

Knockdown of PHOX2B in the retrotrapezoid nucleus reduces the central CO₂ chemoreflex in rats

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Silvia Cardani, Tara A. Janes, William Betzner, Silvia Pagliardini 

Department of Physiology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton AB T6G2E1 Canada

• Women and Children's Health Research Institute, University of Alberta, Edmonton AB T6G2E1 Canada •

Neuroscience and Mental Health Institute, University of Alberta, Edmonton AB T6G2E1 Canada

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Abstract

PHOX2B is a transcription factor essential for the development of the autonomic nervous system. Heterozygous mutations in the PHOX2B coding region are responsible for the occurrence of Congenital Central Hypoventilation Syndrome (CCHS), a rare neurological disorder characterised by inadequate chemosensitivity and life-threatening sleep-related hypoventilation. Animal studies suggest that chemoreflex defects are caused in part by the improper development or function of PHOX2B expressing neurons in the retrotrapezoid nucleus (RTN), a central hub for CO₂ chemosensitivity.

Although the function of PHOX2B in rodents during development is well established, its role in the adult respiratory network remains unknown. In this study, we investigated whether reduction in PHOX2B expression in chemosensitive neuromedin-B (NMB) expressing neurons in the RTN altered respiratory function. Four weeks following local RTN injection of a lentiviral vector expressing the short hairpin RNA (shRNA) targeting *Phox2b* mRNA, a reduction of PHOX2B expression was observed in *Nmb* neurons compared to both naïve rats and rats injected with the non-target shRNA. PHOX2B knockdown did not affect breathing in room air or under hypoxia, but ventilation was significantly impaired during hypercapnia. PHOX2B knockdown did not alter *Nmb* expression but reduced the expression of both *Task2* and *Gpr4*, two CO₂ sensors in the RTN. We conclude that PHOX2B in the adult brain has an important role in CO₂ chemoreception and reduced PHOX2B expression in CCHS beyond the developmental period may contribute to the impaired central chemoreflex function.

eLife assessment

This **important** study utilizes a virus-mediated short hairpin RNA (shRNA) approach to investigate in a novel way the role of the wild-type PHOX2B transcription factor in critical chemosensory neurons in the brainstem retrotrapezoid nucleus (RTN) region for maintaining normal CO₂ chemoreflex control of breathing in adult rats. The **solid** results presented show blunted ventilation during elevated inhaled CO₂ (hypercapnia) with knockdown of PHOX2B, accompanied by a reduction in expression of Gpr4 and Task2 mRNA for the proposed RTN neuron proton sensor proteins GPR4 and TASK2. These results suggest that maintained expression of wild-type PHOX2B affects respiratory control in adult animals, which complements previous studies showing that PHOX2B-expressing RTN neurons may be critical for chemosensory control throughout the lifespan and with implications for neurological disorders involving the RTN. When some methodological, data interpretation, and prior literature reference issues further highlighting novelty are adequately addressed, this study will be of interest to neuroscientists studying respiratory neurobiology as well as the neurodevelopmental control of motor behavior.

Introduction

The Paired Like Homeobox 2B (PHOX2B) is a transcription factor essential for embryonic differentiation and for the maintenance of the neuronal phenotype of different classes of neurons in both the central and peripheral nervous systems (Brunet & Pattyn, 2002 [DOI](#); Pattyn et al., 1997 [DOI](#), 1999 [DOI](#), 2000 [DOI](#); Stanke et al., 1999 [DOI](#); Tiveron et al., 1996 [DOI](#)). In recent years, the PHOX2B gene has been extensively investigated, not only for its neurodevelopmental role, but also because its heterozygous mutation leads to Congenital Central Hypoventilation Syndrome (CCHS, OMIM 209880; Amiel et al., 2003 [DOI](#)), Hirschsprung's disease (HSCR; OMIM 142623; Berry-Kravis et al., 2006 [DOI](#); Trochet et al., 2005 [DOI](#)), and neuroblastoma (Bourdeaut et al., 2005 [DOI](#); Van Limpt et al., 2004 [DOI](#)). CCHS is a rare (1:200,000 births in France (Trang et al., 2005 [DOI](#)) and 1:148,000 births in Japan (Shimokaze et al., 2015 [DOI](#)) disorder characterised by a failure to respond to both hypercapnia and hypoxia (Amiel et al., 2003 [DOI](#); Di Lascio et al., 2021 [DOI](#); Weese-Mayer et al., 2017 [DOI](#)). The most frequent PHOX2B mutation is an expansion of a polyalanine repeat, ranging from +5 to +13 residues on the C-terminal of PHOX2B, an area that regulates DNA binding affinity and protein solubility (Amiel et al., 2003 [DOI](#); Di Lascio et al., 2016 [DOI](#); Weese-Mayer et al., 2003 [DOI](#)). CCHS symptoms present with varying degrees of severity depending on the expansion of the mutation (Berry-Kravis et al., 2006 [DOI](#); Di Lascio et al., 2018 [DOI](#); Matera, 2004 [DOI](#)). In most mild cases, patients display normal ventilation during wakefulness, hypoventilation during sleep leading to hypercarbia and hypoxemia, as well as a lack of sensitivity and discomfort to these challenges. In the most severe of cases, hypoventilation may also be present while awake.

Respiratory disturbances resulting from the PHOX2B mutations appear to stem from its widespread expression in respiratory-related neurons both during development and into adulthood. Although PHOX2B is absent in respiratory rhythmogenic neurons of the preBötzinger complex, it is highly expressed in adult neurons that are responsible for CO₂ and O₂ chemoreflexes (Kang et al., 2007 [DOI](#)). Among those are the neurons of the retrotrapezoid nucleus (RTN), the locus coeruleus, the carotid bodies and the nucleus of the solitary tract that, together with the dorsal motor nucleus of the vagus nerve and the area postrema, make up the dorsal vagal complex, a structure that provides the major integrative centre for the mammalian autonomic nervous system (Cutsforth-Gregory & Benarroch, 2017 [DOI](#); Guyenet et al., 2019 [DOI](#); Kang et al., 2007 [DOI](#); Zoccal et al., 2014 [DOI](#)). Previous work by our group and others specifically

investigated the role of PHOX2B-expressing RTN neurons by either selective lesions or inactivation of neuronal activity of these neurons (Abbott et al., 2011 [↗](#); Basting et al., 2015 [↗](#); Marina et al., 2010 [↗](#); Nattie & Li, 1994 [↗](#); Souza et al., 2018 [↗](#), 2023 [↗](#); Janes et al., 2024 [↗](#)) and showed that RTN neurons have a key role in central CO₂ chemoreception.

Defects observed in the CO₂-chemoreflex of CCHS transgenic rodent models are primarily caused by the improper development or function of PHOX2B-expressing neurons in the RTN (Dubreuil et al., 2008 [↗](#); Ramanantsoa et al., 2011 [↗](#)), although anatomo-pathological studies in CCHS patients suggest that the respiratory impairment may be caused by a more widespread brain dysfunction (Harper et al., 2015 [↗](#); Nobuta et al., 2015 [↗](#)). Transgenic mice born with PHOX2B knockout fail to develop the RTN, the locus coeruleus (Pattyn et al., 1999 [↗](#)) and catecholaminergic groups in A1, A2, A5 and A7 (Pattyn et al., 2000 [↗](#)), as well as neurons of the nucleus of the solitary tract and the carotid bodies. Mice harbouring heterozygous PHOX2B knockout develop apparently normal but have depressed responses to hypoxia and hypercapnia (Dauger, 2003 [↗](#)) and sleep disordered breathing in the perinatal period (Durand et al., 2005 [↗](#)). Furthermore, the expression of the most common heterozygous PHOX2B mutation found in CCHS patients (PHOX2B +7Ala expansion) in mice resulted in a near complete loss of RTN neurons, impaired CO₂ response and consequent death in the newborn period (Dubreuil et al., 2008 [↗](#); Madani et al., 2021 [↗](#); Ramanantsoa et al., 2011 [↗](#); Takakura et al., 2008 [↗](#)). More selective expression of the mutant PHOX2B in the RTN neurons allowed for survival in the neonatal period and partial recovery of the CO₂ response in adulthood (Ramanantsoa et al., 2011 [↗](#)).

Multiple potential cellular mechanisms have been implicated in the etiology of CCHS, from loss of PHOX2B function in development due to heterozygous mutant PHOX2B expression, to gain of function of the mutated protein that may alter transcriptional activity in neurons, as well as aggregate formation and premature cell death (Bachetti et al., 2005 [↗](#); Di Lascio et al., 2013 [↗](#); Dubreuil et al., 2008 [↗](#); Durand et al., 2005 [↗](#); Goridis et al., 2010 [↗](#); Trochet et al., 2005 [↗](#)). While its role during embryonic neurodevelopment is established, PHOX2B is still expressed in selected neuronal populations in the adult brain (Kang et al., 2007 [↗](#); Stornetta et al., 2006 [↗](#)) and its function, with the exception of the contribution to the maintenance of the noradrenergic phenotype (Fan et al., 2011 [↗](#)), is not yet fully understood. Considering the fundamental role of PHOX2B in CCHS pathogenesis and its expression in key structures for CO₂ chemoreflex responses, it is essential to understand how both wild-type and mutant PHOX2B proteins impact CO₂ homeostasis and ventilation not only across development, but also in adulthood.

In order to have a better understanding of the role of PHOX2B in the CO₂ homeostatic processes we used a non-replicating lentivirus vector of two short-hairpin RNA (shRNA) clones targeting selectively *Phox2b* mRNA to knockdown the expression of PHOX2B in the RTN of adult rats and tested ventilation and chemoreflex responses. In parallel, we also determined whether knockdown of PHOX2B in adult RTN neurons negatively affected cell survival. Finally, we sought to provide a mechanistic link between PHOX2B expression and the chemosensitive properties of RTN neurons, which have been attributed to two proton sensors, the proton-activated G protein-coupled receptor (GPR4) and the proton-modulated potassium channel (TASK-2) (Gestreau et al., 2010 [↗](#); Guyenet et al., 2016 [↗](#); Kumar et al., 2015 [↗](#)). The expression of these sensors is partially overlapping in RTN neurons and the genetic deletion of either impairs the central respiratory chemoreflex with maximal impairment when both sensors are eliminated (Gestreau et al., 2010 [↗](#); Guyenet et al., 2016 [↗](#); Kumar et al., 2015 [↗](#)).

Our results indicate that progressive knockdown of PHOX2B in RTN causes a reduction in the chemoreflex response that was proportional to the decrease in the fraction of *Nmb*/PHOX2B expressing RTN neurons. This effect was associated with a reduction in the expression of *Gpr4* and *Task2* mRNA in *Nmb* expressing cells in which PHOX2B was knocked down, suggesting a role for PHOX2B in the transcription of the proton sensors in RTN neurons.

Results

To directly investigate the role of PHOX2B in RTN neurons, we performed selective knockdown of this protein in adult rats through local injection of a non-replicating lentiviral vector of two short-hairpin RNA (shRNA) clones targeting two different sequences of the *Phox2b* mRNA (n=25) or non-target shRNA as control (NT-shRNA; n=23). Initial experiments utilized 200 nl/injection at each of 4 stereotaxic coordinates (1×10^9 VP/ml; n=14). Four weeks post injection, we assessed ventilatory parameters in room air, hypercapnia, and hypoxia. Interestingly, a small but significant impairment in the CO₂ chemoreflex was observed not only in PHOX2B-shRNA but also in NT-shRNA rats. Specifically, room air breathing was not affected by RTN injection, but allometric V_E during 7% CO₂ was blunted in both NT-shRNA and PHOX2B-shRNA rats when compared to pre-surgical baseline (-21% and -24%, respectively; Supp. Fig. 1A). Analysis of the air convection requirement ratio (CRR; V_E/VO₂) confirmed that chemoreflex impairment during 7% CO₂ exposure was of central origin and not due to lowered metabolism (data not shown). Given that CO₂ chemoreflexes were impaired in both NT-shRNA and PHOX2B-shRNA rats, and histological analysis showed significant reduction of *Nmb*⁺/PHOX2B⁺ RTN cells in NT-shRNA and PHOX2B-shRNA rats (33% and 19% cells remaining, respectively; Suppl. Fig. 1), we concluded that the volume and titre of the shRNA viral constructs triggered off-target effects (McBride et al., 2008; Van Gestel et al., 2014) that cause cell death in RTN neurons.

PHOX2B expression is modestly reduced two weeks following infection without respiratory impairment

Due to the off-target effects obtained with large volume injections, we reduced injection volume of both PHOX2B shRNA (n=17) and NT-shRNA viruses (n=17) (4x100nl/injection; 1×10^9 VP/ml).

Two weeks post-surgery we assessed respiratory function during exposure to room air, hypercapnia and hypoxia. Overall, we observed little change in respiration at this time point. During room air, as well as in 5% and 7.2% CO₂, frequency (f_R), was reduced in every experimental group (naïve, NT-shRNA, PHOX2B-shRNA) compared to baseline (Air: p=0.014; 5% CO₂: p<0.001; 7.2% CO₂: p<0.001; Fig. 1A) but no effect on treatment was observed, suggesting that these changes were likely due to the increase in age and body weight during the experiment (naïve: +32.2%; NT-shRNA: +27.8%; PHOX2B-shRNA: +28.6% body weight). Tidal volume was reduced in the PHOX2B-shRNA rats at 7.2% CO₂ compared to both naïve rats (naïve: 9.6 ± 1.2 ml·kg⁻¹; PHOX2B-shRNA: 8.5 ± 0.9 ml·kg⁻¹; -11%; p=0.022) and baseline conditions (baseline shRNA: 9.6 ± 1.3 ml·kg⁻¹; -11%; p<0.001; Fig. 1B) but it was not different from NT-shRNA.

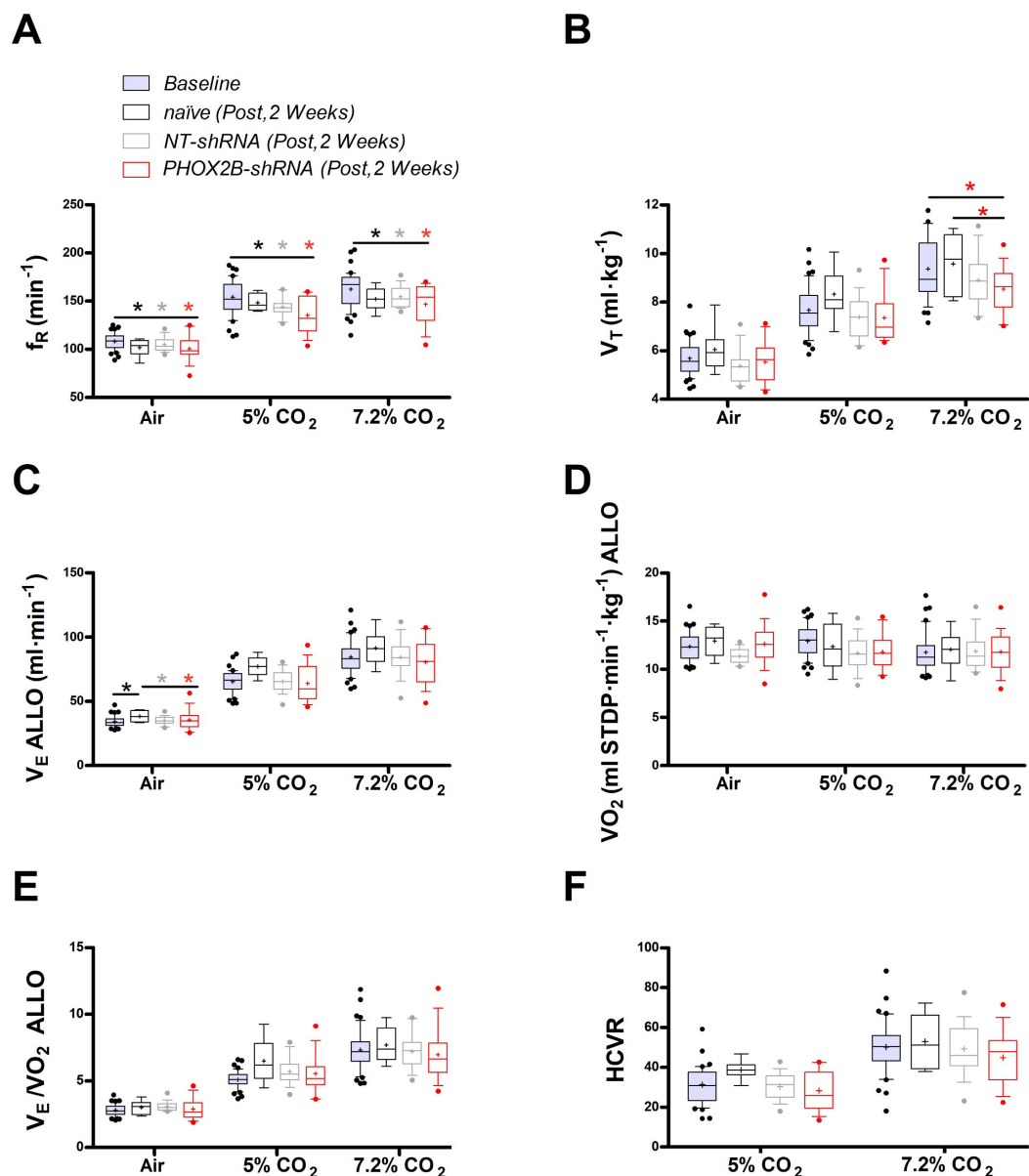


Figure 1.

Respiratory data two weeks post viral shRNA injection.

(A) breathing frequency (f_R), (B) tidal volume (V_T), (C) allometric minute ventilation (V_E ALLO), (D) oxygen consumption (VO_2 ALLO), (E) convective requirement ratio (V_E/VO_2 ALLO), (F) hypercapnic ventilatory response (HCVR absolute change in V_E ALLO vs. corresponding room air) of baseline (pre-surgery, grey filled box), naïve (black n=8), non-target control shRNA (NT-shRNA, grey n=17) and PHOX2B shRNA (PHOX2B-shRNA, red n=17) rats 2-weeks post injection during room air, hypercapnia 5% and 7.2% CO₂. f_R is equally impaired in all experimental group compared to baseline but no treatment effect was observed (A). V_T is significantly impaired following RTN injection in PHOX2B-shRNA group compared to naïve animals ($p=0.022$) and baseline ($p<0.001$) at 7.2% CO₂ (B). V_E ALLO is increased in naïve rats compared to the other treatment groups ($p=0.005$) in room air (C). Boxplots: median, 1st – 3rd quartiles and 10th – 90th percentiles, outliers = dots, “+” indicates arithmetic mean. Bonferroni post-hoc as indicated. Black*, different from naïve; Grey*, different from NT-shRNA; Red*, different from PHOX2B sh-RNA.

Despite similar changes in body weight across treatment groups, allometric ventilation (V_E ALLO) was increased in naïve rats compared to the other treatment groups in room air (naïve: 38.4 ± 4.5 ml·min⁻¹; NT-shRNA: 34.7 ± 2.8 ml·min⁻¹; PHOX2B-shRNA: 34.0 ± 4.5 ml·min⁻¹; $p=0.005$; **Fig. 1C**), but no changes in V_E ALLO were observed in hypercapnia (7.2% CO₂) across treatments (naïve : 91.5 ± 13.2 ml·min⁻¹; NT-shRNA: 84.4 ± 13.7 ml·min⁻¹; PHOX2B-shRNA: 80.5 ± 17.2 ml·min⁻¹). Oxygen consumption (VO_2 ALLO) was not different between experimental conditions or treatment groups (**Fig. 1D**), and V_E/VO_2 ALLO indicated that ventilation was matched with metabolism for each treatment across all gas compositions (naïve: 7.7 ± 1.3 ; NT-shRNA: 7.2 ± 1.3 ; PHOX2B-shRNA: 6.9 ± 2.0 ; **Fig. 1E**). These results suggest that despite a reduction in hypercapnic V_T in the PHOX2B-shRNA rats, overall ventilation was not impaired. Since we observed differences in room air V_E ALLO between treatment groups, we normalized these data to show the hypercapnic ventilatory response (HCVR, i.e., absolute change from room air; **Fig. 1F**). The HCVR was not affected by the PHOX2B shRNA treatment two weeks post-injection (naïve: 53.1 ± 13.4 ml·min⁻¹; NT-shRNA: 49.6 ± 13.2 ml·min⁻¹; PHOX2B-shRNA: 46.5 ± 14.8 ml·min⁻¹).

To determine the extent of PHOX2B knockdown in RTN neurons we combined RNAScope® and immunohistochemistry assays to quantify RTN neurons expressing *Nmb* and PHOX2B (**Fig. 2B**). We reasoned that by assessing the fraction of RTN neurons that express only *Nmb* (compared to *Nmb*⁺/PHOX2B⁺), we would be able to determine the level of PHOX2B knockdown in our experiments. Two weeks post viral injection, we observed a significant decrease (15.2%) in the total number of *Nmb*⁺ RTN neurons (i.e., *Nmb*⁺/PHOX2B⁺ plus *Nmb*⁺/PHOX2B⁻) in NT-shRNA rats compared to naïve rats (naïve; 337.6 ± 11.95 ; $n=4$; NT-shRNA: 286.0 ± 10.44 ; $n=4$; $p<0.001$; **Fig. 2C**). The total number of *Nmb*⁺ RTN neurons in PHOX2B-shRNA rats was reduced compared to naïve rats (PHOX2B-ShRNA 218.8 ± 7.8 $n=4$; -35.2%; $p<0.001$) and compared to NT-shRNA (-23.5%; $p<0.001$).

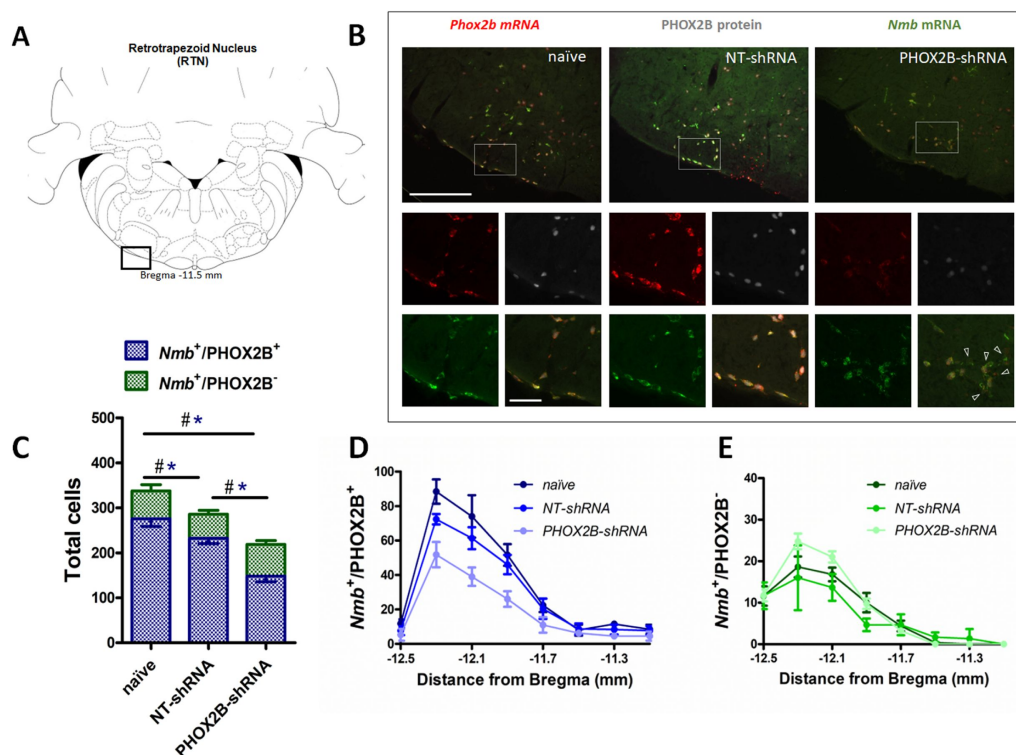


Figure 2.

PHOX2B and *Nmb* expression and total cell count within the RTN area in naïve, NT-shRNA and PHOX2B-shRNA injected rats two weeks post viral shRNA injection.

(A) Schematic and representative image of a transverse brainstem section at the level of the RTN (-11.5 mm distance from Bregma) showing the area of investigation containing RTN neurons. (B) Expression of *Phox2b* mRNA (red), PHOX2B protein (white) and *Nmb* mRNA (green) in RTN *Nmb*⁺/PHOX2B⁺ and *Nmb*⁺/PHOX2B⁻ neurons in naïve (left), NT-shRNA (middle), and PHOX2B-shRNA (right) rats (magnified view inserts below). Arrowheads indicate absence of PHOX2B protein. Scale bar=400µm (top figures), 150µm (inserts below). (C) The number of total cells (*Nmb*⁺/PHOX2B⁺ plus *Nmb*⁺/PHOX2B⁻) comprising the RTN are reduced in PHOX2B-shRNA rats (n=4) as compared to naïve (n=4) and NT-shRNA (n=4), and in NT-shRNA rats as compared to naïve rats (black[#], One-way ANOVA, p<0.001). The number of *Nmb*⁺/PHOX2B⁺ cells are reduced in PHOX2B-shRNA rats as compared to naïve and NT-shRNA (Blue*, One-way ANOVA, p<0.001). The number of *Nmb*⁺/PHOX2B⁻ cells was unchanged across groups. (D,E) Rostral-caudal distribution of *Nmb*⁺/PHOX2B⁺ (D) and *Nmb*⁺/PHOX2B⁻ (E) neurons along the RTN.

In order to assess PHOX2B knockdown, we then quantified the number of neurons expressing either *Nmb* and PHOX2B (*Nmb*⁺/PHOX2B⁺) or *Nmb* only (*Nmb*⁺/PHOX2B⁻). The number of *Nmb*⁺/PHOX2B⁺ neurons in PHOX2B-shRNA rat was reduced by 36.1 % compared to NT-shRNA and by 46.2% compared to naïve rats (*Nmb*⁺/PHOX2B⁺ NT-shRNA: 232.3 ± 11.6 vs naïve: 275.8 ± 17.1; vs PHOX2B-shRNA: 148.3 ± 12.6; p<0.05 and 0.001, respectively; **Fig. 2C,D**). On the contrary, the fraction of *Nmb*⁺/PHOX2B⁻ neurons in PHOX2B-shRNA rat was not different compared to naïve and NT-shRNA rats (*Nmb*⁺/PHOX2B⁻ NT-shRNA: 53.7 ± 8.6; vs naïve: 61.8 ± 13.9; vs PHOX2B-shRNA: 70.5 ± 8.7 cells; **Fig. 2C,E**). These results thus indicate that despite modest loss of *Nmb*⁺ neurons in NT-shRNA and PHOX2B-shRNA treated rats and nearly 50% reduction of *Nmb*⁺/PHOX2B⁺ in PHOX2B-shRNA rats, 2 weeks of shRNA viral infection was not sufficient to impair ventilation.

Four weeks of PHOX2B knockdown impairs the CO₂-chemoreflex

Since the shRNAs integrate into the genome of the infected cells and are continuously produced, it is reasonable to expect that knockdown efficiency is time-dependent, and an extended infection time would result in a higher knockdown effect. To test this hypothesis, a subgroup of rats (naïve, NT-shRNA and PHOX2B-shRNA) were investigated four weeks post-viral injection (**Fig. 3A,B**).

Figure 3.

PHOX2B and *Nmb* expression and total cell count within the RTN area in naïve, NT- shRNA and PHOX2B-shRNA injected rats 4 weeks post viral shRNA injection.

(A) Schematic and representative image of a transverse brainstem section at the level of the RTN (-11.5 mm distance from Bregma) showing the area of investigation containing RTN neurons. (B) Expression of *Phox2b* mRNA (red), PHOX2B protein (white) and *Nmb* mRNA (green) in naïve (left), NT-shRNA (middle), and PHOX2B-shRNA (right) rats (magnified view insert). Arrowheads indicate absence of PHOX2B protein. Scale bar = 400µm (top figures), 150µm (inserts below). (C) The number of total cells ($Nmb^+/PHOX2B^+ + Nmb^+/PHOX2B^-$) comprising the RTN are reduced in PHOX2B-shRNA rats (n=6) as compared to naïve (n=4) (Black[#], One-way ANOVA, p=0.0087) but not to NT-shRNA (n=10). The number of $Nmb^+/PHOX2B^+$ cells are reduced in PHOX2B-shRNA rats as compared to naïve and NT- shRNA (Blue*, one-way ANOVA, p<0.001). The number of $Nmb^+/PHOX2B^-$ cells are increased in PHOX2B-shRNA rats as compared to both naïve and NT-shRNA (Green*, one-way ANOVA p<0.001). (D, E) Rostral-caudal distribution of $Nmb^+/PHOX2B^+$ (D) and $Nmb^+/PHOX2B^-$ (E) neurons along the RTN.

The total number of RTN neurons in PHOX2B-shRNA rats ($Nmb^+/PHOX2B^+$ plus $Nmb^+/PHOX2B^-$; **Fig. 3C**) was significantly reduced compared to naïve rats (naïve: 347.8 ± 12 cells, n=4; PHOX2B-shRNA: 249.5 ± 33.9 cells; n=6; -28.3%; p=0.009) but no difference was observed compared to NT-shRNA rats (294.9 ± 52.8 cells; n=10; -15.4%;). Furthermore, there was no difference in the total number of Nmb^+ cells in RTN between 2 and 4 weeks post infection for both NT-shRNA (week 2: 286 ± 10.4 ; week 4: 294.9 ± 52.8 , p=0.783) and PHOX2B-shRNA rats (week 2: 218.8 ± 7.8 ; week 4: 249.5 ± 33.9 , p=0.118), suggesting that progressive PHOX2B knockdown was not accompanied by increased cell death.

The number of PHOX2B expressing Nmb^+ cells in the RTN was significantly reduced in PHOX2B-shRNA rats by 67.0% and 60.7% compared to naïve and NT-shRNA rats, respectively (naïve: 284.5 ± 19.8 ; NT-shRNA: 238.7 ± 56 ; PHOX2B-shRNA: 93.8 ± 11.9 cells, p<0.001; **Fig. 3D**). Moreover, a significant increase in the fraction of $Nmb^+/PHOX2B^-$ was observed with PHOX2B knockdown (PHOX2B-shRNA: 155.7 ± 22.7 ; naïve: 63.2 ± 12 cells; + 146.1% increase vs naïve; NT-shRNA: 56.2 ± 13.8 cells; + 177% increase vs NT-shRNA, p<0.001; **Fig. 3E**).

The respiratory function prior to histological examination (4 weeks post-viral injection) showed significant differences in PHOX2B-shRNA rats compared to both baseline, naïve and NT-shRNA rats. Similar to what we reported at 2 weeks post-surgery, f_R at 4 weeks was significantly lower in every treatment group compared to baseline recordings (Air: p=0.001; 5% CO₂: p< 0.001; 7.2% CO₂ p< 0.001; **Fig. 4A**).

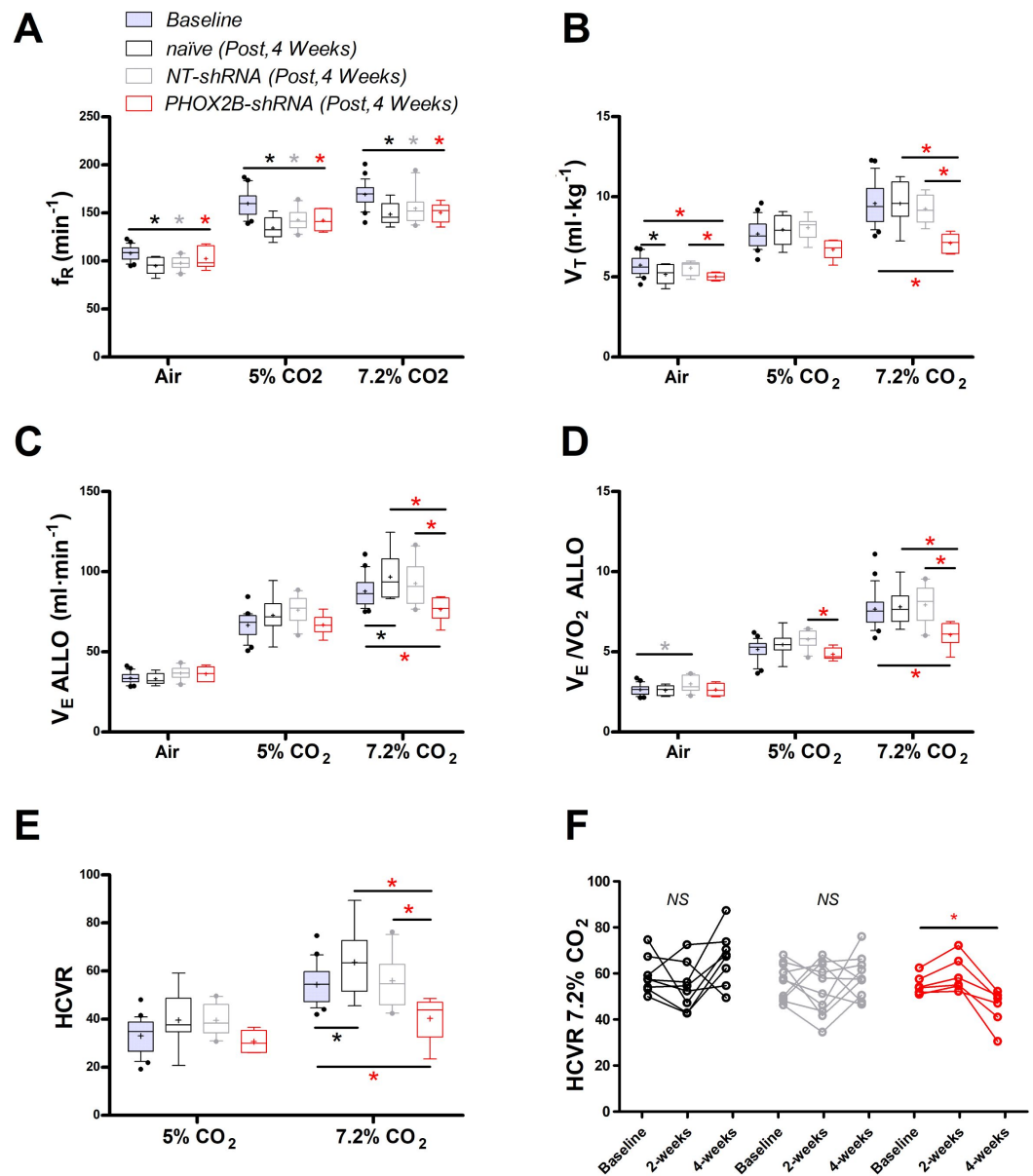


Figure 4.

Respiratory data following 4 weeks post viral shRNA injection.

(A) breathing frequency (f_R), (B) tidal volume (V_T), (C) allometric minute ventilation ($V_E \text{ ALLO}$), (D) convective requirement ratio ($V_E / \text{NO}_2 \text{ ALLO}$), (E) hypercapnic ventilatory response (HCVR, absolute change in $V_E \text{ ALLO}$ vs. corresponding room air), (F) HCVR at baseline, week 2 and week 4 post-viral injections in naïve (black n=8), non-target control shRNA (NT-shRNA, grey n=10) and PHOX2B-shRNA (PHOX2B-shRNA, red n=6). f_R is equally impaired in all experimental group compared to baseline but no treatment effect was observed (A). V_T is significantly impaired following RTN injection in PHOX2B-shRNA group compared to baseline pre-surgery ($p < 0.001$), naïve rats ($p < 0.001$), and NT-shRNA rats ($p = 0.002$) at 7.2% CO_2 . (B). $V_E \text{ ALLO}$ is impaired in PHOX2B-shRNA rats during exposure to hypercapnia (7.2% CO_2) compared to baseline ($p = 0.0025$), naïve rats ($p = 0.007$), and NT-shRNA rats ($p = 0.002$). (C). $V_E / \text{NO}_2 \text{ ALLO}$ is reduced in PHOX2B-shRNA animals compared to NT-shRNA rats both at 5% ($p = 0.023$) and 7.2% ($p = 0.004$) CO_2 (D). HCVR during 7.2% CO_2 is lower in PHOX2B-shRNA rats compared to baseline ($p = 0.007$), naïve rats ($p = 0.001$), and NT-shRNA rats ($p = 0.016$) (E). Boxplots: median, 1st – 3rd quartiles and 10th – 90th percentiles, outliers =

dots, “+” indicates arithmetic mean. Bonferroni post-hoc as indicated. HCVR is significantly impaired only in PHOX2B-shRNA rats 4-weeks post-surgery (One-way ANOVA $p=0.007$) (F). Black*, different from naïve; Grey*, different from NT-shRNA; Red*, different from PHOX2B-shRNA.

During room air, V_T was modestly reduced in naïve rats compared to baseline (baseline: 5.9 ± 0.5 ml·kg⁻¹; week 4: 5.2 ± 0.6 ml·kg⁻¹; -12%; $p=0.001$; **Fig. 4B**). Moreover, V_T in PHOX2B-shRNA rats was reduced compared to NT-shRNA (NT-shRNA: 5.6 ± 0.4 ml·kg⁻¹; PHOX2B-shRNA: 5.3 ± 0.4 ml·kg⁻¹; -10%; $p=0.043$) and to the baseline (6.1 ± 0.7 ml·kg⁻¹; -18%; $p=0.002$). No significant changes were observed at 5% CO₂ although a decrease in V_T occurred at 7.2% CO₂ in PHOX2B-shRNA rats compared to baseline pre-surgery (baseline: 7.7 ± 1.2 ml·kg⁻¹; PHOX2B-shRNA: 6.7 ± 0.6 ml·kg⁻¹; -31%; $p<0.001$), naïve rats (7.9 ± 0.9 ml·kg⁻¹; -26%; $p<0.001$), and NT-shRNA rats (8.1 ± 0.7 ml·kg⁻¹; -23%; $p=0.002$).

Decreased V_T observed for PHOX2B-shRNA led to a ventilation (V_E ALLO) impairment during exposure to hypercapnia (7.2% CO₂, PHOX2B-shRNA vs. NT-shRNA: -17%; $p=0.021$; PHOX2B-shRNA vs. naïve; -21%; $p=0.007$; PHOX2B-shRNA vs. baseline, -18% $p=0.002$; **Fig. 4C**). Since O₂-consumption (VO_2 ALLO) did not change (data not shown) a significant reduction in V_E/VO_2 ALLO confirmed alveolar hypoventilation in PHOX2B-shRNA animals compared to NT-shRNA rats both at 5% (NT-shRNA: 5.8 ± 0.5 ; PHOX2B-shRNA: 4.8 ± 0.4 ; -16%; $p=0.023$) and 7.2% CO₂ (NT-shRNA: 7.9 ± 1.2 ; PHOX2B-shRNA: 6.1 ± 0.8 ; -24%; $p=0.004$; **Fig. 4D**). Moreover, the HCVR (normalized to room air; **Fig. 4E**) at 7.2% CO₂ was lower in PHOX2B-shRNA (40.3 ± 9.4 ml·min⁻¹) rats compared to the pre-surgery baseline (58.1 ± 8.9 ml·min⁻¹; -31%; $p=0.015$), naïve rats (63.6 ± 14.3 ml·min⁻¹; -37%; $p=0.008$) and NT-shRNA rats (56.0 ± 10.4 ml·min⁻¹; -28%; $p=0.016$). Analysis of individual rats in each treatment group over time showed that the HCVR was only consistently impaired in PHOX2B-shRNA rats between weeks 2 and 4 (**Fig. 1F**; $p=0.007$).

Given that previous studies have proposed a role for the RTN in the hypoxic chemoreflex (Barna et al., 2016; Basting et al., 2015; Gourine et al., 2005; Wickström et al., 2004), and we observed impairment of the CO₂-chemoreflex at 4 weeks post-infection, we investigated whether PHOX2B knockdown would also affect respiratory response to hypoxia (10% O₂). The 4 week V_E ALLO was increased relative to baseline values in naïve (baseline: 62.3 ± 5.2 ml·min⁻¹; naïve: 71.0 ± 10.1 ml·min⁻¹; +14%), NT-shRNA (baseline: 71.8 ± 11.1 ml·min⁻¹; NT-shRNA: 75.8 ± 12.8 ml·min⁻¹; +6%) and PHOX2B-shRNA rats (baseline: 65.9 ± 9.8 ml·min⁻¹; PHOX2B-shRNA: 79.3 ± 6.4 ml·min⁻¹; +29%; $p<0.001$). However, analysis of the convective requirement ratio (V_E/VO_2 ALLO) indicates that changes in hypoxic ventilation were due to metabolic adjustments that occurred in all treatment groups (V_E/VO_2 ALLO naïve: 6.5 ± 1.3 ; NT-shRNA: 6.7 ± 1.7 ; PHOX2B-shRNA: 6.6 ± 0.6), suggesting that the hypoxic ventilatory response was not affected by PHOX2B knockdown.

PHOX2B knockdown does not extend to C1 catecholaminergic neurons and does not affect mRNA expression of *Nmb* in RTN neurons

Our data shows that PHOX2B knockdown specifically impairs breathing during hypercapnia. To exclude that the observed impairment was due to unintended knockdown of PHOX2B in adjacent areas of the brain, we quantified the total number of TH⁺/PHOX2B⁺ catecholaminergic C1 neurons located caudal to the RTN in PHOX2B-shRNA rats compared to naïve and NT-shRNA (**Fig. 5A**). No differences were observed in either the TH⁺ cell numbers or in the PHOX2B expression between the 3 treatment groups (naïve: 378.3 ± 23.81 ; NT-shRNA: 391.7 ± 33.08 ; PHOX2B-shRNA: 370.2 ± 33.04 cells).

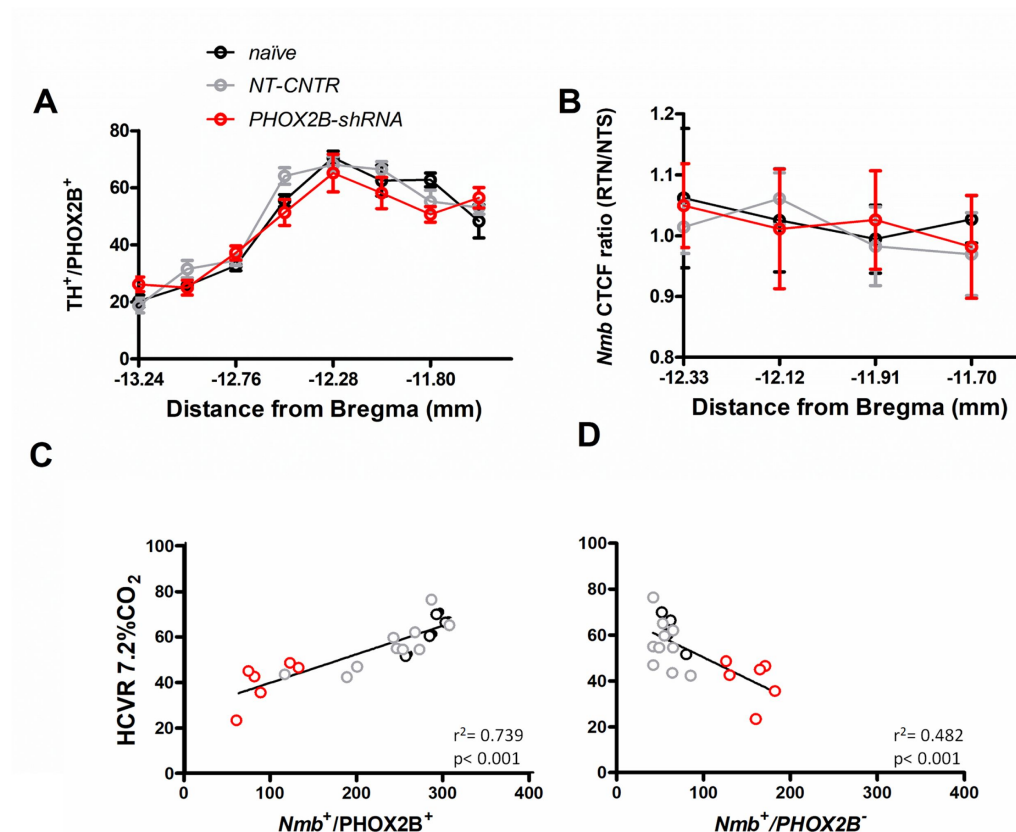


Figure 5.

PHOX2B knockdown in RTN neurons does not alter TH or *Nmb* expression but impairs the hypercapnic ventilatory response.

(A) No differences are observed in the rostral-caudal distribution of TH⁺/PHOX2B⁺ catecholaminergic C1 neurons cells caudal to the RTN between naïve (black, n=4), NT-shRNA (grey, n=10) and PHOX2B-shRNA (red, n=6) rats. (B) Quantification of *Nmb* mRNA fluorescence staining intensity along the rostro-caudal extension of RTN in naïve (black, n=4), NT-shRNA (grey, n=10) and PHOX2B-shRNA (red, n=6) rat calculated as ratio RTN/NTS shows no difference between treatment groups. (D-E) X-Y plot of HCVR during 7.2% CO₂ exposure relative to the number of Nmb⁺/PHOX2B⁺ (D, slope is different from “0” at $p < 0.001$; $r^2 = 0.739$) and Nmb⁺/PHOX2B⁻ (E, $p < 0.001$ for difference between slopes; $r^2 = 0.482$) in the RTN.

NMB is currently considered the most selective anatomical marker for chemosensitive RTN neurons (Shi et al., 2017) and was used in this study to identify RTN location, cell numbers and relative PHOX2B expression. Since changes in the level of the transcription factor PHOX2B could regulate the expression of its target genes, we investigated whether NMB is affected by shRNA knockdown and assessed *Nmb* expression in PHOX2B-shRNA rats compared to both naïve and NT-shRNA rats (Fig 5B). *Nmb* mRNA expression at the level of RTN was calculated as total fluorescence intensity (CTCF) ratio relative to *Nmb* expression at the level of NTS, which served as an internal control (Fig. 5B). We observed no changes in relative fluorescence of *Nmb* in RTN neurons across the 3 groups ($p = 0.608$), suggesting that NMB expression is not affected by PHOX2B knockdown.

To better analyse the association between PHOX2B knockdown in RTN neurons and the attenuation of the HCVR, we evaluated the correlation between the number of *Nmb*⁺/PHOX2B⁺ or *Nmb*⁺/PHOX2B⁻ cells in the RTN and the resulting HCVR using linear regression (**Fig. 5C,D**). Our results indicate that there is a significant correlation between HCVR and number of *Nmb*⁺/PHOX2B⁺ ($r^2 = 0.739$ $p < 0.001$; **Fig. 5C**), and *Nmb*⁺/PHOX2B⁻ ($r^2 = 0.482$ $p < 0.001$ **Fig. 5D**) cells suggesting that the number of PHOX2B expressing cells in the RTN is a good predictor of the chemoreflex response. Moreover, the nature of the correlation between PHOX2B⁻ cells and HCVR is the opposite of PHOX2B⁺, another important indicator that PHOX2B protein contributes to the CO₂ sensing and consequently, the reduction of PHOX2B protein impairs the CO₂-chemoreflex.

Gpr4 and Task2 mRNAs expression is affected by PHOX2B Knockdown

We next examined the mRNA expression of *Gpr4* and *Task2*, two important pH sensors for the central respiratory chemoreflex response in RTN neurons (Gestreau et al., 2010; Guyenet et al., 2016; Kumar et al., 2015). As previously reported, the two pH sensors are expressed in the NMB RTN neurons in partially overlapping populations of chemosensory neurons (Gestreau et al., 2010; Kumar et al., 2015). In naïve rats 91.6 ± 11.2 % of NMB cells were *Gpr4* positive, and 76.6 ± 14.8 % of NMB cells were also *Task2* positive (n=4 rats). These values were not significantly different in either NT-shRNA or PHOX2B shRNA rats (NT-shRNA: 90.0 ± 15.7% *Nmb*⁺/*Gpr4*⁺ and 76.2 ± 21.8% *Nmb*⁺/*Gpr4*⁺, n=10 rats; PHOX2B-shRNA: 89.4 ± 18.3% *Nmb*⁺/*Gpr4*⁺ and 75.6 ± 27.8% *Nmb*⁺/*Gpr4*⁺, n=6 rats).

To further investigate whether the expression of these two sensors was affected by the reduction in PHOX2B protein we quantified the total fluorescence intensity (CTCF) for their mRNA in *Nmb*⁺ cells. Total CFCT for *Gpr4* and *Task2* in PHOX2B-shRNA rats was significantly reduced compared to naïve and NT-shRNA rats (*Gpr4*: -34.4 ± 4.79% vs naïve, -27.9 ± 5.71% vs NT-shRNA $p < 0.001$; *Task2*: -39.0 ± 3.02% vs naïve, -34 ± 2.62% vs NT-shRNA $p < 0.001$; **Fig. 6B,D**). To better understand whether this reduction was ascribed to the specific loss of PHOX2B expression, we compared the mRNA expression levels between *Nmb*⁺/PHOX2B⁻ and *Nmb*⁺/PHOX2B⁺ neurons in PHOX2B-shRNA rats. Both *Gpr4* and *Task2* mRNA expression was reduced in *Nmb*⁺/PHOX2B⁻ cells compared to *Nmb*⁺/PHOX2B⁺ (*Gpr4*: -63.8 ± 3.9%; $p = 0.022$; *Task2*: -63.2 ± 2.77%; $p = 0.029$; **Fig. 6C,E**). Our data suggest that PHOX2B knockdown not only causes a reduction in the hypercapnic respiratory response but also a reduction in the expression of *Gpr4* and *Task2* mRNAs.

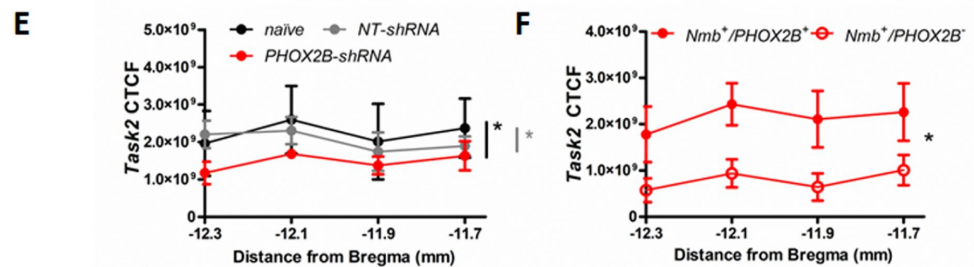
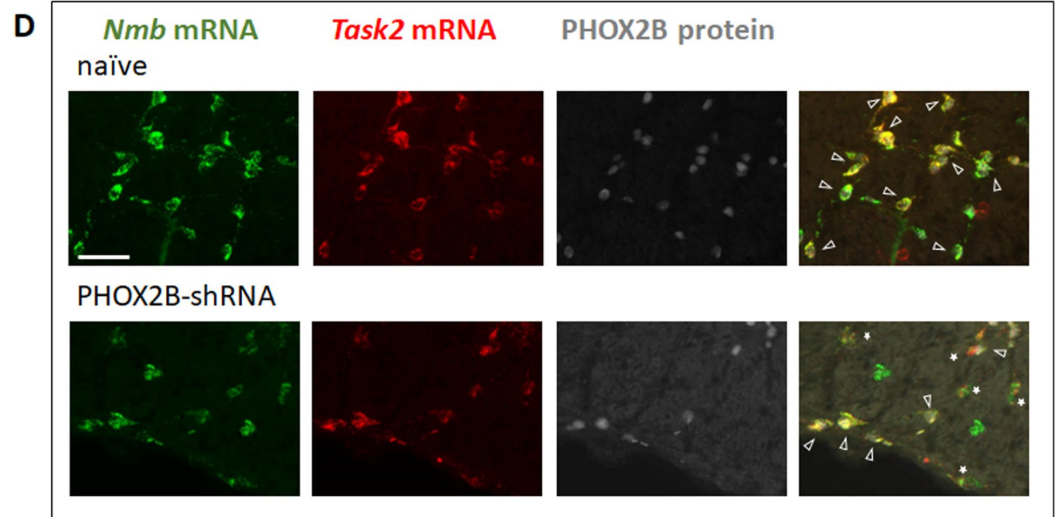
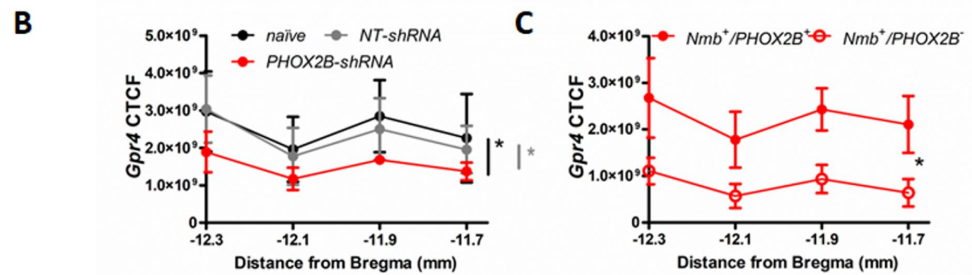
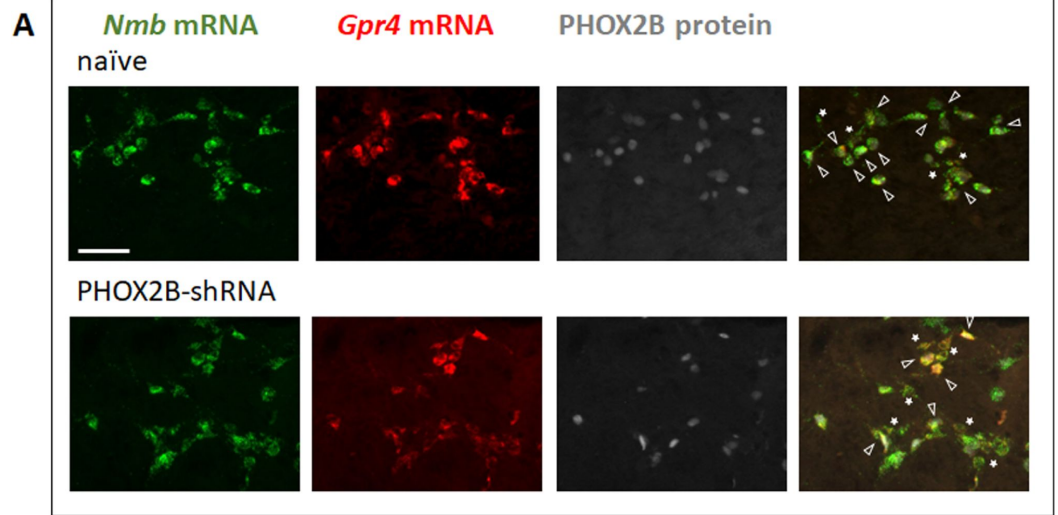


Figure 6.

Gpr4 and Task2 mRNA expression within the RTN area in naïve, and PHOX2B-shRNA injected rats 4 weeks post viral shRNA injection.

(A,D) *Nmb* (green), *Gpr4*, *Task2* mRNA (red) and PHOX2B protein (grey) expression in RTN neurons in naïve (top) and PHOX2B-shRNA (bottom) rats. Scale bar = 150 μ m. Arrowheads indicate colocalization of *Gpr4* (A) and *Task2* (D) with *Nmb*⁺/PHOX2B⁺ neurons. Asterisks indicate colocalization of *Gpr4* (A) and *Task2* (D) with *Nmb*⁺/PHOX2B⁻ neurons. (B,E) Quantification of *Gpr4* and *Task2* mRNA fluorescence staining intensity along the rostro-caudal extension of RTN in naïve (black), NT-shRNA (grey) and PHOX2B-shRNA (red) rats. Black*, different from naïve, Grey*, different from NT-shRNA (C and F) Quantification of *Gpr4* and *Task2* mRNA fluorescence staining intensity along the rostro-caudal extension of RTN in *Nmb*⁺/PHOX2B⁺ (red filled dot) and *Nmb*⁺/PHOX2B⁻ (red empty dot) neurons in PHOX2B-shRNA rats. Mean corrected total cell fluorescence (CTCF) value $\pm$ SEM combined (naïve, n=4; NT-shRNA, n=10; PHOX2B-shRNA, n=6) (see methods for detail). One-way ANOVA repeated measures.

Discussion

The role of the transcription factor PHOX2B in the adult brain is still not fully understood. In this study we reduced the expression of PHOX2B in the key chemosensory structure of the RTN with a selective viral shRNA approach to test whether a knockdown of PHOX2B expression in these neurons negatively impacts ventilation. Two weeks after viral infection we observed a modest reduction of PHOX2B/NMB expressing neurons in the RTN but no significant changes in basal V_E or in the CO₂ chemoreflex. Four weeks after shRNA infection we observed a further reduction in the expression of PHOX2B in RTN neurons and a significant reduction of the CO₂ chemoreflex, while ventilation in normoxia or hypoxia was unaffected. Furthermore, the level of expression of *Gpr4* and *Task2* mRNA, the two proton sensors responsible for the RTN neurons chemosensory properties were significantly reduced in RTN neurons that lacked PHOX2B protein expression, whereas *Nmb* mRNA expression level was unaffected by PHOX2B knockdown. These results suggest that PHOX2B reduction affects RTN neurons transcriptional activity and decreases the CO₂ response, possibly by reducing the expression of key proton sensors in the RTN.

The role of PHOX2B in development and in the adult brain

PHOX2B is a transcription factor with an important role in the development and differentiation of neuronal structures in both the central and peripheral nervous system. Genetic loss of PHOX2B expression in mice shows that PHOX2B is essential for the correct development of all autonomic ganglia in sympathetic, parasympathetic, and enteric nervous system and the distal ganglia of the facial (VII), glossopharyngeal (IX), and vagus (X) cranial nerves (Pattyn et al., 1999), in addition to carotid bodies and the solitary tract nucleus (Dauger, 2003). Furthermore, PHOX2B is involved in the formation of all branchial and visceral hindbrain motor neurons and its absence during embryonic period impairs the development of motoneurons of the facial, trigeminal, ambiguus and dorsal motor nucleus of the vagal nerve. PHOX2B is also necessary for the specification and differentiation of the noradrenergic phenotype in multiple centres in the brain (Brunet & Pattyn, 2002; Pattyn et al., 1999, 2000a).

Interestingly, the expression of PHOX2B persists into adulthood in selected neuronal populations, such as the ones involved in peripheral and central chemoreception, i.e., the carotid bodies, the solitary tract nucleus and the RTN, in addition to neurons in the dorsal motor nucleus of the vagus, the area postrema, the nucleus ambiguus, C1 catecholaminergic neurons and other scattered neurons throughout the brainstem (Stornetta et al., 2006). Such widespread expression within the adult brain suggests an important transcriptional role in neuronal survival and/or function.

With the exception of its role in maintaining the noradrenergic phenotype, its function in the postnatal brain is unknown, in part because a full investigation of its transcriptional activity in vivo has not yet been performed.

Heterozygote polyalanine expansion mutations in the exon 3 of *PHOX2B* are responsible for the majority of cases of CCHS, a rare genetic disease that affect the autonomic nervous system and central CO₂ chemoreflexes (Amiel et al., 2003 [↗](#); Di Lascio et al., 2021 [↗](#); Weese-Mayer et al., 2017 [↗](#)). While studies in transgenic mice expressing the mutated PHOX2B protein suggest that the RTN may not form or be severely impaired (Dubreuil et al., 2008 [↗](#), 2009 [↗](#); Ramanantsoa et al., 2011 [↗](#)), the function of the wild-type protein in these neurons has never been tested in adulthood. Thus, in order to determine whether PHOX2B is necessary for the survival and/or chemosensory function of adult PHOX2B⁺ RTN neurons in vivo, we used a shRNA viral approach to progressively knockdown the PHOX2B protein in these neurons.

Modest reduction of PHOX2B in RTN does not impair ventilation

We identified RTN neurons by the mRNA expression of the neuropeptide, NMB (Shi et al., 2017 [↗](#)) and observed a reduced number in both total *Nmb*⁺ and *Nmb*⁺/PHOX2B⁺ neurons in shRNA rats compared to naïve and NT-shRNA rats two weeks after viral infection. Interestingly, despite the small reduction in total *Nmb*⁺ RTN neurons in both NT-shRNA and PHOX2B-shRNA injected rats, possibly due to some off-target effects due to activation of innate immunity or saturation of the microRNA pathway (McBride et al., 2008 [↗](#); Van Gestel et al., 2014 [↗](#)), no significant impairment in ventilation was observed. Nonetheless, the fraction of *Nmb*⁺ cells expressing PHOX2B was reduced indicating that shRNA knockdown occurred in a fraction of RTN neurons. Even though PHOX2B may be critical for chemosensory function in RTN neurons, the lack of significant ventilatory impairment with a modest reduction of PHOX2B expressing neurons is not surprising. In our previous study (and the ones of others), in which RTN chemosensory neurons were lesioned with Substance P saporin toxin, CO₂-chemoreflex impairment became significant (~30% V_E Allo reduction vs. pre-surgical baseline) only when the sum of *Nmb*⁺/PHOX2B⁺ and *Nmb*⁺/PHOX2B⁻ neurons was reduced by ~63% compared to naïve controls (medium lesion range: 50%-81% loss of *Nmb*⁺ neurons; Janes et al., 2024 [↗](#); Souza et al., 2023 [↗](#)), therefore, even though loss of PHOX2B severely altered the chemosensitive function of NMB cells, we would not expect significant changes in ventilation with a <50% RTN cell loss.

Four weeks of PHOX2B knockdown impairs central CO₂ chemoreception

Four weeks post- shRNA viral injection, the fraction of *Nmb*⁺ cells expressing PHOX2B was further reduced by 67% compared to naïve and by 61% compared to NT-shRNA rats. PHOX2B knockdown was also restricted to RTN neurons, as more caudal C1 TH⁺ neurons and the adjacent FN motoneurons did not show any change in number or PHOX2B expression. Although we did not specifically quantify the relative mRNA expression change in intracellular *Phox2b* levels within infected neurons, we measured the success of PHOX2B knockdown by assessing the overall change in the number of *Nmb*⁺ cells that had no detectable levels of PHOX2B protein in the nuclei. Thus, it is possible that this method led us to underestimate the success of the overall PHOX2B shRNA knockdown in the RTN.

In PHOX2B-shRNA rats we did not observe any respiratory function changes in room air or in hypoxia, similar to RTN lesioning studies (Janes et al., 2024 [↗](#); Souza et al., 2023 [↗](#)). When tested in hypercapnia though, PHOX2B-shRNA rats displayed a reduced HCVR compared to both baseline (i.e., pre-surgery), naïve and NT-shRNA rats. The impairment of V_E Allo was primarily a result of blunted V_T, and analysis of the convective exchange ratio (i.e. V_E/VO₂ ALLO) confirmed hypoventilation in 5% and 7.2% CO₂ only for PHOX2B-shRNA rats, suggesting a specific impairment of chemosensitive properties in RTN neurons with PHOX2B knockdown. These results

are in line with previous studies in which medium RTN lesions (i.e. loss of >60 % *Nmb*⁺ cells) elicited by Saporin toxin injection resulted in a significant CO₂-chemoreflex impairment (Janes et al., 2024 [DOI](#); Souza et al., 2018 [DOI](#)).

Changes in the CO₂ response following 4 weeks of PHOX2B-shRNA treatment could occur because the reduced expression of the transcription factor PHOX2B causes changes in the transcriptional machinery of RTN neurons and alters transcription of CO₂/pH sensing proteins or other key elements for their chemosensitive function. These transcriptional changes could also affect neuronal survival. In fact, a small but significant loss of NMB expressing RTN neurons may contribute to the reduction in the CO₂ response, although multiple studies have demonstrated that significant cell loss must occur in the RTN in order to show a respiratory function impairment (Janes et al., 2024 [DOI](#); Souza et al., 2018 [DOI](#); Nattie & Li, 2002 [DOI](#); Takakura et al., 2008 [DOI](#), 2014 [DOI](#)).

In our experiments, we observed a 35% reduction in total *Nmb*⁺ cells compared to naïve rats and 23% compared to NT-shRNA, possibly due to cell death within the first 14 days post-infection. Interestingly the loss of *Nmb*⁺ cells did not increase with time (although the number of PHOX2B⁺ neurons decreased) and the central chemoreflex at 2 weeks post-surgery was not impaired, suggesting that: i) the observed reduction in overall RTN cell number is not responsible for the CO₂ chemoreflex impairment; ii) PHOX2B expression is most likely not necessary for neuronal survival of RTN neurons; iii) a large reduction in RTN PHOX2B⁺ neurons is necessary to impair the HCVR. The interpretation that PHOX2B expression contributes positively to the chemoreflex is further supported by our linear regression analysis showing that *Nmb*⁺/PHOX2B⁺ cell number was the best predictor of the HCVR ($r^2 = 0.739$). The initial cell loss, which we observed also in NT-shRNA, and more prominently with larger injection volume of virus, may be attributed to some inherent cell death associated with the surgical procedure or, more likely, with the potential off-target toxic effects associated with shRNA procedures (Mcbride et al., 2008 [DOI](#); Van Gestel et al., 2014 [DOI](#)).

PHOX2B knockdown reduces expression of proton sensors in the RTN

Current theories on central respiratory chemosensitivity postulate that changes in CO₂/pH in the RTN are detected through two pH sensors, the proton-activated receptor GPR4 and the pH-sensitive K⁺ channel TASK2 that are expressed in partially overlapping populations of NMB expressing RTN neurons (Kumar et al., 2015 [DOI](#)). Because it is not known whether PHOX2B has any effect on the expression of TASK2, GPR4 or even the expression of the neuropeptide NMB used to anatomically identify RTN neurons, we determined their mRNA levels within the RTN (calculated as CFTC) and observed no changes in relative fluorescence of *Nmb*⁺ mRNA in RTN neurons relative to *Nmb* expression in unrelated cells in the NTS, suggesting that NMB is not affected by PHOX2B shRNA knockdown and that *Nmb* is most likely not a target gene of PHOX2B in adult RTN neurons. Furthermore, we determined the mRNA expression levels of *Task2* and *Gpr4* in both *Nmb*⁺/PHOX2B⁺ and *Nmb*⁺/PHOX2B⁻ neurons in shRNA rats. Our results indicate that the total CTCF for *Gpr4* and *Task2* in PHOX2B-shRNA rats was reduced compared to naïve and NT-shRNA group. Furthermore, there was a significant reduction of both *Gpr4* and *Task2* in *Nmb*⁺/PHOX2B⁻ cells compared to *Nmb*⁺/PHOX2B⁺ cells. Because no good antibodies are currently available to detect protein levels of either NMB, GPR4 or TASK2, our results are only based on changes in mRNA levels, thus we can only speculate that a reduction in *Gpr4* and *Task2* mRNA would translate in a reduction in the protein levels and consequent reduction of RTN neurons chemosensitive properties. Direct electrophysiological recordings in RTN neurons will be able to address the changes in CO₂/pH sensitivity of RTN neurons at cellular level.

PHOX2B knockdown may impair CO₂-sensing through additional transcriptional targets

Since loss of PHOX2B reduces expression of TH and dopamine beta hydroxylase enzymes in catecholaminergic neurons *in vivo* and *in vitro* (Fan et al., 2011 [\[link\]](#)), it is possible that in NMB⁺ RTN neurons, reduction of PHOX2B alters the transcriptional machinery of other proteins (in addition to GPR4 and TASK2) and impairs their function in central chemoreception. For example, a change in the expression of enzymes, neurotransmitters, receptors, and ion channels in RTN neurons could affect the excitatory signal transmission to preBötzinger Complex and the respiratory network specifically during CO₂ challenges (Guyenet et al., 2016 [\[link\]](#)). Further studies will be needed to address and identify additional transcriptional changes induced by PHOX2B knockdown *in vivo*.

An interesting observation from our histological data was the reduction in the overall number of Nmb⁺ cells at two- and four-weeks following shRNA viral injections. Although some cell death may be associated with either surgical procedures or off-target effects associated with the shRNA approach (Mcbride et al., 2008 [\[link\]](#); Van Gestel et al., 2014 [\[link\]](#)), it is intriguing to speculate that loss of PHOX2B expression may have some effects on viability of RTN neurons *in vivo*. Even though this is a possibility, especially with an even more severe knockdown, we did observe a large proportion of Nmb⁺ cells devoid of PHOX2B protein expression, suggesting that absence of PHOX2B is compatible with neuronal survival, at least in our experimental time frame.

Relevance to CCHS and its pathogenic mechanisms

Our results contribute to further our understanding on potential pathogenic mechanisms in CCHS (Di Lascio et al., 2018 [\[link\]](#)). Homozygous knockout mice for PHOX2B die during gestation (Pattyn et al., 1999 [\[link\]](#), 2000 [\[link\]](#)) demonstrating a key role of this transcription factor in cellular proliferation, migration and differentiation during embryonic development, whereas heterozygotes for PHOX2B survive birth and live into adulthood, but display apneas (Durand et al., 2005 [\[link\]](#)) and impairment of the hypoxic and hypercapnic ventilatory responses in the neonatal period (Dauger, 2003 [\[link\]](#)). Here we show that, independent of the PHOX2B role during development, PHOX2B is still required to maintain proper CO₂ chemoreflex responses in the adult brain.

Heterologous expression of the mutant PHOX2B alters development of RTN in mice and impairs their chemoreflex response (Dubreuil et al., 2008 [\[link\]](#), 2009 [\[link\]](#)). The effects of the expression of the mutant PHOX2B on wild-type PHOX2B and its transcriptional activity is not known *in vivo* yet, although *in vitro* data indicates that the mutated PHOX2B protein alters wild-type PHOX2B conformation and its ability to form homo- and hetero-dimers (Di Lascio et al., 2016 [\[link\]](#); Trochet et al., 2005 [\[link\]](#), 2008 [\[link\]](#)), its affinity for DNA and coactivators, as well as its degradation rate (Nagashimada et al., 2012 [\[link\]](#); Wu et al., 2009 [\[link\]](#)). It has also been shown that mutant PHOX2B causes the formation of cytoplasmic aggregates (Bachetti et al., 2005 [\[link\]](#); Trochet et al., 2005 [\[link\]](#), 2008 [\[link\]](#)) and fibrils *in vitro* (Pirone et al., 2019 [\[link\]](#)), and dysregulates the transcriptional function of wild-type PHOX2B (Di Lascio et al., 2013 [\[link\]](#); Parodi et al., 2012 [\[link\]](#)), thus changing expression of important target genes such as DBH, PHOX2A and TLX2 (Bachetti et al., 2005 [\[link\]](#); Di Lascio et al., 2013 [\[link\]](#); Trochet et al., 2005 [\[link\]](#)). Based on this evidence, different CCHS pathogenic mechanisms have been proposed (e.g., PHOX2B loss-of- function, dominant-negative or toxic function of the mutant protein) including gene and cell specific transcriptional dysregulation (Di Lascio et al., 2018 [\[link\]](#), 2021). Interestingly, only a handful of PHOX2B target genes have been identified and the function of PHOX2B and its mutated forms in respiratory control and CCHS pathogenesis is still under investigation.

Our data suggest that, in addition to potential effect of mutated PHOX2B on development and cellular function in CCHS pathogenesis, the expression of wild type PHOX2B has an important role in respiratory control that extends the developmental period and its reduction in CCHS may contribute to the respiratory impairment in this disorder.

Materials and methods

Experiments were performed using male Sprague–Dawley rats (starting weight range 250–350g) born in the University of Alberta animal facility to pregnant dams obtained from Charles River (Senneville, QC). Rats were housed at the University of Alberta Health Sciences Animal Housing Facility and maintained on a 12-hr dark/light cycle with food and water available *ad libitum*. Handling and experimental procedures were approved by the Health Science Animal Policy and Welfare Committee at the University of Alberta (AUP#461) and performed in accordance with guidelines established by the Canadian Council on Animal Care.

ShRNA viral injection in the retrotrapezoid nucleus

In order to knockdown Phox2b expression at the level of RTN neurons, we used a mix of two shRNA clones targeting two different sequences of the Phox2b mRNA carried by a non-replicating lentivirus vector (1×10^9 VP/ml; TRCN000041283: GCCTTAGTGAAGAGCAGTATG and TRCN0000096437: CCTCTGCCTACGAGTCCTGTA; Sigma Aldrich, St. Louis, MA, USA). The shRNAs were designed against the Phox2b mouse sequence (Ref Seq NM_008888) which shares 100% homology with the Phox2b rat sequence (Ref Seq XM_008770167).

Metacam analgesic was administered 1 hour prior to surgery (2mg/kg) to reduce post-surgical pain. Rats were anesthetized with a ketamine/xylazine mixture (100mg/kg + 10mg/kg, respectively; i.p.); the anaesthetic level was assessed by lack of paw pinch reflex and maintenance of a regular breathing rate. Additional anaesthesia was administered as needed. The rat was positioned on a stereotactic apparatus (Kopf Instruments, Tujunga, CA, USA) and a total of four microinjections (200 or 100 nL per injection; two rostro-caudally aligned injections per side) were made lateral to the midline and ventral to the facial motor nucleus under aseptic conditions. Coordinates were as follows in mm from obex (medio-lateral/rostral-caudal/dorso-ventral): ± 1.8 , $+2.0$, -3.5 ; ± 1.8 , $+2.4$, -3.6 . Injections were made using a glass microelectrode (30 μ m diameter tip, Drummond Scientific, PA, USA). Twenty-five rats (8 rats 200nl/injection; 17 rats 100nl/injection) underwent shRNA viral injection (PHOX2B-shRNA) and 23 rats (6 rats 200nl/injection; 17 rats 100nl/injection) received a non-target shRNA viral injection (NT-shRNA; SHC016: MISSION[®] pLKO.1-puro non-Target shRNA Control Plasmid DNA; Sigma Aldrich, St. Louis, MA, USA) to determine any effects of surgery alone on respiratory behaviour. Eight naïve rats, receiving no surgery and maintained in the same housing conditions, were used as additional controls for both respiratory function and anatomical experiments. Following each injection, the glass electrodes were left in place for 3–5 minutes to minimize backflow of virus up the electrode track. At the end of the surgery, the incision was sutured, and rats received local anaesthetic bupivacaine (0.1 mL, s.c.) and were treated with metacam analgesic for 72 hours. Rats recovered for 7 days before the next respiratory function testing.

Data acquisition and analysis of respiratory measurements

Rats were habituated to whole-body plethysmography chambers (Buxco, 5L) once, 3–4 days before baseline recordings. On the day of the experiment, rats were placed in the chamber and testing commenced once the animals were quietly awake (typically 20–30 mins). Gas mixtures were delivered at 1.5 L/min using a GSM-3 (CWE Inc., Ardmore, PA, USA) and monitored using a Gas Analyzer (AD Instruments, Sydney, Australia). Ventilatory parameters were measured during exposure to different air compositions for 8–15 min each. Room air: 21%O₂ + 0%CO₂ (balanced with N₂); Normoxic Hypercapnia: 21%O₂ + 5%CO₂; 21%O₂ + 7.2%CO₂; Normocapnic Hypoxia: 10.6%O₂ + 0% CO₂.

As previously described (Cardani et al., 2022 [\[1\]](#); Janes et al., 2024 [\[2\]](#)) respiratory data were analysed from rats during quiet wakefulness in the last 5 min period of each gas composition using the barometric method (open-flow system) (Cardani et al., 2022 [\[1\]](#); Janes et al., 2024 [\[2\]](#);

Mortola & Frappell, 1998 [↗](#); Seifert et al., 2000 [↗](#)). Raw pressure signals were acquired with a Validyne differential pressure transducer connected to a CD15 carrier demodulator (Validyne Engineering, Northridge, CA, USA) and digitized using a Powerlab 8/35 (AD Instruments, Sydney, Australia). Analysis was done using Labchart 8 (v8.1.19, AD Instruments, Colorado Springs, CO, USA) to determine tidal volume (V_T) and breathing frequency (f_R). The amplitude of the pressure signal was converted to V_T ($\text{ml}\cdot\text{kg}^{-1}$) using the equations of Drorbaug and Fenn 1955 (Drorbaug & Fenn, 1955 [↗](#)) and calibrated against 1 mL of dry air injected into the empty chamber using a rodent ventilator (Harvard Rodent Ventilator Model 683, Holliston, MA, USA $f = 50, 75, 100, 150$ beats $\cdot\text{min}^{-1}$). Humidity and chamber temperature were monitored using a RH-300 water vapour analyzer (Sable Systems, Las Vegas, NV, USA) and a HPR Plus Handheld PIT Tag reader (Biomark, Boise, ID, USA) respectively, depending on the setup. Rectal temperature was read through the temperature probes of a Homeothermic Monitor (507222F, Harvard Apparatus, Holliston, MA, USA). Minute ventilation ($\dot{V}_E$) was calculated as $f_R \times V_T$ and expressed as $\text{ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$. Rats gained, on average, $160 \pm 4\text{g}$ over the experimental paradigm (naive: $158 \pm 19\text{g}$; NT-shRNA: $202 \pm 25\text{g}$; PHOX2B-shRNA: $133 \pm 7\text{g}$); we therefore applied an allometric correction to $\dot{V}_E$ to account for non-linear effects of weight gain (V_E ALLO) (Mortola et al., 1994 [↗](#)).

Metabolic parameters VO_2 and V_E/VO_2 were calculated by pull-mode indirect calorimetry and allometrically scaled to be consistent with ventilation (Mortola et al., 1994 [↗](#)). The % composition of dry gas flowing into, and out of the recording chamber was measured by using an AD Instruments Gas Analyzer (Colorado Springs, CO) and applying the equations of Depocas and Hart (Depocas F, Hart JS, 1957) and Lighton (Lighton, 2021 [↗](#)).

Weight measurements were taken prior to any recording. Rats breathing function was measured the week prior to surgery (baseline recording) and weekly following shRNA injections to determine the time course of ventilatory impairment.

Respiratory data were compared for naive, NT-shRNA and PHOX2B-shRNA pre-and post-injection using a mixed factor ANOVA (independent factor = treatment, repeated measures factor = pre- and post-lesion; **Fig.1** [↗](#), [↗](#)). Post-hoc analysis used the Bonferroni correction factor, with $p < 0.05$ considered to be significant. Ventilatory data are reported as mean $\pm$ standard deviation (SD).

In Situ hybridization (RNAScope) and immunofluorescence

Either 2 or 4-weeks after viral injection, rats were transcardially perfused with saline 0.9% NaCl followed by 4% paraformaldehyde (PFA) and the brains were post-fixed in 4% PFA overnight and cryoprotected in 30% sucrose in 1X Phosphate Buffered Saline (PBS). Brains were then frozen in O.C.T compound (Fisher Scientific) and sectioned on a cryostat (MODEL CM1950, Leica Biosystems, Buffalo grove, IL, USA) at $30\ \mu\text{m}$ and stored in cryoprotectant buffer at -20°C until processing. Sections were mounted on slides for combined RNAScope® in situ hybridization (Advanced Cell Diagnostics-ACD Bio, Newark CA, USA) and immunofluorescence assay and processed as previously detailed (Biancardi et al., 2021 [↗](#); Cardani et al., 2022 [↗](#)). The mRNA expression for *Neuromedin B* (*Nmb*) was used as marker of RTN CO_2 -sensing neurons (Shi et al., 2017 [↗](#)). Slides were incubated with probes for *Nmb* (Rn-NMB-C2 #494791-C2, ACDBio, Newark, CA, USA), *Phox2b* (Rn-Phox2b-O1-C1 #1064121-C1, ACDBio), G-protein-coupled receptor 4 (*Gpr4*) (Mm-Gpr4-C1, #427941, ACDBio), and potassium channel, subfamily K, member 5 (*Kcnk5* or *Task-2*) (Mm-Kcnk5-C3, #427951-C3, ACDBio) for 2 hr at 40°C . *Gpr4* and *Kcnk5* probes are design against the mouse mRNA sequence (Ref Seq NM_175668.4 and NM_021542.4, respectively). However, the alignment of the mouse mRNA sequences with those of rat (*GPR4*: Ref Seq NM_001025680.1; *TASK2*: Ref Seq NM_1039516.2) showed an identity of 94% and 96%, respectively, at the level of the target region (Base Pairs 1030-150) (BLAST® program, National Library of Medicine, National Center for Biotechnology Information, NIH). In parallel, two sections/rat were treated with positive (low copy housekeeping gene), and negative (non-specific bacterial gene) control probes provided by ACDBio. Finally, slides were processed using the RNAScope Multiplex Fluorescent Assay kit V2 (ACDBio) according to the manufacturer's instructions. The probes were visualized using Opal 520

and Opal 570 reagent (1:500 and 1:1000, respectively; PerkinElmer, Woodbridge, ON, CA). PHOX2B immunoreactivity was detected using mouse monoclonal PHOX2B antibody (B- 11: sc-376997, 1:100, Santa Cruz Biotechnology) incubated overnight in 0.3% TritonX-100, 1%NDS in PBS followed by donkey CY5-conjugated anti-mouse IgG (1:200; Jackson Immuno Research Laboratories Inc) in PBS + 1% NDS for 2 hours. Slides were then washed in PBS and cover-slipped with Fluorosave mounting media (EMD Millipore).

We also performed staining for tyrosine hydroxylase (TH) to identify and quantify C1 cells (TH⁺/PHOX2B⁺) following shRNA injection. Briefly, perfusion and tissue fixation/freezing were done as described above. Floating sections were stained with rabbit anti TH antibody (AB152,1:1000, Millipore SIGMA, USA), and mouse anti PHOX2B (B-11: sc-376997, 1:100, Santa Cruz Biotechnology). Antibodies were visualized using donkey CY3 (TH) and CY5 (PHOX2B) conjugated IgG (1:200; Jackson Immuno Research Laboratories Inc). The sections were washed in PBS and mounted on slides and cover-slipped with Fluorosave mounting media.

Cell counting, imaging and data analysis

To quantify the number of neurons *Nmb*⁺/PHOX2B⁻ and *Nmb*⁺/PHOX2B⁺ within the RTN region, we analysed one every 7 sections (210 µm interval; 8 sections in total; from -12.5 to -11.1 mm from Bregma, according to the Paxinos and Watson atlas coordinates (Paxinos & Watson, 2023) and compared total bilateral cell counts of PHOX2B-shRNA rats with non-target control (NT-shRNA) and naïve rats. Cells that express *Nmb* and *Phox2b* mRNA but do not show co-localization with PHOX2B protein were considered *Nmb*⁺/PHOX2B⁻.

The Correct Total Cell Fluorescence (CTCF) signal for *Nmb*, *Gpr4* and *Task2* mRNA was quantified as previously described (Cardani et al., 2022 [\[link\]](#); McCloy et al., 2014 [\[link\]](#)). A Leica TCS SP5 (B-120G) Laser Scanning Confocal microscope was used to acquire images of the tissue. Exposure time and acquisition parameters were set for the naïve group and kept unchanged for the entire dataset acquisition. For each image, three background areas were used to normalize against autofluorescence. We used 4 sections/rat to count *Nmb*, *Gpr4* and *Task2* mRNA CTCF in the RTN area (-12.3 to -11.7 mm from Bregma). For each section two images were acquired with a 20× objective, so that at least fifty cells per tissue sample were available for analysis. To evaluate changes in *Nmb* mRNA expression following PHOX2B knockdown at the level of the RTN, we compared, on the same tissue section, the fluorescence intensity of RTN *Nmb*⁺ cells with the signal of *Nmb*⁺ cells in the NTS (*Nmb* CTCF ratio RTN/NTS) as the latter would not be affected by the shRNA infection and knockdown.

To quantify *Gpr4* and *Task2* mRNA expression in the RTN, we first quantified CTCF for either *Gpr4* or *Task2* mRNA in *Nmb*⁺ RTN neurons in the 3 experimental groups. We then compared CTCF of *Gpr4* and *Task2* mRNA between *Nmb*⁺/PHOX2B⁺ and *Nmb*⁺/PHOX2B⁻ RTN neurons in PHOX2B-shRNA rats to address changes in mRNA expression induced by PHOX2B knockdown.

To assess whether shRNA knockdown affected either the number or the expression of PHOX2B in C1 neurons, we counted the number of TH⁺/PHOX2B⁺ cells along the ventrolateral medulla (-13.2 to -11.5 mm from bregma; 8 sections/rat; 240 µm interval) using an EVOS fluorescent microscope (ThermoFisher Scientific).

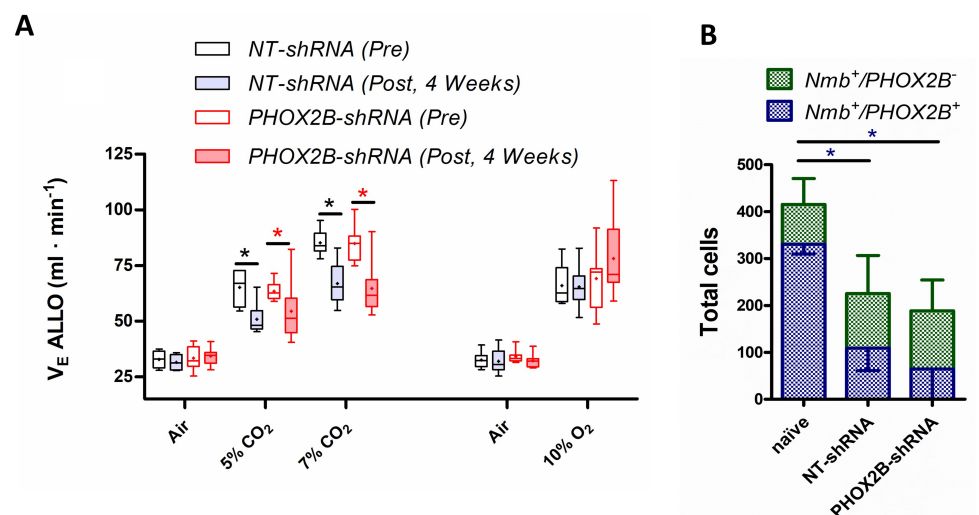
Digital colour photomicrographs were acquired using a Leica TCS SP5 (B-120G) Laser Scanning Confocal microscope. Image J (version 1.54f; National Institutes of Health, Bethesda, MD, USA) and Excel (Microsoft Office 365 for Windows) were used for cell counting and the measurements of fluorescence intensity. Statistical analysis of cell counts, and fluorescent signals was made using one-way ANOVA (Fig. 2C [\[link\]](#), 3C [\[link\]](#), S1B) and repeated-measures ANOVA (Fig 5A, B [\[link\]](#) and 6) with GraphPad Prism 8 Software (GraphPad Software, Inc., San Diego, CA, USA). Linear regressions to determine the effect of cell counts (*Nmb*⁺/PHOX2B⁺, *Nmb*⁺/PHOX2B⁻, total cells) on chemoreflex

magnitude were run using a combined dataset for naive, NT-shRNA and PHOX2B-shRNA rats (SPSS version 13.0; **Fig.5C-D**). p values of < 0.05 were considered significant and data are reported as mean $\pm$ standard deviation (SD).

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Supplementary Fig. 1.

(A) Allometric V_E is equally impaired following RTN injection of non-target control (NT-shRNA $n=6$) and PHOX2B-shRNA ($n=8$). Asterisks, difference from baseline. **(B)** The number of Nmb⁺/PHOX2B⁺ cells comprising the RTN are reduced in NT-shRNA ($n=5$), and shRNA rats ($n=5$) as compared to naive controls ($n=4$; Asterisks, difference from baseline). The number of Nmb⁺/PHOX2B⁻ cells was unchanged in both surgical treatments compared to baseline.

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Article and author information

Silvia Cardani

Department of Physiology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton AB T6G2E1 Canada, Women and Children's Health Research Institute, University of Alberta, Edmonton AB T6G2E1 Canada

Tara A. Janes

Department of Physiology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton AB T6G2E1 Canada, Women and Children's Health Research Institute, University of Alberta, Edmonton AB T6G2E1 Canada

William Betzner

Department of Physiology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton AB T6G2E1 Canada

Silvia Pagliardini

Department of Physiology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton AB T6G2E1 Canada, Women and Children's Health Research Institute, University of Alberta, Edmonton AB T6G2E1 Canada, Neuroscience and Mental Health Institute, University of Alberta, Edmonton AB T6G2E1 Canada

For correspondence: silviap@ualberta.ca

ORCID iD: [0000-0002-1482-9173](https://orcid.org/0000-0002-1482-9173)

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Reviewer #1 (Public Review):**Summary:**

This important study investigated the role of the PHOX2B transcription factor in neurons in the key brainstem chemosensory structure, the retrotrapezoid nucleus (RTN), for maintaining proper CO₂ chemoreflex responses of breathing in the adult rat in vivo. PHOX2B has an important transcriptional role in neuronal survival and/or function, and mutations of PHOX2B severely impair the development and function of the autonomic nervous system and RTN, resulting in the developmental genetic disease congenital central hypoventilation syndrome (CCHS) in neonates, where the RTN may not form and is functionally impaired. The function of the wild-type PHOX2B protein in adult RTN neurons that continue to express PHOX2B is not fully understood. By utilizing a viral PHOX2B-shRNA approach for knockdown of PHOX2B specifically in RTN neurons, the authors' solid results show impaired ventilatory responses to elevated inspired CO₂, measured by whole-body plethysmography in freely behaving adult rats, that develop progressively over a four-week period in vivo, indicating effects on RTN neuron transcriptional activity and associated blunting of the CO₂ ventilatory response. The RTN neuronal mRNA expression data presented suggests the impaired hypercapnic ventilatory response is possibly due to the decreased expression of key proton sensors in the RTN. This study will be of interest to neuroscientists studying respiratory neurobiology as well as the neurodevelopmental control of motor behavior.

Strengths:

1. The authors used a shRNA viral approach to progressively knock down the PHOX2B

protein, specifically in RTN neurons to determine whether PHOX2B is necessary for the survival and/or chemosensory function of adult RTN neurons *in vivo*.

2. To determine the extent of PHOX2B knockdown in RTN neurons, the authors combined RNAScope® and immunohistochemistry assays to quantify the subpopulation of RTN neurons expressing PHOX2B and neuromedin B (Nmb), which has been proposed to be key chemosensory neurons in the RTN.
3. The authors demonstrate that knockdown efficiency is time-dependent, with a progressive decrease in the number of Nmb-expressing RTN neurons that co-express PHOX2B over a four-week period.
4. Their results convincingly show hypoventilation particularly in 7.2% CO₂ only for PHOX2B-shRNA RTN-injected rats after four weeks as compared to naïve and non-PHOX2B-shRNA targeted (NT-shRNA) RTN injected rats, suggesting a specific impairment of chemosensitive properties in RTN neurons with PHOX2B knockdown.
5. Analysis of the association between PHOX2B knockdown in RTN neurons and the attenuation of the hypercapnic ventilatory response (HCVR), by evaluating the correlation between the number of Nmb+/PHOX2B+ or Nmb+/PHOX2B- cells in the RTN and the resulting HCVR, showed a significant correlation between HCVR and number of Nmb+/PHOX2B+ and Nmb+/PHOX2B- cells, suggesting that the number of PHOX2B-expressing cells in the RTN is a predictor of the chemoreflex response and the reduction of PHOX2B protein impairs the CO₂-chemoreflex.
6. The data presented indicate that PHOX2B knockdown not only causes a reduction in the HCVR but also a reduction in the expression of Gpr4 and Task2 mRNAs, suggesting that PHOX2B knockdown affects RTN neurons transcriptional activity and decreases the CO₂ response, possibly by reducing the expression of key proton sensors in the RTN.
7. Results of this study show that independent of the role of PHOX2B during development, PHOX2B is still required to maintain proper CO₂ chemoreflex responses in the adult brain, and its reduction in CCHS may contribute to the respiratory impairment in this disorder.

Weaknesses:

1. The authors found a significant decrease in the total number of Nmb+ RTN neurons (i.e., Nmb+/PHOX2B+ plus Nmb+/PHOX2B-) in NT-shRNA rats at two weeks post viral injection, and also at the four-week period where the impairment of the chemosensory function of the RTN became significant, suggesting some inherent cell death possibly due to off-target toxic effects associated with shRNA procedures that may affect the experimental results.
2. The tissue sampling procedures for quantifying numbers of cells expressing proteins/mRNAs throughout the extended RTN region bilaterally have not been completely validated to accurately represent the full expression patterns in the RTN under experimental conditions.
3. The inferences about RTN neuronal expression of NMB, GPR4, or TASK2 are based on changes in mRNA levels, so it remains speculation that the observed reduction in Gpr4 and Task2 mRNA translates to a reduction in the protein levels and associated reduction of RTN neuronal chemosensitive properties.

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Reviewer #2 (Public Review):**Summary:**

The authors used a short hairpin RNA technique strategy to elucidate the functional activity of neurons in the retrotrapezoid nucleus (RTN), a critical brainstem region for central chemoreception. Dysfunction in this area is associated with the neuropathology of congenital central hypoventilation syndrome (CCHS). The subsequent examination of these rats aimed to shed light on the intricate aspects of RTN and its implications for central chemoreception and disorders like CCHS in adults. They found that using the short hairpin RNA (shRNA) targeting Phox2b mRNA, a reduction of Phox2b expression was observed in Nmb neurons. In addition, Phox2b knockdown did not affect breathing in room air or under hypoxia, but the hypercapnia ventilatory response was significantly impaired. They concluded that Phox2b in the adult brain has an important role in CO₂ chemoreception. They thought that their findings provided new evidence for mechanisms related to CCHS neuropathology. The conclusions of this paper are well supported by data, but careful discussion seems to be required for comparison with the results of various previous studies performed by different genetic strategies for the RTN neurons.

Strengths:

The most exciting aspect of this work is the modelling of the Phox2b knockdown in one element of the central neuronal circuit mediating respiratory reflexes, that is in the RTN. To date, mutations in the PHOX2B gene are commonly associated with most patients diagnosed with CCHS, a disease characterized by hypoventilation and absence of chemoreflexes, in the neonatal period, which in severe cases can lead to respiratory arrest during sleep. In the present study, the authors demonstrated that the role of Phox2b extends beyond the developmental period, and its reduction in CCHS may contribute to the respiratory impairment observed in this disorder.

Weaknesses:

Whereas the most exciting part of this work is the knockdown of the Phox2b in the RTN in adult rodents, the weakness of this study is the lack of a clear physiological, developmental, and anatomical distinction between this approach and similar studies already reported elsewhere (Ruffault et al., 2015, DOI: 10.7554/eLife.07051; Ramanantsoa et al., 2011, DOI: 10.1523/JNEUROSCI.1721-11.2011; Huang et al., 2017, DOI: 10.1016/j.neuron.2012.06.027; Hernandez-Miranda et al., 2018, DOI: 10.1073/pnas.1813520115; Ferreira et al., 2022 DOI: 10.7554/eLife.73130; Takakura et al., 2008 DOI: 10.1113/jphysiol.2008.153163; Basting et al., 2015 DOI: 10.1523/JNEUROSCI.2923-14.2015; Marina et al., 2010 DOI: 10.1523/JNEUROSCI.3141-10.2010). In addition, several conclusions presented in this work are not directly supported by the provided data.

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Reviewer #3 (Public Review):

A brain region called the retrotrapezoid nucleus (RTN) regulates breathing in response to changes in CO₂/H⁺, a process termed central chemoreception. A transcription factor called PHOX2B is important for RTN development and mutations in the PHOX2B gene result in a severe type of sleep apnea called Congenital Central Hypoventilation Syndrome. PHOX2B is also expressed throughout life, but its postmitotic functions remain unknown. This study shows that knockdown of PHOX2B in the RTN region in adult rats decreased expression of Task2 and Gpr4 in Nmb-expressing RTN chemoreceptors and this corresponded with a diminished ventilatory response to CO₂ but did not impact baseline breathing or the hypoxic ventilatory response. These results provide novel insight regarding the postmitotic functions of PHOX2B in RTN neurons.

Main issues:

1. The experimental approach was not targeted to Nmb⁺ neurons and since other cells in the area also express Phox2b, conclusions should be tempered to focus on Phox2b expressing parafacial neurons NOT specifically RTN neurons
2. It is not clear whether PHOX2B is important for the transcription of pH sensing machinery, cell health, or both. If knockdown of PHOX2B knockdown results in loss of RTN neurons this is also expected to decrease Task2 and Gpr4 levels, albeit by a transcription-independent mechanism.

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