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CO₂ sensing by Connexin26 and its role in the control of breathing

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Abstract

Breathing is essential to provide the O₂ required for metabolism and to remove its inevitable CO₂ by product. The rate and depth of breathing is controlled to regulate excretion of CO₂ to maintain the pH of arterial blood at physiological values. A widespread consensus is that chemosensory cells in the carotid body and brainstem measure blood and tissue pH and adjust the rate of breathing to ensure its homeostatic regulation. In this review, I shall consider the evidence that underlies this consensus and highlight historical data indicating that direct sensing of CO₂ also plays a significant role in the regulation of breathing. I shall then review work from my laboratory which provides a molecular mechanism for the direct detection of CO₂ via the gap junction protein connexin26 (Cx26) and demonstrates the contribution of this mechanism to the chemosensory regulation of breathing. As there are many pathological mutations of Cx26 in humans, I shall discuss which of these alter the CO₂ sensitivity of Cx26 and the extent to which these mutations could affect human breathing. I finish by discussing the evolution of the CO₂-sensitivity of Cx26 and its link to the evolution of amniotes.

Key words

Hypercapnia, respiratory chemosensitivity, connexin, hemichannel
1. Blood gases and breathing

We breathe for two essential purposes: the first is to provide oxygen required for metabolism. Adenosine triphosphate (ATP) is the universal store of chemical energy that is used to drive the energetically unfavourable reactions essential for life. To produce ATP via oxidative phosphorylation, we need a constant supply of oxygen. For example, the complete metabolism of one molecule of glucose to CO₂ and H₂O, requires 6 molecules of O₂ and produces 36 molecules of ATP. Without this steady supply of O₂, metabolism would grind to a halt, with ultimately fatal consequences. However, there is a second essential purpose of breathing: to remove CO₂. The complete metabolism of one molecule of glucose, produces 6 molecules of CO₂. In fact, adult humans excrete about 20 moles (or ~880 g) of CO₂ per day. If this were to accumulate in the body, the pH of blood and other fluids would become highly acidic. This is because CO₂ and H₂O can be readily combined by the enzyme carbonic anhydrase (CA) to generate carbonic acid, which rapidly and spontaneously dissociates into a hydrogen ion and a bicarbonate ion:

Equation 1:

\[ \text{CO}_2 + \text{H}_2\text{O} \overset{\text{CA}}{\rightleftharpoons} \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^- \]

The excretion of CO₂ is thus the second essential function of breathing. As air breathing organisms, we face an abundance of O₂ compared to water breathing organisms: one litre of air contains about 210 ml of O₂; by comparison one litre of water at 15 °C will contain only about 7 ml of dissolved O₂. For air breathing animals, oxygen is not a limiting gas; instead the key to survival is the rate at which CO₂ can be removed from blood via breathing. The complete opposite is true for water breathing organisms where O₂ is severely limiting and CO₂ is very readily excreted. As might be expected therefore, water breathing animals largely regulate breathing rates by measuring the partial pressure of O₂ (PO₂), and the ventilation rate of air breathing animals depends primarily on the level of partial pressure of CO₂ in arterial blood (PaCO₂) (1). This fundamental importance of CO₂ and its primacy over O₂ for the regulation of breathing in humans was first established in 1905 by Haldane and Priestley (2).

Equation 1 shows that CO₂ could exert its effect in three possible ways: as molecular CO₂; as pH or as HCO₃⁻. Note that these mechanisms of action are not mutually exclusive. A consensus has emerged, reflected in many textbooks on the subject, that changes in pH are a sufficient stimulus for the detection of changes in PaCO₂ and the consequent actions on breathing. The pH chemosensory hypothesis is made plausible by an abundance of potential molecules available to transduce changes in pH. Although all proteins will be sensitive to pH to some extent, some ion channels and receptors are especially sensitive over a range (pH 7.5 – 6.9) that is physiologically relevant to chemosensory regulation. These include the TASK potassium channel family (3), heteromeric Kir4.1-Kir5.1 inward rectifier K⁺ channels (4), the acid sensing cation channels (ASICs) (5, 6) and G-protein coupled receptors such as GPR4, which can stimulate cAMP production in a pH-dependent manner (7).

Nevertheless, there is longstanding evidence that matters are not quite this simple. Eldridge et al (8) showed that the ventilatory response in cats to inspired CO₂ was greater than the response to an equivalent imposed change in pH at constant PaCO₂. In their discussion, they proposed the following hypothesis (which they favoured over competing explanations):

“… there is an action of molecular CO₂ that is independent of its effects on e.c.f. [H⁺]. This would have to mean that there are two types of receptor sites on the chemosensory cells, or that [H⁺] effects come from locations in the medulla that are separate from the cells that respond to CO₂. Both of these possibilities would imply that during hypercapnia some of the respiratory response is due to the CO₂ effect and some to the simultaneously generated [H⁺].”
In a highly complementary study, Hashim Shams measured both pH and PCO₂ at the ventral surface of the medulla oblongata of the cat (the site of a major component of central chemoreception) while infusing bicarbonate ions into the blood at a rate sufficient to keep the pH constant at the surface of the medulla during the inhalation of CO₂ (9). He found that there was still an increase in breathing proportional to PCO₂ even when extracellular pH was kept constant. When pH was allowed to vary in addition to CO₂ (i.e. no compensating infusion of bicarbonate into the blood) the increase in breathing was roughly double that which occurred during the change in PCO₂ at constant pH. From this Shams concluded that:

“… both H⁺ and CO₂ in interstitial fluid of the ventral medulla oblongata independently stimulate the areas responsible for central chemosensitivity”.

The key implications arising from both of these papers are that: i) there are separate molecular mechanisms for the detection of pH and CO₂ and that both are involved in the regulation of breathing; and ii) there may be separate cells and/or chemosensitive nuclei that specialize in pH detection or CO₂ detection. In this review, I will summarize data that establishes both a molecular mechanism for the detection of CO₂ and the cellular types and areas that appear to perform this function for the regulation of breathing. In so doing, I will give strong support to the far-sighted hypotheses of these authors.

2. Mechanisms underlying the chemosensory control of breathing

2.1 Peripheral versus central

There are two main locations for the detection of the CO₂-linked stimuli that control breathing: the carotid body, and the medulla oblongata in the brain stem (Figure 1). Fernando de Castro (10) suggested that glomus cells within the carotid bodies may detect changes in the composition of the blood. Cornelius Heymans then showed that the carotid bodies could regulate breathing frequency in response to acidosis (via the glomus cell chemoreceptors) (11). The carotid bodies contribute about 30% of the total response to alterations in PaCO₂ (12-14). By utilizing a perfusion technique to control PaCO₂, PaO₂ and pH around the brain stem chemosensors independently from that of the peripheral chemosensors, Schuitmaker et al., (15) established that ventilation was a function only of peripheral pH and not of peripheral PaCO₂ providing strong evidence that the carotid body chemoreceptors detect changes in pH rather than changes in PaCO₂. We now know that carotid body glomus cells use TASK-1 channels, which are pH sensitive, to detect the changes in blood pH that accompany hypercapnia (16) (Figure 1). Some residual pH sensitivity remains in carotid glomus cells in TASK-1 null mice (16), suggesting that a further molecular transducer must also contribute such as GPR4, which is present in the carotid body (17). Central chemosensitivity is unaffected by TASK-1 deletion (18).

The importance of the medulla oblongata, was recognized following the pioneering work of the Mitchell (19) and Loeschcke (20) laboratories. These studies showed that the ventral surface of the medulla oblongata is a key site of chemoreception and that 3 distinct locations (rostral, intermediate and caudal, also respectively called M, S and L areas) are involved. The rostral area is ventral to the 7th nucleus and the retrotrapezoid nucleus (RTN), more recently implicated in chemosensory control, corresponds at least partly to this area (Figure 1). The raphé magnus is also ventral and medial to the 7th nucleus and contains pH-sensitive serotonergic neurons that may be involved in the chemosensory control of breathing (Figure 1). The raphé would thus correspond to the more medial regions of the historically-identified rostral area. The caudal area is close to the nerve rootlets of the 12th nerve and the intermediate area is a small region just anterior to the most anterior rootlet of the 12th nerve. A variety of evidence suggested their importance. Local acidosis of the rostral and caudal areas stimulated breathing via an increase in tidal volume (21). Destructive coagulation of these areas greatly reduced the sensitivity of breathing to CO₂ (22). Electrical stimulation of these areas activated breathing to a greater extent than stimulation of adjacent regions (23).
2.2 pH-sensitive mechanisms

More recent studies have explored the role of the RTN in chemosensory processes (17, 24-27). Some RTN neurons are intrinsically pH-sensitive and the expression of two molecules in these neurons may contribute to chemosensory control of breathing: TASK-2, a pH-sensitive K+ channel (17, 28); and GPR4, a pH-sensitive receptor (17). However, GPR4 is widely expressed in neurons including those of the medullary raphé, peripheral chemosensors, as well as the endothelium (29). While a contribution of GPR4 to chemosensory regulation seems likely, its specific role in the RTN is open to question. Systemic injection (i.e. will affect both peripheral and central chemosensors) of a selective and potent GPR4 antagonist reduced the ventilatory response to CO2. This same antagonist when administered centrally (only affecting central chemosensors) had no effect on the CO2-sensitivity of breathing (29).

Medullary raphé neurons (both serotonergic and non-serotonergic) are sensitive to pH (30-35) and are highly likely to contribute to respiratory chemosensitivity. Consistent with this role, raphé neuron processes are in close proximity to blood vessels (36). Most compellingly, selective chemogenetic silencing of serotonergic raphé neurons reduced by 40% the increase in minute ventilation evoked by hypercapnia (37). The RTN and raphe are likely to act in concert. Chemosensory responses in RTN neurons depend partially on serotonergic inputs from raphé neurons (38) and this suggests that RTN neurons, in addition to being intrinsically pH-sensitive, may act as relays of chemosensory information.

Leaving aside the relative contributions of the RTN and medullary raphé and their possible interactions, the evidence points to at least some of the primary chemosensory cells in both of these nuclei being neurons that utilize pH-sensitive channels or receptors to detect changes in pH and regulate breathing (Figure 1). These cells would therefore correspond to the pH-sensitive pillar of central chemosensory detection postulated by Eldridge et al (8) and Shams (9) more than 30 years ago.

2.3 ATP-dependent mechanisms respiratory chemosensitivity -a potential role for connexin hemichannels in the regulation of breathing

In areas of the ventral medullary surface that correspond to the rostral and caudal chemosensory regions, hypercapnia will induce release of ATP that precedes any adaptive changes in breathing (39). This ATP release contributes to the adaptive regulation of breathing to hypercapnia because application of ATP receptor antagonists will blunt these changes (39). The location of CO2-dependent ATP release at the medullary surface closely corresponds to the distribution of raphé neurons (39, 40). The hypercapnic ATP release might therefore contribute to excitation of the raphé neurons. However there is conflicting data on this point (41, 42), which remains to be resolved, possibly through use of more potent ATP receptor antagonists that are now available.

Given that ATP release occurs very early after the onset of hypercapnia and before any changes in breathing, it is plausible to speculate that it may arise from chemosensory cells. This CO2-dependent ATP release can be recapitulated in vitro in an isolated slice of the ventral medullary surface (43). Under these controlled conditions, ATP release was evoked by an increase in PCO2 at constant extracellular pH (achieved by also increasing HCO3-) (43) and shares this characteristic with the CO2-dependent regulation of breathing discovered by Shams (9). The ATP release occurred in both the rostral and caudal chemosensory regions, and was independent of extracellular Ca2+ (43). By examining the distribution of connexins, Huckstepp et al (43) discovered that Cx26 was preferentially localized in the ventral medulla, and was in fact expressed not in neurons but in glial cells of the marginal zone at the ventral surface of the medulla. This localisation of Cx26 to glial cells is consistent with a wide variety of evidence suggesting that Cx26 is largely absent from neurons and is only expressed in a subset of astrocytes, mainly those close to the margins of the parenchyma (44-46). As a variety of connexin hemichannels have been documented to permit the release of ATP from cells (47-49), Cx26 hemichannels were a favoured candidate for the release of ATP during
hypercapnic stimuli. A range of pharmacological agents with selectivity towards connexins blocked the CO₂ dependent ATP release observed in vitro, suggesting that the ATP release may indeed be mediated via gating of connexin hemichannels (43). These pharmacological agents were somewhat selective for Cx26. These same agents, when used in vivo, reduced the adaptive ventilatory response to CO₂ by a similar amount to blockade of ATP receptors (~20%) and reduced the observed CO₂-evoked ATP release, supporting the involvement of Cx26 in the chemosensory control of breathing (43).

This evidence implicating Cx26 in the CO₂-dependent release of ATP during hypercapnia, raised the possibility that Cx26 might itself be CO₂ sensitive. Expression of Cx26 in HeLa cells caused these cells to exhibit a CO₂-dependent conductance increase that was absent from parental HeLa cells (50). The presence of Cx26 also permitted CO₂-dependent dye loading into, and CO₂-dependent ATP release from, HeLa cells (50). These actions of CO₂ (performed at constant extracellular pH) cannot be explained as an effect of intracellular pH, as acidification is well known to close Cx26 hemichannels (51). Furthermore, CO₂ still gated Cx26 in isolated membrane patches where pH could be controlled on both sides of the membrane (50). The simplest interpretation of these data is that Cx26 is itself directly sensitive to CO₂. Binding of CO₂ to Cx26 hemichannels causes them to open, thus permitting the ATP release, which by acting as a transmitter can mediate adaptive changes in breathing via ATP-sensitive receptors. Glial cells expressing Cx26 in the ventral medulla could contribute the cellular basis of the direct CO₂ sensing involved in the control of breathing described by Eldridge et al (8) and Shams (9).

There is a further potential mechanism of astrocytic pH sensing to consider. Astrocytes in the RTN can release ATP in a pH-dependent manner and influence the rate of breathing thus potentially contributing to the adaptive control of breathing (52). This pH-dependent release of ATP depends on a process involving activation of the sodium bicarbonate transporter (NBCe1) and reversed Na⁺-Ca²⁺ exchange (53).

2.4 The direct action of CO₂ on Cx26

It is possible that the opening effect of CO₂ on Cx26 in HeLa cells could be indirect and mediated via a second protein. The observation that modulation of Cx26 could be observed in isolated patches makes this possibility rather unlikely, but does not eliminate it. The most convincing evidence to demonstrate a direct action of CO₂ on Cx26 would be to demonstrate the ability of mutations of Cx26 to change its CO₂ sensitivity, and ultimately to uncover the mechanism by which CO₂ has this action on Cx26.

Three closely related connexins (Cx26, Cx30 and Cx32) are sensitive to CO₂ (50) and ATP can permeate the hemichannels of all three of these connexins (50, 54, 55). To investigate possible mechanisms of CO₂ sensitivity, we looked for a further related connxin that might not have this sensitivity. The idea being that differences in amino acid sequences between the CO₂-sensitive connexins and a further non-CO₂-sensitive, but related, connxin might illuminate the structural features underlying the interaction with CO₂. We chose Cx31, and found that it lacked sensitivity to CO₂. Our starting supposition for sequence comparison was that CO₂ might carbamylate a lysine residue (50). This is a post-translational protein modification that has largely been overlooked in mammalian physiology. CO₂ carbamylation was originally described as the basis of the Bohr effect whereby CO₂ reduces the affinity of haemoglobin for O₂ enabling it to give up its oxygen to tissue (56). Carbamylation of lysine residues has also been established in RuBisco (57), a key enzyme for photosynthetic carbon fixation, and in microbial beta-lactamases (58, 59). The idea that carbamylation might be a general and important post-translational protein modification involved in physiological regulation was first proposed by George Lorimer (60) and more recently discussed by Louise Meigh (61). This concept can now be explored in a systematic manner via mass spectrometric tools developed by Martin Cann and his team (62).

We compared the sequences of Cx26, Cx30, Cx32 and Cx31, looking for lysine residues that might be present in the three CO₂ sensitive connexins but absent from Cx31. This revealed a lysine residue
and a motif specifically present in the CO2 sensitive connexins (63). We were tremendously aided by an X-ray structure for Cx26 (64), which showed the residues of the motif that we had identified and which we termed the carbamylation motif. The structure showed that the lysine within the motif, K125, is oriented towards R104 in the neighbouring subunit of the hexamer (Figure 2). The gap between the end of the sidechains of the two residues is only 6.5 Å, making it plausible to think that carbamylation of K125 might allow formation of a salt bridge between this residue and R104 to form a “carbamate bridge” between subunits. To provide support for this hypothesis, we simply transplanted the carbamylation motif into Cx31 and demonstrated that it gave a gain of CO2 sensitivity (63). Mutations of the residues K125 and R104 were then made to further test our hypothesis: for example, K125R (arginine not being carbamylatable) destroyed CO2 sensitivity in Cx26, as did R104A (removing the ability to make a salt bridge) (63). The mutations K125E or R104E gave a constitutively open hemichannel that was no longer sensitive to CO2 (63). The mutation K125C gave a hemichannel that could be opened by NO -by nitrosylation of the cysteine residue and formation of a bridge to R104 (65), and the double mutation K125C and R104C gave a hemichannel that was redox sensitive (65).

To summarize, our data definitively show that CO2 has a direct action on Cx26, as mutations of the protein alter its sensitivity to CO2 and can even be used to introduce sensitivity to new ligands. Our data very strongly suggest that CO2 carbamylates a specific lysine residue to cause conformational change and opening of the hemichannel. The very small possibility that CO2 has this effect by some other, as yet unknown, mechanism consistent with our mutational analysis can only be eliminated by direct demonstration of carbamylation e.g. by mass spectrometry methods.

2.5 From structural biology to tools to probe CO2 sensing via Cx26

We have exploited our understanding of how CO2 binds to, and modulates, Cx26 by developing a dominant negative subunit, dnCx26. This subunit carries two mutations: R104A and K125R. The first mutation prevents formation of intersubunit carbamate bridges, while the second prevents binding of CO2. Homomers of dnCx26 (as would be expected) are insensitive to CO2. We have shown, via fluorescence resonance energy transfer, that dnCx26 assembles very efficiently into hexamers with wild type Cx26 (66). HeLa cells that stably express Cx26 (and will dye load in response to CO2), when transfected with dnCx26 lose their CO2 sensitivity (66). Thus, dnCx26 does indeed act as a dominant negative subunit and can remove CO2 sensitivity from the endogenously expressed wild type Cx26 (66). This makes it a very selective genetic tool to probe the contribution of CO2 sensing via Cx26 to physiological processes -indeed it links the motif of CO2 binding to physiological action.

2.6 Genetic evidence for Cx26 and the control of breathing

To exploit dnCx26 as a tool to probe the control of breathing, we generated lentiviral constructs in which a GFAP promoter was used to drive the expression of either wild type Cx26 or dnCx26 followed by an IRES and Clover. Clover is a highly fluorescent green protein and this strategy allowed us to ascertain the expression pattern of our Cx26 constructs without directly tagging the protein which might alter its expression in vivo. When injected into the ventral medulla of the adult mouse, the lentiviral constructs drove selective expression of Cx26 and dnCx26 in GFAP+ cells. The location of expression of dnCx26 was critical. When expressed in the RTN, dnCx26 had no effect on the CO2-sensitivity of breathing (66). Although Cx26 is expressed in this area, it does not contribute to the regulation of breathing and may have some other function. When dnCx26 was expressed caudally in a small area called the caudal parapyramidal area (cPPy) it altered the CO2 sensitivity of breathing (66). dnCx26 had no effect on the adaptive change in respiratory frequency, but instead reduced the adaptive change in tidal volume at moderate levels of inspired CO2. Expression of dnCx26 reduced the change in tidal volume and minute ventilation by about 33% compared to expression of the Cx26 wild type subunit (as a control). When expressed even more caudally, dnCx26 once again had no effect on breathing. Thus, it appears that there is a specific, defined area where the presence of Cx26 mediates CO2-dependent regulation of breathing (Figure 3). We suggest that the glial cells of the cPPy correspond to the chemosensory cells responsible for the direct detection of CO2 first hypothesised by Eldridge et al (8) and Shams (9).
The cPPy has been implicated in the chemosensory control of breathing (67, 68). These previous studies demonstrated the presence of highly pH sensitive serotonergic cells in this area. This raises the fascinating possibility that the CO₂ detection mediated by superficial glial cells that express Cx26, converges with pH detection mediated by colocated serotonergic neurons in the cPPy. A further point of interest is that the key glial cells in the cPPy had an unusual morphology: a very superficial cell body and a long process that projected both rostrally and medially (66).

2.7 Cx26-mediated chemosensing in context

As stated earlier peripheral (carotid body) and central chemoreceptors mediate the CO₂-dependent control of breathing with the former contributing ~30% of the total chemosensory response (12-14). There are two components to the adaptive changes in ventilation - an increase in respiratory frequency, and an increase in tidal volume. These two components combine to increase the rate of ventilation of the lungs: minute ventilation. Broadly, peripheral chemoreceptors appear primarily to increase respiratory frequency, whereas activation of central chemoreceptors has a more powerful effect on tidal volume (21, 69, 70).

Within this overall context (Figure 3), how significant is CO₂ sensing via Cx26? Viral transduction by dnCx26 reduced the mean adaptive ventilatory response to 6% inspired CO₂ by about one third - mainly via a reduction of the increase in tidal volume. As we were unlikely to transduce all of the chemosensitive glial cells in this area, this may be an underestimate of the contribution of this mechanism. As central chemoreceptors mediate about 70% of the adaptive response, CO₂ sensing via Cx26 in the caudal parapyramidal area mediates nearly half of the total central ventilatory response to modest levels of hypercapnia. This compares favourably to the roughly 50% contribution of direct CO₂ sensing to the regulation of breathing via central chemosensors proposed by Shams (9) and suggests that Cx26 in the cPPy may mediate the majority of this component. This contribution of Cx26 to the hypercapnic regulation of breathing is broadly comparable to the chemogenetic inactivation of the entire population of raphé serotonergic neurons (including those in the midbrain and in the medulla oblongata), which gave a 40% reduction in the adaptive ventilatory response at similar levels of inspired CO₂ (37).

Cx26-mediated chemosensing adds a further dimension to the regulation of breathing beyond its proportional contribution to the adaptive reflex to hypercapnia. Although the concentrations of CO₂, HCO₃⁻ and H⁺ are interdependent (Equation 1), local buffering of HCO₃⁻ and H⁺ (53) via a variety of membrane exchangers and transporters (71, 72) can alter both the spatial and temporal dynamics of these potential chemosensory signals. The ability to sense CO₂ directly, via Cx26, provides additional information to determine changes in PCO₂ more accurately than measurement of pH alone (73). Direct measurement of CO₂ might be important during modest levels of hypercapnia, where the systemic pH change will not be large and the actions of transporters and exchangers could locally buffer pH and thus potentially obscure this signal.

3. Cx26 and breathing in humans

Roughly 1-3 per 1000 people have some form of congenital hearing loss. Mutations in Cx26 account for about half of these cases (74). In Caucasian populations the mutations G30del and G35del, which cause loss of functional protein, are the most common and have a prevalence conservatively estimated as around 1:5000 of the general population. In addition to the non-syndromic (and mostly recessive) mutations that cause hearing loss, there are 9 further dominant mutations that cause a severe condition termed Keratitis, Ichthyosis, Deafness Syndrome (KIDS) (75, 76). These mutations are very rare and mostly are idiopathic. In addition to profound hearing loss, KIDS involves skin defects that result in the severest case in loss of the dermal barrier and problems in the eye including corneal defects and photophobia.

Naveed Hussain and Dan Mulkey, treating an infant carrying the Cx26 mutation A88V, which gives a severe form of KIDS, observed and documented that the infant suffered from periods of central
apnoea (77). When they brought this to our attention, we engineered this mutation into Cx26 (Figure 4) and found that it completely abolished the CO₂ sensitivity of the resulting hemichannel. Furthermore, Cx26<sup>ABBV</sup> had a dominant negative effect on the CO₂ sensitivity of HeLa cells stably expressing Cx26<sup>WT</sup> (77), which helps to explain why this mutation is dominant for KIDS. Independently, another group observed that an infant with the same mutation also exhibited central apnoea (78). Given the rarity of these mutations, it seems likely that Cx26 does contribute to the control of breathing in humans.

We have further studied KIDS mutations and their effects on the CO₂ sensitivity of breathing. We found that the mutations N14K and N14Y also abolished CO₂ sensitivity of Cx26 in a dominant manner (79). More recently we have shown that the mutation A40V also abolishes CO₂ sensitivity (80) (Figure 4). We have not yet tested other KIDS mutations, but as 4/9 known KIDS mutations abolish the CO₂ sensitivity of Cx26, screening for central apnoea should be a routine part of the care for KIDS patients.

It is important to note that the KIDS mutations have other effects on the properties of Cx26 and the most popular hypothesis for the aetiology of KIDS is that these mutations give a gain of function - through causing the Cx26 hemichannels to be leaky either by reducing the strength of blockade by Ca<sup>2+</sup> (81) or altering the voltage dependence of the hemichannels such they can open at more negative potentials (82, 83). The fact that deletion of Cx26 does not cause KIDS, gives strong conceptual support to the gain-of-function hypothesis. Whether the loss of CO₂ sensitivity of the KIDS mutant hemichannels also plays a role in exacerbating the wider pathology of KIDS remains unclear at the moment.

We have also examined whether non-syndromic mutations could alter the CO₂ sensitivity of Cx26. Here the picture is mixed, for example: the mutation V84L has no effect on this; M34T reduces the extent of channel opening to CO₂ but does not affect the affinity of Cx26 for CO₂; A88S shifts the affinity of Cx26 to higher levels of CO₂ but does not affect the ability of the hemichannel to open (79). From this, it is plausible to predict that patients carrying M34T or A88S might also have altered control of breathing. Note that the commonest mutations of Cx26 that cause deafness, G30del and G35del, prevent expression of functional Cx26. We would predict that patients with these mutations might also experience periods of central apnoea. We also note that specific combinations of non-syndromic mutations might predispose patients to central apnoea: e.g. M34T/G35del; A88S/G35del. There are no reports in the literature of a link between hearing loss and altered control of breathing. This is perhaps not surprising: only certain mutations of Cx26 might have this effect; and the effects of these mutations might be most profound during sleep when respiratory drive is weakened and it depends more on inputs from central chemoreceptors. The central apnoea that can accompany KIDS has only recently been recognised (77, 78) and, more generally, sleep apnoea is a condition that can be hard to recognise without specialised measurements (84).

4. Evolution of the CO₂ sensitivity in β connexins and its relevance to the control of breathing

An interesting question is how the CO₂ sensitivity of the β connexins (Cx26, Cx30 and Cx32) might have evolved. We have studied this by examining the incidence of the carbamylation motif throughout vertebrate phylogeny, and testing the CO₂-sensitivity of connexins from a wide range of vertebrates (Figure 5). The Cx32 of shark possesses the carbamylation motif and has a CO₂ sensitivity indistinguishable from that of human Cx32, placing the evolution of this motif in the ancestor of all gnathostomes at least 450 million years ago (85). Connexin evolution has been characterised by genome duplications, gene losses, and a series of gene duplications (86). The ancestral gene of Cx32 duplicated to give the extant Cx32 and the Cx26-like gene of fish and amphibia (86). The carbamylation motif in the Cx26-like gene has been lost from most fish, but has been notably retained in lungfish, the closest extant relative to the ancestor of all tetrapods, and amphibia (85). While Cx26-like hemichannels from lungfish and amphibia are not CO₂ sensitive, they differ from the Cx26 gene of mammals, birds and reptiles in having an extended C-terminus. If this C-terminus is truncated to the same length as the very short mammalian C-terminus then the Cx26-
like hemichannel gains CO$_2$-sensitivity (85). Conversely, substituting the longer Cx26-like C-terminus for the short C-terminus of human Cx26, causes loss of the CO$_2$ sensitivity of the hemichannel (85). It seems therefore that the extended C-terminus of the Cx26-like protein prevents hemichannel opening to CO$_2$. However, CO$_2$ still binds to the carbamylation motif of the Cx26-like protein. Instead of opening the Cx26-like hemichannel, CO$_2$ closes the gap junction (85). Interestingly, Cx32 gap junctions are insensitive to moderate levels of CO$_2$ (85). The evolution of the Cx26-like gene therefore gave new functionality: the ability to close a gap junction via a CO$_2$-dependent mechanism.

During the evolution of amniotes, the Cx26-like gene further duplicated to give the Cx26 gene found only in amniotes and the Cx30 gene. The hemichannels of Cx30, which has a long C-terminus, are opened by CO$_2$ (50). Amniote Cx26 is characterised by a short C-terminus and the near universal presence of the carbamylation motif (85). The Cx26 hemichannel is opened by CO$_2$ in reptiles (turtle, gecko), birds (chicken) and mammals (mouse, rat, human, mole rat) and its EC$_{50}$ for CO$_2$ is tuned to the physiology of these animal groups (87). Gap junctions of amniote Cx26 retain the ability to close to CO$_2$, and this also depends on the carbamylation motif (33). All present-day amniotes can trace common ancestry to those that survived the Permo-Triassic catastrophe. This geological event, caused by volcanic activity of the Siberian traps, occurred some 250 MYA, involved an increase in global temperatures of some 6°C, and resulted in extinction of more than 70% of land dwelling forms (88-90). Given the widespread occurrence of the truncated CO$_2$-sensitive Cx26 in amniotes, the simplest hypothesis is that the occurrence of this new Cx26 variant predated this catastrophe (85). Whether this adaptation helped survival of these ancient amniotes during a global extinction event is untestable. Nevertheless, the carbamylation motif and CO$_2$-sensitivity of Cx26 hemichannels are widely expressed across all present-day groups of amniotes (85, 87). As there are only two codons for lysine, it would be very easy to lose the carbamylation motif by genetic drift. Clearly, strong selection pressure has retained this motif and thus Cx26 as a CO$_2$-sensing molecule tuned to respond to changes in PCO$_2$ around the physiological norm.

5. Concluding remarks

Long-standing evidence in the scientific literature has supported a role for CO$_2$ being directly detected by chemosensory cells and regulating breathing independently of pH. Our work has now provided a molecular mechanism (carbamylation of Cx26) and identified glial cells of the cPPy as the cellular substrate of direct CO$_2$ chemosensing. These cells contribute nearly half of the centrally generated chemosensory response to modest levels of hypercapnia. Our discovery of Cx26 and closely related connexin as receptors for CO$_2$ removes a conceptual barrier to thinking about CO$_2$ as a signalling molecule more generally in other physiological contexts. It will be very interesting to see whether CO$_2$ detection by Cx26 could be involved, for example, in the regulation of blood flow, or be significant in the physiology of the cochlea where both Cx26 and Cx30 are strongly expressed. The CO$_2$ sensitivity of Cx32 is also intriguing -this has been retained for more than 450 million years suggesting an important function even in fish. As Cx32 is abundantly expressed in liver, and liver has a significantly elevated PCO$_2$ (91), it is tempting to speculate that CO$_2$ could regulate Cx32 hemichannel function in liver.

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Figures and Legends

Figure 1: Schematic of peripheral and central respiratory chemosensory nuclei. The carotid bodies comprise the peripheral chemosensors and contain glomus cells that respond to changes in arterial pH via TASK-1 channels. GPR4 is present in the carotid, but its specific contribution to pH sensitivity of the glomus cells has not been established. The carotid bodies project to the nucleus tractus solitarius (NTS) in the dorsal medulla oblongata. The NTS projects to the inspiratory rhythm generator (preBötzinger Complex, preBötC) (92) and the retrotrapezoid nucleus (RTN) (93). Overall the carotid bodies contributes to about 30% of the total adaptive ventilatory response to hypercapnia and mainly by evoking a compensatory increase in breathing frequency. The RTN and medullary raphé comprise two important chemosensory areas in the ventral medulla. Both nuclei contain pH-sensitive neurons which detect H+ via GPR4 and additionally TASK-2 in the case of the RTN and project to the inspiratory rhythm generator.
Figure 2: Structure of Cx26 showing the position of residues K125 (blue) and R104 (red) that form the carbamate bridge. The left panel shows the view from the cytoplasmic face along the axis of the pore (shown open). Note how R194 and K125 of adjacent subunits point towards each other and are only 6.5 Å apart. The right panel shows a side view demonstrating the R104 and K125 are in a similar plane. Structure for Cx26 is 2zw3 from the protein database (64).
Figure 3: Revised schematic for respiratory chemosensing to incorporate direct CO$_2$ sensing via Cx26. Specialised glial cells with long dorso-rostral projecting process that express Cx26 are found in the caudal parapyramidal area (cPPy). These processes project towards the preBötzinger complex (preB) and could potentially release ATP at this location to increase breathing. The processes also project medially and could activate the serotonergic neurons of the raphé obscurus (ROb) and raphé pallidus (RPa). Additionally, the cPPY contains serotonergic neurons that could be excited by the local release of ATP from the cPPY glial cells. As the binding site for CO$_2$ is intracellular, CO$_2$ must cross the membrane to cause Cx26 opening and allow the release of ATP.

Abbreviations: VII, 7th (facial) nucleus; NA, nucleus ambiguus; py, pyramids; IO, inferior olive; LRt lateral reticular nucleus; XII, hypoglossal nucleus; NTS, nucleus tractus solitarius.
Figure 4: Location of KIDS mutations that affect CO₂-sensitive of Cx26 relative to the carbamylation motif. Two subunits of Cx26 are shown next to each other. On one subunit, the residues N14, A88 and A40 are shown in magenta. Note that R104 and K125 form the carbamate bridge above the portion of the subunit that connects the N-terminal helix (coloured dark blue). N14 is thus close to the location of the carbamate bridge helping to explain why substitution of a lysine (N14K) or a tyrosine (N14Y) removes CO₂ sensitivity. In contrast the residues A88 and A40 are distant from the site of carbamylation, and it remains unclear why the mutations A88V and A40V abolish CO₂ sensitivity. Structure for Cx26 is 2zw3 from the protein database (64).
Figure 5: Inferred evolution of CO$_2$ sensitivity in β connexins. We propose that the common ancestor of the Cx32 and Cx26-like genes (preCx32) possessed had the carbamylation motif. The carbamylation motif regulated the opening of hemichannels and has been preserved in Cx32 to the present day. Duplication of the ancestral gene gave rise to the Cx26-like gene in which the carbamylation motif had a de novo function - gain of CO$_2$-dependent gap junction closure. However, CO$_2$-dependent opening was lost in the Cx26-like hemichannel. With the evolution of amniotes (grey dashed box), the Cx26-like gene was duplicated to give Cx26 and Cx30. Cx30 gained a long C-terminus and in many cases lost the carbamylation motif. Cx26 in amniotes lost the C-terminus and regained the ability of CO$_2$ to open the hemichannel, and retained CO$_2$-dependent gap junction closure. Green box indicates near-universal presence of carbamylation motif, light green box presence of carbamylation motif in some species but not others. Figure reproduced from (85).