Time- and age-dependent effects of serotonin on gasping and autoresuscitation in neonatal mice

Jianping Chen, Jennifer Magnusson, Gerard Karsenty, and Kevin J. Cummings

Department of Biomedical Sciences, University of Missouri, Columbia, Missouri; and Department of Genetics and Development, Columbia University Medical Center, New York, New York

Submitted 2 January 2013; accepted in final form 2 April 2013

Chen J, Magnusson J, Karsenty G, Cummings KJ. Time- and age-dependent effects of serotonin on gasping and autoresuscitation in neonatal mice. J Appl Physiol 114: 1668–1676, 2013. First published April 4, 2013; doi:10.1152/japplphysiol.00003.2013.—The role of brain stem serotonin (5-hydroxytryptamine, 5-HT) in autoresuscitation in neonatal life is unclear. We hypothesized that a specific loss of 5-HT would compromise gasping and autoresuscitation mainly in the second postnatal week and that acute restoration of 5-HT would reverse the defects. We exposed postnatal day (P)4–5, P8–9, and P11–12 tryptophan-hydroxylase-2 knockout (TPH2−/−) and wild-type littermates (WT) to 10 episodes of anoxia (97% N2, 3% CO2), measuring survival, gasp latency, gasp frequency (fG), and the time required to restore eupnea and heart rate. We also tested P8–9 TPH2−/− mice after restoring 5-HT with a single injection of 5-hydroxytryptophan (5-HTP)-1 h before testing or with multiple injections beginning 24 h before testing. At P4–5 and P8–9, but not at P11–12, gasp latency and the recovery of eupnea were delayed ~2- to 3-fold in TPH2−/− pups compared with WT (P < 0.001). At all ages, TPH2−/− pups displayed reduced gasp fG (~20–30%; P < 0.001) and delayed heart rate recovery (~60%; P = 0.002) compared with WT littersmates. TPH2−/− survival was reduced compared with WT (P < 0.001), especially at P8–9 and P11–12 (P = 0.004). Whereas 1–2 h of 5-HTP treatment improved the gasp latency and fG of P8–9 TPH2−/− pups, improved cardiorespiratory recovery and survival required 24 h of treatment. Our data suggest that 5-HT operates over a long time span (~24 h) to improve survival during episodic severe hypoxia. Early in development (P4–9), 5-HT is critical for both respiratory and cardiovascular components of autoresuscitation; later (P11–12), it is critical mainly for cardiovascular components. Nevertheless, the effect of 5-HT deficiency on survival is most striking from P8 to P12.

Gasing; heart rate; serotonin; development; hypoxia; autoresuscitation; SIDS

SEVERE HYPOXEMIA, as occurring during obstructive apnea or by the continued rebreathing of exhaled gas, elicits cardiorespiratory inhibition characterized by primary apnea and severe bradycardia. Although this inhibition is adaptive in that it conserves energy in times of oxygen deprivation, it must eventually be reversed to sustain life. Reversal is achieved via autoresuscitation, a process beginning with gasps (stage I of autoresuscitation), large-amplitude, low-frequency breaths emerging from primary apnea, to rapidly increase pulmonary gas exchange and sympathetic outflow, both of which are required to restore tissue oxygenation, heart rate, blood pressure (stage II autoresuscitation), and eupnea (stage III autoresuscitation) (15, 18, 19). Compared with adults, gasping and autoresuscitation are probably summoned more often in early infancy, and particularly in those born premature, because immature control systems lead to repetitive episodes of obstructive apnea, bradycardia, and hypoxemia (4, 12). The ability of animals to autoresuscitate wanes with development, reflecting the selective pressure existing in early life to maximize the efficacy of these processes (16). A failure of gasping to restore heart rate during apparent severe hypoxia is evident in some cases of the sudden infant death syndrome (SIDS) (36, 38).

Recent evidence suggests that serotonergic neurons contribute to gasping and autoresuscitation. Mice and rats harboring a genetic [Pet-1 deficient (6, 14, 20)] or pharmacologically induced [5,7-dihydroxytryptamine (5,7-DHT) treated (9)] loss of 5-hydroxytryptamine (5-HT) neurons, respectively, have delayed gasping and recovery of eupnea and heart rate that compromise survival. In Pet-1-deficient mice this phenotype is most evident at the beginning of the second postnatal week (6). Although compelling, these data fail to resolve two important questions: first, are the autoresuscitation defects exhibited by Pet-1−/− and 5,7-DHT-treated rats due specifically to a loss of 5-HT? Pet-1 deficiency leads to an arrested development of serotonergic neurons with vastly reduced innervation of target fields (20). Because substance P, thyrotropin-releasing hormone, and even glutamate are colocalized and released by these terminals (22, 23, 30), the specific role of 5-HT in gasping and autoresuscitation in early life is unclear. Second, what are the temporal dynamics of the action of 5-HT within brain stem regions promoting gasping and autoresuscitation? Given the diversity of serotonergic signaling, 5-HT could act either acutely as a neurotransmitter or over a longer time frame to regulate the expression or activity of other signaling molecules. Understanding how 5-HT promotes gasping and autoresuscitation, including its temporal dynamics, could help efforts to reduce SIDS, because among other defects SIDS is associated with reduced 5-HT content and tryptophan-hydroxylase-2 (TPH2) activity (13).

The purpose of our current study is to examine in more detail the specific role of 5-HT in gasping and autoresuscitation during the first 2 wk of postnatal life. We hypothesized that 1) mice with a specific loss of 5-HT would have defects in gasping and/or the recovery of eupnea and heart rate that would impair their ability to survive episodic anoxia; 2) these defects would be most evident in the second postnatal week; and 3) acute restoration of 5-HT would reverse these defects. To address these hypotheses we utilized mice with a specific loss of 5-HT from the brain stem (TPH2−/−). To delineate the temporal dynamics of 5-HT...
action, we restored the central 5-HT content of TPH2−/− mice using timed injections of the product of TPH2 and 5-HT precursor, 5-hydroxytryptophan (5-HTP), to bypass the genetically induced loss of 5-HT.

MATERIALS AND METHODS

Animals. We tested pups from heterozygous (TPH2+/−) breeders maintained on a mixed C57Bl/6 and 129Sv genetic background. All animals were provided food and water ad libitum and were housed with a 12:12-h light-dark cycle and a room temperature of 21–23°C. We tested a total of 88 pups from 15 breeding pairs. Pups were chosen randomly from each litter, with genotype unknown (experimenter blinded). Data from wild-type (WT: TPH2+/− and TPH2+/+) pups were combined because we found neither a difference in tissue 5-HT content nor any cardiorespiratory variable between these two groups.

Table 1 shows the different groups of animals studied. Uninjected animals were studied at postnatal day (P)4–5, P8–9, or P11–12. Four additional groups of P8–9 animals were used to test the effects of 5-HTP on the autoregulation of TPH2−/− pups. We injected TPH2−/− either once with 40 mg/kg 5-HTP (in saline, 10 μl/kg body wt sc) 1–2 h before testing or four times with 20 mg/kg 5-HTP over the 24 h before testing (~9:00 AM and 2:00 PM each day), including one injection right before testing. To control for handling and vehicle injection, we also injected both WT and TPH2−/− with saline four times starting 24 h before testing. We did not inject WT pups with 5-HTP, because this would produce a group of animals with higher-than-normal levels of 5-HT and its by-products. We chose these doses of 5-HTP on the basis of previous work demonstrating that a single 40 mg/kg injection of 5-HTP can reverse behavioral abnormalities in TPH2−/− mice within 1 h of its administration (26).

Finally, to rule out the possibility that gasping and autoresuscitation defects displayed by TPH2−/− pups were solely the result of reduced feeding and/or stunted growth, we also tested two WT pups that by chance were not feeding properly (see also Discussion, Methodological considerations). All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Missouri (Columbia, MO).

The generation and characteristics of the TPH2−/− mice used in this study have been previously described (40). For genotyping, a tissue sample (either ear or tail) was taken from each pup at the end of each experiment. Genotyping on isolated DNA was performed according to a previous study using primers 5′-ACT GGC TTG TTT GGA TGA GG and 5′-GCC ATT TTG GGC GTT TAA GG to generate a 420-base pair wild-type product and primers 5′-GAG GAC AGA TAA CCC CAA GC and 5′-GGC CAT TAA CTG GGG TAA CG to generate a 367-base pair knockout fragment. PCR for wild-type and knockout fragments were performed separately.

Table 1. Summary of experimental groups and number of animals tested in each

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Uninjected</th>
<th>Vehicle</th>
<th>Injected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT TPH2−/−</td>
<td>TPH2−/−</td>
<td>5-HTP</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>TPH2−/−</td>
<td>TPH2−/−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1 h)</td>
<td>(24 h)</td>
</tr>
<tr>
<td>P4–5</td>
<td>14</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>P8–9</td>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>P11–12</td>
<td>9</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Tryptophan-hydroxylase-2 knockout (TPH2−/−) mice and their wild-type (WT) littermates were either uninjected or injected with vehicle (saline). TPH2−/− mice were also injected once with 5-hydroxytryptophan (5-HTP) 1–2 h before testing [TPH2−/− (1 h)] or 4 times with 5-HTP beginning 24 h before testing [TPH2−/− (24 h)] at postnatal day (P)4–5, P8–9, and P11–12. x, None tested.

Wild-type PCR conditions were 94°C (3 min) (1 cycle), followed by 94°C (30 s), 63°C (45 s), and 72°C (1 min) (29 cycles). Knockout PCR conditions were 94°C (3 min) (1 cycle), followed by 94°C (45 s), 63°C (45 s), and 72°C (1 min) (35 cycles).

Tissue monoamine quantification. High-pressure liquid chromatography (HPLC) was used to determine 5-HT content. After the death of pups during the anoxia trials, whole brains were immediately extracted, and medullas were isolated and stored at −80°C until analysis. Medullas were homogenized using a tissue dismembrator in 100–750 μl of 0.1 M TCA containing 10−2 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. Samples (20 μl) of the supernatant were injected using an autosampler (model 2707; Waters, Milford, MA) onto a 100 × 4.60-mm HPLC column (Phenomenex, Torrance, CA). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1 M TCA, 10−2 M sodium acetate, 10−4 M EDTA, and 10.5% methanol (pH 3.8). Solvent was delivered at 0.6 ml/min using a Waters 515 HPLC pump, HPLC control and data acquisition were managed by Empower software (Waters).

Experimental setup. Measurements were made using a head-out system where respiratory flow is measured via a mask and pneumotach (8). Body temperature, measured with a thermocouple and held at 36 ± 0.5°C in all animals throughout the experiment, was controlled by perfusing a jacketed, glass chamber (~40 ml) with warmed water pumped from a programmable water bath (Fisher Scientific, Pittsburgh, PA). The snout of the animal was sealed away from the body chamber using a polyether material (Impregum F polyether impression material; 3M, St. Paul, MN). A pump (AEI Technologies, Naperville, IL) was used to pull air or anoxic gas (97% N2–3% CO2), at a flow of 75 ml/min, through the pneumotach and mask (volume: 3 ml), a vertical column of Drierite (W. A. Hammond Drierite, Xenia, OH) to remove moisture, and finally through an O2 analyzer (AEI Technologies, Pittsburgh PA) to enable the calculation of metabolic rate (VO2). The mask was open to the atmosphere via the pneumotach, and pressure within the mask, measured with a water column, was negligible. Animals were fitted with a small vest made from tensor bandage, which held in place the ends of two silver wires acting as surface electrodes to detect R waves for the calculation of heart rate. The presence of the vest had no detectable effect on either heart rate or respiratory parameters.

Inspiratory and expiratory airflows were detected by connecting both side-arms of the pneumotachograph to a differential pressure transducer (Validyne Engineering, Northridge, CA). Integration of the flow trace provided respiratory volume. The system was calibrated by injecting 25 and 50 μl of air into the head chamber with a micropipette.

All analog signals were recorded and analyzed in LabChart 7 (ADInstruments, Colorado Springs, CO) using a PowerLab data acquisition system (ADInstruments).

Experimental protocol. Pups were individually removed from the litter and weighed. Pups were then instrumented with ECG leads and a rectal probe and were placed within in the preheated chamber (ambient temperature ~34–35°C). The snout of the animal was then sealed in the mask, and the mask was placed via the surrounding rubber gasket into the anterior portion of chamber. The animal was then allowed to settle for ~10 min until target body temperature was reached (~35.5–36.5°C). We then recorded baseline variables for 5 min, followed by 10 episodes of anoxia (~30 s each, until primary apnea) interspersed with 5 min of room air to allow autoresuscitation via gasping and for cardiorespiratory values to return to normal. Given the small volume of the mask and high flow rate, the time for gas washout within the mask is ~3–4 s, permitting the first gasp to occur in room air following primary
For some TPH2−/− animals, primary apnea lasted >2 min; in these instances we provided animals with up to 10 min of room air for recovery before the next challenge.

Data and statistical analyses. Mean data are expressed ±SE. Values measured were heart rate, tidal volume (Vt), eupneic and gasping frequency (f0), ventilation (Ve; the product of Vt and eupneic f0), coefficient of variation in the respiratory period (CV%), metabolic rate [Vo2 = (0.21 – fractional O2 exhausted from mask) × flow (ml/min/mass [kg])] and the ventilatory equivalent (Ve/Vo2). Heart rate and breathing were determined with LabChart 7 using peak detection on the respiratory and ECG traces. After each challenge we measured the delay to the emergence of gasping after primary apnea (i.e., gasp latency), the instantaneous frequency of the first gasp (gasp f1), the variation of the respiratory period; VeO2, metabolic rate; CV%, coefficient of variation of the respiratory period; f0, eupneic respiratory frequency (breaths/min); CV%, coefficient of variation, baseline heart rate was not different between the genotypes (P = 0.54). Brain stem 5-HT concentration (5-HTC) was significantly reduced in TPH2−/− pups compared with WT (Table 2; P < 0.001). As has been reported in other models of 5-HT deficiency in the neonatal period (2, 10), TPH2−/− pups weighed less than WT (Table 2; P < 0.001).

We show raw records depicting typical respiratory and heart rate responses of un.injected WT and TPH2−/− pups to episodes of anoxia (Fig. 1). After hypoxia-induced apnea and bradycardia, gasping was delayed and the f0 reduced in the TPH2−/− pup compared with its WT littermate (compare Fig. 1, A and B, left; note scale bars on y-axes). In addition, TPH2−/− pups experience a slower recovery of both heart rate and eupnea. WT and TPH2−/− animals eventually succumbed to anoxia after a progressive run-down of gasping, albeit after fewer episodes in TPH2−/− animals than in WT (Fig. 1, A and B, right).

At P4–5 and P8–9, gasp latencies were ~2- to 3-fold longer in TPH2−/− pups compared with WT (Fig. 2A; genotype effect: P < 0.001). By P11–12, the gasp latency of TPH2−/− pups was no longer different from that of WT (age × genotype: P = 0.03). At all ages, the f0 was reduced in TPH2−/− pups, irrespective of age (Fig. 2D; genotype effect: P < 0.002). To address whether the gasping defects of TPH2−/− pups were a function of their small size, we took advantage of two WT pups that by chance were growth restricted, presumably from infrequent feeding or poor milk quality. These pups weighed 2.02 and 2.30 g, respectively (mean weight of P4–5 WT group: 3.7 ± 0.3 g). Although statistical analyses were not possible with only two animals, both gasp latency and eupneic recovery time were close to WT values (Fig. 2E).

Associated with their autoretsuscitation defects was a marked inability of TPH2−/− pups to survive episodic anoxia (Fig. 3). WT pups survived 8, 5.5, and 6.5 episodes at P4–5, P8–9, and P11–12, respectively, whereas TPH2−/− pups survived 4, 2.5, and 2 episodes (Fig. 3, A, C, and E; genotype: P < 0.001). Each of the two growth-restricted WT pups survived 10 episodes (not shown). Although there was no significant effect of

Table 2. Baseline cardiorespiratory variables of TPH2−/− and WT littersmates at P4–5, P8–9, and P11–12

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>n</th>
<th>[5-HT]</th>
<th>Mass</th>
<th>HR</th>
<th>VeB</th>
<th>VeC</th>
<th>Gasp f0</th>
<th>Ve/Vo2</th>
<th>Vo2</th>
<th>Ve/Vo2</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4–5</td>
<td>WT</td>
<td>14</td>
<td>6.9 ± 0.9</td>
<td>3.7 ± 0.3</td>
<td>909 ± 73</td>
<td>4.8 ± 0.6</td>
<td>197 ± 10</td>
<td>36 ± 7</td>
<td>33.2 ± 1.8</td>
<td>28.0 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>TPH2−/−</td>
<td></td>
<td>14</td>
<td>0.4 ± 0.2</td>
<td>2.4 ± 0.1</td>
<td>1,026 ± 38</td>
<td>9.6 ± 0.6</td>
<td>113 ± 6</td>
<td>55 ± 5</td>
<td>29.7 ± 1.7</td>
<td>35.9 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>P8–9</td>
<td>WT</td>
<td>10</td>
<td>6.0 ± 0.2</td>
<td>5.9 ± 0.2</td>
<td>612 ± 19</td>
<td>2.8 ± 0.2</td>
<td>250 ± 10</td>
<td>30 ± 6</td>
<td>23.8 ± 1.2</td>
<td>21.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>TPH2−/−</td>
<td></td>
<td>10</td>
<td>0.5 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>573 ± 20</td>
<td>6.0 ± 0.7</td>
<td>166 ± 11</td>
<td>48 ± 6</td>
<td>26.4 ± 1.9</td>
<td>37.3 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>P11–12</td>
<td>WT</td>
<td>9</td>
<td>8.6 ± 0.5</td>
<td>612 ± 40</td>
<td>678 ± 83</td>
<td>2.8 ± 0.5</td>
<td>259 ± 16</td>
<td>24 ± 5</td>
<td>28.5 ± 1.6</td>
<td>23.5 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>TPH2−/−</td>
<td></td>
<td>10</td>
<td>3.9 ± 0.4</td>
<td>603 ± 21</td>
<td>837 ± 63</td>
<td>4.5 ± 0.4</td>
<td>193 ± 14</td>
<td>26 ± 3</td>
<td>24.6 ± 2.0</td>
<td>36.4 ± 4.4</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE (n = no. of animals) for TPH2−/− and WT littersmates at P4–5, P8–9, and P11–12. [5-HT], tissue serotonin concentration (ng/mg protein); HR, heart rate (beats/min); Ve, ventilation (ml/min × kg−1); Vt, tidal volume (ml/kg); f0, eupneic respiratory frequency (breaths/min); CV%, coefficient of variation, baseline heart rate was not different between the genotypes (P = 0.54). Brain stem 5-HT concentration (5-HTC) was significantly reduced in TPH2−/− pups compared with WT (Table 2; P < 0.001). As has been reported in other models of 5-HT deficiency in the neonatal period (2, 10), TPH2−/− pups weighed less than WT (Table 2; P < 0.001).
Ages on WT pups, the survival of $TPH2^{-/-}$ pups was significantly higher at P4–5 than at older ages (Fig. 3A; post hoc: $TPH2^{-/-}$ at P4–5 vs. P8–9 + P11–12 combined: $P = 0.004$). The survival of both WT and $TPH2^{-/-}$ pups at P4–5 and P8–9 increased with decreasing gasp latency (Fig. 3, B and D). The relationship was strongest at P8–9 (Fig. 3D; WT: $R^2 = 0.53$, $P = 0.01$; $TPH2^{-/-}$: $R^2 = 0.58$, $P = 0.01$) than at P4–5 (Fig. 3B; WT: $R^2 = 0.30$, $P = 0.04$; $TPH2^{-/-}$: $R^2 = 0.28$, $P = 0.02$). No significant relationship existed between these two variables in P11–12 animals (Fig. 3F).

To address the hypothesis that the autoresuscitation defects exhibited by $TPH2^{-/-}$ mice would be reversed with acute restoration of 5-HT, we gave systemic injection(s) of the 5-HT precursor 5-HTP to $TPH2^{-/-}$ pups either 1–2 h [$TPH2^{-/-}$-5-HTP (1 h)] or 24 h [$TPH2^{-/-}$-5-HTP (24 h)] before testing. In separate WT and $TPH2^{-/-}$ pups, we injected vehicle alone (WT-veh and $TPH2^{-/-}$-veh). Medullary [5-HT] was increased in both $TPH2^{-/-}$-5-HTP (1 h) and $TPH2^{-/-}$-5-HTP (24 h) compared with un.injected $TPH2^{-/-}$ pups (Tables 2 and 3; $P < 0.001$ with un injected $TPH2^{-/-}$). $TPH2^{-/-}$ pups injected with 5-HTP had reduced VT and increased $f_0$ compared with vehicle-injected $TPH2^{-/-}$ pups, yet they still hyperventilated relative to vehicle-injected WT pups (Table 3; $P < 0.01$ and $P = 0.02$ for $TPH2^{-/-}$-5-HTP (1 h) and $TPH2^{-/-}$-5-HTP (24 h) compared with $TPH2^{-/-}$-veh, respectively). $TPH2^{-/-}$-5-HTP (24 h) pups were significantly heavier than the $TPH2^{-/-}$-veh pups (Table 3; $P = 0.002$). Baseline variables of WT and $TPH2^{-/-}$ were not influenced by vehicle injection (compare data in Tables 2 and 3).

Representative tracings showing the effects of 5-HTP on autoresuscitation variables are shown in Fig. 4A. As with uninjected pups, when compared with WT-veh pups, $TPH2^{-/-}$-veh pups had markedly prolonged gasp latency (Fig. 4B; $P < 0.001$), slower gasp $f_0$ (Fig. 4C; $P < 0.01$), and a slower recovery of eupnea (Fig. 4D; $P < 0.001$) and heart rate (Fig. 4E; $Q = 3.41$, Kruskal-Wallis on ranks with Dunn’s post hoc). 5-HTP shortened the gasp latency of $TPH2^{-/-}$ pups, whether after 1–2 or 24 h of treatment (Fig. 4B; $P = 0.01$ and $P < 0.001$, respectively, compared with $TPH2^{-/-}$-veh). Compared with that in $TPH2^{-/-}$-veh pups, gasp $f_0$ was significantly elevated in $TPH2^{-/-}$-5-HTP (1 h) vs. $TPH2^{-/-}$-veh (2 h; $P < 0.001$), but not in those treated for 24 h (Fig. 4C; $P = 0.23$). However, the delayed recovery of eupnea observed in $TPH2^{-/-}$ pups was hastened by 24 h of 5-HTP treatment (Fig. 4D; $P = 0.04$ compared with $TPH2^{-/-}$-veh), but not by 1–2 h of 5-HTP treatment ($P = 0.66$). Twenty-four hours of 5-HTP treatment had a marginal effect on the time required for heart rate recovery (Fig. 4E; $TPH2^{-/-}$-5-HTP (24 h) not significantly different from WT or $TPH2^{-/-}$ values).

Similar to that of their uninjected counterparts, the survival of $TPH2^{-/-}$-veh pups was severely compromised compared with WT-veh (Fig. 5; median: 2 episodes survived compared with 10 episodes for WT-veh; $P < 0.001$). The survival of $TPH2^{-/-}$ pups was increased by 24 h of 5-HTP treatment (Fig. 5; median = 4 episodes; $P < 0.00006$ compared with $TPH2^{-/-}$-veh). In contrast, there was no effect of 1–2 h of 5-HTP treatment on the survival of $TPH2^{-/-}$ pups (median = 2 episodes survived for both $TPH2^{-/-}$-5-HTP and $TPH2^{-/-}$-veh). However, unlike $TPH2^{-/-}$-veh pups, none of the $TPH2^{-/-}$ pups treated for 1–2 h survived past the second anoxic episode, resulting in a slight but significant difference in their respective survival curves (Fig. 5; $P = 0.03$; post hoc $TPH2^{-/-}$-5-HTP (1 h) vs. $TPH2^{-/-}$-veh).

**Fig. 1.** Representative cardiorespiratory tracings. Raw data show respiratory volume (Resp. Vol.) and heart rate (HR) responses of a wild-type (WT) and a tryptophan-hydroxylase-2 knockout ($TPH2^{-/-}$) pup. During the first episode of anoxia (left), the primary apnea (solid double-headed arrow) is considerably shorter in the WT (A) compared with its $TPH2^{-/-}$ littermate (B), as is gasp frequency thereafter (gases are marked by asterisks with the gasp period highlighted by a dashed arrow). The recovery of a eupneic breathing pattern (downward arrow) is delayed in the $TPH2^{-/-}$ pup. After 6 (WT) or 3 ($TPH2^{-/-}$) episodes of anoxia (right), both animals succumb via a run-down of gassing that fails to elevate HR. Note that the scale bar in B (left) is 10 s, whereas that in A (left) is 5 s. The beginning of the primary apnea in B is not shown given its long duration. BPM, beats/min; V·s, volt-seconds.
DISCUSSION

We have shown that a specific loss of brain stem 5-HT compromises gasping and autoresuscitation in neonatal mice. Autoresuscitation was compromised in TPH2−/− pups irrespective of their age, albeit through different mechanisms at each age. At P4–5 and P8–9, reduced survival of TPH2−/− pups was associated with a delay in the initiation of gasping, reduced gasp frequency, and delayed recovery of eupnea and heart rate. Despite relatively normal gasping, P11–12 TPH2−/− pups survived 2 fewer episodes than P4–5 TPH2−/− pups. Whereas TPH2−/− gasping was improved by 1–2 h of 5-HTP treatment, it was only when 5-HT content was restored for 24 h that the autoresuscitation and survival of TPH2−/− mice was improved. These results build on those of previous studies using animals deficient in 5-HT neurons and suggest that, in mice, 5-HT makes a significant contribution to gasping and perhaps other cardiovascular or autonomic components of autoresuscitation in the second postnatal week.

Mechanisms of death. There is little doubt that WT and TPH2−/− pups alike are subjected to cumulative effects of severe hypoxia by our experimental paradigm, which uses repetitive exposures to anoxia over a short period of time. Others have also shown that wild-type animals can survive only a limited number of anoxic episodes (7, 16). In some animals there is an eventual disassociation of atrial and ventricular depolarization that prevents heart rate recovery during gasping. In other animals death is preceded not by atrioventricular block but by a “run-down” of gasping that fails to elevate heart rate (16, 17). In our present study, the death of WT and TPH2−/− pups alike was preceded by a run-down of gasping with no sign of tachycardia (Fig. 1). Thus it seems likely that an eventual reduction in cardiac output and/or blood pressure preceded (and caused) the failure of autoresuscitation eventually displayed by both genotypes.

Although both genotypes eventually succumb, we have demonstrated that TPH2−/− pups survive only half the number of anoxic episodes tolerated by WT littermates. Our data suggest that defects within regions of the respiratory network that promote gasping contribute to the death of TPH2−/− pups at P4–5 and P8–9. This is similar to previous observations in Pet-1−/− mice (7, 14). However, gasping defects cannot explain the compromised survival of TPH2−/− pups at P11–12, a phenotype distinct from that described in Pet-1−/− mice (6).
may be that the low survival of older \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash} pups is a function of a reduced ability to lower V\textsubscript{O}2 and forgo processes with high ATP demands (29) or a reduced ability to release adrenal catecholamines (42), that is, if these processes are dependent on 5-HT. Alternatively, \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash} pups may have reduced sympathetic drive during gasping, thereby compromising the increase in cardiac output and blood pressure necessary for recovery from severe hypoxemia. Indeed, a delayed recovery of heart rate persists in \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash} pups at P11–12, and adult \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash} mice are hypotensive during resting conditions (2).

Effects of timed restoration of 5-HT content in \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash} mice. Acute restoration of brain stem 5-HT completely restores the eupneic and gasp \(f_n\) of P8–9 \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash} pups. This finding supports previous work demonstrating an effect of 5-HT on respiratory frequency and/or rhythm generation (21, 34, 37). However, both the initial generation of the gasp and the improvement of cardiorespiratory recovery and survival require 5-HT to be restored for 24 h. This latter finding was somewhat surprising given the acute effects of 5-HT\textsubscript{2A} receptor antagonists on fictive gasping in vitro (39). Our data suggest that within the respiratory network, 5-HT can operate acutely or over a longer time frame to promote respiratory activity. It may be that serotonergic inputs, over time, facilitate gasping and autoresuscitation by inducing the expression or activation of other neuromodulators or their receptors. Glutamate receptors (either NMDA or AMPA) are a possibility.

Table 3. Baseline cardiorespiratory variables of \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash} and WT littersmates injected with vehicle and 5-HTP at P8–9

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>[5-HT]</th>
<th>Mass</th>
<th>HR</th>
<th>(V_e)</th>
<th>(V_t)</th>
<th>(f_n)</th>
<th>CV%</th>
<th>(V_{O2})</th>
<th>(V_{E}/V_{O2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-veh</td>
<td>7</td>
<td></td>
<td>6.1 ± 0.2</td>
<td>602 ± 4</td>
<td>666 ± 61</td>
<td>2.9 ± 0.3</td>
<td>229 ± 12</td>
<td>26 ± 4</td>
<td>28.3 ± 1.4</td>
<td>23.5 ± 2.0</td>
</tr>
<tr>
<td>\textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash}veh</td>
<td>7</td>
<td>2.9* ± 0.2</td>
<td>559 ± 23</td>
<td>960* ± 29</td>
<td>6.9* ± 0.7</td>
<td>145* ± 12</td>
<td>60* ± 12</td>
<td>26.5 ± 1.8</td>
<td>37.6* ± 3.9</td>
<td></td>
</tr>
<tr>
<td>\textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash}5-HTP (1 h)</td>
<td>8</td>
<td>17.6 ± 1.5</td>
<td>3.4* ± 0.2</td>
<td>643* ± 7</td>
<td>856 ± 74</td>
<td>3.3* ± 0.3</td>
<td>260* ± 14</td>
<td>29* ± 7</td>
<td>24.0 ± 1.0</td>
<td>35.3* ± 2.8</td>
</tr>
<tr>
<td>\textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash}5-HTP (24 h)</td>
<td>9</td>
<td>6.0 ± 1.6</td>
<td>3.9*+ ± 0.2</td>
<td>582 ± 16</td>
<td>1056* ± 60</td>
<td>4.8*+ ± 0.4</td>
<td>228*+ ± 8</td>
<td>40 ± 6</td>
<td>31.7* ± 1.1</td>
<td>33.4* ± 1.7</td>
</tr>
</tbody>
</table>

Data are means \(±\) SE (\(n = \) no. of animals) for \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash} pups given 5-HTP 1–2 h [\textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash}5-HTP (1 h)] or 24 h before testing [\textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash}5-HTP (24 h)] as well as vehicle controls (WT-veh and \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash}veh) at P8–9. Variables and units are as defined in Table 2. *\(P < 0.05\), significantly different from WT-veh. †\(P < 0.05\), significantly different from \textit{TPH2}\textsuperscript{\textasciitildedash}/\textasciitildedash}veh.
because the expression of both can be modulated by 5-HT in other contexts (1, 5).

Methodological considerations. The results of our rescue experiments using 5-HTP injections come with caveats. First, there is recent evidence that the development of neuronal networks is altered in $TPH2^{-/-}$ mice (28). This may also involve altered expression of 5-HT receptors; it could be that $TPH2^{-/-}$ mice are sensitized to the effects of 5-HT restoration because of a preexisting upregulation of excitatory 5-HT receptors at postsynaptic sites or downregulation of inhibitory receptors presynaptically. With this in mind, it is overly simplistic to consider that $TPH2^{-/-}$ mice are returned to a “wild-type” state following 5-HTP administration. Furthermore, it is possible that 5-HTP restores gasping and autoresuscitation in $TPH2^{-/-}$ mice via alternative pathways. However, the only other possible molecule upregulated by 5-HTP is melatonin, and to our knowledge, there are no central effects of melatonin on gasping or autonomic responses to hypoxia/anoxia.

As has been noted previously in other neonatal rodents lacking 5-HT (7), $TPH2^{-/-}$ pups hyperventilate relative to their WT littermates because of elevated VT. Because the hyperventilation of $TPH2^{-/-}$ pups was not normalized by 5-HTP, this phenotype may reflect permanent changes within respiratory or metabolic control networks resulting from a deficiency in 5-HT beginning in early embryogenesis (28).
Although hyperventilation and subsequently reduced \( \text{P}CO_2 \) could influence the ability of \( \text{TPH}^2/- \) pups to autoreuscitate, others have demonstrated that the appearance of primary apnea and gasping are mainly dictated by the arterial \( \text{PO}_2 \), with little influence by \( \text{P}CO_2 \) (19). Indeed, notwithstanding hyperventilation and hypopcapnia, \( \text{TPH}^2/- \) pups have relatively normal gasping at P11–12. Nevertheless, these issues highlight the need for animal models in which 5-HT levels can be reduced in a dose- and/or time-dependent manner before testing (e.g., inducible \( \text{TPH}^2/- \) or pharmacological approaches that specifically reduce \( \text{TPH}^2 \) activity); in this way, negative effects of 5-HT deficiency on development would be minimized.

Direct comparisons between \( \text{TPH}^2/- \) pups treated for 1–2 h and those treated for 24 h are also complicated by the fact that 5-HT concentrations are ~3-fold higher in the former compared with the latter group. Supraphysiological concentrations of 5-HT could lead to presynaptic inhibition, the downregulation of 5-HT receptors, or other adaptive responses not present in the other groups. We note, however, that eupnoic \( f_b \) and gasping were normalized in \( \text{TPH}^2/- \) animals having received a single injection, suggesting that the increase in 5-HT is having an excitatory effect on respiratory neurons in this group. Furthermore, although the gasp latency of \( \text{TPH}^2/- \) was not normalized by a single injection of 5-HP, it was reduced compared with that of untreated \( \text{TPH}^2/- \) pups.

\( \text{TPH}^2/- \) mice are clearly growth restricted compared with WT littermates. This has been documented in other \( \text{TPH}^2/- \) mouse lines and has been attributed to reduced levels of circulating IGF-1 (2) and reduced feeding and/or appetite (41). Although we did not measure stomach contents or feeding behavior in our \( \text{TPH}^2/- \) pups, we note that \( \text{TPH}^2/- \) pups receiving 24 h of 5-HP injections gained more weight than the other two \( \text{TPH}^2/- \) groups and, at least anecdotally, had more milk present in their stomachs. Thus we cannot exclude the possibility that the rescue of \( \text{TPH}^2/- \) pups given 24 h of 5-HP injections was due to greater energy intake. Reduced glycogen stores have been shown to be associated with reduced ability to survive episodic anoxia (11). However, we found that gasping and survival were not affected in two underfed, growth-restricted WT pups.

**Summary and clinical implications.** Our data suggest that a specific loss of 5-HT from the brain stem severely compromises the ability of neonatal mice to withstand even two or three episodes of anoxia. Although it remains to be tested, the specific pharmacodynamics of 5-HT receptor activation (i.e., a long time constant) may prevent any deleterious effects on gasping or autoreuscitation caused by a sudden loss of 5-HT or serotonergic neuron function. A relatively long time constant also raises the possibility that 5-HT alters the expression of other neuromodulators or receptors (including 5-HT receptors) that promote autoreuscitation. The reduced heart rate responses of our P11–12 \( \text{TPH}^2/- \) mice, along with previous evidence of autonomic dysfunction in 5-HT-deficient mice and rats (2, 3, 6, 24), raise the possibility that 5-HT deficiency compromises autonomic responses to oxygen deprivation, possibly those responsible for the restoration of blood pressure occurring during the gasping period (18).

Sudden infant death syndrome (SIDS) is the leading cause of death between 1 mo and 1 yr of age (25). Cardiorespiratory recordings obtained from SIDS cases immediately prior to death show failed autoreuscitation where gasping fails to restore heart rate (35, 36, 38). Conditions at the death scene (32) and comprehensive histological evidence (summarized in Ref. 25) also suggest that infants eventually succumbing to SIDS had experienced several episodes of hypoxia before death. Also, many SIDS cases display intrathoracic petechiae at autopsy, suggesting large respiratory efforts and/or extreme transpulmonary pressure gradients that could be associated with obstruction (19). Among other neurotransmitter defects (25), histopathological evidence suggests dysfunction within the serotonergic system of the brain stem (27, 31, 33). This includes reduced 5-HT content and \( \text{TPH}^2 \) activity (13). Our data build on previous work and suggest that 5-HT deficiency may predispose an infant to SIDS through compromised gasping and/or effects in other physiological responses promoting survival during repeated episodes of oxygen deprivation. The serotonergic system may be a fruitful target for approaches aimed at reducing the incidence of SIDS.

**ACKNOWLEDGMENTS**

HPLC determinations were performed by the CMN/KC Neurochemistry Core Lab at Vanderbilt University. The CMN/KC Neurochemistry Core Lab is supported by Vanderbilt Kennedy Center for Research on Human Development, Vanderbilt Conte Center for Neuroscience Research, and The Vanderbilt Center for Molecular Neuroscience.

**GRANTS**

Funding for this study was provided in part from a University of Missouri Research Board Grant and the University of Missouri Richard E. Wallace Incentive Grant (both to K. J. Cummings).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

J.C., J.M., and K.J.C. performed experiments; J.C., J.M., G.K., and K.J.C. approved final version of manuscript; J.M., G.K., and K.J.C. edited and revised 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.
REFERENCES


