Inhibition of medullary raphé serotoninergic neurons has age-dependent effects on the CO₂ response in newborn piglets

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Messier, Michelle L., Aihua Li, and Eugene E. Nattie. Inhibition of medullary raphé serotoninergic neurons has age-dependent effects on the CO₂ response in newborn piglets. J Appl Physiol 96: 1909–1919, 2004. First published January 29, 2004; 10.1152/japplphysiol.00805.2003.—Medullary raphé serotoninergic neurons are chemosensitive in culture and are situated adjacent to blood vessels in the brain stem. Selective lesioning of serotoninergic raphé neurons decreases the ventilatory response to systemic CO₂ in awake and sleeping adult rats. Abnormalities in the medullary serotoninergic system, including the raphé, have been implicated in the sudden infant death syndrome (48). In this study, we ask whether serotoninergic neurons in the medullary raphé and extra-raphé regions are involved in the CO₂ response in unanesthetized newborn piglets, 3–16 days old. Whole body plethysmography was used to examine the ventilatory response to 5% CO₂ before and during focal inhibition of serotoninergic neurons by 8-hydroxy-2-di-n-propylyaminotetralin (8-OH-DPAT), a 5-HT₁A receptor agonist. 8-OH-DPAT (10 or 30 mM in artificial cerebrospinal fluid) decreased the CO₂ response in wakefulness in an age-dependent manner, as revealed by a linear regression analysis that showed a significant negative correlation (P < 0.001) between the percent change in the CO₂ response and piglet age. Younger piglets showed an exaggerated CO₂ response. Control dialysis with artificial cerebrospinal fluid had no significant effect on the CO₂ response. Additionally, 8-OH-DPAT increased blood pressure and decreased heart rate independent of age (P < 0.05). Finally, sleep cycling was disrupted by 8-OH-DPAT, such that piglets were awake more and asleep less (P < 0.05). Because of the fragmentary sleep data, it was not possible to examine the CO₂ response in sleep. Inhibition of serotoninergic medullary raphé and extra-raphé neurons decreases ventilatory CO₂ sensitivity and alters cardiovascular variables and sleep cycling, which may contribute to the sudden infant death syndrome.

These cells are situated adjacent to blood vessels in the brain stem; 8-hydroxy-2-di-n-propylyaminotetralin; 5-HT₁A receptor; homeostatic physiology; sudden infant death syndrome

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The goal of these experiments was to study the CO₂ response before and during reversible inhibition of serotonergic medullary raphe and extra-raphe neurons. 8-Hydroxy-2-di-n-propylaminotetralin (8-OH-DPAT) was used because it is a specific agonist of the 5-HT₁₆ receptor, an autoreceptor located on serotonergic raphe neurons (43). Activation of this receptor results in neuronal hyperpolarization and thus a decrease in cell firing. We tested the hypothesis that specific inhibition of medullary raphe serotonergic neurons with 8-OH-DPAT [10 or 30 mM in artificial cerebrospinal fluid (aCSF)] would decrease the ventilatory response to 5% inspired CO₂ in unanesthetized newborn piglets. To our knowledge, no studies to date have examined the CO₂ response in an unanesthetized newborn animal after specific inhibition of medullary raphe serotonergic neurons.

MATERIALS AND METHODS

Animal care and maintenance. Newborn Yorkshire or Duroc piglets of either sex, ages 3–16 days old and weighing between 1.4 and 4.0 kg on the day of surgery, were housed in a farrowing crate with sow in a controlled light (12:12-h light-dark cycle) and temperature (22 ± 1°C) environment 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 animals. Cefazolin (20 mg/kg iv) was given before surgery, and dexamethasone (1–2 mg/kg iv) was given before implantation of the microdialysis guide tube into the brain stem. Buprenorphine (0.1 mg/kg im) was given immediately postoperatively for analgesia. Piglets received the antibiotic Baytril (34 mg) mixed in with piglet formula each day postsurgery until euthanasia. Bacitracin antibiotic ointment was applied topically to incisions daily. The animals tolerated surgery well and were healthy, as evidenced by active ambulation and increased weight gain postoperatively. Piglets were returned to the farrowing crate with the sow when not being studied.

Surgical preparation. Chronic instrumentation is similar to previously described methods (12, 14, 44). Briefly, piglets were anesthetized (2% isoflurane in O₂) and artificially ventilated for the duration of surgery. They were kept hypoxic and ventilated at a level to maintain a PCO₂ between 35 and 40 Torr. A heating pad was used to maintain body temperature between 37 and 39°C. An umbilical catheter (3.5 Fr) was inserted 7.5 cm into a branch of the femoral artery with the other end tunneled subcutaneously and exited out the back. This catheter was used to monitor blood pressure. It was flushed daily, and the dead space was filled with heparin (1,000 U/ml) to prevent clotting. In some animals, a thermistor was implanted subcutaneously into the abdomen, ~1–2 cm lateral to the midline. In these piglets, the thermistor exited out the back with the arterial catheter. In other piglets, a temperature probe for telemetry was implanted at the same location as described for the thermistor.

Piglets were mounted in a Kopf stereotactic frame, and the skull was exposed by a midline incision. Stereotaxic measurements that included bregma, lambda, and a fiducial mark on the right ear bar were taken, and the midline was predicted using a regression equation (12). Electroencephalogram (EEG) electrodes were screwed into the left frontal and right parietal bones. A common electrode was screwed into the right frontal bone. Electrooculogram (EOG) electrodes were positioned lateral to the left eye and superior to the orbit of the right eye. A bipolar electrode was used to record neck electromyogram (EMG) activity. A ground electrode was implanted subcutaneously in the neck. Brass connectors attached to the end of the electrodes were plugged into two plastic pedestals. The microdialysis guide tube with stylet was inserted though a burr hole that was drilled along the sagittal suture ~3–4 mm rostral to lambda. The guide tube was cemented to the skull together with the plastic pedestals with cranio-plastic cement (Plastics One).

Whole body plethysmography. Whole body plethysmography (2, 20) was performed as modified by Pappenheimer (49), Jacky (30), and Curran et al. (12). A pressure transducer (Validyne) was used to measure respiratory-related pressure deflections that result from inspiration and expiration. Large pressure fluctuations were avoided by creating a leak between the plethysmograph and a reference chamber. Analog signals from the pressure transducer were digitized, sampled at 1 kHz by a computer data-acquisition system (PowerLab, ADInstruments), and stored for offline analysis. A flowmeter (Hastings) was used to maintain a constant and balanced flow of inlet and outlet gas through the chamber via high-resistance inlet and outlet ports. A high flow rate through the plethysmograph (~8 l/min) prevented animals from rebreathing CO₂ and maintained high inspired O₂ levels. Inlet gas was warmed to 38°C and humidified. An O₂ analyzer (Applied Electrochemistry) was used to sample both inlet and outlet O₂. These measurements were used in conjunction with gas flow rate to calculate oxygen consumption (VO₂). A CO₂ analyzer (CWI) was used to measure inlet and outlet CO₂. CO₂ challenges were performed by using a gas mixer (Bird) to bleed 40% CO₂ into the plethysmograph inflow until the inspired CO₂ reached 5%. It took ~3 min for the plethysmograph to equilibrate to 5% CO₂. Neither baseline plethysmograph pressure nor flow rate was affected by the CO₂ challenge. Before the start of each experiment, the plethysmograph was calibrated with triplicate injections of 1, 2, 3, and 5 ml of air.

Experimental protocol. On the day of an experiment, piglets received either 8-OH-DPAT (10 or 30 mM in aCSF) or aCSF (control). Experiments were begun ~18–24 h postoperatively and were performed within the first 7 days after surgery. Alertness, ambulation, and nursing were criteria used to ensure that piglets had recovered from surgery. In some instances, piglets were used for a different experimental protocol that was performed on a separate day. Thirty-two animals received 8-OH-DPAT. The ventilatory data from five of these animals were not included in data analysis because these animals became behaviorally excited during 8-OH-DPAT dialysis, thereby making it difficult to accurately measure breathing. Of the remaining 27 animals, 11 received 10 mM 8-OH-DPAT and 16 received 30 mM 8-OH-DPAT. There were no discernable differences in effect between the two 8-OH-DPAT concentrations so the results were pooled.

Piglets were placed in the prone position in a sling that was suspended from a metal frame inside the plethysmograph. Respiratory, mean arterial blood pressure (MAP), body temperature (Tb), and sleep cycles were continuously recorded (YSI-Life Sciences). Piglets were videotaped during the experiment for later analysis of behavioral sleep state. On the day of an experiment, the stylette was replaced with a microdialysis probe that was continuously dialyzed with aCSF at a flow rate of either 8.5 or 4.0 µl/min. There were no discernable differences in effect between the different flow rates, so the results were pooled. The plethysmograph, with the piglet in it, was equilibrated over a 40-min period, during which time the inspired CO₂ and O₂, humidity, and plethysmograph air temperature were allowed to stabilize, and the piglet was allowed to acclimate to the box. After this equilibration, ~20 min of baseline measurements were taken with the piglet breathing room air (baseline I). A 5% CO₂ challenge was then performed, in which inspired CO₂ was raised to 5% for ~15 min (predrug CO₂ challenge). The box was then flushed of the CO₂, and, after an equilibration period, another 20 min of baseline measurements were taken with the piglet breathing room air (baseline II). At this point, aCSF microdialysis was stopped and either 10 or 30 mM 8-OH-DPAT microdialysis was begun. For control experiments, aCSF was continuously dialyzed. Drug dialysis lasted ~30 min, 15 min of which were with the piglet breathing room air, followed by a 15 min 5% CO₂ challenge. The experiment was then ended and the piglet was either returned to the sow or euthanized.
Neuroanatomy. At the end of an experiment, fluorescein [10 or 30 mM in aCSF; molecular weight (MW) = 332] a fluorescent dye with a similar MW to 8-OH-DPAT (MW = 328) was microdialyzed at the probe placement site for the same length of time as drug dialysis. The brain stem was quickly removed, frozen on dry ice, and sectioned at 50 μm in a Reichert-Jung cryostat. Sections were viewed under a fluorescence microscope (Olympus), allowed to dry, and then stained with cresyl violet. Fluorescent and cresyl violet images were photographed in the computer program ImagePro. These anatomic techniques allowed for an approximation of drug spread and for determination of probe location within the brain stem, respectively.

Data analysis. Data were analyzed as previously described by Messier et al. (44), Curran et al. (12), and Darnall et al. (14). Briefly, MAP and heart rate (HR) were derived from arterial blood pressure recordings (MATLAB). Tidal volume (VT), respiratory rate (fR), and minute ventilation (VE) were computed from plethysmograph respiratory pressure fluctuations. All signals were resampled at 1 kHz. EEG and EOG signals were filtered with a band pass of 0.3–30 Hz. The EEG record was divided into 5-s epochs, and power spectral analysis was performed (delta, 0.5–4.0 Hz; theta, 5.0–9.0 Hz; sigma, 10.0–14.0 Hz). The EMG signal was filtered with a band pass of 10–100 Hz (Grass).

Animals were videotaped for behavioral sleep analysis. Each 5-s epoch of the videotape was scored for eye openings and body movements. Wakefulness was defined as a period of low to moderate EEG amplitude and delta power, eyes open or closed, moderate EMG activity, and occasional body movements. Non-rapid eye movement (NREM) sleep was defined as a period of high EEG amplitude and delta power, with eyes closed, reduced EMG tone, and infrequent body movements. Rapid eye movement (REM) sleep was defined as a period of low EEG amplitude and delta power, decreased VT and MAP, absent EMG tone, eyes closed, rapid eye movements, and limb twitches. Although not all criteria were always available, the remaining criteria were enough for determination of sleep state.

Breaths were selected from representative sleep states (quiet wakefulness, REM sleep, REM sleep) during room air breathing and throughout the 15 min 5% CO2 challenge. Breaths from baseline I and baseline II were averaged together because breathing was not significantly different between baselines. Periods of active wakefulness, in which piglets were moving, were omitted from ventilatory analysis. During quiet wakefulness, each period selected consisted of an average of 1 ± 0.2 min (mean ± SE) of data, made up of an average of 61 ± 11 breaths. Periods were averaged together (e.g., all periods of wakefulness during baseline) for each animal. Data from all piglets were averaged together because breathing was not significantly different between baselines I and II. Two-way repeated-measures ANOVA was used to examine the interaction between 8-OH-DPAT and 5% CO2. Sleep disruption during room air breathing was analyzed by paired t-tests. The appropriate post hoc tests were performed when statistical significance (P < 0.05) was reached. Data are in the form of means ± SE.

RESULTS

Location of microdialysis probes. Probe placement was determined anatomically by microdialysis of fluorescein. Probes were located in our region of interest if the fluorescein spread was found in the midline medullary raphe or in extra-raphe (lateral) regions, extending from the pontomedullary border to the brain stem-spinal cord junction (Fig. 1A). The probe locations for the 27 animals that we report on are shown in Fig. 1B.

Control experiments. To establish baseline breathing patterns and CO2 responses, aCSF (control) experiments were performed on 13 of the 27 animals that were treated with 8-OH-DPAT. In these experiments, in which aCSF was continuously microdialyzed, ventilation was measured while piglets breathed room air or 5% CO2, initially at baseline and then again during a test period (Table 1). Baseline VE measured during room air breathing was not significantly different from VE measured during room air breathing within the test period (paired t-test, P = 0.076). Baseline VE measured with piglets breathing 5% CO2 was not significantly different from VE measured with piglets breathing 5% CO2 during the test period (paired t-test, P = 0.924). Likewise, VT measured both during room air breathing (paired t-test, P = 0.079) and during the 5% CO2 challenge (paired t-test, P = 0.893), as well as HR measured during room air breathing (paired t-test, P = 0.376) and during 5% CO2 breathing (paired t-test, P = 0.543), were not significantly different between baseline measurements and the test period.

8-OH-DPAT experiments. To determine the effects of 8-OH-DPAT on ventilation, we measured the CO2 response before and during 8-OH-DPAT microdialysis into the medullary raphe. As a group (n = 27), 8-OH-DPAT did not significantly affect VE measured during room air breathing, VE while breathing 5% CO2, or the CO2 response (the ΔVE value when comparing breathing at 5% CO2 vs. room air breathing) (Table 2). This finding surprised us, because we hypothesized that inhibition of medullary raphe serotonergic neurons with 8-OH-DPAT would decrease the CO2 response.

Although the overall ventilatory response to 5% CO2 was unaffected by 8-OH-DPAT, some of the animals did show a marked decrease in their CO2 response with drug treatment. This finding led us to separate the animals into two groups on the basis of their response to 5% CO2 during 8-OH-DPAT microdialysis: group I included animals that showed a 5% or greater decrease in the CO2 response during 8-OH-DPAT microdialysis (n = 14), and group II included animals that did not meet group I criteria (n = 13).

Not surprisingly, the CO2 response in the group I animals was significantly decreased by 27% during 8-OH-DPAT dial-
analyses (paired t-test, $P < 0.001$) (Fig. 2A). $\dot{V}_E$ measured while piglets breathed 5% $CO_2$ was decreased by 16% during 8-OH-DPAT dialysis (paired t-test, $P < 0.05$), because of significant decreases in $V_T$ (10%) and $f_R$ (8%) (paired t-tests, $P < 0.05$). $\dot{V}_E$ measured during room air breathing was not significantly different during 8-OH-DPAT dialysis compared with baseline measurements (paired t-test, $P = 0.992$). $f_R$ while breathing room air was not significantly different between baseline and the 8-OH-DPAT test period (paired t-test, $P = 0.430$). However, $V_T$ during room air breathing was significantly lower during 8-OH-DPAT dialysis compared with predrug baseline measurements (paired t-test, $P = 0.024$) but only by 6%.

The $CO_2$ response in the group II animals was significantly increased by 19% during 8-OH-DPAT dialysis (paired t-test, $P = 0.002$) (Fig. 2B). $\dot{V}_E$ during 5% $CO_2$ breathing was increased by 11% because of a nonsignificant increase in $V_T$ (paired t-test, $P = 0.095$) and a significant increase in $f_R$ (8%) (paired t-test, $P = 0.002$). $\dot{V}_E$ measured during room air breathing was not significantly different between baseline and 8-OH-DPAT dialysis (paired t-test, $P = 0.864$). Likewise, $V_T$ in room air breathing (paired t-test, $P = 0.469$) and $f_R$ in room air breathing (paired t-test, $P = 0.443$) were similar at baseline and during the 8-OH-DPAT test period.

The group-averaged $\dot{V}O_2$ ($n = 22$) measured with piglets breathing room air was significantly increased, but only by 6%, during 8-OH-DPAT dialysis compared with baseline (paired t-test, $P = 0.036$) (Table 2). This overall small but significant increase in $\dot{V}O_2$ was the result of small but not significant increases in groups I and II. $\dot{V}O_2$ measured during breathing of room air (predrug, 19.3 ± 1; 8-OH-DPAT, 20.2 ± 2 ml $O_2$ · kg$^{-1}$ · min$^{-1}$) and of 5% $CO_2$ (predrug, 20.9 ± 1; 8-OH-DPAT 21.1 ± 2 ml $O_2$ · kg$^{-1}$ · min$^{-1}$) was increased, but not significantly, during 8-OH-DPAT treatment compared with baseline measurements in group I animals ($n = 12$) (paired t-tests, $P > 0.05$). Likewise, $\dot{V}O_2$ measured during breathing of room air (predrug, 19.5 ± 2; 8-OH-DPAT, 20.9 ± 2 ml $O_2$ · kg$^{-1}$ · min$^{-1}$) and of 5% $CO_2$ (predrug, 21.4 ± 2; 8-OH-DPAT, 23.1 ± 2 ml $O_2$ · Kg$^{-1}$ · min$^{-1}$) was increased, but not significantly, during 8-OH-DPAT dialysis compared with baseline measurements in group II animals ($n = 10$) (paired t-tests, $P > 0.05$).

$T_b$ measured during room air breathing or at the 5% $CO_2$ challenge was not significantly different between baseline and the 8-OH-DPAT test period (paired t-tests, $P > 0.05$) (Table 2).

Differences between group I and group II animals. To investigate the differences between group I and group II animals, we first looked at microdialysis probe placement (Fig. 1B). Although there were slight differences in probe location [e.g., midline ($n = 20$) vs. lateral ($n = 7$)], these differences did not explain the different $CO_2$ responses during 8-OH-DPAT treatment (unpaired t-test, $P = 0.416$).

### Table 1. Ventilatory parameters, $\dot{V}O_2$, and $T_b$ measured in the aCSF control experiments

<table>
<thead>
<tr>
<th>Ventilation</th>
<th>Room Air</th>
<th>5% $CO_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Test period</td>
</tr>
<tr>
<td>$\dot{V}_E$, ml·kg$^{-1}$·min$^{-1}$</td>
<td>492.4±53.4</td>
<td>543.8±60.3</td>
</tr>
<tr>
<td>$V_T$, ml/kg</td>
<td>11.0±1.09</td>
<td>11.7±1.23</td>
</tr>
<tr>
<td>$f_R$, breaths/min</td>
<td>47.1±4.60</td>
<td>49.0±5.07</td>
</tr>
<tr>
<td>$\dot{V}O_2$, ml $O_2$·kg$^{-1}$·min$^{-1}$</td>
<td>17.6±3.1</td>
<td>18.0±1.6</td>
</tr>
<tr>
<td>$T_b$, °C</td>
<td>38.2±0.3</td>
<td>38.3±0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 13$ piglets. $\dot{V}O_2$, metabolic rate; $T_b$, body temperature; aCSF, artificial cerebrospinal fluid; $\dot{V}_E$, minute ventilation; $V_T$, tidal volume; $f_R$, respiratory rate. Measurements were obtained in quiet wakefulness.
We then examined fluorescein spread to determine whether there was a difference between the two groups (Table 3). Data from three piglets were omitted from group I and data from two piglets were omitted from group II because there was no measurable fluorescein spread in these cases. We measured the average cross-sectional area of spread on an average of 7 sections per piglet over a rostrocaudal length of 1,447 μm that contained the largest fluorescein spread. Also calculated was the length (rostrocaudal extent) of spread. Volume of spread was then calculated by multiplying area and length values. From this quantification we did not find a significant difference between the two groups.

Having not found any difference in probe placement or fluorescein spread between group I and group II animals, we next examined piglet age (Table 4). We found a significant difference in that group I animals were ~4 days older than group II animals (unpaired t-test, \(P = 0.002\)). The “responder” group compared with the “nonresponder” group consisted of older piglets.

To further examine the role of age in the different response to \(\text{CO}_2\) during 8-OH-DPAT dialysis, we plotted the percent change in \(\dot{V}_E\) (our index of the \(\text{CO}_2\) response) as a function of piglet age (Fig. 3A). A linear regression analysis revealed a significant negative correlation (\(P < 0.001\)) between the two variables. In fact, 37% of the decrease in the \(\text{CO}_2\) response with 8-OH-DPAT treatment can be attributed to piglet age. The percent change in \(\dot{V}_E\) was also plotted against piglet age for the aCSF controls (\(n = 13\)) (Fig. 3B). The linear regression analysis was not significant (\(P = 0.302\)). There was no relationship among aCSF control piglets between the change in the \(\text{CO}_2\) response before and during “treatment” and piglet age.

MAP and HR. To determine whether 8-OH-DPAT altered MAP and HR, we examined both variables before and during
Table 3. Quantification of fluorescein spread in the 8-OH-DPAT experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Area, cm²</th>
<th>Length, cm</th>
<th>Volume, µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>11</td>
<td>0.010±0.003</td>
<td>0.557±0.45</td>
<td>6.1±2.3</td>
</tr>
<tr>
<td>Group II</td>
<td>11</td>
<td>0.007±0.002</td>
<td>0.443±0.39</td>
<td>3.1±1.1</td>
</tr>
<tr>
<td>P value (unpaired t-test)</td>
<td>0.413</td>
<td>0.070</td>
<td>0.254</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE.

8-OH-DPAT microdialysis (Fig. 4). Because there was no significant correlation between age and the % change in ΔMAP ($r = 0.282; r^2 = 0.079; P = 0.242$) or % change in ΔHR ($r = 0.090; r^2 = 0.008; P = 0.715$), data from all animals were pooled. MAP was significantly increased by 3% during room air breathing (paired $t$-test, $P < 0.05$) and significantly increased by 2% at the 5% CO2 challenge (paired $t$-test, $P < 0.05$) compared with baseline MAP ($n = 19$). HR measured with piglets breathing 5% CO2 was significantly decreased by 8% during 8-OH-DPAT dialysis compared with baseline HR (paired $t$-test, $P < 0.05$) ($n = 19$). Neither MAP nor HR measured during room air breathing or while breathing 5% CO2 were significantly different during the aCSF test period compared with baseline measurements in the aCSF control group (paired $t$-tests, $P > 0.05$) ($n = 11$).

Sleep cycling. Fourteen of the 27 animals that received 8-OH-DPAT were excluded from sleep analysis either because they did not meet the inclusion criteria ($n = 2$) or because of unscorable sleep data ($n = 12$). Thus the group averages are made up of data from the remaining 13 animals. Data from 7 of 13 aCSF control animals are also reported.

An unexpected finding from our experiments was a disruption of sleep cycling. To determine the extent of sleep disruption, we quantified the percentages of time spent awake, in NREM sleep, and in REM sleep according to previously described methods (14, 44). Only those piglets that cycled through NREM sleep in both of the predrug baselines (e.g., baseline I and baseline II) were included in sleep analysis. There was no difference in the percentages of time spent awake, in NREM sleep, or in REM sleep between these baselines, so the data were combined (one-way repeated-measures ANOVAs, $P > 0.05$). Both the predrug 5% CO2 challenge and the CO2 challenge performed during 8-OH-DPAT dialysis woke piglets, as seen by a greater percentage of time spent awake and less time spent in NREM and REM sleep (one-way repeated-measures ANOVA, $P < 0.001$; Tukey’s post hoc multiple comparison procedure, $P < 0.05$). Because there was not a significant interaction between 8-OH-DPAT and 5% CO2 (two-way repeated-measures ANOVA, $P = 0.419$), only sleep obtained during room air breathing is presented (Fig. 5). Piglets spent a greater percentage of time awake during 8-OH-DPAT dialysis (paired $t$-test, $P < 0.001$) and less time in NREM sleep (paired $t$-test, $P = 0.018$) and in REM sleep (paired $t$-test, $P = 0.024$) compared with baseline measurements (Fig. 5A). This sleep disruption is an effect of 8-OH-DPAT treatment, because the percentages of time spent awake (paired $t$-test, $P = 0.521$), in NREM sleep (paired $t$-test, $P = 0.809$), and in REM sleep (paired $t$-test, $P = 0.258$) were similar at baseline measurements and during the aCSF test period in the aCSF control experiments (Fig. 5B).

DISCUSSION

Main findings. Focal and reversible inhibition of serotonergic neurons in the medullary raphé and extra-raphé regions with 8-OH-DPAT had no overall significant effect on the CO2 response in 27 3- to 16-day-old piglets. However, further analysis showed that a subset of piglets that did demonstrate a reduced CO2 response were older. Linear regression analysis showed that 8-OH-DPAT treatment significantly affected the
CO₂ response in an age-dependent manner over the first 16 days of life, such that the drug exaggerated the response in younger piglets and decreased the response in older animals. Additionally, 8-OH-DPAT increased blood pressure and disrupted sleep cycling independent of age. Dialysis with aCSF in some of the same piglets had no such effects. Because of the fragmentary sleep data, it was not possible to examine the CO₂ response in sleep.

Neuroanatomy. Serotonergic neurons are located in the medullary raphe and in nearby extra-raphe regions of the medulla in the newborn piglet brain stem (47a). 5-HT₁₄ autoreceptors are also located in these regions, as evidenced by [³H]8-OH-DPAT binding (47a). Because the fluorescein spread in our experiments was visualized in these areas, we are confident that 8-OH-DPAT was microdialyzed near endogenous receptors on serotonergic neurons located in our region of interest. We note that the fluorescein spread may be an inexact estimate of 8-OH-DPAT diffusion from the microdialysis membrane because fluorescein does not have an affinity to a specific receptor.

The probe sites of the 27 animals reported in this study are all within our region of interest (Fig. 1). Thus it was initially surprising when 14 of these animals showed a decreased CO₂ response with 8-OH-DPAT treatment, whereas 13 showed either no change or an increased CO₂ response with 8-OH-DPAT. One possible explanation for this could be subtle differences in the depth of the microdialysis probe tip within the brain stem, (e.g., how dorsal the probe was positioned from the ventral surface). Lalley (37, 38) showed in decerebrate or anesthetized cats that electrical stimulation (100 Hz) of the medullary raphe results in either excitation or depression of phrenic motoneurons depending on where in the raphe the stimulation occurs; inhibition of phrenic nerve activity was seen during stimulation of the raphe obscurus and raphe magnus, whereas excitation of phrenic nerve activity was seen during stimulation of the more ventrally located raphe pallidus. However, we do not believe that different probe locations explain our results, because the microdialysis locations were similar in both groups of animals. Furthermore, those probes that were located more dorsal in the brain stem were not preferentially associated with a different response than those that were located more ventral. Bernard et al. (3) showed that focal acidification of the medullary raphe with acetazolamide stimulated phrenic nerve activity in anesthetized rats. However, not all acetazolamide injections stimulated ventilatory output. Furthermore, those injections that had no effect on phrenic nerve activity appeared to be located at similar rostro-caudal and dorsoventral locations as those injections that did

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**Fig. 4.** Mean arterial blood pressure (MAP) and heart rate (HR) measured while piglets breathed room air or 5% CO₂. Neither MAP nor HR was affected in the aCSF control experiments (n = 11 awake piglets) (paired t-tests, P > 0.05). However, there were significant differences in MAP and HR in the 8-OH-DPAT experiments (n = 19 awake piglets) (paired t-tests, P < 0.05). *Significant increase in MAP during 8-OH-DPAT microdialysis with piglets breathing room air; †significant increase in MAP during 8-OH-DPAT microdialysis with piglets breathing 5% CO₂; ‡significant decrease in HR with piglets breathing 5% CO₂.

**Fig. 5.** Mean ± SE values for the percentage of time spent awake (solid bars), in non-rapid eye movement (NREM) sleep (open bars), and in rapid eye movement (REM) sleep (shaded bars). A: sleep cycling with piglets breathing room air before (baseline) and during 8-OH-DPAT microdialysis into the medullary raphe (n = 13). Duration of baseline: 22 ± 1 min; duration of 8-OH-DPAT dialysis: 14 ± 1 min. 8-OH-DPAT disrupts sleep cycling, as evidenced by a significant increase in wakefulness (*), and significant decreases in NREM (†) and REM (‡) sleep (paired t-tests, P < 0.05). B: sleep cycling in the aCSF control experiments with piglets breathing room air before (baseline) and during the aCSF test period (n = 7). Duration of baseline: 19 ± min; duration of aCSF test period: 16 ± 2 min. Sleep cycling is not disrupted in the control experiments (paired t-tests for awake, NREM, and REM, P > 0.05).
elicits a response. This suggests that there are nonchemosensitive raphé neurons intermingled with chemosensitive ones. However, because the probe locations were similar for both group I and group II animals, we do not think that the differential responses exhibited by these groups were because of differences in brain stem regions affected by 8-OH-DPAT treatment.

The fluorescence volumes calculated in this study (Table 3) are similar to previously published data from our laboratory (12, 44). That there was not a significant difference in fluorescence spread between the group I and group II animals suggests that differences in drug spread cannot explain the differential CO₂ responses between the two groups. However, there was a trend toward a larger fluorescence spread in the group I animals, suggesting that a larger fluorescence spread, and thus a larger drug spread, may be necessary to see a decreased CO₂ response with 8-OH-DPAT treatment.

Ventilation. To our knowledge, no studies to date have examined the CO₂ response in newborn animals with specific inhibition of serotonergic neurons in and near the medullary raphé. Adult rat medullary raphé serotonergic neurons are chemosensitive in culture (63, 65). Medullary raphé serotonergic neurons are involved in the CO₂ response in awake adult cats, as the unit activity of these cells is increased in response to systemic hypercapnia (8–10%) (60). Finally, selective lesioning of medullary raphé serotonergic neurons with a novel serotonergic neurotoxin decreases the CO₂ response in awake and sleeping adult rats (47). Although these studies do not substantiate our findings of an age-dependent decrease in CO₂ sensitivity, they do support the hypothesis that medullary raphé serotonergic neurons are chemosensitive and involved in the CO₂ response.

The ventilatory response to CO₂ undergoes postnatal development in some species, including the rat. Although the CO₂ response is functional in these animals, CO₂ sensitivity declines over the first postnatal week and reaches a nadir around postnatal day 7, before returning at approximately postnatal day 10 (56, 66). This is in contrast to newborn piglets, in which CO₂ sensitivity is fully mature at birth and does not change with age (52, 68, 69). This was demonstrated in anesthetized piglets between postnatal 0–5 days and 23–29 days (52), as well as in anesthetized piglets between postnatal days 0 to 11 (68, 69). A likely difference in postnatal CO₂ sensitivity between the neonatal rat and newborn piglets can be attributed to the fact that the rat is much less developed at birth compared with the piglet. Although the magnitude of the CO₂ response is fully mature at birth in the newborn piglet, this does not mean that the medullary raphé is involved in the CO₂ response at this point. Wang and Richerson (64) showed that chemoreception develops with age in medullary raphé neurons, because cells studied in brain slices and in tissue culture that are younger than 12 days are less frequently stimulated by CO₂ than are those cells that are 12 days and older (64). Our data suggest that serotonergic raphé neurons are important in central chemoreception but only after a few days of age. We believe that our finding of an age-related decrease in the CO₂ response after 8-OH-DPAT treatment is due to inhibition of serotonergic neurons, not aging itself. This age-related finding was confirmed statistically by a significant (P < 0.001) negative correlation between the percent change in the CO₂ response with piglet age. The control animals, made up of a subset of those animals treated with 8-OH-DPAT (n = 13), did not show this relationship. Linear regression analyses for 8-OH-DPAT and aCSF piglet groups were examined separately because comparison of responses between the two groups was complicated by the large interpiglet variability of the initial CO₂ response values.

Our protocol was not designed to look at the CO₂ response as a function of piglet age. The experimental design was to study the CO₂ response in each piglet before and during treatment. However, we demonstrated a significant difference between the age of group I animals vs. the age of group II animals (Table 4, P = 0.002) that were treated with 8-OH-DPAT. Our linear regression data applied independently to the two treatment groups indicate that 8-OH-DPAT decreased the CO₂ response in older piglets (Fig. 3A) whereas aCSF has no such effect (Fig. 3B). Conversely, younger animals exhibited an enhanced CO₂ response during 8-OH-DPAT treatment. We cannot rule out the possibility that 8-OH-DPAT may have stimulated excitatory postsynaptic 5-HT₁A receptors in these piglets. Further study of age-dependent effects in piglets would be optimized by experiments that examine age and treatment effects within piglets.

The extent of medullary raphé inhibition and piglet age may be important determinants of the experimental outcome. Our results demonstrate that focal manipulation of the raphé results in small but significant decreases in the CO₂ response. This is in contrast to studies in which a substantial portion of the raphé is inhibited or lesioned by drug treatment. Dreshaj et al. (18) placed three to five microinjections (300 nl each) of either lidocaine or ibotenic acid along the rostrocaudal axis of the medullary raphé. Multiple microinjections along this axis with large drug volumes substantially reduced in the CO₂ response by 50% or more in decerebrate piglets. No detailed anatomy was provided in this study, so it is not possible to determine the region of drug spread. However, it is conceivable that the entire rostrocaudal axis of the raphé may have been inhibited. This suggests that a larger region of the raphé must be inhibited or lesioned before large decreases in CO₂ response are observed. Our results also indicate that age plays a role in the different response to drug treatment. In support of this, the decerebrate piglets studied by Dreshaj et al. were between 14 and 20 days old. The piglets used in the present experiments were between 3 and 16 days old. It may be that Dreshaj et al. were able to show a large decrease in the CO₂ response because of the older piglets used in that study.

MAP and HR. In our experiments, 8-OH-DPAT (10 or 30 mM) increased MAP independent of age (n = 19). Microinjection of 8-OH-DPAT into the raphé can produce variable results on MAP depending on dose used and injection location within the raphé. For example, microinjections of 3 or 6 mM 8-OH-DPAT into the raphé magnus and raphé pallidus decreased MAP and HR in anesthetized adult rats (59), whereas microinjections of 8 mM 8-OH-DPAT into the raphé obscurus increased MAP but did not alter HR in anesthetized adult rats (19). Additionally, intracerebroventricular administration of 8-OH-DPAT in unanesthetized adult rats increased MAP and HR at low doses (30 μM) but decreased MAP and HR at high doses (10 mM) (16). Therefore, differences in drug concentration, route of administration, and species differences appear to influence experimental outcomes.
Arousal. 8-OH-DPAT effects on sleep in the literature are inconsistent because the route of administration (e.g., intravenous vs. intraperitoneal vs. microdialysis), dose given, and 5-HT1A receptors affected by drug treatment (presynaptic vs. postsynaptic) (6, 21, 51). In the present experiments, 8-OH-DPAT disrupted sleep cycling, which was characterized by a significant increase in the percentage of time spent awake and significant decreases in the percentages of time spent in NREM and REM sleep. The increased wakefulness cannot be attributed to a fall in body temperature, because 8-OH-DPAT did not alter this variable (Table 2). Microdialysis of muscimol into the medullary raphe (44) and the retrotrapezoid nucleus (14) results in wakefulness in piglets (12, 14). 8-OH-DPAT dialysis into the nucleus paragigantocellularis lateralis also results in fragmented sleep in piglets (15). Why sleep cycling is disrupted by muscimol and 8-OH-DPAT remains to be elucidated.

The sleep circuitry in the brain is complex. Moreover, the medullary raphe has not been traditionally associated with sleep determination. It is the dorsal raphe nucleus that has been extensively studied in the sleep literature because of its projections to REM-generating neurons in the pedunculopontine and laterodorsal tegmentum of the midbrain (61). It is unlikely that the medullary raphe is mediating its effects on wakefulness through the dorsal raphe nucleus because there are few connections between the rostral and caudal raphe (61). One reason why we may have seen sleep disruption with 8-OH-DPAT dialysis could be through projections from the raphe to sleep and/or arousal related regions. Detailed anatomic analyses, using a variety of tract tracers, have demonstrated connections between subregions of the medullary raphe and brain regions associated with REM sleep (27, 53), NREM sleep (10), and wakefulness (36). A role for the raphe in the modulation of NREM sleep has been postulated (34). Alternatively, the medullary raphe sends extensive descending projections to the spinal cord, innervating both the dorsal horn and the ventral horn (7), where it is involved in the modulation of sensory and motor output (33). During wakefulness, serotonergic raphe neurons are involved in repetitive motor activities, such as breathing. Inhibition of motor activities (as evidenced by a decreased CO2 response) may lead to enhanced sensory processing, resulting in the arousal seen in our piglets.

8-OH-DPAT dosage. The initial dose of 8-OH-DPAT chosen for this study was 10 mM. However, when we did not see any apparent effect with this dose (because we had not discovered the age-related findings), we increased the dose to 30 mM. Although these doses may seem high, they are consistent with doses used by other investigators in the caudal brain stem (5, 19, 59). Furthermore, with the 10-fold drop in the drug concentration that occurs with the microdialysis technique (17), the brain tissue was most likely seeing a maximum drug concentration of 3 mM. It is believed that there are fewer 5-HT1A inhibitory autoreceptors located on medullary raphe serotonergic neurons compared with rostral raphe serotonergic neurons, which explains the faster firing rate of the medullary serotonergic cells (24, 58, 60). Furthermore, caudal raphe neurons are less responsive to 5-HT1A agonists (e.g., lysergic acid diethylamide and 5-methoxy-N,N-di-methyltryptamine) than are dorsal raphe neurons (25, 31). Thus, while those investigators who study the rostral raphe nuclei can treat with 10 μM-100 μM 8-OH-DPAT (50, 57), we needed to use a higher dose in the caudal raphe to elicit effects.

Relevance to SIDS. 8-OH-DPAT decreased the CO2 response in an age-dependent manner. This decrease was evident between postnatal days 6 and 8. This time frame appears to represent a period when a shift occurred from a 8-OH-DPAT-induced enhanced CO2 response in younger piglets to a decreased CO2 response in older animals. This implies an age-related shift in serotonergic neuron function in chemoreception. We can loosely correlate this to SIDS, in which there is a critical period during which the majority of SIDS cases occurs, notably 2–4 mo of age (67). Interestingly, whereas the CO2 response was decreased in an age-dependent manner with 8-OH-DPAT, sleep cycling disruption was independent of piglet age, as were the cardiovascular effects observed. This suggests that the neurons inhibited by 8-OH-DPAT may have different functions (CO2 sensing vs. sleep modulation), differ in cell phenotype (e.g., serotonin with different coexisting neuropeptides), or have different anatomical projections. In support of this, Bernard (4) showed in anesthetized rats that excitation of the medullary raphe by excitatory amino acid microinjection sometimes elicited a cardiovascular response, whereas other times it elicited both cardiovascular and respiratory responses. This suggests that neuronal groups involved in different homeostatic processes may be separately organized or intermingled together within the raphe, and different results can be obtained depending on whether these groups are stimulated alone or in combination.

Serotonergic neurons in the medullary raphe and at nearby extra-raphe locations are connected anatomically (70–72) and are involved in the modulation of respiration, central chemoreception, upper airway integrity, and cardiovascular control (3, 22, 46, 63). A decrease in serotonergic receptor binding (5-HT1A-D and 5-HT2) in these medullary nuclei has been reported in SIDS (48), suggesting that a deficiency in the medullary serotonergic system can be fatal (35). Dysfunction of this serotonergic system during a critical period of development could prevent an infant from responding to life-threatening challenges in sleep. In conclusion, medullary raphe serotonergic neurons are involved in central chemoreception in newborn piglets, and disruption of their function in humans could contribute to the pathogenesis of SIDS. It seems possible that the altered chemoreception uncovered in our study may reflect a broader array of dysfunction present when serotonergic neurons are abnormal. Sudden death, something difficult to imagine solely as the result of decreased ventilatory CO2 sensitivity, could be promoted by a range of functional abnormalities attributable to altered serotonergic neuron function.

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J Appl Physiol • VOL 96 • MAY 2004 • www.jap.org
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