Connexin26 mediates CO₂-dependent regulation of breathing via glial cells of the medulla oblongata

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Breathing is highly sensitive to the PCO₂ of arterial blood. Although CO₂ is detected via the proxy of pH, CO₂ acting directly via Cx26 may also contribute to the regulation of breathing. Here we exploit our knowledge of the structural motif of CO₂-binding to Cx26 to devise a dominant negative subunit (Cx26DN) that removes the CO₂-sensitivity from endogenously expressed wild type Cx26. Expression of Cx26DN in glial cells of a circumscribed region of the mouse medulla - the caudal parapyramidal area - reduced the adaptive change in tidal volume and minute ventilation by approximately 30% at 6% inspired CO₂. As central chemosensors mediate about 70% of the total response to hypercapnia, CO₂-sensing via Cx26 in the caudal parapyramidal area contributed about 45% of the centrally-mediated ventilatory response to CO₂. Our data unequivocally link the direct sensing of CO₂ to the chemosensory control of breathing and demonstrates that CO₂-binding to Cx26 is a key transduction step in this fundamental process.
Breathing is a vital function that maintains the partial pressures of O$_2$ and CO$_2$ in arterial blood within the physiological limits. Chemosensory reflexes regulate the frequency and depth of breathing to ensure homeostatic control of blood gases.$^1$ Historically, the ventral surface of the medulla oblongata has been recognised as an important location of central respiratory chemosensors.$^{2-6}$ Recent work has focussed on two populations of neurons thought to contribute to the chemosensory control of breathing: those of the retrotrapezoid nucleus (RTN)$^{7-11}$ and the medullary raphé$^{12-16}$.

According to traditional consensus, CO$_2$ is detected via the change in pH, and pH is a sufficient stimulus for all adaptive changes in breathing in response to hypercapnia.$^6$ pH-sensitive K$^+$ channels (TASKs and KIRs) are potential transducers. Although TASK-1 in the peripheral chemosensors of the carotid body (CB) contributes to overall pH/CO$_2$ chemosensitivity,$^{17}$ TASK-1 does not appear to play a role in central pH/CO$_2$ chemosensing.$^{18}$ By contrast, TASK-2 may act as a central sensor of pH and contribute to adaptive changes in breathing.$^{11,19}$

Recently, a pH sensitive receptor, GPR4, has been linked to central chemosensitivity in the RTN. Complete deletion of this gene (from all tissues) greatly reduced the CO$_2$ chemosensitivity of pH and contribute to adaptive changes in breathing.$^{11,19}$ Nevertheless, the role of direct sensing of CO$_2$ in the regulation of breathing remains uncertain because genetic evidence linking Cx26 hemichannels can be opened by removal of extracellular Ca$^{2+}$ in mice.$^{11}$ However, GPR4 is widely expressed in neurons including those of the medullary raphé and peripheral chemosensors, as well as the endothelium.$^{20}$ Moreover, systemic injection of a selective GPR4 antagonist modestly reduced the ventilatory response to CO$_2$, but this same antagonist when administered centrally had no effect on the CO$_2$ sensitivity of breathing.$^{20}$ A mechanism of pH-dependent release of ATP from ventral medullary glial cells may also contribute to the CO$_2$-dependent regulation of breathing.$^{21}$

There is considerable evidence that CO$_2$ can have additional independent effects from pH on central respiratory chemosensors.$^{22-24}$ Connexin26 (Cx26) is known to be present at the ventral medullary surface in the caudal and rostral chemosensory areas.$^{25,26}$ We have shown that CO$_2$ directly binds to Cx26 hemichannels and causes them to open.$^{27,28}$ We have identified the critical amino acid residues that are necessary and sufficient for this process.$^{28,29}$ Cx26 hemichannels can be gated by a number of stimuli. They are opened by voltage at potentials $>-20$ mV$^{30}$ and closed by acidification.$^{31}$ Like all connexins, Cx26 hemichannels can be opened by removal of extracellular Ca$^{2+}$. However, the CO$_2$-dependent opening of Cx26 hemichannels can occur in the absence of membrane depolarisation and at physiological levels of extracellular Ca$^{2+}$.$^{27-29,33}$

This direct gating of Cx26 is an important mechanism that underlies CO$_2$-dependent ATP release$^{26,27,34,35}$ and provides a potential mechanism for the direct action of CO$_2$ on breathing. Nevertheless, the role of direct sensing of CO$_2$ in the regulation of breathing remains uncertain because genetic evidence linking Cx26 to the control of breathing has been lacking, and the cells that could mediate direct CO$_2$ sensing via Cx26 have not been identified.

In this study, we have addressed both of these issues by exploiting our knowledge of the binding of CO$_2$ to Cx26 to devise a dominant-negative subunit (Cx26$^{DN}$) that removes CO$_2$-sensitivity from endogenous wild-type (WT) Cx26 hemichannels. By using a lentiviral construct to drive the expression of Cx26$^{DN}$ in glial cells of the ventral medulla, we have obtained evidence that links CO$_2$-dependent modulation of Cx26 in glial cells present in a small circumscribed area of the ventral medulla to the adaptive control of breathing.

**Results**

**Rationale for the design of Cx26$^{DN}$**. CO$_2$ causes Cx26 hemichannels to open via carbamylation of K125 and subsequent formation of a salt bridge between the carbamylated lysine side chain and R104 of the neighbouring subunit (Fig. 1a)$^{28}$. As Cx26 hemichannels are hexameric, there are potentially six binding sites for CO$_2$ suggesting the potential for highly cooperative binding of CO$_2$. Indeed, Cx26 is steeply sensitive to changes in PCO$_2$ around its physiological level of about 40 mmHg (Fig. 1b)$^{28}$. We reasoned that introducing two mutations: K125R to prevent CO$_2$-dependent carbamylation and R104A to prevent salt bridge formation (“carbamate bridges”) to a neighbouring carbamylated subunit, should produce a dominant-negative subunit (Fig. 1c). If such a subunit coassembled into the Cx26 hexamer, it would likely have a dominant-negative action as it would remove the capacity to form at least two out of the six possible carbamate bridges (Fig. 1c).

**Cx26$^{DN}$ coassembles with Cx26$^{WT}$**. To determine whether the dominant-negative subunit will effectively coassemble into a hexamer with the WT subunit, we used acceptor depletion Förster resonance energy transfer (FRET; Fig. 2). We exploited the Clover-mRuby2 FRET pair$^{28}$ by tagging Cx26$^{WT}$ and Cx26$^{DN}$ with Clover (donor) and mRuby2 (acceptor). When mRuby2 was bleachled in HeLa cells coexpressing either Cx26$^{WT}$-Clover and Cx26$^{WT}$-mRuby2 or Cx26$^{DN}$-Clover and Cx26$^{DN}$-mRuby2, the fluorescence of the Clover was enhanced where these two fluorophores were colocalised (Fig. 2). The FRET efficiency for Cx26$^{WT}$-Clover and Cx26$^{DN}$-mRuby2 or Cx26$^{WT}$-Clover and Cx26$^{DN}$-mRuby2 was very similar (Fig. 3). This suggests that the Cx26$^{DN}$ subunit interacts closely with Cx26$^{WT}$. In principle, this could be because a homomeric hexamer of Cx26$^{WT}$ was sufficiently close to a homomeric hexamer of Cx26$^{DN}$ by random association in the membrane rather than assembly of a heteromeric hexamers comprised of both types of subunits. We...
therefore also looked at FRET interactions between Cx43WT and Cx26WT. These two connexin subunits do not form heteromeric hexamers, but homomeric hexamers of the two types could come close to each other in the plasma membrane by random association. We found that the FRET efficiency of this interaction was much lower than that of Cx26WT-Cx26WT or Cx26WT-Cx26DN (Figs. 2 and 3). This suggests that Cx26DN does indeed coassemble into a hexamer with Cx26WT. This hypothesis gains further support from our analysis which shows that the FRET efficiency for Cx26WT-Cx26DN is negatively correlated with the donor-to-acceptor (D-A) ratio (Supplementary Fig. 1), which is indicative of assembly into heteromeric hemichannels.37

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**Fig. 2** FRET signal between connexin variants. Example images of acceptor depletion Förster resonance energy transfer (ad-FRET) experiments. HeLa cells were co-transfected with equal amounts of DNA transcripts (to express connexin-fluorophore constructs) and PFA fixed after 48 h. Two channels were recorded: 495–545 nm (Clover, green) and 650–700 nm (mRuby2, magenta), and images were acquired sequentially with 458- and 561-nm argon lasers, respectively. Photobleaching was performed using the 561-nm laser for 80 frames at 100% power, targeting ROIs. Three combinations of connexin–Clover (donor, green) and connexin–mRuby2 (acceptor, red) are shown before and after bleaching. Colocalisation is shown in white in the overlay images, along with bleached areas (white ovals) and background references (yellow ovals). Following acceptor bleaching, ROIs in Cx26WT-Clover + Cx26WT-mRuby2 and Cx26WT-Clover + Cx26DN-mRuby2 samples show enhanced fluorescence intensity of donor (Clover, green) and reduced fluorescence intensity of acceptor (mRuby2, magenta). ROIs in Cx43WT-Clover + Cx26WT-mRuby2 samples show almost no change in fluorescence intensity of donor (Clover, green) and reduced fluorescence intensity of acceptor (mRuby2, magenta). Colocalisation measurements decrease mainly due to photobleaching of mRuby2 and subsequent elimination of its fluorescence. All co-transfection combinations showed statistically significant colocalisation at the highest resolution for the images. Colocalisation threshold calculations were carried out in ImageJ using the Costes method;100 iterations, omitting zero-zero pixels in threshold calculation. Statistical significance was calculated for entire images and individual ROIs, with no difference between either calculation (p value = 1 for all images, with a significance threshold of p > 0.95). Scale bar, 10 μm.

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**Fig. 3** FRET efficiency of coexpressed connexin variants. a Box and whisker plots showing the difference in FRET efficiency (%E) across different connexin co-expression connexin samples. FRET efficiency was calculated from background-adjusted ROIs as: %E = 100 × (cloverpost – cloverpre)/cloverpost. One-way ANOVA was carried out in SPSS. Post hoc testing revealed all individual comparisons to be significant at **p < 0.001, with the exception of the comparison between Cx26WT + Cx26WT and Cx26WT + Cx26DN data sets, which were not significantly different. Each dot represents a different ROI. b Box and whisker plot showing mRuby2 bleaching efficiency during the acceptor depletion step. While a small amount of bleaching occurred in untargeted regions (presumably due to light scattering and/or reflection), targeted ROIs received drastically greater bleaching. Importantly, all targeted regions showed highly similar bleaching efficiencies. Bleaching efficiency was calculated from background-adjusted ROIs as: Bleaching = (1 – (mRuby2post/mRuby2pre)). Boxes show the interquartile range, the median is indicated by the horizontal line within the box, and the mean is indicated by the cross within the box. Range bars show minimum and maximum values.
that lentiviral construct drove expression of the Cx26 variants into the ventral medulla oblongata showed no impact on the CO₂ sensitivity of cells expressing them. We therefore concluded that Cx26DN hemichannels, which carry both of these mutations, are insensitive to CO₂ (Supplementary Fig. 2). This important observation shows that if Cx26DN were to form homeric hemichannels in vivo we would have no impact on the CO₂ sensitivity of cells expressing them.

To assess the capacity of Cx26DN to act as a dominant-negative subunit with respect to CO₂ sensitivity, we transfected this subunit into HeLa cells that stably expressed Cx26WT. We then used a dye loading assay to assess how the sensitivity of these HeLa cells to CO₂ changed with time. Four days after transfection, the HeLa cells that coexpressed Cx26DN with Cx26WT were as sensitive to CO₂ as those HeLa cells that only expressed Cx26WT (Fig. 4). However, 6 days after transfection, the HeLa cells that coexpressed Cx26DN were insensitive to CO₂ (Fig. 4). We conclude that Cx26DN has a dominant-negative effect on the CO₂ sensitivity of Cx26WT and exerts this effect by coassembling into heteromeric hexamers with Cx26WT in the manner hypothesised in Fig. 1.

In vivo expression of Cx26DN blunts the CO₂ sensitivity of breathing. The Cx26DN subunit has the potential to be a genetic tool that could remove CO₂ sensitivity from Cx26 and thus probe this aspect of Cx26 function without deleting the Cx26 gene. This has the advantage of leaving other signalling roles of Cx26 intact and also of linking any CO₂-dependent physiological functions to the CO₂-binding site of Cx26 itself. We therefore created a lentiviral construct that contained either Cx26DN or Cx26WT (a control for the Cx26DN construct) under the control of a bidirectional cell-specific glial fibrillary acidic protein (GFAP) promoter38 (Supplementary Fig. 3a). Rather than tagging a fluorescent protein to the C-terminus of the Cx26 variants, we expressed Clover behind an internal ribosome entry site (IRES) so that the cells that expressed the Cx26 variants could be identified and quantified (Supplementary Fig. 3a). Stereotaxic injection of the lentiviral construct into the ventral medulla oblongata showed that lentiviral construct drove expression of the Cx26 variants selectively in GFAP+ cells (Supplementary Fig. 3b).

We used bilateral stereotaxic injections of the lentiviral constructs into the medulla oblongata to assess the effect of Cx26DN on the CO₂ sensitivity of breathing by means of whole-body plethysmography (Supplementary Fig. 4). Pilot experiments suggested that transduction of glial cells at the ventral medullary surface in a region ventral and medial to the lateral reticular nucleus reduced the adaptive changes in breathing to hypercapnia 3 weeks after injection of the virus (Supplementary Fig. 5). From this pilot work, we designed a study to achieve a statistical power of 0.8 at a significance level of 0.05 in which we injected 12 mice with Cx26WT and 12 mice with Cx26DN and followed how the CO₂ sensitivity of breathing changed with time following the lentiviral injection (Fig. 5a). Two weeks after viral transduction, the change in tidal volume to 6% CO₂ in the Cx26DN-injected mice was less than that of the Cx26WT-injected mice (two-way mixed-effects analysis of variance (ANOVA), p = 0.008; post hoc one-tailed t test, p = 0.002). The change in minute ventilation was also less in the Cx26DN-injected mice compared to the Cx26WT-injected mice (two-way mixed-effects ANOVA, p = 0.015; post hoc one-tailed t test, p = 0.007). There was no readily discernable difference in the changes in respiratory frequency to CO₂ between the Cx26WT- and Cx26DN-injected mice. Three weeks following the viral injection, these differences had disappeared, presumably due to some compensatory mechanism within the respiratory networks39,40. Following the pilot study, we were able to place our injection more medially into the caudal parapyramidal area. More accurately targeting the injection of virus particles to the correct area likely led to the quicker onset of the phenotype, as transfection of the relevant cells would thus have been more efficient and rapid.

Post hoc tissue staining confirmed the location of the transduced glial cells and highlighted cells at the very surface of the ventral medulla that had long processes projecting rostrally and dorsally into the parenchyma of the medulla (Fig. 5b). The difference between Cx26WT- and Cx26DN-transduced mice could not be attributed to the increased expression of Cx26WT and hence CO₂ sensitivity, as sham operated mice (performed as part of the same study) showed no difference in CO₂ sensitivity from the Cx26WT mice (Supplementary Fig. 7). In both the pilot and main experiments, the effect of Cx26DN was to alter the relationship of tidal volume and minute ventilation vs inspired CO₂: specifically, Cx26DN reduced the increase in both of these parameters that occurs at 6% inspired CO₂ by ~30% compared to the control (Cx26WT, see Supplementary Figs. 5 and 6 and Supplementary Tables 1 and 2).

Expression of Cx26DN in glial cells in this location ventral and medial to the lateral reticular nucleus appeared to be uniquely able to alter the CO₂ sensitivity of breathing. Transduction of glial cells in the RTN (Supplementary Fig. 8) or even more caudally in the medulla (Supplementary Fig. 9) had no effect on the CO₂ sensitivity of breathing. We therefore conclude that Cx26 in a circumscribed population of GFAP+ cells ventral and medial to the lateral reticular nucleus contributes to the CO₂-dependent regulation of breathing. To our knowledge, this is the first mechanistic evidence that shows a direct effect of CO₂ on breathing and links the structural biology of CO₂ binding to Cx26 to the regulation of breathing. While the lentiviral construct transduced typical astrocytes (e.g., Fig. 5b), it consistently transduced glial cells that had a soma at the very ventral edge of the medulla. These glial cells were unusual in that they had very long processes, which extended rostrally (Figs. 5b and 6) and also medially (Fig. 6).

Discussion

By devising a dominant-negative subunit Cx26DN, which coassembles with endogenously expressed Cx26WT to remove CO₂ sensitivity from the resulting heteromeric hemichannels, we have demonstrated a clear link between Cx26-mediated CO₂ sensing and the regulation of breathing. Our study also links the structural motif of CO₂ binding in Cx26—the carbamylation motif—to the CO₂-dependent regulation of breathing. CO₂ carbamylation happens spontaneously and was originally described as the basis of the CO₂ Bohr effect41. Carbamylation of lysine residues has also been established in RuBisco42, a key enzyme for photosynthetic carbon fixation, and in microbial beta-lactamases43,44. CO₂-dependent carbamylation as a general and important post-translational protein modification involved in physiological regulation was proposed by George Lorimer45. It is clear from the known examples, and systematic application of mass spectrometric tools46, that only specific lysine residues in some proteins are able to be carbamylated. Our data now suggest that CO₂...
carbamoylation plays an important physiological role in the control of breathing.

The direct and independent role of CO₂ sensing in central chemoreception was first suggested by Shams23. The data in this paper provide unequivocal evidence to demonstrate a molecular mechanism and role for direct CO₂ sensing in the control of breathing. This molecular mechanism and pathway functions independently from any secondary changes in pH that could result from the altered balance between CO₂ and HCO₃⁻ during hypercapnia. Our data further suggest that the role of Cx26 and direct CO₂ sensing is restricted to an area of the ventral medullary surface in the caudal brain stem—ventral and medial to the lateral reticular nucleus. This area corresponds to the classically described caudal chemosensing area3,4,47 and in particular a subregion that has been more recently studied and termed the “caudal parapyramidal” area. The caudal parapyramidal area contains serotonergic neurons that are highly pH sensitive48,49. Interestingly, our lentiviral vector consistently transduced glial cells at the very ventral surface of the medulla in this region (Fig. 7). Our previous work has shown, with the aid of a knock-in reporter, that Cx26 is expressed in GFAP⁺ cells with cell bodies at the very surface of the parenchyma (Fig. 10 of ref. 26). Our current work is consistent with this finding and shows that these superficial GFAP⁺ cells have a cell body with a flattened edge that forms

Fig. 4 Cx26DN removes CO₂ sensitivity in HeLa cells stably expressing Cx26WT. Dye loading under 35 mmHg PCO₂ (control) or 55 mmHg PCO₂ (hypermcapnic) conditions revealed how the CO₂ sensitivity of HeLa cells stably expressing Cx26 (Cx26-HeLa cells) changes over time after transfection with Cx26DN. a, b Representative dye-loading images of Cx26-HeLa cells at day 4 and day 6 days after cells were either untreated a or transected with Cx26DN b. In b, the inset represents a Zero Ca²⁺ control to demonstrate the presence of functional hemichannels even when the HeLa cells showed no CO₂-dependent dye loading. c, d Cumulative probability distributions comparing mean pixel intensity for each condition at day 4 and day 6 (untreated Cx26-HeLa cells, Cx26-HeLa cells transected with Cx26DN, n > 40 cells per treatment repeat, with at least 5 independent repeats for each treatment). The cumulative distributions show every data point (cell fluorescence intensity measurement). e Median change in pixel intensity caused by 55 mmHg PCO₂ and Zero Ca²⁺ from baseline (35 mmHg) over days 4, 5, and 6 post-transfection. At day 6, median pixel intensities (from 7 independent repeats) were compared using the Kruskal-Wallis ANOVA (χ² = 9.85, df = 2, p = 0.007)** and post hoc with Mann-Whitney U test (Cx26WT vs Cx26WT + Cx26DN, W = 51, p = 0.003***; Cx26WT + Cx26DN Zero Ca²⁺ vs Cx26WT + Cx26DN, W = 46, p = 0.002***). Each circle represents one independent replication (independent transfections and cell cultures). Boxes show the interquartile range, the median is indicated by the horizontal line within the box, and the whisker is 1.5 times the interquartile range. Scale bars, 40 μm.
part of the very surface of the marginal glial layer and long dorsally directed processes that extended both rostrally and medially (Figs. 5b and 6). The cell body is ideally placed to detect changes in PCO2 in the cerebrospinal fluid (CSF), but their processes projecting both rostrally and medially could contact neurons of the preBötzinger complex to directly alter inspiratory activity or neurons of the raphé obscurus or pallidus, which detect changes in pH and are part of the chemosensory network50–52.

Our findings are robust and reproducible. We discovered the importance of Cx26 in the caudal parapyramidal area in a pilot experiment (Supplementary Fig. 5) and used these data to design a properly powered experiment to test the importance of Cx26 in this area. For practical reasons, the groups of mice for the experiment reported in Fig. 5 were divided into subcohorts of six per treatment group. In each of these subcohorts, the effect of Cx26DN replicated that seen in the pilot experiment. We also observed the effect of Cx26DN at 1.5, 2, and 2.5 weeks; however, by 3 weeks post-transduction the effect of Cx26DN on breathing had disappeared. This is presumably due to compensation within the respiratory network, which is known to be highly plastic39.

For example, the effects of CB denervation are largely reversed over the course of 2 weeks53; mice engineered to express a mutant Phox2b gene selectively in neurons of the RTN have no CO2 chemosensitivity for the first 9 days postnatally but recover about
40% of their CO₂ chemosensitivity by 4 months. Interestingly, the recovered chemosensitivity in these mice was mediated almost exclusively via an adaptive increase in tidal volume, rather than frequency—it is therefore tempting to speculate that this could have arisen via the progressive postnatal expression of Cx26 in glial cells of the caudal parapyramidal area. There are several ways that the respiratory and chemosensory networks could compensate for the effects of Cx26DN, the most obvious being via strengthening of other chemosensory inputs such as those from the raphé, RTN, or CBs. However, a more subtle compensatory mechanism might be through upregulation of ATP receptors on the neurons downstream from the chemosensory glia in the caudal parapyramidal area. It is unlikely that we transduced every chemosensitive glial cell in this area, hence an upregulation of ATP receptors could maximise the effects of the non-transduced glial cells that remain chemosensitive.

Overexpression of Cx26WT by itself did not enhance the chemosensitivity of breathing. Our construct drives the expression in GFAP+ cells, which could either be part of the chemosensory network or outside of it. For those glial cells within the chemosensory network, it may be that the endogenous levels of expression are sufficient and additional expression of Cx26 can give no further gain of chemosensitivity—a “ceiling” effect. Cx26WT expression in chemosensory cells outside of the network may be ineffective in enhancing chemosensitivity because these cells do not project to (and release ATP in) the correct locations to alter breathing.

Cx26DN and Cx26WT colocalise to gap junction plaques and the FRET analysis shows that they coassemble into heteromeric
The total chemosensory response is mediated peripherally and the volume1. These two components combine to increase the rate of

— two components to the adaptive changes in ventilation

solitarius 49, their potential activation via release of ATP from the raphé obscurus, raphé pallidus, and the nucleus tractus otonergic neurons. As these serotonergic neurons project area8,11,56 it is mechanistically simpler to understand how the CO2-gated release of ATP via Cx26 hemichannels could result in enhanced neuronal excitation and hence the adaptive changes in ventilation, compared to CO2-gated loss of gap junction communication between glial cells.

The RTN has received extensive attention as a chemosensory area8,11,26–58. It is interesting that Cx26DN expression in the RTN had no effect on chemosensitivity of breathing. CO2-dependent ATP release (most probably via Cx26) has been observed in vivo from the rostral ventral surface of the medulla, but this is more medial and probably closer to the Raphé magnus rather than the RTN, which is substantially more lateral26,34. Our data would suggest that chemosensory mechanisms within the RTN are completely independent of Cx26 and may not involve the direct sensing of CO2. The observation of pH-dependent ATP release from RTN astrocytes21 via a membrane process involving the sodium bicarbonate transporter (NBCe1) and reversed Na⁺–Ca²⁺ exchange59 supports this hypothesis.

There is compelling evidence to support the serotonergic medullary raphé neurons as an important mediator of respiratory chemosensitivity. Selective DREADD-mediating silencing of these neurons gave a substantial reduction (~40%) of the adaptive change in ventilation to a 5% inspired CO2 challenge15. Recently, chemosensory responses in RTN neurons have been shown to depend partially on serotonergic inputs from raphé neurons and RTN neurons have been proposed as relays of chemosensory information rather than primary chemosensors90. Interestingly, the serotonergic raphé system extends caudally and will be medial to the chemosensory glial cells that we have identified in this study. As the surface glial cells that we transduced with Cx26DN have long processes that project both rostrally and medially, it is plausible that these cells could excite these raphé neurons via release of ATP or other gliotransmitters. Furthermore, there are additional pH-sensitive serotonergic neurons in the caudal parapyramidal area80 that are likely to be intermingled with the glial cells that we describe in this study. CO2-dependent ATP release from these glial cells could potentially excite the neighbouring serotonergic neurons. As these serotonergic neurons project widely to other chemosensory areas of the medulla including the raphé obscurus, raphé pallidus, and the nucleus tractus solitarius49, their potential activation via release of ATP from CO2-sensitive glia in the same region gives a further mechanism by which this chemosensory signal could converge with that mediated via other chemosensory neurons (including the pH-dependent chemosensory pathway) and be propagated within the brain stem neural networks to facilitate breathing.

Peripheral (mainly CB) and central chemoceptors mediate the CO2-dependent control of breathing. Overall, about 30% of the total chemosensory response is mediated peripherally and the remainder via central chemoceptors61. In addition, there are two components to the adaptive changes in ventilation—an increase in respiratory frequency and an increase in tidal volume1. These two components combine to increase the rate of ventilation of the lungs: minute ventilation. Broadly, peripheral chemoceptors appear primarily to increase respiratory frequency, whereas activation of central chemoceptors have a more powerful effect on tidal volume62,63. However, ATP release from astrocytes in the preBotzinger complex does modulate changes in respiratory frequency in response to elevated CO264.

The widely accepted explanation for the chemosensitivity of breathing posits that pH-sensitive neurons detect changes in the pH of arterial blood and then excite the medullary networks that control breathing. It is clear that the peripheral chemoceptors are exclusively sensitive to pH92. pH-sensitive TASK-1 channels contribute to the detection of pH changes in blood in the CB glomus cells and thus contribute to peripheral chemoreception17. Centrally, neurons from the RTN, the caudal parapyramidal area48, and the medullary raphe56,66 respond to changes in pH most probably via TASK-211,19,67–70 and potentially GPR41,12,92.

Given this multiplicity of evidence supporting the role of pH detection on regulation of breathing, why should CO2 sensing via Cx26 be particularly important? First, the contribution of central direct CO2 sensing to the regulation of breathing is about 50% of the central generated chemosensory response23. Viral transduction by Cx26DN reduced the mean adaptive ventilatory response to 6% inspired CO2 by about 30%—mainly via a reduction of the increase in tidal volume. It is unlikely that we transduced all of the chemosensitive glial cells in this area, so this may be an underestimate of the contribution of this mechanism. Pharmacological blockade of Cx26 in the medulla, with imperfectly selective and potent agents, gave a reduction in the adaptive ventilatory response in anaesthetised and ventilated rats of about 20%26. The Cx26DN transduction is clearly more effective than the prior pharmacological approach—it is highly selective and will completely remove Cx26-mediated CO2 sensitivity from the glial cells in which it is expressed.

As central chemoceptors mediate about 70% of the adaptive response, CO2 sensing via Cx26 in the caudal parapyramidal area mediates just under half of the total central ventilatory response to modest levels of hypercapnia. A further comparison to give physiological context is that this contribution from Cx26 is slightly smaller than, but broadly comparable to, the DREADD-mediated inactivation of the entire population of raphé serotonergic neurons, which gave a 40% reduction in the adaptive ventilatory response at similar levels of inspired CO215. The physiological importance of this contribution to chemosensing is further confirmed by the evolutionary conservation of the carbamylation motif for >400 million years and CO2 sensitivity in Cx26 in all amniotes29.

The contribution of Cx26 to respiratory chemosensitivity occurred over an intermediate (~6%), but physiologically important, range of inspired CO2. There was no detectable effect of Cx26DN on the ventilatory response to 3% inspired CO2. This is unlikely to reflect the properties of Cx26, as it is sensitive to changes in PCO2 from 20 to 70 mmHg27,33, so would very likely be capable of detecting the small change in systemic PCO2 resulting from a 3% CO2 challenge. Cx26 contributes only to the adaptive change in tidal volume during hypercapnia (Fig. 5). The ventilatory response to 3% CO2 involved only a small change in tidal volume (an increase of 0.34 µl/g over VT at 0% CO2 in Cx26WT-injected mice, Fig. 5 and Supplementary Table 1). If Cx26 contributed about 30% of this increase, the expected difference in the VT between Cx26WT- and Cx26DN-injected mice would be ~0.1 µl/g. Although there is a trend toward a reduced change in VT at 3% CO2 (Fig. 5 and Supplementary Table 1), our experiments were insufficiently powered to detect such a small difference and hence cannot reveal any possible contribution of Cx26 at this level of inspired CO2. A further factor in determining the overall contribution of cells that express Cx26 to respiratory
control will be the way they and other populations of chemosensory cells, both central and peripheral, connect to the neuronal circuits controlling breathing.

At 9% inspired CO₂, Cx26-mediated chemosensing makes no contribution to the regulation of breathing, and the ventilatory responses to these higher levels are most likely exclusively mediated by changes in pH. This may be because the acidification caused by the higher level of inspired CO₂ can counteract the CO₂-dependent opening of Cx26 hemichannels. Cx26 hemichannels are closed by strong acidification. We have previously reported that acidification reduces the conductance change evoked by a PCO₂ stimulus in Cx26-expressing HeLa cells. Furthermore, the same increase in PCO₂ at pH 7.35 evokes about half the ATP release from the chemosensory cells in the ventral medulla compared to that evoked at pH 7.5. This is presumably because acidification makes carboxylation of the critical lysine residue harder to achieve. The caudal parapyramidal area (Fig. 7) was the only location in which we found a contribution of Cx26 to respiratory chemosensitivity. This area has been previously described as containing pH-sensitive serotonin neurons. The caudal parapyramidal area is thus sensitive to both chemosensory stimuli and is a potential point of convergence of the CO₂-mediated and pH-mediated chemosensory signals. In the rostral medulla, our injections of Cx26DN were too lateral to test whether Cx26 expression in more medial glial cells might also contribute to activation of the pH-sensitive raphé magnus neurons, but this would be an interesting hypothesis to investigate.

To conclude, the development of Cx26DN as a tool to remove CO₂ sensitivity from endogenously expressed Cx26 has provided the first genetic evidence, to our knowledge, for the involvement of Cx26 in the control of breathing. Our data provide a mechanistic link between the binding of CO₂ to a structural motif on Cx26 and shows that direct sensing of CO₂ by glial cells in a circumscribed area of the ventral medulla contribute nearly half of the centrally generated adaptive ventilatory response to CO₂.

Methods

Animals. All animal procedures were evaluated by the Animal Welfare and Ethical Review Body of the University of Warwick and carried out in strict accordance with the Animals (1986) Scientific Procedures Act of the UK under the authority of Licence PC07CHE03. Mice were randomly assigned to their groups (Cx26WT, Cx26K125R, and sham). A total of 80 mice were used in this study. Pilot experiment, and RTN: Male and female mice aged 12–17 weeks were used. The mice had a fixed Cx26 allele on a C57BL/6 background (EMMA strain 00245). These mice were not crossed with any cre lines so had normal WT expression of Cx26.

Main caudal parapyramidal experiment: C57BL6 WT male mice aged 12–17 weeks were used.

Very caudal parapyramidal experiment: C57BL6 WT Male mice aged 12–20 weeks were used.

Cell lines. HeLa DH (obtained from ECACC) and stable Cx26-expressing HeLa cells (gift from K. Willecke) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, 50 μg/mL penicillin-streptomycin, and 3 mM CaCl₂ at 37 °C.

Construction of connexin gene constructs. Connexin43 DNA sequence from Rattus norvegicus was purchased from Addgene (mCherry-Cx43-7, plasmid #50023, gifted by Michael Davidson) and subcloned into a PUC 19 vector such that the transcript would be 3′ (downstream) of Cx43 (as with Cx26 constructs). Dominant-negative mutant Cx26 (Cx26R104A) DNA with R104A K125R mutations was produced through two steps. First, Cx26 DNA with the K125R mutation and omitted STOP codon (to allow for fusion proteins) was synthesised by Genscript USA from the Cx26 sequence (accession number NM_001004099.1) and subsequently subcloned into a PUC 19 vector such that the transcript would form a fusion protein with mCherry at the C-terminus of Cx26. Second, the R104A mutation was introduced by Agilent Quikchange site-directed mutagenesis, using PUC19-Cx26(K125R) DNA as the template. Primer sequences for the mutagenesis were as follows: Cx26(R104A) forward 5′ GGC CTA CCG GAG ACA GCA AAA GAA AGC GAA GTT CAT GAA GG 3′, Cx26(R104A) reverse 5′ CCT TCA TGA ACT TCG CTT TCT TCT GGT GTC GCC GGT AGC CC 3′. Fusion and characterisation of the Cx26 and mRuby2 protein fluorophores used in these experiments was published by ref. 36. mRuby2 DNA was purchased from Addgene (mRuby2-C1, plasmid #54768, gifted by Michael Davidson) and subcloned into a PUC 19 vector so that it was 3′ of whichever connexin we engineered to be 5′ (upstream) of mRuby2. Clover DNA was a gift from Sergey Kasparov, Bristol and was subcloned into a PUC 19 vector so that it was 3′ of whichever connexin we engineered to be 5′ (upstream) of Clover.

HeLa cell transfection. To express the desired constructs, HeLa cells were transiently transfected with 0.5 μg of DNA of each pCAG-connexin-fluoresphore construct to be co-expressed (1 μg total), using the GeneJuice transfection agent (Merck Millipore).

Experimental artificial CSF (aCSF) solutions. Control (35 mmHg PCO₂): 124 mM NaCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 3 mM KCl, 10 mM D-glucose, 1 mM MgSO₄, 2 mM CaCl₂. This was bubbled with 95%O₂/5%CO₂ and had a final pH of ~7.4. Hypercapnic (55 mmHg PCO₂): 100 mM NaCl, 50 mM NaHCO₃, 1.25 mM NaH₂PO₄, 3 mM KCl, 10 mM D-glucose, 1 mM MgSO₄, 2 mM CaCl₂. This was bubbled with sufficient CO₂ (approximately 9%), balance O₂, to give a final pH of ~7.4.

Hypercapnic (55 mmHg PCO₂): 124 mM NaCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 3 mM KCl, 10 mM D-glucose, 1 mM MgSO₄, 2 mM CaCl₂. This was bubbled with 95%O₂/5%CO₂ and had a final pH of ~7.4.

Dye-loading assay. We used a dye-loading protocol that has been developed and extensively described in our prior work. To perform the dye loading, washed in control aCSF were then exposed to either control or hypercapnic solution containing 200 μM 5(6)-carboxyfluorescein (CFB) for 10 min. Subsequently, cells were transferred to control solution with 200 μM CFB for 5 min, before being washed in control solution without CFB for 30–40 min to remove excess extracellular dye. A replacement coverslip of HeLa cells was used for each condition. For each coverslip, mCherry staining was imaged to verify Cx26 expression.

To quantify fluorescence intensity, cells were imaged by epifluorescence (Scientifica Slice Scope (Scientifica Ltd, Uckfield, UK)), Cairn Research OptoLED illumination (Cairn Research Limited, Faversham, UK), x60 water Olympus immersion objective, NA 1.0 (Scientifica). Hamamatsu ImageEM EMCCD camera (Hamamatsu Photonics K.K., Japan), and Metalluor software (Cairn Research). Using ImageJ, regions of interest (ROIs) were drawn around individual cells and the mean pixel intensity for each ROI was obtained. The mean pixel intensity of the background fluorescence was also measured in a representative ROI, and this value was subtracted from the measures obtained from the cells. This procedure was used to subtract the background fluorescence from every pixel of all of the images displayed in the figures. At least 40 cells were measured in each condition, and the mean pixel intensities were plotted as cumulative probability distributions.

The experiments were replicated independently (independent transfections) at least five times for each Cx26 variant and condition. All experiments performed at room temperature.

FRET data capture and analysis. For FRET analysis, HeLa cells co-transfected with Cx-Clover (donor) and Cx-mRuby2 (acceptor) 72 h after transfection were washed 3× with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (PFA) for 10 min, washed 5× with PBS, and imaged in PBS at 4 °C. C. FRET studies were carried out within 2 weeks of fixation. They examined with a Zeiss LSM 710 Confocal microscope; C-Apochromat ×63/1.20 W Korr M27. Two channels were recorded: 495–545 nm (clover) and 650–784 nm (mRuby2), and images were acquired sequentially with 458- and 561-nm argon lasers, respectively. Imaging parameters for the clover channel are as follows: power, 30.0; pinhole, 78.5; gain (master), 700; digital offset, 0; digital gain, 1.0. Imaging parameters for the mRuby2 channel are as follows: power, 30.0; pinhole, 78.5; gain (master), 750; digital offset, 0; digital gain, 1.0. Photobleaching was performed using the 561-nm laser (as it only excites mRuby2) for 80 frames at 100% power, targeting ROIs. Image acquisition was as follows: ROIs were selected and drawn, including a background region and an ROI that was not to be bleached; pre-bleaching images were acquired for each channel; mRuby2 was photobleached; post-bleaching images were acquired for each channel. Acquisition parameters were kept identical across samples to allow comparison of results.

ROIs were then placed on each background-adjusted ROI were used to calculate FRET efficiency (E), bleaching efficiency (B), relative donor abundance (D), relative acceptor abundance (A), relative donor–acceptor ratio (DA ratio), relative acceptor...
quantity (A level), and donor–acceptor distance (R) as follows:

\[ E = \frac{\text{Clover}_{\text{post}} - \text{Clover}_{\text{pre}}}{\text{Clover}_{\text{pre}}} \]  

(1)

\[ D = \frac{\text{Clover}_{\text{post}} + \text{mRuby}_{\text{2}}_{\text{pre}}}{\text{Clover}_{\text{pre}}} \]  

(2)

\[ A = \frac{\text{mRuby}_{\text{2}}_{\text{pre}}}{\text{Clover}_{\text{pre}}} \]  

(3)

\[ DA \text{ ratio} = \frac{\text{Clover}_{\text{post}} + \text{mRuby}_{\text{2}}_{\text{pre}}}{\text{mRuby}_{\text{2}}_{\text{pre}}} \]  

(4)

\[ A \text{ level} = \text{mRuby}_{\text{2}}_{\text{pre}} \]  

(5)

Colocalisation threshold calculations were carried out in ImageJ using the Costes method. 100 iterations, omitting zero–zero pixels in threshold calculation.

**Lentivirus (LV) design and production.** Two LV constructs were designed to introduce either the Cx26320 or Cx26317 gene into the host cell genome (Supplementary Fig. 3). Constructs are ~4900 bp in length. The sequence of interest, consisting of the Cx26 (or Cx26077) immediately followed by an IRES and clover sequence (Cz626073xEDClever). The IRES sequence was from the encephalomyocarditis virus. LV constructs were produced and packaged by Cyagen Biosciences (USA) using the third-generation packing system. Constructs had a titre 10^8 IU/mL, as confirmed by quantitative PCR on genomic DNA extracted from the infected cells.

**Stereotaxic viral transduction.** Anaesthesia was induced by inhalation of isoflurane (4%). The mouse was then placed on a thermocoupled heating pad (TCAT-2LV, Physitemp) to maintain body temperature at 33 °C and headfixed into a stereotaxic frame. A face mask was used to maintain anaesthesia (isoflurane, intranasal, 0.5–2.5%). Atropine was provided (subcutaneous, 0.05 mg/kg) before surgery to reduce pleural effusion. adequacy of anaesthesia was assessed by respiratory rate, body temperature, and pedal withdrawal reflex. Preoperative Meloxicam (subcutaneous, 2 mg/kg) and postoperative Buprenorphine (intra-peritoneal, 0.05 mg/kg) were provided for analgesia. If any animal showed signs of pain in the days following surgery, additional analgesia was administered as required. No animals displaying signs of pain or receiving analgesia were used in plethysmographic recordings.

To maintain consistent placement of the injection pipette, the intra-aural line was adjusted so that the bregma was level to a point on the skull 2 mm caudal to bregma. Two small holes were made in the interparietal plate to allow for bilateral injection of virus particles via a micropipette lowered into the correct position via a stereotaxic manipulator. The injections were performed manually, using a 1-μl syringe, at a rate ~200 nl/min. A total of 350–400 nl of undiluted virus or saline was injected per side of the brain; the experimenter was blind to the injection solution. Co-ordinates (mm) relative to bregma were: RTN: 5.7 and 10.7 ventral, injection arm at 0° to vertical; caudal parapyramidal area: 5.95 caudal, 0.8 lateral, injection arm at 9° to vertical; caudal parapyramidal area: 5.95 caudal, 0.8 lateral, injection arm at 9° to vertical; ventral surface of the medulla oblongata in 0.1 mm caudal to 10.7 ventral. Co-ordinates were confirmed by post hoc immunostaining for viral-driven expression of fluorophores. Data were only included from mice whose injection sites were within the correct location.

**Immunohistochemistry.** Mice were culled by overdose of isoflurane or intraperitoneal injection of sodium pentobarbital (~100 mg/kg) and transcardially perfused with PFA (4%). The brain was harvested and post-fixed in 4% PFA for 24 h at 4 °C (to further increase tissue fixating), before being transferred to 30% sucrose (for cryoprotection) until the brain sunk to the bottom of the sucrose solution. Transcardial injection of sodium pentobarbital (>100 mg/kg) and transcardially perfused (4%) resulted in 24-well plates, and stored in PBS. For immunostaining, free-floating sections were incubated in PBS containing 0.1% Triton X-100 (Sigma-Aldrich, UK) (0.1% PBS-T) and the appropriate primary antibodies: goat anti-choline acetyltransferase (1:100) (Merck UK, ab144), chicken anti-GFAP (1:500) (abcam, ab61674), and rabbit anti-GFAP (1:500) (abcam, ab68428). Sections were then washed in PBS for 6 × 5 min before incubation at room temperature for 2.5–4 h in 0.1% PBS-T containing the appropriate secondary antibodies: donkey anti-goat Alexa Fluor 594 (1:2500) (abcam, ab150312), goat anti-rabbit Alexa Fluor 594 (1:2500) (abcam, ab150080), and goat anti-chicken Alexa Fluor 488 (1:2500) (abcam, ab150169). After secondary antibody incubation, sections were again washed in PBS for 6 × 5 min before mounting onto polylysine-coated slides (PolySine, VWR). Mounted slices were left to dehydrate overnight before applying coverslips using either Aqua-Poly/Mount (Polysciences Inc., Germany) or Fluoroshield with 4,6-diamidino-2-phenylindole (Sigma-Aldrich, UK). All steps were performed at 21 °C and during any period of incubation or washing, sections were gently agitated on a shaker.

**Whole-body plethysmography.** A custom built plethysmograph, constructed from a plexiglass box (0.5 l), was equipped with an air-tight lid, a pressure transducer to detect the breathing movements of the mouse, and gas inlets and outlets to permit gas flow through the chamber. A heated (via a water bath) mixture of O2 (–20%), N2 (80%), and CO2 (0–9%) flowed through the chamber regulated to a rate of 1 liter/min. The amount of O2 and CO2 in the mixture was measured, just prior to entry into the chamber, by a gas analyser (Hitech Instruments, GIR250 Dual Sensor Gas Analyser). Pressure signals were recorded with an NL108FT – Disposable Physiological Pressure Transducer (Digitimer) amplified and filtered using the NeuroLog system connected to a 1401 interface and acquired on a computer using the Spike2 software (Cambridge Electronic Design). Airflow measurements were used to calculate: tidal volume (VT: signal trough at the end of expiration subtracted from the peak signal during inspiration, converted to ml following calibration with a 1-ml syringe), respiratory frequency (fR breaths per minute), and minute ventilation (Ve) (calculated as VT × fR). The temperature inside the plethysmograph was maintained at ~31 °C, thermoneutral for C57BL/6 mice. The experimenter was only unblinded to the identity of the injected virus once acquisition and analysis of all plethysmographic recordings had been performed.

**Statistics and reproducibility.** For the dye-loading experiments, box-whisker plots were made and statistical tests were performed on the median change in pixel intensity within each replicate was a different transfection of the cells. The statistical package R was used for this analysis. For the FRET data, each replicate was a single ROI, and linear regression analysis and plots were carried out in GraphPad Prism. The plethysmographic data, each replicate was a single mouse, was analysed in SPSS via a two-way mixed-effects, repeated-measures ANOVA. Multiple post hoc comparisons were checked by the false discovery rate method, with the maximum allowed false discovery rate set to 0.05. All reported comparisons pass this test. All post hoc comparisons were one sided as there was a clear a priori hypothesis that Cx26DN should decrease CO2 sensitivity. Power calculations for the experiment in Fig. 5 were based on the pilot data of Supplementary Fig. 5 and were performed in GPower 3.1 for a two-way ANOVA with repeated measures, within-and-between-factor interactions. Further statistical details can be found in the relevant figure legends.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All data generated or analysed during this study are included in this published article. The source data used to generate the charts in the paper are provided in Supplementary Data 1–3.

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Competing interests
The authors declare no competing interests.

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