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RAPID REPORT

Brainstem serotonin deficiency in the neonatal period: autonomic dysregulation during mild cold stress

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Non-technical summary In adult animals serotonergic neurones contribute to heat production (thermogenesis) in response to severe cold stress, as well as the respiratory response to increasing carbon dioxide. We show that in neonatal life, serotonin in the brainstem is absolutely essential for the thermogenic and heart rate responses to very mild (5°C) environmental cooling, probably by aiding the response of the sympathetic nervous system. In contrast, the respiratory response to increasing carbon dioxide is unaffected by a 90% loss of serotonin. Human infants with brainstem serotonin deficiency may be prone to a variety of homeostatic deficits owing to a reduced sympathetic response to mild cold stress.

Abstract Based on previous studies in adult animals, devoid of 5-HT neurones, showing altered thermoregulation in cold stress (4°C) and a reduced ventilatory response to CO_2, we hypothesized that neonatal mice lacking 60–70% of their 5-HT neurones (*Pet-1*$^{-/-}$) would have: (1) a reduced thermogenic response to a mild drop in ambient temperature (T_A), (2) reduced $\dot{V}_E$ and heart rate (HR) responses to mild cooling that reflect this reduced thermogenic response, and (3) a reduced ventilatory response to CO_2 after postnatal day 12 (P12), when 5-HT neurones become chemosensitive *in vitro*. We first determined that a 60–70% loss of 5-HT-positive neurones results in a ∼90% loss of 5-HT from the brainstems of *Pet-1*$^{-/-}$ animals. We then subjected *Pet-1*$^{-/-}$ and wild-type (WT) mice ($N = 5$) to mild environmental cooling ($T_A = 29$°C) at ∼P12. T_A was initially held at 34°C for ∼20 min, reduced to 29°C over 15 min and held for an additional 10 min at steady state, and then returned to 34°C. From 34°C to 29°C, there was a robust increase in $\dot{V}_{O_2}$ in P12 WT, but not *Pet-1*$^{-/-}$ animals (68 ± 19.9% *versus* −16 ± 8%, respectively; $P = 0.002$). On average, body temperature (T_B) dropped 1.1°C more in *Pet-1*$^{-/-}$ compared to WT animals ($P = 0.03$). HR remained unchanged in WT but dropped 22 ± 2.3% in *Pet-1*$^{-/-}$ animals ($P = 0.01$). Genotype had no effect on tail temperature (T_T), either at 34°C or 29°C. After cooling, values for $\dot{V}_{O_2}$ and HR of *Pet-1*$^{-/-}$ animals were no different to values predicted by Q_{10} effects alone, while values of WT animals were greater than predicted. $\dot{V}_E$ increased in WT with cooling, while it decreased in *Pet-1*$^{-/-}$ animals ($P = 0.002$). Still, *Pet-1*$^{-/-}$ animals hyperventilated relative to WT (increased $\dot{V}_E/\dot{V}_{O_2}$) irrespective of T_A ($P = 0.002$). As tested in a separate group of pups, there was no difference in the ventilatory response to CO_2 between WT and *Pet-1*$^{-/-}$ animals, either at P5 or P15. We conclude that during neonatal life in mouse pups: (1) brainstem 5-HT is critical for the thermogenic response to a mild drop in environmental temperature probably via a sympathetically-mediated increase in brown fat metabolism; (2) reduced thermogenesis probably contributes to the reduced HR and $\dot{V}_{O_2}$ observed with 5-HT deficiency; and (3) the presence of some brainstem 5-HT is sufficient for an appropriate ventilatory response to hypercapnia up until P15. Infants with reduced brainstem 5-HT could be prone to cardiovascular and respiratory abnormalities resulting from compromised thermogenesis.

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Abbreviations 5-HT, 5-hydroxytryptamine; HR, heart rate; T_A, ambient temperature; T_B, body temperature; T_T, tail temperature.

Introduction

In homeotherms, an acute drop in environmental temperature induces an increase in sympathetic outflow to the brown fat and heart, which increases $\dot{V}_{O_2}$ and HR (Morrison *et al.* 2008). The physiological mechanism in adults involves serotonergic neurones in the raphe pallidus that are activated by cold stress via descending pathways from the preoptic and dorsomedial hypothalamus (Martin-Cora *et al.* 2000; Rathner *et al.* 2001). These in turn activate presympathetic neurones in the intermediolateral cell column that project to post-sympathetic neurones innervating the brown fat and heart (Madden & Morrison, 2006, 2010; Ootsuka & Blessing, 2006; Nakamura & Morrison, 2007; Morrison *et al.* 2008). A complete loss of central 5-HT in adult *Lmx1b*$^{-/-}$ and *Tph2*$^{-/-}$ mice compromises thermoregulation during severe cold challenge (Hodges *et al.* 2008; Alenina *et al.* 2009) and the reduced $\dot{V}_{O_2}$ could explain the reduced $\dot{V}_E$ of *Lmx1b*$^{-/-}$ neonates compared to WT at reduced T_A (Hodges *et al.* 2009). The extent to which brainstem 5-HT neurones contribute to thermogenesis in neonatal life during a more modest, physiologically relevant drop in environmental temperature is unknown. In this study, we measure the thermogenic response of neonatal mice, previously shown to be lacking ∼60–70% of their 5-HT-positive neurones (*Pet-1*$^{-/-}$; Hendricks *et al.* 2003), testing the hypothesis that reduced brainstem 5-HT compromises the thermogenic response to a mild cold stress. Given that $\dot{V}_E$ and $\dot{V}_{O_2}$ are normally tightly coupled (Frappell *et al.* 1992), and that sympathetic neurones innervating the brown fat and heart can be activated in parallel during cold stress, we also hypothesized that the $\dot{V}_E$ and HR of *Pet-1*$^{-/-}$ neonates would be reduced relative to wild-type pups during mild cooling.

Serotonergic neurones in adult animals contribute to the ventilatory response to increasing CO_2/H^+ within the brainstem parenchyma, either directly as CO_2 chemosensors or through positive interactions with other chemosensitive sites in the brainstem (Dias *et al.* 2008; Hodges *et al.* 2008; Nattie & Li, 2010). Data related to the role of 5-HT neurones in the CO_2 response of unanaesthetised neonatal animals is conflicting. *Pet-1*$^{-/-}$ neonates have a normal ventilatory response to CO_2 at P4.5. Piglets with acute inhibition of 5-HT neurones and rat pups fed a tryptophan-deficient diet (resulting in a ∼50% decrease in medullary 5-HT content) show an increased response early in postnatal life then a decreased response with development (Messier *et al.* 2004; Penatti *et al.* 2010). The reduced response in older animals is consistent with the observation *in vitro* that CO_2 sensitivity emerges with development (Wang & Richerson, 1999). We hypothesize that the hypercapnic ventilatory response will be reduced in *Pet-1*$^{-/-}$ neonates older than P12, the age at which rodent 5-HT neurones become sensitive to CO_2 *in vitro* (Wang & Richerson, 1999).

In this study, we first measured the degree of 5-HT deficiency resulting from a loss of 60–70% of 5-HT-positive neurones from the brainstem of *Pet-1*$^{-/-}$ animals, and then assessed the consequences of this deficiency on their thermogenic, ventilatory and HR responses to a mild and brief drop in T_A. We also measured the hypercapnic ventilatory response in separate groups of *Pet-1*$^{-/-}$ and WT animals. We demonstrate an apparent requirement for brainstem 5-HT neurones for cooling-induced thermogenesis, with consequences for both respiratory and heart rate control.

Methods

Animals

We used five litters from five different *Pet-1*$^{+/-}$ breeding pairs. All animals were studied in the unanaesthetised state. Dams were provided food and water *ad libitum* and were housed with a 12 h light–dark cycle (lights on from 06.00 to 18.00 h) at a T_A of 21–23°C. To assess monoamine content, high-pressure liquid chromatographic (HPLC) analysis was performed on the brainstems (medulla and pons) of *Pet-1*$^{-/-}$ animals and WT ($n = 4$ of each) at ∼1 week of age. Prior to tissue harvesting, animals were killed with a lethal intra-peritoneal dose of a ketamine–xylazine mixture. There is no difference between *Pet-1*$^{+/+}$ and $^{+/-}$ animals with respect to 5-HT cell counts (Hendricks *et al.* 2003). We have not observed any difference between *Pet-1*$^{+/+}$ and $^{+/-}$ animals with respect to weight or 5-HT content (including the current data), so these two genotypes are grouped together (WT) for comparison with *Pet-1*$^{-/-}$ animals. To examine thermoregulatory ability, *Pet-1*$^{-/-}$ animals ($n = 5$; 2 males, 3 females) and WT ($n = 5$; 3 males, 2 females) were studied at P12, at $T_A = 34$°C and 29°C. We chose P12

animals because younger WT animals did not display a robust increase in $\dot{V}_{O_2}$ with cooling, and in our experience, older animals respond to cooling with excessive body movement which confounds the assessment of the autonomic thermoregulatory response. Average weights for WT and *Pet-1*$^{-/-}$ animals were 6.5 ± 0.3 g and 4.7 ± 0.2 g, respectively.

For examining the CO_2 response, we studied two additional groups of animals: one at P5 (10 WT: 8 male, 2 female; 8 *Pet-1*$^{-/-}$: 2 male, 6 female) and another at P15 (6 WT: 2 male, 4 female; 9 *Pet-1*$^{-/-}$: 6 male, 3 female). Average weights for WT and *Pet-1*$^{-/-}$ animals at P5 were 2.8 ± 0.1 g and 2.1 ± 0.1 g, respectively, and at P12 were 8.2 ± 0.5 g and 5.4 ± 0.2 g, respectively. Heart rate data from these animals during normoxic, normocapnic conditions have appeared in a previous publication (Cummings *et al.* 2010). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Dartmouth College.

Genotyping

Genotyping on isolated DNA was performed using primers: 5′-CGC ACT TGG GGG GTC ATT ATC AC-3′, 5′-CGG TGG ATG TGG AAT GTG TGC-3′ and 5′-GCC TGA TGT TCA AGG AAG ACC TCG G-3′ according to a previous study (Hendricks *et al.* 2003). PCR was performed using an initial 5 min denaturing step at 95°C, followed by 35 cycles of 94°C for 1 min, 62°C for 30 s and 72°C for 50 s. PCR products generated were a wild-type allele and knockout allele of 209 and 361 base pairs, respectively.

Experimental setup

Experiments were performed using a setup described previously (Cummings & Frappell, 2009). Briefly, the animal chamber (volume ∼40 ml) was constructed from a water-jacketed glass cylinder. T_A was precisely controlled by changing the temperature of the water perfusing the glass chamber. Breathing was measured with a head-out system consisting of a mask and a pneumotach. This method provides accurate measurements of V_T (and hence $\dot{V}_E$) in neonatal animals (Mortola & Frappell, 1998). The head chamber was made by fitting a section of vinyl over the end of a syringe tube (volume ∼3 ml), held in place with another rubber gasket that fitted into the anterior end of the chamber. The snout of the animal was placed into a small hole in the vinyl and sealed with polyether material (Impregum F Polyether Impression material, 3M, St Paul, MN, USA).

A downstream pump (AEI Technologies, Naperville, IL, USA) connected to the outlet port of the mask pulled air or 5% CO_2 (balanced with air or hyperoxia) through the pneumotach and mask at a flow of 100 ml min^{-1}. $\dot{V}_{O_2}$ in air was determined by pulling the expired gas through an O_2 analyser (AEI Technologies, Pittsburgh, PA, USA). CO_2 was delivered directly from a tank to the surrounds of the pneumotach through the open end of a 50 cc syringe placed over the end of the pneumotach. In this way, the downstream pump pulled the gas through the mask with a very fast wash-in time. T_B was continually monitored with a fine rectal thermocouple (Omega Engineering, Stamford, CT, USA). ECG was obtained with two surface electrodes embedded in a small vest worn by the animal. The electrodes rested on the ventral surface of the animal, displaced from each other ∼1 cm in the anterior–posterior and medial–lateral axes. Thermocouples and ECG leads exteriorized by way of a hole in a rubber gasket (Terumo Medical Corp., Japan) in the posterior end of the chamber.

Inspiratory and expiratory airflows were detected by connecting both side-arms of the pneumotach to a differential pressure transducer (Validyne Engineering, Northridge, CA, USA). Integration of the flow trace provided respiratory volume, calibrated by injecting and withdrawing known volumes of air (0.025, 0.05 ml) at the end of each experiment. The pneumotach responded in a linear fashion to these volumes.

Experimental protocols

General. Experimentation was performed while blinded to genotype. Pups were removed from the litter and immediately weighed. Fur from the snout was removed as well as a small (0.2 cm × 0.5 cm) area on the dorsal surface of the tail, at approximately the midpoint for placement of a thermocouple. Animals were then instrumented with ECG leads contained in a small vest made from tensor bandage. A rectal thermocouple was inserted ∼1 cm and lightly glued to the base of the tail, and a separate thermocouple was fixed to the midpoint of the tail with a small drop of glue and then covered with a small bead of Impregum F. The snout of the animal was sealed into the mask. Animals rested comfortably in the chamber; no change in HR was observed in animals after being placed in the mask.

Protocol A: Thermoregulation (P12). Each animal was allowed to equilibrate to a T_A of 34 ± 0.2°C for 20 min. T_A was then reduced from 34°C to 29°C (Fig. 2*A*). T_A was measured with a thermocouple placed within the chamber, ∼1 cm off the upper surface of the chamber at the midpoint. Because of the hole in the rubber gasket at the rear of the chamber, a small temperature gradient existed within the chamber; T_A directly over the tail was measured during each experiment and found to be ∼27.5°C when T_A at the chamber midpoint was 29°C. Most of the decrease in T_A during the cooling step occurred over

the first 5 min after changing the temperature of the water perfusing the chamber. After 25 min at 29°C, T_A was subsequently increased back to 34°C over the course of 15 min. We measured V_T, f, $\dot{V}_E$, the co-efficient of respiratory variation (CV% = (standard deviation of the respiratory period/average period) × 100), $\dot{V}_{O_2}$, T_B, T_T and HR in each animal during the last 5 min of each T_A.

Protocol B: Ventilatory CO_2 responses (P5 and P15). The T_B of each animal was increased to 36.0 ± 0.2°C over the course of ∼20 min. Unlike Protocol A, these animals were held at a euthermic T_B of 36.0°C for the entire protocol (requiring a higher T_A in *Pet-1*$^{-/-}$ compared to WT animals). We chose to clamp T_B rather than T_A as T_B has known effects on the ventilatory response to CO_2 (Cummings & Frappell, 2009). Resting $\dot{V}_E$ was measured for 10 min in normoxic, normocapnic conditions. The gas drawn through the mask was then changed to either normoxic hypercapnia (5% CO_2, 21% O_2, balance N_2) or hyperoxic hypercapnia (5% CO_2, 95% O_2). Hypercapnia was maintained for 2 min. We measured V_T, f and $\dot{V}_E$ in the last minute of normocapnia and hypercapnia.

Data analysis

All analog signals were recorded and analysed in Labchart 6 (ADInstruments, Colorado Springs, USA) using Powerlab data acquisition system (ADInstruments). Heart rate and breathing were analysed using peak detection on the respiratory and R-wave traces. Heart rate was measured continually. Mass-specific $\dot{V}_{O_2}$ was determined using the formula ($\dot{V}_{O_2}$ = (0.21 − fractional O_2 exhausted from mask) × flow (ml min^{-1})/mass (kg)). Data are expressed as means ± SE.

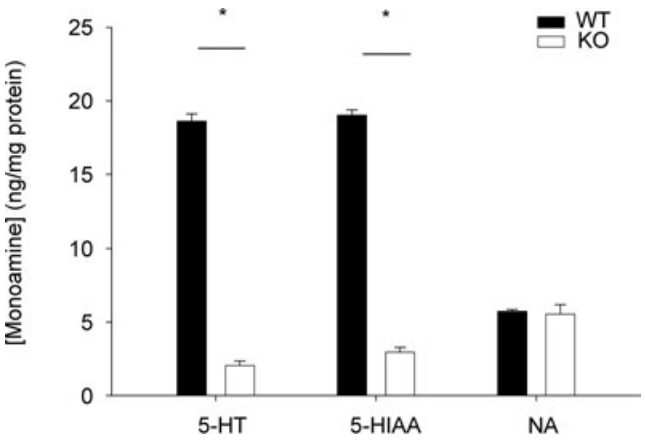

Figure 1. *Pet-1*$^{-/-}$ animals (KO) have reduced brainstem 5-HT
Shown is the 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and noradrenaline (NA) content from the medulla and pons of wild-type WT animals ($n = 4$) and *Pet-1*$^{-/-}$ animals ($n = 4$) at P7.

Statistical analysis

Pet-1$^{-/-}$ animals and many other 5-HT-deficient rodent models are invariably smaller than WT throughout neonatal life (Erickson *et al.* 2007; Alenina *et al.* 2009; Cummings *et al.* 2009, 2010), and this was the case with the current study. To assess the independent contribution of body mass to thermoregulation, we conducted two separate analyses of variance (ANOVA). In one, we performed a two-factor, repeated-measures ANOVA, with genotype as the between-subjects independent variable and T_A as the within-subjects independent variable. In a second two-factor, repeated-measures ANOVA, we replaced genotype as the between-subjects variable with mass. Tukey's *post hoc* tests were performed when significant effects were found. Because T_B responses to ambient cooling were not the same between genotypes, for each genotype we compared the observed $\dot{V}_{O_2}$ and HR after cooling with values predicted from a Q_{10} effect alone (assuming a Q_{10} co-efficient of 2). A two-factor, repeated-measures ANOVA was used to assess the effects of factor 1 (genotype) and factor 2 (observed or predicted response) on $\dot{V}_{O_2}$ and HR.

Results

Figure 1 shows that the brainstem 5-HT and 5-HIAA content of *Pet-1*$^{-/-}$ animals was reduced ∼90% compared to WT ($P < 0.001$). WT and *Pet-1*$^{-/-}$ animals had the same brainstem noradrenaline content. Figure 2*A* shows the raw metabolic and T_B responses of each animal to the drop in T_A. The change in the fractional concentration of O_2 leaving the mask (mask O_2) over the course of the cooling step reflects the change in $\dot{V}_{O_2}$. From 34°C to 29°C, the $\dot{V}_{O_2}$ of all five WT animals increased, while there was no significant change in the $\dot{V}_{O_2}$ of any *Pet-1*$^{-/-}$ animals (Fig. 2*A*). An analysis of the mean data confirms a differential effect of cooling that depends on genotype: the $\dot{V}_{O_2}$ of WT animals increased 68 ± 19%, while the $\dot{V}_{O_2}$ of *Pet-1*$^{-/-}$ animals declined 16 ± 8% (genotype × T_A interaction: $P = 0.002$; Fig. 2*B*). T_B was slightly but significantly lower in *Pet-1*$^{-/-}$ animals compared to WT at T_A of 34°C ($P = 0.003$; Fig. 2*C*). After ambient cooling, the T_B of *Pet-1*$^{-/-}$ animals fell 4.2°C while that of WT fell 3.1°C (genotype × T_A interaction: $P = 0.03$, Fig. 2*C*). T_T also dropped in all animals during cooling, but unlike T_B, the fall in T_T was the same in both genotypes (Fig. 2*D*). The HR of WT animals remained unchanged from 34°C to 29°C, while the HR of *Pet-1*$^{-/-}$ animals dropped 22 ± 2.3% (genotype × T_A interaction: $P = 0.01$, Fig. 2*E*).

Mass also had a statistically significant effect on both T_B and $\dot{V}_{O_2}$ responses of WT and *Pet-1*$^{-/-}$ animals ($T_A \times$ mass interaction effect: $P < 0.05$ for both T_B and $\dot{V}_{O_2}$; Fig. 3*A* and *B*). Rather than a reduced sympathetic response to the brown fat and heart, the reduced $\dot{V}_{O_2}$ and HR responses of *Pet-1*$^{-/-}$ animals may have been due to their smaller size and larger drop in T_B (i.e. Q_{10} effect). To address this, we compared the $\dot{V}_{O_2}$ and HR responses of each animal with the responses expected from Q_{10} effects alone. The $\dot{V}_{O_2}$ and HR of *Pet-1*$^{-/-}$ animals after cooling are not different from values that would result from the Q_{10} effect alone, while both $\dot{V}_{O_2}$ (Fig. 3*C*; $P < 0.001$) and HR (Fig. 3*D*; $P = 0.02$)

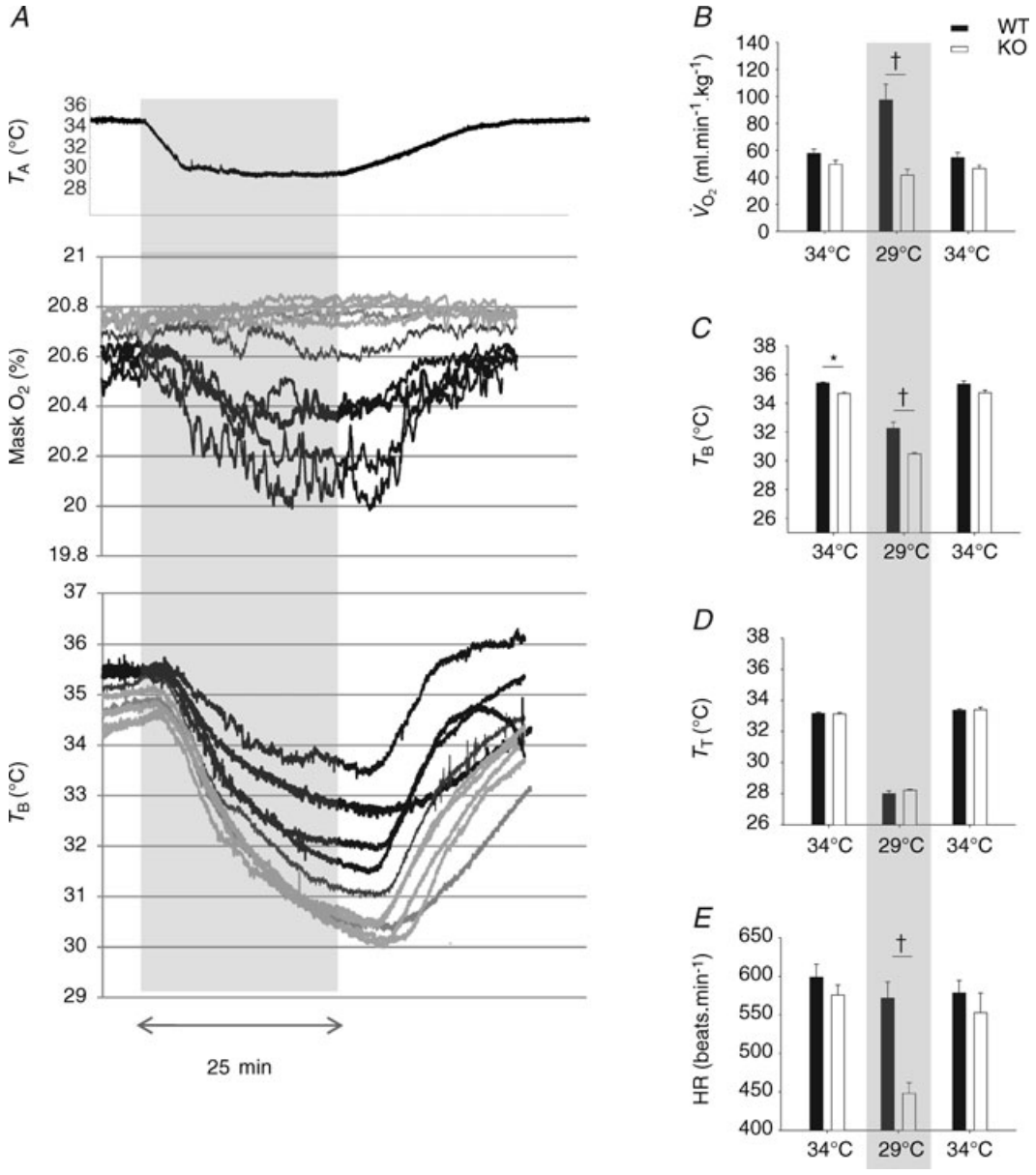

Figure 2. Reduced thermogenesis and heart rate (HR) in *Pet-1*$^{-/-}$ (KO) neonates during ambient cooling
A, raw traces showing the changes in the fractional concentration of O_2 leaving the mask (mask O_2) and body temperature (T_B) in *Pet-1*$^{-/-}$ animals ($n = 5$, grey lines) and littermates (WT) ($n = 5$, black lines) as ambient temperature (T_A) is cooled from 34°C to 29°C over 25 min (shaded region). *B*, at 34°C, metabolic rate ($\dot{V}_{O_2}$) is not significantly different in *Pet-1*$^{-/-}$ animals compared to WT. The disparity between genotypes becomes apparent as T_A is reduced to 29°C (shaded region). *C*, at 34°C, T_B is slightly but significantly lower in *Pet-1*$^{-/-}$ animals compared to WT. As is the case with $\dot{V}_{O_2}$, the disparity becomes greater with cooling to 29°C. *D*, there were no differences in tail temperature (T_T) between *Pet-1*$^{-/-}$ and WT animals at either T_A. Note: T_T is less than 29°C because of a T_A gradient within the chamber (T_A was 1.5°C lower at the tail end compared to the middle). *E*, there is a significantly greater reduction in HR with cooling in *Pet-1*$^{-/-}$ animals compared to WT. All data in *B–E* are means ± SE. *Significant genotype effect, $P < 0.05$; †genotype × T_A interaction, $P < 0.05$.

responses of WT animals are greater than those that would result from Q_{10} alone.

$\dot{V}_E/\dot{V}_{O_2}$ was significantly elevated in P12 *Pet-1*$^{-/-}$ animals compared to WT (genotype: $P = 0.002$; Fig. 4*A*). This was evident at both T_A values, despite $\dot{V}_E$ decreasing in *Pet-1*$^{-/-}$ and increasing in WT animals from 34°C to 29°C (genotype × T_A interaction: $P = 0.002$; Fig. 4*B*). The drop in $\dot{V}_E$ in *Pet-1*$^{-/-}$ animals after cooling resulted from reductions in both V_T and f (genotype × T_A interaction: $P = 0.04$ and $P = 0.02$, respectively; Fig. 4*C* and *D*). *Pet-1*$^{-/-}$ animals had more respiratory variability than WT animals, irrespective of T_A (genotype: $P = 0.006$; Fig. 4*E*).

With T_B held at 36.0°C, the room air $\dot{V}_E$ of P5 and P15 *Pet-1*$^{-/-}$ animals was the same as WT (Fig. 5), and there was no effect of genotype on $\dot{V}_E/\dot{V}_{O_2}$ (not shown). At both ages, *Pet-1*$^{-/-}$ and WT animals had the same ventilatory response to both normoxic (Fig. 5*A*) and hyperoxic (Fig. 5*B*) hypercapnia. This is reflected in a similar percentage increase in $\dot{V}_E/\dot{V}_{O_2}$ from room air to hypercapnia in both genotypes (Fig. 5*C*).

Discussion

Given evidence from adult animals, we hypothesized that the thermogenic response of *Pet-1*$^{-/-}$ neonates – sustaining a 90% loss of brainstem 5-HT – would be reduced compared to WT. Not only was our hypothesis confirmed but surprisingly, *Pet-1*$^{-/-}$ animals at P12 were devoid of any measurable increase in $\dot{V}_{O_2}$ during a mild (5°C) drop in T_A. Associated with this ablation of the $\dot{V}_{O_2}$ response during cooling in *Pet-1*$^{-/-}$ animals was a considerably larger drop in T_B compared to WT. That T_T was the same between WT and *Pet-1*$^{-/-}$ animals at reduced T_A suggests that 5-HT loss does not compromise tail vasoconstriction, an important heat-conserving mechanism in rodents. The negative effect of brainstem 5-HT loss on the $\dot{V}_{O_2}$ response to cooling is reflected in both the HR and $\dot{V}_E$ responses: both HR and $\dot{V}_E$ of *Pet-1*$^{-/-}$ animals fall with cooling while in WT HR is maintained and $\dot{V}_E$ increases. Based on these data we propose that a loss of brainstem 5-HT in neonatal life eliminates the sympathetically mediated $\dot{V}_{O_2}$ and HR responses to

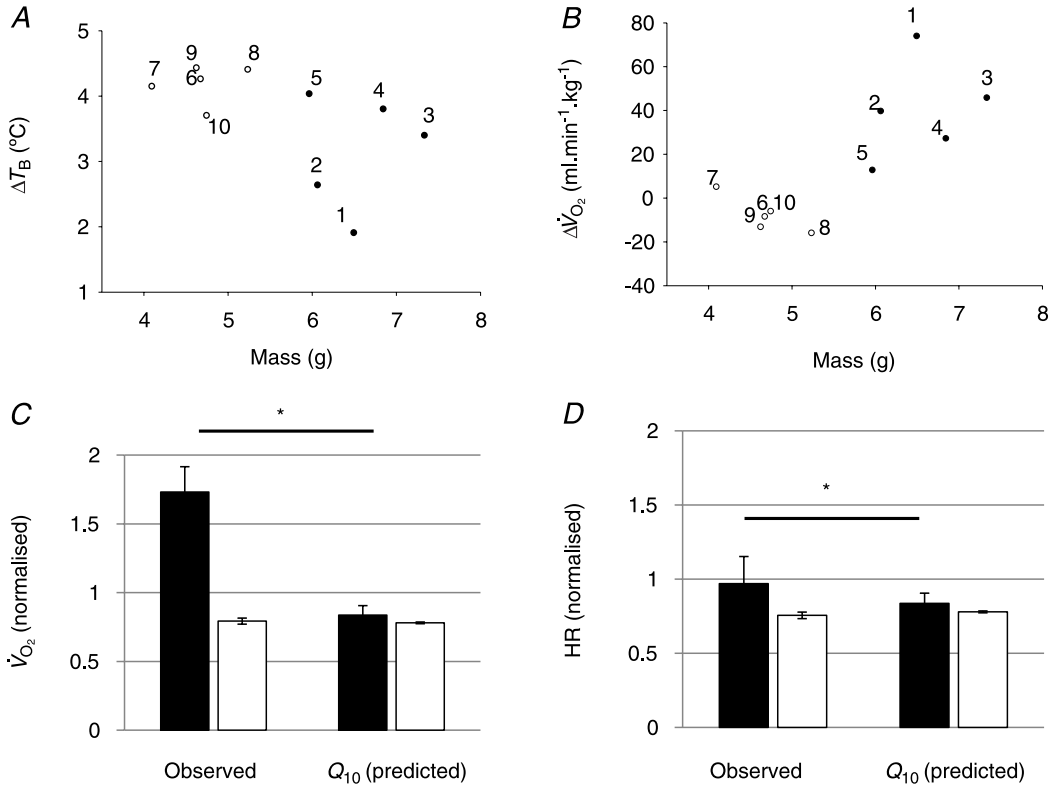

Figure 3. Effects of body size and Q_{10} on thermogenesis and HR during ambient cooling
A and *B*, WT animals (filled circles, nos. 1–5) weigh more than *Pet-1*$^{-/-}$ animals (open circles, nos. 6–10), and mass has a significant relationship with T_B and $\dot{V}_{O_2}$ (2-factor repeated-measures ANOVA: $P < 0.05$ for each). *C* and *D*, $\dot{V}_{O_2}$ and HR after cooling (normalised to baseline values) for WT (filled bars), and *Pet-1*$^{-/-}$ (open bars). Shown are observed values and values predicted from the Q_{10} effect alone (assuming Q_{10} co-efficient of 2 and with a drop in T_B of 4.2°C in *Pet-1*$^{-/-}$ and 3.1°C in WT). Data in *C* and *D* are means ± SE. *Significant difference exists between observed WT values for $\dot{V}_{O_2}$ and HR and the values predicted from the Q_{10} effect alone. No such difference exists between the observed and predicted values for *Pet-1*$^{-/-}$ animals.

mild and brief cooling, with consequences for ventilatory control.

Brainstem 5-HT has the potential to influence both non-shivering and shivering thermogenesis. Bulbospinal 5-HT neurones innervate the intermediolateral cell column (Bowker *et al.* 1981), and activation of 5-HT receptors in this region enhances brown fat-mediated thermogenesis in adults (Madden & Morrison, 2010). Other data obtained from conscious piglets show that pharmacological inhibition of 5-HT neurones inhibits shivering thermogenesis (Hoffman *et al.* 2007; Brown *et al.* 2008). Adult *Lmx-1b*$^{-/-}$ mice (devoid of 5-HT neurones) do retain partial non-shivering and shivering thermogenesis in the severe cold (Hodges *et al.* 2008). Shivering thermogenesis, at least in neonatal rats, does not normally begin until the start of the third postnatal week (Jansky, 1973). Although we cannot completely discount an effect on shivering thermogenesis, it is likely that the effects we describe in *Pet-1*$^{-/-}$ animals at P12 are primarily the result of absent non-shivering thermogenesis. We cannot glean which 5-HT neurones are responsible for reduced thermogenesis in *Pet-1*$^{-/-}$ animals, or whether the effects are due to a developmental or physiological deficit; the lesion to the 5-HT system in *Pet-1*$^{-/-}$ animals is widespread throughout the brainstem raphe system (Hendricks *et al.* 2003). Bulbospinal 5-HT neurones

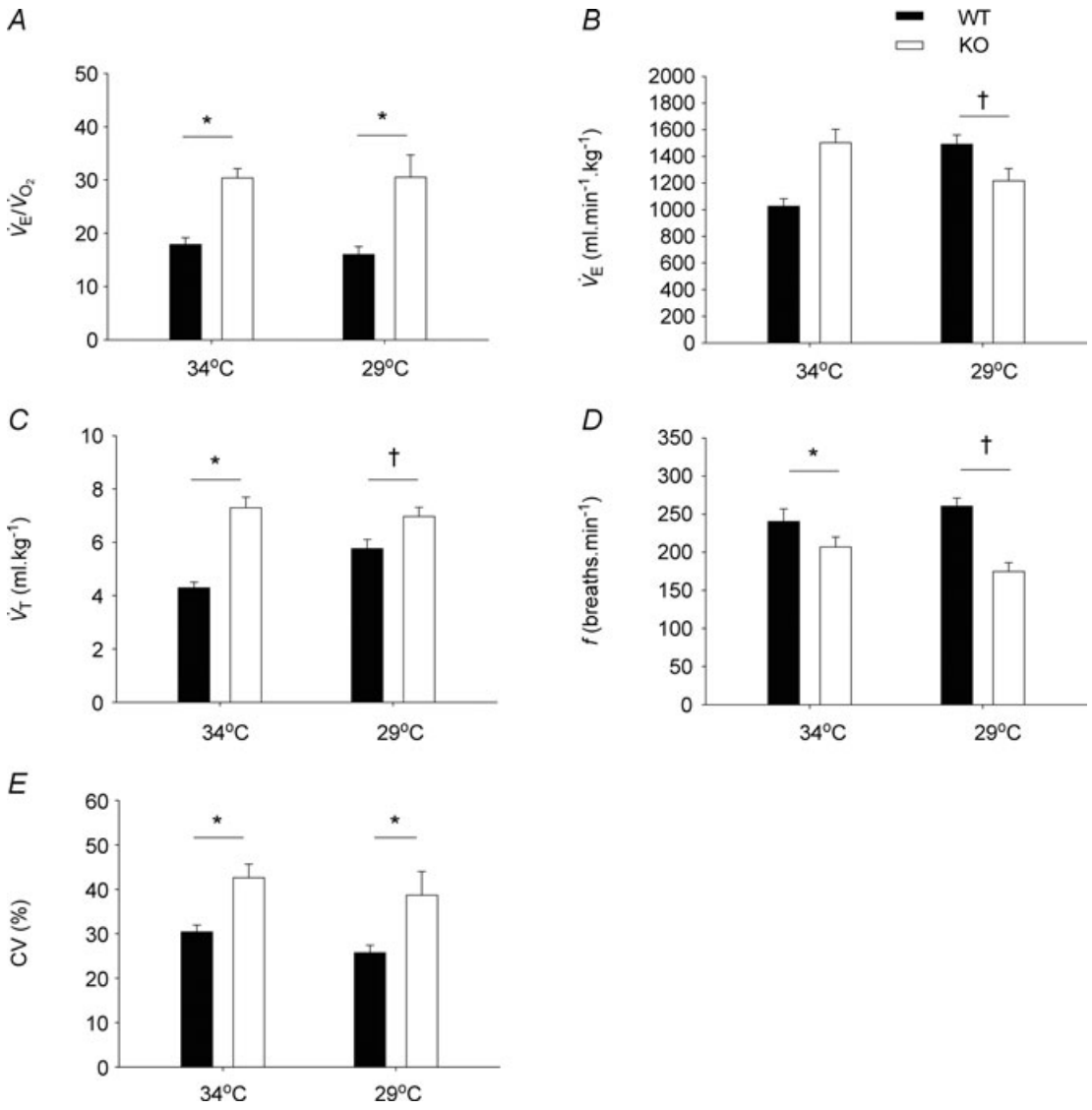

Figure 4. Hyperventilation in P12 *Pet-1*$^{-/-}$ animals
A, $\dot{V}_E/\dot{V}_{O_2}$ is elevated in P12 *Pet-1*$^{-/-}$ animals, irrespective of T_A. *B*, from $T_A = 34°C$ to 29°C, $\dot{V}_E$ decreases in *Pet-1*$^{-/-}$ animals, but increases in WT animals. *C* and *D*, $\dot{V}_E$ is selectively reduced in *Pet-1*$^{-/-}$ animals from a reduction in tidal volume (V_T) and respiratory frequency (f) *E*, respiratory variability, as measured by the co-efficient of variation in the respiratory period (CV%) is higher in *Pet-1*$^{-/-}$ animals at both T_A. All data are means ± SE. *Significant genotype effect, $P < 0.05$; †genotype × T_A interaction, $P < 0.05$.

originating in the raphe pallidus make an important physiological contribution to the sympathetic activation of the brown fat (Bowker *et al.* 1981; Allen & Cechetto, 1994; Nakamura & Morrison, 2007; Brown *et al.* 2008; Madden & Morrison, 2010), so a deficit in this neuronal population probably underpins the absence of thermogenesis in *Pet-1*$^{-/-}$ animals.

In addition to their compromised thermoregulation, the HR of *Pet-1*$^{-/-}$ animals fell considerably with ambient cooling, while that of WT remained unchanged. This observation is consistent with findings from anaesthetised adult rats in which both brown fat-mediated thermogenesis and HR are inhibited during acute skin cooling after application of muscimol (a $GABA_A$ receptor agonist) or 8-OH-DPAT (a 5-HT_{1A} receptor agonist) to the raphe pallidus (Nakamura & Morrison, 2007). T_B fell more in *Pet-1*$^{-/-}$ animals than WT with ambient cooling, leaving open the possibility that an effect of Q_{10} contributed to their reduced $\dot{V}_{O_2}$ and HR responses to ambient cooling. That in WT, but not *Pet-1*$^{-/-}$ animals, there was a significant difference between the observed $\dot{V}_{O_2}$ and HR after cooling and values predicted from Q_{10} alone suggests that 5-HT neurones are essential for the increase in sympathetic drive to the brown fat and

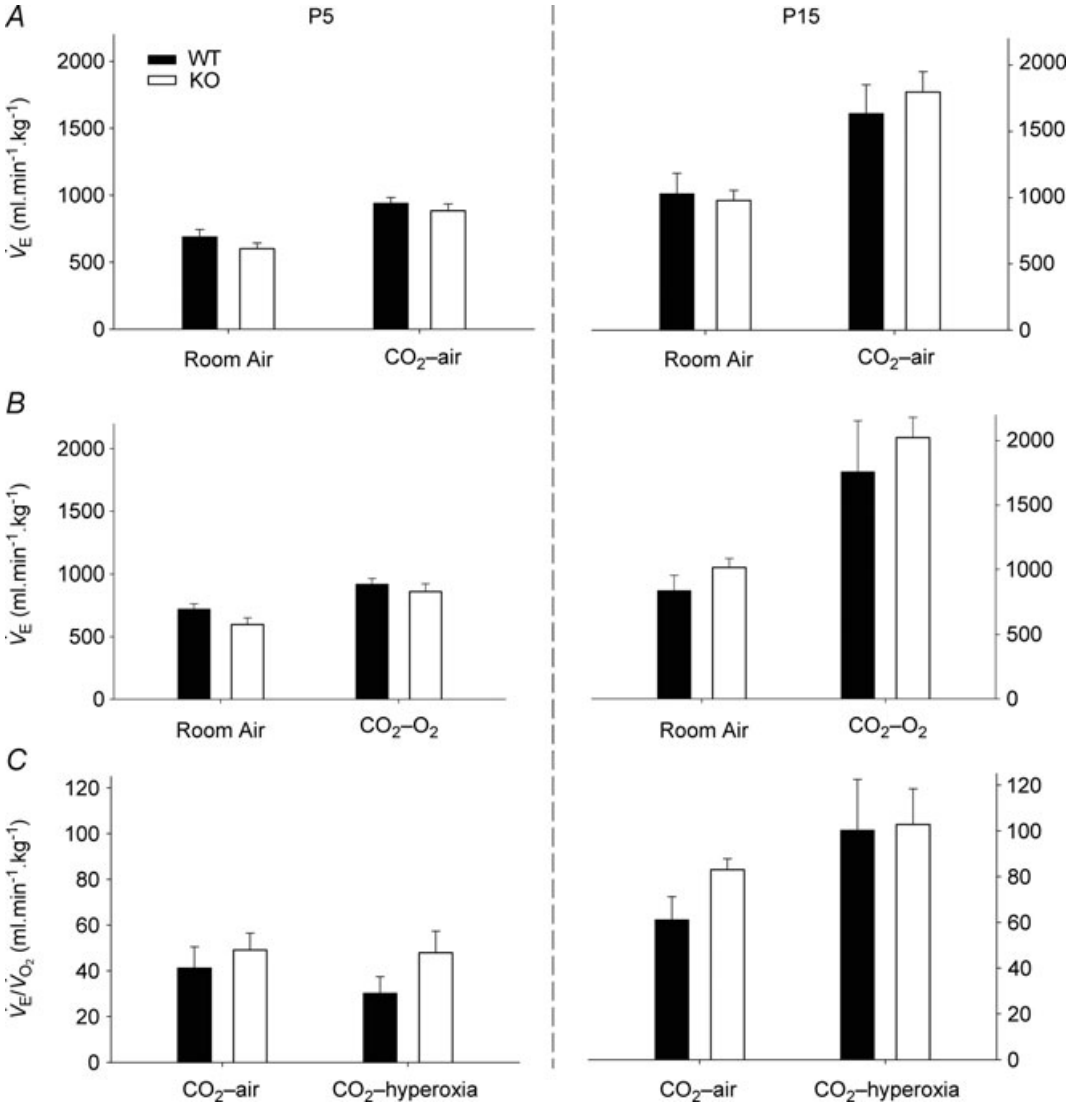

Figure 5. A loss of brainstem 5-HT neurones has no effect on the ventilatory response to CO_2 at P5 or P15

A, ventilation ($\dot{V}_E$) of WT and *Pet-1*$^{-/-}$ (KO) animals at P5 and P15, during room air and after 2 min of 5% CO_2 balanced with air (CO_2–air). *B*, $\dot{V}_E$ of WT and *Pet-1*$^{-/-}$ animals during room air and after 2 min of 5% CO_2 balanced with O_2 (CO_2–O_2). *C*, increase in $\dot{V}_E/\dot{V}_{O_2}$ (% change) after 2 min of either CO_2–air or CO_2–O_2 in each genotype at P5 and P15. All data are means ± SE.

heart with ambient cooling. Whether the sympathetic regulation of thermogenesis and heart rate during ambient cooling is served by the same set(s) of brainstem 5-HT neurones is another question we cannot answer with the current data, and is a matter of speculation even in adult animals (Ootsuka & Blessing, 2006). Brainstem 5-HT may be especially important during neonatal life for the appropriate sympathetic response to a variety of physiological stressors. An in-depth examination of the heart rate responses to other forms of physiological stress that may impact neonatal survival is warranted (e.g. hypoxia).

Others have shown that during the first two postnatal weeks, neonatal rodents with a genetically induced loss of brainstem 5-HT have reduced, unstable breathing (Erickson *et al.* 2007; Cummings & Frappell, 2009; Hodges *et al.* 2009; Cummings *et al.* 2010). The inhibitory effect of cooling on the $\dot{V}_E$ of *Pet-1*$^{-/-}$ animals is probably due to their reduced $\dot{V}_{O_2}$; $\dot{V}_E/\dot{V}_{O_2}$ did not change in either genotype with cooling although it was higher in *Pet-1*$^{-/-}$ at both T_A values. *Lmx-1b*$^{-/-}$ neonates lacking nearly all serotonergic neurones have reduced breathing, including apnoeas nearly 1 min long (Hodges *et al.* 2009). Although reduced $\dot{V}_{O_2}$ could contribute to the *Lmx-1b*$^{-/-}$ phenotype, the more severe respiratory dysfunction observed in *Lmx1b*$^{-/-}$ mice compared to *Pet-1*$^{-/-}$ mice may result from a loss of co-expressed factors (e.g. substance P, thyrotropin releasing factor) along with 5-HT. This may also explain why the CO_2 response is reduced in *Lmx1b*$^{-/-}$ mice but not in *Pet-1*$^{-/-}$ neonates. Alternatively, 5-HT neurones may contribute more to the CO_2 response at later points in development or at more extreme levels of hypercapnia (Hodges *et al.* 2008; Nattie & Li, 2010; Penatti *et al.* 2010).

P12 *Pet-1*$^{-/-}$ animals hyperventilate (increased $\dot{V}_E/\dot{V}_{O_2}$) compared to WT. This implies that these animals are hypocapnic and potentially alkalotic relative to controls. We did not observe hyperventilation in P5 or P15 *Pet-1*$^{-/-}$ animals when T_B was held at 36°C. T_B is unlikely to be playing a role, as further hyperventilation did not occur in P12 *Pet-1*$^{-/-}$ animals when T_B was reduced after ambient cooling. Alternatively, *Pet-1*$^{-/-}$ animals may only hyperventilate during a narrow window of postnatal life. Others have shown that a loss of brainstem 5-HT induces hyperventilation in rats and goats (Olson *et al.* 1979; Mitchell *et al.* 1983). The influence of brainstem 5-HT on the matching of $\dot{V}_E$ and $\dot{V}_{O_2}$ seems to depend on both species and stage of development. Our observations emphasize the need for accurate measurements of V_T and $\dot{V}_{O_2}$ when assessing factors influencing respiratory control during the neonatal period. This is troublesome in small, developing mammals where the T_B–T_A difference is narrowed, and in which there exists a considerable amount of airway resistance that, when using whole-body arrangements, contaminates the respiratory signal (Mortola & Frappell, 1998).

We have demonstrated that at a stage of development that may be close to the newborn period in humans (Clancy *et al.* 2001), brainstem 5-HT is essential for the thermogenic, HR and ventilatory responses to a mild and brief environmental cooling. In unanaesthetised neonatal animals, compromised non-shivering thermogenesis should be considered when assessing the effects of 5-HT deficiency on respiratory and HR control, especially at environmental temperatures below thermoneutrality. With previous findings in mind, we propose that brainstem 5-HT deficiency compromises, in a broad manner, the sympathetic responses to a variety of physiological stressors. Human infants with brainstem 5-HT deficiency may be prone to a variety of homeostatic deficits involving thermoregulatory, respiratory and cardiovascular control. Some of these deficits could manifest in the sudden infant death syndrome, recently linked to a deficiency in medullary 5-HT (Duncan *et al.* 2010).

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Author contributions

K.J.C., A.L. and E.E.N. contributed to the conception, design and analysis of experiments including interpretation of data, and drafting/revising/approving the final version of the manuscript for publication.

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