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Diphtheria toxin treatment of *Pet-1-Cre* floxed diphtheria toxin receptor mice disrupts thermoregulation without affecting respiratory chemoreception

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Abstract

In genetically-modified *Lmx1b*$^{f/f/p}$ mice, selective deletion of LMX1B in *Pet-1* expressing cells leads to failure of embryonic development of serotonin (5-HT) neurons. As adults, these mice have a decreased hypercapnic ventilatory response and abnormal thermoregulation. This mouse model has been valuable in defining the normal role of 5-HT neurons, but it is possible that developmental compensation reduces the severity of observed deficits. Here we studied mice genetically modified to express diphtheria toxin receptors (DTR) on *Pet-1* expressing neurons (*Pet-1-Cre*/Floxed DTR or Pet1/DTR mice). These mice developed with a normal complement of 5-HT neurons. As adults, systemic treatment with 2 – 35 μg diphtheria toxin (DT) reduced the number of tryptophan hydroxylase immunoreactive (TpOH-ir) neurons in the raphe nuclei and ventrolateral medulla by 80%. There were no effects of DT on baseline ventilation (V_E) or the ventilatory response to hypercapnia or hypoxia. At an ambient temperature (T_A) of 24°C, all Pet1/DTR mice dropped their body temperature (T_B) below 35°C after DT treatment, but the latency was shorter in males than females (3.0 ± 0.37 vs 4.57 ± 0.29 days, respectively; $p < 0.001$). One week after DT treatment, mice were challenged by dropping T_A from 37°C to 24°C, which caused T_B to decrease more in males than in females (29.7 ± 0.31°C vs 33.0 ± 1.3°C, p <

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We declare that we have read and abided by the statement of ethical standards for manuscripts submitted to *Neuroscience*.

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0.01). We conclude that the 20% of 5-HT neurons that remain after DT treatment in Pet1/DTR mice are sufficient to maintain normal baseline breathing and a normal response to CO_2, while those affected include some essential for thermoregulation, in males more than females. In comparison to models with deficient embryonic development of 5-HT neurons, acute deletion of 5-HT neurons in adults leads to a greater defect in thermoregulation, suggesting that significant developmental compensation can occur.

Keywords

serotonin; chemoreception; thermoregulation; gender differences

Introduction

The 5-HT system is involved in mammalian respiratory drive, respiratory rhythm generation (Dekin et al., 1985, Pena and Ramirez, 2002, Feldman et al., 2003, Hodges and Richerson, 2008a, Hodges et al., 2008, Hodges et al., 2009, Ptak et al., 2009, Hodges and Richerson, 2010) and the ventilatory response to CO_2 (Richerson, 1995, Wang et al., 1998, Wang and Richerson, 1999, Wang et al., 2001, Nattie et al., 2004, Li and Nattie, 2008, Corcoran et al., 2009, Ray et al., 2011). In addition, the 5-HT system, especially the raphe pallidus (RPa), has been associated with thermoregulation (Martin-Cora et al., 2000, Zaretsky et al., 2003, Morrison et al., 2008, Naumenko et al., 2009, Madden and Morrison, 2010, Hale et al., 2011, Morrison and Nakamura, 2011, Naumenko et al., 2011, Morrison et al., 2014). Adult mice genetically modified so that nearly all central 5-HT neurons are absent during adulthood (*Lmx1b*$^{f/f/p}$ mice) have previously been shown to breathe normally in room air (21% O_2 and 0% CO_2) and thermoregulate normally when T_A is 22°C, but have a 50% decrease in the ventilatory response to CO_2 and rapidly become hypothermic when exposed to a T_A of 4°C (Hodges et al., 2008). Similarly, male *Pet-1* null mice, in which there is a 70% decrease in 5-HT neurons, have normal baseline ventilation, whereas in 5-HT transporter (5-HTT) knockout mice baseline ventilation is increased (Gobbi et al., 2001, Li and Nattie, 2008, Hodges et al., 2011). However, in both of these strains thermoregulation is normal at a T_A of 22°C. In each of the above mouse strains the defects are present from embryonic life onwards, giving an opportunity for developmental compensation. In contrast, RC::PDi transgenic mice express a modified muscarinic acetylcholine receptor under control of the promoter for the 5-HTT (*Slc6a4*), and 5-HT neurons are normal throughout development, but upon systemic treatment with the exogenous agonist clozapine-N-oxide (CNO) their membrane potential is reversibly hyperpolarized (Ray et al., 2011). This leads to a large drop in T_B even when T_A is 22°C, whereas there is no effect on baseline ventilation parameters. These results suggest that developmental compensation occurs in *Lmx1b*$^{f/f/p}$, *Pet-1* null and 5-HTT knockout mice. In contrast, there should not be any developmental compensation in RC::PDi mice. However, CNO treatment only suppresses 5-HT neuron firing by ≈50%, which could potentially explain why CNO only suppresses the hypercapnic ventilatory response by 50% in RC::PDi mice (Ray et al., 2011). Therefore, for different reasons each of these approaches could have given results that underestimated the actual contribution of 5-HT neurons to central respiratory chemoreception.

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Our principal objective in the present work was to use the DTR/DT system to acutely delete a large percentage of central 5-HT neurons in adult mice and study the effect on thermoregulation and ventilation in the absence of compensatory developmental changes. DT is a protein with a molecular weight of ± 62,000 D that causes cell death by inhibition of protein synthesis, and must be transported into cells by DTR to be effective (Moskaug et al., 1989, Collier, 2001). Wild type mouse cells are at least 103–105 times more resistant to DT than human cells. Thus, engineered expression of the high-affinity human DTR by a particular cell type in mice is a powerful means of selectively depleting that population of cells *in vivo* upon systemic DT administration (Palmiter et al., 1987, Saito et al., 2001, Cha et al., 2003, Buch et al., 2005). In Pet1/DTR mice, we expected that DTRs would be expressed selectively in central 5-HT neurons. This was based on the previous demonstration that DTR is expressed selectively in cells that express Cre recombinase at some time in the life of floxed DTR mice (Palmiter et al., 1987, Buch et al., 2005), and the previous finding that *Pet-1* is expressed selectively in central 5-HT neurons (Hendricks et al., 1999). We obtained evidence in support of this assumption using immunohistochemistry and Nissl staining, which confirmed that DT administration eliminated a large percentage of TpOH-ir neurons and an equally large number of Nissl stained neurons in the raphe nuclei.

Experimental procedures

All experiments were done in accordance with guidelines of the National Institutes of Health for animal care and use and were approved by the Yale University Animal Care and Use Committee.

Animals

Pet1/DTR mice were generated by mating ePet-Cre mice (Scott et al., 2005) with Cre-inducible DTR transgenic mice (iDTR) (Buch et al., 2005). In iDTR mice, DTR is expressed upon Cre recombinase-mediated excision of a STOP cassette (Buch et al., 2005). We expected that Cre recombinase would be expressed selectively in 5-HT neurons, because in ePet-Cre mice Cre expression is under control of the enhancer region of *Pet-1*. Genotyping of Pet1/DTR mice was done using the strategy as previously reported (Saito et al., 2001, Cha et al., 2003). Tail samples were digested and PCR was performed using the following Flox primers: DTR-1 sense, ACCATGAAGCTGCTGCCGTC and DTR-2 antisense, ATCAGTGGGAATTAGTCATGC. CRE primers: CRE-1 sense, 5′ATTTGCCTGCATTACCGGTCG 3′ and CRE-2 antisense, 5′CAGCATTGCTGCTGTCACTTGGTC 3′. The PCR product was analyzed by agarose gel electrophoresis. The band size for DTR is 600 bp and for Cre is 375 bp. Bands are absent for both wild-type (WT) genotypes.

Adult male Pet1/DTR mice (87 ± 4 days old; 27.9 ± 0.7 g; n = 24), female Pet1/DTR mice (104 ± 6 days old; 20.2 ± 0.8 g; n = 14) and their male WT (93 ± 5 days old; 28.5 ± 0.9 g; n = 11) and female WT (94 ± 9 days old; 21.1 ± 0.5 g; n = 7) littermates were studied. All animals received food and water *ad libitum* and were housed on a 12 hr light/dark cycle in the Yale Animal Care Facility.

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Diphtheria toxin protocol

We tested the effects of systemic administration of DT (Sigma, DO564) using doses of 5, 50 and 250 μg/kg i.p. in 100 μl of normal saline solution. DT was administered 1, 3 or 5 times per week, for 2, 4 or 6 weeks in order to find a protocol for DT administration that caused a specific effect on Pet1/DTR mice without nonspecific effects on WT mice. The effects were independent of the dosing schedule and only on the cumulative dose so the results are presented as a function of the total amount of DT (DT_T). Since DT led to a drop in T_B, animals were housed at an T_A of 30 C as needed to prevent hypothermia.

Immunohistochemistry

Mice were deeply anesthetized with pentobarbital and then perfused with phosphate buffered saline (PBS; 20 ml), followed by 4% paraformaldehyde in phosphate buffer (25 ml over 10 minutes). Brains were removed and left in fixative overnight, then for cryoprotection were placed in 30% sucrose in PBS for 2–3 days. Brainstems were sectioned in the coronal plane (25 μm) on a cryostat (CM30505, Leica). Sections were permeabilized and blocked with 3% horse serum and 0.4% Triton X-100 in PBS, then incubated overnight with a primary antibody against TpOH (1:2000, mouse monoclonal; Sigma T-0678) in blocking solution (3% horse serum in PBS). Sections were incubated with biotinylated horse anti-mouse IgG (1:1000; Vectastain Elite ABC kit, Vector Laboratories), followed by ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories) for 50 min and then Nova Red peroxidase substrate (NovaRed™, Vector Laboratories) for 5–7 min. Sections were dehydrated with successive ethanols and xylene, and coverslipped with Permount mounting medium (UN1294, Fisher Scientific). The numbers of TpOH-ir neurons were counted from 17 Pet1/DTR (male = 11, female = 6) and 13 WT (male = 9, female = 4) mice using a brightfield microscope (DMI 6000B, Leica). One slice was studied out of every 4 slices from bregma –5.80 mm to bregma –7.64 mm (Paxinos and Franklin, 2001). TpOH-ir neurons were counted separately in the raphe obscurus (ROb), raphe magnus (RMg), RPa and ventrolateral medulla (VLM) as shown in Figure 1A.

Nissl Staining

Every 4th section was mounted on a slide and used for Nissl staining. Slides were placed in 37% formalin and 100% ethyl alcohol (EtOH) 1:1 for 5 minutes, and rehydrated in 95% EtOH for 3 min, 70% EtOH for 2 min, rinsed twice in distilled water for 3 min and stained with Cresyl Violet (1 mg/ml) for 20 min. After staining, sections were rinsed twice in water for 2 min, then dehydrated in 70% EtOH for 2 min, in 1% glacial acetic acid in 95% EtOH for 5 min, in 95% EtOH for 3 min and twice in 100% EtOH for 3 min each. To lighten the sections, xylene was applied twice for 5 minutes each, after which slides were coverslipped with Permount mounting media (UN 1294, Fisher Scientific). The Nissl stained cells were counted using a brightfield microscope (DMI 6000B, Leica) using the same boundaries as shown in Figure 1A.

Whole-body plethysmography

Prior to the first injection of DT, and one week after the final injection of DT, standard whole-body plethysmography was used as described previously to measure V_E, respiratory

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frequency (f_R), tidal volume (V_T) and oxygen consumption (VO_2) in awake Pet1/DTR and WT mice of both sexes (Hodges and Richerson, 2008b, Hodges et al., 2008). All experiments were performed in the daytime (light) period and studies before and after DT administration were conducted at the same time of day for each mouse. V_T was calibrated by injecting 300 μl of air into the plethysmography at a rate of 100 strokes/min using a mechanical ventilator (Minivent; Harvard Apparatus; Holliston, MA). The mice were monitored during more than 30 min in normoxia (21% O_2- balance N_2), 10 min in 5% hypercapnia (5% CO_2-21% O_2- balance N_2), 10 min in 7% hypercapnia (7% CO_2-21% O_2-balance N_2), 10 min in hyperoxic hypercapnia (7% CO_2 −50% O_2-balance N_2), 20 min in normoxia and 10 min in hypoxia (10% O_2- balance N_2). T_B was measured using a telemetric temperature probe (IPTT 6007) subcutaneously implanted between the scapulae under isoflurane anesthesia at least 5 days before the first experiment. Data were analyzed from the last 5 min of exposure to each gas using Matlab software off-line (The MathWorks; Natick, MA). Data were only analyzed during periods of quiet wakefulness when mice were not sleeping, walking, running, grooming, licking, eating or sniffing, and when the eyes were open.

Quantification of changes in ventilation

The ventilatory responses to changes in O_2 and CO_2 were quantified in three separate ways. 1) V_E during the stimulus was calculated as a percentage of baseline V_E. 2) The change in V_E induced by the stimulus (in ml/min/gm) was the difference between V_E during a stimulus and V_E at baseline. 3) The mean value of V_E during a stimulus was also compared to the mean value of V_E at baseline. The same methods were used to compare changes of other parameters as well.

Statistics

Data are presented as mean values ± SEM. The threshold for statistical significance was set to $p < 0.05$ and determined by one-way or two-way ANOVA followed by Bonferroni's multiple comparison test (Prism 4, GraphPad Software).

Results

DT decreased the number of TpOH-ir neurons in Pet1/DTR mice

DT injections resulted in a much smaller number of TpOH-ir neurons in Pet1/DTR mice (Fig. 1B). We tested three protocols: 1) DT 5 μg/kg i.p. 2 times daily for 2–4 weeks; 2) DT 50 μg/kg i.p. 1–2 times per week for 1–3 weeks, or; 3) DT 250 μg/kg i.p. 1–2 times per week for 1–2 weeks. The protocols using DT_T from 2 to 35 μg all resulted in a decrease in the number of 5-HT neurons in each of the four nuclei studied (Fig. 1C); however, non-specific effects were seen when the higher doses of DT were administrated as a single injection, or when they were administered as 2 or 3 split doses. When the highest levels of DT_T were given, undesirable effects occurred in Pet1/DTR mice, and surprisingly also in WT mice. In these cases, mice of both genotypes appeared sick, lost weight and had an increase in mortality. The protocol least likely to show nonspecific effects was 50 μg/kg DT once a week for 2–3 weeks (1.5 – 2 μg DT_T). More than three injections at that dose or three injections at a higher dose caused an increase in mortality of both Pet1/DTR and WT mice.

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The mortality of Pet1/DTR mice was less than 10% with a DT_T of 4 μg (n = 14), and increased progressively with DT_T, being 23% and 57% with a DT_T of 15 μg and 25 μg, respectively. The mortality of WT mice was 20% with a DT_T of 30 μg (n = 10). No WT mice died after DT_T less than 30 μg.

When data were pooled from animals with DT_T injections between 2 and 35 μg it was found that there was a significant reduction in TpOH-ir neurons in Pet1/DTR mice by 85% in ROb, 83% in RMg, 77% in RPa and 81% in VLM (Fig. 1D). The number of TpOH-ir neurons present in Pet1/DTR mice (n = 17) was 2.5 ± 0.5, 2.8 ± 0.6, 1.8 ± 0.4 and 1.3 ± 0.3 per 25 μm thick slice in the ROb, RMg, RPa and VLM, respectively. In comparison, there were 16.7 ± 0.4, 16.6 ± 0.6, 7.7 ± 0.3 and 7.0 ± 0.2 TpOH-ir neurons/25 μm thick slice in WT mice injected with DT (n = 13, $p < 0.0001$). There were no differences in the numbers of TpOH-ir neurons between WT mice injected with DT versus Pet1/DTR mice that did not receive injections (n = 5; fig 1D). The highest doses of DT led to high mortality in Pet1/DTR mice as well as in WT mice, making it difficult to collect data from mice given the highest doses.

DT decreased the total number of neurons present in the raphe nuclei of Pet1/DTR mice

The mechanism of action of DT/DTR has been well-defined as causing cell death due to prevention of protein synthesis (Collier, 2001). However, it is theoretically possible that some neurons were still alive after our treatment, but their protein synthesis was so impaired that TpOH levels were too low to be detected using immunohistochemistry. Therefore, we examined whether the decrease in TpOH immunoreactivity after DT treatment was associated with a decrease in the total number of neurons in the medullary raphe. Nissl staining was performed on sections from the same brains that were used for immunohistochemistry. Photomicrographs of the ROb and RPa showed fewer Nissl stained cells in Pet1/DTR mice than in WT mice after both were treated with DT (Fig. 2A and B). We counted cells within 100 μm of the midline in sections from untreated Pet1/DTR mice (220 ± 13, n=5), DT treated WT mice (204 ± 8, n=5) and DT treated Pet1/DTR mice (162 ± 7, n=8) (Fig. 2C). There were many neurons in this region that were not serotonergic, but the average reduction in the number of Nissl stained cells was nearly equal to the average reduction in the number of TpOH-ir neurons, consistent with the reduction in TpOH-ir neurons being due to death of 5-HT neurons.

Baseline ventilation and chemoreception were not affected by treatment with DT

There were no differences in resting ventilation between untreated male or female Pet1/DTR mice or WT mice (Table 1). There were no effects on V_E, VO_2, V_T or f_R after 4 μg DT_T under baseline conditions in normoxia (Table 1). It was not possible to exceed that dose of DT without inducing non-specific effects such as a drop in temperature and death in WT mice, and yet it was ineffective in eliminating all serotonin neurons in Pet1/DTR mice, in that 20% of the TpOH-ir neurons were spared after that dose or larger doses (n = 17) (Fig. 1C and D).

The ventilatory responses to chemoreceptor stimuli were also tested, and the responses were quantified using three different methods (see Methods). The absolute values of ventilation

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before and during hypoxia, hypercapnia and hyperoxic hypercapnia are shown in table 2 for the different groups before DT. There was no difference between groups for any of the measures. We also found no effect on changes in V_E (Fig. 3; measured as percentage increase or the absolute change) or other ventilatory parameters (data not shown) in response to hypoxia, hypercapnia or hyperoxic hypercapnia after DT treatment among male or female Pet1/DTR mice (n = 12 each) in comparison to male or female WT mice (n = 7 each).

Since Pet1/DTR mice decreased their T_B after administration of DT it was necessary to raise T_A in the plethysmography chamber from 30°C to 33 – 34°C in order to maintain a normal T_B of Pet1/DTR mice. Before DT administration, T_B during plethysmography for male and female Pet1/DTR mice was 37.5 ± 0.2°C (n=12) and 37.4 ± 0.2°C (n=12), respectively. After DT administration, with T_A maintained at 33 – 34°C, T_B during plethysmography was 36.8 ± 0.2°C (n=7) and 37.3 ± 0.1°C (n=7), for male and female Pet1/DTR mice, respectively.

DT caused male Pet1/DTR mice to be unable to maintain normal T_B

Male Pet1/DTR mice housed at T_A of 24°C dropped their T_B below 35°C after one injection of DT at 50 μg/kg. A single injection of DT at 50 μg/kg caused T_B of Pet1/DTR male mice (n = 9) to decrease from 37.4 ± 0.20°C to 34.8 ± 0.26°C ($p = 0.02$). After the first injection, animals were housed at T_A of 30°C so they were able to maintain T_B near normal, during which a second injection caused T_B to decrease to 32.6 ± 0.59 °C ($p < 0.0001$). Neither of these two injections at 50 μg/kg had any effect on T_B of male WT mice (n = 9). T_B of male WT mice was 37.62 ± 0.23°C at baseline, 37.5 ± 0.19°C after the first DT injection and 37.3 ± 0.20°C after the second DT injection.

Acute deletion of 5-HT neurons affected thermoregulation in males more than in females

We measured the latency from the first DT injection (50 μg/kg i.p.) to when T_B dropped below 35°C. As shown in Figure 4A, the latency in female Pet1/DTR mice (4.67 ± 0.33 days; n = 9) was longer than that in male Pet1/DTR mice (3.11 ± 0.31 days; n = 9; $P < 0.001$). After DT injections, if T_B dropped below 35°C, T_A was increased from 30°C first to 32°C, then 34°C and then 36.5°C, reaching the highest of these temperatures needed to maintain T_B at ≈37°C.

Thermoregulation was then tested by decreasing T_A from 36.5°C to 24°C and measuring changes in T_B (Fig. 4B). Over a period of 2.5 hr, T_B of male mice decreased to 29.7 ± 0.31°C (n=9), whereas that of females only decreased to 33.0 ± 1.34°C (n=9; $p < 0.001$). In contrast to this differential effect of 5-HT neuron deletion on thermoregulation in female vs male mice, there were no gender differences in the number of TpOH immunoreactive neurons between female Pet1/DTR mice (n = 6) and male Pet1/DTR mice (n = 7) after DT injections in any region studied (Fig. 4C).

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Discussion

DTR mice were used as a genetic tool for temporal control of 5-HT neuron deletion

Acute deletion of 5-HT neurons allowed us to test their role in brain function, without compensation that often occurs with conventional knockout mice. These data show that 20% of central 5-HT neurons are capable of maintaining normal breathing in room air and a normal response to hypercapnia and hypoxia in adult Pet1/DTR mice. However, after 80% of central 5-HT neurons were lesioned with DT injections, mice were not able to maintain T_B within a normal range, even when housed at an ambient temperature of 32 °C. 5-HT neurons were found to play a greater role in regulating T_B in males than in females since there was a faster and larger fall in T_B in males than in females after DT injection.

DT treatment had no effect on baseline breathing or chemoreception

The baseline ventilatory parameters presented here were consistent with previous data of f_R, V_T and V_E in a range of 180 – 225 breath min^{-1}, 8 – 15 μl g^{-1} and 1.5 – 3.5 ml min^{-1} g^{-1}, respectively (Morin-Surun et al., 2001, Li and Nattie, 2008, Hodges et al., 2011). The 5-HT system is involved in respiratory drive and chemoreception (Dekin et al., 1985, Richerson, 1995, Wang et al., 1998, Wang and Richerson, 1999, Wang et al., 2001, Pena and Ramirez, 2002, Feldman et al., 2003, Nattie et al., 2004, Hodges and Richerson, 2008a, Hodges et al., 2008, Li and Nattie, 2008, Corcoran et al., 2009, Hodges et al., 2009, Ptak et al., 2009, Hodges and Richerson, 2010, Niebert et al., 2011, Ray et al., 2011). Nevertheless, we did not observe any significant change in breathing in normoxia, hypercapnia or hypoxia between male or female mice after deletion of 5-HT neurons with systemic administration of DT. The chemosensory response to CO_2 and O_2 of Pet1/DTR mice was similar to that previously observed in mice (Morin-Surun et al., 2001, Hodges et al., 2008, Li and Nattie, 2008, Ray et al., 2011). It is possible that this technique may lead to a result that is hard to interpret, because of the nonspecific effects and increased mortality with the highest doses of DT (Saito et al., 2001, Luquet et al., 2005, Bennett and Clausen, 2007). Unlike other genetically modified mice affecting the 5-HT system that are able to compensate during development, such as the Lmx1b$^{f/f/p}$, 5-HTT knockout and Pet1 knockout mice (Gobbi et al., 2001, Hodges et al., 2008, Li and Nattie, 2008, Hodges et al., 2009, Cummings et al., 2010, Hodges et al., 2011), Pet1/DTR mice develop normally. However, the response of adult Pet1/DTR mice also differs from adult RC::PDi mice, which had a reduced response to CO_2 after acute silencing of 5-HT neurons after normal development (Ray et al., 2011). One possibility for the difference is that a larger percentage of 5-HT neurons might have been affected in RC::PDi mice, but there are no data available on what percentage of 5-HT neurons are inhibited by CNO in that model. Another possibility is that the delay between DT treatment and loss of TpOH-ir neurons of a few days is long enough for there to be compensation, whereas the acute inhibition of 5-HT neurons in RC:PDi mice occurs so quickly that compensation does not occur. A third possibility is that the subset of 5-HT neurons that are spared in Pet1/DTR mice after DT treatment are a different subset than those that are unaffected by CNO in RC:PDi mice. This is feasible because DTR expression is driven by the enhancer region of *Pet-1* in Pet1/DTR mice, whereas expression of Di is driven by the promoter for the 5-HTT in RC:PDi mice. The neurons affected by these two genetic influence may only be partially overlapping. In fact, the 30% of 5-HT neurons that

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are spared in *Pet-1* knockout mice have somata that are distributed across the B1–B9 cell groups, but have projections to only a selective subset of brain regions, including the nucleus tractus solitarius, nucleus ambiguus, and ventrolateral medulla (Kiyasova et al., 2011), each of which is involved in breathing. This could explain why breathing is much less affected in *Pet-1* knockout mice than in *Lmx1b$^{f/f/p}$* mice even though 70% of 5-HT neurons are deleted in the former. This relatively lower dependence on *Pet-1* of 5-HT neurons that project to respiratory nuclei may mean they express DTRs at a lower level than other 5-HT neurons, so that the effect of 5-HT neurons on breathing is relatively normal in Pet1/DTR mice after DT treatment.

Serotonergic mechanisms involved in thermoregulation

Pet1/DTR and RC::PDi mice both exhibited a decrease in T_B. In RC::PDi mice the fall in T_B occurred immediately after central 5-HT neurons were silenced, whereas in Pet1/DTR mice the fall in T_B occurred over time in accordance with the time it took for 5-HT neurons to be deleted by the toxin. The data presented here support the hypothesis that the 5-HT system is required for normal thermoregulation. Several areas of the hypothalamus have been implicated as important thermosensitive regions that could evoke physiological responses (Adair, 1977, Boulant, 2006). Interactions between the hypothalamus and 5-HT system can explain why a decrease in 5-HT neurons produces abnormal regulation of T_B. For example, there are serotonergic fibers and terminals throughout the hypothalamus, as well as 5-HT_{2C} and 5-HT_{1B} receptors (Steinbusch, 1981, Mirkes and Bethea, 2001, Makarenko et al., 2002). In addition, the RPa interacts with descending hypothalamic drive to control generation of heat by brown adipose tissue (BAT) (Madden and Morrison, 2010, Hale et al., 2011, Morrison and Nakamura, 2011), and there are direct projections to the spinal cord which help regulate the shivering response to cold (Cano et al., 2003). In fact, genetic deletion of 5-HT neurons in *Lmx1b$^{f/f/p}$* mice leads to severely impaired BAT thermogenesis and abnormal shivering. Recently, it has been shown that there is potent modulation of BAT thermogenesis by orexin released from the lateral hypothalamus directly into the RPa (Tupone et al., 2011). Thus, there are several anatomical sites where dysfunction of 5-HT neurons could interfere with temperature sensation, BAT thermogenesis and shivering and lead to abnormal regulation of body temperature.

In contrast to the severe deficit seen here in adult Pet1/DTR mice after deletion of 80% of 5-HT neurons, there is a much less severe defect in adult Lmx1b$^{f/f/p}$ mice even though they lack ~100% of central 5-HT neurons, in *Pet-1* null mice that lack ~70% of central 5-HT neurons and in 5-HTT knockout mice (Hodges et al., 2008, Li and Nattie, 2008, Hodges et al., 2011). The latter three models all maintain a normal T_B when T_A is at normal room temperature. One possible reason for the difference is that the brain in the latter three transgenic mouse lines may have compensated during development for the lack of normal 5-HT system function.

There is a decrease in number of 5-HT neurons in Pet1/DTR mice, Lmx1b$^{f/f/p}$ mice and *Pet-1* null mice, presumably leading to a decrease in brain extracellular 5-HT levels, whereas 5-HTT knockout mice likely have an excess of brain extracellular 5-HT levels, and yet all four mouse lines have abnormal thermoregulation. The excess of 5-HT in 5-HTT

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knockout mice might lead to an increase in autoinhibition of 5-HT neurons due to activation of 5-HT_{1A} inhibitory autoreceptors. This could explain why 5-HT neurons show reduced firing rate (Gobbi et al., 2001), as well as a reduction in the density, expression and desensitization of 5-HT_{1A} receptors (Li et al., 2000, Bouali et al., 2003). Although this may seem paradoxical, treatment by 5-HTT blockers has been shown to cause such changes both *in vivo* (Gartside et al., 1995, Romero et al., 2003) and in brain slices (Evans et al., 2008). These papers show that even though SSRIs cause a decrease in firing rate of 5-HT neurons, there is still an increase in extracellular [5-HT]. The sustained increase in extracellular [5-HT] could lead to desensitization of some downstream receptors. The decrease in firing rate of 5-HT neurons could reduce the release of co-localized neuropeptide neurotransmitters. In both cases, the functional effects would be similar to those that occur with a decrease in the number of 5-HT neurons.

It is possible that compensatory changes occur in Lmx1b$^{f/f/p}$ mice, *Pet-1* null mice and 5-HTT knockout mice, and may reduce the severity of the phenotypes resulting from these different defects in the 5-HT system. Compensatory changes could also occur in other neurotransmitter systems, such as norepinephrine or acetylcholine, to make up for the missing serotonin. If compensatory changes do occur in mice with a deficient 5-HT system, these changes might be expected to be greatest in the Lmx1b$^{f/f/p}$ mice since they have the most radical defect – a complete absence of central 5-HT neurons. Those compensatory changes could explain why Lmx1b$^{f/f/p}$ mice have normal T_B at 22°C. However, there is no compensation by chronic metabolic acid/base changes, because arterial blood gases and pH are not different between Lmx1b$^{f/f/p}$ and WT mice (Hodges et al., 2008). There are also no changes in norepinephrine or dopamine levels in Lmx1b$^{f/f/p}$ mice, indicating that neither of these two systems have compensated for the absence of 5-HT (Zhao et al., 2006). There still remains the possibility that compensation occurs in other systems such as the cholinergic or glutamatergic systems. Further studies at different ages during development may define compensatory mechanisms in Lmx1b$^{f/f/p}$ and other transgenic mice, so that the magnitude of the contribution of the 5-HT system to thermoregulation, ventilation and chemoreception can be determined more accurately.

Thermoregulation is more dependent on 5-HT neurons in male than in female mice

Gender differences have been reported in rats for ventilatory control including the hypercapnic ventilatory response (HCVR), as well as ethanol-induced hypothermia and circadian temperature (Taylor et al., 2006, Behan and Wenninger, 2008, Taylor et al., 2009, Wenninger et al., 2009). Therefore, many respiratory and thermoregulatory studies have focused only on males to eliminate differences between the two genders as a confounding factor. Some studies have linked an altered 5-HT system with gender differences in ventilation or thermoregulation (Li and Nattie, 2008, Hodges et al., 2011). Here we found further evidence of gender differences in regulation of brain function by the 5-HT system as DT treatment has different effects on T_B in males than in females. These findings suggest that 5-HT neurons are more important for thermoregulation in males compared to females. Several lines of evidence suggest possible interactions between sex hormones and the 5-HT system. It is possible that 5-HT neuron activity could be regulated by sex hormones, which could be acting directly on progestin receptor or estrogen receptor beta in several raphe

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nuclei (Bethea, 1993, Gundlah et al., 2001). Furthermore, ovarian steroids enhance serotonin neuron function by causing an increase in transcription and translation of TPH-2 and 5-HTT, and a decrease of $5HT_{1A}$ receptors and monoamine oxidase A (Lu and Bethea, 2002, Lu et al., 2003, Sanchez et al., 2005, Tokuyama et al., 2008, Bethea et al., 2009). These changes could explain, in part, why the females in our study were less affected than males even though they had the same number of remaining 5-HT neurons. Moreover, convergence of hormones and 5-HT in the hypothalamus and release of different hormones from the hypothalamus after stimulation of 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors has been shown (Bluet Pajot et al., 1995, Bagdy, 1996, Mirkes and Bethea, 2001), pointing out that not only is 5-HT activity regulated by sex hormones, but 5-HT neurons may also regulate hypothalamic activity, which is involved in homeostatic processes including hormonal release and thermoregulation.

Off target effects prevented deletion of more than 80–85% of 5-HT neurons

We expected that Pet1/DTR mice would express DTRs selectively in all 5-HT neurons, because the *Pet-1* enhancer region causes Cre recombinase expression almost exclusively in 5-HT neurons (Scott et al., 2005), and *Pet-1* is thought to be expressed selectively in all 5-HT neurons both during development and in adults (Hendricks et al., 1999). Even if *Pet-1* was only expressed transiently during development that should have resulted in transient Cre recombinase expression, and thus permanent DTR expression. The DTR transgene should have only been expressed in cells that expressed Cre recombinase at some time in their life (Buch et al., 2005), so it should not have been expressed in cells other than 5-HT neurons.

DT injections at low doses impaired thermoregulation of Pet1/DTR mice but not that of WT mice. At these doses there was not any difference in baseline ventilation or in the ventilatory response to hypercapnia or hypoxia between Pet1/DTR and WT mice. As the dose was increased, nonspecific effects confounded the use of this genetic approach, because when doses were used that were high enough to lesion more than 85% of 5-HT neurons, defects in thermoregulation were induced in WT mice, as well as an increase in their mortality.

In this study, male and female mice both retained approximately 15% of 5-HT neurons in ROb, 17% in RMg, 23% in RPa and 19% in VLM. We did not find a difference in the effect of DT on survival of 5-HT neurons between different nuclei of the raphe. It was not determined whether the surviving 5-HT neurons were a subset characterized by specific projections, as has been observed in *Pet-1* knockout mice (Kiyasova et al., 2011). As described above, the loss of 5-HT neuron cell bodies is distributed across many different raphe nuclei, but 5-HT innervation is preserved in medullary nuclei involved in control of breathing. Projections are also preserved to a variety of brain areas involved in stress responses, with dense innervation of the basolateral amygdala, the paraventricular nucleus of the hypothalamus, the intralaminar thalamic nuclei, the ventral part of the periaqueductal gray, and the parabrachial nucleus (Kiyasova et al., 2011). Defining whether the neurons that survived in Pet1/DTR mice after DT treatment have a specific phenotype (possibly the same subset as those that survive in Pet-1 knockouts) might contribute to understanding why there was a large effect on T_B and not on breathing. It is possible that a subset of 5-HT neurons are thermosensitive and may reinforce the mechanisms of thermoregulatory control

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by the hypothalamus or modulate descending synaptic drive from the hypothalamus to effector organs involved in thermogenesis. It is already known that stimulation of 5-HT_{1A}, 5-HT_3 and 5-HT_7 receptors can lead to hypothermia, and the mechanisms involved differ(Popova et al., 2008, Naumenko et al., 2009, 2011). However, it remains unknown how 5-HT neurons contribute to T_B regulation and the underlying mechanisms for hypothermia produced by failure of the 5-HT system.

Conclusions

The data presented here confirm the importance of the 5-HT system in thermoregulation and demonstrate that failure of the 5-HT system affects the body temperature in males more than females. Acute deletion of 5-HT neurons by DT treatment of adult Pet1/DTR mice leads to a greater defect in thermoregulation than when 5-HT neurons are prevented from developing during embryogenesis (e.g. Lmx1b$^{f/f/p}$ and *Pet-1* knockouts), pointing to the potential for significant compensation in the thermoregulatory system.

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Highlights

1. Pet1/DTR mice express diphtheria toxin receptors on serotonin neurons
2. Systemic diphtheria toxin causes loss of 80% of serotonin neurons
3. Ventilation was not affected after deletion of 80% of central serotonin neurons
4. Diphtheria toxin did cause a severe defect in thermogenesis
5. 5-HT neurons that were spared may be those that are important for chemoreception

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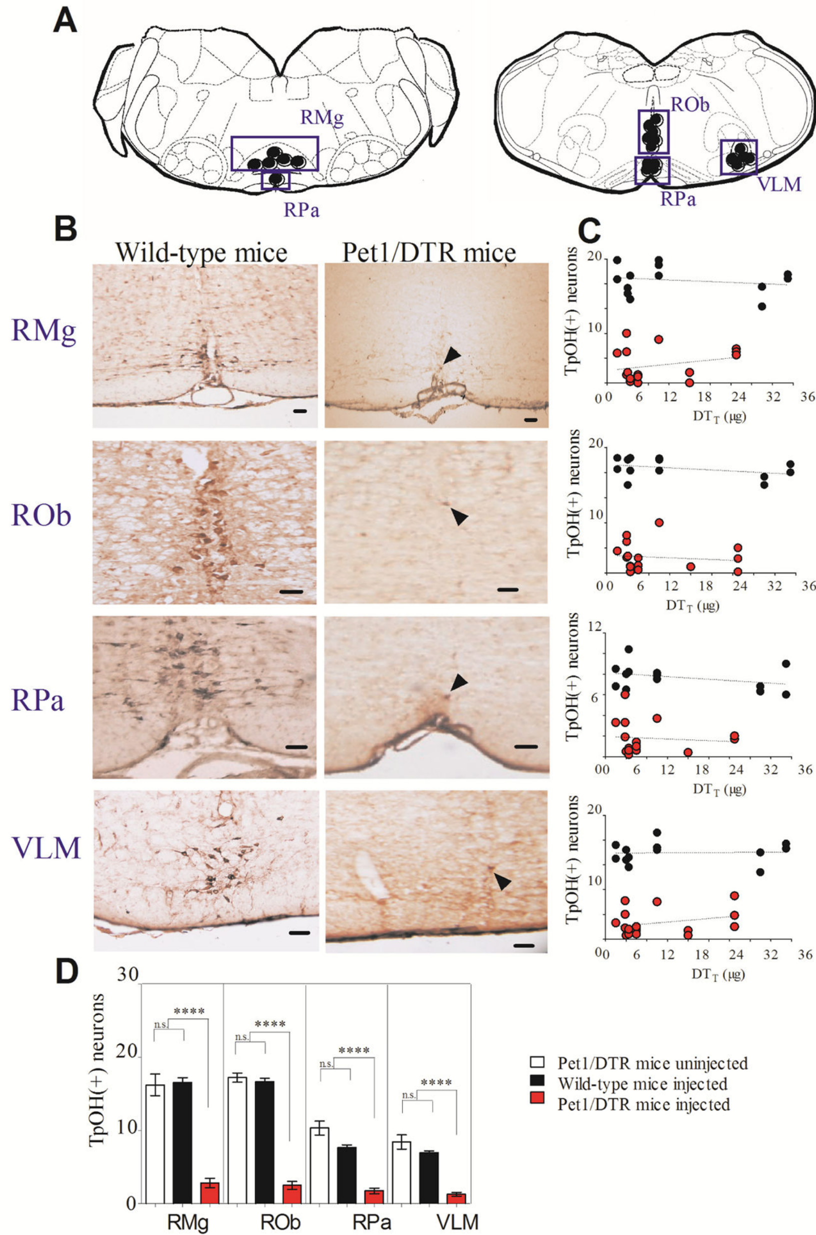

Figure 1. Systemic diphtheria toxin reduces the number of TpOH-ir neurons in the brainstem of Pet1/DTR mice but not WT mice

A: Schematic representation of the locations used to count the number of TpOH-ir neurons in the RMg, RPa ROb, and VLM using coordinates described in the text as distance from Bregma (B). Adapted from Paxinos & Franklin, 2001. **B**: TpOH-ir neurons in the RMg, ROb, RPa and VLM from WT and Pet1/DTR mice after 4 μg DT_T. Black bars, 100 μm. **C**: Number of TpOH-ir neurons/25 μm thick slice in Pet1/DTR mice (red circles, n = 17) and WT mice (black circles, n = 13) as a function of dose of DT_T from 2–36 μg. **D**: Summary of the number of TpOH-ir neurons from Pet1/DTR mice injected with DT (red bar; n = 17 compared with WT mice injected with DT (black bar; n = 13 and Pet1/DTR mice that did not receive any injection (empty bar; n = 5. ****, $p < 0.0001$). No differences were found

between DT treated WT mice and untreated Pet1/DTR mice. ($p > 0.05$, 1 way ANOVA, Bonferroni's multiple comparison test).

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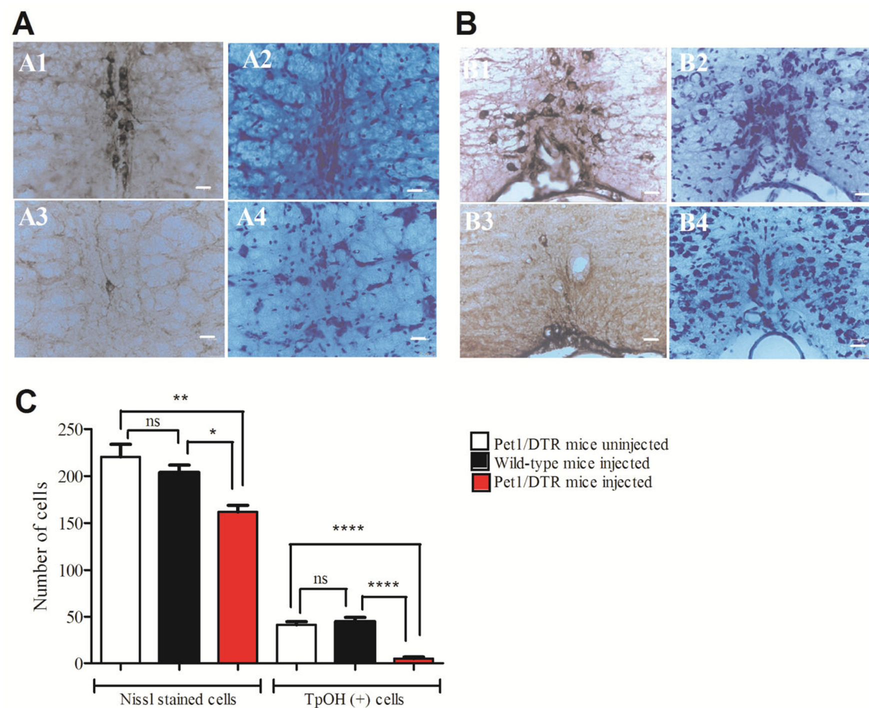

Figure 2. Nissl staining demonstrated that systemic DT caused death of neurons in the raphe nuclei of the medulla

A & B: Photomicrographs of ROb (A) and RPa (B) showed fewer TpOH-ir neurons (A3 & B3) and Nissl stained cells (A4 & B4) in Pet1/DTR mice after systemic treatment with DT than in WT mice after the same treatment (A1, A2, B1 & B2). White bars 20 μm. **C:** Cells were counted within 100 μm of the midline in untreated Pet1/DTR mice (220 ± 13, n=5), WT mice treated with DT (204 ± 8, n=5) and Pet1/DTR mice treated with DT (162 ± 7, n=8). *, ** and **** $p < 0.05$, 0.01 and 0.0001 respectively. 1 way ANOVA, Bonferroni's multiple comparison test.

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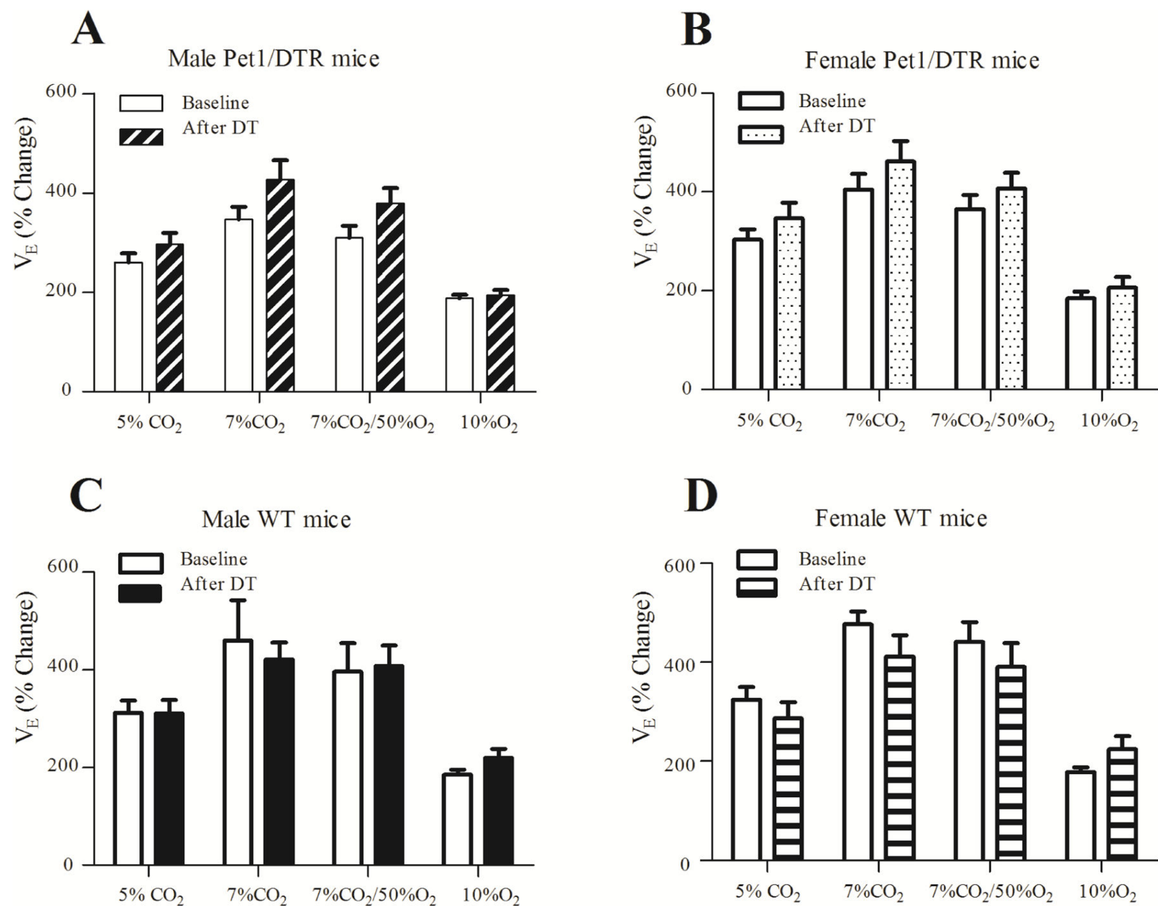

Figure 3. The responses to hypercapnia and hypoxia were not affected after 80–85% of 5-HT neurons were deleted

After two injections of DT (4 μg DT_T), there was no effect on V_E at baseline or in response to 5% and 7% hypercapnia, hypercapnia-hyperoxia or hypoxia. **A:** Pet1/DTR male mice. **B:** Pet1/DTR female mice. **C:** WT male mice. **D:** WT female mice. 1 way ANOVA, Bonferroni's multiple comparison test.

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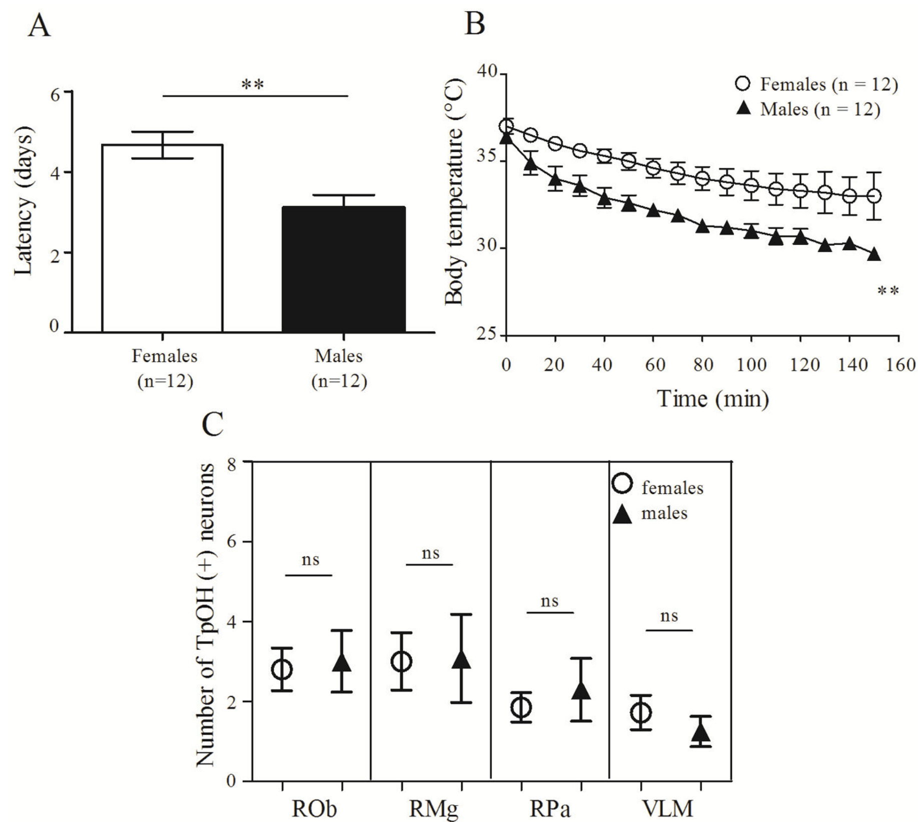

Figure 4. Deletion of 5-HT neurons in adult Pet1/DTR mice affects thermoregulation in males more than in females

A: Latency from the first injection of DT at 50 μg/kg to the time that T_B dropped below 35°C. The latency in female Pet1/DTR mice was longer than in male Pet1/DTR mice (**, $p = 0.0035$, $t = 3.421$ and $df = 16$, t test). **B:** Body temperature dropped more in male than in female Pet1/DTR mice when T_A was changed from 37°C to 24°C (****, $p < 0.001$, $F = 110.9$. 2 way ANOVA). **C:** There were no gender differences in the number of TpOH-ir neurons in ROb, RMg, RPa or VLM from Pet1/DTR mice (n=6 females and n=7 males. 1 way ANOVA, Bonferroni’s multiple comparison test).

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Table 1

Ventilation and oxygen consumption in room air at baseline and after 4 μg DT_T for WT and Pet1/DTR mice.

	VO_2 (ml min^{-1} g^{-1})	V_E (ml min^{-1} g^{-1})	V_T (ml g^{-1})	f_R (min^{-1})
Male WT				
Baseline	0.056 ± 0.004	2.44 ± 0.32	0.015 ± 0.001	157 ± 12
Post DT	0.044 ± 0.005	2.11 ± 0.22	0.014 ± 0.001	153 ± 14
Female WT				
Baseline	0.047 ± 0.005	2.18 ± 0.14	0.016 ± 0.001	134 ± 9
Post DT	0.045 ± 0.004	2.38 ± 0.39	0.017 ± 0.002	140 ± 14
Male Pet1/DTR				
Baseline	0.056 ± 0.004	1.98 ± 0.13	0.013 ± 0.001	149 ± 6
Post DT	0.041 ± 0.004	1.94 ± 0.16	0.014 ± 0.001	141 ± 7
Female Pet1/DTR				
Baseline	0.058 ± 0.003	2.45 ± 0.18	0.016 ± 0.001	143 ± 4
Post DT	0.049 ± 0.003	2.09 ± 0.09	0.016 ± 0.001	134 ± 8

DT_T, total amount of diphtheria toxin. Values are means ± SEM. VO_2, oxygen consumption; V_E, minute ventilation; V_T, tidal volume, and; f_R, respiratory frequency.

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Table 2

Baseline ventilatory response to hypercapnia or hypoxia for WT and Pet1/DTR mice.

	VO_2 (ml min^{-1} g^{-1})	V_E (ml min^{-1} g^{-1})	V_T (ml g^{-1})	f_R (min^{-1})
Male WT				
21% O_2/5% CO_2	0.049 ± 0.003	7.29 ± 0.62	0.024 ± 0.002	297 ± 9
21% O_2/7% CO_2	0.043 ± 0.002	10.10 ± 0.99	0.031 ± 0.003	325 ± 6
50% O_2/7% CO_2	0.034 ± 0.008	9.00 ± 1.02	0.029 ± 0.001	303 ± 9
10% O_2/0% CO_2	0.064 ± 0.007	3.79 ± 0.50	0.015 ± 0.001	248 ± 14
Female WT				
21% O_2/5% CO_2	0.039 ± 0.003	7.05 ± 0.64	0.028 ± 0.002	248 ± 7
21% O_2/7% CO_2	0.034 ± 0.004	10.27 ± 0.48	0.035 ± 0.002	292 ± 5
50% O_2/7% CO_2	0.030 ± 0.006	9.56 ± 0.93	0.034 ± 0.003	280 ± 6
10% O_2/0% CO_2	0.058 ± 0.004	3.81 ± 0.56	0.017 ± 0.002	223 ± 12
Male Pet1/DTR				
21% O_2/5% CO_2	0.049 ± 0.003	5.15 ± 0.47	0.019 ± 0.002	264 ± 6
21% O_2/7% CO_2	0.043 ± 0.003	6.69 ± 0.50	0.023 ± 0.002	291 ± 5
50% O_2/7% CO_2	0.045 ± 0.009	5.99 ± 0.46	0.022 ± 0.002	272 ± 5
10% O_2/0% CO_2	0.060 ± 0.006	2.90 ± 0.17	0.013 ± 0.001	225 ± 6
Female Pet1/DTR				
21% O_2/5% CO_2	0.049 ± 0.002	7.12 ± 0.38	0.027 ± 0.001	265 ± 8
21% O_2/7% CO_2	0.044 ± 0.002	9.44 ± 0.56	0.032 ± 0.002	294 ± 9
50% O_2/7% CO_2	0.055 ± 0.010	8.53 ± 0.53	0.031 ± 0.001	274 ± 9
10% O_2/0% CO_2	0.056 ± 0.006	3.44 ± 0.23	0.016 ± 0.001	217 ± 7

Values are means ± SEM. VO_2, oxygen consumption; V_E, minute ventilation; V_T, tidal volume, and; f_R, respiratory frequency.