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RESEARCH ARTICLE

The Molecular Circadian Clock of Phox2b-expressing Cells Drives Daily Variation of the Hypoxic but Not Hypercapnic Ventilatory Response in Mice

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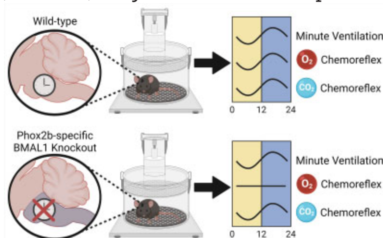
Abstract

While the suprachiasmatic nucleus (SCN) controls 24-h rhythms in breathing, including minute ventilation (V_E), the mechanisms by which the SCN drives these daily changes are not well understood. Moreover, the extent to which the circadian clock regulates hypercapnic and hypoxic ventilatory chemoreflexes is unknown. We hypothesized that the SCN regulates daily breathing and chemoreflex rhythms by synchronizing the molecular circadian clock of cells. We used whole-body plethysmography to assess ventilatory function in transgenic BMAL1 knockout (KO) mice to determine the role of the molecular clock in regulating daily rhythms in ventilation and chemoreflex. Unlike their wild-type littermates, BMAL1 KO mice exhibited a blunted daily rhythm in V_E and failed to demonstrate daily variation in the hypoxic ventilatory response (HVR) or hypercapnic ventilatory response (HCVR). To determine if the observed phenotype was mediated by the molecular clock of key respiratory cells, we then assessed ventilatory rhythms in BMAL1$^{fl/fl}$; Phox2b$^{Cre/+}$ mice, which lack BMAL1 in all Phox2b-expressing chemoreceptor cells (hereafter called BKOP). BKOP mice lacked daily variation in HVR, similar to BMAL1 KO mice. However, unlike BMAL1 KO mice, BKOP mice exhibited circadian variations in V_E and HCVR comparable to controls. These data indicate that the SCN regulates daily rhythms in V_E, HVR, and HCVR, in part, through the synchronization of the molecular clock. Moreover, the molecular clock of Phox2b-expressing cells is specifically necessary for daily variation in the hypoxic chemoreflex. These findings suggest that disruption of circadian biology may undermine respiratory homeostasis, which, in turn, may have clinical implications for respiratory disease.



Key words: Phox2b; ventilation; hypercapnic ventilatory response; hypoxic ventilatory response; circadian rhythms; BMAL1

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Introduction

Many physiological processes exhibit circadian rhythmicity, including measures of respiratory function.[1] Minute ventilation (V_E), for example, peaks during the active phase in rodents[2–4] and humans.[5] The daily rhythm in V_E is controlled by the master circadian clock in the brain, the suprachiasmatic nucleus (SCN),[3] and oscillates independent of sleep–wake state[2] and activity level.[6] Despite the SCN being crucial for organizing daily ventilatory behavior, the specific mechanisms by which the circadian clock organizes daily rhythms in breathing are not well understood. Moreover, the extent to which the circadian clock controls other aspects of breathing, such as ventilatory chemoreflex, remains unknown. Indeed, ventilatory responses to hypercapnia and hypoxia also exhibit daily variation in rodents[7,8] and in humans.[5,9] Ventilatory chemoreflex is influenced by clock-controlled variables such as sleep–wake state.[10,11] However, daily variation in the hypercapnic ventilatory response (HCVR) persists independent of sleep–wake state,[5,7] suggesting circadian regulation of ventilatory chemoreflex is influenced by other clock-derived mechanisms that are yet to be elucidated.

One mechanism by which the SCN may control or modulate ventilatory behavior is by synchronizing the molecular, circadian clock within key respiratory tissues or cell populations. Through direct neuronal projections and endocrine signaling, the SCN sends timing signals to organize the expression of clock genes in all cells and tissues.[12] These local, molecular rhythms are driven by a transcriptional–translational feedback loop of clock genes, which produce a ~24-h rhythm in expression. Clock genes, including the core clock gene BMAL1, oscillate rhythmically within the respiratory network of the brainstem.[13] However, it is unknown whether the cyclic expression of clock genes contributes to daily variation in breathing behavior. Within the respiratory network, the genetic marker Paired Like Homeobox 2b (Phox2b) is expressed within many neural populations involved in respiratory control and chemoreception, including the retrotrapezoid nucleus (RTN), the nucleus of tractus solitarius (NTS), the locus coeruleus (LC), C1 adrenergic neurons of the rostral ventrolateral medulla (RVLM), the nucleus ambiguus (NA), and the carotid bodies (CB).[14–16] Mutation of the Phox2b gene results in congenital central hypoventilation syndrome—a disease characterized by a dramatic reduction in breathing accompanied by an imbalance in blood gas levels.[16,17] Removal of Phox2b expression results in death within hours after birth due to severe breathing difficulties.[18] Despite Phox2b-expressing cells being pivotally involved in ventilatory function and chemoreception,[17,19] it is unknown to what extent the molecular clock within this population modulates daily rhythms in ventilation and ventilatory chemoreflex.

In the present study, we used whole-body plethysmography to determine the role of the molecular clock in regulating daily rhythms in ventilation and chemoreflex. We found that mice lacking a functional molecular clock exhibit a blunted daily rhythm in V_E and fail to demonstrate day–night variation in the hypoxic ventilatory response (HVR). Loss of the molecular clock also results in an overall decrease in the HCVR and a sex-specific absence of circadian variation of HCVR. We further demonstrate that daily variation of HVR is mediated exclusively by the molecular clock within Phox2b-expressing cells. To our knowledge, this is the first study to demonstrate that the molecular circadian clock contributes to daily variation in V_E and ventilatory chemoreflex. Furthermore, these data are the first to identify that the molecular clock of Phox2b-expressing cells is necessary for daily variation in the HVR of mice.

Materials and Methods

All methods were reviewed, approved by, and performed according to the guidelines of the Institutional Animal Care and Use Committee of Marquette University (Milwaukee, WI, USA).

Animals

Heterozygous BMAL1 knockout (KO) mice (Strain# 009100)[20] were purchased from the Jackson Laboratory (JAX) and used to generate homozygous BMAL1 KO mice and wild-type control littermates. $BMAL1^{fl/fl}$ (Strain# 007668)[21] and $Phox2b^{cre/+}$ (Strain# 016223)[22] founder mice were purchased from JAX and bred together to create the BKOP line. All mouse strains obtained from JAX were on a C57Bl/6 J background. For all studies, we used age-matched male and female littermates (11–34 wk old) from the F2 and F3 generations. Experimental BKOP mice ($BMAL1^{fl/fl}$; $Phox2b^{cre/+}$) were compared to littermate controls. We found no differences in V_E, HVR, or HCVR between $BMAL1^{fl/fl}$; $Phox2b^{+/+}$ and $BMAL1^{+/+}$; $Phox2b^{cre/+}$ mice (Supplementary Figure S1), therefore, data from these two genotypes were pooled into one control group. All mice were single-housed and provided water and 21% kcal/fat chow diet (PicoLab Mouse Diet 20) *ad libitum*. Mice were housed on a 12-h light: 12-h dark, light–dark cycle with lights onat 8 AM and lights off at 8 PM. ZT0 and ZT12 were defined as light onset and dark onset, respectively. Additional experiments were performed under constant darkness to remove the organizational effects of light.

Ventilatory Measurements

All ventilatory data were collected using whole-body plethysmography (Scireq/Emka Technologies, Paris, France). For assessment of 24-h V_E, mice were acclimated to the chambers for 1 h prior to data collection. Chamber temperature and humidity readings were recorded alongside ventilatory signals. The Drorbaugh and Fenn equation[23] was used to apply a volume correction factor to pressure signals on a breath-by-breath basis. IOX 2.10 software was used to generate real-time ventilatory measures. Threshold requirement settings included a minimum breathing flow rate of 0.5 mL/s, deviation of 80% or less between the inspiratory and expiratory volumes of a putative breath as well as adherence to the following mouse-specific breathing parameters: inspiratory time (50–2000 ms), expiratory time (50–1000 ms), tidal volume (0.04–0.63 mL), and respiratory frequency (10–650 bpm). These parameters auto-excluded any pressure surges due to arousal or behaviors associated with elevated activity (grooming, sniffing, etc.). Breaths were averaged into 30-min bins to create a 24-h ventilatory profile. Food and water were provided *ad libitum* in the chambers during data collection. For assessment of HCVR and HVR, mice were acclimated to the plethysmograph chambers for 30 min prior to recording. Mice received a room air equivalent (21% O_2, 79% N_2, 0% CO_2) of compressed gas during 4–5 min of baseline breathing followed by 4–5 min of hypercapnia (21% O_2, 74% N_2, 5% CO_2) or hypoxia (10% O_2, 90% N_2, 0% CO_2). The HCVR and HVR were calculated as the slope of V_E between inspired 0%–5% CO_2 and 21%–10% O_2, respectively. HCVR and HVR were measured at ZT6 (light phase) and ZT14 (dark phase). If necessary, mice were kept in a quiet

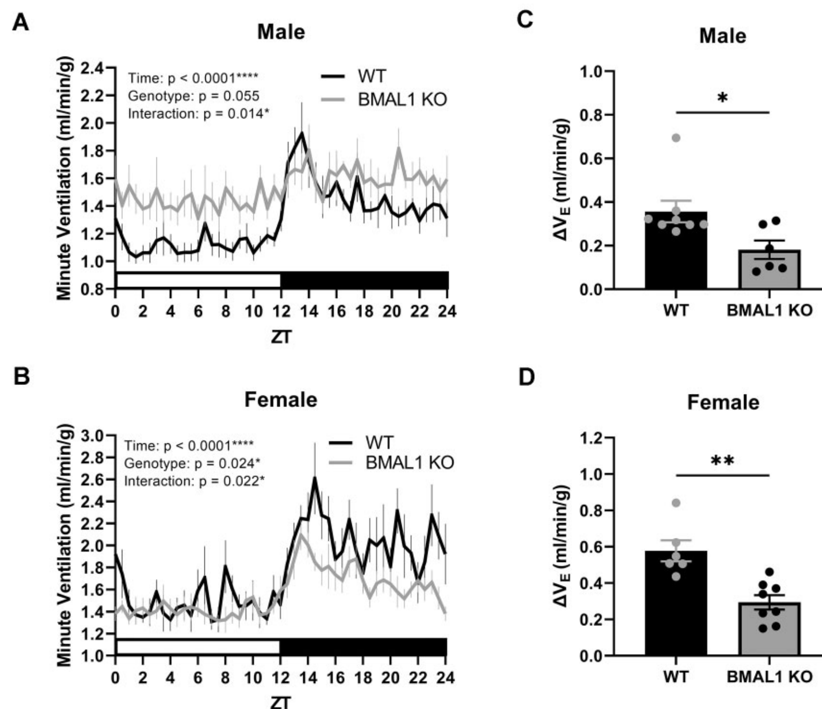

Figure 1. The daily rhythm in V_E is blunted in BMAL1 KO mice. (A) The 24-h V_E of male wild-type mice (black line, $n = 8$) was significantly different relative to BMAL1 KO mice (gray line, $n = 6$). (B) The 24-h V_E of female wild-type mice (black line, $n = 6$) was significantly different relative to BMAL1 KO mice (gray line, $n = 8$). The change in mean V_E (ie, ΔV_E) between light and dark phases of male (C) and female (D) BMAL1 KO mice was decreased relative to wild-type littermates. (A and B) Repeated measures 2-way ANOVA with Sidak's post-hoc. (C and D) Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. Abbreviations: WT, wild-type; KO, knockout; V_E, minute ventilation; and ZT, zeitgeber time.

wakeful state during the brief, 4–5 min assessment of HCVR and HVR by gently tapping on the chamber to prevent sleep-induced changes in breathing.

Indirect Calorimetry

For assessment of daily patterns in VO_2 and VCO_2, mice were housed in Promethion home-monitoring cages (Sable Systems, North Las Vegas, NV, USA). All mice were acclimated to their cages at least one full day prior to data collection. Oxygen consumption and carbon dioxide production rates were continuously recorded and averaged into 30-min bins to create 24-h profiles.

Statistical Methods

All data are presented as the mean ± SEM. Group differences were tested using *t*-tests (2-tailed), and 2- or 3-way analyses of variance (ANOVA), as appropriate, with post-hoc tests and repeated measures as indicated using GraphPad Prism 9. Rhythmicity and acrophase of V_E was determined by cosinor fit analysis using CircWave V1.4 software.[24]

Results

Loss of BMAL1 Attenuates the Daily Rhythm in V_E

To investigate the role of the molecular clock in driving daily ventilatory rhythms, we measured 24-h V_E in wild-type mice and mice with genetic deletion of the core clock gene BMAL1 (Figure 1). As expected, wild-type mice exhibited a daily rhythm in V_E that peaked during the animals' active phase in the dark ($P < 0.0001$; Supplementary Figure S2A and B). However, both male (Figure 1A) and female (Figure 1B) BMAL1 KO mice demonstrated altered 24-h patterns of V_E relative to wild-type littermates. While BMAL1 KO mice exhibited a significant 24-h V_E rhythm according to cosinor fit analysis ($P < 0.0001$; Supplementary Figure S2A and B), the change in mean V_E between the light and dark phases was reduced, indicative of a dampened V_E rhythm (Figure 1C and D). We also noted that biological sex played a prominent role in the daily V_E of BMAL1 KO mice, as there was a significant interaction effect of sex and genotype on daily V_E (repeated measures 3-way ANOVA, $P < 0.01$). In male BMAL1 KO mice, an increase in mean V_E during the light phase primarily contributed to the attenuated V_E rhythm (Supplementary Figure S2C). Whereas in female BMAL1 KO mice, the attenuated V_E rhythm primarily stemmed from a decrease in V_E during the dark phase (Supplementary Figure S2D). The body weight of BMAL1 KO mice did not differ from wild-type, sex-matched controls (Supplementary Figure S3A). However, daily mean V_E of BMAL1 KO mice was significantly different than wild-type controls in a sex-specific manner (Supplementary Figure S3B). Notably, the V_E of BMAL1 KO mice exhibited a similar pattern as the animals' metabolic rate, particularly in males (Supplementary Figure S4), suggesting that in the absence of a functioning molecular clock, the dampened V_E rhythms may be secondary to changes in metabolism.[25] Collectively, these data indicate that loss of molecular time-keeping attenuates the daily rhythm in V_E.

Loss of BMAL1 Disrupts Circadian Variation of Ventilatory Chemoreflex

We next determined the extent to which the molecular clock contributes to daily variation in the ventilatory chemoreflex

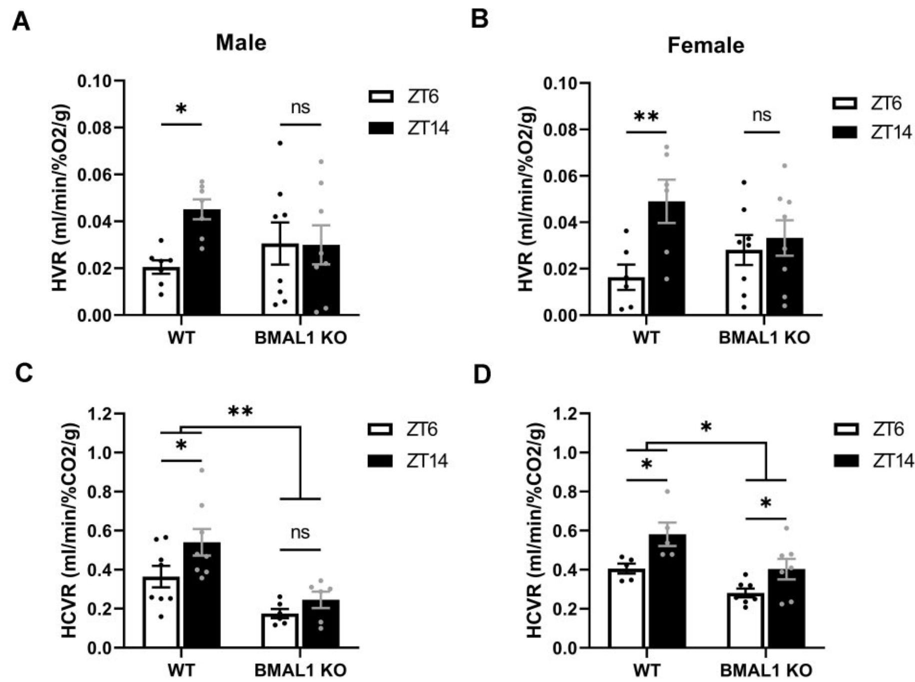

Figure 2. Circadian regulation of ventilatory chemoreflex is largely abolished in BMAL1 KO mice. (A) In contrast to wild-type mice ($n = 7$), male BMAL1 KO mice ($n = 8$) fail to demonstrate day–night variation in HVR. (B) In contrast to wild-type mice ($n = 6$), female BMAL1 KO mice ($n = 8$) fail to demonstrate day–night variation in HVR. (C) Male BMAL1 KO mice ($n = 6$) demonstrate an overall decrease in HCVR compared to wild-type mice ($n = 8$) and a lack of daily variation in HCVR. (D) Female BMAL1 KO mice ($n = 7$) demonstrate an overall decrease in HCVR relative to wild-type mice ($n = 5$) but retain a significant day–night difference in HCVR. (A-D) Repeated measures 2-way ANOVA with Sidak's post hoc. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: WT, wild-type; KO, knockout; ZT, zeitgeber time; ns, not significant; HVR, hypoxic ventilatory response; and HCVR, hypercapnic ventilatory response.

by measuring the ventilatory responses to hypoxia and hypercapnia in wild-type and BMAL1 KO mice (Figure 2). HVR and HCVR were measured during the light phase (ZT6) and dark phase (ZT14) to assess day–night variation. As expected, wild-type mice exhibited significant day–night differences in both HVR and HCVR (Figure 2A-D). In contrast, day–night differences in HVR were completely lost in BMAL1 KO mice (Figure 2A and B). Interestingly, the day–night variation in HCVR was lost in male BMAL1 KO mice (Figure 2C), but not females (Figure 2D). Additionally, the HCVR of BMAL1 KO mice was decreased overall relative to wild-type littermates for both sexes. Taken together, these data demonstrate that circadian regulation of ventilatory chemoreflex is largely abolished in mice lacking a functional molecular clock, with HCVR being disrupted only in male mice.

Loss of BMAL1 Within Phox2b-expressing Cells Does Not Alter Resting Energy Balance

Phox2b is expressed mainly in neural populations involved in respiratory control, including the RTN, NTS, LC, RVLM, NA, and CB (Figure 3A).[14–16] Phox2b-expressing cells maintain blood gas homeostasis by acting as chemoreceptors within the brainstem and periphery.[17,19,26] These chemosensitive cells provide excitatory connections to the respiratory pattern generator to increase ventilation in response to altered blood gas concentration.[27,28] To determine whether Phox2b-expressing cells specifically underly the disruption of ventilatory rhythms in global BMAL1 KO mice, we used a Cre-lox system to generate a transgenic model in which BMAL1 is knocked out from all Phox2b-expressing cells (BKOP; Figure 3A). The body weight of BKOP mice did not differ from wild-type, sex-matched controls (Figure 3B). Additionally, BKOP mice exhibited similar daily patterns of oxygen consumption ($P = 0.63$; Figure 3C) and carbon dioxide production ($P = 0.76$; Figure 3D) compared to littermate controls. These data suggest that deletion of BMAL1 within Phox2b-expressing cells does not alter energy balance under resting conditions.

BMAL1 Expression Within Phox2b-expressing Cells is Not Required to Produce a Daily Rhythm in V_E

Next, we determined the extent to which the molecular clock of Phox2b-expressing cells accounts for the disrupted daily rhythm in V_E of BMAL1 KO mice. To remove any possible confounds with light and its synchronizing effects on rhythms, wild-type and BKOP mice were assessed while held in constant dark conditions. Both male (Supplementary Figure S5A) and female (Supplementary Figure S5B) littermate controls demonstrated robust circadian rhythms in V_E ($P < 0.0001$ for both), with acrophases occurring at approximately CT18. Similarly, male and female BKOP mice maintained robust daily rhythms in V_E comparable to wild-type controls ($P < 0.0001$ for both). Overall, there was no effect of genotype on the daily V_E rhythms of male (Figure 4A, $P = 0.39$) or female (Figure 4B, $P = 0.99$) mice. Additionally, the change in mean V_E between the resting and active phases was similar in male (Figure 4C) and female (Figure 4D) BKOP mice relative to control littermates. Interestingly, we noted a trending interaction effect of time of day, sex, and genotype on V_E (repeated measures 3-way ANOVA, $P = 0.06$). Taken together, these data indicate that BMAL1 expression within Phox2b-expressing cells is not required to produce a daily rhythm in V_E under free-running conditions and that the V_E changes observed in BMAL1 KO mice are likely not due to the lack of a molecular clock within Phox2b-expressing cells.

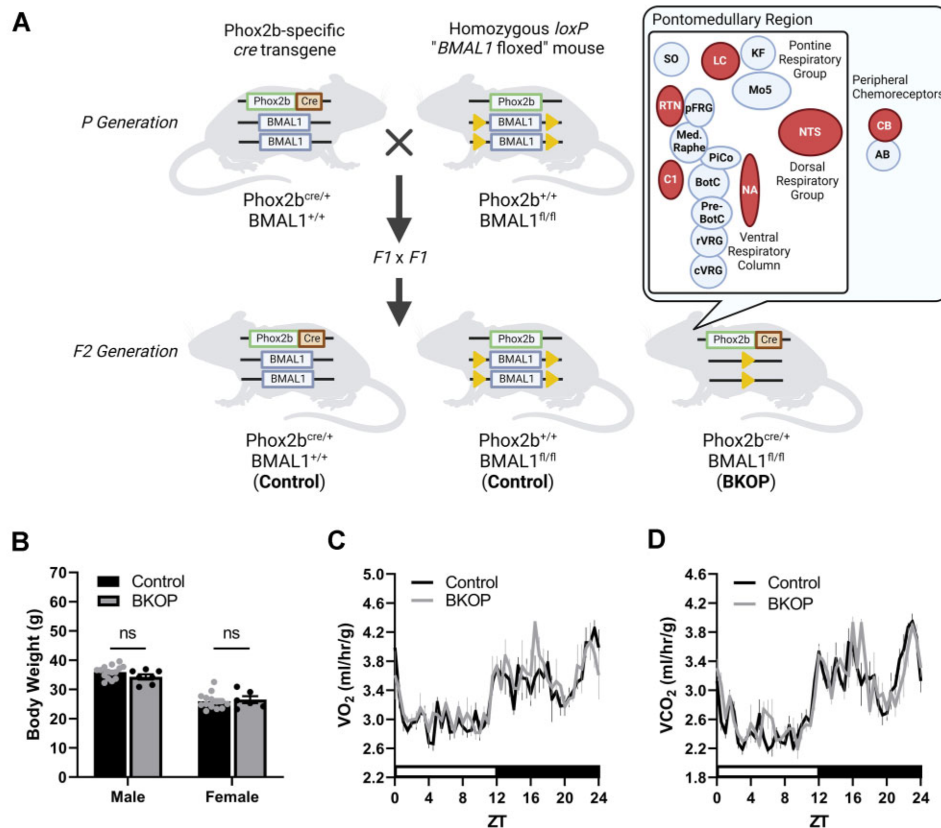

Figure 3. Resting energy balance is unaltered in BKOP mice. (A) Breeding strategy for the BKOP mouse with targeted ponto-medullary and peripheral brain regions. $Phox2b^{cre/+}$ mice were bred with $BMAL1^{fl/fl}$ mice, producing F1 offspring heterozygous for "floxed" BMAL1. F1 mice were bred together to generate F2 offspring with Cre-mediated deletion of BMAL1 from all Phox2b-expressing cells (ie, BKOP). F2 mice lacking either Cre recombinase or LoxP sites were used as controls. Yellow triangles denote LoxP sites. Red shading denotes regions that express Phox2b. Created with Biorender.com. (B) Male ($n = 7$) and female ($n = 6$) BKOP mice are similar in body weight to male ($n = 15$) and female ($n = 14$) wild-type controls at 20 wk of age. (C) BKOP (gray line, $n = 3$) and wild-type (black line, $n = 5$) mice have similar daily patterns of oxygen consumption. (D) BKOP (gray line, $n = 3$) and wild-type (black line, $n = 5$) mice have similar daily patterns of carbon dioxide production. (B) Two-way ANOVA with Sidak's post hoc. (C and D) Repeated measures 2-way ANOVA with Sidak's post hoc. Abbreviations: BKOP, BMAL1 knocked out of Phox2b cells; VO_2, oxygen consumption; VCO_2, carbon dioxide production; ZT, zeitgeber time; ns, not significant; LC, locus coeruleus; KF, Kölliker-Fuse nucleus; Mo5, trigeminal motor nucleus; SO, superior olive; RTN, retrotrapezoid nucleus; pFRG, parafacial respiratory group; Med. Raphe, medullary raphe nuclei; NTS, nucleus tractus solitarius; PiCo, pre-inspiratory complex; BotC, Bötzinger complex; Pre-BotC, preBötzinger complex; C1, adrenergic neurons of the RVLM; NA, nucleus ambiguus; rVRG, rostral ventral respiratory group; and cVRG, caudal ventral respiratory group.

BMAL1 Expression Within Phox2b-expressing Cells is Necessary for Daily Variation of HVR but Not HCVR in Male Mice

Since many Phox2b-expressing cells act as chemoreceptors, we next determined whether lack of a molecular clock within Phox2b-expressing cells would disrupt the day–night variation in ventilatory chemoreflex (Figure 5). Similar to BMAL1 KO mice (Figure 2A), male BKOP mice lacked a day–night difference in HVR (Figure 5A), indicating that the molecular clock specifically within Phox2b-expressing cells is necessary for the daily variation of HVR. However, male BKOP mice demonstrated daily variation in HCVR consistent with wild-type controls (Figure 5B). This daily variation was additionally observed in the absence of light cues (Supplementary Figure S6A). Moreover, male BKOP mice demonstrated a trend for an overall augmentation of HCVR relative to littermate controls ($P = 0.08$). Based on our study's sample size, female mice failed to exhibit significant daily variation in HCVR (Supplementary Figure S6B and C) or HVR (Supplementary Figure S6D), even in littermate controls. Collectively, these results indicate that the molecular clock of Phox2b-expressing cells is required for circadian variation of HVR, but not HCVR, in male mice.

Discussion

Many components of respiratory function exhibit daily rhythms, such as resting ventilation,[2–6] and ventilatory chemoreflex.[7–9] While the SCN is known to regulate some variables of daily breathing,[3] the specific mechanisms are not well understood, and it remains unclear the extent to which the endogenous circadian clock controls other variables of respiratory function such as ventilatory chemoreflex. Here, we performed whole-body plethysmography on global and conditional BMAL1 KO mice to determine the extent that the SCN regulates the daily ventilatory rhythms of mice in a time- and cell-specific manner through its organization of the molecular clock.

In line with prior studies,[2–4] we found that wild-type mice exhibit a daily rhythm in V_E that peaks during the mid-dark phase. However, BMAL1 KO mice demonstrated a blunted daily rhythm in V_E. This agrees with the finding that the daily rhythm in V_E is diminished in SCN-lesioned mice.[3] The presence of a detectable, albeit blunted, V_E rhythm in BMAL1 KO mice is likely attributable to the organizational effects of light independent of the circadian clock. Indeed, some behavioral rhythms such as locomotor activity and metabolic rate become organized into

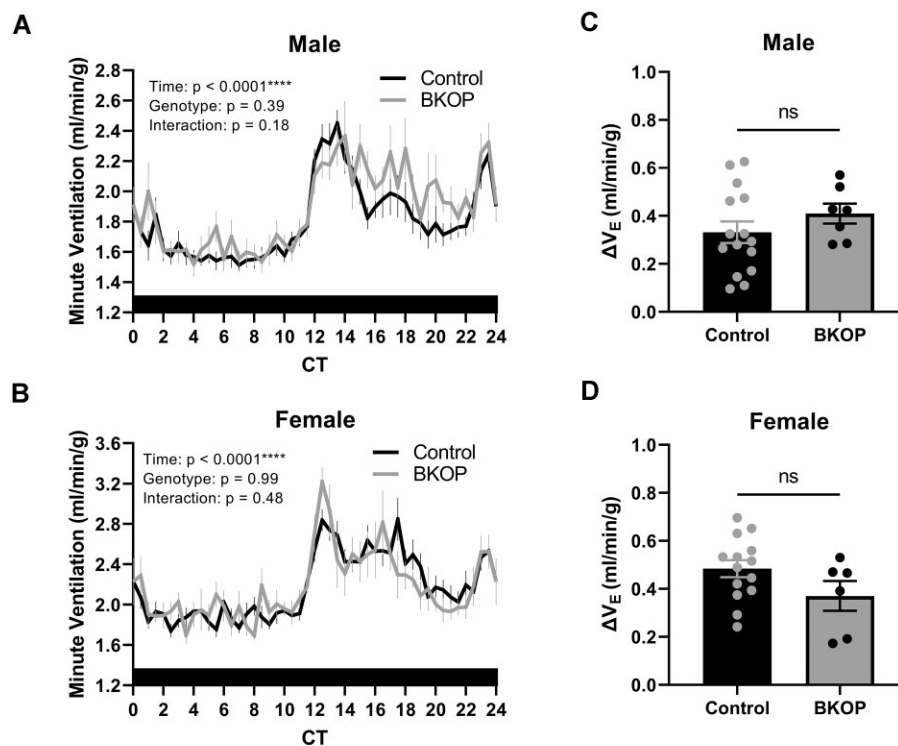

Figure 4. BKOP mice demonstrate a daily rhythm in V_E comparable to wild-type littermates. (A) Male BKOP mice (gray line, $n = 7$) demonstrate a daily rhythm in V_E comparable to wild-type controls (black line, $n = 15$) when held in constant darkness. (B) Female BKOP mice (gray line, $n = 6$) similarly demonstrate a daily rhythm in V_E comparable to wild-type controls (black line, $n = 14$) under constant darkness. The change in mean V_E (ie, ΔV_E) between the resting (CT0-12) and active (CT12-24) phases of male (C) and female (D) BKOP mice was similar relative to control littermates. (A and B) Repeated measures 2-way ANOVA with Sidak's post-hoc. (C and D) Student's *t*-test. $^{****}P < 0.0001$. Abbreviations: BKOP, BMAL1 knocked out of Phox2b cells; CT, circadian time; V_E, minute ventilation; and ns, not significant.

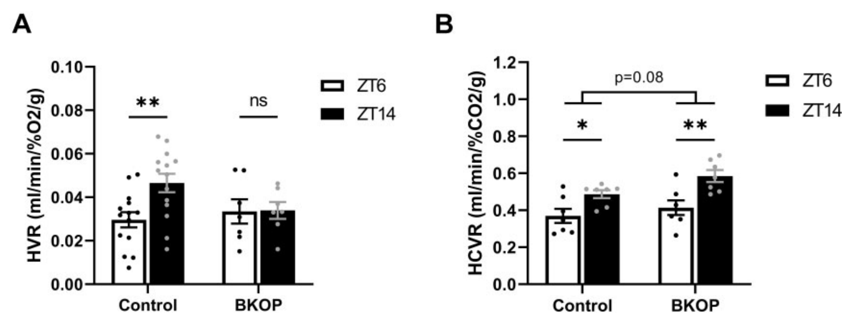

Figure 5. Male BKOP mice lack circadian variation in HVR, but not HCVR. (A) In contrast to wild-type mice ($n = 14$), male BKOP mice ($n = 7$) lack daily variation in HVR. (B) Both male wild-type ($n = 7$) and BKOP mice ($n = 7$) retain a significant day–night difference in HCVR. (A and B) Repeated measures 2-way ANOVA with Sidak's post hoc. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: BKOP, BMAL1 knocked out of Phox2b cells; ZT, zeitgeber time; ns, not significant; HVR, hypoxic ventilatory response; and HCVR, hypercapnic ventilatory response.

24-h patterns when BMAL1 KO mice are housed under standard light:dark but not constant dark conditions.[20,25] Interestingly, we noted a significant sex difference in the daily V_E rhythms of BMAL1 KO mice, with males exhibiting an increase in V_E primarily during the light phase and females exhibiting a decrease in V_E specifically during the dark phase. One limitation of this study is that we did not measure sleep–wake state during the assessment of V_E, which could contribute to sex differences. However, the sexually dimorphic V_E patterns of BMAL1 KO mice were fairly similar to their daily patterns of metabolic rate.[25] This suggests that in the absence of a functional clock, the mouse's sexually dimorphic daily patterns in metabolic rate may partially regulate the daily rhythm in V_E.

Clock genes and genes involved in respiratory plasticity are rhythmically expressed in the caudal medulla of the hindbrain.[13] To our knowledge, this is the first study to selectively perturb the molecular clock exclusively within cells of the neural respiratory network. We found that BKOP mice maintained a daily V_E rhythm comparable to wild-type controls, indicating that the intrinsic time-keeping of Phox2b-expressing cells is not required to organize the daily rhythm in V_E. Of note, our BKOP model did not target many of the inspiratory and expiratory neurons involved in respiratory rhythm or pattern generation, such as the preBötzinger complex. The molecular clock within the respiratory rhythm generator itself may contribute to the daily V_E rhythm, as daily ventilation cycles seem predominantly

driven by respiratory rate rather than tidal volume.[3,4] Moreover, it is unknown whether clock genes in other respiratory structures such as the cervical spinal cord and diaphragm,[13] or the lungs/airway[29,30] impact the daily rhythm in V_E. Molecular clock dysfunction in any number of these non-Phox2b structures may contribute to the disrupted V_E rhythm observed in global BMAL1 KO mice, warranting further investigation.

Furthermore, we found that day–night differences in HVR are completely abolished in BMAL1 KO mice, indicating a prominent influence of the molecular clock on ventilatory chemoreflex. To determine whether the molecular clock of canonical chemoreceptive cells accounts for the disrupted daily variations in HVR of BMAL1 KO mice, we additionally examined HVR in BKOP mice. We found that deletion of BMAL1 within Phox2b-expressing cells completely abolished circadian regulation of HVR. These data indicate that the molecular clock of Phox2b-expressing cells underlies the daily variation in HVR. Circadian regulation of HVR may be mediated by the CB or NTS, which are Phox2b-expressing structures that facilitate the HVR.[15,16] However, the extent of these regions' involvement requires further investigation. Our results additionally indicate that, when responding to hypoxia, non-Phox2b chemoreceptors cannot compensate for the lack of circadian time-keeping of Phox2b chemoreceptors. While the neuromodulatory systems involved in circadian regulation of HVR remain unknown, others have suggested histamine or orexin signaling may play a role.[8,31]

We additionally demonstrated that BMAL1 KO mice exhibit an overall decrease in HCVR relative to wild-type mice. BMAL1 KO mice are known to exhibit metabolic dysfunction, including hyperglycemia and impaired insulin secretion.[32] Impaired glycemic control can impair the HCVR of mice by decreasing their sensitivity to hypercapnia,[33] which may explain the reduction in HCVR of BMAL1 KO mice. Additionally, we found that while males lacked daily variation in HCVR, female BMAL1 KO mice continued to exhibit a day–night difference in HCVR. Additional studies are warranted to determine the mechanism behind this sex-specific effect. In our BKOP model, we found that the molecular clock within Phox2b-expressing cells is not required for circadian regulation of HCVR, as BKOP mice maintained a significant day–night difference in HCVR. While the BKOP model ablates the molecular clock of several CO_2-sensitive neurons, non-Phox2b-expressing chemoreceptor regions such as the serotonergic neurons of the medullary raphe[19] and orexinergic neurons of the hypothalamus[34] are unaffected. It is possible that the molecular clock within these regions compensates for the timing deficiencies of Phox2b-expressing chemoreceptors. One potential mechanism through which this occurs may be via direct connections of serotonergic[35,36] and orexinergic[37] neurons with the preBötzinger complex and phrenic motor nucleus. Notably, Phox2b-expressing RTN neurons of the brainstem also express 5HT2/7 receptors[19] and OX1/2 receptors.[38–40] Therefore, serotonergic and orexinergic neurons may additionally provide timing information to Phox2b-expressing cells to modulate HCVR across the day, despite Phox2b cells lacking an intrinsic clock. Orexin is of particular interest since it modulates HCVR in a vigilance state- and time-dependent manner,[38,41] while serotonin signaling is dispensable for circadian modulation of ventilatory chemoreflex.[42]

Our study is not without limitations. Calculations of HVR and HCVR were based on percentages of inspired O_2 and CO_2 rather than arterial blood gas measures. While this approximation is common in murine studies, inspired gases may not linearly reflect a direct stimulus for ventilatory chemoreflexes. Additionally, we were not able to assess ventilatory responses and metabolic rate simultaneously while animals respond to gas challenges. Therefore, we are not able to determine the mechanism responsible for the observed changes in chemoreflex. BKOP animals may have an impaired chemoreflex and/or an altered acute metabolic response to gas challenges, which indirectly affects chemoreflex. Furthermore, ventilatory chemoreflexes were assessed at only 2 time points to minimize O_2/CO_2-induced phase shifts over multiple recording sessions. Due to this limited temporal resolution, it is possible that chemoreflex rhythms were phase-shifted rather than completely abolished. While day–night differences in ventilatory chemoreflex were found for the female wild-type littermates of BMAL1 KO mice, the female control littermates of BKOP mice did not exhibit day–night differences in HVR or HCVR. Therefore, trends were only reported in males. This may be partly due to the increased variance observed in the chemoreflex data of females as well as effects of background strain. Alternatively, the estrus cycle may have influenced the chemoreflex of females. A recent study found that orexin contributes to the ventilatory chemoreflex of female rats during the active phase, but only during diestrus.[43] Differences in estrus cycle may have contributed to differing chemoreflex trends across experiments.

Collectively, these data demonstrate a crucial role for the molecular circadian clock in regulating daily ventilatory rhythms in mice. We find that ventilatory rhythms are differentially regulated by the molecular clock in a time- and cell-specific manner and are influenced by factors such as biological sex. In addition to resting breathing and ventilatory chemoreflex, the pathological features of some respiratory diseases, including sleep apnea, sudden unexpected death in epilepsy, chronic asthma, obstructive pulmonary disease, and COVID-19, are known to demonstrate circadian variation.[1,44–47] Therefore, the present findings may have therapeutic considerations for several respiratory diseases and their time of day-specific symptomatology. Additional studies are necessary to further elucidate the circadian mechanisms driving daily ventilatory rhythms and how circadian disruption/misalignment may affect ventilatory drive and respiratory disease in humans.

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Supplementary material

Supplementary material is available at the *APS Function* online.

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Conflict of interest statement

No conflicts of interest, financial, or otherwise, are declared by the authors.

Data availability

The data underlying this article are available in the article and in its online supplementary material. Further inquiries can be directed to the corresponding author.

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