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Interaction between defects in ventilatory and thermoregulatory control in mice lacking 5-HT neurons

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Abstract

We have previously shown that mice with near-complete absence of 5-HT neurons (*Lmx1b*$^{f/f/p}$) display a blunted hypercapnic ventilatory response (HCVR) and impaired cold-induced thermogenesis, but have normal baseline ventilation ($\dot{V}_E$), core body temperature (T_{Core}) and hypoxic ventilatory responses (HVR) at warm ambient temperatures (T_{Amb}; 30°C). These results suggest that 5-HT neurons are an important site for integration of ventilatory, metabolic and temperature control. To better define this integrative role, we now determine how a moderate cold stress (T_{Amb} of 25°C) influences ventilatory control in adult *Lmx1b*$^{f/f/p}$ mice. During whole animal plethysmographic recordings at 25°C, baseline $\dot{V}_E$, metabolic rate (V_{O_2}), and T_{Core} of *Lmx1b*$^{f/f/p}$ mice were reduced ($P<0.001$) compared to wild type (WT) mice. Additionally, the HCVR was reduced in *Lmx1b*$^{f/f/p}$ mice during normoxic (−33.1%) and hyperoxic (−40.9%) hypercapnia. However, $\dot{V}_E$ in *Lmx1b*$^{f/f/p}$ mice was equal to that in WT mice while breathing 10% CO_2, indicating that non-5-HT neurons may play a dominant role during extreme hypercapnia. Additionally, ventilation was decreased during hypoxia in *Lmx1b*$^{f/f/p}$ mice compared to WT mice at 25°C due to decreased T_{Core}. These data suggest that a moderate cold stress in *Lmx1b*$^{f/f/p}$ mice leads to further dysfunction in ventilatory control resulting from failure to adequately maintain T_{Core}. We conclude that 5-HT neurons contribute to the hypercapnic ventilatory response under physiologic, more than during extreme levels of CO_2, and that mild cold stress further compromises ventilatory control in *Lmx1b*$^{f/f/p}$ mice as a result of defective thermogenesis.

Keywords

hypercapnia; hypoxia; control of breathing; serotonin

1. Introduction

The primary goal of the respiratory control system is to establish a rate of alveolar gas exchange to match metabolic demand, and as a result ventilation ($\dot{V}_E$) is closely linked to metabolic rate ($\dot{V}_{O_2}$). However, changing environmental conditions such as ambient temperature (T_{Amb}), O_2 availability, or inspired CO_2 leads to a shift in homeostatic strategies in an attempt to

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maintain core body temperature (T_{Core}) and/or blood gases. $\dot{V}_E$ and $\dot{V}_{O_2}$ are close to their minimum when T_{Amb} is near or within the thermoneutral zone (~30–32°C in mice (Gordon, C. J., 1985)). Lowering T_{Amb} initiates mechanisms aimed at heat conservation and generation, which increases $\dot{V}_{O_2}$ and consequently $\dot{V}_E$. These shifts in ventilation and metabolism alter the response strategy to respiratory challenges. For example, in rodents the primary response to hypoxia (and hypercapnia) under warm ambient conditions is a large increase in $\dot{V}_E$, whereas $\dot{V}_{O_2}$ is minimally affected (Saiki, C. et al., 1996). In contrast, under cool ambient conditions the predominant effect of hypoxia is to lower $\dot{V}_{O_2}$ with little or no change in $\dot{V}_E$ (Mortola, J. P. et al., 1995). The hypoxia-induced reduction in $\dot{V}_{O_2}$ results in a decrease in T_{Core}, which can independently lower the sensitivity of the ventilatory control system depending upon the magnitude of the temperature drop (Maskrey, M., 1990). For example, severe hypothermia (decreasing T_{Core} to ~28°C) lowers both the hypoxic and HCVRs in dogs (although interpretation of these data is complicated by the use of anesthesia) (Natsui, T., 1969). Smaller decreases in abdominal temperature (from 37°C to 35°C) in conscious rats using an abdominal heat exchanger leads to little or no change in the response to hypercapnia, whereas increasing T_{Core} augments CO_2 sensitivity (Maskrey, M., 1990). In contrast, hypoxia reduces ventilation in animals under these conditions (Maskrey, M., 1990), suggesting that modest hypothermia has greater effects on ventilatory responses to hypoxia than hypercapnia.

The integration of respiratory, metabolic and thermoregulatory demands is critical for proper blood gas, metabolic and temperature homeostasis, and the hypothalamus and raphé 5-HT system may both represent sites for such integration (Waldrop, T. G. et al., 1986; Hinrichsen, C. F. et al., 1998; Hodges, M. R. et al., 2008b). The preoptic anterior hypothalamus (POAH) contains warm- and cold-sensitive neurons (Griffin, J. D. et al., 1996), and receives afferent inputs from peripheral thermoreceptors (Boulant, J. A. et al., 1974). Additionally, warm-sensitive POAH neurons and lateral hypothalamic hypocretin-producing neurons are also CO_2 sensitive, and lesioning orexin neurons blunts the HCVR (Deng, B. S. et al., 2007; Williams, R. H. et al., 2007; Wright, C. L. et al., 2007). Similarly, raphé 5-HT neurons respond to central (Nason, M. W., Jr. et al., 2006) and peripheral (Martin-Cora, F. J. et al., 2000) cooling and augment cold-induced thermogenesis. 5-HT neurons also contribute to central respiratory chemoreception, and facilitate respiratory rhythm generation and respiratory motor output (Al-Zubaidy, Z. A. et al., 1996; Pena, F. et al., 2002; Richerson, G. B., 2004; Hodges, M. R. et al., 2008a).

We have previously examined ventilatory and thermoregulatory control in *Lmx1b*$^{f/f/p}$ mice, in which *Lmx1b* (LIM homeobox transcription factor 1β) is deleted selectively in neurons that express *Pet1* (plasmacytoma expressed transcript 1). This leads to complete and specific 5-HT neuron loss and central 5-HT depletion, without affecting other monoamine systems (Zhao, Z. Q. et al., 2006; Zhao, Z. Q. et al., 2007). *Lmx1b*$^{f/f/p}$ mice exhibit a severely blunted HCVR and cold-induced thermogenesis despite normal baseline $\dot{V}_E$ and $\dot{V}_{O_2}$, and a normal HVR (Hodges, M. R. et al., 2008b). Those ventilatory measurements were performed at T_{Amb} of 30.0 – 30.8° C (thermoneutral) to prevent confounding effects of changes in T_{Core}. However, the temperature of most animal facilities is maintained near 25°C, which is below thermoneutral temperature for a mouse. Previous measurements of T_{Core} in *Lmx1b*$^{f/f/p}$ mice revealed a failure to maintain T_{Core} at T_{Amb} of 4°C and 12°C, but 24-hour T_{Core} measurements in their home cages at an T_{Amb} of 25°C revealed no differences between genotypes (Hodges, M. R. et al., 2008b). Here we examine ventilation at rest, during hypoxia, and during normoxic and hyperoxic hypercapnia in WT and *Lmx1b*$^{f/f/p}$ mice at an T_{Amb} of 25°C using flow-through plethysmography and show that the defects in thermoregulatory and respiratory control induced by 5-HT system dysfunction combine to cause greater deleterious effects on ventilatory control during hypoxia. In addition, we challenged WT and *Lmx1b*$^{f/f/p}$ mice to pathologically high (10%) levels of CO_2 to determine how important 5-HT neurons are for the HCVR under extreme conditions. All data collected at an T_{Amb} of 30°C have been reported previously

(Hodges, M. R. et al., 2008b), but are included here for direct comparison to those obtained at 25°C.

2. Methods

2.1 Animal model

The generation of *Lmx1b*$^{f/f/p}$ mice has previously been described (Zhao, Z. Q. et al., 2006). 22 female age-matched (6–12 months old, see also Table 1) WT (n = 12) and *Lmx1b*$^{f/f/p}$ (n = 10) mice were used in this study. WT and *Lmx1b*$^{f/f/p}$ littermates were paired during testing when possible.

2.2 Plethysmography

Ventilation and oxygen consumption were measured using standard flow-through plethysmographic techniques (Drorbaugh, J. E. et al., 1955), as described previously (Hodges, M. R. et al., 2008b). Compressed gas mixtures contained: 21% O_2 with 0, 3, 5, 7, or 10% CO_2 (normoxic hypercapnia; balance N_2), 50% O_2 with 0, 3, 5, 7, or 10% CO_2 (hyperoxic hypercapnia; balance N_2), or 10% O_2 (hypoxia; balance N_2). Hypercapnia studies consisted of >20 minutes of baseline, followed by 10-minute exposures of 3, 5, 7, and 10% CO_2, each interrupted by 10-minute baseline measurements. Similarly, hypoxia studies consisted of >20 minutes of baseline followed by 10 minutes of 10% O_2. The plethysmograph was set on top of a telemeter energizer/receiver (Model ER-4000, Mini Mitter, Bend, OR) for continuous measurement of core body temperature. Air temperature (25–26 °C) and humidity (Omega HX-93AV, Omega Engineering Inc., Stamford, CT), animal temperature, breathing-induced pressure oscillations (DC002NDR5, Honeywell International, Morristown, NJ), and O_2 and CO_2 concentrations (Models CD-3A (CO_2) and S3A/I (O_2), AEI Technologies Inc., Naperville, IL) were measured continuously, sampled at 100 Hz, digitized using an A/D converter (PCI-6221, National Instruments, Austin, TX) and monitored/stored on disk using a custom-written data acquisition program (Matlab, The MathWorks, Natick, MA). The outflow gases sampled for O_2 and CO_2 concentrations were dried using a dessication column and measured with continuous flow (200 ml/min) through the analyzers. Oxygen consumption was calculated by subtracting the outflow fraction of O_2 from the inflow fraction of O_2, and multiplying the difference by the chamber flow rate (700 ml/min) measured using a flow meter.

2.3 Telemetry Probe Implantation

The methods for implantation of telemetric temperature probes have been published (Hodges, M. R. et al., 2008b). Briefly, mice were given pre-operative analgesia (meloxicam (1.0 mg/kg I.P.) or buprenorphine (0.1 mg/kg I.P.)) prior to induction and maintenance of anesthesia with 20% (v/v) isoflurane mixed with polyethylene glycol. Telemetric temperature probes (Emitter G2, Minimitter, Bend, OR) were implanted into the abdomen using a ventral midline incision, and the wound was sutured. Mice received 1.7 μg/ml Meloxicam for 2 days, and studied > 7 days post-op.

2.4 Data analysis

All data were analyzed off-line using custom-written software by an individual blind to the animal genotypes. All samples of continuous ventilatory data segments of 6–10 second duration that did not contain sighs, coughs, sniffing or movement artifacts were selected for analysis during the last five-minute period of exposure to each gas mixture for the normoxic and hyperoxic hypercapnia studies. The average number of 6–10 second segments analyzed under control conditions was 35.0 ± 3.3 and 34.9 ± 2.3 for WT and (*Lmx1b*$^{f/f/p}$) mice, respectively, roughly corresponding to 600–1000 breaths analyzed per animal during the control period. Hypoxia data were analyzed during minutes 2–10 of the 10-minute exposure,

and divided into 2-minute segments to evaluate the time-course of the response. Inspiratory time (T_I, seconds), expiratory time (T_E, seconds), inter-breath interval (IBI, seconds, used to calculate respiratory frequency (fR), breaths·minute^{-1}), standard deviation of IBI (seconds), tidal volume (V_T, μl), oxygen consumption ($\dot{V}_{O_2}$, ml·min^{-1}) and minute ventilation ($\dot{V}_E$, ml·min^{-1}, which is the product of V_T and fR), were calculated for all animals under all conditions, with the exception of $\dot{V}_{O_2}$ during hyperoxia due to an inability to accurately measure high (>48%) O_2 concentrations. V_T, $\dot{V}_E$ and $\dot{V}_{O_2}$ were normalized to animal weight.

2.5 Statistics

All data are presented as mean ± SEM. Comparisons were made using a two-way ANOVA (SYSTAT 11, Systat Software, Inc., San Jose, CA) and valid pair-wise comparisons using either a paired t-test or t-test assuming unequal variances (Excel, Microsoft Corp.), when appropriate. The threshold for significance was $P < 0.05$.

All animals were housed and maintained in the Yale Animal Resource Center and all protocols approved by the Yale Animal Care and Use Committee.

3. Results

3.1 Baseline ventilation and metabolic rates are reduced in Lmx1b$^{f/f/p}$ mice

Lmx1b$^{f/f/p}$ mice had significantly reduced mass compared to their WT littermates at the ages studied (Table 1). Therefore, all variables that would be affected by weight differences, such as minute ventilation ($\dot{V}_E$), tidal volume (V_T), and oxygen consumption ($\dot{V}_{O_2}$) were normalized to body weight. In contrast to previous studies where T_{Amb} was held between 30.0 – 30.8°C, in the current study we made all measurements at an T_{Amb} of 25°C. Under these conditions, V_T was equal in WT and *Lmx1b$^{f/f/p}$* mice, but $\dot{V}_E$ was reduced in *Lmx1b$^{f/f/p}$* mice due to a lower breathing frequency at rest (Table 1, Fig. 1A & C). The lower frequency was due to an increased inspiratory time (T_I), with expiratory time (T_E) equal between genotypes. As a result of the longer T_I, ventilatory drive (VT/T_I) was significantly reduced in *Lmx1bf/f/p* mice. In addition, both $\dot{V}_{O_2}$ and T_{Core} were significantly lower in *Lmx1b$^{f/f/p}$* mice relative to WT mice at rest. Therefore, the decrease in $\dot{V}_E$ was largely due to the decreased metabolic demand. The $\dot{V}_E/\dot{V}_{O_2}$ ratio was equal at baseline in WT and *Lmx1b$^{f/f/p}$* mice ($P = 0.18$).

3.2 Ventilatory responses to hypoxia and hypercapnia are blunted in Lmx1b$^{f/f/p}$ mice

We challenged WT and *Lmx1b$^{f/f/p}$* mice to hypoxia and both normoxic ($F_{I_{O_2}} = 0.21$) and hyperoxic ($F_{I_{O_2}} = 0.50$) hypercapnia at T_{Amb} of 25°C to determine the effects of a moderate cold stress on the response to these challenges.

3.2.1 Normoxic hypercapnia

In normoxia, we found significant effects of both genotype ($P \leq 0.001$) and condition ($P \leq 0.001$) on $\dot{V}_E$ and body temperature, and condition, fR, T_I, T_E, VT/T_I effects on V_T ($P < 0.0001$; two-way ANOVA; Fig. 1). Specifically, $\dot{V}_E$ was reduced in *Lmx1b$^{f/f/p}$* mice compared to WT mice when breathing room air, 3, 5 and 7% CO_2 (Fig. 1A). However, there was no difference in $\dot{V}_E$ between genotypes breathing 10% CO_2 in normoxia ($P = 0.39$). We also compared the increase in $\dot{V}_E$ at each level of inspired CO_2 relative to the baseline (Fig. 1B), and found that the increase in $\dot{V}_E$ was significantly reduced breathing 3% and 5% CO_2 in *Lmx1b$^{f/f/p}$* compared to WT mice. The blunted HCVR was due solely to a smaller frequency response in *Lmx1b$^{f/f/p}$* mice (Fig. 1C), with V_T increasing equally in WT and *Lmx1b$^{f/f/p}$* mice (Fig. 1D). There was also a longer T_I in *Lmx1b$^{f/f/p}$* mice ($P < 0.002$), and consequently a smaller V_T/T_I during all CO_2 levels in normoxia ($P \leq 0.002$; data not shown). T_{Core} was significantly reduced in *Lmx1b$^{f/f/p}$* (36.2 ± 0.2°C) relative to WT (37.7 ± 0.1°C) mice at baseline during normoxia,

and remained unchanged from baseline in both genotypes while breathing 3, 5 & 7% CO_2 (Fig. 2E). However, T_{Core} dropped significantly relative to baseline in both genotypes when breathing 10% CO_2, likely due to increased evaporative and convective heat loss due to the hyperpnea. Unlike WT mice, T_{Core} in *Lmx1b*$^{f/f/p}$ mice was significantly lower at an T_{Amb} of 25°C relative to 30°C during plethysmographic recordings (Fig. 1E). The $\dot{V}_E/\dot{V}_{O_2}$ ratio in *Lmx1b*$^{f/f/p}$ mice was equal to WT mice while breathing 3% ($P = 0.09$), 5% ($P = 0.1$) and 7% ($P = 0.085$) CO_2 due to reductions in both $\dot{V}_E$ and $\dot{V}_{O_2}$ (Fig. 1F). However, it is important to note that this measure was significantly reduced in *Lmx1b*$^{f/f/p}$ mice relative to WT mice when measured at an T_{Amb} of 30°C (Hodges, M. R. et al., 2008b).

3.2.2 Hyperoxic hypercapnia

During hyperoxia, there were significant effects of both genotype ($P < 0.01$) and condition ($P \leq 0.03$) on $\dot{V}_E$, f_R, T_I, T_E, V_T/T_I and body temperature, and condition effects on V_T ($P < 0.0001$; two-way ANOVA; Fig. 2). Hyperoxia decreased baseline $\dot{V}_E$ in WT mice from 1.32 ± 0.06 ml · min^{-1} · g^{-1} (normoxia) to 1.15 ± 0.06 ml · min^{-1} · g^{-1} (hyperoxia: $P = 0.015$), but not in *Lmx1b*$^{f/f/p}$ mice, where $\dot{V}_E$ in normoxia was 1.04 ± 0.06 ml · min^{-1} · g^{-1} and 1.05 ± 0.04 ml · min^{-1} · g^{-1} in hyperoxia ($P = 0.907$). However, hyperoxia *per se* had no effect on $\dot{V}_E$ during hypercapnia relative to normoxia in both WT and *Lmx1b*$^{f/f/p}$ mice ($P > 0.05$, two-way ANOVA). *Lmx1b*$^{f/f/p}$ mice had a lower $\dot{V}_E$. than WT mice while breathing 3, 5, and 7% CO_2, but $\dot{V}_E$ was not different when breathing room air or 10% CO_2 (Fig. 2A). The change in $\dot{V}_E$ relative to baseline was also reduced in *Lmx1b*$^{f/f/p}$ mice breathing 3, 5, and 7% CO_2, but not 10% CO_2 (Fig. 2B). *Lmx1b*$^{f/f/p}$ mice also had a lower breathing frequency than WT mice under all conditions in hyperoxia, with no differences in V_T (Fig. 2C & D). Additionally, T_I was greater ($P \leq 0.02$) and V_T/T_I less ($P \leq 0.0006$) in *Lmx1b*$^{f/f/p}$ mice relative to WT mice at rest and at all CO_2 levels tested in hyperoxia (data not shown). Similar to results in normoxia, T_{Core} was significantly lower in *Lmx1b*$^{f/f/p}$ mice (36.4 ± 0.1°C) relative to WT (37.8 ± 0.1°C) mice at baseline during hyperoxia (Fig. 2E). T_{Core} also dropped significantly from baseline when breathing 10% CO_2 in both genotypes. However, there were no differences in T_{Core} between genotypes at an T_{Amb} of 30°C (Fig. 2E).

3.2.3 Hypoxia

In contrast to our previous experiments in which T_{Amb} was 30°C (Hodges, M. R. et al., 2008b), there were several differences in the response of *Lmx1b*$^{f/f/p}$ and WT mice to hypoxia when T_{Amb} was 25°C (Fig. 3). There were significant effects of both genotype and condition on $\dot{V}_E$, fR, T_I, T_E, and body temperature, and there were genotype effects on V_T/T_I ($P < 0.05$; ANOVA). Specifically, $\dot{V}_E$ (Fig. 3A), fR (Fig. 3B), $\dot{V}_E/\dot{V}O_2$ (Fig. 3D) and V_T/T_I were reduced throughout minutes 2 – 10 of the hypoxic challenge in *Lmx1b*$^{f/f/p}$ mice, with no effects on V_T (Fig. 3C). Interestingly, we did not observe a significant decrease in $\dot{V}_E$ or $\dot{V}_{O_2}$ in *Lmx1b*$^{f/f/p}$ mice relative to WT mice during the control period before the hypoxia challenges as seen prior to the hypercapnia challenges, despite a similar difference in T_{Core}. This may be related to variability in the time required for some animals to shift from an exploratory, active behavior to quiet wakefulness. However, both WT and *Lmx1b*$^{f/f/p}$ mice significantly decreased $\dot{V}_{O_2}$ during hypoxia, with no difference between genotypes (Fig. 3E). This result indicates that the lower $\dot{V}_E/\dot{V}_{O_2}$ during hypoxia was due to a larger reduction in $\dot{V}_E$ than in $\dot{V}_{O_2}$. T_{Core} in both WT and *Lmx1b*$^{f/f/p}$ mice significantly decreased over time during hypoxia, but the initial temperature was lower in *Lmx1b*$^{f/f/p}$ mice compared to WT mice (Fig. 3F).

We then compared these data to those obtained from WT and *Lmx1b*$^{f/f/p}$ mice studied at an T_{Amb} of 30°C (Hodges, M. R. et al., 2008b). For comparison, we normalized the data obtained during hypoxia at both ambient temperatures to the control period, and expressed it as % of control. We found no differences in $\dot{V}_E$ (% control; Fig. 4A) or $\Delta\ \dot{V}_{O_2}$ (relative to room air breathing; Fig. 4B) between WT and *Lmx1b*$^{f/f/p}$ mice during hypoxia studied at an T_{Amb} of

either 25 or 30°C. However, T_{Amb} had significant effects on the HVR. At 25°C both genotypes responded to hypoxia with a small increase in $\dot{V}_E$ and large decrease in $\dot{V}_{O_2}$. In contrast, at 30°C they responded to hypoxia with a large increase in $\dot{V}_E$ and small decrease in $\dot{V}_{O_2}$. We also found no difference in $\dot{V}_E/\dot{V}_{O_2}$ (% control) between WT and *Lmx1b*$^{f/f/p}$ mice during hypoxia at 30°C, but $\dot{V}_E/\dot{V}_{O_2}$ was lower in *Lmx1b*$^{f/f/p}$ mice compared to WT mice during hypoxia at 25°C due to the smaller increase in $\dot{V}_E$(Fig. 4C). T_{Core} was not different between WT and *Lmx1b*$^{f/f/p}$ mice studied at 30°C while breathing room air and during hypoxia, and T_{Core} did not change in either genotype in response to hypoxia (Fig. 4D). In contrast, WT and *Lmx1b*$^{f/f/p}$ mice studied at an T_{Amb} of 25°C both had a lower T_{Core} compared to an T_{Amb} of 30°C, and at the lower T_{Amb} there was a significant decrease in T_{Core} over time in response to hypoxia.

4. Discussion

Mice in which CNS 5-HT neurons have been genetically deleted (*Lmx1b*$^{f/f/p}$: (Zhao, Z. Q. et al., 2006) have previously been characterized, and have severe deficits in the HCVR and cold-induced thermogenesis (Hodges, M. R. et al., 2008b). When studied at an T_{Amb} of 30°C, these mice have normal baseline $\dot{V}_E$, $\dot{V}_{O_2}$, T_{Core} and HVR. We show here that when studied under mild cold stress (T_{Amb} of 25°C combined with convective heat loss due to airflow in the plethysmograph), *Lmx1b*$^{f/f/p}$ mice continue to display an attenuated HCVR, but now also have decreased baseline $\dot{V}_E$, $\dot{V}_{O_2}$, T_{Core}, and ventilatory response to hypoxia. These results are consistent with the conclusion that the primary defects in *Lmx1b*$^{f/f/p}$ mice are a reduced HCVR and thermogenic capabilities, and that the decreased baseline ventilation and HVR are secondary to dysfunctional thermogenesis. Thus, a mild cold stress further compromises ventilatory control as a consequence of the thermoregulatory deficit in mice with 5-HT system dysfunction.

4.1 Methodological considerations

Mammals respond to a decreased T_{Amb} by initiating mechanisms that drive heat conservation and generation, which increases $\dot{V}_{O_2}$ and consequently ventilation (Mortola, J. P., 2005). Indeed, both WT and *Lmx1b*$^{f/f/p}$ mice had increased baseline ventilation and $\dot{V}_{O_2}$ under conditions of mild cold stress relative to measurements at 30°C, but $\dot{V}_{O_2}$ and T_{Core} were lower in *Lmx1b*$^{f/f/p}$ mice compared to WT mice in these cooler conditions. This indicates that 25°C is below the lower critical temperature for these mice, and is consistent with our previous observations of thermoregulatory dysfunction (Hodges, M. R. et al., 2008b). However, we previously found no difference in T_{Core} in WT and *Lmx1b*$^{f/f/p}$ mice measured during 24-hour recordings in their home cages at 25°C. This indicates that a mildly decreased T_{Amb} is not sufficient alone to decrease T_{Core} in *Lmx1b*$^{f/f/p}$ mice. It is therefore important to consider the design of the plethysmographic measurements.

We used a flow through plethysmographic chamber with a flow rate of 700 ml·min^{-1} to ensure rapid gas changes while retaining a large enough drop in O_2 concentration to allow accurate $\dot{V}_{O_2}$ measurements. This high flow rate would be expected to cause substantial convective heat loss, and a greater thermal challenge than would occur by decreasing T_{Amb} alone. The attenuated thermogenic response to cold would lead to an exaggerated drop in T_{Core} in *Lmx1b*$^{f/f/p}$ mice. In addition, the small dimensions of the plethysmograph chamber allowed for free, but somewhat restricted movement, which itself can affect T_{Core} and $\dot{V}_{O_2}$ (Cinelli, P. et al., 2007). This could influence T_{Core} in a variety of ways, including increasing heat generation due to stress-induced activation of the hypothalamic-pituitary-adrenal axis and catecholamine release (Harris, R. B. et al., 2002), while decreasing heat generation due to reduced motor activity. The convective airflow combined with lower T_{Amb}, restricted movement, and the

known thermogenic defects in *Lmx1b*$^{f/f/p}$ mice likely contributed to a significantly lower T_{Core}.

4.2 Primary and secondary effects of an absence of 5-HT neurons

In our previous work (Hodges, M. R. et al., 2008b) we found that the primary effect of near-complete absence of 5-HT neurons was a blunted hypercapnic response and impaired cold-induced thermogenesis. In contrast, there was no effect on baseline $\dot{V}_E$, $\dot{V}_{O_2}$ and T_{Core}. Here, we show that under cool conditions *Lmx1b*$^{f/f/p}$ mice display reduced $\dot{V}_E$, $\dot{V}_{O_2}$ and T_{Core} at baseline, and decreased $\dot{V}_E/\dot{V}_{O_2}$ ratio during hypoxia. We conclude that the lower ventilation at rest and during hypoxia under conditions of moderate cold stress is due to the decreased T_{Core} in *Lmx1b*$^{f/f/p}$ mice, which is secondary to a primary defect in heat generation (Hodges, M. R. et al., 2008b). While the concept of decreased body temperature blunting the ventilatory response to hypoxia is not novel, these data suggest that when the 5-HT system is not working normally a modest thermal challenge has secondary deleterious effects on ventilatory control as a result of the primary defect in thermogenesis.

Proper coupling of ventilation and metabolism is particularly important during a hypoxic challenge, where the integrated response normally includes both hyperpnea and hypometabolism. At an T_{Amb} near the thermoneutral range, WT and *Lmx1b*$^{f/f/p}$ mice respond to hypoxia with a robust hyperpnea and only a small decrease in metabolism, whereas when T_{Amb} is cooler both genotypes exhibited only a small increase in ventilation and a large decrease in $\dot{V}_{O_2}$. In addition, both genotypes maintained a constant T_{Core} during hypoxia at 30°C, but T_{Core} decreased significantly in both genotypes during hypoxia at 25°C. This suggests that *Lmx1b*$^{f/f/p}$ mice retain the ability to shift strategies in changing ambient conditions, but are clearly less effective in maintaining T_{Core}. In addition, the data suggest that 5-HT neurons do not contribute directly to the HVR or hypoxia-induced hypothermia *per se*, suggesting that the mechanism by which hypoxia inhibits $\dot{V}_{O_2}$ (and subsequently decreases T_{Core}) is independent of raphé 5-HT neurons.

4.3 Effects on the hypercapnic ventilatory response

At an T_{Amb} of 30°C, the HCVR of *Lmx1b*$^{f/f/p}$ mice is reduced by 42.2% in normoxia and by 51.6% in hyperoxia (Hodges, M. R. et al., 2008b). Under those conditions, there were no differences between *Lmx1b*$^{f/f/p}$ and WT mice in $\dot{V}_{O_2}$ or T_{Core} at baseline or during hypercapnia, indicating that the deficit in the HCVR is a direct result of the absence of 5-HT and/or 5-HT neurons, and not an indirect effect from altered thermoregulation. Here we found that ventilation while breathing 3, 5 and 7% inspired CO_2 was reduced by 33.1% in normoxia and by 40.9% in hyperoxia at an T_{Amb} of 25°C, slightly less but similar to our previous findings. This result is similar to previous reports showing a greater effect of mild hypothermia on the HVR compared to the HCVR (Maskrey, M., 1990).

We previously found that relative to wild type mice, *Lmx1b*$^{f/f/p}$ mice have a significantly smaller increase in ventilation when challenged with 5% and 7% CO_2, but there was no difference in ventilation at baseline or in response to 3% CO_2 at 30°C (Hodges, M. R. et al., 2008b). Here we found that the ventilatory response to 3%, 5% and 7% CO_2 was less in *Lmx1b*$^{f/f/p}$ than WT mice at 25°C. However, there was no difference in ventilation between genotypes breathing 10% CO_2 in both normoxia and hyperoxia at 25°C. These data support the hypothesis that 5-HT neurons make their greatest contribution to central chemoreception at low levels of CO_2 that would occur under physiological conditions, consistent with their high degree of intrinsic chemosensitivity to small changes in pH *in vitro* (Richerson, G. B., 1995; Wang, W. et al., 2001; Bradley, S. R. et al., 2002; Wang, W. et al., 2002; Richerson, G. B., 2004). The data are also consistent with the hypothesis that non-5-HT neurons make their greatest contributions under conditions of severe hypercapnia (Nattie, E., 1999).

In contrast to our previous findings at a T_{Amb} of 30°C, the decreased ventilation during hypercapnia was not accompanied by a significant reduction in the $\dot{V}_E/\dot{V}_{O_2}$ ratio, indicating that under moderate cold stress *Lmx1b*$^{f/f/p}$ mice retain the ability to generate hyperpnea proportional to metabolism during hypercapnia. The reason that different results were obtained at the two temperatures is unclear, but there are several considerations. One possibility is that a subset of non-5-HT chemoreceptors (including peripheral chemoreceptors) play a greater role at colder temperature as a result of an interaction between temperature regulation and chemoreception. Another possibility is that cold exposure leads to an increase in tonic respiratory drive from non-5-HT sources, which could mask the established primary deficit in $\dot{V}_E$ relative to metabolism (Hodges, M. R. et al., 2008b). A third is that 5-HT neurons mediate a decrease in oxygen consumption in response to hypercapnia during cold exposure but not under thermoneutral conditions. Finally, it is important to note that it is difficult to accurately measure O_2 consumption in a small species such as a mouse, and even small errors in measurement of $\dot{V}_{O_2}$ could lead to inability to detect real differences. The p values comparing these data were close to the threshold for significance, preventing us from ruling out a small, but real deficit in the $\dot{V}_E/\dot{V}_{O_2}$ ratio of *Lmx1b*$^{f/f/p}$ mice. However, the existing data suggest that there may be a greater degree of compensation for the loss of 5-HT neurons under thermal stress relative to thermoneutral conditions. The source of this compensation is unclear, but it may include an increase in the contribution of peripheral or other central chemoreceptors.

Consistent with developmental compensation, hyperoxia decreased resting ventilation in WT mice relative to normoxia, but had no effect on resting ventilation in *Lmx1b*$^{f/f/p}$ mice. This suggests that there may be altered carotid body function or central processing of carotid body input in response to the loss of 5-HT neurons. If there is compensation by peripheral chemoreceptors (or possibly by non-5-HT central chemoreceptors) this would lead to an underestimation of the role of 5-HT neurons in the HCVR. As discussed previously (Hodges et al, 2008), this role of 5-HT neurons in the HCVR likely includes both a direct role in sensing CO_2 and an indirect role in enhancing chemosensitivity of non-5-HT neurons.

4.4 Summary and Conclusions

Ventilation and T_{Core} can be regulated normally in *Lmx1b*$^{f/f/p}$ mice under conditions of minimal environmental stress, but both become abnormal when challenged. This is consistent with the idea that 5-HT neurons play a major role in respiratory and thermoregulatory homeostasis under conditions of environmental stress. Abnormalities in the 5-HT system have been identified in sudden infant death syndrome (SIDS) (Paterson, D. S. et al., 2006), which is thought to be due to the inability of a vulnerable infant to maintain homeostasis when challenged by an exogenous stressor (Kinney, H. C., 2005). Based on these new, and previous (Richerson, G. B., 2004; Hodges, M. R. et al., 2008b) data, we conclude that: 1) 5-HT neurons contribute significantly to the HCVR and cold-induced thermogenesis; 2) 5-HT neurons participate in the integration of ventilatory and metabolic demands, and; 3) A mild thermal stress can lead to worsening of the deficits in ventilatory control that are caused by an abnormal 5-HT system.

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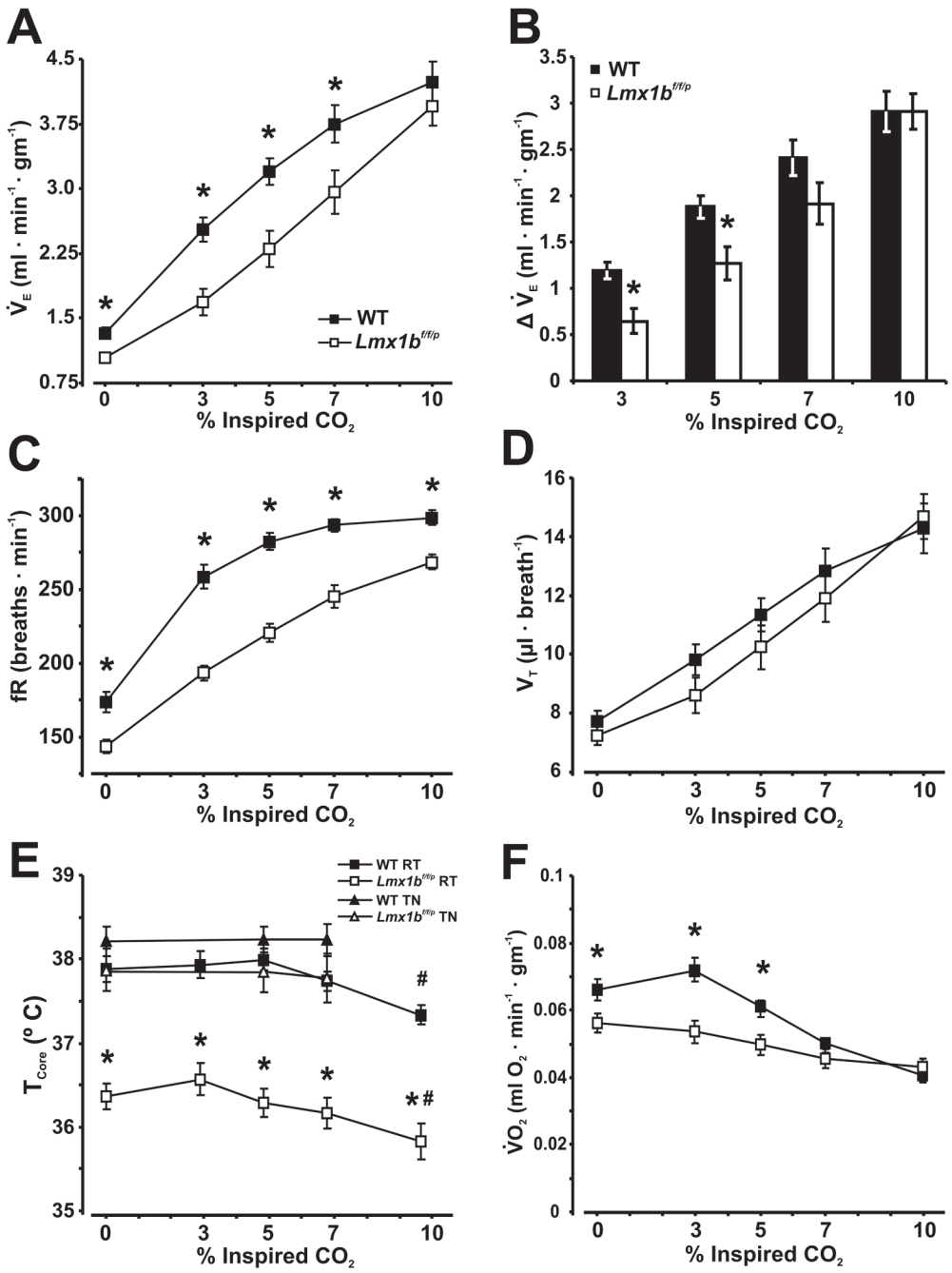

Figure 1. The normoxic hypercapnic ventilatory response and body temperature are reduced in *Lmx1b*$^{f/f/p}$ mice at 25°C

A, Minute ventilation ($\dot{V}_E$), **B**, change in ventilation from baseline, **C**, respiratory frequency (fR), **D**, tidal volume (V_T), **E**, core temperature (T_{Core}) and **F**, oxygen consumption ($\dot{V}_{O2}$) at rest and breathing 0%, 3%, 5%, 7% and 10% inspired CO_2 in normoxia (F_{IO_2} = 0.21) at room temperature (RT: 25°C; A–D, F) or thermoneutral (TN: 30°C (Hodges, M. R. et al., 2008b)) in WT (n=12) and *Lmx1b*$^{f/f/p}$ mice (n=10). Two-way ANOVA (genotype and condition, or ambient temperature as factors) or unpaired *t*-test, * denotes P<0.05 for WT versus *Lmx1b*$^{f/f/p}$, # denotes P<0.05 for 10% CO_2 versus baseline. Data are mean ± SEM.

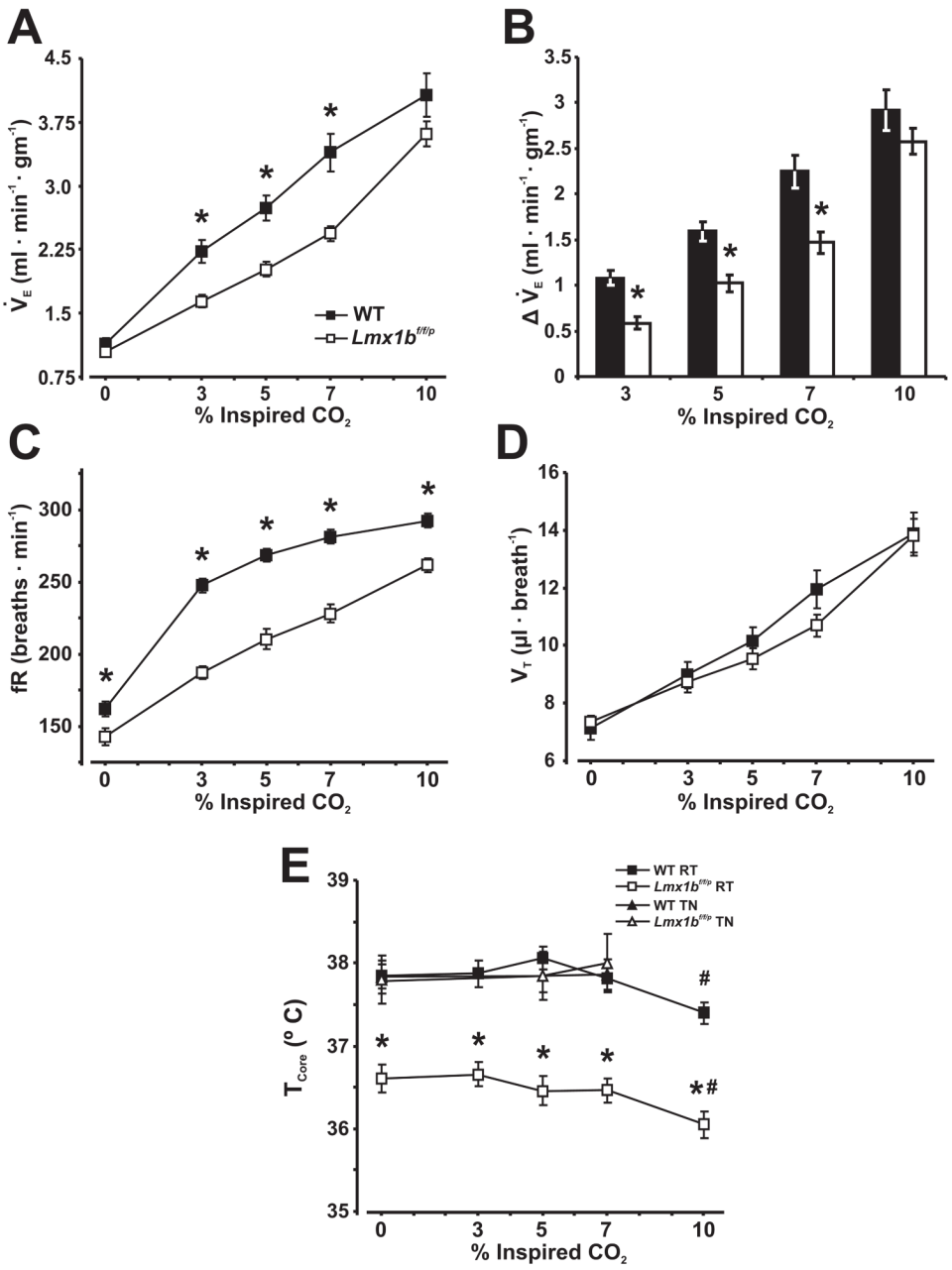

Figure 2. The hyperoxic hypercapnic ventilatory response and body temperature are reduced in *Lmx1b*$^{f/f/p}$ mice at 25°C

A, Minute ventilation ($\dot{V}_E$), **B**, change in ventilation from baseline, **C**, respiratory frequency (fR), **D**, tidal volume (V_T), and **E**, core temperature (T_{Core}) at rest and breathing 0%, 3%, 5%, 7% and 10% inspired CO_2 in hyperoxia (F_{IO_2} = 0.5) at room temperature (RT: 25°C) or thermoneutral (TN: 30°C (Hodges, M. R. et al., 2008b)) in WT (n=12) and *Lmx1b*$^{f/f/p}$ mice (n=10). Two-way ANOVA (genotype and condition, or ambient temperature as factors) or unpaired *t*-test, * denotes P<0.05 for WT versus *Lmx1b*$^{f/f/p}$, # denotes P<0.05 for 10% CO_2 versus baseline. Data are mean ± SEM.

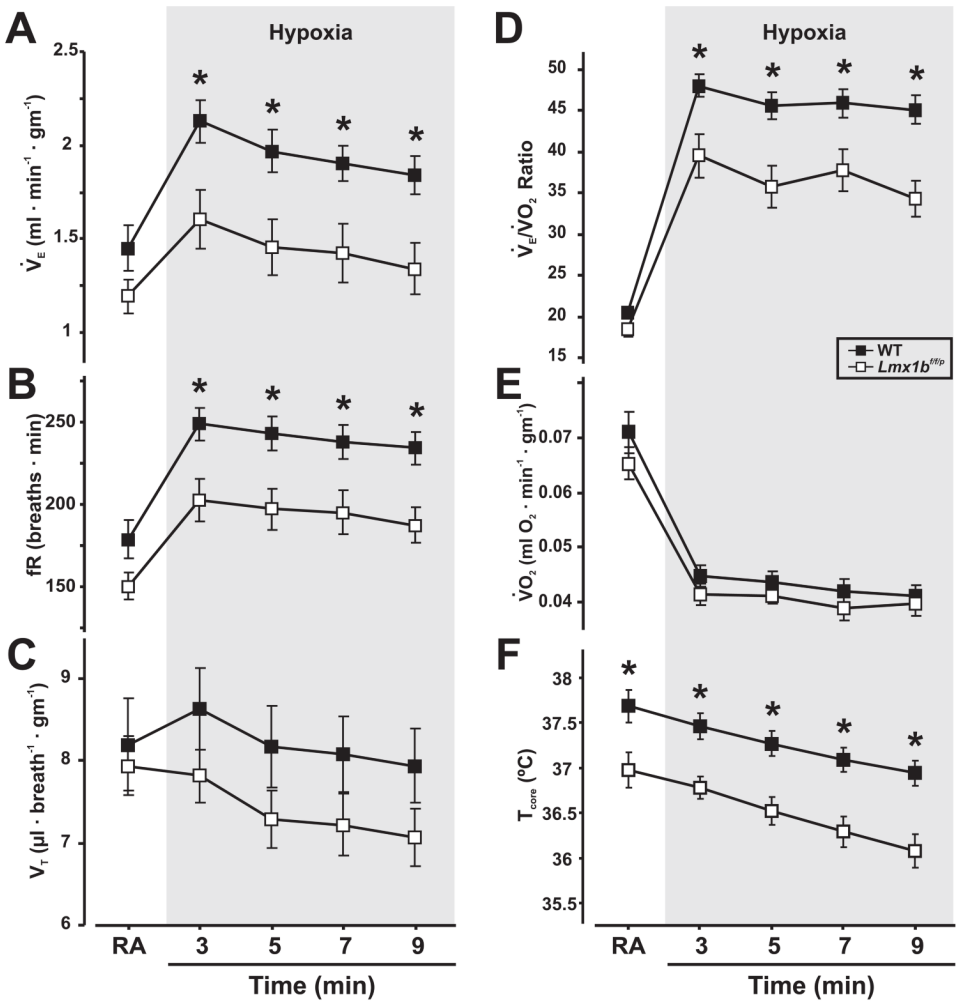

Figure 3. *Lmx1b*$^{f/f/p}$ mice have a blunted hypoxic ventilatory response at 25°C

A, Minute ventilation (V_E), **B**, respiratory frequency (fR), **C**, tidal volume (V_T), **D**, $\dot{V}_E/\dot{V}_{O_2}$ ratio, **E**, $\dot{V}_{O_2}$, and **F**, core temperature (T_{Core}) breathing room air (RA) and during minutes 2–10 of a 10-minute hypoxia challenge (F_{IO_2} = 0.1) in WT (n=9) and *Lmx1b*$^{f/f/p}$ mice (n=7). Two-way ANOVA (genotype and time as factors) and unpaired *t*-test, * denotes P<0.05 for WT versus *Lmx1b*$^{f/f/p}$ mice. Data are mean ± SEM.

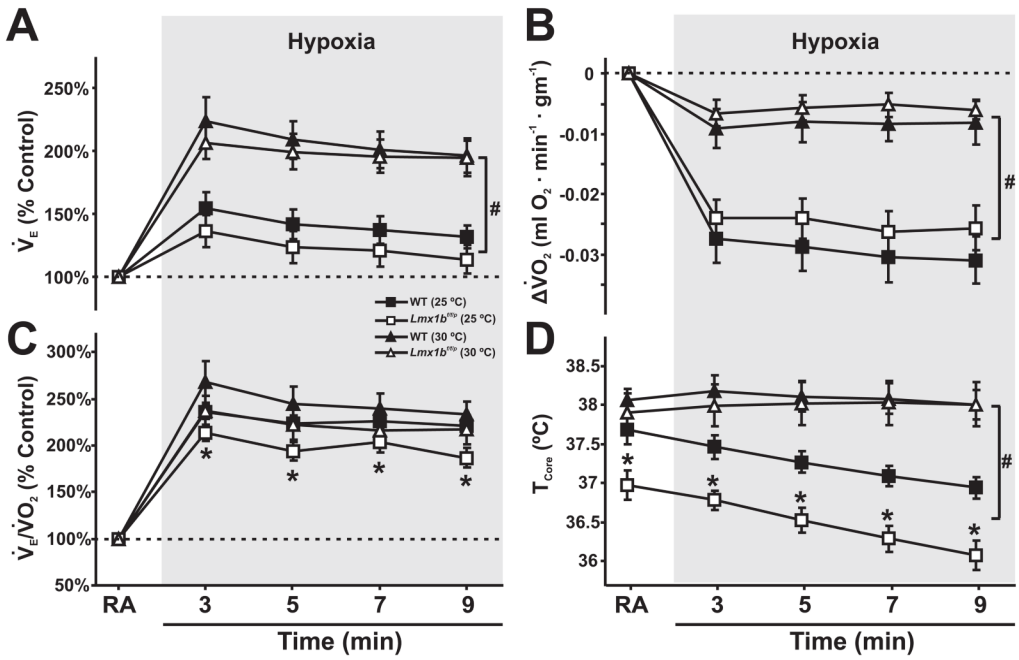

Figure 4. The ventilation to oxygen consumption ratio is reduced at 25°C due to a reduced body temperature in *Lmx1b*$^{f/f/p}$ mice

A, Minute ventilation (V_E; % control), B, $\dot{V}_{O_2}$, C, $\dot{V}_E/V_{O_2}$ ratio (% control), and D, core temperature (T_{Core}) in WT (solid symbols) and *Lmx1b*$^{f/f/p}$ (open symbols) mice at T_{Amb} of 25° C (squares) and 30°C (triangles (Hodges, M. R. et al., 2008b)). Note that both genotypes shift ventilatory and metabolic strategies during hypoxia at different T_{Amb}, and that the $\dot{V}_E/V_{O_2}$ ratio is reduced under cool conditions. Two-way ANOVA (genotype and time, or T_{Amb} as factors) and unpaired *t*-test, * denotes P<0.05 for WT versus *Lmx1b*$^{f/f/p}$ mice, # denotes P<0.05 for 25°C versus 30°C. Data are mean ± SEM.

Table 1

Baseline parameters in WT and *Lmx1b*$^{f/f/p}$ mice

Parameter	Wild Type	*Lmx1b*$^{f/f/p}$	
Age (days)	263.6 ± 9.9	264.6 ± 17.5	
Weight (gm)	33.0 ± 1.6	28.5 ± 1.1	*
T_{Core} (°C)	37.9 ± 0.2	36.4 ± 0.2	**
$\dot{V}_E$ (ml · min^{-1} gm^{-1})	1.32 ± 0.06	1.04 ± 0.06	**
fR (breaths · min^{-1})	172.9 ± 6.9	143.6 ± 4.7	**
V_T (μl · breath^{-1} · gm^{-1})	7.71 ± 0.37	7.24 ± 0.33	
T_I(sec^{-1})	0.1 ± 0.004	0.175 ± 0.007	**
T_E (sec^{-1})	0.255 ± 0.012	0.255 ± 0.009	
V_T/T_I(ml · breath^{-1}·sec^{-1})	2.55 ± 0.14	1.20 ± 0.08	**
$\dot{V}_{O_2}$ (ml O_2 min^{-1}·gm^{-1})	0.066 ± 0.003	0.056 ± 0.003	*
$\dot{V}_E/\dot{V}_{O_2}$	20.8 ± 1.3	19.0 ± 0.7	

All values are mean (± SEM); Age (at the time of study), core temperature (T_{Core}), minute ventilation ($\dot{V}_E$), frequency (fR), tidal volume (V_T), inspiratory time (T_I), expiratory time (T_E), ventilatory drive (V_T/T_I), oxygen consumption ($\dot{V}_{O_2}$), convection requirement ($\dot{V}_E/\dot{V}_{O_2}$). All measurements were obtained while breathing room air at an ambient temperature of 25°C. Comparisons were made with an unpaired *t*-test.

* denotes P<0.05,

** denotes P<0.005.