Connexin26 hemichannels with a mutation that causes KID syndrome in humans lack sensitivity to CO$_2$.
Abstract
Mutations in connexin26 (Cx26) underlie a range of serious human pathologies. Previously we shown that Cx26 hemichannels are directly opened by CO$_2$ (Meigh et al., 2013). However the effects of human disease-causing mutations on the CO$_2$ sensitivity of Cx26 are entirely unknown. Here, we report the first connection between the CO$_2$ sensitivity of Cx26 and human pathology, by demonstrating that Cx26 hemichannels with the mutation A88V, linked to Keratitis-Ichthyosis-Deafness syndrome, are both CO$_2$ insensitive and associated with disordered breathing in humans.

Connexin26 (Cx26) is one of 21 connexin genes found in humans (Cruciari & Mikalsen, 2006). The canonical function of connexins is to form gap junctions in which two hexameric connexons, or hemichannels, in closely apposed membranes dock together to form an intercellular channel. However connexins can also function as hemichannels, thereby providing large conductance channels, which allow passage of small molecules such as ATP into the extracellular space (Stout et al., 2004; Wang et al., 2013). We have recently shown that Cx26 hemichannels are directly sensitive to CO$_2$ (Huckstepp et al., 2010a; Meigh et al., 2013). When CO$_2$ binds to Cx26, it carbamylates K125, forms a salt bridge to R104 and opens the hemichannel (Meigh et al., 2013). Cx26 hemichannels are thus a source of CO$_2$-gated ATP release (Huckstepp et al., 2010a).

Mutations of Cx26 are the commonest cause of non-syndromic hearing loss (Cohn & Kelley, 1999; Kelley et al., 2000; Xu & Nicholson, 2013). Some of these mutations cause loss of functional protein, while other mutations result in gap junctions and hemichannels with altered properties. However the effect of these mutations on the CO$_2$ sensitivity of Cx26 has never been examined. Some missense mutations of Cx26 cause serious pathologies in humans, such as the very rare ectodermal disorder, Keratitis-Ichthyosis-Deafness (KID) syndrome. KID syndrome involves a combination of deafness, visual impairment, and dermatological abnormalities (Caceres-Rios et al., 1996). About 100 cases have been reported in the literature, and of these around 70% are caused by de novo mutations in Cx26, with the remainder being inherited in an autosomal dominant manner or via germ line mosaicism (Sbidian et al., 2010). To date there are 9 missense mutations that can cause KID syndrome (Xu & Nicholson, 2013). The severity of the
symptoms of KID syndrome depends on the particular mutation in Cx26 (Janecke et al., 2005; Jonard et al., 2008).

The mutation, Cx26\(^{A88V}\), is linked to a very severe form of KID syndrome, which is fatal in infancy (Haruna et al., 2010; Koppelhus et al., 2010). In one of the original reports linking Cx26\(^{A88V}\) to KID syndrome, the patient required mechanical ventilation (Koppelhus et al., 2010), suggesting a possible effect of the mutation on the neural control of breathing. In KID syndrome caused by a different missense mutation (G45E), which is fatal within the first year of life, there are also reports of breathing problems. One patient required mechanical ventilation immediately after birth (Janecke et al., 2005) and a second died from breathing failure (Sbidian et al., 2010). Nevertheless, without detailed recordings of cardiorespiratory activity, it is not possible to know whether these patients experienced inadequate central respiratory drive. For other mutations linked to KID syndrome there are no reports of abnormal breathing in the literature.

The reason why the A88V and G45E mutations should cause such pervasive and severe pathology remains unclear as subunits of Cx26\(^{A88V}\) and Cx26\(^{G45E}\) form both functional gap junctions and hemichannels (Gerido et al., 2007; Mhaske et al., 2013). Expression of Cx26\(^{A88V}\) in HeLa cells gives rise to enhanced hemichannel-mediated currents (compared to wild type Cx26, Cx26\(^{WT}\)) at positive transmembrane potentials and in the absence of extracellular Ca\(^{2+}\), leading to the suggestion that this mutation represents a gain of function (Mhaske et al., 2013). The G45E mutation, also causes enhanced hemichannel activity in the absence of extracellular Ca\(^{2+}\), and increased permeability to Ca\(^{2+}\) (Gerido et al., 2007; Sanchez et al., 2010). A gain of function has therefore been suggested as underlying the actions of this mutation too. Although the absence of extracellular Ca\(^{2+}\) opens connexin hemichannels, this condition is unlikely to occur in physiological systems. Thus the consequences of the A88V and G45E mutations on physiologically relevant gating of Cx26 remain unclear.

We identified a patient with KID syndrome caused by a heterozygous Cx26 A88V mutation. This patient failed to breathe spontaneously at birth and initially required mechanical ventilation. Later when he started to breathe spontaneously, he continued to demonstrate periods of apnea and bradycardia. A pneumogram performed at a post-menstrual age of 40 weeks showed abnormal persistence of central apnea lasting ≥ 20 seconds and accompanied by periods of bradycardia and prolonged oxygen desaturation (Figure 1). This respiratory pattern is abnormal for the age of the infant and is suggestive of blunted chemosensory control of breathing. Given
the previously described role of Cx26 in mediating the CO₂-dependent drive to breathe (Huckstepp et al., 2010b; Wenker et al., 2012), we considered whether the mutation A88V might alter the CO₂-sensitivity of Cx26.

We introduced the A88V mutation into Cx26 and then tested the CO₂ sensitivity of Cx26A88V hemichannels expressed in HeLa cells via an established and sensitive dye-loading protocol (Huckstepp et al., 2010a; Meigh et al., 2013). Under conditions of normal extracellular Ca²⁺, HeLa cells expressing wild type Cx26 hemichannels readily load with carboxyfluorescein when exposed to a moderately hypercapnic saline (PCO₂ 55 mmHg) (Huckstepp et al., 2010a; Meigh et al., 2013). However HeLa cells expressing Cx26A88V showed no such CO₂-dependent dye loading even when exposed to higher levels of PCO₂ (70 mmHg, Figure 2). The failure to exhibit CO₂-dependent dye loading was not due to a lack of functional hemichannels as the positive control of removing extracellular Ca²⁺, which opens all connexin hemichannels, caused robust dye loading (Figure 2). Surprisingly therefore, the conservative mutation A88V caused Cx26 hemichannels to lose their sensitivity to CO₂. As this mutation is far from the residues involved in CO₂ binding (K125 and R104), the mechanism for the loss of CO₂ sensitivity is unclear.

As the missense mutations which underlie KID syndrome act in a dominant manner (Jonard et al., 2008; Xu & Nicholson, 2013), we tested whether the expression of Cx26A88V subunits might have a dominant negative action on the CO₂ sensitivity of Cx26WT. We transfected HeLa cells that stably expressed Cx26WT with the Cx26A88V subunit and documented their sensitivity to CO₂ following transfection. Four days after transfection with Cx26A88V, the HeLa cells still exhibited sensitivity to CO₂ (Figure 3a), but this was reduced compared to the Cx26WT HeLa cells that had not been transfected with Cx26A88V (Figure 3b). Five and six days after transfection, the HeLa cells showed no sensitivity to CO₂ (Figure 3a). Nevertheless functional hemichannels were still present as the removal of extracellular Ca²⁺ caused dye loading (Figure 3a). The loss of CO₂ sensitivity was not simply a consequence of days in culture, as Cx26WT HeLa cells that had not been transfected with Cx26A88V retained their sensitivity to CO₂ over the whole period examined (Figure 3b). We therefore conclude that Cx26A88V subunits are able to act in a dominant negative manner to cause loss of CO₂ sensitivity from wild type Cx26 hemichannels.

This is the first instance in which a mutation linked to serious human pathologies has been demonstrated to abolish the CO₂ sensitivity of Cx26. This in turn suggests that Cx26-mediated CO₂ sensing may be important for human physiology in the range of contexts that are
associated with the diverse pathologies linked to this mutation. In the closely related β connxin, connxin30 (Cx30), the mutation A88V connected to Clouston’s Syndrome (Bosen et al., 2014), may result in constitutively open Cx30 hemichannels (Essenfelder et al., 2004). However Cx30 is also opened by CO$_2$ (Huckstepp et al., 2010a) and the effect of this mutation on the CO$_2$ sensitivity of Cx30 has not yet been investigated. There are no reports in the literature of disordered breathing in patients with Clouston’s syndrome.

Previous studies suggesting that the A88V mutation gave a gain of function in Cx26, examined hemichannel function in the absence of extracellular Ca$^{2+}$ (Mhaske et al., 2013). As the CO$_2$ sensitivity of the mutated hemichannel was not specifically examined in this previous study, it is likely that both sets of findings are correct—an enhancement of macroscopic hemichannel currents (Mhaske et al., 2013), and a loss of CO$_2$ sensitivity. However under physiological conditions of normal extracellular Ca$^{2+}$ and in the presence of physiological CO$_2$/HCO$_3^-$ buffering, we suggest that A88V should be considered as a loss-of-function mutation that effectively removes the capacity for CO$_2$-evoked ATP release via Cx26 hemichannels.

Our report is the first to document altered central respiratory drive in a KID syndrome patient. In rodents, CO$_2$-sensitivity of Cx26 contributes to the chemosensory control of breathing (Huckstepp et al., 2010b; Wenker et al., 2012). Although we do not know if the loss of CO$_2$ sensitivity in Cx26 contributed to the aberrant respiratory drive exhibited by this patient, these results are consistent with this possibility, and represent the first evidence to suggest that Cx26 hemichannels are a requisite component of the drive to breathe in humans. Overall the ability of physiological levels of PCO$_2$ to permit ATP release via Cx26 hemichannels may be important in the epidermis, cochlea and brain. Investigation of whether the absence of this mechanism of ATP release in patients with Cx26$^{A88V}$ contributes to the serious pathological abnormalities that they suffer would seem to be warranted.

Materials and Methods

Case study

The Institutional Review Board of the Connecticut Children’s Medical Center considered this under the category of a case report and thus exempt from formal review.
Mutant connexin production

Puc19 Cx26<sup>A88V</sup> was produced from wild type Cx26 via the Quikchange protocol using the following primers: forward 5’ TGT CCA CGC CGG TCC TCC TGG TAG C 3’ reverse 5’ GCT ACC AGG AGG ACC GGC GTG GAC A 3’. Cx26<sup>A88V</sup> was subcloned into a pCAG-GS mCherry vector for mammalian cell transfection. Successful mutation of Cx26 was confirmed by sequencing which also verified that apart from the desired mutation the sequence was identical to the wild type.

HeLa cell culture

HeLa cells were cultured by standard methods in DMEM, 10% FCS with addition of 3 mM CaCl<sub>2</sub>. For experimentation, cells were plated onto coverslips at a density of 5 x 10<sup>4</sup> cells per well. Transient transfections were performed using the genejuice protocol.

Solutions used

Control aCSF: 124 mM NaCl, 26 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 3 mM KCl, 10 mM D-glucose, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>.

Zero Ca<sup>2+</sup> aCSF: 124 mM NaCl, 26mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 3 mM KCl, 10 mM D-glucose, 1 mM MgSO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 1 mM EGTA.

Hypercapnic (55mmHg CO<sub>2</sub>) aCSF: 100 mM NaCl, 50 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 3 mM KCl, 10 mM D-glucose, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>.

Hypercapnic (70mmHg CO<sub>2</sub>) aCSF: 70 mM NaCl, 80 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 3 mM KCl, 10 mM D-glucose, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>.

Hypercapnic aCSF was saturated with sufficient CO<sub>2</sub> (the remaining balance being O<sub>2</sub>) to adjust the final pH (pH 7.5) to that of the control aCSF removing any potential effects of changes in extracellular pH.

All other solutions were saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>.

Dye loading protocols

Coverslips plated with HeLa cells transiently transfected with Cx26<sup>A88V</sup> were exposed to Hypercapnic aCSF (55mmHg or 70mmHg) containing 200 µM CBF for 10 minutes. This was
followed by control aCSF with 200 µM CBF for 5 minutes and a 30 minute wash with control aCSF to ensure that all dye is removed from the outside of the cells.

A control comparison was used to establish any baseline loading occurring in the absence of a stimulus. HeLa cells expressing Cx26<sup>A88V</sup> were exposed to 200 µM CBF in control aCSF for 15 minutes, followed by 30 minutes of washing.

A zero Ca<sup>2+</sup> positive control was also performed to ensure functional connexin hemichannels were being expressed. Cx26<sup>A88V</sup> expressing HeLa cells were exposed to 200 µM CBF in zero Ca<sup>2+</sup> aCSF for 10 minutes. This was followed by control aCSF with 200 µM CBF for 5 minutes and 30 minutes of washing with aCSF.

**Imaging and analysis**

For each condition cells were imaged by epifluorescence (Scientifica Slice Scope, Cairn Research OptoLED illumination, 60x water Olympus immersion objective, NA 1.0, Hamamatsu ImageEM EMCCD camera, Metafluor software). Using ImageJ, the extent of dye loading was measured by drawing a region of interest (ROI) around individual cells and calculating the mean pixel intensity for the ROI. 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. All of the images displayed in the figures reflect this procedure in that the mean intensity of the pixels in a representative background ROI has been subtracted from every pixel of the image. The analysis of the CO<sub>2</sub> sensitivity of Cx26<sup>A88V</sup> was performed as 5 independent repetitions in which at least 40 cells were measured in each condition, and the mean pixel intensities plotted as cumulative probability distributions.

**References**


Figure Legends

Figure 1. Incidence of central sleep apnea in a patient with Cx26\textsuperscript{A88V}. Recording of cardiorespiratory activity during sleep from an infant at a post-menstrual age of 40 weeks diagnosed with KID syndrome. Traces of nasal air flow, thoracic movement, electrocardiogram (ECG), heart rate (HR) and arterial O\textsubscript{2} saturation show that this patient exhibited a prolonged period during which no effort was made to breathe and this was followed by pronounced bradycardia and arterial O\textsubscript{2} desaturation, all of which are characteristic of central sleep apnea. Unfortunately, at 2 months of age this patient died from overwhelming sepsis.

Figure 2 Cx26\textsuperscript{A88V} hemichannels are no longer sensitive to CO\textsubscript{2}. Top) Images of HeLa cells expressing Cx26\textsuperscript{A88V} under control, hypercapnic and zero Ca\textsuperscript{2+} conditions. The cells were exposed to 200 µM carboxyfluorescein (CBF) for 5 minutes under each condition before being washed. Some low background loading of CBF is seen under control conditions. In presence of CO\textsubscript{2} no loading is seen. The positive control of removal of extracellular Ca\textsuperscript{2+} causes robust dye loading demonstrating the presence of functional hemichannels. Bottom) Cumulative probability distributions of pixel intensity of HeLa cells expressing Cx26\textsuperscript{A88V} under control, hypercapnia (two levels of PCO\textsubscript{2}) and zero Ca\textsuperscript{2+}. Only the removal of extracellular Ca\textsuperscript{2+} causes dye loading as shown by the rightward shift of the curve to higher pixel intensities (p=0.004, Mann Whitney U test compared to control). These distributions show all of the measurements made (minimum 40 cells each from 5 independent repetitions).

Figure 2 Supplement 1. HeLa cells transfected with the empty pCAG-GS mCherry vector show no sensitivity to CO\textsubscript{2} and do not dye load when exposed to zero Ca\textsuperscript{2+} aCSF. a) Cumulative probability distributions of pixel intensity for HeLa cells transfected with pCAG-GS mCherry under control, hypercapnia and zero Ca\textsuperscript{2+} conditions. The cells were exposed to 200 µM CBF for 5 minutes under each condition before being washed. The graphs show all of the measurements from 4 independent repetitions for each condition. b) When transfected with pCAG-GS mCherry, the HeLa cells exhibit diffuse red fluorescence from expression of the mCherry. This contrasts with the punctate fluorescence seen flowing transfection with pCAG-GS Cx26-mCherry (inset). Scale bars 20 µm.
**Figure 3** Cx26\(^{A88V}\) hemichannels act in a dominant negative manner to remove CO\(_2\) sensitivity from Cx26\(^{WT}\). a) Cumulative probability distributions for CO\(_2\)-dependent dye loading in HeLa cells that stably express Cx26\(^{WT}\), which have been transfected with Cx26\(^{A88V}\). 4 days after transfection with Cx26\(^{A88V}\) the cells still exhibit significant sensitivity to 55 mmHg PCO\(_2\) stimulus (p=0.048 CO\(_2\) compared to control, Mann Whitney U test). 5 and 6 days after transfection the CO\(_2\) sensitivity of the HeLa cells was abolished. On all three days, the positive control of zero Ca\(^{2+}\) caused dye loading, demonstrating the presence of functional hemichannels. The graphs show all of the measurements made from 5 independent repetitions of the experiment. b) Comparison of the sensitivity to CO\(_2\) of HeLa cells stably expressing Cx26\(^{WT}\) which have been transfected with Cx26\(^{A88V}\) (Cx26\(^{WT}\) + Cx26\(^{A88V}\), n=5) with those that have not (Cx26\(^{WT}\), n=7). In the absence of transfection, the Cx26\(^{WT}\)-expressing HeLa cells retain sensitivity to CO\(_2\) on all three days. By contrast Cx26\(^{A88V}\) causes significantly depressed CO\(_2\) sensitivity 4 days after transfection (p=0.001), and loss of sensitivity on days 5 (p=0.024) and 6 (p=0.001). Comparisons of Cx26\(^{WT}\) with Cx26\(^{WT}\) + Cx26\(^{A88V}\) via Mann Whitney U test, and False Discovery Rate procedure for multiple comparisons (Curran-Everett, 2000). Error bars upper and lower quartiles.