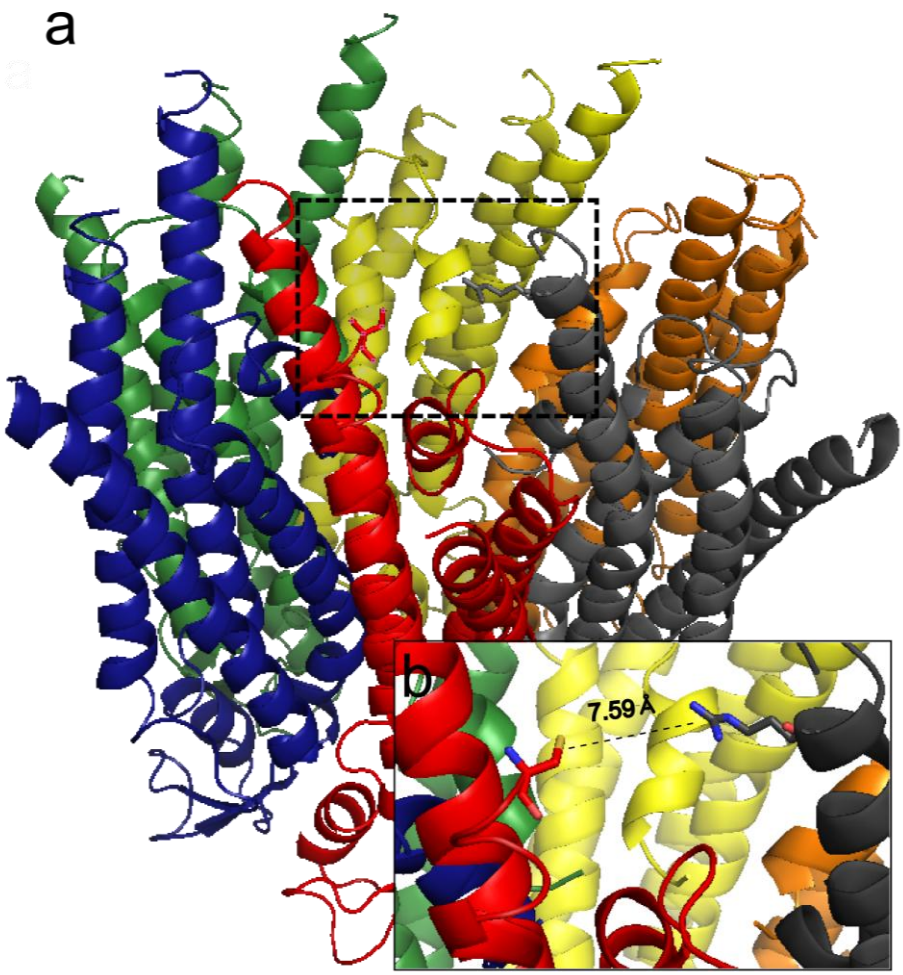

Figure 6 Structure of Cx26 K125C mutant. a) Cx26 hexamer with dotted line highlighting region of interest. b) Magnification shows cysteine 125 (red) and arginine 104 (grey). The distance between these residues is 7.59Å.

We found that 100µM nitrite caused dye loading in Cx26 K125C expressing cells (p=0.008 compared to control, Figure 7). Importantly, we found that WT Cx26 has

no endogenous sensitivity to nitrite, with no increase in dye loading being seen when in WT Cx26-expressing cells (Figure 7). The dye loading in Cx26 K125C-expressing cells was significantly greater than that of the WT Cx26-expressing cells (P=0.004, Figure 7). Taken together these results demonstrate that WT Cx26 does not respond to NO_2 with channel opening but this new functionality can be conferred by mutation of lysine 125 to a cysteine residue.

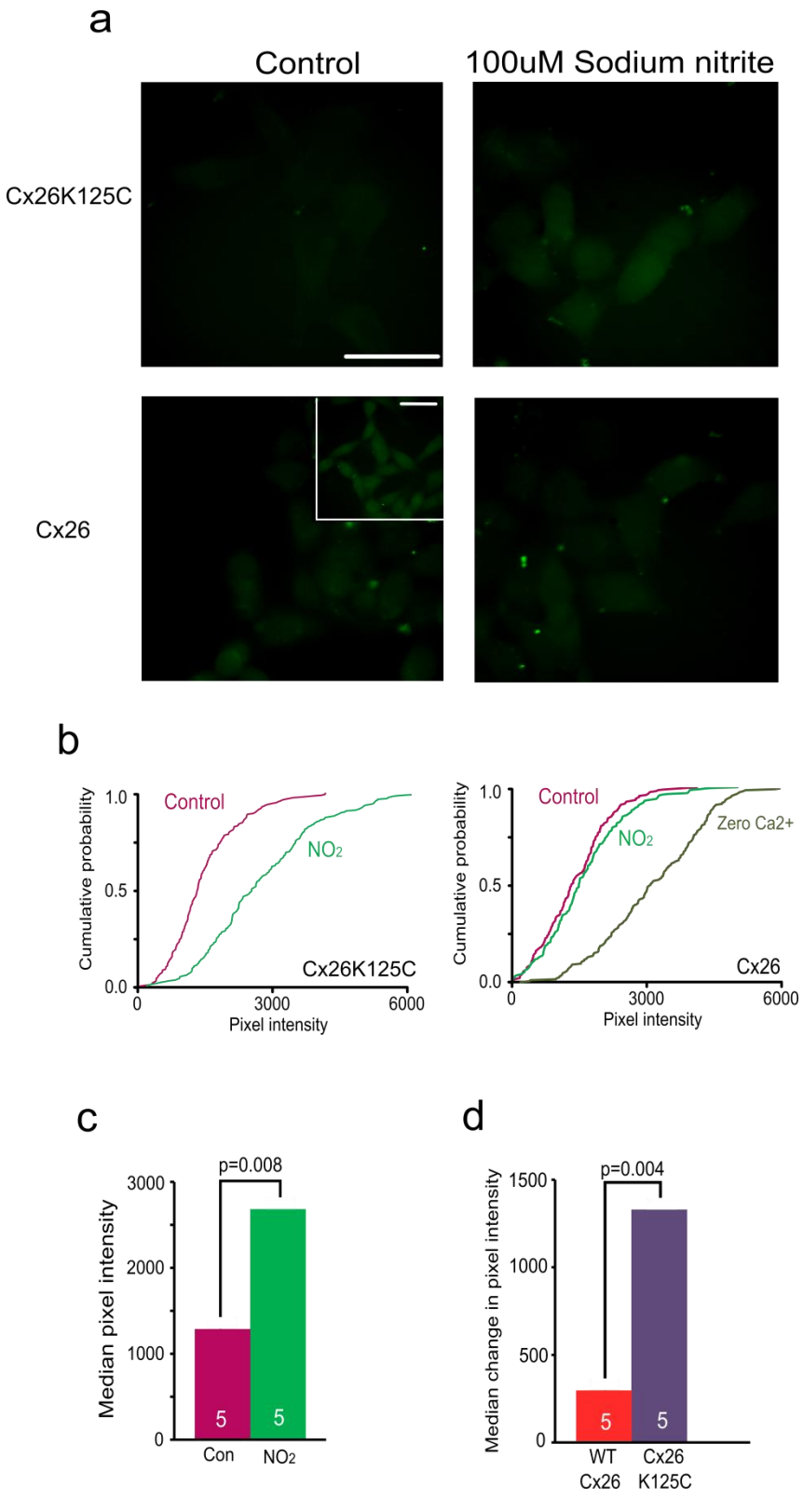

Figure 7 Cx26 K125C displays NO_2 dependent channel opening. a. Dye loading images for Cx26K-C and Cx26 under control and 100µM Sodium nitrite. All scale bars 50µm. Inset shows zero Ca^{2+} loading. b. Cumulative probability distributions for Cx26K-C and Cx26 combining all results over 5 repetitions. c. Median pixel intensity data for control and NO_2 conditions for Cx26K-C displaying Mann-Whitney statistical test data. d. Median

change in pixel intensity between control and NO_2 conditions comparing Cx26K-C to WT Cx26 displaying Mann-Whitney statistical test data.

Our ability to transform the ligand sensitivity of Cx26 by a single mutation has two important implications. Firstly, it suggests that the molecule is very flexible as the SNO bridge can form over a greater distance than the carbamate bridge (7.6 Å versus 6.5 Å). Secondly, the ability of this alternative SNO bridge formation to open the hemichannel supports our original hypothesis of carbamate bridge formation is a key step in native Cx26 hemichannel gating. This study is also exciting because it is the first time a new ligand sensitivity has been engineered into a connexin and strengthens the idea that at least some of the connexins can act as receptors.

6.4.2 Redox sensitivity can be conferred to Cx26 by replacement of the salt bridge interaction with a cysteine bond

Given that substitution of lysine 125 by cysteine gives a channel openable by nitrite, the obvious next step is to make arginine 104 a cysteine too. This would give the chance of disulphide bridge formation between the two residues. If this were to occur the new channel should be sensitive to intracellular redox state. We therefore produced a Cx26 mutant in which both Lysine 125 and Arginine 104 were replaced by cysteine residues (Figure 8). The structure of the new mutant can be seen in Figure 9.

```
Cx26          91 VAMHVAYRRH EKKRKFMKGE IKNEFKDIEE IKTQKVRIEG SLWWTYTTSI FFRVIFEAVF 150
Cx26 K125C    91 VAMHVAYRRH EKKCKFMKGE IKNEFKDIEE IKTQCVRIEG SLWWTYTTSI FFRVIFEAVF 150
  R104C
```

Figure 8. Sequence comparison of Cx26 and Cx26 K125C R104C. Mutation of residues 125 and 104 to cysteine residues. These residues should be capable of forming a disulphide bond making the channel redox sensitive.

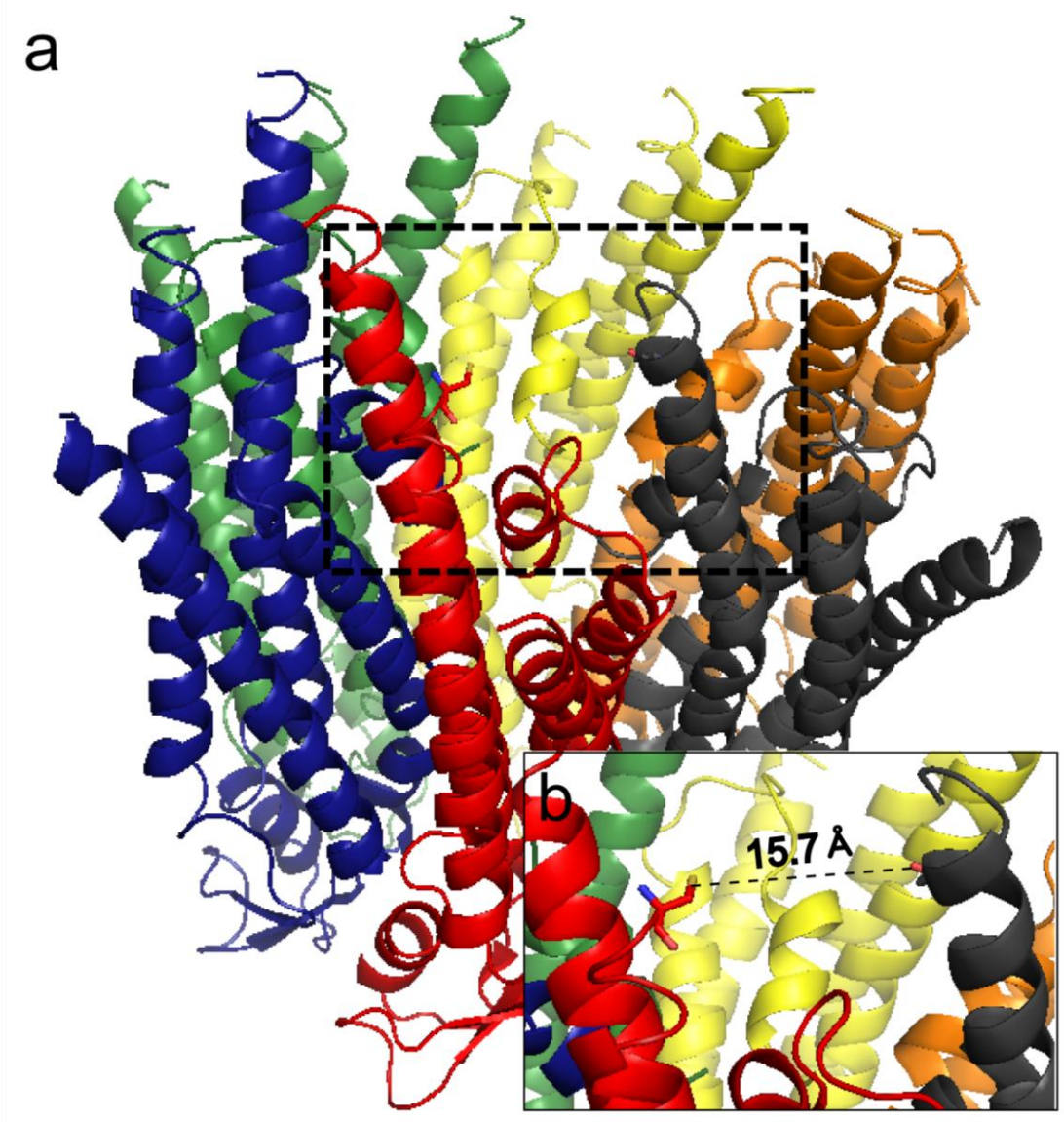

Figure 9 Structure of Cx26 K125C R104C mutant. a) Cx26 hexamer with region of interest highlighted by dotted box. b) Mutated cysteine residues shown in red and grey. The distance between these residues is 15.7Å

To test the ability of Cx26 K125C R104C to respond to intracellular redox we needed a way to manipulate this variable. The concentration of glutathione (GSH) is a key

controller of intracellular redox. Due to its structural similarity to cysteine, N-Acetyl—L-cysteine (NAC) can be used to enhance the rate of GSH synthesis, and to replenish its cellular concentration (Figure 10) (Miners *et al.*, 1984). As previously shown in Figure 3, the oxidised form of GSH, glutathione disulphide (GSSG) can promote the formation of disulphide bonds. Elevated levels of GSH reverse this reaction leading to cleavage of disulphide bonds. The use of NAC should therefore lead to the loss of the intersubunit interaction in this system and reduce the redox state leading us to expect channel closure.

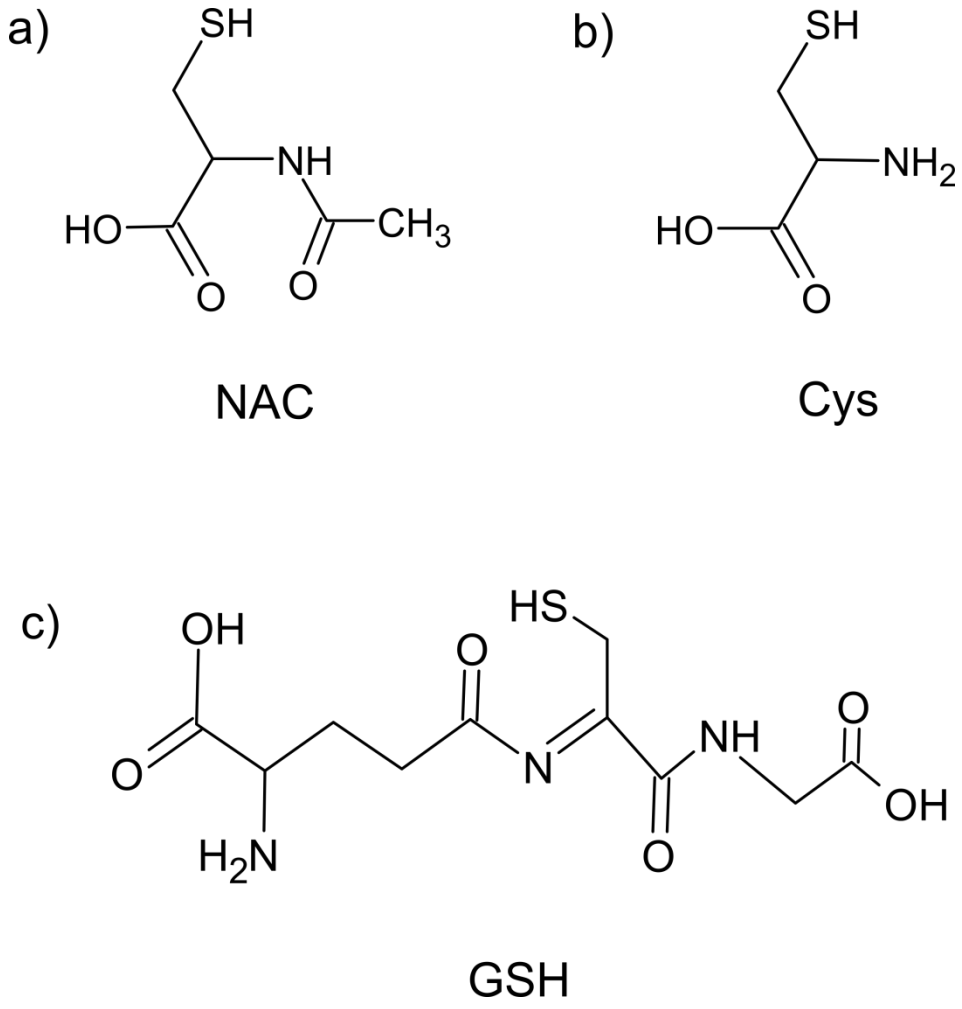

Figure 8 Structure of NAC. N-Acetyl –L-Cysteine (a) can be used to produce cysteine (b). Cysteine can be used to replenish glutathione levels (c).

The converse, inducing intracellular oxidization, can be performed by inhibiting the synthesis of GSH. We therefore used L-Buthionine Sulfoximine (BSO) to inhibit the key synthetic enzyme of gamma-glutamylcysteine synthetase, which catalyses the first step of GSH synthesis by conjugating a glutamate and a cysteine residue (Richman & Meister, 1975; Griffith & Meister, 1979; Puri & Meister, 1983). The reduction of GSH levels in cells, prevents disulphide bond reversal and promotes the formation of disulphide bonds. For our mutated Cx26, we anticipate that this would lead to the formation of a disulphide bond between the cysteine residues at 125 and 104 on the neighbouring subunit.

We found that there was no increase in dye loading when cells were exposed to the reduced aCSF. (Figure 11). However when cells were exposed to the oxidised aCSF, there was a large increase in dye loading compared to control experiments ($p=0.016$, Fig 11a, b,d). This redox sensitivity is not a property of WT Cx26, as cells expressing Cx26 showed no redox sensitive dye loading ($p=0.421$, Figure 11e).

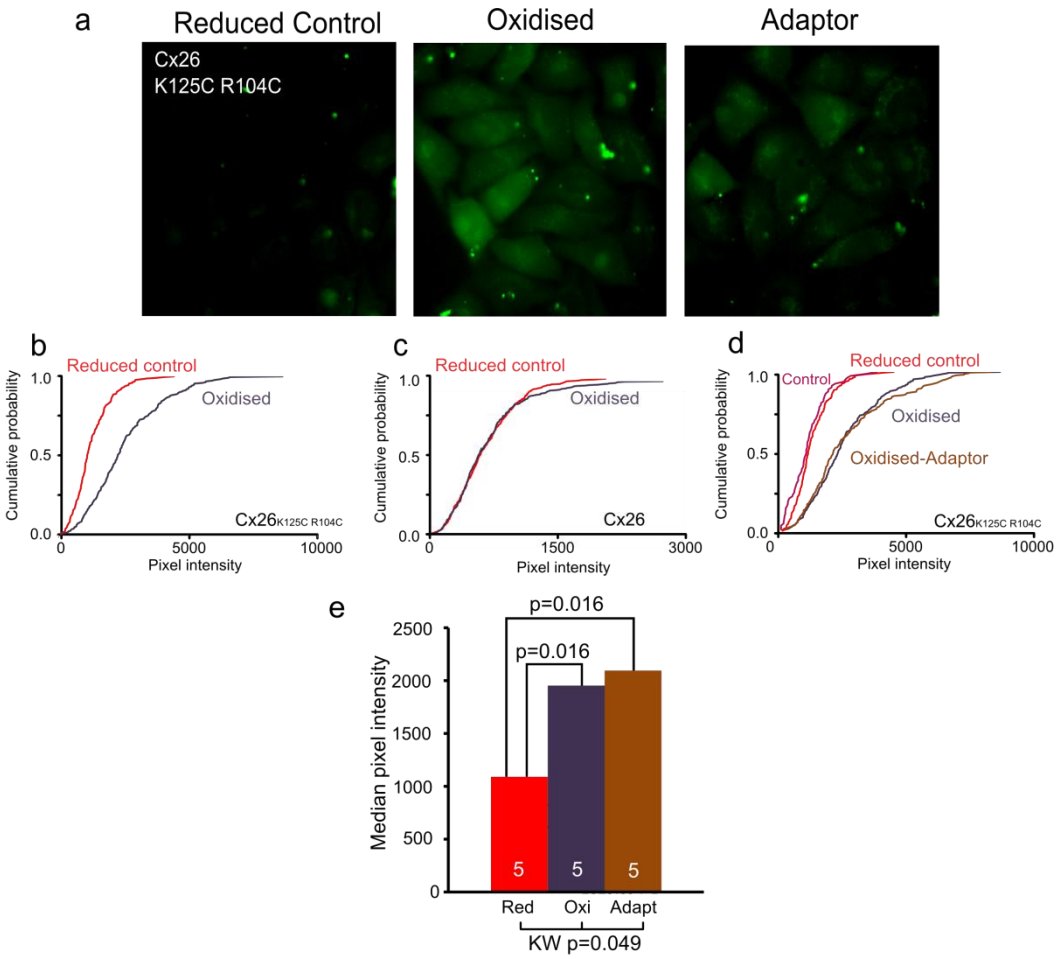

Figure 11 Cx26 K125C R104C is redox sensitive. a) Dyeloading images for Cx26 K-C R-C. An oxidising environment was shown to lead to increased dyeloading, indicating channel opening through disulphide bond formation. b) Cumulative probability distribution of all data for 5 repetitions for Cx26 K-C R-C mutant. c) Cumulative probability distribution demonstrates comparable loading with presence or absence of MTSES adaptor molecule. d) Median pixel intensities for reduced, oxidised and adaptor conditions displaying Mann-Whitney and Kruskal-Wallis statistical test data. e) Cumulative probability distribution for WT Cx26 demonstrating a lack in redox sensitivity.

We also tested the efficacy of an adaptor molecule, MTSES, under oxidation conditions. This molecule has a sulphoxide group on each end allowing it to bridge large gaps between cysteine residues (Figure 3). However the presence of the adaptor molecule caused no change to the dye loading, suggesting that the protein is sufficiently flexible to enable disulphide bond formation between C125 and C104, although these are predicted to be nearly 16 Å apart in the crystal structure of the protein (Figure 11).

6.4.3 Patch clamp recordings confirm redox sensitivity of Cx26 K125C R104C

Whole cell patch clamp recordings were made from Cx26 K125C R104C-expressing HeLa cells. Once again we used the oxidised and reduced aCSF to manipulate intracellular redox. Oxidation should allow the formation of disulphide bonds causing the opening of the channel, which would be evident as an increase in the whole cell conductance during the patch clamp recording.

We observed a slow increase in whole cell conductance occurring approximately 1 minute after application of BSO (oxidized aCSF, Figure 12). By contrast the NAC present in the reduced aCSF leads to channel closure due to increased glutathione synthesis resulting in reduction of disulphide bonds. On transfer to the reduced aCSF, a slow decrease in whole cell conductance was seen approximately 3 minutes after NAC application. Interestingly removal of NAC revealed a small conductance change suggesting that the native redox state of the HeLa cells is slightly oxidized compared to the highly reduced state induced by inclusion of NAC.

By contrast to Cx26 K125C R104C, WT Cx26 demonstrated no change in whole cell conductance in response to either BSO or NAC, while still demonstrating CO_2 sensitivity (Figure 12). These results confirm novel redox sensitivity has been introduced into Cx26 K125C R125C.

We found that the changes in response to alteration of redox state of the whole cell conductance of HeLa cells expressing Cx26 K125C R104C were rather slow compared to those induced by CO_2 in HeLa cells expressing WT Cx26. There are two

potential reasons for this. For BSO to take effect it must firstly cross the membrane and inhibit gamma-glutamylcysteine synthetase. Secondly, for disulphide bond formation to occur the intracellular stores of glutathione which could in the mM range must be significantly depleted (Maher, 2005). Similarly, to achieve a reduced intracellular redox state, NAC must enter the cell and then be used to replenish GSH levels. This could take several minutes. In contrast, CO_2 must only travel across the cell membrane to take effect, explaining the fast dynamics of channel opening by this stimulus.

As well as the changes in channel conductance described above, an acceleration of the increase in whole cell conductance was observed when BSO was replaced by NAC. If the only effect of BSO were to cause channel opening by promotion of disulphide bridge formation, its removal should lead to channel closure rather than accelerated opening. That we did not see this occurring immediately, could be explained if BSO had other effects that opposed the redox sensitive opening of the hemichannel. Upon removal of BSO, it would take time to wash out the BSO from the intracellular milieu, and hence its actions on GSH levels would persist for some minutes. If the other effects of BSO such as a direct block of the open hemichannel channel were removed rather quickly this would explain the continued and accelerating increase in whole cell conductance for a few minutes following the removal of BSO. The putative additional action of BSO in this system is currently unknown.

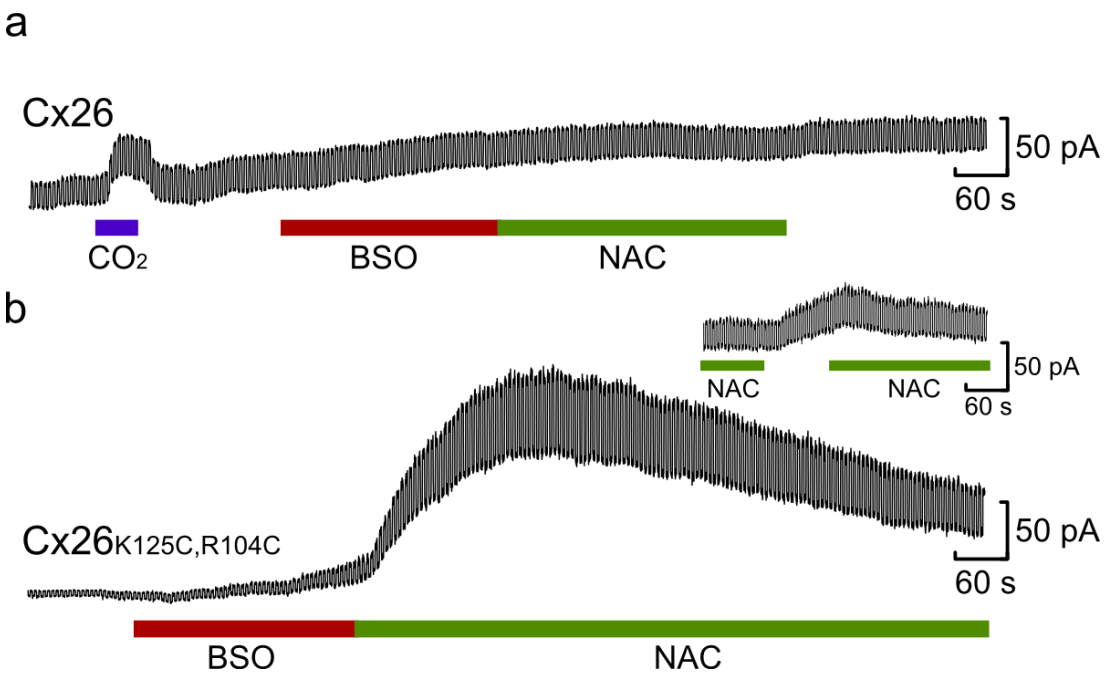

Figure 12 Cx26 K125C R104C is redox sensitive unlike WT Cx26 a) WT Cx26 is not sensitive to changes in redox potential. The sensitivity of these cells to CO_2 confirms the connexin channels to be functioning correctly. b) Cx26 K125C R104C responds to BSO application with channel opening. This is reversed by NAC application. Inset demonstrates channel opening upon NAC wash indicating the natural redox potential of the cells.

6.5 Discussion

6.5.1 Cx26 as a ligand gated receptor

NO/NO_2 is known to be an important signalling molecule. The similarity between CO_2 and NO_2 offers an intriguing possibility for the adaption of the function of Cx26. Exposure of Cx26 K125C to 100µM sodium nitrite produced channel opening, as demonstrated via the dye loading assay. In contrast, WT Cx26 demonstrates no channel opening in response to NO_2. We also demonstrated that redox sensitivity can be introduced by the formation of a disulphide bond between connexin subunits. This bond was shown to form in the absence of the MTSES adaptor protein, despite a 15.7Å gap between residues (Figure 9). Similarly, an electrostatic interaction was able to form from NO_2 to Arginine 104 despite the gap between them being approximately 7.59Å (10.5Å distance between residues minus 1.21Å

N=O, 1.7Å N-S)(Figure 6) (Allen *et al.*, 1987). Salt bridge formation can only occur when the distance between residues is less than 4Å. Therefore both of these results indicate a large degree of flexibility in the Cx26 hemichannel (Kumar & Nussinov, 2002). As stimulus driven channel opening is thought to be due to the channel becoming locked in the open state, these results indicate the hemichannel is able to move between an open and closed conformation in the absence of any stimulus. As described in chapter 4 this could be explained by the flickering channel opening known to be exhibited by Cx26 coupled with the rotational theory for channel opening (Gonzalez *et al.*, 2006; Huckstepp *et al.*, 2010a).

The results from the experiments performed in this chapter indicate two important things. Firstly these experiments demonstrate our ability to adapt connexin channel function to a number of different stimuli. Connexin hemichannels have previously been demonstrated to be sensitive to changes in pH, voltage and extracellular Ca^{2+}. With all of these stimuli a physical blockade of the channel has been proposed as the mechanism of action (Oh *et al.*, 2004; Locke *et al.*, 2011; Zonta *et al.*, 2012). The response of Cx26 to CO_2, and of these mutants to their respective stimuli, however results from direct binding leading to a conformational change causing opening of the channel. These results therefore represent the first evidence of connexin hemichannels having the capacity to act as ligand-gated receptors. This work also represents the first occasion where ligand-gated receptors have been altered to respond to a stimulus unrelated to their native sensitivity. Other receptors have been re-engineered to be activated by new ligands such as the so-called DREADD receptors. In these receptors the muscarinic receptor was altered to respond to

clozapine-N-Oxide (CNO), while at the same time losing its endogenous sensitivity to acetylcholine. However, CNO is structurally similar to another endogenous muscarinic receptor ligand, clozapine, and as such our work reflects a unique result in introducing ligand gated receptor activation to a stimulus unrelated to that expressed endogenously (Armbruster *et al.*, 2007). As our modified Cx26 required only one mutation to lose sensitivity to the natural ligand (CO_2) and gain sensitivity to NO/NO_2 and one further additional mutation to gain sensitivity to intracellular redox potential, this makes it a remarkably quick and simple method for introducing a completely novel sensitivity into a cell.

6.5.2 Cx26 as NO_2 and redox sensors

The mutant connexin hemichannels produced in this chapter have lost their ability to respond to CO_2 and instead respond to NO_2 or redox state. As such these channels could potentially be used as sensors for these molecules; the gated hemichannel allowing the possibility of the release of a detectable molecule in response to NO_2 or a change in redox condition. These could be molecules that can be visualised, such as a dye or fluorophore, or a molecule detectable by biosensors e.g. ATP (Gourine *et al.*, 2005a). There are however already detector molecules for these 2 stimuli. For nitric oxide, fluorescent probes use transition metals to bind a fluorophore. When nitric oxide is present the fluorophore is released from the transition metal and can be visualised (Lim & Lippard, 2007). For detecting changes in redox potential, reduction-oxidisation sensitive GFPs are available. These molecules are green fluorescent protein adapted to have cysteine residues at the appropriate positions required to produce a disulphide bond. Upon oxidation,

disulphide bond production causes a change in fluorescence that can be visualised (Hanson *et al.*, 2004). As such the available methods of the detection of these molecules are more sophisticated than the method produced here and at this level of development Cx26 mutations do not offer a viable alternative to GFP probes. The physiological role of NO/NO_2 signalling is a topic of great research interest. In Chapter 5 we speculated on the ability to use viral transfection of connexins to effect cell signalling and demonstrated that connexin delivery in this way has the potential to effect native CO_2 sensitivity. It could also be possible to use expression of Cx26 K125C to alter NO/NO_2 sensitivity of specific groups of cells and thus affect a novel additional cell signalling pathway within a physiological system. Although a specific role in which this sensor could be used is yet to present itself, it has the potential to be a relevant tool in future research.

6.5.3 Confirmation of earlier findings

Both the NO_2 and redox sensitive connexins produced were devised based on our hypothesised mechanism of channel opening, these results are important as further supporting our findings from chapters 3 and 4. The adaption of the connexin channel to bind NO_2 in what was previously the CO_2 binding site was shown to be capable of producing the same result as seen previously with CO_2, namely channel opening. This indicated that the presence of a negative charge due to NO_2 or CO_2 binding is equivalent in this system. This confirms that an intersubunit salt bridge is capable of producing channel opening in Cx26. As only lysine 125 has been adapted to bind NO_2 by mutation to a cysteine residue, the change in NO_2 sensitivity between WT Cx26 and Cx26K125C indicates residue 125 is the binding site for NO_2

and therefore confirms this position as the binding site for CO_2. The redox experiments performed also confirm our previous hypothesis. Here we demonstrate that a disulphide bond formed between residues 125 and 104 on neighbouring subunits is capable of producing channel opening. This confirms our prior experimentation that reveals a salt bridge interaction between these 2 residues is responsible for CO_2 sensitivity in Cx26.

Chapter 7 -Discussion

The work in this thesis aimed to elucidate the mechanism of CO_2-senitivity in Cx26 by answering three main questions:

- Does CO_2 bind to Cx26 directly?
- If so how?
- How does this binding produce a conformational change?

During the course of this thesis I have identified a CO_2 binding site on Cx26 in the form of lysine 125. Though sequence comparison this residue was identified to be present only in CO_2-sensitive connexins. Using point mutation of both a CO_2-sensitive and an insensitive connexin, this residue was shown to be required for the previously established CO_2 dependent channel opening seen in Cx26. Another residue, arginine 104 was also shown to be required for channel opening which we hypothesise represents a salt bridge interaction that causes the conformational change required for channel opening. This work therefore answers all of the above questions, both confirming the direct nature of CO_2 binding and providing strong evidence for the mechanism involved in channel opening. The implications of these finding will be discussed in this chapter.

7.1 CO_2 directly binds to Connexin 26

Having identified CO_2 binds directly to Cx26 to open the hemichannel there are several implications for the functions of connexins and connexin hemichannels.

7.1.1 Hemichannels in signalling

As discussed in the introduction, connexin hemichannels have been demonstrated to be capable of signalling (Stout *et al.*, 2002; Pearson *et al.*, 2005). This does however seem counterintuitive as common sense suggests that opening large, non-specific pores in the cell membrane would lead to the loss of metabolites and ions from the cell, leading to its death. During these experiments a constitutively open mutant was expressed in HeLa cells (Cx26K125E). These cells remained alive and healthy throughout cell culture and experimentation indicating that the presence of hemichannels does not lead to cell death. In these cells, dye passed into these cells without elevated CO_2 as a stimulus, indicating the channels to be open. Despite a wash lasting for 30 minutes, enough dye remained in the cells to be visualised. This indicates that carboxyfluorescein, and potentially other intracellular content, become trapped within the cell. This could be due to compartmentation of metabolites within the cell. It is now known that over half the volume of a typical cell is made up of internal compartments separated from the rest of the cell by plasma membrane (Alberts *et al.*, 2002). This restriction of intracellular movement coupled with the presence of cytoskeleton and other proteins is likely to make the interior of the cell almost crystalline and not of the fluid nature previously described.

7.1.2 Connexins as receptors

The identification of Cx26 as a receptor for CO_2 introduces a more general concept of connexins as ligand gated channels. Due to their role as gap junctions, connexins are present in all tissues of the body. The connexin expressed is however tissue

specific, suggesting specific connexins perform specific roles in different cells. We could speculate that isolated hemichannels may exist to act as receptors, the ligands of which determine which connexin is expressed in which tissue. As our findings support an intracellular CO_2 binding site we may expect the ligands for these channels to be small molecules capable of passing through the membrane or those present within the cells. This could include molecules similar in structure to CO_2 or perhaps amino acids and ATP.

Supporting the ability of other connexins to act as ligand gated receptors, Cx36 has been shown to respond to changes in NO/NO_2 with channel opening (N. Dale, H. Benford unpublished study). Much like the response to CO_2, this appears to be connexin specific with Cx26 shown to be insensitive to NO/NO_2 both in the work outlined in this thesis and in the previous experiments. Although the mechanism involved is yet to be deciphered this offers the exciting possibility of another ligand gated connexin receptor. Cx36 is expressed in the sympathetic preganglionic neurons and is known to be involved with cardiovascular responses (Marina *et al.*, 2008), as is the NO/NO_2 signalling molecule (Chowdhary & Townend, 1999).

7.1.3 Implications for CO_2 chemosensitivity of breathing

The presence of a CO_2 binding site in Cx26 identifies it as the first protein known to be expressed in key chemosensitive areas (Huckstepp *et al.*, 2010b) that is capable of directly sensing CO_2. Despite a multitude of evidence supporting direct modulation of respiration by CO_2 (Eldridge *et al.*, 1985; Harada *et al.*, 1985; Shams, 1985), this mechanism of CO_2 chemosensitivity has previously been dismissed. This has been due to the belief that CO_2 can freely diffuse through plasma membranes

and through its reaction with H_2O decrease internal pH (Thomas, 1976; Boron, 2010). Here I have demonstrated that the sequence of Cx26 determines the sensitivity of this channel to CO_2 and consequently the interaction is a direct one and not through the proxy of pH.

Harada et al demonstrated a difference in CO_2 chemosensitivity between CO_2 detection and pH detection with CO_2 producing a more transient stimulus (Harada *et al.*, 1985). Having established the mechanism of Cx26 channel opening, can we shed any light on the reason for this? In pH CO_2 detection, a decrease in internal pH is thought to inhibit K^+ channels leading to depolarisation of the cell (Filosa *et al.*, 2002). In this situation action potentials will continue to fire while the channels remain inhibited and the membrane depolarisation remains above the threshold level. However in direct CO_2 signalling there is a finite number of Cx26 hemichannels each with a finite number of binding sites. There is also a physical signalling molecule that is released which could hypothetically become depleted. We could speculate that occupation of all available CO_2 sites coupled with ATP depletion could lead to a more transient ventilatory response to direct CO_2 binding than to pH. Another possibility arises from the ability of connexin channels to be gated by numerous stimuli. Although CO_2 causes opening of the Cx26, these channels are also closed by decreases in pH (Spray *et al.*, 1982) which will accompany an increase in CO_2 in vivo. The transient nature of the response may represent the limited time frame that CO_2 can induce channel opening before changes in pH cause them to close. This difference in time frame may reflect the

fact that CO_2 is more mobile and can easily cross the blood brain barrier where as H^+ cannot.

7.1.4 Cx26 in chemosensitivity

Although Cx26 may seem an unusual candidate for a CO_2 receptor, further investigation into its properties leave little doubt for its role in chemosensitivity. Firstly, Cx26 is expressed in precisely the best place for detecting systemic CO_2 changes from the medulla. This involves being specifically expressed within the first 300µm of the surface and around penetrating blood vessels (Figure 11). As a result the CO_2 levels detected are not influenced by local metabolism which would artificially boost the CO_2 detected.

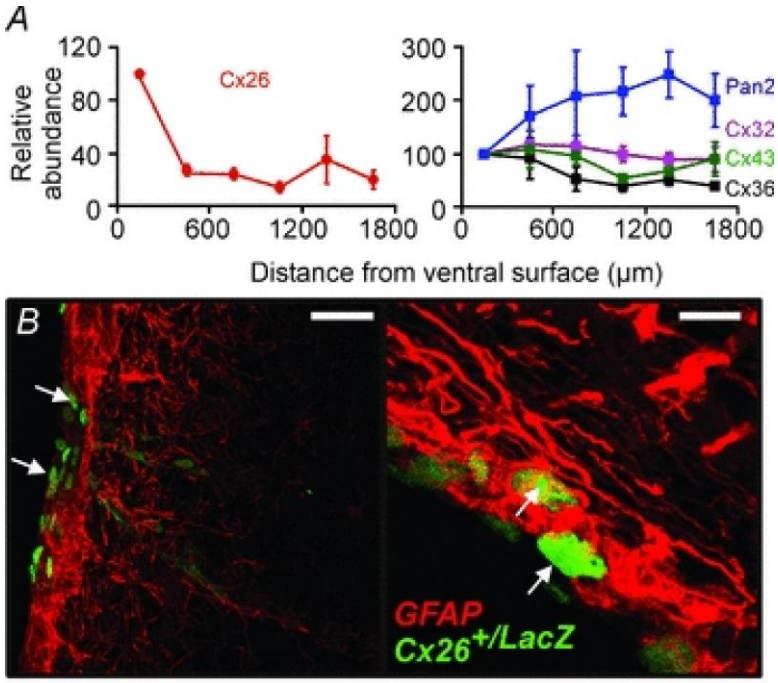

Figure 9 Cx26 is specifically expressed close to the surface of the ventral medulla where as other gap junction proteins are uniformly distributed throughout. Reproduced from (Huckstepp *et al.*, 2010b)

Previous work by Huckstepp et al also revealed the CO_2-sensitivity of Cx26 to be perfectly adapted to mammalian physiology. As a comparison we can consider GPCR proteins which act as CO_2 sensors in insects and C.elegans (Jones *et al.*, 2007; Yao & Carlson, 2010; Hallem *et al.*, 2011). In these species rather than controlling respiration, CO_2 levels are monitored for feeding or social activities (Suh *et al.*, 2004; Hallem & Sternberg, 2008). GPCRs in insects are known to respond to increases in CO_2 between 0.01 and 0.3mM (Turner & Ray, 2009; Yao & Carlson, 2010). As a result these channels are likely to be fully saturated at mammalian CO_2 levels which are around 40mmHg or 1-2mM and therefore GPCRs would not be capable of detecting small changes in CO_2 around this level. Patch clamp studies investigating channel opening of Cx26 at various CO_2 concentrations revealed sensitivity in this protein to be centred around 40mmHg and to have a very steep dose response relationship for CO_2 (Huckstepp *et al.*, 2010a) (Figure 2). This indicates that Cx26 is most sensitive to changes in CO_2 at physiological levels relevant to mammals and can therefore detect small changes in CO_2 despite the relatively high basal level CO_2 concentration. This high basal level of CO_2 is due to air breathing animals being poor at the excretion of CO_2, leading to its retention (Nattie, 1999). Animals using gills are much better at this process and this can be seen by comparing frogs, which display a basal level of 40mmHg like other air breathers, and tadpoles, which have the reduced blood CO_2 of 30mmHg (Nattie, 1999). As CO_2 sensitivity in Cx26 is highly adapted to air-breathing animals this suggests Cx26 may have evolved this feature sometime after animals moved onto the land.

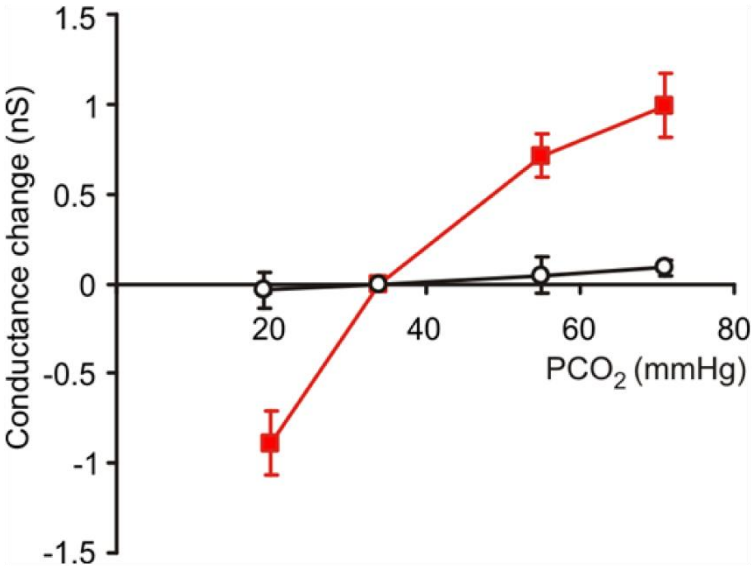

Figure 2 Cx26 is most sensitive to changes around 40mmHg Reproduced from (Huckstepp *et al.*, 2010a) Patch clamp data for Cx26 indicating channel opening over a range of CO_2 levels. Cx26 (red) is most sensitive to changes in CO_2 around 40mmHg. WT HeLa cells (black) show no CO2 dependent channel opening.

7.1.5 Potential functions of Cx26 as a CO_2 receptor in other regions and organs

As CO_2-sensitivty of Cx26 is dependent on the presence of the protein alone, it follows that CO_2-sensitivity could be important anywhere the protein is found. In the brain, Cx26 is found near penetrating blood vessels (Huckstepp *et al.*, 2010b) which would allow it to detect systemic CO_2 accurately in any part of the brain. Cx26 has also been identified in the bottom two layers of the meninges, the leptomeninges, a protective membrane around the surface of the brain (Huckstepp *et al.*, 2010b). As such Cx26 could be present throughout the entire surface of the brain with CO_2 sensitivity possible at any of these sites. Although this work has focussed on the changes in breathing associated with elevated CO_2, there are several other consequences to hypercapnia. CO_2 is known to cause vasodilatation and cardiovascular responses which aim to decrease blood pH by aiding CO_2

excretion from the lungs (Richardson *et al.*, 1961). Panic and anxiety responses are known to be evoked in high CO_2 environments which discourage the individual from entering areas with dangerous CO_2 levels (Papp *et al.*, 1993). Arousal from sleep can also occur if environmental CO_2 levels increase or if problems with ventilation prevent proper CO_2 excretion (Haxhiu *et al.*, 2001).

Cx26 could also be important in areas where CO_2 is not yet known to have a direct signalling role for example in the periphery. Cx26 is known to be expressed in various organ systems such as the skin (Goliger & Paul, 1994), cochlea (Kikuchi *et al.*, 1995) and liver (Green *et al.*, 1988) with no potential roles for CO_2 currently identified. In other organ systems, however, a role for CO_2 is less speculative. For example, Cx26 is expressed in the alveoli of the lung (Carson *et al.*, 1998). There is an unknown mechanism by which CO_2 can relax the parenchymal tissue of the lung (Carson *et al.*, 1998; Emery *et al.*, 2007). It is tempting to speculate that this mechanism could be mediated by Cx26.

The expression of Cx26 is also highly regulated in the uterus. One possible function may be during implantation of the blastocyst, where Cx26 is upregulated at the sites of future implantation. As PCO_2 affects the success of implantation it is tempting to speculate that the CO_2 produced by blastocytes may be detected by Cx26 and trigger cell signalling that contributes to the processes of implantation (Torbit & Weitlauf, 1975; Grummer *et al.*, 1994).

7.2 CO_2 binding occurs at residue lysine 125

In this work we have demonstrated that the CO_2 binding site is on the intracellular side of the membrane, with lysine 125 being found near the transmembrane domain boundary of the third transmembrane region (Maeda *et al.*, 2009). This indicates that CO_2 must be able to cross the plasma membrane of these cells.

7.2.1 CO_2 membrane transport

It was previously thought that CO_2 and other gases were able to freely diffuse through plasma membranes (Boron, 2010). This however has been disputed as some membranes show no permeability to CO_2 perhaps due to integrated membrane proteins impeding CO_2 transport in some cells (Boron, 2010). Other work has taken this further indicating that only specialised membranes containing especially low cholesterol content would be capable of CO_2 diffusion (Itel *et al.*, 2012). There are three potential ways by which CO_2 could move into the cell: the plasma membrane could be permeable to CO_2, CO_2 could enter through a specialised channel or CO_2 entered through Cx26 itself.

CO_2-sensitivity of Cx26 has been identified in both HeLa cells and glial cells in the medulla (Huckstepp *et al.*, 2010b). Following the assumption that CO_2 does not freely pass through all membranes we would have to assume that both of these membranes exhibit suitable properties to allow CO_2 diffusion. As CO_2 detection by Cx26 appears to be important in the chemosensory response of the medulla (Huckstepp *et al.*, 2010b) it could be possible that the membranes of these cells are adapted for gas transport. HeLa cells, however, as far as we know are not important

in CO_2 detection and as such there would be unlikely to have specialised membranes.

This same argument also applies for specialised channels. Aquaporin channels, which are involved in the transport of water, are also known to transport CO_2 (Boron, 2010). It has been suggested that these channels may be specifically expressed in tissues requiring gas transportation. We could expect high expression of aquaporins in the medulla where CO_2 detection is important but may not expect this same expression in HeLa cells.

As connexin hemichannels are large, non specific pores it could be possible that CO_2 could move through them into the cells. Passage of CO_2 would however require the connexin channel to be open and we may expect the channels to be closed at all times when not exposed to stimulus. Our results however appear to suggest that is not true. In chapter 2 Figure 6, we determined that zero Ca^{2+} did not cause channel opening in parental HeLa cells. This experiment also lets us compare the control loading of parental HeLa cells (expressing no connexins) and Cx26. We see that control loading for parental HeLa cells is lower than those cells containing connexins. This increase in control level is also seen in Cx31 (non CO_2-sensitive) and therefore does not reflect channel opening in response to the lower PCO_2 of the control aCSF. Instead this suggests that connexin channels allow a small amount of dye influx even in the absence of a stimulus. This could be due to incomplete closure of the channel or due to spontaneous opening events. Either of these mechanisms could allow the movement of CO_2 into the cell.

Membrane properties and aquaporin content of HeLa cells are to my knowledge unknown and as such the exact mechanism of CO_2 transport cannot be asserted conclusively. However based on the arguments above, the author supports CO_2 movement through the Cx26 channel itself.

7.2.2 The conservation of lysine 125

Despite the myriad of other CO_2/H^+ sensing molecules, the selective removal of Cx26 in areas of known CO_2 chemosensitivity greatly reduces the adaptive changes in breathing to hypercapnia (N. Dale, G. Koentges, X. Zhang, J. Zhang unpublished study). Previous experiments, such as Shams et al, have also demonstrated elevated CO_2 in the absence of a pH change to cause a change in respiration (Eldridge *et al.*, 1985; Shams, 1985). Together these indicate that loss of direct CO_2 detection can have profound effects on CO_2 chemosensitivity even if pH detection mechanisms are maintained. Assuming Cx26 to be the only CO_2 receptor, a loss of lysine 125 would reflect the loss of the ability to respond to an entire branch of the H^+/CO_2 threat and as such would be highly disadvantageous. In line with this we have found that the CO_2 binding site and the surrounding sequence, which is important for correct orientation of this residue, are highly conserved in all species tested including primates, rodents, carnivores, sea mammals and to a lesser extent birds (Figure 3).

```
Human Cx26          81   LIFVSTPALLVAMHVAYRRHEKKRKFIKGEIKSEFKDIEEIKTQKVRIEGSLWWTYTSSIFFRVIFEAAFMYVFYVMYDG  160
Mouse Cx26          81   LIMVSTPALLVAMHVAYRRHEKKRKFMKGEIKNEFKDIEEIKTQKVRIEGSLWWTYTTSIFFRVIFEAVFMYVFYIMYNG  160
Rat Cx26            81   LIMVSTPALLVAMHVAYRRHEKKRKFMKGEIKNEFKDIEEIKTQKVRIEGSLWWTYTTSIFFRVIFEAVFMYVFYIMYNG  160
Chicken Cx26        81   LIFVSTPALLVAMHVAYTRHERKRRFRSG----DKINIEELKNEKIHIRGPLWWTYTCSIFFRIVFEAVFMYVFYYMYDG  156
Cat Cx26            81   LIFVSTPALLVAMHVAYRRHEKKRKFIKGEIKNEFKDIEEIKSQKVRIEGSLWWTYTSSIFFRVIFEAVFMYVFYIMYDG  160
Killer whale Cx26   81   LIFVSTPALLVAMHVAHYRHEKKRKFMKGEIKSEYKDIEEIKSQKVRIEGSLWWTYTGSIFFRVIFEAAFMYVFYVMYDG  160
```

Figure 10 Lysine 125 is conserved in all species examined

7.2.3 Cx26 and sea mammals

Analysis of the sequence of Cx26 in dolphins and killer whales reveals that the residues important for CO_2-sensitvity are conserved in these species. This indicates that Cx26 CO_2-sensitvity had already evolved when air breathing species returned to the water. Coupled with our knowledge that Cx26 is suited for CO_2 chemosensitivity in air breathing animals we can place the evolution of Cx26 CO_2-sensitvity between 530 and 53 million years ago.

Sea mammals are also of interest due to the strange environment in which they live. During prolonged diving blood CO_2 levels will rise but a drive to breathe must be suppressed. It can therefore be assumed that these animals exhibit a reduced level of CO_2 sensitivity, with higher levels of CO_2 required to cause chemosensory responses. Reduced CO_2 sensitivity is seen in other animals such as those living in burrows where poor ventilation and overcrowding lead to elevated CO_2. In these animals reduced CO_2 sensitivity is thought to be due to insensitivity to H^+ but as more evidence appears that both H^+ and CO_2 play a large role in CO_2 chemosensitivity we cannot discount a role for Cx26.

Sequence comparison of killer whale and dolphin Cx26 to the human sequence reveals several subtle changes (Figure 4). In this comparison the human sequence was used nesting rodents such as mice or rats could theoretically also exhibit reduced CO_2 chemosensitivity.

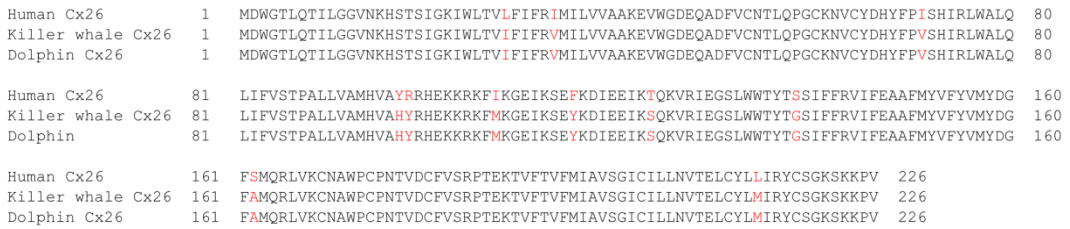

Figure 4 Sequence differences of Cx26 in human and sea mammals

In chapter 4 we demonstrated that the Cx26R104E mutant lacked CO_2-sensitvity despite the presence of the lysine 125 binding site. We surmised that this single amino acid change could sufficiently change the environment around lysine 125 to cause it to exist in the NH_3^+ form and therefore prevent carbamylation. We could also imagine a situation where a change in protein structure could reduce but not eliminate the restricted hydration environment around the lysine 125, pushing the equilibrium of NH_2/NH_3^+ towards the protonated form. CO_2 binding would therefore be possible, but would require increased CO_2 levels to produce a response. This could explain the reduced CO_2-sensitivity seen in some species. The residues involved with maintaining the restricted hydration around lysine 125 are currently unknown, but are likely to involve the c-terminal tail or the loops connecting the transmembrane helices. These areas were however unresolved in the crystal structure produced by Maeda et al due to the high flexibility of these residues (Maeda *et al.*, 2009). As CO_2 binding to Cx26 is thought to lock the channel in its open state, and may therefore reduce the flexibility of the protein, it may be possible to gain an improved crystal structure of Cx26-CO_2 allowing the resolution of the residues of interest. Using this improved crystal structure, coupled with known sequence differences between Cx26 in normal sensitivity and reduced

sensitivity animals it may be possible to investigate the role of Cx26 in these specialised CO_2 detecting systems.

7.2.4 Cx26 Mutations

The importance of maintaining Cx26 CO_2-sensitivity can also be seen when investigating known Cx26 mutants. Mutations in Cx26 are known to be responsible for several forms of non-syndromic deafness i.e. forms of deafness that appear independently in the absence of other symptoms. There are also an increasing number of mutations of Cx26 associated with the syndromic form of deafness, those forms of deafness that occur with other defects such as in the eyes, bones and connective tissue (Garretsen *et al.*, 1997; Burke *et al.*, 2013). There are two forms of mutation responsible for these defects: missense mutations associated with a milder form of the disease and premature stop codons which lead to profound hearing loss.

Interestingly there are no missense mutations involving residues 125 or 104 described for either of these classes of disease (Ballana *et al.*2013). This could indicate the importance of these residues in Cx26, with mutations that affect the CO_2-sensitivity causing death of the individual and as a consequence the removal of these mutations from the population. Patients with congenital central hypoventilation syndrome (CCHS) and sudden infant death syndrome (SIDS), both thought to be linked to defects in chemosensitivity, often die in their sleep if not artificially ventilated (Weesemayer *et al.*, 1992). When awake, breathing is thought to be consciously adapted in situations that are likely to produce high CO_2, such as

during exercise, without a strong reliance on the CO_2 chemosensitive system (Gozal & Simakajornboon, 2000). When unconscious, removal of CO_2 depends entirely on CO_2 chemosensitivity and if this is defective the sufferer may suffocate. Mutations that effect CO_2 detection in Cx26 are likely to follow this same pathology.

Several stop codon mutations do however truncate the protein before residue 125. Cx26 produced by these patients would lack CO_2-sensitivity and as such we may expect these mutations to have been selected out of the population also. This however is not what we see, with patients with these mutations surviving into adulthood. This difference in survival may be explained by looking at the resulting protein from these different types of mutations. Stop codon mutations occur early in the protein sequence e.g. residues 24, 44 and 57 (Ballana *et al.*) and as such the Cx26 produced is severely truncated and is unlikely to function as a gap junction protein. Missense mutation proteins in comparison are full length and depending on the mutation involved would still be able to function in some of their roles. We could therefore see the stop codon mutants as effectively a loss of Cx26 rather than a defect. Connexins 32 and 30 are structurally similar to Cx26 with both proteins also exhibiting CO_2-sensitivity (Huckstepp *et al.*, 2010a). These proteins are often expressed in the same tissues as Cx26 and in these cells form heteromeric channels with this protein (Mese *et al.*, 2007; Yum *et al.*, 2007). It could be possible that the loss of Cx26 leads to an up-regulation of Cx32 or Cx30 to compensate for the lack gap junction coupling in these cells. The presence of these connexins would also compensate for the CO_2 chemosensory response albeit at a lower degree of sensitivity.

7.3. Binding causes restriction of the channel biasing it toward an open state

7.3.1 Supporting the Cx26 crystal structure

Our hypothesised mechanism for CO_2 dependent opening of Cx26 was based on the assumption that lysine 125 is orientated towards arginine 104 of a neighbouring subunit. This assumption arises from the 2zw3 crystal structure produced by Maeda et al (Maeda *et al.*, 2009). The loss of CO_2 sensitivity demonstrated with removal of arginine 104, the electrostatic interaction formed with a negative charge at residue 125 and the ability to mimic this interaction with a disulphide bond all support the validity of the published crystal structure.

7.3.2 CO_2 dependent closure of connexins

In previous studies by Huckstepp et al the CO_2-sensitvity of different connexins was shown to vary. Cx26, 30 and 32 respond to elevated CO_2 with channel opening where as Cx36 is not sensitive to changes in CO_2. Interestingly Cx43 appeared to close in response to elevated CO_2 (Huckstepp *et al.*, 2010a). Although Cx43 contains a sequence similar to the conserved region present Cx26, in this protein it is present around lysine 144. In addition to this the positively charged arginine 104 is replaced with a neutral leucine residue thus preventing the intersubunit interaction important in Cx26 (Figure 5). Taken together this indicates that CO_2 dependent closure of connexin channels must occur by an alternative mechanism. Increases in CO_2 are known to cause decreases in pH which can close connexin channels. However, external pH in these experiments was kept constant and did not decrease upon application of CO_2 (Huckstepp *et al.*, 2010a). As with the Cx26

experiments, the independence to pH changes coupled with the sensitivity to CO_2 when expressed in HeLa cells indicates direct CO_2 modulation. The mechanism of CO_2 dependent channel closure is currently unknown.

```
Cx43  3    DWSALGKLLDKVQAYSTAGGKVWLSVLFIFRILLLGTAVESAWGDEQSAFRCNTQQPGCE  62
Cx26  2    DWGTLQTILGGVNKHSTSIGKIWLTVLFIFRIMILVVAAKEVWGDEQADFVCNTLQPGCK  61

Cx43  63   NVCYDKSFPISHVRFWVLQIIFVSVPTLLYLAHVFYVMRKEEK LNKKEEELKVAQTDGVN  122
Cx26  62   NVCYDHYFPISHIRLWALQLIFV STPALLVAMHVAY-RRHEKKRKFIKGEIKSEFKD---  117

Cx43  123  VDMHLKQIEIKKFKYGIEEHG KVKMRGGLLRTYIISILFKSIFEVAFLLIQWYIY-GFSL  181
Cx26  118   ----IEEIKTQ----------KVRIEGSLWWTYTSSIFFRVIFEAAFMYVFYVMYDGFSM  163

Cx43  182  SAVYTCKRDPCPHQVDCFLSRPTEKTIFIIFMLVVSLVSLALNIIELFYVFFK  234
Cx26  164  QRLVKCNAWPCPNTVDCFVSRPTEKTVFTVFMIAVSGIRILLNVTELCYLLIR  216
```

Figure 5 Sequence alignment of Cx26 and Cx43. The loss of the positive residue at position 104 coupled with the movement of the conserved sequence suggests channel closure to CO_2 does not occur through the same mechanism as channel opening.

7.3.3 Gating of connexin channels

The mechanisms involved in connexin channel closure are disputed, with three main contenders as outlined in the introduction to this thesis. These are physical blockade by a separate molecule or the C-terminal tail (Locke *et al.*, 2011; Zonta *et al.*, 2012), plugging of the channel with the N-terminus (Maeda *et al.*, 2009) or rotational of the connexin subunits (Muller *et al.*, 2002). The results demonstrated in this thesis appear to support a fourth, distinct mechanism. The elastic network model of Cx26 indicates the subunits of the connexin channel move to physically occlude the channel. The flexible nature of Cx26 is confirmed by our experiments involving Cx26K125E and Cx26K125CR104A which found that an intersubunit salt bridge was able to form between the residues of interest despite them being separated by more than 4Å, the cut off point for forming electrostatic interactions

(Kumar & Nussinov, 2002). This suggests that connexin hemichannels are not static structures and that the subunits are highly mobile closing the channel by moving to block the central pore. In the introduction we discussed how the mechanism of connexin gating may depend on the stimulus involved. As CO_2 has never previously been investigated as a stimulus for connexin gating this may explain why this mechanism has never been witnessed before.

7.4. Tools for further research

As well as determining the mechanism for the CO_2-sensitvity of Cx26 this work has produced a number of tools that could prove useful in future research. In chapter 6 we described the production of Cx26 K125C which is NO/NO_2 sensitive and Cx26 K125C R104C which is sensitive to changes in redox potential. Due to sophisticated GFP-based sensors for both of these stimuli are unlikely to provide this role. However, in chapter 5 we demonstrated our ability to change the sensitivity of connexin expressing cells by the introduction of non-native connexin subunits. Using this same mechanism it may be possible to introduce novel sensitivity into cells in vivo allowing signalling pathways to be investigated.

The ability of dnCx26 to remove CO_2-sensitvity in isolated Cx26 expressing HeLa cells supports the idea of adapting this system for use in vivo. In this system, dnCx26 could be expressed specifically in the tissue of interest using viral specific promoters. This would knock out CO_2-sensitivity, but not connexin expression, in the tissue of interest allowing the role of CO_2 in these processes to be investigated. As Cx26 can act as a CO_2 receptor independently and that this protein is found in various tissues throughout the body this tool could prove it invaluable in

deciphering the role of Cx26 CO_2 detection in other tissues. Cx26 is also capable of producing heteromeric channels with Cx30 and 32 (Mese *et al.*, 2007; Yum *et al.*, 2007). As both of these connexin are known to be CO_2-sensitive it follows that these heteromeric channels should also be CO_2-sensitive, especially since the residues identified to be involved in the CO_2-sensitvity of Cx26 would be present in all subunits of these channels. Loss of a number of these subunits should prevent CO_2 sensitivity and as such our dnCx26 could also be used to probe the CO_2-sensitivity of these heteromeric channels.

5. Summary of results

In this thesis I have demonstrated that Cx26 hemichannels are opened by elevated CO_2 due to direct binding to lysine 125. The negative charge formed from this carbamate reaction causes an electrostatic interaction which restricts channel movement biasing the channel towards its open state. This represents identification of the first CO_2 receptor and provides the key missing link to how CO_2 itself can act as the stimulus in CO_2 chemosensitivity.

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