Plasticity in breathing and arterial blood pressure following acute intermittent hypercapnic hypoxia in infant rat pups with a partial loss of 5-HT neurons

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ABSTRACT

The role of serotonin (5-HT) neurons in cardiovascular responses to acute intermittent hypoxia (AIH) has not been studied in the neonatal period. We hypothesized that a partial loss of 5-HT neurons would reduce arterial blood pressure (BP) at rest, increase the fall in BP during hypoxia, and reduce the long term facilitation of breathing (vLTF) and BP following AIH. We exposed 2 week-old, 5,7-dihydroxytryptamine (5,7-DHT)-treated and controls to AIH (10% O₂; n=13 control, 14 treated), acute intermittent hypercapnia (AIHC; 5% CO₂; n=12 and 11), or acute intermittent hypercapnic hypoxia (AIHH; 10% O₂, 5% CO₂; n=15 and 17). We gave five 5-min challenges of AIH and AIHC, and twenty ~20 sec challenges of AIHH to mimic sleep apnea. Systolic (sBP), diastolic (dBp), mean arterial pressure (MAP), heart rate (HR), \( \dot{V}_e \) and metabolic rate (\( \dot{V}_{O_2} \)) were continuously monitored. 5,7-DHT induced an ~35% loss of 5-HT neurons from the medullary raphe. Compared to controls, pups deficient in 5-HT neurons had reduced resting sBP (~6 mmHg), MAP (~5 mmHg), HR (56 beats/min), and experienced a reduced drop in BP during hypoxia. AIHH induced vLTF in both groups, reflected in increased \( \dot{V}_e \) and \( \dot{V}_e / \dot{V}_{O_2} \), and decreased arterial PCO₂. The sBP of pups deficient in 5-HT neurons, but not controls, was increased 1 hr following AIHH. Our data suggest that a relatively small loss of 5-HT neurons compromises resting BP and HR, but has no influence on ventilatory plasticity induced by AIHH. AIHH may be useful for reversing cardio-respiratory defects related to partial 5-HT system dysfunction.
INTRODUCTION

Neonatal mammals are prone to respiratory and cardiovascular instabilities due to relatively immature control systems and hence can experience repeated episodes of hypoxia and hypercapnia. In addition to the ventilatory response, chemo- and baroreflex- mediated cardiovascular responses are necessary during hypoxia to increase blood flow to critical tissues in order to meet metabolic demands. The maintenance of arterial blood pressure (BP) in the face of hypoxia-induced vasodilation is a key component of the integrated response; ultimately any change in BP depends on the ventilatory, autonomic, and neurohumoral responses to hypoxia (25). BP of adult rats typically falls in response to acute hypoxia, notwithstanding the concurrent, baroreflex-mediated tachycardia (20, 26, 38). Acute hypercapnia, on the other hand, generally has modest effects on BP (30, 38). There have been few studies that have examined the effects of hypoxia and hypercapnia on BP in the neonatal period; in lambs hypoxia elicits a mild decrease in BP with a concomitant increase in HR (33, 36).

Serotonergic neurons residing within the medullary raphe nuclei help maintain cardio-respiratory homeostasis by projecting to, and modifying the activity of, cardiovascular, respiratory and autonomic neurons in the brainstem and spinal cord (21, 37). Adult mice with a specific loss of central 5-HT have reduced heart rate (HR) and BP (1). In neonatal rat pups, a systemic loss of 5-HT (i.e. from the CNS, autonomic ganglia and blood) leads to a premature, severe loss of BP during severe hypoxia (42). However, the specific role of 5-HT neurons in the control of resting BP, and the BP responses to moderate hypoxia and hypercapnia in the neonatal period has not previously been explored.

Acute intermittent hypoxia (AIH) or asphyxia induces an increase in ventilatory and sympathetic motor activity that can persist for hours after the termination of the stimulus (i.e. ventilatory (vLTF) and sympathetic long-term facilitation (sLTF) (40, 41)). It is generally thought that these forms of plasticity are adaptive, ostensibly reducing the chances of apnea and ensuring appropriate oxygen transport to the most metabolically active tissues. There is ample evidence from adult animals that the expression of vLTF depends on, among other factors, serotonergic projections from the medullary raphe to the phrenic motor nucleus (8, 23). There is
some evidence that sLTF also depends on 5-HT (8). Although vLTF has been shown in neonatal rats in response to AIH (18), the degree to which this phenomenon depends on 5-HT neurons is unknown. Further, the effects of AIH on the long-term regulation of blood pressure at an age roughly equivalent to infancy (i.e. effects owing at least partially to sLTF) have not been investigated.

We are particularly interested in the role of 5-HT neurons in the acute and long-term regulation of BP during and following hypoxia because the Sudden Infant Death Syndrome (SIDS) is associated with, among other immunohistochemical defects, a ~30% reduction in 5-HT content and increased numbers of immature 5-HT neurons (10, 31). In addition, there are records that suggest SIDS cases succumb following one of possibly several hypoxic and hypercapnic episodes (27, 34). It may be that even a relatively small lesion to medullary 5-HT system predisposes an infant to sudden death because of compromised cardiovascular responses to the hypercapnic hypoxia occurring during sleep apnea, or from a failure to elicit long-lasting cardiovascular and respiratory adaptations to these challenges (i.e. LTF). This study addresses the hypotheses that a partial loss of 5-HT neurons exacerbates the drop in BP during hypoxia and reduces the magnitude of both vLTF and the increase in BP following repetitive hypoxic episodes.
**METHODS AND MATERIALS**

**Ethical Approval**

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Missouri at Columbia, MO.

**Animals and treatments**

We used a total of 37 vehicle (saline) control Sprague-Dawley rat pups along with 39 pups treated with 5,7-dihydroxytryptamine (5,7-DHT). Pups were derived from 10 breeding pairs. Males and females were used, as in preliminary experiments we did not see any effect of gender. Dams were fed *ad libitum* on standard rat chow, and kept on a 12hr light-dark cycle. On any given day we tested at least one treated and one control littermate.

**Surgery (5,7-DHT injection)**

5,7-DHT was injected into postnatal day (P)10-12 rat pups, as previously described (5); experiments were performed 4 days later at P14-16. Prior to 5,7-DHT injection, pups were injected with desipramine (50 ul of a 10 mg/ml solution, in saline, *i.p.*) in order to preserve catecholaminergic neurons. While under 1.5-2% isoflurane pups were injected (*i.c.v.*, via the cisterna magna) with 3μl of 13 mg/ml 5,7-DHT (40 μg total) or vehicle (0.9% saline). We previously found that this dose resulted in an incomplete loss of 5-HT neurons (*personal observation*). After each injection, tissues were sutured and pups were allowed to recover in a small chamber over a heating pad and returned to the dam. Treated pups nearly always lost 1-2 grams in the 24 hrs following surgery, before gaining weight over the following 3 days (albeit at a reduced rate compared to controls). Nevertheless, we observed that treated pups always behaved normally in the litter, with no signs of dehydration, lethargy, or any other behavioural abnormalities.

**Surgery (femoral catheter)**
We implanted a femoral catheter into pups four days following 5,7-DHT or vehicle injection (P14-16), as previously described (42). Prior to surgery, the tip of the PE 10 catheter was heated, stretched to the appropriate diameter, and prefilled with heparinized saline solution (100 ul/ml). Under 2% isoflurane a ~1 cm skin incision was made in the left groin and a 20X dissecting microscope was used to dissect out the left femoral artery. The artery was then tied with 5-0 surgical suture just distal to the epigastric branch. An incision was made on the left femoral artery for insertion of a PE 10 catheter. The catheter tip was advanced ~0.6 to 0.8 cm, close to the inguinal ligament. Tissues were sutured using 5-0 surgical silk.

**Experimental Setups**

**Whole-body plethysmography**

Similar to previous studies in our lab, we used whole body plethysmography to measure cardiorespiratory variables of interest (42). For these whole-body measurements, we used a jacketed glass chamber (volume=100 ml) attached to a programmable water bath/pump (Fisher Scientific, Pittsburgh, PA) to hold chamber temperature constant at ~30-31°C, monitored with a thermocouple (Omega Engineering Inc., Stanford, CT) inserted into the chamber. Air was provided by a wall outlet and hypoxia (10% O2, balance N2) and hypercapnia (5% CO2, balance air) from pre-mixed cylinders. Air, hypoxia and hypercapnia were passed through a flask of water to control for the effects of day-to-day variability in humidity on the magnitude of the respiratory signal (*see below*). Hypercapnic hypoxia (10% O2, 5% CO2, balance N2) was produced by blending N2, O2 and CO2 from cylinders. The three gases were passed through a flowmeter with three independent channels and a common output (Cole-Parmer, Vernon Hills, IL). With O2 (AEI Technologies, Pittsburgh, PA) and CO2 analysers (CWE, Inc., Ardmore, PA) we empirically determined the ratios of N2, O2 and CO2, as well as the duration of their application, that were sufficient to take the % inspired O2 from ~21 to 10%, and the % inspired CO2 from 0 to 5%, in ~20-25 sec. This third protocol was designed to more closely mimic blood gas perturbations associated with sleep apnea. All gases were sent through a final flowmeter (Cole-Parmer) in order
to regulate the flow to ~300 ml/min (hypoxia and hypercapnia) or 600 ml/min (hypercapnic hypoxia). In addition to its higher flow, hypercapnic hypoxia was delivered directly into the chamber without humidification in order to further minimize gas wash-in time. Chamber pressure was kept close to atmospheric by pulling gas from the opposite end of the chamber via wall vacuum, also run through a flowmeter to precisely balance the gas flows. Inflow and outflow gas were run through 20G needles in order to create a nearly-sealed chamber to maximize the pressure signal related to breathing (i.e. minimizing the “leak” of pressure from the chamber), while still being able to deliver continual flow of gas. In order to determine $\dot{V}_{O_2}$ during normoxia, hypoxia or hypercapnia, a separate pump (AEI Technologies, Naperville, IL) pulled a sub-sample of gas through the $O_2$ analyzer, which was single-point calibrated at the start of each experiment with humidified room air to prevent an overestimation of $\dot{V}_{O_2}$ resulting from $O_2$ being diluted with $H_2O$ from the lung. We could not determine $\dot{V}_{O_2}$ in pups exposed to AIHH because of the high flow rate and short wash-in time for the gas.

A differential pressure transducer (Validyne Engineering, Northridge, CA), connected to the animal chamber and a reference chamber was used to ensure the near-atmospheric pressure within the chamber, and to detect changes in chamber pressure related to breathing. The pressure signal generated during inspiration is due to the heating and humidification of the inspired air. To calculate $V_T$, we used the equation describing the relationship between this pressure signal and the heating and humidification of a volume of air entering the lungs (9). Body temperatures were measured in a subset of treated and control pups following experimentation, and both were found to be ~36°C. To account for thermal drift within the chamber during the experiment, the two chambers were connected with a ~10cm length of small-diameter tubing. The femoral catheter was fed through a 20G needle and attached to a blood pressure transducer mounted outside the chamber, calibrated at the beginning of each experiment day with a sphygmanometer. Analog signals from respiratory and arterial pressure transducers were fed into a Powerlab data acquisition system (ADInstruments, Colorado Springs, CO) and analyzed in Labchart 7.3.7 (ADInstruments).

**Experimental Protocol**
We monitored MAP, HR and breathing in treated and control pups at P14-16 (four days following 5,7-DHT injection), at rest and during hypoxia, hypercapnia and hypercapnic hypoxia. Prior to the first challenge, pups were allowed to warm up in the chamber for at least 20 min. We started recordings data when pups displayed full arousal from anesthesia and full recovery and stability of HR, breathing, BP and metabolic rate ($\dot{V_o}$). We first recorded variables for 15 min in room air. In a cohort of control and treated pups (n=14 control and 11 treated from Group 1 and 2, see below) baseline PaO$_2$ and PaCO$_2$ were measured using ~90 ul of whole blood, collected via the arterial catheter and immediately transferred to a blood gas analyser (ABL80Flex Co-Ox; Radiometer, Brønshøj, Denmark). Pups were then subjected to one of three protocols: intermittent hypoxia (AIH), hypercapnia (AIHC) or hypercapnic hypoxia (AIHH). Each protocol lasted a total of 120 min. In initial experiments, pups were exposed to five 5-min episodes of AIH (Group 1: n=13 control, 14 treated), or AIHC (Group 2: n=12 control, 11 treated). Episodes were interspersed with 5 min of room air. Arterial blood gases were measured during the last episode of hypoxia (n=6 control and 6 treated) or hypercapnia (n=8 control, 5 treated). A third protocol used a more severe stimulus that better reflects conditions experienced during sleep apnea: Twenty ~20-25 sec challenges of intermittent hypercapnic hypoxia (AIHH; Group 3: n=15 control, 17 treated). Each episode of hypercapnic hypoxia was interspersed with 2 min of room air. We were unable to measure blood gases in this group to the transient nature of the stimulus. For all pups we continued recording variables for 60 min following the last challenge. At this point a final arterial blood sample was taken from four pups (2 from each group) to obtain PaCO$_2$ in order to validate observed changes in $\dot{V_e}/\dot{V}_o$. At the end of the experiment, animals were anesthetized and perfused with 4% PFA to fix brainstem tissue for immunohistochemistry.

**Immunohistochemistry**
Brainstems were cut on a vibratome (VT 1000S, Leica) at 30-40um and free-floating sections were stored in cryoprotectant until immunostaining. The medulla was sectioned starting caudal to the emergence of the raphe obscurus and ending at roughly the dorsal cochlear nucleus. Immunohistochemistry was performed on every sixth section; upon staining, one row of tissue sections was transferred to PBS for washing. Sections were rinsed 3 times in PBS for 5 minutes each. The sections were then blocked with 10% normal donkey serum (NDS) (Millipore, Temecula, CA) in 0.3% Triton in PBS for 30 minutes and washed 3 times (5 minutes each) in PBS. Sections were incubated overnight in goat-anti-5-HT (1:1000) (ImmunoStar, Hudson, WI) and mouse-anti-TH (1:1000) (Millipore) in 1% NDS and 0.3% Triton in PBS. After the overnight incubation period, sections were washed 3 times in PBS (10 minutes each) and incubated for 2 hours with respective secondary antibodies (donkey anti-goat Alexa 647 and donkey anti-mouse Alexa 488; both 1:200) (Jackson ImmunoResearch, West Grove, PA). After secondary antibody binding, the sections were washed 3 times (10 minutes each) in PBS, mounted on 0.5% gelatin slides, dried and coverslipped with Prolong Gold (Life Technologies, Grand Island, NY).

Immunohistochemistry images were acquired on an Olympus BX51 microscope containing a three-axis motorized stage (Ludl Electronic Products Ltd., Hawthorne, NY) with a monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ) along with Neurolucida software (Version 10, MicroBrightField, Willston, VT) using Cy2 and Cy5 filters. Images were acquired individually and were subsequently combined and analyzed with Image J (version 1.46r; National Institutes of Health) for 5-HT and TH containing neurons.

**Data and Statistical Analysis**

Baseline variables were determined across 1 min of quiet room air breathing, prior to the first challenge. For Groups 1 and 2, we calculated respiratory frequency ($f_B$, min$^{-1}$), tidal volume ($V_T$, ml/kg), $\dot{V}_{\dot{E}}$ ($f_B \times V_T$; ml.min$^{-1}$.kg$^{-1}$), $\dot{V}_{O_2}$ (determined using the formula: $\dot{V}_{O_2} = (\text{fractional } O_2 \text{ inflow} - \text{fractional } O_2 \text{ outflow}) \times \text{flow (mL.min}^{-1}\text{/ mass (kg)}$, ventilatory equivalent ($\dot{V}_{\dot{E}}/\dot{V}_{O_2}$), systolic arterial blood pressure (sBP), diastolic arterial
blood pressure (dBP), mean arterial pressure (MAP, mmHg), and HR (beats.min⁻¹) from the raw respiratory and BP traces. Baseline blood gases, pH and haematocrit were also determined in a subsets of Group 1 and 2 animals. Variables were measured from the last min of each of the 5 normoxic, pre-challenge periods, as well as the last min of each of the 5 min hypoxic or hypercapnic challenges. Due to the short wash-in and wash-out periods of hypercapnic hypoxia (Group 3), and the transient nature of the BP response, within each of the 20 challenges the maximum $\dot{V}_E$, BP, and HR responses were measured within a 5 sec window (see Fig. 7). Every attempt was made to measure variables from signals that were not contaminated by movement artefact.

For some animals, the arterial pressure signal was lost during the protocol. This was associated with movement and/or a change in the pup’s body position within the chamber and kinking of the catheter. In addition, the catheters of some control and treated pups became dislodged following movement within the chamber and therefore had to be sacrificed prior to completing the protocol. We indicate animal numbers within the Results and Figures, where appropriate.

The average number of 5-HT-positive neurons within the midline raphe pallidus and obscurus was determined for each pup, using 5-10 sections per animal. Care was taken to ensure that, between groups, counts were done on sections taken from the same rostral-caudal position within the medulla. TH-positive neurons were counted along the extent of the ventrolateral medulla and nucleus of the solitary tract. The rostral-caudal position of each section was confirmed using easily identifiable landmarks (eg. inferior olives, area postrema, 4th ventricle), and referring to Paxinos and Watson’s Rat Brain Stereotaxic Atlas (32).

We analysed the effect of 5,7-DHT on baseline parameters using Student’s two-tailed t-tests. Effects of each gas and 5,7-DHT treatment on the change in $\dot{V}_E$, $\dot{V}_O$, $\dot{V}_E/\dot{V}_O$, MAP, HR, and blood gases were assessed with 2-factor repeated-measures ANOVAs. These tests included data for all animals during the last min of each of the five (or 20) gas challenges and the intervening normoxic, normocapnic periods. To discern whether each gas elicited plasticity (i.e. changes in the variable from baseline to 1 hr post-challenges), we used 2-factor repeated-measures ANOVAs on data from the baseline period and from 1 hr following the last challenge (i.e.
only from animals surviving the whole protocol). In line with the changes in $\dot{V}_e / \dot{V}_{O_2}$, hypocapnia appeared in all four pups (2 control and 2 treated) 1 hr following AIHH; thus PaCO$_2$ data were combined for statistical analyses. When significant main effects were found, we used Tukey’s post hoc analyses for pair-wise comparisons. Differences between groups with respect to 5-HT- and TH-positive cell counts were determined with a Student’s t-test.
RESULTS

1. Effects of a partial loss of 5-HT neurons on resting variables

5,7-DHT treatment reduced the number of 5-HT-positive neurons in the raphe obscurus and pallidus by 33% and 37%, respectively (P<0.001 for both; Fig. 1a-c), with no effect on the number of TH-positive neurons in the VLM or NTS (Fig. 1d). On the day of testing, pups with reduced 5-HT neurons weighed ~20% less than controls (Veh: 31.1 ± 0.8g; 5,7-DHT treated: 24.6 ± 0.7g; P<0.001). However, as pups behaved normally following 5,7-DHT injection, this finding is unlikely to be due to gross malnutrition or dehydration. There was a tendency for the $\dot{V}_O_2$ of treated pups to be slightly lower than controls, but this effect did not reach statistical significance (P=0.06; Table 1). Treatment had no significant influence on resting $f_B$ (control: 121 ± 5; treated: 111 ± 5 breaths/min; P=0.14), $V_T$ (control: 11.3 ± 0.7; treated: 11.2 ± 0.9; P=0.94), $\dot{V}_E$ (Table 1) or $\dot{V}_E/\dot{V}_O_2$ (Table 1). The lack of an effect on $\dot{V}_E/\dot{V}_O_2$ is also reflected in the lack of an effect of treatment on resting $PaCO_2$ (Table 1). However, baseline $PaO_2$ was slightly but significantly elevated in treated pups compared to controls (Table 1). Finally, HR (P<0.001), sBP (P=0.01) and MAP (P=0.01) were all significantly reduced in treated pups compared to controls (Table 1).

2. Effects of a partial loss of 5-HT neurons on cardio-respiratory responses to acute intermittent hypoxia (Group 1: AIH)

Typical BP and HR responses of a control and treated pup to a single episode of hypoxia are shown in Fig. 2. While hypoxia induced hypotension in both groups, the loss of BP experience by treated pups was on average ~9 mmHg less than controls (treatment x hypoxia: P=0.02; Fig. 3a), solely because of a reduced drop in sBP (treatment x hypoxia: P=0.002; not shown). In the 1 hr following hypoxia there were no significant changes in BP in either treated or control pups (P=0.69; Fig. 3a). In both groups the drop in BP was accompanied by tachycardia (Fig. 3a). Unlike BP, a loss of 5-HT neurons had no influence on the magnitude of
the HR response (Fig. 3a). However, the increase in HR relative to the fall in BP (ΔHR/ΔBP) was more than 3-
times greater in 5-HT-deficient pups compared to controls (P<0.001; Fig. 3b). Over the 1hr following AIH, the
HR of treated pups remained significantly lower compared to controls, with no evidence of plasticity (Fig. 3a).

For both groups, hypoxia elicited a significant increase in $\dot{V}_e$. However, control pups had a slightly but
significantly greater hypoxic ventilatory response compared to pups deficient in 5-HT neurons (treatment x gas:
P=0.03; Fig. 4a), solely because the increase in $f_B$ was ~40 breaths/min greater in controls that in treated pups
(treatment x gas: P<0.001). Relative to baseline values, there was no change in the $\dot{V}_e$ of either control or
treated pups in the hr following intermittent hypoxia (P=0.11; Fig. 4a). Hypoxia had no significant influence on
$\dot{V}_O_2$ either acutely or 1 hr following the challenges (Fig. 4b). The increase in $\dot{V}_e/\dot{V}_O_2$ across the five hypoxic
episodes was not significantly influenced by a loss of 5-HT neurons (Fig. 4c), reflected also by an equivalent
decrease in PaCO₂ between the two groups during hypoxia (Table 2). Compared to baseline, $\dot{V}_e/\dot{V}_O_2$ was
unaltered 1 hr following AIH (P=0.16; Fig. 4c).

3. Effects of a partial loss of 5-HT neurons on cardio-respiratory responses to acute intermittent
hypercapnia (Group 2: AIHC)

Acute hypercapnia led to a small but statistically significant increase in BP (P<0.001), and this effect
was not influenced by a loss of 5-HT neurons (Fig. 5). In both groups, BP was unaltered 1 hr following AIHC
(P=0.75; Fig. 5). Accompanying the increase in BP during acute hypercapnia was a slight but significant
decrease in HR in control and treated pups (P<0.001; Fig. 5). There was no significant influence of a loss of 5-
HT neurons on ΔHR/ΔBP (not shown). The HR of control pups remained higher than treated pups 1 hr post-
AIHC; however there was no significant change in HR in either group over this period (Fig. 5).

In response to acute hypercapnia, $\dot{V}_e$ increased to the same extent in both groups (gas: P<0.001; Fig.
6a). As was the case with AIH, there was no evidence of vLTF 1 hr following AIHC (P=0.87; Fig. 6a). In both
groups there was a slight but significant increase in $\dot{V}_O_2$ over the course of the 5 challenges (P<0.001; Fig. 6b),
but after 1 hr $\dot{V}_O_2$ was not statistically different from baseline values (P=0.13; Fig. 6b). As with $\dot{V}_e$, acute
hypercapnia induced a significant hyperventilation, but 1 hr following AIHC $\dot{V}_e/\dot{V}_O_2$ had not changed from
baseline values (P=0.37).

4. Effects of a partial loss of 5-HT neurons on cardio-respiratory responses to acute intermittent
hypercapnic hypoxia (Group 3: AIHH)

Group 3 animals were exposed to twenty ~20 sec episodes of AIHH in order to more closely mimic
conditions associated with sleep apnea. Importantly, the total duration of this protocol was the same as AIH and
AIHC protocols. We measured the peak ventilatory, BP and HR responses (within a 5 sec window) to each
episode of hypercapnic hypoxia (Fig. 7). Similar to AIH, AIHH led to a significant drop in BP (gas: P<0.001,
and the drop was smaller in pups with a partial loss of 5-HT neurons compared to controls (treatment x gas:
P=0.005; Fig. 8a). 1 hr following AIHC, the BP of control pups had not changed relative to pre-challenge BP.
Surprisingly, however, and unlike the AIH or AIHC protocols, AIHH only induced a rise in sBP and MAP in
pups deficient in 5-HT neurons (treatment x time interaction: P=0.001 and 0.01, respectively; Fig. 8a,b & 9). In
both groups, acute AIHH increased HR, but 1 hr after the challenges HR was unchanged from baseline (Fig. 8a).

On average, acute hypercapnic hypoxia increased $\dot{V}_e$ ~3-fold, and a partial loss of 5-HT neurons did not
influence the response (Fig. 10a). Unlike AIH and AIHC, AIHH induced vLTF in both control pups and pups
deficient in 5-HT neurons (P<0.001; Fig. 10 a,b). In both groups an increase in both $f_B$ and $V_T$ contributed to
vLTF (P=0.02 for both). In addition, in both groups AIHH induced a long-term depression of $\dot{V}_O_2$ (P=0.004;
Fig. 10c), a unique feature of this paradigm. As a result, $\dot{V}_e/\dot{V}_O_2$ nearly doubled in both groups from the
beginning to the end of the AIHH protocol (P<0.001; Fig. 10d), reflected also in a significant decline in PaCO$_2$
(Fig. 10e). Thus, among our three paradigms, AIHH was the only one to elicit long-term plasticity in breathing and blood pressure regulation, and these effects were not dependent on the presence of a full complement of 5-HT neurons. On the contrary, AIHH induced an increase in BP only in pups deficient in 5-HT neurons.
In this study, we treated two-week old rat pups with 5,7-DHT to induce a partial loss of 5-HT neurons, with the goal of modelling the partial 5-HT system dysfunction described in SIDS cases. We examined the consequences of this partial lesion for BP and the control of breathing during and following acute intermittent hypoxia, hypercapnia and hypercapnic hypoxia. Our hypotheses were that a partial loss of 5-HT neurons would compromise BP regulation at rest and in response to acute hypoxia, and that 5-HT-neurons are not only important for respiratory LTF but also for the increase in BP following acute intermittent hypoxia. Most of the previous studies exploring the role of 5-HT neurons in cardiorespiratory homeostasis have used rodent models harboring extensive lesions to the medullary 5-HT system (4, 5, 12). Our findings reveal that cardiorespiratory homeostasis is altered in infant rat pups following even a relatively small (~35%) loss of medullary 5-HT neurons. A loss of these neurons led to reduced resting sBP, MAP and HR, as well as a reduced fall in BP during acute hypoxia. In addition, we found that AIHH induced respiratory and metabolic plasticity at this age, characterized by increased $\dot{V}_E$, decreased $\dot{V}_{O_2}$, and decreased PaCO$_2$. A small loss of 5-HT neurons had no impact on this response.

Implications for a partial loss of 5-HT neurons on cardiorespiratory homeostasis at rest in infant rat pups

Despite normal feeding and overall behavior, pups deficient in 5-HT neurons were significantly lighter than controls. Although we have no obvious explanation, we note that a loss of 5-HT neurons or content in the neonatal period is consistently associated with reduced growth rate and body mass (3-5, 12). Resting BP values were similar to those reported previously for rat pups at this age (13). We found that in resting, normoxic conditions, infant rat pups deficient in 5-HT neurons had reduced sBP, MAP and HR compared to their control littermates. Reduced MAP has been observed in adult mice totally devoid of central 5-HT (1), but to our knowledge this is the first report indicating a role for 5-HT neurons in resting BP in rodents of an age roughly equivalent to infancy. Since only sBP was reduced following a loss of 5-HT neurons suggests that at this age,
central 5-HT signalling contributes to the sympathetic outflow to the cardiac ventricles, as has been found by others in adult animals (24). HR of treated pups is significantly reduced compared to controls, a finding that has been previously reported in neonatal rodents deficient in 5-HT neurons (6). Reduced sympathetic drive to the sinoatrial node could also explain this finding; however, given that 5-HT neurons send projections to nuclei containing cardiac vagal neurons (16), and that 5-HT activates a number of receptors within these nuclei (17), it is equally possible that there is increased vagal drive with a loss of 5-HT signalling. A partial loss of 5-HT neurons had no influence on resting \( f_B \), \( V_T \), \( \dot{V}_e \), \( \dot{V}_e / \dot{V}_o \), or \( \text{PaCO}_2 \). However, we know from several previous studies that a more extensive loss of 5-HT neurons leads to reduced \( f_B \) and apnea (6, 12). Interestingly we did find that the \( \text{PaO}_2 \) of treated pups was \(~8\) mmHg greater than controls. Given there was no effect of 5-HT neuron loss on resting \( \text{PaCO}_2 \), it may be that 5-HT neurons influence the autonomic control of pulmonary vascular resistance and hence the proper matching of lung \( \dot{V}_e \) and perfusion. \( \dot{V}_e \)-perfusion mismatch minimally affects \( \text{PaCO}_2 \) because the arterio-venous PCO\(_2\) gradient is much smaller than the PO\(_2\) gradient.

Effects of a loss of 5-HT neurons on the cardiovascular and respiratory responses to acute hypoxia

We hypothesized that the fall in BP elicited by AIH or AIHH would be greater in pups deficient in 5-HT neurons. This hypothesis was based on data from a previous study from our lab in which we systemically injected rat pups with a chemical inhibitor of tryptophan hydroxylase (6-fluorotryptophan) to acutely reduce central 5-HT content. In that study, we found that treated pups had a much greater loss of BP during repeated episodes of severe hypoxia (42). However, in the current study we found that pups with reduced 5-HT neurons actually had a reduced fall in BP in response to moderate hypoxia. This discrepancy may be explained by the treatment (major loss of 5-HT content then, partial depletion of 5-HT neurons now) and/or the severity of the hypoxic stimulus (anoxia then, moderate hypoxia now) used between the two studies. Perhaps more importantly, the systemic application of 6-fluorotryptophan presumably decreased 5-HT levels in the blood,
autonomic ganglia and vascular endothelium, tissues that not only store and release 5-HT but that also help
regulate vasomotor tone and hence BP (39).

We also demonstrate that in pups deficient in 5-HT neurons the increase in HR relative to this drop in
BP during hypoxia was ~3-fold greater than that experienced by controls. Given that the hypoxic stimulus was
the same as experienced by control pups (Table 2), this finding suggests that the baroreflex is enhanced by a
partial a loss of medullary 5-HT signalling. Supporting this idea are studies demonstrating that 5-HT, acting on
5-HT3 receptors within the nucleus of the solitary tract, inhibits the cardiac component of the baroreflex (2, 28).
As the absolute increase in HR in treated pups was not greater than in controls, it may be that 5-HT neurons
influence the sympathetic component of the baroreflex regulating vasomotor tone.

We found that pups deficient in medullary 5-HT neurons exhibited a reduced ventilatory response to
hypoxia over the course of the five challenges, due to a reduced hypoxic $f_B$ response. However, we note that
neither the increase in $\dot{V}_e / \dot{V}_O_2$ nor the fall in PaCO$_2$ during hypoxia were affected by a loss of 5-HT neurons.
Thus, pups deficient in 5-HT neurons hyperventilate to the same degree as controls.

**Effects of a loss of 5-HT neurons on the plasticity of breathing and blood pressure**

A major goal of this study was to resolve the effects of a relatively minor loss of 5-HT neurons on
cardiovascular and respiratory plasticity in response to intermittent hypoxia and/or hypercapnia. Our most
salient finding in this regard is that in both control and treated pups alike, AIHH elicited vLTF 1 hr following
AIHH. AIHH also induced a long-term depression of $\dot{V}_O_2$. In both groups, the increase in $\dot{V}_e$ and decrease in
$\dot{V}_O_2$, resulted in a near-doubling of $\dot{V}_e / \dot{V}_O_2$, from baseline to 1 hr after the challenges. Arterial blood gas analysis
of four samples confirmed that PaCO$_2$ was significantly reduced following AIHH. These findings advance
those from previous studies that have demonstrated vLTF in the neonatal period (11, 18, 35), suggesting that the
increase in $\dot{V}_e$ following AIHH is not simply the result of increased metabolic drive. Rather, AIHH induces a
leftward relocation along the isometabolic line defining the relationship between PCO₂ and alveolar ventilation, reducing plant gain to help prevent apnea following a transient ventilatory overshoot (7).

The magnitude of vLTF was not influenced by a ~35% loss of 5-HT neurons. And, to our surprise, following AIHH an increase in BP was only observed in pups deficient in 5-HT neurons. This suggests that 5-HT neurons, by some unknown mechanism, actually constrain the development of sympathetic LTF following AIHH. We can only speculate on the underlying mechanisms for this finding. Recently it has been shown that 5-HT signalling through 5-HT₇ receptors, via PKA activation, can constrain the development of phrenic LTF (15), as can adenosinergic signalling through A₂A receptors (14). It may be that there is more activity through these pathways in control pups. Alternatively it may be that, unlike pups deficient in 5-HT neurons, there exists a “ceiling effect” in control pups whereby serotonergic pathways contributing to resting BP are saturated and not amenable to plasticity.

Methodological Considerations

Although we were not specifically interested in whether vLTF was a unique effect of hypercapnic hypoxia, it is possible we could have observed vLTF following AIH if we had used the same experimental paradigm; i.e. twenty ~20 sec challenges, rather than five 5-min challenges. Indeed, others have demonstrated vLTF in neonatal rat pups using protocols involving brief exposures to many episodes of hypocapnic hypoxia (18). In addition, give the technical limitations we encountered, we have not included a group exposed only to air alone (i.e. time control). However, the lack of cardiorespiratory plasticity following either AIH or AIHC (protocols of the same duration) suggests minimal influence from time alone on our measured variables.

Although we injected 5,7-DHT into the cisterna magna it is nevertheless possible that midbrain 5-HT neurons, those that send projections to the forebrain, were also lesioned. This is an important caveat given that nuclei in the hypothalamus (e.g. the paraventricular nucleus) project to the medulla and have well-described effects on cardiorespiratory control (19). In addition, this rostral group of neurons innervates regions of the pons and midbrain that are involved in sleep state transitions and arousal (29), so it may be that the
cardiovascular phenotypes we describe in pups deficient in neurons is at least in part due to altered sleep regulation.

Finally, it is worth pointing out that 5-HT neurons co-release Substance P and Thyrotropin-Releasing Hormone, among others, the cardiovascular effects we describe in treated pups may not be specifically due to a loss of 5-HT signalling.

**Perspectives and Significance**

We have shown that even a minor loss of 5-HT neurons reduces the resting HR and BP of infant rat pups. Another novel finding is that infant rat pups display cardiovascular and respiratory plasticity following AIHH. Hyperventilation develops 1 hr following the termination of the stimulus, by way of both increased \( V_e \) and reduced \( V_O^2 \). This response effectively stabilizes breathing by reducing plant gain and hence the chances of PaCO\(_2\) dropping below the apneic threshold during a transient ventilatory overshoot. We also show that 5-HT neurons are not necessary for the development of this plasticity. On the contrary, at this age signaling from 5-HT neurons – by some unknown mechanism – constrains the increase in BP following AIHH. Based on these findings, an intriguing possibility is that AIHH can be utilized as a strategy to reverse other cardiorespiratory defects resulting from a loss of, or dysfunction within, 5-HT neurons.

Ultimately our goal is to better understand the role of 5-HT system dysfunction in the pathophysiology of SIDS. Previous recordings from SIDS cases indicate that infants die during one of likely several severely hypoxic episodes; death is preceded by severe bradycardia and presumably low blood pressure. There is evidence that the vast majority of SIDS cases have one or more 5-HT abnormalities within the brainstem 5-HT system, including a \(~30\%\) reduction in 5-HT content and increased numbers 5-HT neurons, most of which are of a granular, immature phenotype (31). Our experiments were therefore designed around the notion that the medullary 5-HT system is partially down-regulated in SIDS. There are other immunohistochemical abnormalities, beyond the increased numbers of 5-HT neurons, that others cite as evidence that the 5-HT system is actually upregulated in SIDS (22, 31). Nevertheless, we have shown that even a relatively minor depletion of
5-HT neurons alters cardiovascular function in a way that might increase the risk of sudden death for an infant who is confronted with other intrinsic or environmental stressors.

Acknowledgments

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References


35. Reid IM and Solomon IC. Intermittent hypoxia-induced respiratory long-term facilitation is dominated by enhanced burst frequency, not amplitude, in spontaneously breathing urethane-anesthetized neonatal rats. *Progress in brain research* 212: 221-235, 2014.


Figure 1. Immunohistochemical analyses of medullary slices.  
(a) Shown are epifluorescent photomicrographs of representative coronal sections (bregma -12.8 mm) stained for 5-HT (red) and tyrosine hydroxylase (green), from control (Veh) (a) and a littermate (b) treated centrally with 5,7-dihydroxytryptamine (5,7-DHT).  
(c) 5,7-DHT treatment resulted in a 33% and 37% loss of 5-HT-positive neurons from raphe obscurus (ROb, area within the solid lines), and raphe pallidus (RPa, area within dashed line), respectively. (d) Compared to control, 5,7-DHT treatment did not significantly affect the number of tyrosine hydroxylase-positive neurons within the nucleus of the solitary tract (NTS) or ventrolateral medulla (VLM).

Figure 2. Representative traces showing the cardiovascular response to an acute episode of hypoxia.  
Shown are the responses of arterial blood pressure (BP), mean arterial pressure (MAP) and heart rate (HR) in a control (top) and 5,7-DHT-treated pup (bottom). 5 min period of hypoxia is denoted with a black bar. Arrows indicate the hypoxia-induced fall in MAP and concomitant increase in HR.

Figure 3. Cardiovascular responses to acute intermittent hypoxia (Group 1).  
(a) Responses of heart rate (HR) and mean arterial pressure (MAP) during each of the five episodes of hypoxia (shaded) the intervening normoxic periods, and 1 hr following the challenges, of vehicle (closed symbols, n=13) and 5,7-DHT-treated littermates (open symbols, n=14). 8 control and 9 treated pups survived the entire protocol and maintained reliable systolic and diastolic BP readings. (b) Change in HR per mmHg change in MAP in response to acute hypoxia. All data are means ± S.E. ¹ significant effect of treatment; ² significant effect of hypoxia; ³ significant interaction between treatment and hypoxia. Significance evaluated at P<0.05.

Figure 4. Respiratory responses to acute intermittent hypoxia (Group 1).  
(a) Ventilatory (\( V_e \)) responses of control (closed circles, n=13) and 5,7-DHT-treated pups (open circles, n=14) during each of the five episodes of
hypoxygen (shaded), the intervening normoxic periods, and 1 hr following the challenges. 11 control and 10 treated pups survived the entire protocol. b) Responses of metabolic rate ($\dot{V}_{\text{O}_2}$) in control and treated pups during and following the hypoxic challenges. (c) Ventilatory equivalent ($\dot{V}_e/\dot{V}_{\text{O}_2}$) of control and treated pups during and following the challenges. All data are means ± S.E. $^1$ significant effect of treatment; $^2$ significant effect of hypoxia; $^3$ significant interaction between treatment and hypoxia. Significance evaluated at P<0.05.

**Figure 5. Cardiovascular responses to acute intermittent hypercapnia (Group 2).** Responses of heart rate (HR) and mean arterial pressure (MAP) during each of the five episodes of hypercapnia (shaded), the intervening normocapnic periods, and 1 hr following the challenges, of vehicle (closed symbols, n=12) and 5,7-DHT-treated littermates (open symbols, n=11). 8 control and 7 treated pups survived the entire protocol and maintained reliable systolic and diastolic BP readings. All data are means ± S.E. $^1$ significant effect of treatment; $^2$ significant effect of hypoxia. Significance evaluated at P<0.05.

**Figure 6. Respiratory responses to acute intermittent hypercapnia (Group 2).** (a) Ventilatory ($\dot{V}_e$) responses of control (closed circles, n=12) and 5,7-DHT-treated pups (open circles, n=11) during each of the five episodes of hypercapnia (shaded), the intervening normocapnic periods, and 1 hr following the challenges. 9 control and 7 treated pups survived the entire protocol. b) Responses of metabolic rate ($\dot{V}_{\text{O}_2}$) in control and treated pups during and following the hypercapnic challenges. (c) Ventilatory equivalent ($\dot{V}_e/\dot{V}_{\text{O}_2}$) of control and treated pups during and following the challenges. All data are means ± S.E. $^2$ significant effect of hypercapnia. Significance evaluated at P<0.05.

**Figure 7. Representative traces showing the cardiorespiratory responses to hypercapnic hypoxia.** Shown is the raw blood pressure trace (BP), mean arterial pressure (MAP), heart rate (HR) and breathing (V) of a
vehicle control pup (top) and a 5,7-DHT-treated littermate (bottom) before, during and following an ~20 sec exposure to acute hypercapnic hypoxia. Arrows denote the initiation and termination of the exposure. Note that due to the wash-in time the maximum responses of BP and HR and breathing are slightly delayed relative to the gas exposure. Boxes indicate the 5 sec segments analyzed for BP (1), HR (2) and breathing (3). The respiratory trace was not identifiable for ~3 sec when gases were being switched, due to small pressure fluctuations.

**Figure 8. Cardiovascular responses to acute intermittent hypercapnic hypoxia (Group 3).** (a) Responses of mean arterial pressure (MAP) and heart rate (HR) during the 1st, 5th, 10th, 15th and 20th episodes of hypercapnic hypoxia (shaded), the intervening normocapnic normoxic periods, and 1 hr following the challenges, of vehicle control (closed symbols, n=15) and 5,7-DHT-treated littermates (open symbols, n=17). 9 control and 14 treated survived the entire protocol and with reliable systolic and diastolic BP readings. Average systolic (sBP, b) and diastolic (dBP, c) arterial blood pressure at baseline (time=0 min) and 60 minutes following the last exposure (time=105 min). All data are means ± S.E. ¹ significant effect of treatment; ² significant effect of hypercapnic hypoxia. ³significant interaction between treatment and hypercapnic hypoxia. Significance evaluated at P<0.05.

**Figure 9. Representative blood pressure and respiratory traces pre- and post acute intermittent hypercapnic hypoxia.** Shown are raw blood pressure (BP) and respiratory volume (V_T) traces for a control (top) and 5,7-DHT-treated pup (bottom) prior to (pre-AIHH) and after (post-AIHH) acute intermittent hypercapnic hypoxia. Note the increase in V_T from pre-AIHH to post-AIHH as well as the increase in systolic blood pressure in the treated pup.

**Figure 10. Respiratory responses to acute intermittent hypercapnic hypoxia (Group 3).** (a) Ventilatory (V_e) responses of control (closed circles, n=15) and 5,7-DHT-treated pups (open circles, n=17) during the 1st,
5th, 10th, 15th and 20th episodes of hypercapnic hypoxia (shaded), the intervening normocapnic normoxic periods, and 1 hr following the challenges. (b) Average \( \dot{V}_e \) at baseline (time=0 min) and 60 min after (time=105 min) the last hypercapnic hypoxic exposure. 10 control and 16 treated pups survived the entire protocol. Average metabolic rate (\( \dot{V}_{O_2} \), c), ventilatory equivalent (\( \dot{V}_e / \dot{V}_{O_2} \), d) and arterial partial pressure of CO\(_2\) (PaCO\(_2\), e) of control and treated pups at baseline (time=0 min) and 60 minutes following the last hypercapnic hypoxic exposure (time=105 min). All data are means ± S.E. \(^1\)significant effect of treatment; \(^2\) significant effect of hypercapnic hypoxia. Significance evaluated at P<0.05.
Table 1. Resting variables of control (Veh), and 5,7-Dihydroxytryptamine (5,7-DHT)-treated P14-P16 rat pups. 

- $\dot{V}_e$: ventilation (ml.min$^{-1}$,kg$^{-1}$); $\dot{V}_{O_2}$: metabolic rate (ml.min$^{-1}$,kg$^{-1}$); $\dot{V}_e/\dot{V}_{O_2}$: ventilatory equivalent; MAP: mean arterial pressure (millimeters of mercury, mmHg); sBP: systolic arterial blood pressure (mmHg); dBP: diastolic arterial blood pressure (mmHg); HR: heart rate (beats.min$^{-1}$), PaO$_2$: partial pressure of arterial O$_2$ (mmHg); PaCO$_2$: partial pressure of arterial CO$_2$ (mmHg). Shown are mean data ± S.E. *: significant difference between vehicle and 5,7-DHT treated pups (p<0.05).
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<td>PaCO2</td>
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**Table 2. Blood gas analyses.** Values for arterial partial pressure of O₂ (PaO₂) and CO₂ (PaCO₂) for 5,7-DHT treated pups and vehicle controls during resting conditions (Base; from Groups 1-3), during resting conditions prior to hypoxia (Base (Hx)) or hypercapnia (Base (Hc)) and during last min of the 5th hypoxic (Hx) or hypercapnic (Hc) exposure. * significant difference between vehicle and 5,7-DHT treated pups (P<0.05). Note: There was no significant effect of a loss of 5-HT neurons on the magnitude of the fall in PaO₂ during hypoxia or the rise in PaCO₂ during hypercapnia. Values are averages ± S.E.
Figure 1

a

b

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c

d

# 5-HT+ neurons

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TH+ neurons

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Figure 2

Veh

5,7-DHT

BP (mmHg)

MAP (mmHg)

HR (BPM)
Figure 3

(a) Graph showing changes in HR (beats.min\(^{-1}\)) and MAP (mmHg) over time (min).

(b) Bar graph showing the change in ΔHR / ΔMAP (beats.min\(^{-1}\).mmHg\(^{-1}\)) over time.
Figure 4

(a) 

VE (ml.min⁻¹.kg⁻¹)

(b) 

VO₂ (ml.min⁻¹.kg⁻¹)

(c) 

VE/VO₂

Time (min)
Figure 5
Figure 7

Veh

5,7-DHT
Figure 8

(a) Heart Rate (HR) and Mean Arterial Pressure (MAP) over time.

(b) Systolic Blood Pressure (SBP) over time.

(c) Diastolic Blood Pressure (dBP) over time.
Figure 9

- **Veh – pre AIHH**
  - BP (mmHg)
  - $V_T$ (V)

- **Veh – post AIHH**
  - BP (mmHg)
  - $V_T$ (V)

- **5,7-DHT – pre AIHH**
  - BP (mmHg)
  - $V_T$ (V)

- **5,7-DHT – post AIHH**
  - BP (mmHg)
  - $V_T$ (V)
Figure 10

(a) 

VE (ml min\(^{-1}\) kg\(^{-1}\))

(b) 

VE (ml min\(^{-1}\) kg\(^{-1}\))

(c) 

\(\dot{V}O_2\) (ml min\(^{-1}\) kg\(^{-1}\))

(d) 

\(\dot{V}E/\dot{V}O_2\)

(e) 

\(PaCO_2\) (mmHg)

Time (min)

---

VE (ml min\(^{-1}\) kg\(^{-1}\))

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\(\dot{V}O_2\) (ml min\(^{-1}\) kg\(^{-1}\))

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\(PaCO_2\) (mmHg)

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Time (min)