Ventilatory response to hypercapnia and hypoxia after extensive lesion of medullary serotonergic neurons in newborn conscious piglets


Department of Physiology, Dartmouth Medical School, Lebanon, New Hampshire; Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, Connecticut; and Department of Pathology, Children’s Hospital Boston, Boston, Massachusetts

Submitted 29 March 2006; accepted in final form 26 May 2006

Penatti, E. M., A. V. Berniker, B. Kereshi, C. Cafaro, M. L. Kelly, M. M. Niblock, H. G. Gao, H. C. Kinney, A. Li, and E. E. Nattie. Ventilatory response to hypercapnia and hypoxia after extensive lesion of medullary serotonergic neurons in newborn conscious piglets. J Appl Physiol 101: 1177–1188, 2006.—Acute inhibition of serotonergic (5-HT) neurons in the medullary raphe (MR) using a 5-HT1A receptor agonist had an age-dependent impact on the CO2 response of piglets (33). Our present study explored the effect of chronic 5-HT neuron lesions in the MR and extra-raphe on the ventilatory response to hypercapnia and hypoxia in piglets, with possible implications on the role of 5-HT in the sudden infant death syndrome. We established four experimental groups. Group 1 (n = 11) did not undergo any treatment. Groups 2, 3, and 4 were injected with either vehicle or the neurotoxin 5,7-dihydroxytryptamine in the cisterna magna during the first week of life (group 2, n = 9; group 3, n = 11) or second week of life (group 4, n = 10). Ventilation was recorded in response to 5% CO2 (all groups) and 12% O2 (group 2) during wakefulness and sleep up to postnatal day 25. Surprisingly, the piglets did not reveal changes in their CO2 sensitivity during early postnatal development. Overall, considerable lesions of 5-HT neurons (up to 65% decrease) in the MR and extra-raphe had no impact on the CO2 response, regardless of injection time. Postlesion raphe plasticity could explain why we observed no effect. 5,7-Dihydroxytryptamine-treated males, however, did present a lower CO2 response during sleep. Hypoxia significantly altered the frequency during sleep in lesioned piglets. Further studies are necessary to elucidate the role of plasticity, sex, and 5-HT abnormalities in sudden infant death syndrome.

5,7-dihydroxytryptamin; sudden infant death syndrome; raphe; plasticity; serotonin

SEROtonIC (5-HT) NEURONS in the medullary raphe (MR) region (the raphe magnus, pallidus, and obscurus) and extraraphe (ER) region (the parapyramidal and juxtafacial paragigantocellularis lateralis) modulate sensory processing, homeostatic regulation, sleep, cardiovascular control, and motor output (5, 8, 23, 28, 55).

The role of 5-HT neurons in early postnatal life is of particular interest because of their potential link to the sudden infant death syndrome (SIDS). There are two major findings that correlate SIDS with 5-HT. First, abnormalities in medullary 5-HT neurons have consistently been described in many SIDS cases. These include decreased 5-HT receptor binding (24–26, 40, 41) and increased number of 5-HT neurons (43). It is hypothesized that abnormalities in medullary 5-HT neurons can affect several protective responses to certain stresses, such as hypercapnia, hypoxia, asphyxia, thermal imbalances, and reflex apnea. If the inhibition occurs during sleep in an unstable developmental period, the event could prove life-threatening and lead to SIDS. Second, there exists a genetic risk factor for SIDS: the prevalence of the long allele polymorphism in the promoter region of the 5-HT transport protein (SERT) gene (36, 58). An in vitro study showed that this polymorphism decreases the 5-HT level in the synapse, since it excites the SERT (27).

Several studies have demonstrated that the MR is chemosensitive in vivo. In conscious adult rats and goats, for instance, acidification of the MR using CO2 microdialysis increases ventilation (Ve) (19, 37). Acute and extensive disruption of the raphe substantially reduces the “CO2 response” in decerebrated piglets (10). Nonspecific inhibition of the MR using GABA_A agonist microdialysis reduced the CO2 response by 18% in wakefulness and disrupted sleep cycling in conscious piglets (32). Additionally, selective lesioning of 5-HT neurons in the MR of conscious adult rats with a novel 5-HT neurotoxin, SERT antibody-saporin conjugate, decreased the ventilatory response to 7% CO2 by 15% in wakefulness and by 18% in non-rapid eye movement (NREM) sleep (38). The acute inhibition of 5-HT neurons in the MR of adult rats, using the 5-HT1A receptor agonist (+/-)-8-hydroxy-2-(dipropylamino)-tetralin (DPAT), decreased the CO2 response (51).

Central respiratory chemosensitivity of MR neurons in rats increases with age; the response of these neurons to acidosis in vitro is immature in rats younger than P12 compared with older rats (56). Moreover, previous data from our laboratory demonstrated that acute inhibition of 5-HT neurons in the MR with DPAT lowered the CO2 response in newborn piglets older than 10 days, but had the opposite effect in younger piglets (33). These age-dependent observations could relate to SIDS, so it was necessary to clarify whether 5-HT neurons in the MR modulate central chemoreception in early postnatal life.

The potential role of MR 5-HT neurons in the ventilatory response to hypoxia is not yet understood. It has been demonstrated that the discharge of the nucleus of the solitary tract (NTS) neurons can be inhibited by activation of the nucleus raphe magnus (NRM) (44). A recent study in conscious rats determined that nonspecific lesioning of neurons in the MR region by microinjection of ibotenic acid leads to an increased

Address for reprint requests and other correspondence: E. M. Penatti, Dept. of Physiology, Dartmouth-Hitchcock Medical Center, Borwell Bldg., Lebanon, NH 03756–0001 (e-mail: eliana.m.penatti@Dartmouth.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.jap.org

First published June 8, 2006; doi:10.1152/japplphysiol.00376.2006.
ventilatory response to 7% O$_2$ (14). Based on these data, it appears that MR neurons may activate an inhibitory pathway to the NTS during hypoxia. However, the phenotype of these MR neurons has not been well characterized.

We had three primary objectives in the present study: to evaluate the normal piglet response to CO$_2$ in the first 3 wk of life during wakefulness and NREM sleep (group 1); to verify whether substantial and irreversible lesioning of 5-HT neurons in the MR and ER regions (as opposed to acute inhibition with DPAT) in conscious newborn piglets decreases the ventilatory response to CO$_2$ in either wakefulness or NREM sleep; and to determine whether 5-HT neurons in the MR and ER regions modulate the ventilatory response to hypoxia. Irreversible lesions of 5-HT neurons were created with the neurotoxin 5,7-dihydroxytryptamine (DHT). Intracerebroventricular administration of DHT, combined with systemic pretreatment of desipramine to protect noradrenergic neurons, has a selective neurotoxic effect on central 5-HT neurons (2). Three DHT lesion groups (groups 2, 3, and 4), which differed from each other by the time of drug injection and duration in the study, were the focus of the present project. The effect of the lesions was evaluated as follows: injection of DHT in the first week of life with follow-up 10–12 days later (group 2); injection of DHT in the second week of life with follow-up 12–15 days later (group 3); injection of DHT in the first week of life and follow-up 18–21 days later (group 4). Groups 2 and 4 were designed to compare the short- and long-term impact of early lesioning.

**MATERIALS AND METHODS**

**Animal Care and Maintenance**

Newborn Yorkshire or Duroc piglets of both sexes, aged 4–12 days old and weighing 2.1–3.6 kg on the day of surgery, were housed in a farrowing crate with the sow in the Animal Research Facility. All surgical procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee of Dartmouth College. Pre- and postoperative care was provided for all piglets. Cefazolin (20 mg/kg iv) and desipramine (5 mg/kg ip) were administered to piglets before surgery. Buprenorphine (0.1 mg/kg im) was given immediately postoperatively for analgesia. Piglets received the antibiotic Ceftaxin (25 mg/kg) in piglet formula each day postsurgery until euthanasia. Bacitracin antibiotic ointment was applied topically to incisions before surgery. Buprenorphine (0.1 mg/kg im) was given immediately after surgery. For challenge experiments. The experiments were performed soon after surgery and were studied in the plethysmograph for the CO$_2$ challenge experiments. The experiments were performed soon after surgery (early measurement) and then repeated several days later (late measurement). In group 2, early measurements for both control and treated piglets were recorded 24 h after surgery. Late measurements occurred at 9.4 ± 2.4 days (mean ± SE; control $n = 5$) and 11.8 ± 1.3 days (treated $n = 4$) after surgery. In group 3, early measurements were taken 1.2 ± 0.8 days (control $n = 5$) and 1.8 ± 1.2 days (treated $n = 6$) after surgery. Late measurements were recorded 13.8 ± 1.2 days (control) and 14 ± 1.0 days (treated) after surgery. In group 4, early measurements occurred 1.4 ± 1.6 days (control $n = 5$) and 1.4 ± 0.6 days (treated $n = 5$) after surgery. Late measurements were taken 19.4 ± 1.6 days (control) and 19.0 ± 1.0 days (treated) after surgery. For group 2, there were just the two experiments: early and late. We conducted experiments in between the early and late recordings with groups 3 and 4, but we decided not to include these data because they did not reveal new findings or different ventilatory trends; therefore, the early and late measurements only were used for consistency. Upon completion of the experiments, the piglets were euthanized with concentrated halothane (5%), and the brain stem was perfused fixed for further immunohistochemistry (IHC) studies.

**Groups 2, 3, and 4**. Piglets received an injection (vehicle or DHT) during surgery and were studied in the plethysmograph for the CO$_2$ challenge experiments. The experiments were performed soon after surgery (early measurement) and then repeated several days later (late measurement). In group 2, early measurements for both control and treated piglets were recorded 24 h after surgery. Late measurements occurred at 9.4 ± 2.4 days (mean ± SE; control $n = 5$) and 11.8 ± 1.3 days (treated $n = 4$) after surgery. In group 3, early measurements were taken 1.2 ± 0.8 days (control $n = 5$) and 1.8 ± 1.2 days (treated $n = 6$) after surgery. Late measurements were recorded 13.8 ± 1.2 days (control) and 14 ± 1.0 days (treated) after surgery. In group 4, early measurements occurred 1.4 ± 1.6 days (control $n = 5$) and 1.4 ± 0.6 days (treated $n = 5$) after surgery. Late measurements were taken 19.4 ± 1.6 days (control) and 19.0 ± 1.0 days (treated) after surgery. For group 2, there were just the two experiments: early and late. We conducted experiments in between the early and late recordings with groups 3 and 4, but we decided not to include these data because they did not reveal new findings or different ventilatory trends; therefore, the early and late measurements only were used for consistency. Upon completion of the experiments, the piglets were euthanized with concentrated halothane (5%), and the brain stem was perfused fixed for further IHC studies.

**Hypoxia Experiments**

Piglets received an injection (vehicle or DHT) during surgery and were studied in the plethysmograph for the hypoxia experiments. The piglets in this protocol were also used for the group 2 hypercapnia experiments; the hypoxia protocol, however, contained one additional
treated piglet. Early measurements were conducted 3.4 ± 0.6 days (control) and 2.4 ± 0.7 days (treated) after surgery. Late measurements occurred 10.4 ± 1.0 days (control) and 11.8 ± 0.2 days (treated) after surgery.

**Plethysmograph Setup**

Piglets were placed into a sling in the prone position; the sling was suspended from a metal frame inside the plethysmograph. Respiration, body temperature, and sleep cycling were recorded continuously. All piglets were videotaped during the experiment for behavioral analysis of sleep state. Group 2 piglets also had EEG and electrocorticogram electrodes to determine sleep state. Monitoring and recording began after a 40-min equilibration period. Approximately 30 min of baseline measurements were taken while the piglet breathed room air.

**Hypercapnia Experiments**

A CO2 challenge was then performed in which inspired CO2 was raised to 5% for 30 min. There was no recovery period in the hypercapnia trials. The experiments were considered complete after the 30-min CO2 challenge, and the piglets were either returned to the sow or euthanized.

**Hypoxia Experiments**

Instead of adjusting CO2, the O2 outflow was lowered to 12% for ~15 min. At the end of the hypoxia period, the box was opened to flush out the hypoxic air from the plethysmograph. The box was then closed to record a 30-min recovery period. The experiments were considered complete after the recovery period, and the piglets were either returned to the sow or euthanized. We selected 30-min intervals for our studies because they proved an appropriate length of time for the piglets to present multiple sleep cycles and thereby yield sufficient NREM sleep data.

**Data Analysis**

Original data collected in PowerLab were reformatted and analyzed with custom programs in MatLab (7). Sleep states were assessed in two ways. Group 2 piglets were evaluated with electrodes and behavior. Wakefulness was defined as a period of moderate EEG amplitude accompanied by irregular limb movements. NREM sleep was defined as a period of high EEG amplitude, closed eyes, and intermitent body shivering. Sleep states for groups 1, 3, and 4 were assessed by behavior only; these piglets did not have sleep electrodes and were thus determined to be in wakefulness or NREM sleep by their physical appearance. Piglets were judged to be awake if they were moving and their eyes were open, or if they appeared drowsy (eyes opening and closing frequently). NREM sleep was characterized by a prolonged quiet period with eyes closed, intermitent shivering episodes, and the animal appeared relaxed in the sling. All experiments were video recorded so that they could be reviewed multiple times. Six out of 18 experiments in group 2 were used to assess the accuracy of behavior sleep scoring. These six experiments, which included early and late measurements from three piglets, were scored with the video recording only and then compared with the score based on behavior and EEG signals. There was no significant difference between the method used for scoring and the percentage of time in wakefulness and in NREM sleep [repeated-measures (RM) ANOVA with EEG vs. behavioral scoring as repeated measures and time and state as categorical variables].

Breaths were selected from representative sleep states (wakefulness and NREM sleep) and according to gas mixture (room air, 5% CO2, or 12% O2). We excluded rapid eye movement (REM) sleep data from the study because many piglets did not cycle through REM, so there was not enough data for proper analysis. During quite wakefulness, each period selected consisted of an average of 2.5 ± 0.3 min (mean ± SE), made up of an average of 186 ± 36 breaths (mean ± SE). Periods were averaged together (e.g., all periods of wakefulness during room air) for each animal. Within each of the four groups, data were then combined for all control and all treated piglets. Data were omitted from analysis when a piglet’s movement interfered with ventilatory assessment.

**Neuroanatomy**

Upon completion of the experiments, which occurred 7–25 days after surgery, depending on the group, piglets were euthanized with a high concentration of halothane (5%) and transcardially perfused with 1,000 ml of warmed saline (36–38°C) followed by 1,000–1,500 ml of chilled 4% paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.3–7.4). The brain stem was removed, immersed overnight in 4% paraformaldehyde, and cryoprotected for 48 h in 30% sucrose. Tryptophan hydroxylase (TPOH) IHC was performed according to previously described methods (39). To verify that DHT specifically lesioned TPOH-immunoreactive (ir) neurons, tyrosine hydroxylase (TH) IHC was performed on tissue sections adjacent to those processed for TPOH. The IHC procedure was similar to that described for TPOH staining, except that a rat anti-mouse monoclonal antibody (1:2,000 dilution) was used for the primary antibody and Tris buffered saline was used for the buffer.

Two negative control experiments were performed. The first method involved omitting the primary antibody from the IHC reaction. The second method stained the cerebellum for TPOH and TH, since there should not be any immunoreactivity for either enzyme in the cerebellum. Positive control experiments were performed by staining rat brain stem sections with TPOH and TH; the primary antibodies for both enzymes recognize a rat antigen.

Cell counting was performed as previously described by Niblock and colleagues (39). TPOH-ir neurons were identified by a dark brown diaminobenzidine precipitate found in the cell body and neuronal processes. Immunoreactive neurons were counted using the computer image program Neuroleucida. Cells were counted in the MR, as well as in the ER medullary locations containing TPOH-ir cells.

The obex was used as a reference point to make cell count comparisons between piglets. TPOH-ir neurons were counted at the obex, as well as at locations every 960 μm rostral from that point to the retinotegmental nucleus at thepons. A 960-μm interval represents approximately one-tenth of the total length of the caudal 5-HT system in the piglet. To account for different brain stem sizes among piglets, the rostral-caudal axis was normalized from the obex to the rostral end of the facial nucleus. These cell counts were compared by RM ANOVA with cell count as the repeated measure and treatment and distance as factors. To estimate the percent decrease in TPOH-ir neurons in the DHT-treated brain stems, cell counts obtained for each section of each piglet were compiled over the rostral-to-caudal region and compared with the control data.

**Statistics**

RM ANOVA was used to analyze ventilatory data. VE, tidal volume (Vt), and respiratory rate (fR) were the RM variables, and state (wakefulness, NREM sleep), treatment (vehicle, DHT), and gas (room air, 5% CO2) were categorical variables. To evaluate the CO2 response and the “hypoxic response”, the change (Δ) in VE, ΔVT, and ΔfR values were calculated and analyzed with the RM ANOVA approach.

There were 6 piglets out of the 11 in group 1 that missed one experiment in a specific period of the protocol: 2 missed an experiment at 10–12 days, 2 missed an experiment at 13–15 days, and 2 missed an experiment at 19–21 days. A linear regression equation, which used comparable values from the same piglet, was used to fill in these missing data for each.

There was one piglet in each of the three surgery groups (groups 2, 3, and 4) that did not have complete cell count data for all of the eight different brain sections. Specifically, 1.3% of all cell count data were
missing. A proportion was established to account for the missing data. First, the ratio of the cell count average from all other piglets in the obex to the cell count average from all other piglets in the given section was determined. Then the cell count value from a similar area in the obex (MR or ER) was divided by the calculated average ratio to determine the blank value. Missing cells in the MR and ER were determined independently and used to calculate the total cell count for that brain section.

RESULTS

5-HT Cell Counts

Figure 1 shows the plots for mean ± SE of the total number of counted TPOH-ir cells, including MR and ER areas, for each of the four groups. Figure 1A depicts the TPOH cell counts for group 1. One animal was excluded from these cell count data, because the ventral surface of the brain stem was damaged during removal, and it was not possible to obtain an accurate count. Figure 1, B, C, and D, represents the TPOH cell counts for groups 2, 3, and 4, respectively. As expected, the cell counts in the DHT-treated animals were significantly lower in groups 2, 3, and 4 (P < 0.01, RM ANOVA with cell count as the repeated measure and treatment and distance as categorical variables). The average of total number of TPOH cells ± SE for each group is as follows: group 2 controls (n = 4) 982 ± 17 vs. treated (n = 4) 370 ± 94, a 62.3% decrease; group 3 controls (n = 4) 1,086 ± 91 vs. treated (n = 6) 384 ± 48, a 64.6% decrease, and group 4 controls (n = 5) 839 ± 70 vs. treated (n = 5) 551 ± 53, a 34.3% decrease.

To verify that the large lesion targeted 5-HT neurons only, tyrosine (Tyr-ir) cell counts from the obex to rostral facial nucleus were performed with three control and three DHT-treated piglets to examine noradrenergic neurons. Control piglets had an average ± SE of 598 ± 149 Tyr-ir cell counts, whereas DHT-treated animals had 576 ± 83 Tyr-ir cell counts. There was no significant difference between the two groups; thus pretreatment with desipramine before the high dose of DHT effectively prevented toxicity to noradrenergic neurons.

Developmental Changes in Normal Piglets During Early Postnatal Life

During the first 3 wk of postnatal life, untreated piglets (group 1) presented a significant increase in their body weight (BW) (P < 0.001, RM ANOVA; P < 0.05 Holm-Sidak post hoc vs. control as first value). As a result of this rapid growth, the O2 uptake (V˙O2) normalized to BW actually decreases dramatically during wakefulness (Fig. 2; P < 0.05, RM ANOVA; P < 0.05 Holm Sidak post hoc vs. control as first value) and NREM sleep (data not shown) in both room air and hypercapnia. The considerable weight gain observed in normal piglets made it difficult to evaluate V˙E, since common ventilatory parameters are normalized to BW. Three separate criteria demonstrate the impact of BW on ventilatory interpretation: absolute V˙E, V˙E normalized to BW, and the ratio of V˙E to V˙O2 (in which the variable BW is excluded).

Ventilatory Measurements for Group 1

Absolute V˙E increased significantly (P < 0.001, RM ANOVA with absolute V˙E as the repeated measure and gas and state as factors) and proved significantly higher in hypercapnia than in room air (Fig. 3A; P < 0.001). The increase in absolute V˙E was due to the absolute V˙T (data not shown), which also varied significantly (P < 0.001, RM ANOVA with V˙T as the repeated measure and gas and state as factors). For both absolute V˙E and absolute V˙T, there was no interaction between the two categorical variables (gas and state). The IR proved relatively constant throughout the period studied, and the IR values were significantly higher by 18 breaths/min during wakefulness (P < 0.05) than NREM sleep in both room air and hypercapnia. Additionally, the IR was statistically higher in hypercapnia by 19 breaths/min compared with room air (P < 0.05).

V˙E normalized to BW decreased significantly (P < 0.001, RM ANOVA with V˙E as the repeated measure and gas and state as factors) and also proved significantly higher in hypercapnia than in room air (Fig. 3B; P < 0.001). This decrease in
\( \dot{V}E \) can be attributed to the significant decrease in normalized \( \dot{V}T \) (\( P < 0.001 \), RM ANOVA with \( \dot{V}T \) as the repeated measure and gas and state as factors; data not shown). As with absolute \( \dot{V}E \) and absolute \( \dot{V}T \), there was no interaction between gas and state for either \( \dot{V}E \) or \( \dot{V}T \).

As outlined, the ventilatory measurements varied significantly, largely because of the increase in BW. It is possible to exclude the BW variable by analyzing the ratio between the normalized \( \dot{V}E \) and the normalized \( \dot{V}O_2 \) (\( \dot{V}E/\dot{V}O_2 \)). The \( \dot{V}E/\dot{V}O_2 \), though, varied significantly (\( P < 0.001 \), RM ANOVA with gas and state as factors) and proved significantly higher in hypercapnia than in room air (Fig. 3C; \( P < 0.001 \)). During room air and hypercapnia, the \( \dot{V}E/\dot{V}O_2 \) was fairly constant in both wakefulness and NREM sleep up to 15–18 days and then sharply decreased. Additionally, there was no significant interaction with gas or state or gas and state together.

Figure 4 illustrates different ways to evaluate the CO\(_2\) response, emphasizing the \( \Delta \) absolute \( \dot{V}E \), \( \dot{V}E \), and \( \dot{V}E/\dot{V}O_2 \). Figure 4A shows a significant increase in the \( \Delta \) absolute \( \dot{V}E \) as a function of time (\( P < 0.001 \), RM ANOVA with \( \Delta \) absolute \( \dot{V}E \) as the repeated measure and state as a factor). Conversely, \( \dot{V}E \) (Fig. 4B) yielded a significant decrease as a function of time in both wakefulness and NREM sleep (\( P < 0.001 \), RM ANOVA with \( \dot{V}E \) as the repeated measure and state as a factor); the response increased sharply in the first 10–12 days, remained fairly constant for a short period, and then decreased after 16–18 days. In all three plots, \( \Delta \) absolute \( \dot{V}E \), \( \dot{V}E \), and \( \dot{V}E/\dot{V}O_2 \), there was no interaction between the CO\(_2\) response and state.

Figure 5 represents the CO\(_2\) response for group 1 as percent increase of \( \dot{V}E \) during wakefulness (Fig. 5A) and NREM sleep (Fig. 5B) as a function of time. Unlike the CO\(_2\) responses presented above (\( \Delta \) absolute \( \dot{V}E \), \( \dot{V}E \), and \( \dot{V}E/\dot{V}O_2 \)), the percent increase of \( \dot{V}E \) remained relatively constant and did not

**Fig. 2.** Normal piglet body weight (BW) and metabolic rate in early postnatal life. Values are means ± SE for BW and consumption of oxygen (\( \dot{V}O_2 \)) as a function of time (\( n = 11 \)). A: BW increased rapidly during the first 3 wk of life (\( P < 0.001 \), RM ANOVA with BW as the repeated measure and time as a factor; \( P < 0.05 \), Holm-Sidak post hoc test of 2nd, 3rd, 4th, and 5th measurements vs. 1st value). B: room air \( \dot{V}O_2 \) during wakefulness is plotted (\( P < 0.05 \), RM ANOVA with \( \dot{V}O_2 \) as the repeated measure and time as a factor; \( P < 0.05 \), Holm-Sidak post hoc test of 3rd, 4th, and 5th measurements vs. 1st value). Similar results for BW and metabolic rate were obtained during non-rapid eye movement (NREM) sleep (data not shown).

**Fig. 3.** Ventilatory measurements for group 1. A: absolute ventilation (\( \dot{V}E \)) measurements at room air and 5% CO\(_2\) are shown for wakefulness (○, room air; □, CO\(_2\)) and NREM sleep (△, room air; ◇, CO\(_2\)). Absolute \( \dot{V}E \) increased significantly over time and proved considerably higher in 5% CO\(_2\) than in room air (\( P < 0.001 \), RM ANOVA with absolute \( \dot{V}E \) as the repeated measure with state and gas as factors). B: same as A, but \( \dot{V}E \) is normalized to BW. \( \dot{V}E \) decreased significantly over time and was considerably higher in hypercapnia than in room air (\( P < 0.001 \), RM ANOVA with \( \dot{V}E \) as the repeated measure with state and gas as factors). C: same as A, but \( \dot{V}E \) is expressed as a ratio over \( \dot{V}O_2 \). There was a significant decrease over time (\( P < 0.001 \), RM ANOVA with \( \dot{V}E/\dot{V}O_2 \) as the repeated measure with state and gas as factors), and there was a significant difference between room air and hypercapnia. There was no interaction between gas and sleep state, according to any of the three ventilatory measures. All values are means ± SE (\( n = 11 \)).
vary significantly over time in either sleep state (RM ANOVA with $\Delta V\dot{E}$ as the repeated measure and state as a factor). Among all the various CO2 response criteria outlined above, the percent increase of $V\dot{E}$ proved a useful and reliable way to evaluate the normal ventilatory change in early postnatal life. Consequently, the CO2 response for all other groups has been presented as the percent increase of $V\dot{E}$.

Ventilatory Measurements for Groups 2, 3, and 4

The initial values for BW, age, and body temperature were as follows: 2.3 ± 0.1 kg, 6.4 ± 0.9 days, and 38.7 ± 0.2°C, respectively, for group 2; 4.3 ± 0.3 kg, 11.6 ± 0.4 days, and 38.1 ± 0.1°C, respectively, for group 3; 2.6 ± 0.2 kg, 6.2 ± 0.6 days, and 38.4°C, respectively, for group 4. Figure 6 illustrates the absolute $V\dot{E}$ for groups 2 (Fig. 6, A and D), 3 (Fig. 6, B and C), and 4 (Fig. 6, C and F) during room air and hypercapnia in wakefulness (A–C) and NREM sleep (D–F). There was a considerable increase of absolute $V\dot{E}$ for all groups, with no significant difference between DHT and vehicle treatment. In all groups, absolute $V\dot{T}$ increased significantly ($P < 0.05$, RM ANOVA with $V\dot{T}$ as repeated measure and gas and treatment as factor) during both wakefulness and NREM sleep (data not shown). As observed in group 1, the fR proved relatively constant throughout the period studied for groups 2 and 4 during both wakefulness and NREM sleep (data not shown). Unlike in groups 2 and 4, in group 3 the fR did not prove constant throughout the period studied, but it decreased significantly ($P < 0.001$, RM ANOVA with fR as the repeated measure with gas and treatment as factors) during both wakefulness and NREM sleep (data not shown). Overall, chronic DHT treatment did not alter absolute $V\dot{E}$ in the three separate lesion groups.

Figure 7 illustrates the CO2 response as percent increase of $V\dot{E}$ for groups 2 (Fig. 7, A and D), 3 (Fig. 7, B and E), and 4 (Fig. 7, C and F) during wakefulness (A–C) and NREM sleep (D–F). In all groups, the CO2 response showed no significant variation in control and treated piglets over time; there was no significant interaction with sleep state in any of the three ventilatory plots. Values are means ± SE ($n = 11$).
together. In essence, there was no evidence that the DHT-treated piglets presented a decreased CO₂ response compared with control piglets as hypothesized, regardless of injection time.

Figure 8 illustrates the percent increase of VT (Fig. 8, A and D), fR (Fig. 8, B and E), and V˙E (Fig. 8, C and F) measured during hypoxia for group 2, in wakefulness (A–C) and NREM sleep (D–F). The baseline values for VT, fR, and V˙E resulted from the average between room air and recovery (posthypoxia challenge) values. It is important to note that our results might have missed the initial peak response to hypoxia because of the 20-min period our setup required to drop from room air to hypoxia; we did not record V˙E until after the plethysmograph reached 12% O₂.

Based on our data, however, neither treatment nor sleep state had a significant impact on percent increase of V˙E or VT, except for VT during NREM sleep; in that case, there was a significant interaction between V˙E and state (P < 0.05, RM ANOVA with VT as the repeated measure and treatment and state as factors). The percent increase of fR did not vary significantly over time, but there was a significant interaction with treatment (P < 0.001, RM ANOVA with fR as the repeated measure and treatment and state as factors) and with treatment and state together (P < 0.05, RM ANOVA with fR as the repeated measure and treatment and state as factors). A post hoc Bonferroni correction was applied and produced a significant difference between control and treated piglets (P < 0.05) for the late measurement in NREM sleep. Whereas the fR for control piglets decreased over time, the fR for treated piglets increased sharply, especially during NREM sleep. Although the percent increase of V˙E and VT did not differ significantly between control and treated piglets, DHT treatment increased significantly the fR during NREM sleep in the late measurement.

Our experimental design did not include sex as an experimental variable. During completion of experiments and data analysis, we learned about sex-specific effects in PET-1 knock-out mice, a transgenic mouse that shows fewer 5-HT neurons in the medulla (17). Male PET-1 knockout mice had a decreased CO₂ response compared with females (18). Based on the transgenic mice data and on the age-dependent effect of DPAT, we hypothesized that substantial and chronic lesioning of 5-HT neurons in the MR and ER regions would dramatically decrease the CO₂ response, especially in male piglets.
To evaluate whether the response to chronic DHT treatment differed between males and females, data from each sex in groups 2, 3, and 4 were combined and analyzed. In group 2, there were four females and one male for the control piglets and three females and one male for treated piglets. In group 3, there were four females and one male for the control piglets, and two females and four males for treated piglets. In group 4, there were three females and two males for control piglets, and three females and two males for treated piglets. Figure 9 portrays the $VE\%$ increase from room air to 5% CO$_2$ as a function of time during wakefulness and NREM sleep, according to sex and sleep state. There was a borderline significant interaction between percent increase $VE\%$ and sex and treatment and state ($P = 0.079$, RM ANOVA with percent increase $VE\%$ as the repeated measure with state, treatment and sex as factors). Our hypothesis in this case was very specific: males, but not females, would have a decreased CO$_2$ response after 5-HT lesions. We were thus able to apply an a priori comparison of means (4) on males and females in the late CO$_2$ response during wakefulness and NREM sleep. DHT-treated males had a significant decrease in their CO$_2$ response in the late measurement but only in NREM sleep ($P < 0.02$, Bonferroni correction with four comparisons).

**DISCUSSION**

**Main Findings**

1) Normal piglets more than double their BW over the first 3 wk of life. The rapid increase of BW in early postnatal piglet life has a significant impact on several methods that are commonly used to assess $VE\%$. The $VE\%$ percent increase proved the best measurement of the CO$_2$ response to compare developmental $VE\%$ changes in normal piglets.

2) We successfully lesioned a large fraction (up to 65%) of 5-HT neurons in the MR and ER regions by the injection of DHT into the cisterna magna. 3) Despite significant lesioning, we could not substantiate the hypothesis that DHT-treated piglets would present a decrease in their response to 5% CO$_2$. 4) When females and males were analyzed independently, DHT-treated males did exhibit a significant decrease in the CO$_2$ response during NREM sleep. 5) DHT treatment resulted in an exaggerated fR response to hypoxia in the treated piglets during NREM sleep.

**Neuroanatomy**

The staining pattern of TPOH-ir neurons in the caudal brain stem of group 1 and control animals from groups 2, 3, and 4...
was remarkably consistent and supported data from previous studies in piglets (39). We established irreversible lesions of 5-HT neurons in the treated animals from groups 2, 3, and 4 with DHT. Although its mechanism is unknown, the 5-HT neurotoxicity of DHT is efficacious, and it has been used widely in several physiological studies (2, 31, 34, 54). In the present study, DHT treatment decreased the number of TPOH-ir neurons in the MR and ER regions up to 65%.

VE: Normal Development During the First Month of Life

The postnatal development of the CO2 response has been well studied in rats. Rats present a triphasic pattern of responsiveness to CO2 during postnatal development (45, 49). The ventilatory response is present in the first 5 days of life, although it fluctuates and actually reaches a nadir around postnatal day 8, sometimes called a “critical period.” Subsequently, the hypercapnic response attains adult levels. The research with piglets has not been as extensive, however, so the patterns during hypercapnia are not understood as well as those in rats. Usually, the VT and VE responses to CO2 gradually increase with age, while the fR response to CO2 fluctuates in younger piglets and increases in older piglets (47). These studies, however, were performed with anesthetized animals, so it is possible some of ventilatory data, especially the fR inconsistencies, resulted from the anesthesia and would not hold true for conscious animals.

In the present study, VE percent increase proved to be the one measure for evaluating the CO2 response that remained relatively constant throughout the first 3 wk of life. We saw no evidence of any critical period for the CO2 response during postnatal development in the conscious piglet compared with rats (45, 49). Our findings demonstrated that the CO2 sensitivity does not change in piglets during the neonatal period.

VE: Effects of Vehicle and DHT Injection Into the Cisterna Magna

Previous study in the laboratory demonstrated that acute inhibition of 5-HT neurons in the MR using DPAT had an age-dependent impact (33). Piglets older than 7–10 days presented a decreased CO2 response, whereas younger piglets had the opposite effect. Based on these results, the aim of our present study was to verify whether chronic lesioning of 5-HT
neurons in the MR and ER, established during the first or second week of life, would exhibit the same effect that was observed with acute inhibition of these neurons.

We could not support the hypothesis that an extensive loss of 5-HT neurons in the MR and ER would decrease the CO₂ response in piglets; there was no significant difference in the ventilatory response to hypercapnia between control and treated piglets. The time of DHT injection, whether it was administered in the first or second week of life, did not affect the response. Additionally, there was no significant change between the short-term response to 5% CO₂ of piglets in group 2 and the long-term response of piglets in group 4.

It is probable that the unchanged CO₂ response in the lesion experiments resulted from an adaptation, redundancy, or plasticity that enabled these piglets to fully recover their central chemosensitivity and compensate for the large lesions. Generally, plasticity varies among species, depends on the age at which the lesion is made, and is greater in conscious animals (13). For example, long-term facilitation following intermittent hypoxia, a model of 5-HT-dependent plasticity, is greater in older female rats (3). Additionally, neonatal goats that received carotid body denervation recovered the reflex completely within 3 mo (29). Neonatal piglets also showed a quick recovery within just 3 wk (29, 48). Moreover, conscious adult goats presented transient attenuation of CO₂ sensitivity after neurotoxic lesions in the MR (20), and their V̇E returned to normal within 2 wk.

Sex and CO₂ Responses

SIDS is reported in males twice as often as in females (16), so it followed that there could have been sex discrepancies in the data. Male PET-1 knockout mice presented a decreased CO₂ response compared with females (18). We analyzed males and females independently in groups 2, 3, and 4 in wakefulness and NREM sleep. Overall, similar to the PET-1 knockout mice data, our study showed that chronic 5-HT lesions caused by DHT significantly decreased the late CO₂ response only in male piglets during NREM sleep. Interestingly, when we analyzed the cell counts data by sex, we found no significant differences in TPOH-ir cell counts between male and female piglets. To emphasize this finding, another set of experiments from our laboratory also revealed that there was no difference in 5-HT cell counts between male and female piglets in a control population (Ref. 39; data not shown). Our results raise the possibility of sex differences in plasticity and the ability to compensate for medullary lesions.

It is difficult to assess the role of sex in the relationship between 5-HT neurons and central chemoreception since, to date, most data in the literature have been obtained from adult male rats. For instance, lesioning just 28% of the 5-HT neurons in adult rats caused a significant decrease in the CO₂ response (38). Also, microdialysis with different concentrations of DPAT into the MR of adult male rats decreased absolute V̇E up to 30% during 7% CO₂ in wakefulness and NREM sleep (51).
Additionally, studies that showed stimulation of VE by focal dialysis of CO₂ in the raphe were performed in adult male rats (37). One previous study demonstrated an age-dependent effect of DPAT on the CO₂ response in conscious newborn piglets, but this research was performed on a female-dominated cohort (12 of 14 piglets were female) (33). Because these aforementioned studies have not examined both sexes equally, the connection between sex, 5-HT neurons, and central chemoreception has not been fully clarified. There is evidence, however, that other neurons participate in central chemoreception. For example, Mulkey and colleagues (35) found that 5-HT neurons near the retrotrapezoid nucleus of adult male rats were not activated by hypercapnia, but rather it was glutamatergic neurons that appeared to be the central chemoreceptors in vivo. There are other central chemoreceptor sites, such as the NTS, the locus coeruleus, the midline MR, the rostral aspect of the ventral respiratory group or the pre-Bötzinger complex, the fastigial nucleus, as well as the carotid body, that can participate in chemoreception during development and in adulthood (12). Further investigation is required to determine the specific role of these various sites in central chemoreception.

### Hypoxia Results

5-HT-containing neurons in the MR region project to the NTS (52). The NTS is the primary termination point of the carotid sinus nerve, which receives afferents from oxygen chemoreceptors in the carotid body (21). Since neurons in the NTS have direct effenter connections with respiratory motor neurons that impact VE (9), it follows that 5-HT neurons in the MR region may play a role in the ventilatory response to hypoxia.

The significant findings in the hypoxia component of our study correlate with the response observed in other trials after nonspecific lesioning. For instance, when anesthetized rats received electrical stimulation or L-glutamate microinjection in the MR, NTS activity decreased, thereby causing a lower hypoxic response (44). In other studies, Gargaglioni and colleagues (14) microinjected ibotenic acid into the NRM of conscious rats and observed an increased ventilatory response to 7% O₂ due to an elevated VE. Previous study from our laboratory has demonstrated that acute inhibition of the rostral MR with the GABAplex receptor agonist muscimol increased the laboratory has demonstrated that acute inhibition of the rostral zone (52). Gargaglioni et al. (14) postulated that the NRM exerts an inhibitory modulation on breathing during hypoxia.

In our study, there was also a heightened response to hypoxia, especially with respect to frequency during NREM sleep. These piglet data support the notion that the MR normally inhibits the response to hypoxia.

### The Relevance to SIDS

In the MR and ER, 5-HT receptor binding is decreased (24–26, 41), and the number and density of 5-HT neurons cells are increased (~2-fold) in SIDS cases compared with controls (43). We investigated the possible relationship between these neurons and central chemoreception during postnatal development in conscious piglets. Piglets were selected for a few reasons. SIDS has a peak incidence at 2–4 mo (15, 24), which is thought to correlate with 8–14 days postnatal age in piglets in terms of the development and distribution of medullary 5-HT neurons (39). Also, the development of sleep cycling and cardiorespiratory control in piglets is thought to parallel that in humans (46). Our data suggest that 5-HT neurons affect CO₂ sensitivity in male newborn piglets during NREM sleep and the frequency response to hypoxia in both sexes, indicating that the 5-HT system plays an important role in the control of breathing during development. How abnormalities in 5-HT neurons may contribute to sudden death remains a perplexing issue.

### ACKNOWLEDGMENTS

We thank Laurie Hildebrandt for wonderful technical assistance and Meghan L. Hewitt for time with running experiments and analyzing data.

### GRANT

This work was supported by National Institute of Child Health and Human Development Grant HD-36379.

### REFERENCES


