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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Submitted 29 May 2015; accepted in final form 5 September 2015

Magnusson J, Cummings KJ. Plasticity in breathing and arterial blood pressure following acute intermittent hypercapnic hypoxia in infant rat pups with a partial loss of 5-HT neurons. Am J Physiol Regul Integr Comp Physiol 309: R1273–R1284, 2015. First published September 9, 2015; doi:10.1152/ajpregu.00241.2015.—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-wk-old, 5,7-dihydroxytryptamine-treated and controls to AIH (10% O2; n = 13 control, 14 treated), acute intermittent hypercapnia (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 acute intermittent hypercapnia, and twenty ~20-s challenges of AIHH to mimic sleep apnea. Systolic BP (sBP), diastolic BP, mean arterial pressure, heart rate (HR), ventilation (Ve), and metabolic rate (V˙O2) were continuously monitored. 5,7-Dihydroxytryptamine induced an ~35% loss of 5-HT neurons from the medullary raphe. Compared with controls, pups deficient in 5-HT neurons had reduced resting sBP (~6 mmHg), mean arterial pressure (~5 mmHg), and HR (56 beats/min), and experienced a reduced drop in BP during hypoxia. AIHH induced vLTF in both groups, reflected in increased Ve and Ve/V˙O2, and increased arterial Pco2. The sBP of pups deficient in 5-HT neurons, but not controls, was increased 1 h 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 cardiorespiratory defects related to partial 5-HT system dysfunction.

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R1273
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 sex. Dams were fed ad libitum on standard rat chow, and kept on a 12:12-h 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. Before 5,7-DHT injection, pups were injected with desipramine (50 µl ip of a 10 mg/ml solution in saline) to preserve catecholaminergic neurons. While under 1.5–2% isoflurane, pups were injected (intracerebroventricular, via the cisterna magna) with 3 µl 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 g in the 24 h following surgery, before gaining weight over the following 3 days (albeit at a reduced rate compared with controls). Nevertheless, we observed that treated pups always behaved normally in the litter, with no signs of dehydration, lethargy, or any other behavioral abnormalities.

Surgery (femoral catheter). We implanted a femoral catheter into pups 4 days following 5,7-DHT or vehicle injection (P14-16), as previously described (42). Before surgery, the tip of the PE10 catheter was heated, stretched to the appropriate diameter, and prefilled with heparinized saline solution (100 µl/ml). Under 2% isoflurane, a ~1-cm skin incision was made in the left groin, and a ×20 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 epigastic branch. An incision was made on the left femoral artery for insertion of a PE-10 catheter. The catheter tip was advanced ~0.6–0.8 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 laboratory, 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, 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 premixed 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 analyzers (CWE, 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 s. 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) 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 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 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. To determine metabolic rate (V02) during normoxia, hypoxia, or hypercapnia, a separate pump (AEI Technologies, Naperville, IL) pulled a subsample of gas through the O2 analyzer, which was single-point calibrated at the start of each experiment with humidified room air to prevent an overestimation of V02 resulting from O2 being diluted with H2O from the lung. We could not determine V02 in pups exposed to acute intermittent hypercapnic hypoxia (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 were 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 tidal volume (VT), 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 ~10-cm length of small-diameter tubing. The femoral catheter was fed through a 20G needle and attached to a BP transducer mounted outside the chamber, calibrated at the beginning of each experiment day with a sphygmomanometer. 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 mean arterial pressure (MAP), HR, and breathing in treated and control pups at P14-16 (4 days following 5,7-DHT injection), at rest and during hypoxia, hypercapnia, and hypercapnic hypoxia. Before the first challenge, pups were allowed to warm up in the chamber for at least 20 min. We started recording data when pups displayed full arousal from anesthesia and full recovery and stability of HR, breathing, BP, and V02. 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 groups 1 and 2, respectively, see below), baseline arterial PO2 (PaO2) and arterial PCO2 (PaCO2) were measured using ~90 µl of whole blood, collected via the arterial catheter and immediately transferred to a blood-gas analyzer (ABL80Flex Co-Ox; Radiometer, Brønshøj, Denmark). Pups were then subjected to one of three protocols: AIHH, acute intermittent hypercapnia (AIHC), or AIHC. Each protocol lasted a total of 120 min. In initial experiments, pups were exposed to five 5-min episodes of AIHH (group 1: n = 13 control, 14 treated), or AIHH (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- to 25-s challenges of 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 due 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 \( P_{aCO_2} \), to validate observed changes in the ventilatory equivalent (\( VE/V_{O_2} \)). At the end of the experiment, animals were anesthetized and perfused with 4% paraformaldehyde to fix brain stem tissue for immunohistochemistry.

**Immunohistochemistry.** Brain stems were cut on a vibratome (VT 1000S, Leica) at 30–40 \( \mu \)m, 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 three times in PBS (10 min each). The sections were then blocked for 1 h in 1% normal donkey serum and 0.3% Triton in PBS for 30 min and washed three times (5 min each) in PBS. The sections were incubated overnight in goat-anti-5-HT (1:1,000) (Immunostar, Hudson, WI) and mouse-anti-tyrosine hydroxylase (TH; green), from control [vehicle (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 with control, 5,7-DHT treatment did not significantly affect the number of TH-positive neurons within the nucleus of the solitary tract (NTS) or ventrolateral medulla (VLM). Values are means ± SE. \( *P < 0.05 \), significant difference between treated and control pups.

**Table 1.** Resting variables of control and 5,7-DHT-treated postnatal days 14–16 rat pups

<table>
<thead>
<tr>
<th>Group</th>
<th>( \text{V}_E, \text{ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1} )</th>
<th>( \text{V}_O_2, \text{ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1} )</th>
<th>( \text{V}_E/\text{V}_O_2 )</th>
<th>MAP, mmHg</th>
<th>sBP, mmHg</th>
<th>dBP, mmHg</th>
<th>HR, beats/min</th>
<th>( P_{aO_2}, \text{mmHg} )</th>
<th>( P_{aCO_2}, \text{mmHg} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>1.321 ± 0.110</td>
<td>35.8 ± 2.8</td>
<td>41.5 ± 2.8</td>
<td>65.2 ± 1.2</td>
<td>82.1 ± 1.7</td>
<td>46.3 ± 1.2</td>
<td>458 ± 7</td>
<td>89.9 ± 3</td>
<td>29.7 ± 1.1</td>
</tr>
<tr>
<td>5,7-DHT</td>
<td>1.119 ± 0.100</td>
<td>28.4 ± 2.8</td>
<td>45.0 ± 3.7</td>
<td>60.8 ± 1.4*</td>
<td>76.0 ± 2.1*</td>
<td>46.7 ± 1.7</td>
<td>402 ± 8*</td>
<td>100.1 ± 3*</td>
<td>29.0 ± 1.2</td>
</tr>
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</table>

Values are means ± SE. Veh, vehicle control; 5,7-DHT, 5,7-dihydroxytryptamine; \( \text{V}_E \), ventilation; \( \text{V}_O_2 \), metabolic rate; \( \text{V}_E/\text{V}_O_2 \), ventilatory equivalent; MAP, mean arterial pressure; sBP, systolic arterial blood pressure; dBP, diastolic arterial blood pressure; HR, heart rate; \( P_{aO_2} \), partial pressure of arterial O\(_2\); \( P_{aCO_2} \), partial pressure of arterial CO\(_2\). \*Significant difference between vehicle and 5,7-DHT-treated pups \((P < 0.05)\).
mined using the formula: $\dot{V}O_2 = (\text{fractional } O_2 \text{ inflow} - \text{fractional } O_2 \text{ outflow}) \times \text{flow (ml/min)/mass (kg)}$, $\dot{V}E/\dot{V}O_2$, systolic arterial BP (sBP), diastolic arterial BP (dBP), MAP (mmHg), and HR (beats/min) from the raw respiratory and BP traces. Baseline blood gases, pH, and hematocrit were also determined in a subsets of group 1 and 2 animals. Variables were measured from the last minute of each of the five normoxic, prechallenge periods, as well as the last minute of each of the 5-min hypoxic or hypercapnic challenges. Because of 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-s window (see Fig. 7). Every attempt was made to measure variables from signals that were not contaminated by movement artifact.

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 killed before the protocol was completed. 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 (NTS). The rostral-caudal position of each section was confirmed using easily identifiable landmarks (e.g., inferior olives, area postrema, fourth ventricle), and referring to Paxinos and Watson’s rat brain stereotaxic atlas (32).

We analyzed 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 V̇E, V̇O₂, V̇E/V̇O₂, MAP, HR, and blood gases were assessed with two-factor repeated-measures ANOVAs. These tests included data for all animals during the last minute 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-h postchallenges), we used two-factor repeated-measures ANOVAs on data from the baseline period and from 1 h following the last challenge (i.e., only from animals surviving the whole protocol). In line with the changes in V̇E/V̇O₂, hypocapnia appeared in all four pups (2 control and 2 treated) 1 h following AIHH; thus Paco₂ data were combined for statistical analyses. When significant main effects were found, we used Tukey’s post hoc analyses for pairwise comparisons. Differences between groups with respect to 5-HT- and TH-positive cell counts were determined with a Student’s t-test.

RESULTS

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. 1, A–C), with no effect on the number of TH-positive neurons in the ventrolateral medulla or NTS (Fig. 1D). On the day of testing, pups with reduced 5-HT neurons weighed ~20% less than controls (vehicle: 31.1 ± 0.8 g; 5,7-DHT treated: 24.6 ± 0.7 g; 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 V̇O₂ 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 fB (control: 121 ± 5; treated: 111 ± 5 breaths/min; P = 0.14), Vr (control: 1000 ml/min/kg; control: 31.1 ± 0.8 g; 5,7-DHT treated: 24.6 ± 0.7 g; 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 V̇O₂ 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 fB (control: 121 ± 5; treated: 111 ± 5 breaths/min; P = 0.14), Vr (control: 140 ml/min/kg; P < 0.05).
controls (treatment by treated pups was, on average, induced hypotension in both groups, the loss of BP experience a single episode of hypoxia are shown in Fig. 2. While hypoxia Cardiorespiratory Responses to AIH (Group 1: AIH)

Effects of a Partial Loss of 5-HT Neurons on Cardiorespiratory Responses to AIH (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 that in controls (treatment × hypoxia: P = 0.02; Fig. 3A), solely because of a reduced drop in sBP (treatment × hypoxia: P = 0.002; not shown). In the 1 h 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 three times greater in 5-HT-deficient pups compared with controls (P < 0.001; Fig. 3B).

Over the 1 h following AIH, the HR of treated pups remained significantly lower compared with controls, with no evidence of plasticity (Fig. 3A).

Fig. 5. Cardiovascular responses to acute intermittent hypercapnia (group 2). Responses of HR and MAP during each of the five episodes of hypercapnia (shaded), the intervening normocapnic periods, and 1 h following the challenges, of Veh (●, n = 12) and 5,7-DHT-treated littersmates (○, n = 11) are shown. Eight control and seven treated pups survived the entire protocol and maintained reliable sBP and DBP readings. Values are means ± SE. *Significant effect of treatment. †Significant effect of hypoxia. Significance evaluated at P < 0.05.

Table 2. Blood-gas analyses

<table>
<thead>
<tr>
<th></th>
<th>Veh</th>
<th>5,7-DHT</th>
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<tbody>
<tr>
<td></td>
<td>Base</td>
<td>Base (Hx)</td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>PaO2, mmHg</td>
<td>92.1 ± 3.2</td>
<td>89.9 ± 3.2</td>
</tr>
<tr>
<td>PaCO2, mmHg</td>
<td>29.7 ± 1.1</td>
<td>29.3 ± 1.1</td>
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</table>

Values are averages ± SE; n, no. of animals. PaO2 and PaCO2 values are shown for 5,7-DHT-treated pups and vehicle controls during resting conditions (Base; from groups 1–3), during resting conditions before hypoxia [Base (Hx)] or hypercapnia [Base (Hc)], and during the last minute of the fifth 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 serotonin neurons on the magnitude of the fall in PaO2 during hypoxia or the rise in PaCO2 during hypercapnia.

Fig. 6. Respiratory responses to acute intermittent hypercapnia (group 2). A: V̇E responses of control (●, n = 12) and 5,7-DHT-treated pups (○, n = 11) during each of the five episodes of hypercapnia (shaded), the intervening normocapnic periods, and 1 h following the challenges. Nine control and seven treated pups survived the entire protocol. B: responses of V̇O2 in control and treated pups during and following the hypercapnic challenges. C: V̇E/V̇O2 of control and treated pups during and following the challenges. Values are means ± SE. *Significant effect of hypercapnia. Significance evaluated at P < 0.05.
For both groups, hypoxia elicited a significant increase in $V_{\dot{E}}$. However, control pups had a slightly but significantly greater hypoxic ventilatory response compared with pups deficient in 5-HT neurons (treatment $\times$ gas: $P = 0.03$; Fig. 4A), solely because the increase in $f_B$ was $\sim 40$ breaths/min greater in controls than in treated pups (treatment $\times$ gas: $P < 0.001$). Relative to baseline values, there was no change in the $V_{\dot{E}}$ of either control or treated pups in the hour following intermittent hypoxia ($P = 0.11$; Fig. 4A). Hypoxia had no significant influence on $V_{\dot{O}_2}$, either acutely or 1 h following the challenges (Fig. 4B). The increase in $V_{\dot{E}}/V_{\dot{O}_2}$ 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 $P_{\text{aCO}_2}$ between the two groups during hypoxia (Table 2). Compared with baseline, $V_{\dot{E}}/V_{\dot{O}_2}$ was unaltered 1 h following AIH ($P = 0.16$; Fig. 4C).

Effects of a Partial Loss of 5-HT Neurons on Cardiorespiratory Responses to AIHC (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 h 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 <

Fig. 7. Representative traces showing the cardiorespiratory responses to hypercapnic hypoxia. Shown are the raw BP trace, MAP, HR, and breathing [tidal volume ($V_{\text{T}}$)] of a Veh control pup (top) and a 5,7-DHT-treated littermate (bottom) before, during, and following an $\sim 20$-s 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-s segments analyzed for BP (1), HR (2), and breathing (3). The respiratory trace was not identifiable for $\sim 3$ s when gases were being switched, due to small pressure fluctuations. Scale bar on $x$-axis = 10 s.
As with V\textsuperscript{E}, acute hypercapnia induced a significant hyperventilation of this paradigm. As a result, V\textsubscript{E}/V\textsubscript{O2} nearly doubled in both groups from the beginning to the end of the AIHH protocol (Fig. 10, A). Unlike AIH and AIHC, AIHH induced an increase in BP (gas: P < 0.001; Fig. 6B), but after 1 h V\textsubscript{O2} was not statistically different from baseline values (P = 0.13; Fig. 6B). As with V\textsubscript{E}, acute hypercapnia induced a significant hyperventilation, but 1 h following AIHC V\textsubscript{E}/V\textsubscript{O2} had not changed from baseline values (P = 0.37).

Effects of a Partial Loss of 5-HT Neurons on Cardiorespiratory Responses to AIHH (Group 3: AIHH)

Group 3 animals were exposed to twenty 20-s episodes of AIHH 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-s 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 with controls (treatment \times gas: P = 0.005; Fig. 8A). One hour following AIHC, the BP of control pups had not changed relative to prechallenge BP. Surprisingly, however, and unlike the AIH or AIHC protocols, AIHH induced a rise in sBP and MAP only in pups deficient in 5-HT neurons (treatment \times time interaction: P = 0.001 and 0.01, respectively; Figs. 8, A and B, and 9). In both groups, acute AIHH increased HR, but 1 h after the challenges HR was unchanged from baseline (Fig. 8A).

On average, acute hypercapnic hypoxia increased V\textsubscript{E} approximately threefold, 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 (treatment; P < 0.001; Fig. 10, A and B). In both groups, an increase in both f\textsubscript{R} and V\textsubscript{T} contributed to vLTF (P = 0.02 for both). In addition, in both groups, AIHH induced a long-term depression of V\textsubscript{O2} (P = 0.004; Fig. 10C), a unique feature of this paradigm. As a result, V\textsubscript{E}/V\textsubscript{O2} 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 P\textsubscript{ACO2} (Fig. 10E). Thus, among our three paradigms, AIHH was the only one to elicit long-term plasticity in breathing and BP 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.

DISCUSSION

In this study, we treated 2-wk-old rat pups with 5,7-DHT to induce a partial loss of 5-HT neurons, with the goal of modeling 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 AIH, AIHC, and AIHH. 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 important not only for respiratory LTF, but also for the increase in BP following AIH. 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 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 V\textsubscript{E}, decreased V\textsubscript{O2}, and decreased P\textsubscript{ACO2}. 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. Al-
though 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 with 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, this suggests that, at this age, central 5-HT signaling 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 with 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 signaling. A partial loss of 5-HT neurons had no influence on resting fB, V̇T, VT/V̇O₂, or P aCO₂. However, we know from several previous studies that a more extensive loss of 5-HT neurons leads to reduced fB and apnea (6, 12). Interestingly we did find that the P aCO₂ of treated pups was ~8 Torr greater than that of controls. Given there was no effect of 5-HT neuron loss on resting P aCO₂, it may be that 5-HT neurons influence the autonomic control of pulmonary vascular resistance and hence the proper matching of lung V̇E and perfusion. V̇E-perfusion mismatch minimally affects P aCO₂ because the arteriovenous P C O₂ gradient is much smaller than the P O₂ 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 laboratory 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 present 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 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 approximately threefold 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 loss of medullary 5-HT signaling. Supporting this idea are studies demonstrating that 5-HT, acting on 5-HT₃ receptors within the NTS, 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.
entire protocol. Average \( V\dot{O}_2 \) (see data combined) at baseline (time 0 min) and 60 min after (time 105 min) are shown. Values are means ± SE. 1|Significant effect of treatment. 2|Significant effect of hypercapnic hypoxic exposure (time 105 min) and 60 min following the last hypercapnic hypoxic exposure (time 105 min) are shown. Values are means ± SE. 1|Significant effect of treatment. 2|Significant effect of hypercapnic hypoxic exposure. Significance evaluated at \( P < 0.05 \).

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 hypocapnia fB response. However, we note that neither the increase in \( V_E/V_O2 \) nor the fall in \( P_{aCO2} \) 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 BP

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 h following AIHH. AIHH also induced a long-term depression of \( V_O2 \). In both groups, the increase in \( V_E \) and decrease in \( V_O2 \) resulted in a near-doubling of \( V_E/V_O2 \) from baseline to 1 h after the challenges. Arterial blood-gas analysis of four samples confirmed that \( P_{aCO2} \) 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 \( V_E \) 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 \( P_{aCO2} \) and alveolar ventilation, reducing plant gain to help prevent apnea following a transient ventilatory overshoot (7).

The magnitude of vLTF was not influenced by an \( \sim 35\% \) 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 signaling through 5-HT7 receptors, via PKA activation, can constrain the development of phrenic LTF (15), as can adenosinergic signaling 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 \( \sim 20\)-s 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, given 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 (34a). 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 signaling.

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 h following the termination of the stimulus, by way of both increased $V_{\text{E}}$ and reduced $V_{\text{O}_2}$. This response effectively stabilizes breathing by reducing plant gain and hence the chances of $P_{\text{aCO}_2}$ 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 $BP$. There is evidence that the vast majority of SIDS cases have one or more 5-HT abnormalities within the brain stem 5-HT system, including an ~30% reduction in 5-HT content and increased numbers of 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 downregulated 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 abnormal beyond the increased numbers of 5-HT neurons, or other cardiorespiratory defects resulting from a loss of, or dysfunction within, 5-HT neurons.

ACKNOWLEDGMENTS

The authors thank Dr. Eileen Hassel (University of Missouri) for assistance with immunohistochemistry, Dr. Craig Enter (University of Missouri) for use of a blood gas analyzer, and Ms. Jane Chen (University of Missouri) for technical assistance and animal husbandry.

GRANTS

Funding for this work was provided by an American Heart Association Scientist Development Grant (14SDG18560022; principal investigator: K. J. Cummings) and University of Missouri College of Veterinary Medicine Faculty Research Grants.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.M. and K.J.C. conception and design of research; J.M. and K.J.C. performed experiments; J.M. and K.J.C. analyzed data; J.M. and K.J.C. interpreted results of experiments; J.M. and K.J.C. prepared figures; J.M. and K.J.C. drafted manuscript; J.M. and K.J.C. edited and revised manuscript; J.M. and K.J.C. approved final version of manuscript.

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