Neuroscience

Knockdown of PHOX2B in the retrotrapezoid nucleus reduces the central CO_2 chemoreflex in rats

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2
April 11, 2024 (this version)

Reviewed preprint version 1
February 14, 2024

Posted to preprint server
December 14, 2023

Sent for peer review
December 13, 2023

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

Abstract

PHOX2B is a transcription factor essential for the development of different classes of neurons in the central and peripheral 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 CO_2 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 it was associated with reduced the expression of both *Task2* and *Gpr4*, two CO_2 sensors in the RTN. We conclude that PHOX2B in the adult brain has an important role in CO_2 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 viral-mediated short hairpin RNA (shRNA) approach to investigate in a novel way the role of the wild-type PHOX2B transcription factor expressed in critical chemosensory neurons in the brainstem retrotrapezoid nucleus (RTN) region for maintaining normal CO2 chemoreflex control of breathing in adult rats. The **convincing** results show blunted ventilation during elevated inhaled CO2 (hypercapnia) with knockdown of PHOX2B, accompanied by a reduced expression of Gpr4 and Task2 mRNA for the proposed RTN neuron proton sensor proteins GPR4 and TASK2. These results indicate that maintained expression of wild-type PHOX2B affects respiratory control in adult animals, complementing 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, which 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; Pattyn et al., 1997, 1999, 2000; Stanke et al., 1999; Tiveron et al., 1996). 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), Hirschsprung's disease (HSCR; OMIM 142623; Berry-Kravis et al., 2006; Trochet et al., 2005), and neuroblastoma (Bourdeaut et al., 2005; Van Limpt et al., 2004). CCHS is a rare (1:200,000 births in France (Trang et al., 2005) and 1:148,000 births in Japan (Shimokaze et al., 2015) disorder characterised by a failure to respond to both hypercapnia and hypoxia (Amiel et al., 2003; Di Lascio et al., 2021; Weese-Mayer et al., 2017). 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; Di Lascio et al., 2016; Weese-Mayer et al., 2003). CCHS symptoms present with varying degrees of severity depending on the expansion of the mutation (Berry-Kravis et al., 2006; Di Lascio et al., 2018; Matera, 2004). 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 CO_2 and O_2 chemoreflexes (Kang et al., 2007). 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 to the dorsal vagal complex, a structure that provides the major integrative centre for the mammalian autonomic nervous system (Cutsforth-Gregory & Benarroch, 2017; Guyenet et al., 2019; Kang et al., 2007; Zoccal et al., 2014). 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 CO_2 chemoreception.

Defects observed in the CO_2-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 results in a near complete loss of RTN neurons, impaired CO_2 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 CO_2 response in adulthood (Ramanantsoa et al., 2011).

Multiple potential cellular mechanisms have been implicated in the aetiology 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 CO_2 chemoreflex responses, it is essential to understand how both wild-type and mutant PHOX2B proteins impact CO_2 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 CO_2 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 *Nmb* neurons of the RTN negatively affected their cell survival. Finally, we sought to provide a mechanistic link between PHOX2B expression and the chemosensitive properties of *Nmb* neurons of the RTN, 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 *Nmb* neurons of the RTN and the genetic deletion of either one 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 of the RTN 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 ($1x10^9$ 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 CO_2 chemoreflex was observed not only in PHOX2B-shRNA but also in NT-shRNA rats compared to pre-surgical baseline (allometric V_E in 7% CO_2: -21% and -24%, respectively; Supp. Fig. 1A). Because histological analysis also 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; $1x10^9$ 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% CO_2, frequency (f_R), was reduced in every experimental group (naïve, NT-shRNA, PHOX2B-shRNA) compared to baseline (Air: p=0.014; 5% CO_2: p< 0.001; 7.2% CO_2 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 long-term experiment (naïve: +32.2%; NT-shRNA: +27.8%; PHOX2B-shRNA: +28.6% change in body weight). Tidal volume was reduced in the PHOX2B-shRNA rats at 7.2% CO_2 compared to both naïve rats (naive: 9.6 ± 1.2 ml·kg^{-1}; PHOX2B-shRNA: 8.5 ± 0.9 ml·kg^{-1}; -11%; p= 0.022) and baseline pre-surgery conditions (baseline: 9.6 ± 1.3 ml·kg^{-1}; -11%; p< 0.001; **Fig. 1B**) but it was not different from NT-shRNA.

Despite similar changes in body weight across treatment groups, allometric ventilation (V_E ALLO) was higher 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% CO_2) 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 in V_E from room air; **Fig. 1F**). The HCVR was also 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

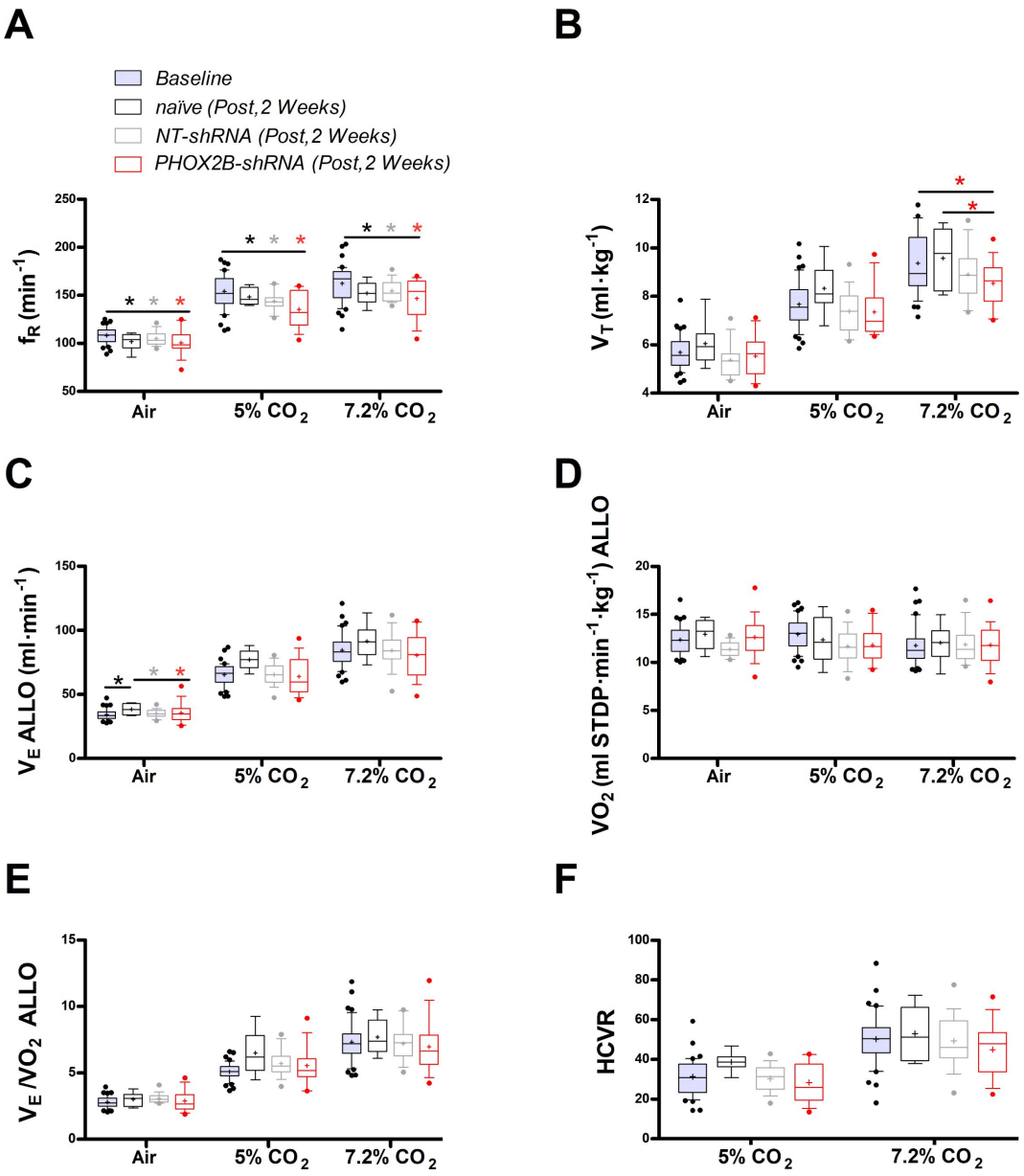

Figure 1.

Respiratory data two weeks post viral PHOX2B 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% CO_2. 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% CO_2 (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- shRNA.

Nmb$^+$/PHOX2B$^+$), we would be able to determine the level of PHOX2B knockdown in our experiments. Two weeks post viral injection, we observed a small 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* cells of the RTN in PHOX2B-shRNA rats was further reduced compared to both naïve rats (PHOX2B-ShRNA 218.8 ± 7.8 n=4; -35.2%; p<0.001) and to NT-shRNA (-23.5%; p<0.001).

We then quantified the number of neurons in the RTN 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 a reduction in the proportion of *Nmb* neurons expressing PHOX2B in PHOX2B-shRNA rats, 2 weeks of PHOX2B shRNA viral infection was not sufficient to impair ventilation.

Four weeks of PHOX2B knockdown impairs the CO_2-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**).

The total number of *Nmb* neurons in the RTN of 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 the progressive PHOX2B knockdown was specific for PHOX2B-shRNA treatment and it was not accompanied by an increased cell death beyond the first 2 weeks.

The number of *Nmb*$^+$/PHOX2B$^+$ 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 ± 12cells; + 146.1% increase vs naïve; NT-shRNA: 56.2 ± 13.8cells; + 177% increase vs NT-shRNA, p<0.001; **Fig. 3E**), confirming the efficiency of the PHOX2B knockdown in the *Nmb* cells of the RTN.

The respiratory function prior to histological examination (4 weeks post-viral injection) showed significant differences in PHOX2B-shRNA rats (n=6) compared to both pre-surgery baseline, naïve (n=8) and NT-shRNA rats (n=10). Similar to what we reported at 2 weeks post-surgery, f_R at 4 weeks was lower in every treatment group compared to baseline recordings (Air: p=0.001; 5% CO_2: p< 0.001; 7.2% CO_2 p< 0.001; **Fig. 4A**).

During room air, V_T was 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 pre-surgery baseline (6.1 ± 0.7 ml·kg^{-1}; -18%; p=0.002). No significant

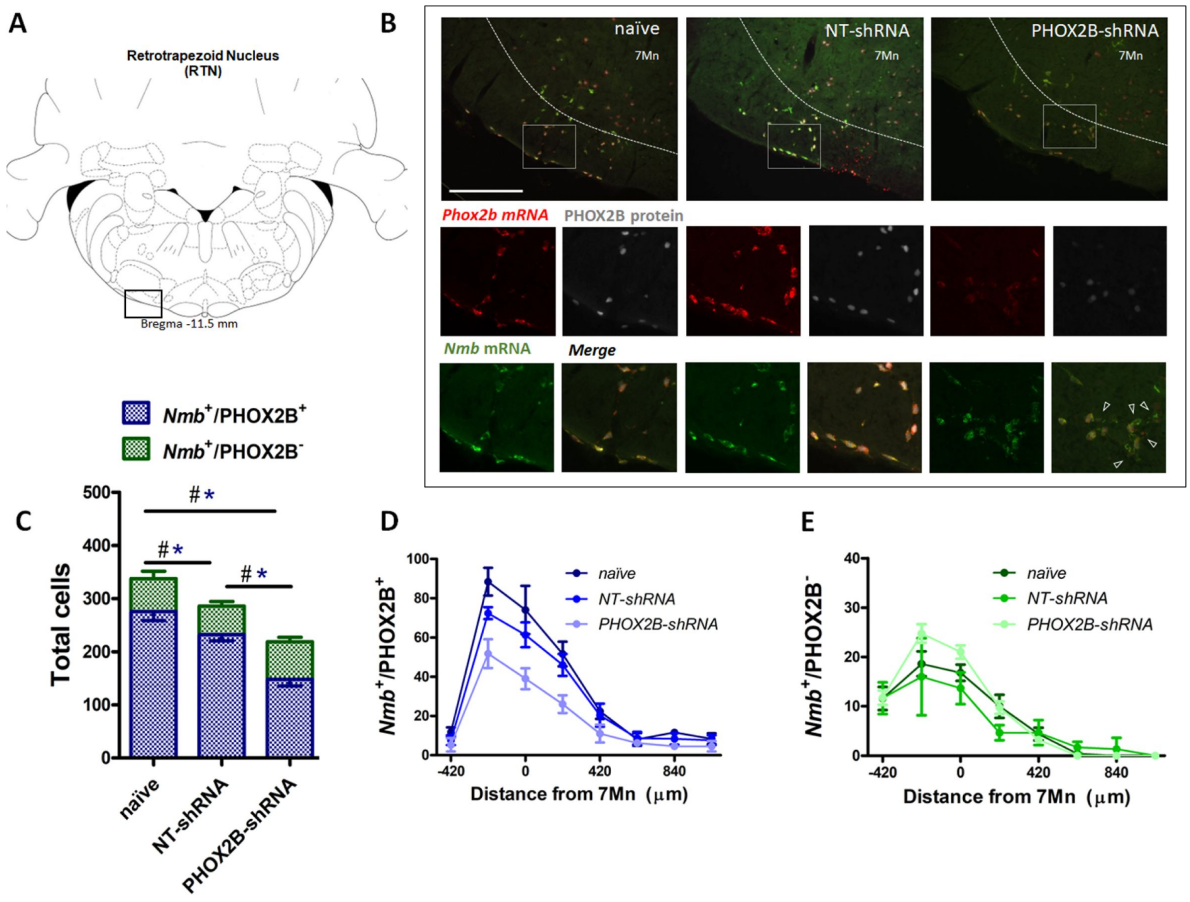

Figure 2.

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 0.001). The number of *Nmb*$^{+}$/PHOX2B^{-} cells was unchanged across groups. **(D,E)** Rostral-caudal distribution (distance from the caudal tip of the facial nucleus, 7Mn) of *Nmb*$^{+}$/PHOX2B^{+} (D) and *Nmb*$^{+}$/PHOX2B^{-} (E) neurons along the RTN.

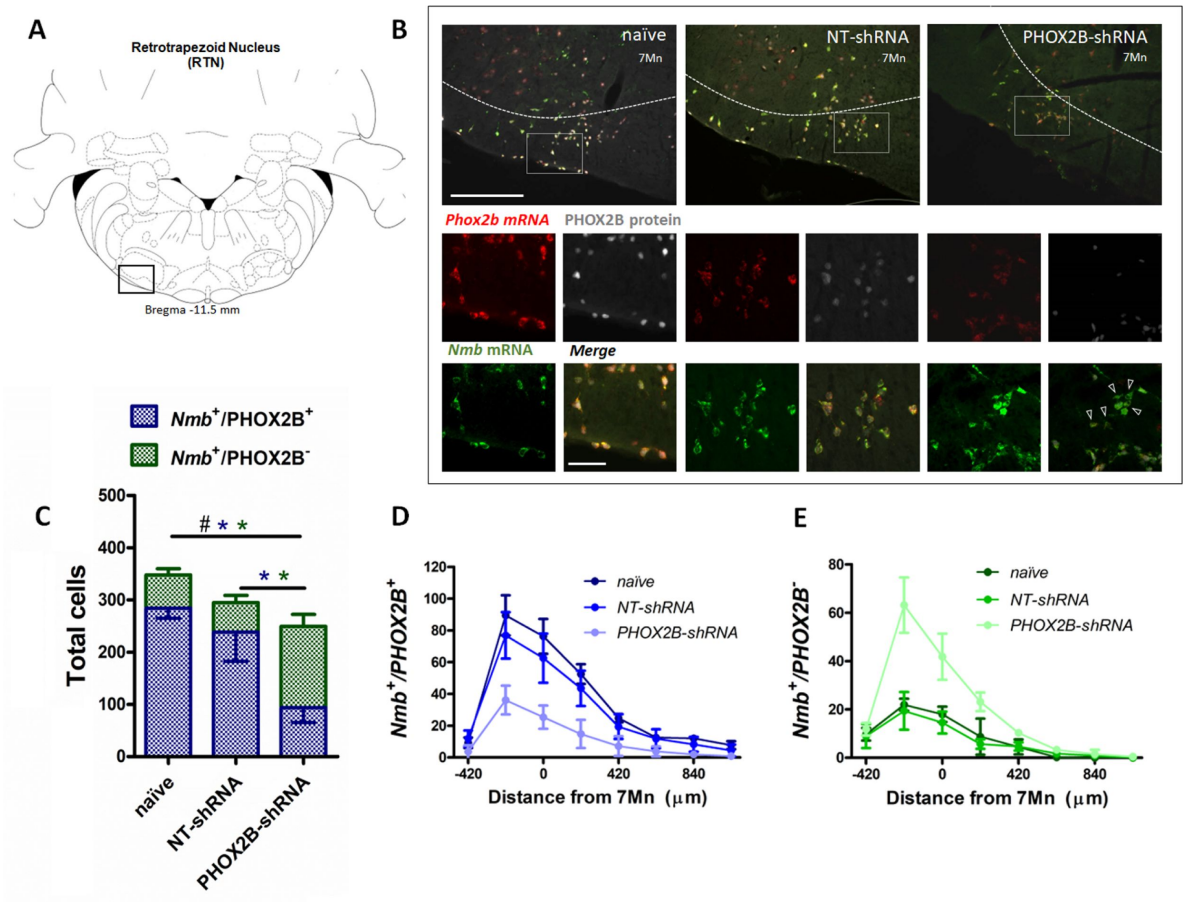

Figure 3.

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 (distance from the caudal tip of the facial nucleus, 7Mn) of Nmb^+/$PHOX2B^+$ (D) and Nmb^+/$PHOX2B^-$ (E) neurons along the RTN.

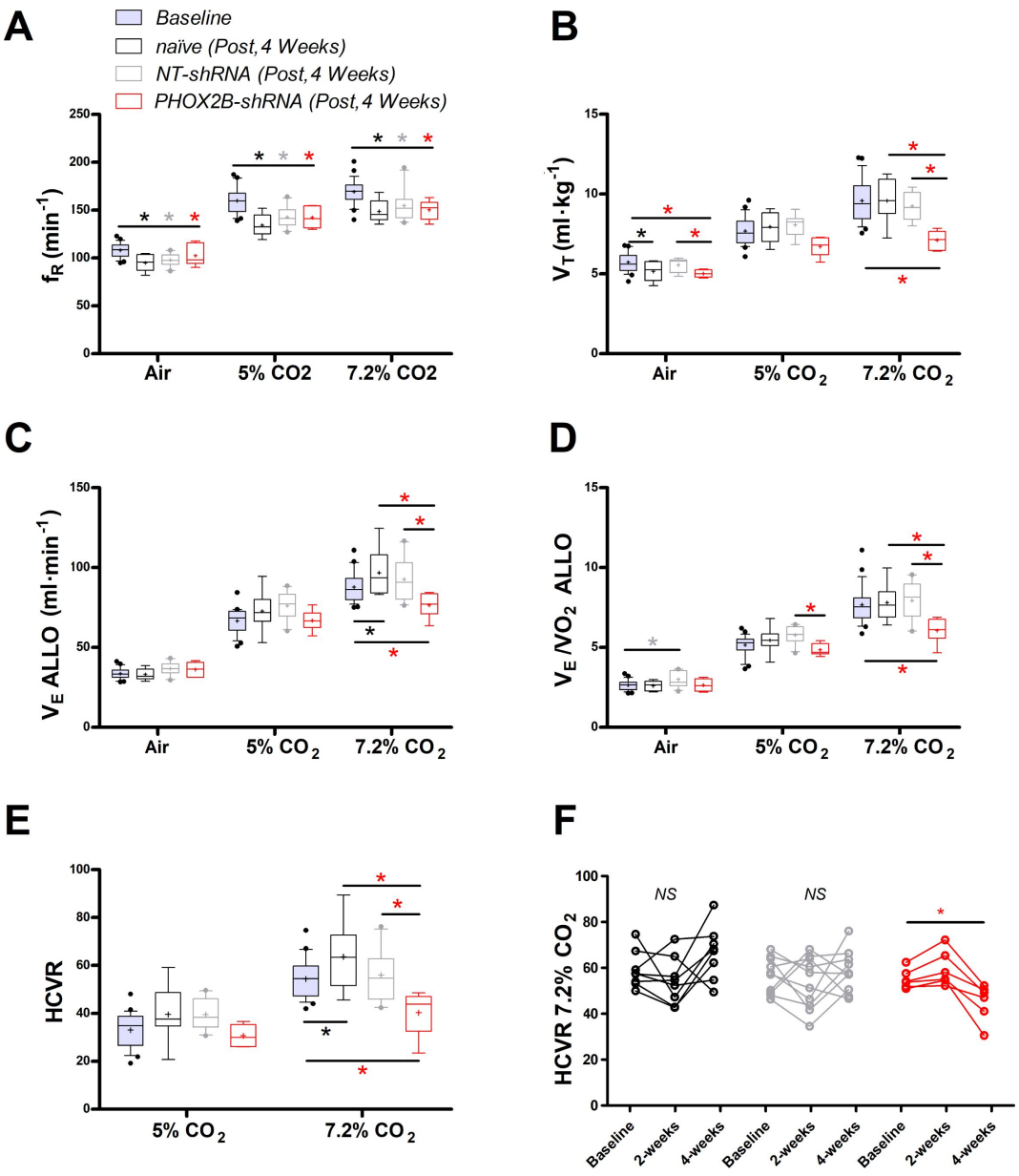

Figure 4.

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 ALLO), **(D)** convective requirement ratio (V_E/VO_2 ALLO), **(E)** hypercapnic ventilatory response (HCVR, absolute change in V_E 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 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/VO_2 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.

changes were observed at 5% CO_2, although a decrease in V_T occurred at 7.2% CO_2 in PHOX2B-shRNA rats compared to pre-surgery baseline (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% CO_2, 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 O_2-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% CO_2 (NT-shRNA: 7.9 ± 1.2; PHOX2B-shRNA: 6.1 ± 0.8; -24%; p=0.004; **Fig. 4D**). Moreover, the HCVR (**Fig. 4E**) at 7.2% CO_2 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 CO_2-chemoreflex at 4 weeks post-infection, we investigated whether PHOX2B knockdown would also affect respiratory response to hypoxia (10% O_2). 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 (VE/VO_2 ALLO naive: 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

To exclude that the observed chemoreflex 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 in PHOX2B-shRNA rats compared to naïve and NT-shRNA (**Fig. 5A,B**). No differences were observed in either the TH^+ cell numbers or in the TH^+/$PHOX2B^+$ neurons between the 3 treatment groups (TH^+/$PHOX2B^+$ cells: naïve: 378.3 ± 23.81; NT-shRNA: 391.7 ± 33.08; PHOX2B-shRNA: 370.2 ± 33.04 cells) (**Fig. 5B**).

Neuromedin B 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 a target gene of PHOX2B and it is affected by PHOX2B knockdown. We assessed *Nmb* expression in individual RTN cells of PHOX2B-shRNA rats and compared its cellular expression to both naïve and NT-shRNA rats (**Fig 5B**). Neuromedin B mRNA expression was calculated as total fluorescence intensity (CTCF) ratio in single cells of the RTN relative to the single cell *Nmb* expression measured at the level of NTS *Nmb* cells, which served as an internal control (**Fig. 5D**). We observed no changes in relative fluorescence of *Nmb* in RTN neurons across the 3 groups (p=0.608), suggesting that *Nmb* levels in RTN neurons are not affected by PHOX2B knockdown.

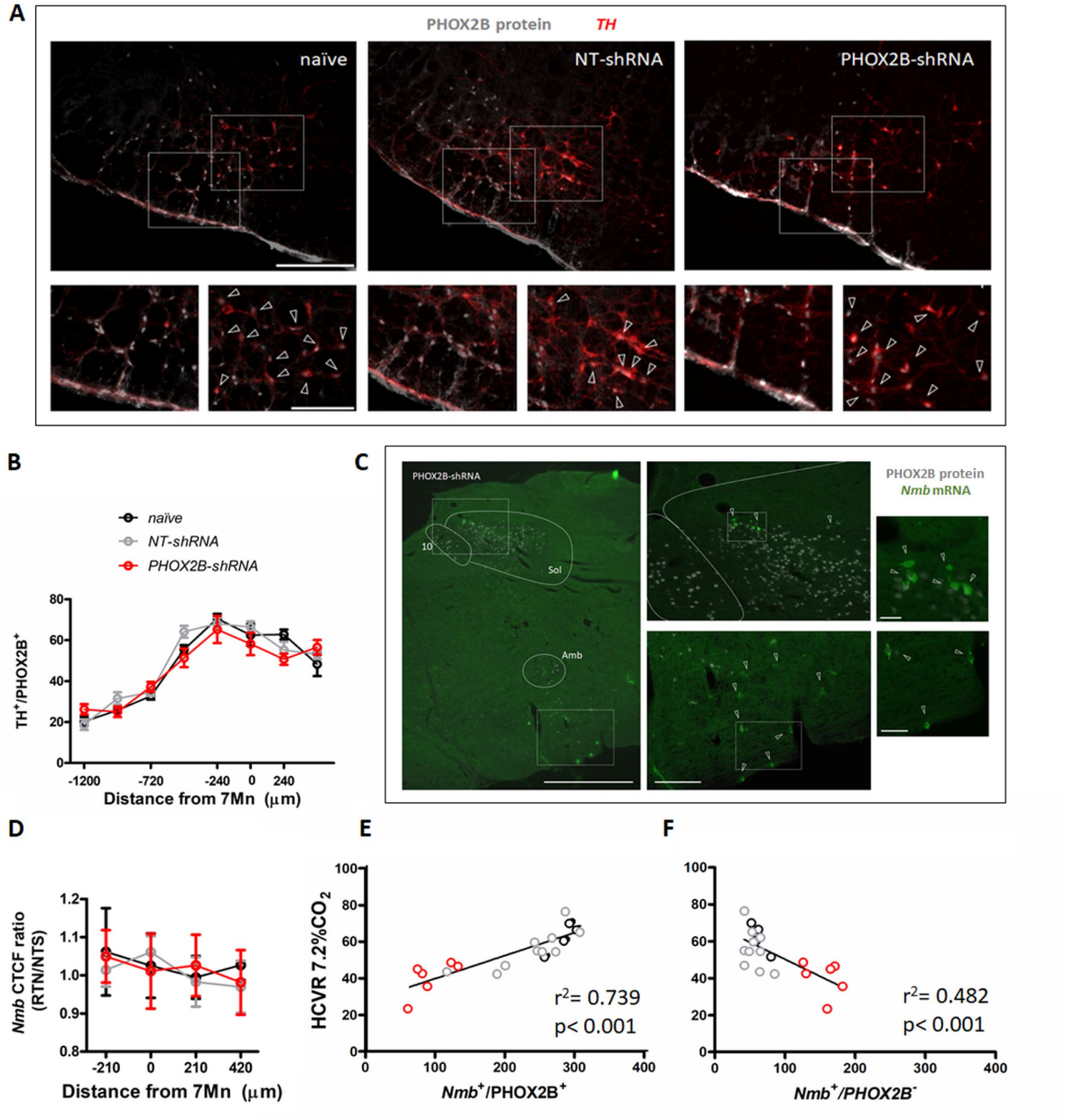

Figure 5.

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

PHOX2B protein (white) and TH (red) expression in C1 neurons of naïve (left), NT-shRNA (middle), and PHOX2B-shRNA (right) rats. Magnified view at the bottom. Arrowheads indicate the colocalization of PHOX2B and TH protein in C1 neurones cells. Scale bar = 400μm (top figures), 150μm (bottom figures). **(B)** 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. **(C)** PHOX2B protein (white) and *Nmb* mRNA (green) expression in NT-shRNA rat at the level of NTS and RTN regions. Magnified view on the left. Arrowheads indicate cells with *Nmb* mRNA expression. Scale bar = 400μm (right figures), 150μm (left figures). **(D)** Quantification of single cells *Nmb* mRNA fluorescence 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 average ratio between RTN and NTS cells shows no difference between treatment groups. Data are shown as average cell fluorescence value at different rostro-caudal levels. **(D-E)** X-Y plot of HCVR during 7.2% CO_2 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.

To better analyse the effect of PHOX2B knockdown on 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. 5E,F**). Our results indicate that there is a significant correlation between HCVR and number of Nmb$^+$/PHOX2B$^+$ (r^2 = 0.739 p<0.001; **Fig. 5E**), and Nmb$^+$/PHOX2B$^-$ (r^2 = 0.482 p<0.001 **Fig. 5F**) 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 may contribute to the CO_2 sensing and consequently, the reduction of PHOX2B protein impairs the CO_2-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* cells of the RTN 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), suggesting that the fraction of *Nmb* cells expressing the two pH sensors were not affected by the PHOX2B shRNA treatment.

Because it is not yet known whether PHOX2B controls the expression of GPR4 AND TASK2, we quantified their single cell mRNA expression (CTCF) in *Nmb* cells of the RTN. Averaged cell fluorescence for *Gpr4* and *Task2* mRNA 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 their single cell 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 HCVR but also a reduction in *Gpr4* and *Task2* mRNA levels in cells that are affected by PHOX2B knockdown.

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 CO_2 chemoreflex. Four weeks after shRNA infection we observed a further reduction in the expression of PHOX2B in *Nmb* neurons and a significant reduction of the CO_2 chemoreflex, while ventilation in normoxia or hypoxia was unaffected. Furthermore, the level of expression of *Gpr4* and *Task2* mRNAs, 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 the transcriptional activity of *Nmb* neurons in the RTN and decreases the CO_2 response, possibly by reducing the expression of key proton sensors in the RTN.

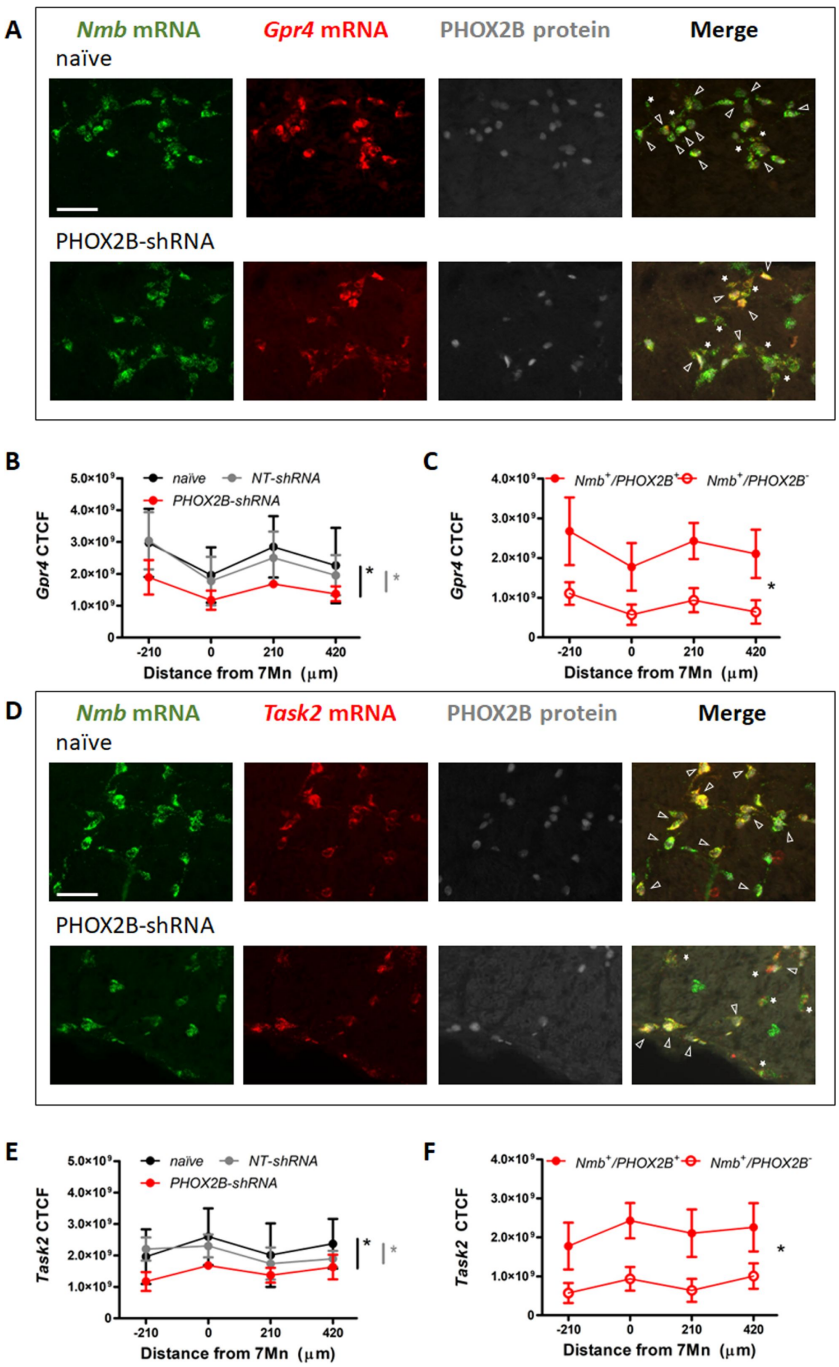

Figure 6.

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 µm. 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 single cells *Gpr4* and *Task2* mRNA fluorescence 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 single cells *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. Data are shown as average cell fluorescence value at different rostro-caudal levels of the RTN. Mean corrected total cell fluorescence (CTCF) value ± SEM combined (naïve, n=4; NT-shRNA, n=10; PHOX2B-shRNA, n=6) (see methods for detail). One- way ANOVA repeated measures.

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 the 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 neurons 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 CO_2 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 beyond the post-natal period. Thus, in order to determine whether PHOX2B is necessary for the survival and/or chemosensory function of adult PHOX2B$^+$/*Nmb*$^+$ 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

Although the PHOX2B shRNA approach was not selective for *Nmb* neurons, we used small volumes and localized injections to target the RTN. 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 PHOX2B shRNA treated rats compared to naïve and NT-shRNA rats two weeks after viral infection. Interestingly, despite the small reduction in total *Nmb* cells of the RTN 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. 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, CO_2-chemoreflex impairment became significant (~30% V_E Allo reduction vs. pre-surgical baseline) only when *Nmb* neurons of the RTN (*Nmb*$^+$/PHOX2B$^+$ and *Nmb*$^+$/PHOX2B$^-$ neurons) were 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 in a fraction of *Nmb* cells, we would not expect significant changes in ventilation only with a <50% RTN cell impairment or loss.

Four weeks of PHOX2B knockdown impairs central CO_2 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 adjacent C1 TH$^+$ neurons did not show any change in number of TH$^+$/PHOX2B$^+$ expressing cells, although we can't exclude that some C1 cells may have been infected, and their relative PHOX2B expression levels were slightly reduced. To support the lack of significant alterations associated with the possible loss of C1 function was the lack of significant changes in the hypoxic response that has been shown to be dependent on C1 neurons (Malheiros-Lima et al., 2017).

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, as some RTN neurons may still have detectable levels of PHOX2B, albeit decreased.

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; Guyenet et al., 2019). 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/VO_2 ALLO) confirmed hypoventilation in 5% and 7.2% CO_2 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 CO_2-chemoreflex impairment (Janes et al., 2024; Souza et al.,2018). Hence, killing RTN neurons or knocking down PHOX2B in *Nmb* cells of the RTN gave comparable results.

Changes in the CO_2 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 CO_2/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 CO_2 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; Souza et al.,2018; Nattie & Li, 2002; Takakura et al., 2008, 2014).

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 fraction of Nmb$^+$/PHOX2B$^+$ neurons decreased) and the central chemoreflex at 2 weeks post-surgery was not impaired, suggesting that: i) the observed reduction in overall *Nmb* cell number in RTN occurring in the first 2 weeks is not responsible for the CO_2 chemoreflex impairment that emerges 4 weeks post-infection; ii) PHOX2B expression is most likely not necessary for neuronal survival of adult *Nmb* neurons of the RTN; iii) a large reduction in *Nmb* neurons expressing PHOX2B$^+$ is necessary to impair the HCVR (as we observed at 4 weeks). 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 (although not observed in similar studies performed by the same investigators, Janes et al., 2024) or, more likely, with the potential off-target toxic effects associated with shRNA procedures (Mcbride et al., 2008; Van Gestel et al., 2014).

PHOX2B knockdown reduces mRNA expression of proton sensors in the Nmb cells of the RTN

Current theories on central respiratory chemosensitivity postulate that changes in CO_2/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). Because it is not known whether PHOX2B has any influence on the expression of TASK2, GPR4 or even the expression of the neuropeptide NMB used to anatomically identify RTN neurons, we quantified their mRNA at cellular levels (calculated as CFTC) and observed no changes in relative fluorescence of *Nmb* mRNA in the RTN relative to *Nmb* levels in unrelated cells in the NTS, suggesting that *Nmb* expression 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 although the fraction of *Nmb* cells of the RTN expressing *Gpr4* and *Task2* did not change across treatment, the levels of *Gpr4* and *Task2* in *Nmb* neurons of PHOX2B-shRNA rats was reduced compared to the naïve and NT-shRNA groups. Furthermore, there was a significant reduction of both *Gpr4* and *Task2* levels within *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 CO_2/pH sensitivity of RTN neurons at cellular level.

PHOX2B knockdown may impair CO_2-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), it is possible that in NMB cells of the RTN, 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 CO_2 challenges (Guyenet et al., 2016). 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 PHOX2B 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; Van Gestel et al., 2014), 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). Homozygous knockout mice for PHOX2B die during gestation (Pattyn et al., 1999, 2000) 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) and impairment of the hypoxic and hypercapnic ventilatory responses in the neonatal period (Dauger, 2003). Here we show that, independent of the PHOX2B role during development, PHOX2B is still required to maintain proper CO_2 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, 2009). The effects of the expression of the mutant PHOX2B on wild-type PHOX2B protein 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; Trochet et al., 2005, 2008), its affinity for DNA and coactivators, as well as its degradation rate (Nagashimada et al., 2012; Wu et al., 2009). It has also been shown that mutant PHOX2B causes the formation of cytoplasmic aggregates (Bachetti et al., 2005; Trochet et al., 2005, 2008) and fibrils *in vitro* (Pirone et al., 2019), and dysregulates the transcriptional function of wild-type PHOX2B (Di Lascio et al., 2013; Parodi et al., 2012), thus changing expression of important target genes such as DBH, PHOX2A and TLX2 (Bachetti et al., 2005; Di Lascio et al., 2013; Trochet et al., 2005). 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, 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 ($1x10^9$ 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/rostro-caudal/dorso-ventral): ±1.8, +2.0,-3.5; ±1.8,+2.4,-3.6. Injections were made using a glass microelectrode (30 µm 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 and viral constructs 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%O_2 + 0%CO_2 (balanced with N_2); Normoxic Hypercapnia: 21%O_2+ 5%CO_2; 21%O_2 + 7.2%CO_2; Normocapnic Hypoxia: 10.6%O_2+ 0% CO_2.

As previously described (Cardani et al., 2022; Janes et al., 2024) 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; Janes et al., 2024; 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 (ml·kg^{-1}) using the equations of Drorbaug and Fenn 1955 (Drorbaugh & 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·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 x V_T and expressed as ml·min^{-1}·kg^{-1}. Rats gained, on average, 160 ± 4g over the experimental paradigm (naive: 158 ± 19g; NT-shRNA: 202 ± 25g; PHOX2B-shRNA: 133 ± 7g); 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 every recording session. 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**,4). Post-hoc analysis used the Bonferroni correction factor, with $p<0.05$ considered to be significant. Ventilatory data are reported as mean ± 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 µm 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 *Nmb*+/PHOX2B- and *Nmb*+/PHOX2B+ neurons within the RTN region, we analysed one every seven sections (210 µm interval; 8 sections/rat in total) along the rostrocaudal distribution of the RTN on the ventral surface of the brainstem and compared total bilateral cell counts of

PHOX2B-shRNA rats with non-target control (NT-shRNA) and naïve rats. Cells that expressed *Nmb* and *Phox2b* mRNAs but did not show co-localization with PHOX2B protein were considered *Nmb*+/PHOX2B-.

The Corrected Total Cell Fluorescence (CTCF) signal for *Nmb, Gpr4* and *Task2* mRNAs was quantified as previously described (Cardani et al., 2022; McCloy et al., 2014). Briefly, 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. The collected images were then analysed by selecting a single cell at a time and measuring the area, integrated density and mean grey value (McCloy et al., 2014). For each image, three background areas were used to normalize against autofluorescence. We used 4 sections/rat (210 µm interval) to count *Nmb*, *Gpr4* and *Task2* mRNA CTCF in the core of the RTN area where several *Nmb* cells could be identified. For each section, two images were acquired with a 20× objective, so that at least fifty cells per tissue sample were obtained for the mRNA quantification analysis. To evaluate changes in *Nmb* mRNA expression levels following PHOX2B knockdown at the level of the RTN, we compared, the fluorescence intensity of each RTN *Nmb* cell (223.2 ± 37.1 cells/animal) with the average fluorescent signal of *Nmb* cells located dorsally in the NTS (4.3 ± 1.2 cells/animal) (*Nmb* CTCF ratio RTN/NTS) as we reasoned that the latter would not be affected by the shRNA infection and knockdown.

To quantify *Gpr4* and *Task2* mRNA expression in *Nmb* cells of the RTN, we first quantified single cell CTCF for either *Gpr4* (200.7 ± 13.2 cells/rat) or *Task2* (169.6 ± 10.3 cells/rat) mRNA in *Nmb* cells of the RTN in the 3 experimental groups (naïve, NT shRNA and PHOX2B shRNA) independent of their PHOX2B expression. We then compared CTCF values of *Gpr4* and *Task2* mRNA between Nmb^{+}/$PHOX2B^{+}$ and Nmb^{+}/$PHOX2B^{-}$ RTN neurons in PHOX2B-shRNA rats to address changes in their mRNA expression induced by PHOX2B knockdown.

To assess whether shRNA knockdown affected either the number or the expression of PHOX2B in TH expressing C1 neurons, we counted the number of TH^{+}/$PHOX2B^{+}$ and TH^{+}/$PHOX2B^{-}$ cells along the ventrolateral medulla (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**, **3C**, S1B) and repeated-measures ANOVA (**Fig 5B**, D 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.5E**-F). *p* values of < 0.05 were considered significant and data are reported as mean ± standard deviation (SD).

Acknowledgements

Concept and experimental design, S.P. and S.C.; Data collection, S.C., W.B. and S.P.; Data analysis, S.C. and T.A.J.; Manuscript preparation, S.C., T.A.J. and S.P. S.C. is supported by the Canadian Lung Association BaO Fellowship. T.A.J. is supported by the Canadian Institutes for Health Research (CIHR) Postdoctoral Fellowships. S.P. is supported by a Women and Children's Health Research Institute Innovation Grant and CIHR Project scheme grant.

References

1. Abbott S. B. G., Stornetta R. L., Coates M. B., Guyenet P. G (2011) **Phox2b-Expressing Neurons of the Parafacial Region Regulate Breathing Rate** *Inspiration, and Expiration in Conscious Rats* https://doi.org/10.1523/JNEUROSCI.3280-11.2011

2. Amiel J. *et al.* (2003) **Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome** *Nature Genetics* **33**:459–461 https://doi.org/10.1038/ng1130

3. Bachetti T., Matera I., Borghini S. (2005) **Distinct pathogenetic mechanisms for PHOX2B associated polyalanine expansions and frameshift mutations in congenital central hypoventilation syndrome** *Human Molecular Genetics* **14**:1815–1824 https://doi.org/10.1093/hmg/ddi188

4. Barna B. F., Takakura A. C., Mulkey D. K., Moreira T. S (2016) **Purinergic receptor blockade in the retrotrapezoid nucleus attenuates the respiratory chemoreflexes in awake rats. Acta Physiologica (Oxford** *England* **217**:80–93 https://doi.org/10.1111/APHA.12637

5. Basting T. M., Burke P. G. R., Kanbar R., Viar K. E., Stornetta D. S., Stornetta R. L., Guyenet P. G (2015) **Hypoxia Silences Retrotrapezoid Nucleus Respiratory Chemoreceptors via Alkalosis** *Journal of Neuroscience* https://doi.org/10.1523/JNEUROSCI.2923-14.2015

6. Berry-Kravis E. M., Zhou L., Rand C. M., Weese-Mayer D. E (2006) **Congenital central hypoventilation syndrome PHOX2B mutations and phenotype** *American Journal of Respiratory and Critical Care Medicine* **174**:1139–1144 https://doi.org/10.1164/rccm.200602-305OC

7. Biancardi V., Saini J., Pageni A., Prashaad M., Funk H., Pagliardini G. D. (2021) **Mapping of the excitatory, inhibitory, and modulatory afferent projections to the anatomically defined active expiratory oscillator in adult male rats** *Journal of Comparative Neurology* **529**:853–884 https://doi.org/10.1002/CNE.24984

8. Bourdeaut F., Trochet D., Janoueix-Lerosey I., Ribeiro A., Deville A., Coz C., Michiels J. F., Lyonnet S., Amiel J., Delattre O (2005) **Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma** *In Cancer Letters* **228** https://doi.org/10.1016/j.canlet.2005.01.055

9. Brunet J. F., Pattyn A (2002) **Phox2 genes - From patterning to connectivity** *In Current Opinion in Genetics and Development* **12**:435–440 https://doi.org/10.1016/S0959-437X(02)00322-2

10. Cardani S., Janes T. A., Saini J. K., Di Lascio S., Benfante R., Fornasari D., Pagliardini S. (2022) **Etonogestrel Administration Reduces the Expression of PHOX2B and Its Target Genes in the Solitary Tract Nucleus** *International Journal of Molecular Sciences* **23** https://doi.org/10.3390/ijms23094816

11. Cutsforth-Gregory J. K., Benarroch E. E (2017) **Nucleus of the solitary tract, medullary reflexes, and clinical implications** *Neurology* **88**:1187–1196 https://doi.org/10.1212/WNL.0000000000003751

12. Dauger S (2003) **Phox2b controls the development of peripheral chemoreceptors and afferent visceral pathways** *Development* **130**:6635–6642 https://doi.org/10.1242/dev.00866

13. Depocas F., Sanford Hart J. **Use of the Pdzlling Oxygen Analyzer for Memwement of Oxygen conszlm~tion of Ahmds iti Open-Circuit Systems ad in a Short-Lag, Closed-Cirwit Appma tml**

14. Di Lascio S., Bachetti T., Saba E., Ceccherini I., Benfante R., Fornasari D. (2013) **Transcriptional dysregulation and impairment of PHOX2B auto-regulatory mechanism induced by polyalanine expansion mutations associated with congenital central hypoventilation syndrome** *Neurobiology of Disease* **50**:187–200 https://doi.org/10.1016/j.nbd.2012.10.019

15. Di Lascio S., Belperio D., Benfante R., Fornasari D. (2016) **Alanine expansions associated with congenital central hypoventilation syndrome impair PHOX2B homeodomain-mediated dimerization and nuclear import** *Journal of Biological Chemistry* **291**:13375–13393 https://doi.org/10.1074/jbc.M115.679027

16. Di Lascio S., Benfante R., Cardani S., Fornasari D. (2018) **Advances in the molecular biology and pathogenesis of congenital central hypoventilation syndrome—implications for new therapeutic targets** *Expert Opinion on Orphan Drugs* **0**:1–13 https://doi.org/10.1080/21678707.2018.1540978

17. Di Lascio S., Benfante R., Cardani S., Fornasari D. (2021) **Research Advances on Therapeutic Approaches to Congenital Central Hypoventilation Syndrome (CCHS)** *Frontiers in Neuroscience* **14** https://doi.org/10.3389/FNINS.2020.615666

18. Di Lascio S., Benfante R., Di Zanni E., Cardani S., Adamo A., Fornasari D., Ceccherini I., Bachetti T. (2018) **Structural and functional differences in PHOX2B frameshift mutations underlie isolated or syndromic congenital central hypoventilation syndrome** *Human Mutation* https://doi.org/10.1002/humu.23365

19. Drorbaugh J. E., Fenn W. O (1955) **A barometric method for measuring ventilation in newborn infants** *Pediatrics* **16**:81–87 https://doi.org/10.1542/peds.16.1.81

20. Dubreuil V., Ramanantsoa N., Trochet D., Vaubourg V., Amiel J., Gallego J., Brunet J.-F., Goridis C (2008) **A human mutation in Phox2b causes lack of CO2 chemosensitivity, fatal central apnea, and specific loss of parafacial neurons** *Proceedings of the National Academy of Sciences* **105**:1067–1072 https://doi.org/10.1073/pnas.0709115105

21. Dubreuil V., Thoby-Brisson M., Rallu M., Persson K., Pattyn A., Birchmeier C., Brunet J.-F., Fortin G., Goridis C (2009) **Defective Respiratory Rhythmogenesis and Loss of Central Chemosensitivity in Phox2b Mutants Targeting Retrotrapezoid Nucleus Neurons** *Journal of Neuroscience* **29**:14836–14846 https://doi.org/10.1523/JNEUROSCI.2623-09.2009

22. Durand E., Dauger S., Pattyn A., Gaultier C., Goridis C., Gallego J (2005) **Sleep-disordered breathing in newborn mice heterozygous for the transcription factor phox2b** *American Journal of Respiratory and Critical Care Medicine* **172**:238–243 https://doi.org/10.1164/rccm.200411-1528OC

23. Fan Y., Huang J., Duffourc M., Kao R. L., Ordway G. A., Huang R., Zhu M. Y (2011) **Transcription factor Phox2 upregulates expression of norepinephrine transporter and dopamine β-hydroxylase in adult rat brains** *Neuroscience* **192**:37–53 https://doi.org/10.1016/j.neuroscience.2011.07.005

24. Gestreau C. *et al.* (2010) **Task2 potassium channels set central respiratory CO2 and O2 sensitivity** *Proceedings of the National Academy of Sciences* **107**:2325–2330 https://doi.org/10.1073/pnas.0910059107

25. Goridis C., Dubreuil V., Thoby-brisson M., Fortin G (2010) **Phox2b, congenital central hypoventilation syndrome and the control of respiration** *Seminars in Cell & Developmental Biology* **21** https://doi.org/10.1016/j.semcdb.2010.07.006

26. Gourine A. V, Llaudet E., Dale N., Spyer K. M (2005) **Behavioral/Systems/Cognitive Release of ATP in the Ventral Medulla during Hypoxia in Rats: Role in Hypoxic Ventilatory Response** *Journal of Neuroscience* https://doi.org/10.1523/JNEUROSCI.3763-04.2005

27. Guyenet P. G. *et al.* (2016) **Proton detection and breathing regulation by the retrotrapezoid nucleus** *Journal of Physiology* **594**:1529–1551 https://doi.org/10.1113/JP271480

28. Guyenet P. G., Stornetta R. L., Souza G. M. P. R., Abbott S. B. G., Shi Y., Bayliss D. A (2019) **The Retrotrapezoid Nucleus: Central Chemoreceptor and Regulator of Breathing Automaticity** *Trends in Neurosciences* **42**:807–824 https://doi.org/10.1016/J.TINS.2019.09.002

29. Harper R. M., Kumar R., Macey P. M., Harper R. K., Ogren J. A (2015) **Impaired neural structure and function contributing to autonomic symptoms in congenital central hypoventilation syndrome** *Frontiers in Neuroscience* **9** https://doi.org/10.3389/FNINS.2015.00415

30. Janes T. A., Cardani S., Saini J. K., Pagliardini S (2024) **Etonogestrel promotes respiratory recovery in an in vivo rat model of central chemoreflex impairment** *Acta Physiologica* https://doi.org/10.1111/apha.14093

31. Kang B. J., Chang D. A., Mackay D. D., West G. H., Moreira T. S., Takakura A. C., Gwilt J. M., Guyenet P. G., Stornetta R. L (2007) **Central nervous system distribution of the transcription factor Phox2b in the adult rat** *Journal of Comparative Neurology* **503**:627–641 https://doi.org/10.1002/cne.21409

32. Kumar N. N. *et al.* (2015) **Regulation of breathing by CO2 requires the proton-activated receptor GPR4 in retrotrapezoid nucleus neurons** *Science* **348**:1255–1260 https://doi.org/10.1126/science.aaa0922

33. Lighton J. R. B. (2021) **Measuring metabolic rates: a manual for scientists**

34. Madani A. *et al.* (2021) **Obstructive Apneas in a Mouse Model of Congenital Central Hypoventilation Syndrome** *American journal of respiratory and critical care medicine* **204**:1200–1210 https://doi.org/10.1164/rccm.202104-0887OC

35. Malheiros-Lima MR, Takakura AC, Moreira TS (2017) **Depletion of rostral ventrolateral medullary catecholaminergic neurons impairs the hypoxic ventilatory response in conscious rats** *Neuroscience* **351**:1–14 https://doi.org/10.1016/j.neuroscience.2017.03.031

36. Marina N., Abdala A. P., Trapp S., Li A., Nattie E. E., Hewinson J., Smith J. C., Paton J. F. R., Gourine A. V (2010) **Essential Role of Phox2b-Expressing Ventrolateral Brainstem Neurons in the Chemosensory Control of Inspiration and Expiration** *Journal of Neuroscience* **30**:12466–12473 https://doi.org/10.1523/JNEUROSCI.3141-10.2010

37. Matera I (2004) **PHOX2B mutations and polyalanine expansions correlate with the severity of the respiratory phenotype and associated symptoms in both congenital and late onset Central Hypoventilation syndrome** *Journal of Medical Genetics* **41**:373–380 https://doi.org/10.1136/jmg.2003.015412

38. Mcbride J. L. *et al.* (2008) **Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi** *Proceedings of the National Academy of Sciences of the United States of America* **105**:5868–5873 https://doi.org/10.1073/pnas.0801775105

39. McCloy R. A., Rogers S., Caldon C. E., Lorca T., Castro A., Burgess A (2014) **Partial inhibition of Cdk1 in G2 phase overrides the SAC and decouples mitotic events** *Cell Cycle* **13**:1400–1412 https://doi.org/10.4161/CC.28401/SUPPL_FILE/KCCY_A_10928401_SM0001.ZIP

40. Mortola J. P., Frappell P. B. (1998) **On the barometric method for measurements of ventilation, and its use in small animals** *Can J Physiol Pharmacol.* **76**:937–944 https://doi.org/10.1139/cjpp-76-10-11-937

41. Mortola J. P., Matsuoka T., Saiki C., Naso L (1994) **RESPIRATION PHYSIOLOGY Metabolism and ventilation in hypoxic rats: effect of body mass** *Respiration Physiology* **97**:225–234

42. Nagashimada M., Ohta H., Li C., Nakao K., Uesaka T., Brunet J. F., Amiel J., Trochet D., Wakayama T., Enomoto H (2012) **Autonomic neurocristopathy-associated mutations in PHOX2B dysregulate Sox10 expression** *Journal of Clinical Investigation* **122**:3145–3158 https://doi.org/10.1172/JCI63401

43. Nattie E. E., Li A (1994) **Retrotrapezoid nucleus lesions decrease phrenic activity and CO2 sensitivity in rats** *Respiration Physiology* **97**:63–77 https://doi.org/10.1016/0034-5687(94)90012-4

44. Nattie E. E., Li A (2002) **Substance P-saporin lesion of neurons with NK1 receptors in one chemoreceptor site in rats decreases ventilation and chemosensitivity** *The Journal of Physiology* **544**:603–616 https://doi.org/10.1113/JPHYSIOL.2002.020032

45. Nobuta H. *et al.* (2015) **Dysregulation of locus coeruleus development in congenital central hypoventilation syndrome** *Acta Neuropathologica* **130**:171–183 https://doi.org/10.1007/s00401-015-1441-0

46. Parodi S., Di Zanni E., Di Lascio S., Bocca P., Prigione I., Fornasari D., Pennuto M., Bachetti T., Ceccherini I. (2012) **The E3 ubiquitin ligase TRIM11 mediates the degradation of congenital central hypoventilation syndrome-associated polyalanine-expanded PHOX2B** *Journal of Molecular Medicine* **90**:1025–1035 https://doi.org/10.1007/s00109-012-0868-1

47. Pattyn A., Hirsch M. R., Goridis C., Brunet J. F (2000) **Control of hindbrain motor neuron differentiation by the homeobox gene Phox2b. Development (Cambridge** *England* **127**:1349–1358 https://doi.org/10.1242/DEV.127.7.1349

48. Pattyn A., Morin X., Cremer H. (1999) **The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives** *Nature* **399**:366–370

49. Pattyn A., Morin X., Cremer H., Goridis C., Brunet J. F (1997) **Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development (Cambridge** *England* **124**:4065–4075

50. Paxinos G. (2007) **The rat brain in stereotaxic coordinates**

51. Pirone L., Caldinelli L., Di Lascio S., Di Girolamo R., Di Gaetano S., Fornasari D., Pollegioni L., Benfante R., Pedone E. (2019) **Molecular insights into the role of the polyalanine region in mediating PHOX2B aggregation** *FEBS Journal* **286**:2505–2521 https://doi.org/10.1111/FEBS.14841

52. Ramanantsoa N. *et al.* (2011) **Breathing without CO2 Chemosensitivity in Conditional Phox2b Mutants** *Journal of Neuroscience* **31**:12880–12888 https://doi.org/10.1523/JNEUROSCI.1721-11.2011

53. Seifert E. L., Knowles J., Mortola J. P (2000) **Continuous circadian measurements of ventilation in behaving adult rats** *Respiration Physiology* **120**:179–183

54. Shi Y., Stornetta R. L., Stornetta D. S., Suna Onengut-Gumuscu X., Farber E. A., Turner S. D., Guyenet P. G., Bayliss D. A (2017) **Neuromedin B Expression Defines the Mouse Retrotrapezoid Nucleus** *Journal of Neuroscience* https://doi.org/10.1523/JNEUROSCI.2055-17.2017

55. Shimokaze T., Sasaki A., Meguro T., Hasegawa H., Hiraku Y., Yoshikawa T., Kishikawa Y., Hayasaka K (2015) **Genotype-phenotype relationship in Japanese patients with congenital central hypoventilation syndrome** *Journal of Human Genetics* **60**:473–477 https://doi.org/10.1038/jhg.2015.65

56. Souza G. M. P. R., Kanbar R., Stornetta D. S., Abbott S. B. G., Stornetta R. L., Guyenet P. G (2018) **Breathing regulation and blood gas homeostasis after near complete lesions of the retrotrapezoid nucleus in adult rats** *Journal of Physiology* **596**:2521–2545 https://doi.org/10.1113/JP275866

57. Souza G. M. P. R., Stornetta D. S., Shi Y., Lim E., Berry F. E., Bayliss D. A., Abbott S. B. G (2023) **Neuromedin B-Expressing Neurons in the Retrotrapezoid Nucleus Regulate Respiratory Homeostasis and Promote Stable Breathing in Adult Mice** *Journal of Neuroscience* **43**:5501–5520 https://doi.org/10.1523/JNEUROSCI.0386-23.2023

58. Stanke M., Junghans D., Geissen M., Goridis C., Ernsberger U., Rohrer H (1999) **The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons** *Development* **126**:4087–4094 https://doi.org/10.1242/dev.00165

59. Stornetta R. L., Moreira T. S., Takakura A. C., Kang B. J., Chang D. A., West G. H., Mulkey D. K., Bayliss D. A., Guyenet P. G (2006) **Expression of Phox2b by Brainstem Neurons Involved in Chemosensory Integration in the Adult Rat** *Journal of Neuroscience* **26**:10305–10314 https://doi.org/10.1523/JNEUROSCI.2917-06.2006

60. Takakura A. C., Barna B. F., Cruz J. C., Colombari E., Moreira T. S (2014) **Phox2b-expressing retrotrapezoid neurons and the integration of central and peripheral chemosensory control of breathing in conscious rats** *Experimental Physiology* **99** https://doi.org/10.1113/expphysiol.2013.076752

61. Takakura A. C., Moreira T. S., Stornetta R. L., West G. H., Gwilt J. M., Guyenet P. G (2008) **Selective lesion of retrotrapezoid Phox2b-expressing neurons raises the apnoeic threshold in rats** *The Journal of Physiology* **586** https://doi.org/10.1113/JPHYSIOL.2008.153163

62. Tiveron M. C., Hirsch M. R., Brunet J. F (1996) **The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system** *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* **16**:7649–7660 https://doi.org/10.1523/JNEUROSCI.16-23-07649.1996

63. Trang H., Dehan M., Beaufils F., Zaccaria I., Amiel J., Gaultier C (2005) **The French Congenital Central Hypoventilation Syndrome Registry** *Chest* **127**:72–79 https://doi.org/10.1378/chest.127.1.72

64. Trochet D., de Pontual L., Estêvao M. H., Mathieu Y., Munnich A., Feingold J., Goridis C., Lyonnet S., Amiel J. (2008) **Homozygous mutation of the PHOX2B gene in congenital central hypoventilation syndrome (Ondine's Curse)** *Human Mutation* **29**:770–770 https://doi.org/10.1002/humu.20727

65. Trochet D., Hong S. J., Lim J. K., Brunet J. F., Munnich A., Kim K. S., Lyonnet S., Goridis C., Amiel J (2005) **Molecular consequences of PHOX2B missense, frameshift and alanine expansion mutations leading to autonomic dysfunction** *Human Molecular Genetics* **14**:3697–3708 https://doi.org/10.1093/hmg/ddi401

66. Van Gestel M. A., Van Erp S., Sanders L. E., Brans M. A. D., Luijendijk M. C. M., Merkestein M., Pasterkamp R. J., Adan R. A. H. (2014) **shRNA-induced saturation of the microRNA pathway in the rat brain** *Gene Therapy* **21**:205–211 https://doi.org/10.1038/GT.2013.76

67. Van Limpt V., Schramm A., Lakeman A., Van Sluis P., Chan A., Van Noesel M., Baas F., Caron H., Eggert A., Versteeg R. (2004) **The Phox2B homeobox gene is mutated in sporadic neuroblastomas** *Oncogene* **23**:9280–9288 https://doi.org/10.1038/sj.onc.1208157

68. Weese-Mayer D. E., Berry-Kravis E. M., Zhou L., Maher B. S., Silvestri J. M., Curran M. E., Marazita M. L (2003) **Idiopathic congenital central hypoventilation syndrome: Analysis of genes pertinent to early autonomic nervous system embryologic development and identification of mutations in PHOX2b** *American Journal of Medical Genetics* https://doi.org/10.1002/ajmg.a.20527

69. Weese-Mayer D. E., Rand C. M., Zhou A., Carroll M. S., Hunt C. E. (2017) **Congenital central hypoventilation syndrome: A bedside-to-bench success story for advancing early diagnosis and treatment and improved survival and quality of life** *Pediatric Research* **81** https://doi.org/10.1038/pr.2016.196

70. Wickström H. R., Berner J., Holgert H., Hökfelt T., Lagercrantz H (2004) **Hypoxic response in newborn rat is attenuated by neurokinin-1 receptor blockade** *Respiratory Physiology and Neurobiology* **140**:19–31 https://doi.org/10.1016/j.resp.2004.01.008

71. Wu H., Su Y., Hung C., Hsieh W., Wu K (2009) **Interaction Between PHOX2B and CREBBP Mediates Synergistic Activation : Mechanistic Implications of PHOX2B Mutants Human Mutation** *Human Mutation* https://doi.org/10.1002/humu.20929

72. Zoccal D. B., Furuya W. I., Bassi M., Colombari A, Colombari D. S., Hildreth E., Putnam C. (2014) **The nucleus of the solitary tract and the coordination of respiratory and sympathetic activities** *Front. Physiol.* https://doi.org/10.3389/fphys.2014.00238

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

Copyright

Editors

Reviewing Editor
Jeffrey Smith
National Institute of Neurological Disorders and Stroke, Bethesda, United States of America

Senior Editor
Sacha Nelson
Brandeis University, Waltham, United States of America

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 unknown. By utilizing a viral PHOX2B-shRNA approach for the 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 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 reduces the HCVR and the expression of Gpr4 and Task2 mRNAs. This suggests 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) This study's results show that independent of its role 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.

(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 the 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.

https://doi.org/10.7554/eLife.94653.2.sa2

Reviewer #2 (Public Review):

Summary:

This significant research explored how the PHOX2B transcription factor functions within neurons located in the retrotrapezoid nucleus (RTN), a crucial brainstem chemosensory area, to sustain appropriate CO2 chemoreflex reactions related to breathing in adult rats when observed in a living state. By applying a viral shRNA technique to selectively suppress PHOX2B in RTN neurons, the authors present compelling evidence of deteriorating ventilatory reactions to increased CO2 levels. This impairment progresses over a four-week period in vivo, hinting at disruptions in RTN neuron transcriptional processes and a consequent dulling of CO2-induced breathing responses. The data on RTN neuronal mRNA expression indicates that the weakened hypercapnic ventilatory response may stem from reduced levels of crucial proton sensors within the RTN. This research holds relevance for neuroscientists focused on the neurobiology of respiration and the neurodevelopmental regulation of motor functions.

Strengths:

The authors employed a shRNA viral strategy to systematically reduce PHOX2B protein levels, targeting RTN neurons specifically, to assess the importance of PHOX2B for the survival and chemosensory capabilities of adult RTN neurons in a living organism. The findings of this research underscore that beyond its developmental role, PHOX2B remains essential for sustaining accurate CO2 chemoreflex reactions in the adult brain. Furthermore, its diminished presence in Congenital Central Hypoventilation Syndrome (CCHS) could be a factor in the respiratory deficiencies observed in the condition. This study highlights the critical ongoing function of PHOX2B in adult physiology and its potential impact on respiratory health, offering valuable insights for the scientific and medical communities involved in treating and understanding respiratory disorders.

Weaknesses:

N/A

https://doi.org/10.7554/eLife.94653.2.sa1

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 postmitotic functions of PHOX2B in RTN neurons.

I have two main concerns and several points of clarification.

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's not clear whether PHOX2B is important for 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.

Other points:

(3) All individual data points should be visible in floating bar graphs in Figs 1 and 4. For example, I don't see any dots for naïve animals in any of the panels in Fig. 1.

(4) the C1 and facial partly overlap with the RTN at this level of the medulla and these cells should appear as Phox2b+/Nmb- cells so it is not clear to me why these cells are not evident in the control tissue in figs 2B and 3B. Also, some of the bregma levels shown in Fig. 5A overlap with Figs 2-3 so again it's not clear to me how this non-cell type specific viral approach was targeted to Nmb cells but not near by TH+ cells. Please clarify.

(5) How do you get a loss of Nmb+ neurons (Figs 2-3) with no change in Nmb fluorescence (Fig. 5B)? In the absence of representative images these results are not compelling and should be substantiated by more readily quantifiable approaches like qPCR.

https://doi.org/10.7554/eLife.94653.2.sa0

Author response:

The following is the authors' response to the original reviews.

> ***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.

Public Reviews:

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.

Thank you for sharing the excitement for our study showing novel findings on the contribution of PHOX2B to the chemoreflex response and activity of adult RTN neurons. We believe that reporting the results on cell death following shRNA viral injections, potentially due to some off-target effects, are important to share with the scientific community to help plan experiments of similar kind in various fields of neuroscience.

Thanks for pointing out your concerns about cell quantification, we have edited the methods and results section to add clarity about our analytical procedure.

As we discussed in the manuscript, we were only able to assess mRNA levels of Nmb, Gpr4, Task2 as current available antibodies for the 3 targets are still unreliable. Future studies will benefit from the analysis of changes at protein levels and possibly electrophysiological recordings to verify that chemosensitive properties of RTN neurons are impaired due to reduction of PHOX2B expression. We discuss these limitations in the discussion.

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.*

Thanks for the feedback on or manuscript. We have further highlighted in our discussion the previous developmental work aimed at determining the role of PHOX2B in embryonic development. Our study was triggered by the fascinating observations that despite its important role in development of the central and peripheral nervous system, PHOX2B is still present in the adult brain and its function in adult neurons is unknown, thus we aimed to investigate its role in the adult RTN by knocking down its expression with a shRNA approach. Therefore, in our model knockdown of PHOX2B does not affect development of the RTN. Previous studies (mentioned by the reviewer, as well as cited in the manuscript) have focused on investigating 1) the role of PHOX2B in the developmental period, 2) the physiological changes associated with the transgenic expression of mutant forms of PHOX2B in relation to CCHS, 3) the killing or the acute silencing/excitation of neuronal activity of PHOX2B+ RTN neurons. Our study had a different aim: to test whether the transcription factor PHOX2B had a physiologically relevant role in adult RTN neurons. In this experimental approach PHOX2B is not altered throughout embryonic or postnatal development. By knocking down PHOX2B in the Nmb+ cells of the RTN our results show a reduction in chemoreflex response and mRNA expression of protein sensors. Hence, we conclude that PHOX2B alters the function of Nmb+ RTN neurons, possibly through transcriptional changes including the reduction in Gpr4 and Task2 mRNA expression.

> ***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.*

Although we did not specifically target Nmb+ neurons, we performed viral injections within the area where neurons expressing PHOX2B and Nmb are localized (i.e., the RTN region). We carefully quantified the impact of PHOX2B knockdown on Nmb expressing neurons, as well as the effects on the adjacent TH expressing C1 population and FN neurons (figure 5). As reported in the results section, significant changes in the numbers of PHOX2B expressing neurons was only observed at the site of injection in PHOX2B+/Nmb+ neurons. We did not observe changes in the total number of C1 cells (TH+/PHOX2B+), in the number of TH cells coexpressing PHOX2B, or in the hypoxic ventilatory response (which is dependent on the health status of C1 neuron). We have updated figure 5 to show representative expression of PHOX2B in TH+ neurons in the ventral medulla to complement our cell count analysis. To address potential effects on other cell populations we have edited our discussion as follows:

“PHOX2B knockdown was also restricted to RTN neurons, as adjacent C1 TH+ neurons did not show any change in number of TH+/PHOX2B+ expressing cells, although we cannot exclude that some C1 cells may have been infected and their relative PHOX2B expression levels were reduced. To support the lack of significant alterations associated with the possible loss of C1 function was the absence of significant changes in the hypoxic response that has been shown to be dependent on C1 neurons (Malheiros-Lima et al., 2017).”

Where appropriate, we have substituted “RTN” with “Nmb expressing neurons of the RTN” throughout the manuscript.

We have clarified in the methods and results section how we quantified Task2 and Gpr4 mRNA expression. The quantification was performed on a pool of single cells (200-250/rat) expressing Nmb. Hence, the overall reduction is not a result of general fluorescence loss in the RTN region, but specifically assessed in single cells expressing Nmb. This is therefore independent of the reduction of the total number of Nmb cells.

We propose that cell death is not a direct effect of PHOX2B knockdown, but rather it is associated with the injection of the viral constructs that have been already reported to promote some off-target effects (as reported in the manuscript). While modest cell death is observed only in the first two weeks post-infection, it does not increase further between 2 and 4 weeks post infection, when the reduction in PHOX2B (not associated with a further reduction in Nmb+ cells, hence no further cell death in RTN) is evident and the respiratory chemoreflex is impaired. These results suggest that 1) reduction of PHOX2B is not responsible for cell death; 2) it is the reduction of PHOX2B levels that promotes chemoreflex impairment. Given the observation that Nmb cells with no detectable PHOX2B protein show reduced

expression of Task2 and Gpr4 mRNA, we propose that one of the possible mechanisms of chemoreflex impairment in PHOX2B shRNA rats is the reduction of Task2 and Gpr4. In the discussion we also suggest possible additional mechanisms that can be investigated in further studies.

> ***Recommendations for the authors:***.
>
> *In revising this manuscript, the authors should carefully address the issues raised by the reviewers to substantially improve the manuscript and solidify the reviewers' general assessment of the potential importance of this work.*
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Major concerns:*
>
> *(1) The cell counts for Nmb+/PHOX2B+ and Nmb+/PHOX2B- RTN neurons are a critical component of the study, and it is unclear how the tissue sampling procedures (eight sections per animal) for quantifying numbers of cells expressing proteins/mRNAs throughout the extended RTN region bilaterally has been validated to accurately represent the full expression patterns in the RTN under the experimental conditions. It is possible that the sampling/quantification procedures used may be adequate, but validation is important. Also, quantification of the CTCF signal for Nmb, Gpr4, and Task2 mRNA is an important component of this study, but only four sections/rats were used.*

Thank you for pointing out your concern on our quantification method. We have clarified in the methods section the procedure for cell counting and quantification of the CTCF signal. We have sampled the area of the RTN in order to identify Nmb cells of RTN.

We have edited the methods section as follows:

“To quantify Nmb+/PHOX2B- and Nmb+/PHOX2B+ neurons within the RTN region, we analysed one in every seven sections (210 μm interval; 8 sections/rat in total) along the rostrocaudal distribution of the RTN on the ventral surface of the brainstem and compared total bilateral cell counts of PHOX2B-shRNA rats with non-target control (NT-shRNA) and naïve rats. Cells that expressed Nmb and Phox2b mRNAs but did not show co-localization with PHOX2B protein were considered Nmb+/PHOX2B-.

The Corrected Total Cell Fluorescence (CTCF) signal for Nmb, Gpr4 and Task2 mRNAs was quantified as previously described (Cardani et al., 2022; McCloy et al., 2014). Briefly, 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. The collected images were then analysed by selecting a single cell at a time and measuring the area, integrated density and mean grey value (McCloy et al., 2014). For each image, three background areas were used to normalize against autofluorescence. We used 4 sections/rat (210 μm interval) to count Nmb, Gpr4 and Task2 mRNA CTCF in the core of the RTN area where several Nmb cells could be identified. For each section two images were acquired with a 20× objective, so that at least fifty cells per tissue sample were obtained for the mRNA quantification analysis. To evaluate changes in Nmb mRNA expression levels following PHOX2B knockdown at the level of the RTN, we compared, the fluorescence intensity of each RTN Nmb+ cell (223.2 ± 37.1 cells/animal) with the average fluorescent signal of Nmb+ cells located dorsally in the NTS (4.3 ± 1.2 cells/animal) (Nmb CTCF ratio RTN/NTS) as we reasoned that the latter would not be affected by the shRNA infection and knockdown.

To quantify Gpr4 and Task2 mRNA expression in Nmb cells of the RTN, we first quantified single cell CTCF for either Gpr4 (200.7 ± 13.2 cells/animal) or Task2 (169.6 ± 10.3 cells/animal) mRNA in Nmb+ RTN neurons in the 3 experimental groups (naïve, NT shRNA and PHOX2B

shRNA) independent of their PHOX2B expression. We then compared CTCF values of Gpr4 and Task2 mRNA between Nmb+/PHOX2B+ and Nmb+/PHOX2B- RTN neurons in PHOX2B-shRNA rats to address changes in their mRNA expression induced by PHOX2B knockdown.

> *(2) Furthermore, to evaluate changes in Nmb mRNA expression following PHOX2B knockdown at the level of the RTN, it is stated in Materials and Methods "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)". How this was accomplished is unclear, considering the non-overlapping locations of the RTN and rostral NTS. Providing images would be helpful.*

The first sections containing Nmb cells in the ventral medulla also express few Nmb cells in the dorsal medulla. We used those cells as reference for fluorescence levels since they would not be affected by the viral infection. Similar cells are also present in the brains of mice and reported in the Allen Brain atlas (https://mouse.brain-map.org/experiment/show/71836874). We have clarified our procedure in the methods section (see above) and included a sample image of Nmb in both ventral and dorsal regions in Figure 5.

> *(3) The staining for tyrosine hydroxylase (TH) to identify and quantify C1 cells (TH+/PHOX2B+) following shRNA injection provides important information, and it would be useful to show images of histological examples to accompany Fig. 5A.*

We included in figure 5A a sample image of C1 neurons used for our TH quantification.

> *Minor:*
>
> *(1) Provide animal ns in the text of the Results section for the four weeks of PHOX2B knockdown.*

They have been included.

> *(2) Please state in the legends for Figures 2 & 3, which images are superimposition images.*

We have in the figure information about merged images.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *This manuscript by Cardani and colleagues attempts to address whether a reduction in Phox2b expression in chemosensitive neuromedin-B (NMB)-expressing neurons in the RTN alters respiratory function. The authors used a short hairpin RNA technique to silence RTN chemosensor neurons. The present study is very interesting, but there are several major concerns that need to be addressed, including the main hypothesis.*
>
> *Major*
>
> *(1) Page 6, lines 119-121: I did not grasp the mechanistic property described by the authors in this passage, nor did I understand the experiments they conducted to establish a mechanistic link between Phox2b and the chemosensitive property. Could the authors provide further clarification on these points?*

We believe the reviewer refers to this paragraph: “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)."

The rationale for running these experiments is based on the fact that it is well known in the literature that PHOX2B is an important transcription factor for the development of several neuronal populations. PHOX2B Knockout mice die before birth and heterozygous mice have some anatomical defects, but respiration is only impaired in the early post-natal period. While many developmental transcription factors are generally downregulated in the post-natal period, PHOX2B is still expressed in some neurons into adulthood. What is the function of PHOX2B in these fully developed neurons? We do not know as we do not yet know the entire set of target genes that PHOX2B regulates in the adult brain. Hence we decided to test what would happen if we knocked down the PHOX2B protein in the Nmb neurons of the RTN, an area that is critical for central chemoreception and involved in the presentation of CCHS. Our results show that reduction of PHOX2B blunts the CO2 chemoreflex response and reduces mRNA expression of Task2 and Gpr4, two pH sensors that have been shown to be key for RTN chemosensitive properties. We also show that the Nmb mRNA and cell survival are not affected by PHOX2B knockdown and we propose that the reduced CO2 chemoreflex may be attributed to a reduction of chemosensory function of Nmb neurons of the RTN due to partial loss of Gpr4 and Task2.

> *(2) It is imperative for the authors to enhance the description of their hypothesis, as, from my perspective, the contribution of the data to the field is not clearly articulated. Numerous more selectively designed experiments were conducted to investigate the role of Phoxb-expressing neurons at the RTN level and their involvement in respiratory activity. In summary, the current study appears to lack novelty.*

We respectfully disagree with this statement. We believe we have adequately summarized previous work, although we realize we can't reference every single publication on this topic. As described above, the developmental role of PHOX2B has been elegantly investigated in mouse embryonic studies (extensively cited in the manuscript). Furthermore, very interesting studies have shown that when the CCHS defining mutant PHOX2B protein (+7Ala PHOX2B) and other mutations linked to CCHS have been transgenically expressed in mice through development, severe anatomical defects are observed and respiratory function is impaired (extensively cited in the manuscript). We have also cited papers relevant to this study that describe the role of PHOX2B/Nmb RTN neurons and the pH protein sensors in the CO2 chemoreflex. If we missed some papers that the reviewer deems essential in the context of this study we will be happy to include them.

We are not aware of other studies that have investigated the specific role of the PHOX2B protein in the adult RTN in the absence of confounding developmental pathogenesis (i.e. in an otherwise 'healthy' animal), and of no other studies that looked at the effects on the RTN proton sensors and Nmb expression following PHOX2B knockdown. Hence we believe that our results are novel and, in our opinion, very interesting.

> *(3) On pages 13 and 14 (Results section), I am seeking clarity on the novelty of the findings. Doug Bayliss's prior work has already demonstrated the role of Gpr4 and Task2 on Phox2b neurons in regulating ventilation in conscious rodents.*

Bayliss' group has elegantly demonstrated that Gpr4 and Task2 are the two proton sensors in the PHOX2B/Nmb neurons of the RTN that have a key role in chemoreception (cited in the manuscript). The novelty of our findings is that we show that a reduction in PHOX2B protein is associated with a reduction of mRNA levels of Gpr4 and Task2. This is a novel finding.

Currently, we do not know what transcriptional activity PHOX2B has in adult RTN neurons (i.e., what gene targets PHOX2B has in this cell population and many others) and here we propose that Nmb is not a gene target of PHOX2B while Gpr4 and Task2 are.

> *(4) The authors assert that the transcription factor Phox2b remains not fully understood. While I concur, the present study falls short of fully investigating the actual contribution of Phox2b to breathing regulation. In other words, the knockdown of Phox2b neurons did not add much to the knowledge of the field.*

We respectfully disagree with the reviewer. With the exception of very few target genes, the transcriptional role of PHOX2B beyond the embryonic development is poorly understood. No mechanistic connection has been made before between the transcriptional activity of PHOX2B with the expression of proton sensors in the RTN. Other groups have investigated the role of stimulating or depressing the neuronal activity of PHOX2B/NMB neurons in the RTN showing a key role of RTN on respiratory control, but these prior studies did not test whether changing the expression of the PHOX2B protein in these neurons had a role on respiratory control and the central chemoreflex. No other study has investigated the role of the PHOX2B protein within the RTN cells, with the exception of PHOX2B knockout mice or transgenic expression of the mutated PHOX2B that are relevant for CCHS. Again, these previous studies were done on a background of developmental impairment and to the best of our knowledge did not seek to show any association between PHOX2B expression and expression of Gpr4 or Task2.

> *(5) I recommend removing the entire section entitled "The role of Phox2b in development and in the adult brain." The authors merely describe Phox2b expression without contextualizing it within the obtained data.*

Because reviewers raised the issue about not including important information about the role of PHOX2B in development and respiratory control we prefer to keep the section.

> *(6) Are the authors aware of whether the shRNA in Phox2b/Nmb neurons truly induced cell death or solely depleted the expression of the transcription factor protein? Do the chemosensitive neurons persist?*

This is an excellent question that we tried to address with our study. As we report in figures 2 and 3, we propose that some cell death is occurring as an off-target effect within the first 2 weeks post-infection, likely due to off-target action of the shRNA approach and not dependent on the reduction of PHOX2B expression (discussed in the manuscript). This is further evidenced by our Fig.S1 data in which higher concentrations of shRNA led to more cell death, indicative of off-target effects. We do not believe it is a consequence of our surgical procedure as we do not see similar cell loss when injecting vehicle or other control solutions (unpublished work; Janes et al., 2024).

During the first 2 weeks post-surgery the proportion of Nmb+/PHOX2B- cells does not change compared to control rats or non-target shRNA (knockdown is not yet visible at protein level). Four weeks post-injection, there is no further cell death (assessed by the total number of NMB cells), whereas the fraction of NMB cells that express PHOX2B is reduced (and the fraction of NMB not expressing PHOX2B is increased), suggesting that the reduction of PHOX2B protein in Nmb cells is not correlated with cell loss/survival whereas the impairment that we observe in terms of central chemoreception is possibly due to the progressive decrease of PHOX2B expression in these neurons.

> *(7) In Figures 2 and 3, it is noteworthy that the authors observe peak expression at a very caudal level. In rats, the RTN initiates at the caudal end of the facial, approximately 11.6*

> *mm, and should exhibit a rostral direction of about 2 mm.*

In our experience the Nmb cells on the ventral surface of the medulla peak in number around the caudal tip of the facial nucleus in adult SD rats (Janes et al., 2024). To add clarity to the figure we reported cell count distribution data in relation to the distance from caudal tip of the facial.

> *Minor*
>
> *(1) I would like to suggest that the authors correct the recurring statement throughout the manuscript that Phox2b is essential only for the development of the autonomic nervous system. In my view, it also plays a crucial role in certain sensory and respiratory systems.*

We have addressed this in the manuscript.

> *(2) Page 4, lines 59-60: Out of curiosity, do the data include information from different countries?*

This data refers to information from France and Japan. Currently it is estimated that there are 1000-2000 CCHS patients worldwide.

> *(3) Page 7, lines 129-131: In my understanding, the sentence is quite clear; if we knock down the PHOX2B gene, we are expected to reduce or even eliminate the expression of Gpr4 or Task2. Am I right?*

This is what we propose from the results of this study. We would like to point out that the transcriptional activity of PHOX2B (i.e., what genes PHOX2B regulate) in adult neurons has not yet been fully investigated. With the exception of few target genes (e.g., TH, DBH) the transcriptional activity of PHOX2B in neurons is not yet known. Here we report novel findings that suggest that Gpr4 and Task2 are potential target genes of PHOX2B in RTN neurons.

> *(4) The authors mentioned that NT-shRNA also impacts CO2 chemosensitivity. Could this effect be attributed to mechanical damage of the tissue resulting from the injection?*

Just to clarify, we observe some impairment in chemosensitivity when NT-shRNA was injected in “larger” (2x 200ul/side) volume. No impairment was observed in NT-shRNA when we injected smaller volumes (2x 100ul/side). Physical damage could be a possibility although in our experience (unpublished work; Janes et al, 2024, Acta Physiologica) injections of similar volume of solution performed by the same investigator in the same brain area and experimental settings did not produce a physical lesion associated with respiratory impairment. Hence we attribute the unexpected results with larger volumes to toxic effects associated with the shRNA viral constructs.

> *(5) In the reference section, the authors should review and correct some entries. For instance, Janes, T. A., Cardani, S., Saini, J. K., & Pagliardini, S. (2024). Title: "Etonogestrel Promotes Respiratory Recovery in an In Vivo Rat Model of Central Chemoreflex Impairment." Running title: "Chemoreflex Recovery by Etonogestrel." Some references contain the journal, pages, and volume, while others lack this information entirely.*

We have updated references. Janes et al., 2024 has now been published in Acta Physiologica.

> *(6) Why does the baseline have distribution points, whereas the other boxplots do not?*

We have clarified in the figure legend that, to be fair to the presentation of our results, the data points shown in some of the boxplot graphs do not refer to entire baseline data but only the ones that are outliers.

In our Box-and Whisker-Plots, whiskers represent the 10th and 90th percentiles, showing the range of values for the middle 80% of the data. Individual data values that fall outside the 10th/90th percentile range are represented as single point (outliers).

> ***Reviewer #3 (Recommendations For The Authors):***
>
> - *What is the rationale behind dedicating the first paragraph of results to discussing an artifact?*

We think that it is important to report off target effects of shRNA viral constructs as concentration and volumes of viruses injected in various studies vary considerably and other investigators may attempt to use larger volumes of viruses to obtain more considerable or faster knockdown but would obtain erroneous conclusions if appropriate tests are not performed.

Furthermore, because some readers could question whether we injected enough virus to knockdown the expression of PHOX2B, and may wonder if with a larger amount of virus we would increase knockdown efficiency, we wanted to show that, in our opinion, we used the maximum amount of virus to knockdown PHOX2B without causing toxic effects or physiological changes that are not dependent on PHOX2B knockdown.

> - *All individual data points should be visible in floating bar graphs in Figures 1 and 4. For example, I don't see any dots for naïve animals in any of the panels in Figure 1.*

We have clarified in the figure legend that, to be fair to the presentation of our results, the data points shown in some of the boxplot graphs do not refer to entire baseline data but only the ones that are outliers.

In our Box-and Whisker-Plots, whiskers represent the 10th and 90th percentiles, showing the range of values for the middle 80% of the data. Individual data values that fall outside the 10th/90th percentile range are represented as single point (outliers).

> - *Please include specific F and T values along with DF.*

We have included a table with all the specific values in the supplementary section as Table 1.

> - *The C1 and facial partly overlap with the RTN at this level of the medulla and these cells should appear as Phox2b+/Nmb- cells so it is not clear to me why these cells are not evident in the control tissue in Figures 2B and 3B. Also, some of the bregma levels shown in Figure 5A overlap with Figures 2-3 so again it is not clear to me how this non-cell type specific viral approach was targeted to Nmb cells but not nearby TH+ cells. Please clarify.*

In our experience, C1 TH cells are located slightly medial to the Nmb cells and they spread much more caudally than Nmb cells of the RTN. We focused our small volume injection in the core of the RTN to target Nmb cells but we also assessed PHOX2B knockdown in TH C1 cells by counting the PHOX2B/TH cells across treatment groups. Although we can't exclude subtle changes in the C1 population, we did not observe changes in the total number of C1 cells

(TH+/PHOX2B+), in the number of TH cells expressing PHOX2B, or in the hypoxic ventilatory response (which is dependent on the health status of C1 neuron). We have updated figure 5 to show representative expression of PHOX2B in TH+ neurons in the ventral medulla to complement our cell count analysis. To address potential effects on other cell populations we have edited our discussion as follows:

"PHOX2B knockdown was also restricted to RTN neurons, as adjacent C1 TH+ neurons did not show any change in number of TH+/PHOX2B+ expressing cells, although we cannot exclude that some C1 cells may have been infected and their relative PHOX2B expression levels were reduced. To support the lack of significant alterations associated with the possible loss of C1 function was the absence of significant changes in the hypoxic response that has been shown to be dependent on C1 neurons (Malheiros-Lima et al., 2017)."

> - *To confirm, Nmb is not expressed in the NTS, and this region was chosen as a background, right?*

In order to systematically analyze Nmb mRNA expression we decided to use measurement of fluorescence relative to Nmb neurons present in the dorsal brainstem. Here cells are sparse but we used them as reference fluorescence since they would not be affected by the ventral shRNA injection. Similar cells are also present in the brains of mice and reported by the Allen Brain atlas (https://mouse.brain-map.org/experiment/show/71836874). We have clarified our procedure in the methods section (see above) and included a sample image of Nmb in both ventral and dorsal in Figure 5.

> - *How do you get a loss of Nmb+ neurons (Figs 2-3) with no change in Nmb fluorescence (Fig. 5B)? In the absence of representative images these results are not compelling and should be substantiated by more readily quantifiable approaches like qPCR.*

We have clarified in the methods and results section our analytical procedure to assess PHOX2B and Nmb expression. Figure 2 and 3 display the results of counting numbers of Nmb+ cells in the RTN. Figure 5B reports the average of total cell fluorescence measured inside Nmb+ cells, not an average fluorescence measurement of the area of the ventral medulla. Basically, our results show that we have less Nmb cells that express PHOX2B but the overall Nmb mRNA fluorescence (expression) in Nmb cells relative to Nmb fluorescence in cells of the dorsal brainstem is the same.

We have edited the methods as follows:

"The Corrected Total Cell Fluorescence (CTCF) signal for Nmb, Gpr4 and Task2 mRNAs was quantified as previously described (Cardani et al., 2022; McCloy et al., 2014). Briefly, 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. The collected images were then analysed by selecting a single cell at a time and measuring the area, integrated density and mean grey value (McCloy et al., 2014). For each image, three background areas were used to normalize against autofluorescence. We used 4 sections/rat (210 µm interval) to count Nmb, Gpr4 and Task2 mRNA CTCF in the core of the RTN area where several Nmb cells could be identified. For each section two images were acquired with a 20× objective, so that at least fifty cells per tissue sample were obtained for the mRNA quantification analysis. To evaluate changes in Nmb mRNA expression levels following PHOX2B knockdown at the level of the RTN, we compared the fluorescence intensity of each RTN Nmb+ cell (223.2 ± 37.1 cells/animal) with the average fluorescent signal of Nmb+ cells located dorsally in the NTS (4.3 ± 1.2

cells/animal) (Nmb CTCF ratio RTN/NTS) as we reasoned that the latter would not be affected by the shRNA infection and knockdown. “

A single cell qPCR analysis would be definitely ideal but a qPCR from dissected tissue would not help us determine whether within a cell there was a reduction in Nmb mRNA levels.

> - *The boxed RTN region in these examples is all over the place. It the RTN should be consistently placed along the ventral surface under the facial and pprox.. equal distance from the trigeminal and pyramids.*

We have update the figures to consistently present the areas of interest where Nmb cells are located and images are taken.

> - *Fluorescent in situ typically appears as discrete puncta so it is not clear to me why that is not the case here.*

Our images are taken at low magnification (20X) where it is difficult to distinguish the single mRNA molecules. However, is it possible to appreciate the differences between the grainy fluorescent signal in the in situ hybridization assay (RNAScope) and the smoother signal of protein detection in the immunofluorescence assay.

> - *Can TUNEL staining be done to confirm loss of Nmb neurons is due to death and not re-localization?*

Does the reviewer mean “cell migration” with relocalization? We do not expect that this would occur in our experiments. Although TUNEL in the first week post-infection could be useful to determine cell death in our tissue, we do not expect a cell migration of neurons within the brain as our viral shRNA injections are performed in adult rats when developmental processes are already concluded.