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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 of 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 minute 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, $p < 0.01$). 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.

Key words: 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 et al., 2008, 2009; Hodges and Richerson, 2008a, 2010; Ptak et al., 2009) and the ventilatory response to CO_2 (Richerson, 1995; Wang et al., 1998, 2001; Wang and Richerson, 1999; 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, 2014; Naumenko et al., 2009, 2011; Madden and Morrison, 2010; Hale et al., 2011; Morrison and Nakamura, 2011). 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 ambient temperature (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 minute ventilation (V_E), whereas in 5-HT transporter (5-HTT) knockout mice V_E is increased (Gobbi et al., 2001; Li and Nattie, 2008; Hodges et al., 2011). However, in both of these strains

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Abbreviations: 5-HTT, 5-HT transporter; BAT, brown adipose tissue; CNO, clozapine-N-oxide; DT, diphtheria toxin; DTR, diphtheria toxin receptors; EtOH, ethyl alcohol; f_R, respiratory frequency; HCVR, hypercapnic ventilatory response; PBS, phosphate-buffered saline; ROb, raphe obscurus; RMg, raphe magnus; RPa, raphe pallidus; SSRI, selective serotonin reuptake inhibitor; T_A, ambient temperature; T_B, body temperature; TpOH-ir, tryptophan hydroxylase immunoreactive; V_E, minute ventilation; VLM, ventrolateral medulla; VO_2, oxygen consumption; V_T, tidal volume; WT, wild-type.

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thermoregulation is normal at a T_A of 22 °C. In each of the above mouse strains the defects are present from embryonic life onward, 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 body temperature (T_B) even when T_A is 22 °C, whereas there is no effect on V_E 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 (HCVR) 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.

Our principal objective in the present work was to use the diphtheria toxin receptors (DTR)/diphtheria toxin (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 Da 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 tryptophan hydroxylase-immunoreactive (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, ACCATGAAG CTGCTGCCGTC and DTR-2 antisense, ATCAGTGGG AATTAGTCATGC. CRE primers: CRE-1 sense, 5′ATTT GCCTGCATTACCGGTCG 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-h light/dark cycle in the Yale Animal Care Facility.

Diphtheria toxin protocol

We tested the effects of systemic administration of DT (Sigma, DO564, St Louis, MO, USA) using doses of 5, 50 and 250 μg/kg i.p. in 100 μl of normal saline solution. DT was administered one, three or five 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 a 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 min). 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, Wetzlar, Germany). 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, St Louis, MO, USA) 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,