Maternal dietary tryptophan deficiency alters cardiorespiratory control in rat pups

Eliana M. Penatti,1 Alexis E. Barina,1 Sharat Raju,1 Aihua Li,1 Hannah C. Kinney,2 Kathryn G. Commons,3 and Eugene E. Nattie1
1Department of Physiology, Dartmouth Medical School, Lebanon, New Hampshire; and Departments of 2Pathology and
3Anesthesia, Children’s Hospital Boston and Harvard Medical School, Boston, Massachusetts

Submitted 12 July 2010; accepted in final form 18 October 2010

Penatti EM, Barina AE, Raju S, Li A, Kinney HC, Commons KG, Nattie EE. Maternal dietary tryptophan deficiency alters cardiorespiratory control in rat pups. J Appl Physiol 110: 318–328, 2011. First published October 21, 2010; doi:10.1152/japplphysiol.00788.2010.—Malnutrition during pregnancy adversely affects postnatal forebrain development; its effect upon brain stem development is less certain. To evaluate the role of tryptophan [critical for serotonin (5-HT) synthesis] on brain stem 5-HT and the development of cardiorespiratory function, we fed dams a diet ~45% deficient in tryptophan during gestation and early postnatal life and studied cardiorespiratory variables in the developing pups. Deficient pups were of normal weight at postnatal day (P)5 but weighed less than control pups at P15 and P25 (P < 0.001) and had lower body temperatures at P15 (P < 0.001) and P25 (P < 0.05; females only). Oxygen consumption (V̇O2) was unaffected. At P15, deficient pups had an altered breathing pattern and slower heart rates. At P25, they had significantly lower ventilation (V̇e) and V̇e-V̇O2 ratios in both air and 7% CO2. The ventilatory response to CO2 (% increase in V̇e/V̇O2) was significantly increased at P5 (males) and reduced at P15 and P25 (males and females). Deficient pups had 41–56% less medullary 5-HT (P < 0.01) compared with control pups, without a difference in 5-HT neuronal number. These data indicate important interactions between nutrition, brain stem physiology, and age that are potentially relevant to understanding 5-HT deficiency in the sudden infant death syndrome.

chemoreception; carbon dioxide; control of breathing; serotonin; sudden infant death syndrome

MALNUTRITION DURING PREGNANCY and early postnatal life is well recognized to seriously impair forebrain development in the offspring (13, 17, 18, 31, 33). Studies in humans and animal models indicate widespread deficits in neuronal proliferation, differentiation, survival, synaptogenesis, and myelination in the forebrain (16–18, 33, 48). Yet the effects of early malnutrition upon the development of the brain stem and homeostatic functions mediated by it are poorly understood and incompletely characterized (43). Of special interest is the relationship of dietary deficiencies during gestation to the sudden infant death syndrome (SIDS), given that the underlying pathophysiological process of SIDS is thought to originate during gestation (24–26) and SIDS predominantly affects infants living in poverty, in whom poor nutrition during pregnancy is known to be a major problem (1, 25, 30).

SIDS is the sudden death of an infant at ≥12 postnatal months that remains unexplained by a complete autopsy, death scene investigation, and review of the clinical history (49). Typically, a seemingly healthy infant is found dead after a sleep period, with the unwitnessed death occurring during sleep itself or during one of the many arousals that occur throughout the night (25, 26). Studies of cardiorespiratory variables in infants who subsequently die of SIDS demonstrate multiple abnormalities in homeostasis, including episodic apnea and bradycardia (22, 23, 32, 41, 47), and impaired gasping (41). Recently, we reported (12) that SIDS is associated with a deficiency of serotonin (5-HT) in regions of the medulla oblongata that are comprised of 5-HT cell bodies and their projection sites and are critical for respiratory and autonomic control. The 5-HT deficiency in the SIDS cases is characterized by a 26% decrease in levels of 5-HT measured by high-performance liquid chromatography (HPLC) and a 22% decrease in levels of tryptophan hydroxylase (TPH), the key biosynthetic enzyme for 5-HT, measured by Western blot (12). This partial deficiency appears to be associated with a constellation of secondary abnormalities in 5-HT receptor and transporter binding and 5-HT cell density and maturation that have likewise been reported in the medulla in SIDS infants in several independent data sets (12, 24, 29, 38, 39).

The possibility that SIDS is a disorder produced by medullary 5-HT deficiency led us to ask in an animal model whether a 5-HT-related dietary deficiency in gestation and early postnatal life can cause a deficiency of medullary 5-HT as well as associated abnormalities in homeostatic control. 5-HT is synthesized in the brain from tryptophan, an essential amino acid, with TPH as the rate-limiting enzyme (14, 15). Systemic tryptophan is taken up at the blood-brain barrier by neutral amino acid transporters (14, 46), and medullary 5-HT levels are known to be quite labile and dependent on tryptophan intake (14, 15). In the present study, we tested the hypothesis that maternal tryptophan deficiency produced by a diet deficient in tryptophan fed to rat dams just before and during pregnancy and extending into early postnatal life [to postnatal day (P)25] results in medullary 5-HT deficiency in the pups and is associated with alterations in cardiorespiratory regulation in early postnatal life. We focused on analysis of ventilation, metabolic rate, and heart rate (HR) in air and in 7% carbon dioxide (CO2) in male and female pups at P5, P15, and P25, i.e., the time frame possibly homologous to SIDS risk in the human infant (see DISCUSSION). We also measured the levels of 5-HT with HPLC, the number of medullary 5-HT neurons, and the intensity of 5-HT and TPH immunostaining/5-HT neuron at P25, i.e., the end point of the delivery of the tryptophan-deficient diet. The cellular analysis was performed in the same pups that underwent physiological assessment, thereby allowing for the potential demonstration of homeostatic deficits in pups with a documented medullary 5-HT deficiency.
MATERIALS AND METHODS

All experimental protocols were within the guidelines of the National Institutes of Health for animal use and care and were approved by the Dartmouth College Institutional Animal Use and Care Committee.

Experimental groups and diets. Sprague-Dawley adult female rats were fed isocaloric, normal-protein, amino acid-based diets in which tryptophan was normal or reduced to values ~55% of required (Harlan Teklad). The TD.99366 control diet was composed (g/kg) of the following ingredients: 3.5 L-alanine, 12.1 L-arginine HCl, 6.0 L-asparagine, 3.5 L-aspartic acid, 3.5 L-cystine, 40.0 L-glutamic acid, 23.3 glycine, 4.5 L-histidine HCl, monohydrate, 8.2 L-isoleucine, 11.1 L-leucine, 18.0 L-lysine HCl, 8.2 L-methionine, 7.5 L-phenylalanine, 3.5 L-proline, 3.5 L-serine, 8.2 L-threonine, 1.8 L-tryptophan, 5.0 L-tyrosine, 8.2 L-valine, 351.68 sucrose, 150.0 corn starch, 150.0 maltodextrin, 80.0 soybean oil, 30.0 cellulose, 35.0 mineral mix, 8.2 calcium phosphate, monobasic, monohydrate, 13.0 vitamin mix, 2.5 choline bitartrate, and 0.02 tertiary butylhydroquinone, an antioxidant. The TD.08125 tryptophan-deficient diet was equivalent to the TD.99366 diet except that it was reduced in tryptophan to 1 g/kg (~55% of the estimated nutritional requirement). To keep an isonitrogenous/isocaloric modification of TD.99366, L-glutamic acid was increased to 41.18 g/kg. The animals were acclimated with the new food for at least 2 wk before mating and continued receiving the special diet throughout the pregnancy up to P25. There were four groups of litters: two from dams who received the amino acid control diet (control) and two from dams who received the tryptophan-restricted diet (tryptophan deficient). One set of diet and control litters was used for physiology at different ages: P5, P15, and P25. After evaluation at P25 the brains were used for immunohistochemistry. Separate groups of control and diet-treated litters were used for medullary tissue analysis of monoamine levels at P25. These were controls for another experiment in the laboratory at that time, and the pregnant dams had subcutaneous saline-releasing minipumps implanted at 4–5 days of gestational age.

The dams and pups were kept in the Animal Research Facility at Dartmouth in controlled temperature and light conditions with lights on from 7 AM to 7 PM and lights off from 7 PM to 7 AM. All experiments were performed between 9 AM and 5 PM. Food and water were available ad libitum to the dam. The rat pups were separated from the dam for the physiology experiments and were immediately brought back to the litter upon their completion, usually within 20 min. A total of 36 pups from both sexes were studied physiologically from two litters under the control diet (8 males and 10 females) and three litters under the tryptophan-deficient diet (9 males and 9 females).

Head-out and whole body plethysmography under normoxia and hypercapnia. Physiological studies were conducted by using either a head-out (7, 8, 35) or a whole body (4, 28, 40) plethysmograph to measure ventilation (Vt), tidal volume (VT), respiratory frequency (f), and oxygen consumption (VO2). The rectal temperature was measured before and after each experiment with a small thermistor probe. The volume of the head-out plethysmograph used to measure ventilation in P5 rat pups was ~39 ml (2.5-cm diameter, 8-cm-long cylinder). For the P15 and P25 pups, we measured ventilation with a whole body plethysmograph with a volume of ~685 ml (7.5-cm diameter, 14.5-cm-long cylinder) connected to a reference chamber of the same size. The inflow gas for the plethysmograph was humidified and matched to the outflow connected to a vacuum system. The flow rate was ~1 l/min for the whole body plethysmograph; ~100 ml/min of outflow gas served O2 and CO2 analyzers (S3-A from Applied Electrochemistry and Capstar-100 from CWE, respectively). In the head-out box, the chamber with the pup’s head was not sealed and was constantly flushed by either room air or the 7% CO2 challenge gas at a flow rate of ~0.5 l/min. A port very close to the pup’s head was connected to the O2 and CO2 analyzers, which, because of the flow rate of the gas analyzer sampling, obtained expired gas from the pup’s nose. We measured chamber pressure by transducer and calibrated the plethysmograph with 0.1-ml injections for the head-out plethysmograph and with 0.2-ml injections for the whole body plethysmograph. The chamber temperature for both the head-out and whole body plethysmographs was measured by a thermomter continuously, and it was kept at ~30°C by heat lamp. The measurement of breathing for the whole body plethysmograph during air or 7% CO2 was recorded for ~3 min.

The same pups were studied at all three ages. HR and Vt were measured during room air and hypercapnia (7% CO2 challenge) periods. The measurement of HR was performed with a telemetric device (CTA-F40 from Data Systems International) with two ECG leads that were placed on the pup’s chest skin and kept in place with an elastic bandage. After the ECG leads were placed the pup was placed inside the box, and measurements of breathing and HR were recorded when the animal was settled and calm. For the head-out box, VT was directly proportional to the pressure deflection caused by the pup’s thorax movements, and it was calculated based on the calibration of the box with a known volume of air. For the whole body box, VT was calculated with the plethysmograph temperature at that time and the pup’s averaged temperature from measurements before it was placed in the box and after it was removed from the box. Breathing frequency (f) was counted and used to estimate Vt per breath.

Medullary tissue 5-HT and metabolite levels. For analysis of medullary tissue 5-HT, as well as noradrenaline, epinephrine, 3,4-dihydroxyphenylacetic acid (DOPAC, a dopamine metabolite), dopamine, 5-hydroxyindoleacetic acid (5-HIAA, a 5-HT metabolite), and homovanillic acid (HVA, a catecholamine metabolite), pups were killed by pentobarbital overdose and the medullae were removed and frozen en bloc. Ten control (5 male, 5 female) and 10 diet-exposed (5 male, 5 female) P26 pups were used for this analysis. In an effort to measure 5-HT in regions heavily concentrated with 5-HT neurons, we dissected a triangular wedge of tissue from the midline with the base at the ventral medullary surface. This tissue wedge included the majority of medullary 5-HT neurons, which are heavily concentrated in the raphe obscurus, raphe magnus (RM), and raphe pallidus. The tissue was shipped in a frozen state and with medullary levels of 5-HT, and its metabolites were measured by HPLC at the Neurochemistry Core, Vanderbilt University School of Medicine (Nashville, TN) according to published protocols (https://medschool.mc.vanderbilt.edu/cmn/node/12).

TPH and 5-HT immunohistochemistry and data analysis. On completion of the physiological experiments, the pups were deeply anesthetized with ketamine and xylazine (100 mg/kg and 15 mg/kg, respectively) for perfusion fixation of the brain. After perfusion of 4% paraformaldehyde via the right heart ventricle, brains were removed and equilibrated in 30% sucrose for at least 24 h before being frozen on crushed dry ice. Brains were sectioned frozen at 40 μm thick with a rotary microtome with a chuck cooled with a Peltier device. Sections were collected in 0.1 M phosphate-buffered saline (PBS) and processed floating. For immunohistochemistry, primary antisera were diluted together in buffer containing 0.1% bovine serum albumin (BSA), 0.3% Triton X-100, and PBS. Primary antisera included anti-TPH raised in mouse, which recognizes both isofoms of TPH, i.e., TPH1 and TPH2 (Chemicon/Millipore, Billerica, MA; 1:1,000), and anti-5-HT raised in rabbit (Chemicon/Millipore; 1:10,000). Tissue sections were incubated with both of these primary antisera together for 24 h at room temperature or for 2–3 days in the cold room (4°C) with mild agitation. After rinsing several times with PBS, the tissue was incubated with fluorophore-conjugated secondary antisera diluted to 1:100 in 0.1% BSA, 0.3% Triton X-100, and PBS. Secondary antisera included Alexa 488-conjugated donkey anti-sheep and Alexa 647-conjugated donkey anti-rabbit (Invitrogen, Carlsbad, CA). After rinsing, immunolabeled tissue sections were mounted on glass slides, dried, and coverslipped with mounting medium containing 90% glycerol and 10% PBS pH 8.5–9.
We first tested the hypothesis that TPH and 5-HT immunostaining is decreased in the RM in the tryptophan-deficient pups compared with age-matched control pups. We used 18 control (8 male, 10 female) and 18 diet-exposed (9 male, 9 female) P26 pups for this analysis. The tissue sections from control and tryptophan-deficient pups were processed in parallel as matched pairs or matched quadruplets with aliquots of the same reagents. The tissue sections dually immunolabeled for both TPH and 5-HT were photographed with conventional fluorescence illumination and a ×10 objective. For each immunohistochemical run, tissue was photographed at the same time with the same illumination and exposure. For each rat, every fourth section was processed. Of these, at least three sections containing RM were photographed. In each photograph of the RM four to seven cells were randomly selected. For each of these cells a small circle, ~10 μm² in area, was placed over the soma in a location that did not overlap the nucleus. Within this circle, the intensity of TPH and 5-HT immunolabeling was measured with Slidebook image acquisition software. In addition, areas of the tissue section where there was little detectable TPH or 5-HT immunolabeling were sampled to determine background fluorescence intensities. For these sampled areas, the mean intensity of each fluorescent channel was determined. Background fluorescence intensity values were subtracted from somatic intensity values for each cell for both Alexa 488 and Alexa 647. The intensity of somatic staining per cell was then averaged for each section of RM sampled. Subsequently, the intensity of each section was averaged together, yielding an individual animal average. Individual averages of immunolabeling intensity were then normalized to their pair-matched control(s) sampled identically, and the average percent deviation from control values was determined.

We next tested the hypothesis that the number of 5-HT neurons, identified by TPH immunostaining, is decreased in the RM in the tryptophan-deficient pups compared with age-matched control pups. To quantify the number of cells in the RM, the same sections were analyzed, and the average number of cells per section was determined for each rat. For this analysis, the same photographs of RM were analyzed by visualizing TPH immunolabeling. All immunolabeled cells that were dispersed in a triangular pattern above the pyramids were counted from each section. Individual averages were used to calculate a group average.

Statistical analysis. An unpaired t-test was used to analyze the data, comparing VE, V̇t, respiratory rate, V̇O₂, HR, brain tissue amine levels, and number of TPH-immunolabeled cells between control and tryptophan-deficient diet groups in males and in females at each postnatal age. Intensity of immunolabeling between groups was compared with a pair-matched t-test. For the CO₂ response data (% increase in V̇E/V̇O₂; see Fig. 5) a two-way repeated-measures ANOVA was applied with age and diet as factors and a Bonferroni post hoc test.

RESULTS

Effect of tryptophan-deficient diet on body weight, body temperature, and oxygen consumption. The body weights of rat pups from dams fed the tryptophan-deficient diet were the same as those of pups from dams fed the control diet at P5 but were significantly less at P15 and P25 in both males and females (P < 0.001; Fig. 1, top). Basal body temperature was...
significantly lower in the tryptophan-deficient pups (Fig. 1, middle) at P15 in males and females ($P < 0.001$) and at P25 in females ($P < 0.05$). $V_O_2$ was not significantly different between the two groups at any of the three ages studied (Fig. 1, bottom).

**Effects of tryptophan-deficient diet on ventilation and heart rate while breathing air or 7% CO$_2$ at P5.** Exposure to the tryptophan-deficient diet had no significant effects on $V_E$, the ratio of $V_E$ to $V_O_2$, $V_T$, $f$, or HR while breathing air or 7% CO$_2$ in male or female pups at P5 (Fig. 2).

**Effects of tryptophan-deficient diet on ventilation and heart rate while breathing air or 7% CO$_2$ at P15.** At P15, tryptophan-deficient diet-exposed pups had increased $V_E$ breathing air (males, $P < 0.01$; females, $P < 0.01$) and 7% CO$_2$ (females, $P < 0.05$) compared with age-matched control pups (Fig. 3). This reflected a combination of a greater $V_T$ breathing air (males, $P < 0.01$; females, $P < 0.05$) and 7% CO$_2$ (males and females, $P < 0.01$) together with a tendency for a slower $f$, which reached significance during the breathing of 7% CO$_2$ (males, $P < 0.01$; females, $P < 0.01$). Ventilation normalized.
to metabolic rate ($V_{E}/V_{O_2}$) was significantly increased during air breathing in diet-exposed male pups ($P < 0.03$), an effect that was of borderline significance in females ($P < 0.07$). There was no significant effect during 7% CO2 exposure. HR was significantly lower at P15 while breathing air in males ($P < 0.01$) and females ($P < 0.05$).

**Effects of tryptophan-deficient diet on ventilation and heart rate while breathing air or 7% CO2 at P25.** At P25, diet-exposed pups had significantly decreased $V_{E}$ breathing air (male, $P < 0.05$; female, $P < 0.01$) and 7% CO2 (male, $P < 0.001$; female, $P < 0.01$) (Fig. 4). This lower $V_{E}$ reflected a combination of a smaller $V_{T}$ breathing air (males, $P < 0.05$; females, $P < 0.05$) and 7% CO2 (males, $P < 0.001$; females, $P < 0.01$) together with a slower f, which reached significance during the breathing of air in females ($P < 0.01$) and 7% CO2 in males ($P < 0.05$) and females ($P < 0.01$). When ventilation was normalized to each pup’s metabolic rate, $V_{E}/V_{O_2}$ was also significantly lower in the diet-exposed pups breathing air (males and females, $P < 0.001$) and 7% CO2 (males and females, $P < 0.01$). At P25 there was no difference in HR between treated and control pups.

**Effects of tryptophan-deficient diet on CO2 response at P5, P15, and P25.** In the control and diet-exposed pups, there was a significant effect on the CO2 response (represented as % increase in $V_{E}/V_{O_2}$) of both age and diet in both sexes ($P < 0.001$; 2-way repeated-measures ANOVA with age and diet as factors) and there was a significant interactive effect in males ($P < 0.001$) and a borderline significant interactive effect in females ($P < 0.06$) (Fig. 5). Post hoc testing (Bonferroni) indicated significant effects in males at P5 (increased response: $P < 0.001$) and in both sexes at P15 (decreased response: males, $P < 0.001$; females, $P < 0.03$).
and P25 (decreased response: males, \( P < 0.001 \); females, \( P < 0.01 \)).

Effect of tryptophan-deficient diet on 5-HT and 5-HIAA content in medulla. Analysis by HPLC of medullary tissue from wedge-shaped samples of the medullary raphé of P25 pups showed significantly decreased levels of 5-HT and its metabolite, 5-HIAA, in diet-exposed males (\( P < 0.01 \) and \( P < 0.001 \), respectively) and females (\( P < 0.001 \) and \( P < 0.001 \), respectively) compared with control pups (Fig. 6). The average decrease in 5-HT was 41% in males and 56% in females; that of 5-HIAA was 56% in males and 74% in females. There were no significant differences in norepinephrine, epinephrine, DOPAC, dopamine, or HVA.

Effect of tryptophan-deficient diet on tryptophan hydroxylase, 5-HT content, and number of 5-HT neurons in raphé magnus. Medullary tissue sections from RM of P25 diet-exposed and control pups were analyzed for the intensity of immunolabeling for TPH and 5-HT per cell (Fig. 7). For this, the intensity of immunolabeling was measured within individual cell soma, but not in axon terminal fields where additional tissue 5-HT is located. In addition, we counted the number of TPH-labeled cells per section within RM. The intensity of

---

Fig. 4. \( V_{E} \), \( V_{E}/V_{O_{2}} \), \( V_{T} \), RR, and HR at postnatal age P25 in rat pups from dams fed a normal diet (dark bars) or a tryptophan-deficient diet (light bars). Significant differences between control and tryptophan-deficient diet treatment (\( t \)-test): * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \); \( n \) in each case is as in Fig. 1.
somatic TPH immunolabeling per cell in diet-exposed pups was the same as in control pups for both males and females (Fig. 7). For males, the intensity of 5-HT immunolabeling measured in the soma was decreased 30% or more in eight of nine diet-exposed pups compared with their controls \((P < 0.05)\). The interactive effect \((age \times diet)\) was significant in males \((P < 0.001)\) and of borderline significance in females \((P < 0.06)\). Post hoc testing (Bonferroni) showed significance in males at P5, P15, and P25 \((P < 0.01)\) and in females at P15 \((P < 0.03)\) and P25 \((P < 0.01)\).

**DISCUSSION**

While the detrimental consequences on cognitive development due to malnutrition in early life are well recognized, little is known of the effects of malnutrition upon homeostatic control regulated by the brain stem. Of special interest are the effects of deficiencies in nutrients that are critical to the synthesis of neurotransmitters involved in brain stem control of homeostasis, such as the amino acid tryptophan required for the synthesis of 5-HT. 5-HT is used by groups of medullary neurons that influence respiratory patterning, gasping, thermal stability, chemosensitivity, HR, and blood pressure (25). In this study, we framed the issue of the effects of nutritional deficiency upon homeostatic control in early life in the context of SIDS, given that SIDS is associated with medullary 5-HT deficiency, likely originates in gestation, and is related to poverty (12, 24–26, 29, 30, 38, 39). Our major findings are that 1) dietary deficiency of tryptophan during gestation and early postnatal life \((PO–P25)\) results in decreased tissue levels of 5-HT measured at P25 in the midline medulla, the key site of the medullary 5-HT system and a region relevant to homeostatic control; 2) decreased medullary 5-HT levels in tryptophan-deficient pups are associated with homeostatic deficits, confirming that optimal homeostasis depends on proper diet; and 3) several homeostatic deficits are age specific, suggesting that different vulnerable periods may exist for different functions. Indeed, these key findings related to diet and brain stem neurochemistry and the interaction of diet with vulnerable periods in the regulation of homeostasis are relevant to the issue of malnutrition and homeostasis in early life beyond the SIDS problem.

**Technical limitations of physiological studies.** The major technical limitation in physiological studies of developing rodents involves the measurement of \(V_t\). We employed a head-out plethysmograph with a neck seal for the P5 pups, the
This technique accurately and reliably measures \( \Delta V_t \) by the pressure changes created in the closed body chamber by lung expansion and contraction (7, 8, 35). As the pups grow and become more active, however, they are more difficult to study in the head-out chamber, and we then employ a whole body plethysmograph in which we place the entire pup at P15 and P25. This technique measures \( \Delta V_t \) via the gas laws applied to the change in chamber pressure that occurs as the inspired gas within the sealed chamber is warmed and further wetted when the inspirate moves from the cooler ambient temperature to the warmer pup temperature (4). However, in smaller animals (all ages studied here) with rapid breathing frequencies, the change in chamber pressure can also reflect a second physical event, the rarefaction and compression of the gas as it flows through the tiny airways (35, 36). Thus, at P15, and less likely at P25, the “tidal volume” that we estimate can be in part due to a pressure change related to airway caliber and resistance (35, 36). This limitation must be put into the context of the value of obtaining ventilatory data from relatively unstressed, unanesthetized pups.

The ideal experimental design in studies using rodent neonates would be to assign one pup from each litter to an experimental group. To minimize costs, both in terms of financial and animal welfare issues, we have chosen to accept the limitation of using all pups from each litter. An additional limitation of our study design is the necessary stress of the brief maternal separation along with handling of the pup. This may contribute to the slightly higher \( \dot{V}O_2 \) and \( Ve/V\dot{O}_2 \) observed in our results compared with previously published data (45).

**Tryptophan depletion-induced effects on pup medullary 5-HT levels.** Tryptophan is critical for the generation of several compounds in the brain, including 5-HT, products of the kynurenine pathway, and the pineal hormone melatonin (44).
In this study, we focused on the consequences of tryptophan depletion for 5-HT synthesis and function, while recognizing that deficiencies in related compounds may contribute to the brain stem-mediated outcomes. We ascertained the effect of the maternal dietary tryptophan deficiency on the pups by measuring the level of medullary raphé 5-HT from tissue samples obtained at P25. The 5-HT levels were reduced by 41% and 56% in males and females, respectively. The major 5-HT metabolite, 5-HIAA, was similarly reduced, indicating that the decrease in the 5-HT levels was due to decreased synthesis rather than rapid degradation and turnover. The dietary effect of tryptophan depletion was specific to 5-HT and did not affect other biogenic amines in the medullary tissue samples. Maternal tryptophan depletion did not change the number of medullary 5-HT neurons, as measured by cell counts of TPH-immunoreactive cells, nor did it affect the amount (intensity) of TPH immunolabeling per cell. Yet the amount of 5-HT immunolabeling per cell was decreased in the tryptophan-depleted pups, most consistently in males. These observations are consistent with a previous study reporting no change in TPH cell number in the RM in dietary-depleted pups at the same age (37). Gonzalez et al. (16) reported, however, that a tryptophan-depletion diet results in a decrease in 5-HT cell number. Our results could explain this finding, since these investigators counted cells with 5-HT immunolabeling (16) and decreased immunolabeling of 5-HT per cell as we found may cause some cells to fall below the level of detection, resulting in a lower cell count. In our study, the decreased 5-HT labeling per cell body provides additional support for the HPLC data showing decreases in 5-HT and its metabolites in total tissue. Interestingly, the effect on 5-HT immunolabeling was not as consistent in females as it was in males, suggesting that males and females may adapt to tryptophan depletion differently. The reduction in the medullary 5-HT levels in the pups was only partial as expected because the maternal dietary depletion was also partial. When we applied a diet with 100% depletion of tryptophan, the dams lost weight quickly and did not conceive. The partial 5-HT reduction observed in our study stands in contrast to the genetically engineered LmxB conditional knockout mice with a complete reduction in brain stem 5-HT neurons (19, 20). Given that the depletion of medullary 5-HT levels is partial in SIDS infants (12), the dietary-depleted pups appear to represent a closer animal model of the 5-HT defect in SIDS infants.

Tryptophan depletion-induced effects on pup growth. Pups from dams fed the tryptophan-deficient diet had body weights similar to control pups at P5 but had significantly lower body weights at P15 (−30%) and P25 (−40%) in both sexes. Other rodent models with reduced brain 5-HT levels also exhibit growth retardation during the postnatal period. LmxB conditional knockout mice with complete absence of 5-HT neurons exhibit growth retardation from −P4 until −P55. At −P20 these mice have −40% lower body weights, an effect that begins to lessen by P25. These mice have severely abnormal breathing and excess mortality in early postnatal life (20). Transgenic mice lacking TPH2, the brain-specific form of TPH, have almost absent brain tissue 5-HT levels, a 50% mortality in the first 4 wk of life, and growth retardation that begins at −P6 and ends at −P64, i.e., after weaning they “catch up” (2). At −P25, these mice have −50% lower body weight than control pups (2). Thus normal amounts of brain 5-HT are necessary for optimal growth during early postnatal life but not during gestation or after a certain postnatal age; with severe or near-total absence of 5-HT there is early life mortality and growth retardation (2, 20). In our tryptophan-depletion diet model, we see no early life mortality but do detect growth retardation. The mechanism for this growth retardation is unknown. Mice lacking TPH2 drink milk and have normal digestion, are able to compete with littermates for milk, and can vocalize normally to alert mothers. They have, however, a deficiency in serum levels of insulin-like growth factor (IGF) until −2 mo of age (2). It is not clear how the 5-HT deficiency is linked to IGF.

Age-specific respiratory and/or autonomic effects in tryptophan-depleted rat pups. In this study, the maternal dietary depletion of tryptophan extended from conception through pregnancy into the postnatal period to P25. Nevertheless, the effects were characterized by different patterns at specific ages, that is, the effects were neither uniform nor present at all the postnatal ages analyzed. Despite maternal pre- and postnatal tryptophan deficiency, there were no respiratory, HR, or temperature effects at P5. There was, however, in males a small but significant increase in the CO2 response measured as percent increase in Ve/Vo2. At P15, a distinct complex of abnormalities was detected, including 1) decreased body temperature; 2) decreased HR at rest while breathing air; 3) larger Vt and slower breathing frequencies while breathing air or 7% CO2; 4) greater Ve values in air for males and in air and 7% CO2 for females; and 5) a decreased CO2 response expressed as percent increase in Ve/Vo2 in both sexes. The level of Ve relative to the metabolic rate while breathing air was unaffected. At P25 a different constellation of physiological abnormalities was detected, with 1) decreased body temperature in females; 2) no changes in HR; 3) decreased baseline Ve breathing room air in males and in females; 4) −40% decreased Ve/Vo2 while breathing air in males and females, indicating hypoventilation; 5) 40–50% decreased absolute value of Ve and absolute value of Ve/Vo2 while breathing 7% CO2 in males and females; and 6) −17% decreased CO2 response expressed as percent increase in Ve/Vo2 in males and females due to decreases in Vt and f. Thus the pups at P25 have a decreased drive to breathe in air and a lessened response to CO2.

The development of the brain occurs in successive stages, with different structures and functions maturing in orderly and predictable sequences according to different rates and timing. Human and animal studies indicate that malnutrition imposed at a critical period of brain development is likely to cause permanent impairments in brain structure and function. Dobbing and Sands (10, 11) have emphasized that in humans this critical period extends from the later half of pregnancy to about 2 postnatal years, i.e., the age range in which malnutrition is most common worldwide. Although the precise developmental sequences of the respiratory, HR, CO2 response, and temperature parameters mediated by the medullary 5-HT system in rat pups is unknown, our control data indicate dramatic changes in them between P5, P15, and P25. The absence of an effect of tryptophan depletion (environmental factor) at P5 suggests that the medullary 5-HT system is not a prerequisite for homeostatic function at this age. The demonstration of different profiles of physiological abnormalities at P15 and P25 suggests that the medullary 5-HT system is at different stages of
development, the requirement for 5-HT varies according to the stage, and the effect of the deficiency of tryptophan is consequently different. The observation that HR is affected at P15 but not P5 or P25, for example, suggests that the influence of 5-HT on cardiac regulation is most pronounced at this age and P15 is the vulnerable period for 5-HT deficiency upon cardiac patterning. Rats with 5-HT lesions induced at P2 demonstrate age-dependent changes in HR and spontaneous bradycardia (7), as do pet-1 null transgenic mice with reduced numbers of medullary 5-HT neurons (8). The observation that an abnormality in CO2 sensitivity is present at P15 and P25 is consistent with cell culture data showing that 5-HT neurons become CO2 sensitive at P12 or later and that the function of 5-HT neurons in central chemoreception becomes more manifest at ~P12 (6).

The question arises of what age in the rodent is homologous to the peak window of risk for SIDS in the human infant, i.e., 2–4 postnatal months, and whether the vulnerable period of P15–P25 to tryptophan deficiency in rat pups corresponds to the human vulnerable period of SIDS. We cannot pinpoint an exact postnatal age in the rat that coincides with a given postnatal age in the human infant, but we can examine age dependence over a compressed developmental period. For example, others have outlined key rodent postnatal development periods (9); phase 1: early development, from P0 to P10, with eyes closed, no hearing, Hering-Breuer and laryngeal inhibitory reflexes present (34), and baroreflex present (42); phase 2: transitional period, from P11 to P14, with eyes open and hearing present (3); and phase 3: late development, from P15 to P21, when sleep EEG becomes adultlike (5). Within this entire developmental window of P5–P25 we hope to capture age-dependent changes that could be relevant for the age-dependent vulnerability to SIDS. A review of rat postnatal brain stem development indicates that between P11 and P14 there is a major neurochemical reorganization of neurons involved in the control of breathing as well as in other vital functions such as vision, hearing, temperature control, and sleep state (27).

In conclusion, we report abnormal profiles of homeostatic function in rat pups exposed to maternal tryptophan deficiency during pregnancy and the early postnatal period that are age specific. These data indicate a tight link between diet, vulnerable developmental periods, and physiological dysfunction that is relevant to the conceptualization of all dietary deficiencies of the central nervous system in early life. Moreover, our data indicate that maternal dietary depletions may have adverse effects on the development of the brain stem and brain stem-mediated function in offspring that are just as detrimental as their effects on the forebrain and cognition, with dietary tryptophan depletion potentially contributing to SIDS and explaining in part the prevalence of SIDS among impoverished populations. The age-dependent effects of tryptophan depletion on respiratory function provide an interesting correlate to the age dependence of SIDS and suggest a plausible mechanism by which malnutrition may increase the risk of SIDS. These dietary data suggest that dietary deficiencies in amino acids that form neurotransmitters important for homeostasis may contribute to the high mortality of malnourished neonates and infants living in poverty (30).

**GRANTS**

This work was supported by National Institute of Child Health and Human Development Grant PO1-HD-036379.

**DISCLOSURES**

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

**REFERENCES**


