Brain stem serotonin protects blood pressure in neonatal rats exposed to episodic anoxia

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Yang HT, Cummings KJ. Brain stem serotonin protects blood pressure in neonatal rats exposed to episodic anoxia. J Appl Physiol 115: 1733–1741, 2013. First published October 17, 2013; doi:10.1152/japplphysiol.00970.2013.—In neonatal rodents, a loss of brain stem serotonin [5-hydroxytryptamine (5-HT)] in utero or at birth compromises anoxia-induced gasping and the recovery of heart rate (HR) and breathing with reoxygenation (i.e., autoreuscitation). How mean arterial pressure (MAP) is influenced after an acute loss of brain stem 5-HT content is unknown. We hypothesized that a loss of 5-HT for ~1 day would compromise MAP during episodic anoxia. We injected 6-fluorotryptophan (20 mg/kg ip) into rat pups (postnatal days 9–10 or 11–13, n = 22 treated, 24 control), causing a ~70% loss of brain stem 5-HT. Pups were exposed to a maximum of 15 anoxic episodes, separated by 5 min of room air to allow autoreuscitation. In younger pups, we measured breathing frequency and tidal volume during episodes, separated by 5 min of room air to allow autoreuscitation. In younger pups, we measured breathing frequency and tidal volume using “head-out” plethysmography and HR from the electrocardiogram. In older pups, we used whole body plethysmography to detect gasping, while monitoring MAP. Gasp latency and the time required for respiratory, HR, and MAP recovery following each episode were determined. Despite normal gasp latency, breathing frequency and a larger tidal volume (P < 0.001), 5-HT-deficient pups survived one-half the number of episodes as controls (P < 0.001). The anoxia-induced decrease in MAP experienced by 5-HT-deficient pups was double that of controls (P = 0.017), despite the same drop in HR (P = 0.48). MAP recovery was delayed ~10 s by 5-HT deficiency (P = 0.001). Our data suggest a loss of brain stem 5-HT leads to a pronounced, premature loss of MAP in response to episodic anoxia. These data may help explain why some sudden infant death syndrome cases die from what appears to be cardiovascular collapse during apparent severe hypoxia.

blood pressure; gasping; hypoxia; serotonin; SIDS

IN MAMMALS, REDUCED ALVEOLAR ventilation or rebreathing can cause death quickly, owing to high aerobic demands coupled with scant systemic oxygen stores. As an adaptive response to severe hypoxemia (i.e., when brain stem tissue PO2 falls below ~10 Torr), heart rate (HR), blood pressure, and breathing are suppressed (11, 13). This inhibition, culminating in primary apnea and bradycardia, is predominantly a direct effect of severe hypoxia on respiratory neurons and cardiac pacemaker activity with little if any vagal influence (12, 15). Survival depends on subsequent behavioral and/or physiological responses that ultimately reverse this inhibition. Young mammals are particularly adept at doing this via a sequence of respiratory and cardiovascular responses, collectively termed “autoreuscitation”; that together reoxygenate the sinoatrial node to restore HR, blood pressure, and eventually normal breathing (11, 13). Gasping is the respiratory component of autoreuscitation, emerging from primary apnea to rapidly increase pulmonary gas exchange. During severe hypoxia, both tonic and respiro-phasic sympathetic discharges also increase to preserve blood pressure in the face of a drastic, hypoxia-induced bradycardia (38), a response critical for survival (15, 42). While neurotransmitter systems controlling the gasping and HR responses to severe hypoxia have been identified (12, 40), we know little about the neurotransmitter systems controlling blood pressure.

5-Hydroxytryptamine (5-HT) is a neurotransmitter of interest with respect to blood pressure control during severe hypoxic conditions. Rodents missing brain stem serotonergic neurons beginning either in utero (Pet-1–/– mice) or shortly after birth [rat pups treated intracerebroventricularly with 5,7-dihydroxytryptamine (5,7-DHT), a 5-HT neurotoxin] die more easily than their control littermates when exposed to repeated episodes of anoxia (4, 7). Compared with their respective control littermates, the gasping of 5,7-DHT rat pups is much less affected than that of Pet-1–/– mice; instead, the failed autoreuscitation of 5,7-DHT rats is more closely associated with a progressive deterioration in their ability to restore HR across successive anoxic episodes (7). In the first postnatal week, tryptophan hydroxylase-2-deficient (TPH2–/–) mice having a specific loss of central 5-HT content (without a loss of neurons) also have major defects in gasping and HR recovery that compromise survival (3). However, by the second postnatal week, TPH2–/– mice resemble 5,7-DHT rats in that their failed autoreuscitation is more closely related to failed HR recovery than an inability to gasp (3). Together, these findings suggest that, in the second postnatal week, 5-HT has a physiological role in the maintenance and/or restoration of blood pressure and HR, independent from its role in gasping at younger ages. Mice lacking neuronal 5-HT are hypotensive under resting conditions (1), but there is little information related to the role of 5-HT in the maintenance and restoration of blood pressure during severely hypoxic conditions.

The effects on an acute loss of brain stem 5-HT on the respiratory (gasp) and cardiovascular (blood pressure and HR) responses to anoxia in the neonatal period are an issue germane to the sudden infant death syndrome (SIDS) and its etiology. SIDS is highly associated with defects within the brain stem 5-HT system, including an ~30% loss of 5-HT content (9, 18, 30). And there is decades-old epidemiological, physiological, and pathological evidence suggesting hypoxia is an important, and perhaps even common, trigger for SIDS (17, 24, 25, 28, 29). An especially intriguing finding is that some SIDS cases fail to autoreuscitate during severely hypoxic conditions; like 5-HT-deficient rodents, these infants can gasp but nonetheless fail to restore HR (33, 39).

We undertook the present work as an extension of our and others’ previous studies of mice and rats lacking brain stem...
5-HT or serotonergic neurons since early embryogenesis or just after birth (4, 7, 10). Based on our recent findings (3, 7), we hypothesized that an acute loss of 5-HT in the second postnatal week would compromise the cardiovascular components of autoresuscitation, but not gasping. By inducing an acute loss of 5-HT just before the anoxic episode(s), we avoided the known effects of long-term 5-HT deficiency on growth and the development of cardiorespiratory circuits (1, 23).

MATERIALS AND METHODS

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 24 6-fluorotryptophan (6-FL)-treated Sprague-Dawley rat pups [n = 13 at postnatal days (P) 8–10 and n = 11 at P11–13], along with 22 vehicle controls (vehicle-injected; n = 12 and 10). These pups were derived from 12 breeding pairs. Dams were fed ad libitum on standard rat chow, and kept on a 12:12-h light-dark cycle. Littermates were always used; i.e., for each litter, pups were assigned to either 6-FL or vehicle, and on any given day, at least one pup from each group was tested. For any given litter, treated and control pups were matched with respect to body weight (a reflection of feeding and hence tryptophan intake) rather than sex. Our previous work showed that sex has no effect on any autoresuscitation variables or survival (3, 7). 6-FL was dissolved in very dilute acid (0.1 N HCl), at a concentration of 20 mg/ml. As 6-FL has a relatively short half-life (0.5–1 h), with 5-HT content being restored a few hours following a single injection (2), we decided to give three injections of 6-FL (or vehicle) over a 24- to 36-h period (each injection: 250–300 mg/kg ip). An injection was given every ~8–12 h, the last of which occurred 1–2 h before experimentation. Although systemic injection of 6-FL induces a global loss of 5-HT [including stores located in the periphery (2)], we chose this method as a convenient way to induce a specific, acute loss of central 5-HT and avoid the confounding effects of multiple surgeries over 24 h. After each injection, pups were immediately returned to the litter and dam. Pups appeared to feed and otherwise behave normally following each injection.

Experimental Setups

For these experiments, we used two groups of rat pups, one slightly older (~2 days) than the other, and two different systems to detect cardiorespiratory variables of interest. Our purpose was not to assess the effects of age; indeed, both groups of 5-HT-deficient pups ultimately responded in the same general way to episodic anoxia (see RESULTS). Rather, each group allowed us to accurately determine gas tidal volume (VT). We also measured HR using electrodes embedded within a vest made from material bath/pump to control ambient and, therefore, pump body temperature. Body temperature was held at 36 ± 0.5°C in all animals throughout the experiment. In some cases, ambient temperature was increased 1–2°C during the experiment to keep body temperature constant.

Ventilation (Vt) was measured with a mask connected to a pneumotach, in a head-out arrangement. The head mask was made by fitting a section of vinyl over the end of syringe tube (volume ~5 ml), held in place with a rubber gasket (Terumo Medical) that fit into one end of the glass chamber. Following the removal of fur with a commercially available product (Nair, Church & Dwight, Ewing, NJ), the snout of the animal was placed into a small hole in the vinyl and sealed with polyether material (Impregum F Polyether Impression material, 3M, St. Paul, MN). A pump (AEI Technologies, Naperville, IL) connected to the outlet port of the mask pulled air through the pneumotach and across the animals’ face at a flow of 150 ml/min. The air was subsequently drawn through a small column of Drierite (W. A. Hammond Drierite, Xenia, OH) and then an O2 analyzer (AEI Technologies, Pittsburgh, PA) to allow for the calculation of O2 uptake (VO2). Anoxic gas (97% N2/3% CO2) was delivered directly from a tank to the surroundings of the pneumotach through the open end of a 50-ml syringe placed over the end of the pneumotach, to be pulled through the mask via the downstream pump. Three percent CO2 was added to the inspirate to prevent arterial hypocapnia during hypoxic hyperventilation, as has been done in other studies (7, 12). The time constant for gas wash-in was ~2 s. Given its low resistance (the mask is essentially open to the atmosphere), pressure in the mask is negligible, imparting no respiratory load on the animal. Body temperature was continually monitored with a 36-gauge thermocouple (Omega Engineering, Stamford, CT). Surface ECG was used to measure HR using electrodes embedded within a vest made from tensor bandage.

Inspiratory and expiratory airflows were detected by connecting both side-arms of the pneumotach to a differential pressure transducer (Validyne Engineering, Northridge, CA). Integration of the flow trace provided respiratory volume, calibrated by injecting 50 and 100 ml of air into the sealed chamber with a micropipette, with flow running, at normal respiratory breathing frequency (fB).

Whole body plethysmography (group 2). For whole body measurements, we used a slightly larger water-perfusable glass chamber (volume = 100 ml). A programmable water bath/pump maintained a thermoneutral ambient temperature, measured with a thermocouple inserted into the chamber. For both groups, chamber temperature was held constant at 30–31°C within the thermoneutral range for pups at this age. Normoxic and anoxic gas (0% O2/3% CO2) from the wall (air) or a cylinder, respectively, was passed through a flowmeter before entering the chamber. 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. Twenty-gauge needles, inserted through rubber stoppers and into the chamber, served as critical resistors to minimize the influence of breathing on the flow of gas through the chamber (i.e., maximize the pressure signal related to breathing). Gas flow through the chamber was held constant at 300 ml/min. Changes in chamber pressure related to breathing were detected with a differential pressure transducer (Validyne), connected to the animal chamber on one side and an empty reference chamber on the other. To account for thermal drift, we connected the two chambers via a ~10-cm length of small-diameter tubing. To optimize the gas wash-in time and detection of arterial pressure, we did not humidify the gas in-flow or insert a rectal thermocouple for monitoring body temperature, preventing accurate determination of VT. The femoral catheter was fed through a 20-gauge needle and attached to a blood pressure transducer mounted
outside the chamber, calibrated with a sphygmomanometer. Analog signals from respiratory and arterial pressure transducers were fed into Powerlab data acquisition system (ADInstruments) and analyzed in Labchart 7 (ADInstruments).

**Surgery (P11–13 Pups)**

Pups were anesthetized under 2% isoflurane. Using a ×20 dissecting microscope, a ~1-cm skin incision was made in the left groin. The left femoral artery was carefully dissected out and tied with 3–0 surgical suture just distal to the epigastric branch. An incision was made on the left femoral artery, and a PE-10 catheter (the tip of which was heated and stretched) was inserted into the femoral artery. The catheter was filled with heparinized saline solution (100 µl/ml). The catheter tip was advanced ~0.6–0.8 cm, close to the inguinal ligament. The catheter was exteriorized at the back of the neck through a tunnel under the skin. Tissues were sutured using 3–0 surgical silk.

**Experimental Protocol**

Animals were allowed to rest in the warmed chamber (at least 10 min) until \(f_0\), HR, and \(V_\text{O}_2\) were in steady state. We recorded 10 min of baseline variables and then exposed the pups to a maximum of 15 episodes of environmental anoxia (each given until hypoxic apnea: ~45 s in younger pups, and 60–70 s in older pups due to the larger chamber and longer wash-in time). Each episode was followed by 5 min of room air to allow autoresuscitation. We chose 15 episodes to compare our results with our laboratory’s previous studies (7) and because others have shown that normal rat pups can survive ~15 episodes of anoxia (37).

**Tissue Monoamine Quantification**

5-HT and noradrenaline (NA) content in the medulla oblongata were determined using high-pressure liquid chromatography (HPLC). Following experiments, whole brains were extracted and stored at ~80°C. Medullae were isolated and homogenized using a tissue dismembrator in 100–750 µl of 0.1 M TCA containing 10\(^{-5}\) M sodium acetate, 10\(^{-4}\) M EDTA, 5 ng/ml isoproterenol (as internal standard), and 10.5% methanol (pH 3.8). After centrifugation, the supernatant was removed for HPLC analysis.

HPLC was performed utilizing an Antec Decade II (oxidation: 0.4 (3 mm GC WE, HYREF) electrochemical detector operated at 33°C. Sample (20 µl) of the supernatant were injected using a autosampler (model 2707, Waters, Milford, MA) onto a 100 × 4.60 mm HPLC column (Phenomenex, Torrance, CA). Biogenic amines are eluted with a mobile phase consisting of 89.5% 0.1 M TCA, 10\(^{-4}\) M EDTA, and 10.5% methanol (pH 3.8). After centrifugation, the supernatant was removed for HPLC analysis. Tissue monamines were determined using an Antec Decade II (oxidation: 0.4 (3 mm GC WE, HYREF) electrochemical detector operated at 33°C. Sample (20 µl) of the supernatant were injected using a autosampler (model 2707, Waters, Milford, MA) onto a 100 × 4.60 mm HPLC column (Phenomenex, Torrance, CA). Biogenic amines are eluted with a mobile phase consisting of 89.5% 0.1 M TCA, 10\(^{-4}\) M EDTA, and 10.5% methanol (pH 3.8). After centrifugation, the supernatant was removed for HPLC analysis. Tissue monamines were determined using an Antec Decade II (oxidation: 0.4 (3 mm GC WE, HYREF) electrochemical detector operated at 33°C. Sample (20 µl) of the supernatant were injected using a autosampler (model 2707, Waters, Milford, MA) onto a 100 × 4.60 mm HPLC column (Phenomenex, Torrance, CA). Biogenic amines are eluted with a mobile phase consisting of 89.5% 0.1 M TCA, 10\(^{-4}\) M EDTA, and 10.5% methanol (pH 3.8). After centrifugation, the supernatant was removed for HPLC analysis.

**Data and Statistical Analysis**

In P8–10 pups, we measured the \(f_0\) during eupnea and severely hypoxic conditions (min\(^{-1}\), \(V_\text{r}\) (ml/kg), \(V_\text{e} (f_0 \times V_\text{r}; ml/min·kg\(^{-1}\)), \(V_\text{O}_2\) (ml/min·kg\(^{-1}\)), \(V_\text{e}/V_\text{O}_2\), and HR (beats/min). The first and second gasps were most reliably discernible; hence, we report instantaneous gasp \(f_0\) using the period of the first gasp. Mass-specific \(V_\text{O}_2\) was determined using the formula: \(V_\text{O}_2 = (0.21 \times \text{fractional O}_2\text{ exhausted from mask}) \times \text{flow (ml/min)/mass (kg)}\). In both groups, we determined survival across the 15 anoxic episodes. For the P8–10 group, for every anoxic episode, we determined the gasp latency (duration of primary apnea) and the time required for the recovery of 63% of the preanoxic HR and \(f_0\) (i.e., their respective time constants). Significant differences between groups were assessed with either Student’s two-tailed \(t\)-tests (baseline variables) or repeated-measures ANOVA with data from anoxic episodes 1–7 (when all treated pups are still alive), followed by Tukey’s post hoc analysis. Differences in survival were assessed with a Kaplan-Meier Survival Analysis (Log-Rank).

In P11–13 pups, we were mainly interested in the changes in MAP and HR in response to anoxia. We report the absolute values of these variables during normoxia (eupnea) and primary apnea. The recovery of MAP, while coinciding with HR recovery, was biphasic, having a large overshoot before reaching steady state. We, therefore, report the delay in the start of MAP recovery following the emergence of gasping (MAP latency). For most animals, we could detect arterial pressure across all anoxic episodes. For some animals, the arterial pressure signal was lost during exposure to anoxia; this appeared to be due to random changes in the animal’s body position within the chamber following anoxia-induced arousal. In total, a failure to detect arterial pressure occurred seven times for both treated and control pups (from a total of 134 and 74 anoxic episodes for control and treated pups, respectively) and never occurred more than twice for any one animal. For each of the first 10 anoxic episodes, we report both absolute HR and MAP during eupnea (preepisodes) and during the anoxic episode, as well as the change in these variables from eupnea to anoxia.

The effects of 6-FL treatment on baseline, room air variables were analyzed with Student’s two-sided \(t\)-test. The effects of anoxia on autoregulation variables were tested with a two-factor ANOVA (factors: 6-FL or vehicle and episode no.), followed by Tukey’s post hoc analysis. A repeated-measures analysis was not possible due to some missing values (explained above). Differences in survival were assessed with a Kaplan-Meier Survival Analysis (Log-Rank).

**RESULTS**

**Baseline Cardiorespiratory Variables**

The effects of 6-FL treatment on resting variables are shown in Tables 1 (P8–10) and 2 (P11–13). At P8–10, 6-FL-treated, 5-HT-deficient pups had slightly increased HR compared with control littermates (40 beats/min; \(P < 0.01\)). Despite 6-FL-treated pups having a significantly increased \(V_\text{r}\) compared with littermates (\(P = 0.02\)), treatment had no effect on overall \(V_\text{e}\) (\(P = 0.10\), nor did treatment affect \(V_\text{O}_2\) (\(P = 0.13\)). However, as has been previously discovered in other animal models of 5-HT deficiency (4), \(V_\text{e}/V_\text{O}_2\) was ~39% higher in 5-HT-deficient pups compared with controls (\(P < 0.01\)). At P11–13, 6-FL had no effect on resting HR (\(P = 0.62\)). There was a marginal, yet statistically insignificant (\(P = 0.06\), effect of 6-FL on resting MAP. The \(f_0\) of P11–13-treated pups was reduced ~15% compared with controls (\(P = 0.04\)). At both ages, the body weights were not different between treated and control pups.

**Table 1. Resting variables of Veh and 6-FL-treated postnatal day 8–10 rat pups**

<table>
<thead>
<tr>
<th></th>
<th>(f_0)</th>
<th>HR, beats/min</th>
<th>(V_\text{r}), ml/min·kg(^{-1})</th>
<th>(V_\text{e}), ml/min·kg(^{-1})</th>
<th>(f_0), breaths/min</th>
<th>(V_\text{O}_2), ml/min·kg(^{-1})</th>
<th>(V_\text{e}/V_\text{O}_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>12</td>
<td>25.2 ± 0.9</td>
<td>440 ± 13</td>
<td>1,131 ± 79</td>
<td>8.0 ± 0.5</td>
<td>141 ± 5</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>6-FL</td>
<td>13</td>
<td>23.5 ± 0.7</td>
<td>480 ± 5*</td>
<td>1,426 ± 15</td>
<td>10.6 ± 0.9*</td>
<td>134 ± 6</td>
<td>37 ± 1.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), no. of pups. Veh, vehicle; 6-FL, 6-flourotryptophan; HR, heart rate; \(V_\text{e}\), ventilation; \(V_\text{r}\), tidal volume; \(f_0\), respiratory frequency; \(V_\text{O}_2\), metabolic rate; \(V_\text{e}/V_\text{O}_2\), respiratory equivalent. *Significant difference between Veh and 6-FL-treated pups (\(P < 0.05\)).
Effects of Acute 5-HT Deficiency on Gasping and Recovery of HR and Breathing (Group 1)

6-FL treatment reduced the brain stem 5-HT content of P8–10 pups by ~74% compared with control littersmates, with no significant effects on NA content (Fig. 1A; \( P < 0.001 \)). 5-HT-deficient pups could survive only one-half the number of anoxic episodes (median survived = 7 vs. 14 for control; Fig. 1B; \( P < 0.001 \)). Representative recordings demonstrating the respiratory and HR responses to anoxia are shown for control (Fig. 1, C and D) and treated pups (Fig. 1, E and F). The ability of 5-HT-deficient pups to gasp and restore HR and eupnea was no different than that of controls following the first hypoxic episode (compare Fig. 1C with Fig. 1E); however, with subsequent episodes, a delay in cardiorespiratory recovery became qualitatively evident in 5-HT-deficient pups (compare Fig. 1D with Fig. 1E). 5-HT deficiency had no consequences for gasp latency (Fig. 2A). And the gasp VT of 5-HT-deficient pups was actually greater than that of controls (Fig. 2B; \( P < 0.001 \)). Initially, 5-HT deficiency had no consequences for gasp frequency in both groups (increased significantly prior to death, and this occurred prematurely in 5-HT-deficient pups (treatment \( \times \) episode: \( P < 0.001 \); Fig. 2C; and compare gasping in Fig. 1F and Fig. 1D). The death of 5-HT-deficient pups and controls alike was preceded by a significant delay in the recovery of HR (Fig. 2D) and eupnea (Fig. 2E) (treatment effect: \( P < 0.001 \) for both), but again this occurred prematurely in 5-HT-deficient pups (treatment \( \times \) episode: \( P < 0.001 \) for HR and eupnea). Death occurred when gasping completely failed to restore HR or breathing (not shown).

Effects of Acute 5-HT Deficiency on MAP Responses to Anoxia (Group 2)

6-FL treatment reduced the brain stem 5-HT content of P11–13 pups by ~64% compared with controls, again with no

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Fig. 1. Failed autoresuscitation in postnatal day (P) 8–10 rat pups treated systemically with 6-fluorotryptophan (6-FL). A: in P8–10 rat pups, 6-FL treatment over 24–36 h results in a ~74% loss of serotonin (5-HT) from the medulla oblongata with no effect on noradrenaline (NA) content. B: 6-FL-treated pups can survive only one-half the number of severely anoxic episodes as controls [vehicle (Veh)] (median episode survived). C–F: representative tracings of heart rate (HR) and breathing [respiratory volume (Resp Vol)] in response to episodes of anoxia. In control pups, the time required to restore HR (denoted by shaded box) and for the return of eupnea (denoted by the arrow) after the initiation of gasping (denoted by *) remain unchanged from the first (C) to the sixth episode (D) of anoxia (gas challenge denoted by speckled box). In contrast, for 6-FL-treated, 5-HT-deficient pups, their recovery becomes progressively delayed from the first (E) to the sixth episode (F). Solid bar indicates time scale (30 s). Note that, in F, the scale is slightly different (zoomed out) to show the prolonged respiratory recovery. BPM, beats/min.
significant effects on NA content ($P < 0.001$; Fig. 3A). As with group 1, treated pups survived only 7 episodes, compared with 14 survived by control littermates ($P < 0.001$; Fig. 3B). The death of 5-HT-deficient pups was associated with a larger drop in MAP during primary apnea and a delay in its recovery following the emergence of gasping [compare Fig. 3C (control) with Fig. 3D (6-FL treated)]. This effect became qualitatively apparent only after several anoxic episodes [compare Fig. 4, A and B (control) with Fig. 4, C and D (6-FL-treated)].

There was no effect of 5-HT deficiency on MAP during resting, normoxic conditions (Fig. 5A and Table 2). After the first episode of anoxia, MAP became slightly (~10 mmHg) but significantly elevated in 5-HT-deficient pups compared with controls (treatments: $P < 0.001$; Fig. 5A). Across the first 10 anoxic episodes, we quantified the effects of 5-HT deficiency on MAP during primary apnea and the intervening eupneic periods (Fig. 5, A and B). MAP fell in both groups during primary apnea (episode: $P < 0.001$), but across the episodes the fall became greater in 5-HT-deficient pups (treatment × episode: $P = 0.03$; Fig. 5A). By the eighth anoxic episode, the fall in MAP was ~45 mmHg in 5-HT-deficient pups, compared with ~20 mmHg for control littermates (Fig. 5B). As was observed with HR and respiratory recovery, MAP was eventually compromised in control pups in the episodes immediately preceding death (not shown).

The premature loss of MAP in 5-HT-deficient pups was not owing to prolonged hypoxic apnea: similar to group 1, there was no difference in gasp latency between groups [mean gasp latency: 21.1 ± 1.5 s (control); 17.6 ± 1.3 s (6-FL-treated); $P = 0.07$]. Nor was the relative hypotension in 6-FL-treated pups the result of a selective effect of hypoxia on HR: from episode to episode, the drop in HR was consistently ~350 beats/min in both groups ($P = 0.48$; Fig. 5, C and D). Lastly, 5-HT deficiency was associated with a significant delay, relative to controls, in the recovery of MAP following the initiation of gasping. On average, 5-HT-deficient pups required an additional ~10 s before MAP began increasing during the gasping phase (treatment: $P < 0.001$; treatment × episode: $P < 0.001$; Fig. 5E).

**Fig. 2.** Effect of acute 5-HT deficiency on gasping and autoresuscitation (P8–10). Despite normal gasp latency (A), increased gasp tidal volume (VT; B), and increasing gasp frequency ($f_B$) with successive anoxic episodes (C), the ability of 5-HT-deficient pups to restore HR (D) and eupnea (E) becomes prematurely compromised with successive anoxic episodes. ○, 5-HT-deficient pups; ●, control pups. Values are means ± SE. *$P < 0.05$. **$P < 0.01$.
DISCUSSION

Autoresuscitation involves a combination of behavioral, respiratory (gasing), and cardiovascular (blood pressure and HR) responses that together restore PO2 to preserve life during severely hypoxic conditions. Based on previous studies using rodent models with a severe 5-HT deficiency beginning in utero or just after birth (3, 7), we hypothesized that, in the second postnatal week, an acute loss of 5-HT would compromise the cardiovascular components of autoresuscitation rather than gasing. To test this, we utilized a convenient, pharmacological approach to induce a rapid loss of 5-HT (~60–70%) from the brain stems of P8–13 neonatal rats over ~1 day, testing their ability to autoresuscitate during repeated episodes of anoxia. Our main finding is that an acute 5-HT deficiency compromises MAP pressure during repeated episodes of anoxia. Despite normal gasing, 5-HT-deficient pups experience a progressive, premature deterioration of MAP and HR recovery with successive episodes of anoxia. As a result, 5-HT-deficient pups survive only one-half the number of anoxic episodes as controls.

Recent data suggest that, in normal conditions, central 5-HT signaling serves to protect blood pressure (1). Our data suggest that central 5-HT also preserves MAP during hypoxia-induced primary apnea and aids in its restoration after the transition to gasing. The mechanism(s) for this remains unknown at this stage. The relative hypotension experienced by 5-HT-deficient pups is not the result of a larger bradycardia during severely hypoxic conditions. In any case, bradycardia is predominantly due to direct effects of severe hypoxia on the sinoatrial node and probably not influenced centrally (12).

It is possible that 5-HT deficiency compromises the centrally-mediated increase in tonic and respiro-phasic sympathetic discharge that occurs during severe hypoxia that helps preserve and restore blood pressure (38). As the phenotype of 5-HT-deficient pups emerges only after several episodes, it may be that brain stem (or spinal cord) 5-HT facilitates the longer-term increase in sympathetic discharge occurring with intermittent periods of hypoxia, as has been previously described (8). 5-HT receptors are expressed by premotor, presympathetic neurons in the rostral ventrolateral medulla (22, 34), and specific 5-HT2A receptor agonists typically elicit sympatho-excitation and a pressor response when applied to this area (35). 5-HT can also inhibit the activity of γ-aminobutyric acid within the paraventricular nucleus of the hypothalamus (20), a site whose importance to cardiorespiratory and autonomic regulation in rats seems to increase as hypoxia becomes more severe (16). Notwithstanding the normal drop in HR in response to anoxia, we cannot rule out an effect of 5-HT deficiency on contractility (i.e., stroke volume). However, it has been previously shown that 5-HT, when applied to the rostral ventrolateral medulla, primarily stimulates sympathetic vasomotor neurons to increase peripheral vascular resistance, with no effects on left ventricular contractility (35).

Combined with previous findings, the present data suggest that relative effects of 5-HT deficiency on respiratory and cardiovascular components of autoresuscitation may depend on the timing of the 5-HT deficiency relative to the appearance of anoxic episodes. Previously, we and others showed that a loss of 5-HT beginning in utero (Pet-1−/− and TPH2−/− mice)
leads to dramatic defects in gasping (3, 4, 10). The death of these animals is highly associated with an inability to generate the first gasp, and survival is significantly improved when gasping is hastened pharmacologically (5). In contrast, if 5-HT neurons are lesioned in just after birth, gasping is relatively preserved, and death is more closely associated with a progressive delay in HR recovery with successive anoxic episodes (7). Our present results show that, when a loss of 5-HT content occurs 24 h before the anoxic episodes, the defect(s) in autoresuscitation is solely cardiovascular in nature. Gasping emerges after a normal delay and is at a normal rate (and actually increased volume), yet MAP progressively deteriorates with additional anoxic episodes. The absence of any deleterious effect of acute 5-HT deficiency on gasping agrees with previous studies showing that acute antagonism of 5-HT receptors in situ had little if any consequences on the ability of the intact ponto-medullary respiratory network to generate fictive gasping (43). While speculative, it is likely that the premature, progressive increase in gasp fG displayed by 6-FL-treated, 5-HT-deficient pups over the first few anoxic episodes is due to a more abrupt and severe drop in brain stem PO2 subsequent to a loss of MAP. We and other have shown that the influence of 5-HT on gasping seems to wane with age (3, 4, 41). It may be that an 6-FL-induced loss of 5-HT content would also compromise gasping during the first postnatal week.

Methodological Considerations

We chose systemic application of 6-FL as a way of inducing a rapid loss of 5-HT with minimal stress to the animal. We chose not to apply 6-FL centrally [i.e., via the cisterna magna, a method we have used previously to apply 5,7-DHT to the brain stem (7)]; because 6-FL has a short half-life, this would have required several surgeries over 24 h. Reducing 5-HT content over just 1 day allowed us to circumvent potentially confounding developmental defects that have been described in neonatal mice and rats experiencing a prolonged 5-HT deficiency, including effects on neuronal circuitry (1, 23, 26).

While the failed autoresuscitation of 6-FL-treated pups is likely due to reduced central 5-HT signaling [the phenotype of 6-FL-treated pups is similar to that of rat pups treated centrally with 5,7-DHT (7)], we still do not know the nuclei that depend on 5-HT for the appropriate autonomic response to anoxia. In addition, we cannot rule out the possibility that reduced peripheral 5-HT (likely induced by our treatment) compromises MAP regulation in response to episodic anoxia. 5-HT is produced by a various tissues, including the enterochromaffin cells of the gut (14), which supply the 5-HT originally discovered in the blood (36). Indeed, it has been known for some time that blood 5-HT has vasoconstrictor properties (36), either directly or via 5-HT receptors expressed within sympathetic ganglia or nerves (44). And recently an entire serotonergic system has been
identified in the peripheral arteries (27). We must consider these sites of 5-HT synthesis and activity when interpreting our findings. Regardless, our data may apply to situations where 5-HT content is reduced globally, including reduced intake of dietary tryptophan (31) or increased alcohol intake (32).

**Clinical Implications**

5-HT deficiency is one of several 5-HT system abnormalities that have been identified in the medulla oblongata of SIDS cases. Others include increased numbers of immature serotonergic neurons, reduced 5-HT1A receptor and 5-HT transporter binding (discovered using tissue section autoradiography) (19). While these pathological abnormalities suggest 5-HT system dysfunction, their specific role(s) in the pathogenesis of SIDS has been controversial (21). Failed autoresuscitation has also been documented in SIDS. The failed autoresuscitation we have observed in 5-HT-deficient rodents resembles what has been observed in SIDS cases immediately before death: infants gasp but fail to restore cardiorespiratory function (33, 39). Taken together with previous work, our present results strengthen the case that 5-HT deficiency is an important intrinsic risk factor for SIDS. Even a short-term 5-HT deficiency (1 day) could compromise the ability of an infant to survive a series of severely hypoxic episodes during sleep (e.g., during repeated airway obstruction or while in the prone sleep position) via a loss of blood pressure and eventual circulatory collapse. Conditions or factors that preserve 5-HT content could be considered for strategies aimed at reducing SIDS in at-risk populations.

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### Table 2. Resting variables of Veh and 6-FL-treated postnatal day 11–13 rat pups

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mass, g</th>
<th>fB, breaths/min</th>
<th>HR, beats/min</th>
<th>MAP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>10</td>
<td>30.0 ± 1.3</td>
<td>151 ± 5</td>
<td>466 ± 2</td>
<td>57.0 ± 2.6</td>
</tr>
<tr>
<td>6-FL</td>
<td>11</td>
<td>29.2 ± 1.2</td>
<td>129 ± 6*</td>
<td>474 ± 11</td>
<td>63.7 ± 2.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of pups. MAP, mean arterial pressure. *Significant difference between Veh and 6-FL-treated pups (P < 0.05).
DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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

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