Chronic fluoxetine microdialysis into the medullary raphe nuclei of the rat, but not systemic administration, increases the ventilatory response to CO₂

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Taylor, Natalie C., Aihua Li, Adam Green, Hannah C. Kinney, and Eugene E. Nattie. Chronic fluoxetine microdialysis into the medullary raphe nuclei of the rat, but not systemic administration, increases the ventilatory response to CO₂. J Appl Physiol 97: 1763–1773, 2004. First published July 23, 2004; doi:10.1152/japplphysiol.00496.2004.—In conscious rats, focal CO₂ stimulation of the medullary raphe increases ventilation, whereas interference with serotonergic function here decreases the ventilatory response to systemic hypercapnia. We sought to determine whether repeated administration of a selective serotonin reuptake inhibitor in this region would increase the ventilatory response to hypercapnia in unanesthetized rats. In rats instrumented with electroencephalogram-electromyogram electrodes, 250 or 500 μM fluoxetine or artificial cerebrospinal fluid (aCSF) was microdialyzed into the medullary raphe for 30 min daily over 15 days. To compare focal and systemic treatment, two additional groups of rats received 10 mg·kg⁻¹·day⁻¹ fluoxetine or vehicle systemically. Ventilation was measured in normocapnia and in 7% CO₂ before treatment (day 0), acutely (days 1 or 3), on day 7, and on day 15. There was no change in normocapnic ventilation in any treatment group. Rats that received 250 μM fluoxetine microdialysis showed a significant 13% increase in ventilation in wakefulness during hypercapnia on day 7, due to an increase in tidal volume. In rats microdialyzed with 500 μM fluoxetine, there were 16 and 32% increases in minute ventilation during hypercapnia in wakefulness and sleep on day 7, and 20 and 28% increases on day 15, respectively, again due to increased tidal volume. There was no change in the ventilatory response to CO₂ in rats microdialyzed with aCSF or in systemically treated rats. Chronic fluoxetine treatment in the medullary raphe increases the ventilatory response to hypercapnia in an unanesthetized rat model, an effect that may be due to facilitation of chemosensitive serotonergic neurons.

brain stem; chemoreceptor; serotonin transporter; selective serotonin reuptake inhibitor

THE MEDULLARY RAPHE NUCLEI of the mammalian brain stem, comprised of the raphe magnus, obscurus, and pallidus, are understood to modulate several homeostatic systems throughout the body. Serotonergic neurons that are located both within these nuclei and parapyramidal along the ventral surface of the medulla project to regions in the caudal brain stem and spinal cord involved in sensory, motor, and autonomic functions (12). These neurons have also been implicated in the ventilatory response to CO₂ (11, 33, 34, 40, 43, 54, 55). Moreover, electrical and chemical stimulation of the medullary raphe nuclei affects phrenic nerve output (3, 27), and antidiromic stimulation of the phrenic motor nucleus at the C₄ spinal level has been found to directly activate a population of medullary raphe neurons (21).

With regard to respiration, the medullary raphe nuclei have been shown to be one of several brain stem regions of central chemoreception (4), which can be defined as regions capable of specifically responding to changes in tissue pH and/or Pco₂ by increasing ventilation in the whole animal (38). In one study, microdialysis delivery of artificial cerebrospinal fluid (aCSF) equilibrated with 25% CO₂ to the medullary raphe at the level of the facial nucleus increased frequency and ventilation by 15–20% in an unanesthetized rat model, and the effect was only seen in non-rapid eye movement (NREM) sleep (40). A similar study performed in unanesthetized goats found that microdialysis of aCSF equilibrated with 25 and 80% CO₂ increased ventilation by 8 and 12% respectively; however, in this experiment the ventilatory effect was not observed in NREM or rapid eye movement (REM) sleep (20). These data suggest that medullary raphe chemosensitivity may vary across arousal states, because serotonergic medullary raphe neurons are most active during wakefulness, less active through the stages of NREM sleep, and fall nearly silent during REM sleep (23). Chemosensitivity of medullary raphe neurons has also been demonstrated specifically in primary tissue culture, revealing two morphologically distinct subsets of chemosensitive neurons: one that increases firing rate in response to acidosis/Pco₂ increase and one that is inhibited by it (56). Further in vitro studies exploring the neurochemical phenotypes of chemosensitive medullary raphe neurons revealed that acidosis/Pco₂-stimulated medullary raphe neurons are entirely serotonergic, whereas acidosis/Pco₂-inhibited neurons are not (57). Taken together, these studies suggest that the chemoreceptive properties of the medullary raphe nuclei are functionally linked to serotonergic mechanisms.

There is additional evidence in whole animal studies that suggest serotonergic function in the medullary raphe nuclei is important for the ventilatory response to hypercapnia. Inhibition of medullary raphe neurons by focal microdialysis of the GABAₐ receptor agonist muscimol (10 mM) significantly reduced ventilation by 17% in an unanesthetized piglet model during a 5% CO₂ challenge but not in room air breathing (34). Specific inhibition of serotonergic neurons in the medullary raphe via microdialysis of the serotonin (5-hydroxytryptamine) type 1A (5-HT₁A) autoreceptor agonist 8-hydroxy-2-di-n-propylaminotetraline (8-OH-DPAT) reduced ventilation by 22 and 26% respectively on days 7 and 15, respectively, whereas microdialysis into the medulla of aCSF microdialyzed into the medullary raphe increased ventilation by 10.2% on day 7, and by 17% on day 15, in an unanesthetized piglet model (34). Differences in the magnitude of the effects observed in these studies may reflect differences in the microdialysis delivery or the experimental setup used (34). In addition, the lack of a consistent effect of this method of inhibition in the acute state (34) may reflect the transient nature of serotonergic activity in response to heightened ventilation (40).

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pylaminotryptaline (8-OH-DPAT) was also capable of decreasing the ventilatory response to 5% CO₂ during wakefulness in an unanesthetized piglet model, as long as the piglets were over 10 days old (33). In a focal lesioning study performed recently, targeted killing of serotonergic neurons in the medullary raphe nuclei and parapyramidal regions of a rat model using microinjection of a serotonin transporter antibody-saporin conjugate led to a 15 and 18% decrease in ventilation during 7% CO₂ during sleep and wakefulness, respectively. Immunohistological analysis confirmed that only serotonergic neurons were affected by the treatment and that 28% of these neurons were destroyed using the targeted cytotoxin (43). Therefore, serotonergic neurons in the medullary raphe are capable of influencing ventilation during hypercapnia in fully conscious animal models during sleep and wakefulness.

Due to the fact that disruption of serotonergic neurons leads to a decrease in the ventilatory response to hypercapnia in conscious mammals, and serotonergic neurons in the medullary raphe exhibit chemosensitivity in both in vivo and in vitro studies, we hypothesize in this study that focal facilitation of serotonergic activity in the medullary raphe will lead to an increase in the ventilatory response to hypercapnic stress. In an attempt to increase serotonergic function in only medullary raphe neurons, we chose to microdialyze a solution containing the selective serotonin reuptake inhibitor (SSRI) fluoxetine directly into the medullary raphe nuclei at the level of the facial nucleus. Fluoxetine, like other widely prescribed SSRI antidepressants, is capable of acutely increasing extracellular serotonergic activity in the central nervous system. However, it does not show clinical efficacy until after several days of chronic treatment, suggesting that long-term neurochemical changes are responsible for its therapeutic action (for review, see Ref. 53). Taking this into consideration, we have developed our protocol to measure the ventilatory response to CO₂ in an unanesthetized rat immediately, 1 wk, and 2 wk after daily fluoxetine microdialysis in the medullary raphe. We also considered the effect of sleep and wakefulness on ventilation, because the ability of medullary raphe neurons to influence the ventilatory CO₂ response may vary across different behavioral states. Finally, we wanted to determine whether systemic fluoxetine administration would change the ventilatory response to hypercapnia in a potentially similar manner as daily focal microdialysis using a similar ventilation protocol.

METHODS

Animals and instrumentation. Forty-two adult male Sprague-Dawley rats weighing 300–450 g were housed in a temperature-controlled environment on a 12:12-h light-dark cycle, with the period of light taking place between midnight and noon to facilitate recording of ample sleep and wake data during the working day. Animals received rat chow and water ad libitum. All surgical and experimental protocols were approved by the Institutional Animal Care and Use Committee of Dartmouth College. Rats were allowed to recover for at least 4 days after surgical instrumentation before the start of experimentation.

Rats were anesthetized with ketamine (100 mg/kg im) and xylazine (20 mg/kg ip), and depth of anesthesia was evaluated throughout surgery by response to firm hindpaw pinching. The abdominal surface, neck, and head were shaved and cleaned with betadine and 70% ethyl alcohol. A sterilized telemetric temperature probe (model TA-F20, Data Sciences International, St. Paul, MN) was first inserted into the peritoneal cavity by an incision through the linea alba and the wound was closed.

The head of the animal was then secured and centered on a Kopf stereotaxic apparatus, and the surface of the skull was exposed. Three EEG electrodes were screwed into the right side of the skull: one was placed 2 mm rostral to bregma and 2 mm lateral to the midline, one was placed 2 mm rostral to lambda and 2 mm lateral to the midline, and a ground electrode was placed laterally between the two. Two wire electromyogram (EMG) electrodes and a ground were then inserted into the neck skeletal muscles, and all electrode wires were put into a small six-prong plastic pedestal. In animals receiving microdialysis, a microdialysis probe cannula with a dummy insert was placed 11.0–12.8 mm caudal to bregma and 0 mm from the midline, and 10.4–10.6 mm below the dorsal surface of the cerebellum into the medullary raphe nuclei at the level of the facial nuclei. Skull measurements were in accordance with the rat stereotaxic atlas of Paxinos and Watson (46). The EEG-EMG electrodes and probe cannula were secured onto the surface of the skull with cranioplast cement, and the skull incision was sutured.

Microdialysis. The microdialysis probe used in this study had an 11-mm stainless steel shaft (0.38 mm OD) and a 1-mm cuprophane membrane tip, allowing diffusion of molecules up to 6,000 Da (model CMA/11, CMA Microdialysis, Solna, Sweden). Flow through the probe during dialysis of solutions was 0.05 ml/h. aCSF was used as the drug vehicle and for control experiments, and contained (in mM) 152.0 sodium, 3.0 potassium, 2.1 magnesium, 2.2 calcium, 131.0 chloride, and 26.0 bicarbonate. aCSF was equilibrated with 5% CO₂ before addition of the calcium. Fluoxetine HCl (Sigma, St. Louis, MO) was prepared in aCSF as a 1 mM stock solution and diluted to 250 or 500 µM concentrations directly before microdialysis. Concentrations of fluoxetine in dialysate were chosen on the basis of a previous study using in vivo microdialysis of fluoxetine in rats (59).

Systemic administration of fluoxetine. In the rat groups receiving systemic drug administration, animals were reanesthetized and an osmotic minipump (model Alzet 2ML4, Durect, Cupertino, CA) was surgically placed inside of the abdominal cavity after pretreatment (day 0) recording. Osmotic minipumps allow for constant drug infusion and therefore maintain steady serum concentrations. The concentration of fluoxetine (Eli Lilly, Indianapolis, IN) added to each pump was calculated on the animal’s body weight and the flow rate of the minipumps (manufacturer’s specifications), such that each animal received 10 mg·kg⁻¹·day⁻¹ fluoxetine for the 15-day period. This dose was chosen on the basis of previous studies employing minipumps for fluoxetine delivery in models of antidepressant activity in rats (18, 58). A sham control group of animals were implanted with osmotic minipumps containing vehicle only (75% polyethylene glycol and sodium chloride). Ventilation recording in the two systemic groups was performed to determine acute fluoxetine effects on breathing took place 3 days after osmotic minipump implantation, so animals had time to recover after this second surgery. After the 15-day period, animals were euthanized and the osmotic minipumps were removed. We checked each pump reservoir for any remaining solution and ensured that each animal included in data analysis had received the correct volume of drug or vehicle over the course of the experiment.

Ventilation, V₀₂, temperature, and EEG-EMG measurement. Ventilation was recorded inside a whole body plethysmograph as previously described (41, 42) on the basis of the setup of Jacky (22) and Pappenheimer (45). Briefly, the analog signal from the pressure transducer was digitally converted and sampled at 150 Hz at a computer interface by using the Datapac 2000 system (RUN Technologies, Laguna Hills, CA). The plethysmograph was at atmospheric pressure, with inflow and outflow rates balanced to one another. Inflow rates to the plethysmograph were kept ≥1.4 l/min (flowmeter model 601E, Matheson Tri-gas, Montgomeryville, PA) to prevent excess accumulation of CO₂. Another flowmeter modulated outflow to an in-house vacuum. O₂ and CO₂ within the chamber were sampled (~100 ml/min) in the outflow line by a combined O₂ and CO₂ analyzer (Gemini Respiratory Gas Analyzer, CWE, Ardmore, PA).
Before each experimental day, the plethysmograph was calibrated with five 0.3-ml air injections. O2 consumption (VO2) was measured by applying the Fick principle and calculating the difference in O2 content between inspired and expired gas [VO2 = (Vins × FO2ins) - (Vout × FO2out)], where Vins and Vout represent inflow and outflow flow rates, and FO2ins and FO2out represent the fraction of inflow and outflow O2. VO2 was normalized with body weight and expressed as milliliters per gram per hour. Inflow content of O2 was measured at the beginning of the experiment before the rat was placed into the box. A thermometer inside the chamber provided chamber temperature, and rat body temperature was measured using the analog signal generated by the telemetric temperature probe in the abdomen. EEG and EMG signals were sampled at 150 Hz and filtered at 0.3–50 and 0.1–100 Hz, respectively, and recorded on the computer interface system directly using the Datapac 2000 system.

**Microdialysis experimental protocol.** There were three groups of animals in our experimental design: rats receiving microdialysis of aCSF (n = 8), 250 (n = 8), and 500 μM fluoxetine HCl (n = 10) in aCSF. On *day 0* of the experiment (pretreatment), the rats were placed into the plethysmograph chamber and EEG-EMG were connected. Rats were allowed to acclimatize for at least 30 min and recording of ventilation did not commence until it was clear that the rat was relaxed and no longer exploring. Thirty minutes of ventilation data during room air were recorded to record noticeable periods of sleep and wakefulness. Then the inflow of the plethysmograph was changed to a mixture of 7% CO2 and room air (a moderate hypercapnic stress that produces marked increases in breathing frequency and VT) and 30 min of hypercapnic ventilation commenced when the plethysmograph reached 7% CO2 according to the gas analyzer. On the second day of the experiment (*day 1*), the microdialysis probe was primed with aCSF or fluoxetine solution and placed into the medulla. The animal was again placed into the plethysmograph chamber, and it received 30 min of microdialysis treatment before the ventilation experiment described above was begun. Microdialysis of 250 and 500 μM fluoxetine for 30 min at a flow rate of 0.05 ml/h results in a total of 6.25 and 12.5 nmol fluoxetine, respectively, traveling through the microdialysis probe in the dialysate. From *days 2* to 6 and 8 to 14 of the experiment, the animal received 30-min microdialysis treatments once every 24 ± 2 h. On *days 7* and 15, the ventilation experiment was again performed after 30 min of microdialysis, allowing us to compare pretreatment ventilation values immediately, 1 wk, and 2 wk after microdialysis treatment into the medullary raphe region.

**Systemic administration experimental protocol.** The experimental protocol for rats receiving systemic fluoxetine (n = 8) or vehicle (n = 8) administration was identical to our microdialysis experiments with two exceptions: 1) the acute ventilation experiments were performed on *day 3* after osmotic minipump implantation so that rats were recovered from the second surgery; and 2) obviously, these rats did not receive microdialysis.

**Data analysis.** Sleep analysis was completed by using both the raw EEG-EMG record and the fast Fourier transform of the EEG at 3.6-s-long epochs. Delta, theta, and sigma frequency bands were characterized as 0.3–5, 6–9, and 10–17 Hz, respectively. The ventilation experiments occurred at random times both before and during the dark period began in the housing environment (9 AM to noon) or after the dark period had begun (noon to 5 PM). States of arousal were defined as previously described (40, 41). Wakefulness was characterized when the raw EEG signal was low, EMG activity was present, and delta power was low. NREM sleep was characterized when the raw EEG signal was high, EMG activity was low, delta power was high, and the product of sigma and theta was high. REM periods were brief but characterized by low raw EEG signal, absent EMG activity, low delta power, and high theta-to-delta ratio. Respiration events that occurred during the REM sleep were excluded because the recording period in this study was not long enough to achieve ample REM in all experiments. There were also instances when it was difficult to determine sleep state, so respiration events from those periods were also excluded. When the amount of time spent in each sleep state was analyzed, REM periods were grouped together with the periods when the arousal state was indeterminate.

Ventilation was analyzed by examining individual breath events with the Datapac 2000 software. Sighs, sniffing, and periods of high rat activity (moving, grooming) were eliminated from the analysis, and posthypneapneas were not included in the analysis. The frequency and raw pressure data were grouped into bins of ≥50–400 breath events, and differentiated by quiet wakefulness and NREM states. Data were exported to spreadsheet, and VT and breathing frequency were calculated for each respiratory event using chamber temperature, body temperature, and the barometric pressure. VT and VE were normalized per 100 g rat body weight. VT, VE, and breathing frequency are reported as mean values for quiet wakefulness (quiet wakefulness) and NREM sleep during room air breathing and hypercapnia over the course of each experimental day. All data were analyzed by using one-way repeated-measures (RM) and two-way ANOVAs or t-tests as appropriate (Sigma-Stat, Jandel Scientific). In certain cases, Friedman’s RM ANOVA on ranks had to be performed. Post hoc tests were performed when significant differences between treatments and/or experimental day were found.

**RESULTS**

**Body weight, body temperature, and VO2.** Mean body weights for each treatment group at the start (*day 0*) and conclusion (*day 15*) of the experimental period were as follows (±SE): aCSF microdialysis group, 328.4 ± 17.9 and 338.9 ± 17.7 g; 250 μM fluoxetine microdialysis group, 307.2 ± 22.2 and 325.4 ± 19.3 g; 500 μM fluoxetine microdialysis group, 331.2 ± 17.6 and 347.1 ± 14.6; systemic vehicle group, 343.6 ± 18.3 and 381.5 ± 16.1 g; and systemic fluoxetine group, 310.4 ± 9.2 g and 345.2 ± 6.6 g. The *day 0–15* increases seen in body weight within the three rat groups receiving microdialysis did not differ significantly from each other, nor did the increases seen in the two systematically treated groups (1-way ANOVA with treatment as the factor). Repeated fluoxetine (250 and 500 μM) or aCSF microdialysis into the medullary raphe did not significantly alter core body temperatures in room air or in 7% CO2. Similarly, chronic systemic infusion of vehicle or 10 mg·kg⁻¹·day⁻¹ fluoxetine did not change core body temperatures seen during our ventilation experiments. VO2 during normocapnic breathing, calculated by application of the Fick principle, also did not change significantly within any of our treatment groups during the 15-day experimental period. Statistical analyses of VO2 and core body temperature data were done with one-way RM ANOVA using experiment day as the factor, with each animal serving as its own pretreatment (*day 0*) control.

**Sleep states.** We categorized vigilance states into periods of quiet wakefulness, NREM, and indeterminate (which included any periods of REM) during our ventilation experiments based on EEG-EMG activity as described. It is important to reiterate that these experiments were designed to measure ventilation with regard to sleep state and to be able to detect any potentially large changes in ventilation during quiet wakefulness or NREM states due to drug administration; however, our 30-min recording period during room air and 7% CO2 is not long enough to adequately describe changes in sleep cycling due to fluoxetine. Percent experimental time spent in quiet wakefulness and NREM states during the 30-min room air and 7% CO2 recording periods on the 4 experimental days are shown in Table 1. Percent time in the indeterminate state never
There were no significant changes in percent time in quiet wakefulness, NREM, or indeterminate states over the 15-day experiment in any of the five treatment groups when each animal served as its own control (1-way RM ANOVA with experiment day as the factor). We also found no change in the percent time in quiet wakefulness, NREM, or indeterminate states over the 15 days when aCSF, 250 μM fluoxetine, and 500 μM fluoxetine microdialysis groups or when the systemic vehicle and 10 mg·kg⁻¹·day⁻¹ fluoxetine microdialysis groups were compared with one another (2-way ANOVA with treatment and experiment day as factors).

Ventilation and fluoxetine microdialysis into the medullary raphe. Ventilation data from the rat group that received daily 30-min microdialysis treatments of aCSF alone are shown in Fig. 1. Chronic aCSF microdialysis did not effect $V_e$, $V_t$, or breathing frequency during normocapnia or during 7% CO₂ breathing (1-way RM ANOVA with experiment day as the factor). Ventilation parameters during quiet wakefulness were similar to those seen in NREM.

In rats that received microdialysis treatments of aCSF containing 250 μM fluoxetine, $V_e$, $V_t$, and breathing frequency during room air recording did not change significantly over the experimental period in quiet wakefulness (Fig. 2). Because of technical problems, we were not able to obtain sleep data for four of the rats in this group, preventing statistical analysis of NREM ventilation parameters. During 7% CO₂ breathing, $V_e$ in quiet wakefulness was seen to significantly increase across the experiment days ($P ≤ 0.05$, 1-way RM ANOVA), although post hoc analysis did not reveal any significant changes between day 0 (pretreatment) and days 1, 7, or 15. The increase in $V_e$ observed over the course of the experiment was not due to a change in breathing frequency, but a significant increase in $V_t$ on day 7 compared with day 0 ($P ≤ 0.05$ 1-way RM ANOVA). Dunnett post hoc compared with day 0. On day 15, $V_t$ during 7% CO₂ breathing appeared to remain elevated; however, this was not significant in post hoc analysis. NREM ventilation data during hypercapnia mirrored that of quiet wakefulness, but it could not be analyzed due to the small subject size.

When the concentration of fluoxetine in the dialysate was increased to 500 μM, $V_e$, $V_t$, and breathing frequency during room air breathing did not change over the experimental period in either quiet wakefulness or NREM (Fig. 3). During hypercapnic breathing however, there was a significant increase observed in $V_e$ on days 7 and 15 compared with day 0 in quiet wakefulness and on days 1, 7, and 15 compared with day 0 in NREM ($P ≤ 0.05$ 1-way RM ANOVA, Dunnett post hoc). As seen in the animal group microdialyzed with 250 μM fluoxetine, the increase in $V_e$ was due to an increase in $V_t$ only. In quiet wakefulness, $V_t$ in 7% CO₂ conditions was significantly increased compared with day 0 on days 7 and 15 ($P = 0.005$ Friedman’s RM ANOVA on ranks, Dunnett’s post hoc comparison to day 0). In NREM, $V_t$ was also significantly increased on days 7 and 15 compared with day 0 ($P = 0.01$ 1-way RM ANOVA, Dunnett’s post hoc comparison to day 0). There did not seem to be a definitive change in breathing.

### Table 1. Percent experiment time in QW or non-REM states during 30 min of room air recording

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day 0</th>
<th>Day 1 or 3</th>
<th>Day 7</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QW</td>
<td>NREM</td>
<td>QW</td>
<td>NREM</td>
</tr>
<tr>
<td>aCSF microdialysis</td>
<td>61.3±5.8</td>
<td>35.6±4.2</td>
<td>62.8±3.6</td>
<td>43.4±3.3</td>
</tr>
<tr>
<td>250 μM fluoxetine microdialysis</td>
<td>64.4±6.5</td>
<td>34.5±8.8</td>
<td>68.9±8.8</td>
<td>30.7±8.8</td>
</tr>
<tr>
<td>500 μM fluoxetine microdialysis</td>
<td>62.3±4.9</td>
<td>41.3±5.7</td>
<td>55.1±6.5</td>
<td>42.1±6.6</td>
</tr>
<tr>
<td>Systemic vehicle</td>
<td>67.7±11.2</td>
<td>30.4±10.3</td>
<td>66.4±8.2</td>
<td>31.8±7.9</td>
</tr>
<tr>
<td>Systemic fluoxetine (10 mg·kg⁻¹·day⁻¹)</td>
<td>62.9±6.4</td>
<td>33.3±6.1</td>
<td>59.4±4.5</td>
<td>39.6±4.6</td>
</tr>
</tbody>
</table>

Values are means ± SE given in %. QW, quiet wakefulness; NREM, non-rapid eye movement; aCSF, artificial cerebrospinal fluid. Day 1 applies to fluoxetine microdialysis experiments and day 3 to systemic experiments.
nerve) and between probe tips are shown in Fig. 4. All tips fell in the midline on histological analysis. The locations of the microdialysis cannulas placed within the region of the medullary raphe nuclei were confirmed. During 7% CO₂ breathing (1-way repeated-measures ANOVA), but there was no post hoc significance. Additionally, in the aCSF microdialysis control group, there was no change in the ventilation as a result of microdialysis probe presence in brain stem tissue.

Ventilation and systemic fluoxetine administration. Interestingly, systemic fluoxetine administration did not lead to the same ventilation effect during hypercapnia that was seen with focal fluoxetine administration into the medullary raphe. Neither systemic vehicle (Fig. 6) nor 10 mg·kg⁻¹·day⁻¹ fluoxetine (Fig. 7) infusion via the intraperitoneally placed osmotic minipump led to change in VE, breathing frequency, or VT during room air or during 7% CO₂ conditions in either quiet wakefulness or NREM over the 15-day experimental period (1-way RM ANOVA). With measurement of osmotic minipump reservoir volume at the conclusion of all systemic experiments, it was found that every rat had received the appropriate volume of either vehicle or fluoxetine solution.

Comparison among microdialysis and systemically treated groups. The design of this experiment was such that each animal would serve as its own pretreatment (day 0) control on frequency during hypercapnia, and there was actually a significant decrease in breathing frequency seen in quiet wakefulness on day 15 compared with day 0 (Friedman’s RM ANOVA on ranks, Dunnett post hoc compared with day 0).

The rats considered in the three microdialysis groups all had cannulas placed within the region of the medullary raphe nuclei on histological analysis. The locations of the microdialysis probe tips are shown in Fig. 4. All tips fell in the midline between −11.0 mm (the caudal aspect of the root of the facial nerve) and −12.8 mm from bregma (the level of the inferior olive). A representative brain stem slice taken from an animal that received 15 days of microdialysis treatment is shown in Fig. 5. Tissue damage was observed surrounding the area of the probe; however, it most likely did not interfere with the ability of fluoxetine to access neurons within the medullary raphe, because ventilation effects were still observed as a result of fluoxetine microdialysis on day 15 (the final day of the experiment). Additionally, in the aCSF microdialysis control group, there was no change in the ventilation as a result of microdialysis of a solution into the brain stem or due to long-term microdialysis probe presence in brain stem tissue.

Fig. 2. Absolute ventilation values in rats microdialyzed with 250 μM fluoxetine. Mean (±SE) VE, VT, and f values are shown for 30 min of room air and 30 min of 7% CO₂ recording during quiet wakefulness (n = 8; *) and non-rapid eye movement sleep (n = 4; ○) over the 4 experimental days. Daily 250 μM fluoxetine microdialysis over a 15-day period did not change ventilation during room air (1-way repeated-measures ANOVA), but there was no post hoc significance. During 7% CO₂ breathing in quiet wakefulness, 250 μM fluoxetine treatment led to a significant increase in tidal volume on day 7 compared with day 0 (*P ≤ 0.05, 1-way repeated-measures ANOVA, Dunnett post hoc compared with day 0).

Fig. 3. Absolute ventilation values in rats microdialyzed with 500 μM fluoxetine. Mean (±SE) VE, VT, and f values are shown for 30 min of room air and 30 min of 7% CO₂ recording during quiet wakefulness (n = 10; *) and non-rapid eye movement sleep (n = 10; ○) over the 4 experimental days. Daily 500 μM fluoxetine microdialysis does not change ventilation during room air (1-way repeated-measures ANOVA). During 7% CO₂ breathing, there was a significant increase in VE on days 7 and 15 compared with day 0 in both quiet wakefulness and in non-rapid eye movement sleep sleep (*P ≤ 0.05 in wakefulness and #P ≤ 0.05 in sleep, 1-way repeated-measures ANOVA, Dunnett post hoc compared with day 0). The increase in VE was due to a significant increase in VT during hypercapnia in wakefulness and sleep on days 7 and 15 and not to a change in f. During hypercapnia, f actually was found to be significantly decreased in wakefulness on day 15 compared with day 0.
subsequent experimental days over a 2-wk period. We also had in place an aCSF microdialysis group and a systemic vehicle group to serve as sham controls for multigroup comparisons in our focal and systemic experiments and to evaluate any non-specific effects of microdialysis probe or osmotic minipump placement. The primary ventilation effect that we saw during fluoxetine microdialysis into the medullary raphe was a significant increase in $V_\dot{E}$ during hypercapnia on days 7 and 15 due to an increase in $V_T$. Therefore, we compared the percent change in absolute ventilation during 7% CO$_2$ in aCSF, 250 μM fluoxetine, and 500 μM fluoxetine groups to determine whether the groups differed from one another (Fig. 8). We found that the percent change in $V_\dot{E}$ on days 1, 7, and 15 during hypercapnic breathing was significantly higher in the 500 μM fluoxetine microdialysis group compared with the aCSF microdialysis group in both quiet wakefulness and NREM ($P \leq 0.05$, 2-way ANOVA with treatment and experiment day as factors, Dunnet’s post hoc comparison to aCSF), although...
there was no significant interaction between treatment and experiment day. The 250 μM fluoxetine microdialysis group showed an increased percent change in hypercapnic \( V_e \) in quiet wakefulness and NREM, but this was not significant. We also compared percent change in hypercapnic \( V_e \) during quiet wakefulness and NREM between our systemic vehicle and 10 mg·kg\(^{-1}\)·day\(^{-1}\) fluoxetine groups, but there was no difference found in either treatment or experiment day nor was there an interaction (2-way ANOVA, data not shown).

**DISCUSSION**

The data in this study show that daily focal fluoxetine microdialysis into the medullary raphe nuclei of the rat over a 15-day period can enhance the ventilatory response to moderate hypercapnia during both quiet wakefulness and NREM sleep in an unanesthetized rat model. The increase in hypercapnic ventilation was due to an increase in \( V_t \) and not in breathing frequency, and the response did not become clear until after several days of microdialysis treatments had taken place. Microdialysis of the larger 500 μM concentration led to the larger increase in the hypercapnic response, a 16 and 32% increase in \( V_e \) in 7% CO\(_2\) conditions during wakefulness and sleep on day 7, and a 20 and 28% increase on day 15 (Fig. 8). Systemic fluoxetine treatment of 10 mg·kg\(^{-1}\)·day\(^{-1}\) via osmotic minipump did not change the ventilatory response to hypercapnia (Figs. 6 and 7) with the use of a similar experimental protocol.

To our knowledge, this is the first study that has used repeated daily reverse microdialysis of an SSRI in a region of serotonergic neurons over the course of 2 wk. There have been other studies in which microdialysis was used to deliver fluoxetine to different rat brain regions, for example, the arcuate nucleus and nucleus accumbens (59), the mediodorsal hypothalamus (32), the striatum (30), and the frontal cortex (14). However, these studies only considered the acute effects of fluoxetine microdialysis on neurotransmitter concentrations. In this study, we used chronic fluoxetine microdialysis in an attempt to enhance the function of serotonergic neurons located in the medullary raphe and perhaps to potentiate their chemoreceptive properties. Due to the fact that SSRIs have a delayed onset of therapeutic action of 2–4 wk, we hypothesized that changes in the ventilatory response to \( \text{CO}_2 \) might also be delayed. Indeed, significant increases in hypercapnic ventilation as a result of fluoxetine microdialysis were not present on experimental \( \text{day 1} \), but did become evident on \( \text{days 7 and 15} \), suggesting that longer term neurochemical changes in serotonergic neurons brought about by chronic fluoxetine may be involved.

**Repeated fluoxetine microdialysis effects on medullary raphe serotonergic neurons.** It is still unclear as to how SSRI compounds lead to clinical benefit and why this takes days to weeks to occur. In our present experiments, there are several
mechanistic possibilities to explain how repeated focal fluoxetine microdialysis might affect the way medullary raphe neurons modulate the ventilatory response to CO₂. When administered acutely, SSRIs increase extracellular serotonin due to serotonin transporter blockade at serotonergic cell bodies, which initially decreases serotonergic neuron firing activity due to 5-HT₁A autoreceptor activation (8, 15). With continued SSRI treatment, the 5-HT₁A autoreceptor can be desensitized to heightened extracellular serotonin, allowing serotonergic activity to resume (5). Functional desensitization of somatodendritic 5-HT₁A receptors in serotonergic neurons after chronic SSRI treatment has been described in the dorsal raphe (26, 28, 35, 47), so it is feasible that somatodendritic 5-HT₁A receptors located on chemosensitive medullary serotonergic cell groups may also become desensitized and enhance serotonergic tone postsynaptically after chronic fluoxetine microdialysis.

Desensitization of the 5-HT₁A is not the only mechanism that explains the delayed effects of SSRI treatment. There is indication that the serotonin transporter itself becomes downregulated after chronic SSRI administration (17, 49). Benmansour and colleagues (1, 2) found negligible changes in serotonergic transporter binding and clearance of extracellular 5-HT in the CA3 region of the hippocampus after 4 or 10 days of systemic SSRI treatment via osmotic minipumps in rats. However, after 15 days of chronic treatment, they found an 80–90% decrease in serotonin transporter binding throughout the brain, along with a dramatic decrease in serotonin clearance, and this effect was not explained by a decrease in serotonin transporter gene expression (1, 2). They attributed the downregulation or serotonin transporter to a possible change in subcellular localization of the transporter from the plasma membrane to the cytoplasm. It also has been reported that chronic SSRI treatment alters serotonin synthesis in vivo and in vitro, because sertraline and fluoxetine both lead to upregulation of the gene encoding tryptophan hydroxylase (the enzyme responsible for the rate-limiting step of serotonin synthesis) and increase total serotonin synthesis (24).

We hypothesize that, during repeated fluoxetine microdialysis into the medullary raphe, any of the mechanisms described above could contribute to enhanced responsiveness of medullary raphe serotonergic neurons that participate in central chemoreception. However, further experiments are needed to directly prove that a change in serotonergic function occurred. Considering that chronic systemic fluoxetine administration did not increase ventilation during 7% CO₂ breathing, knowing more about what repeated fluoxetine microdialysis does to medullary raphe serotonergic cell bodies and dendrites to potentially change their chemoreceptive properties would be valuable.

Focal fluoxetine microdialysis into the medullary raphe vs. systemic administration. Understanding why focal fluoxetine treatment of serotonergic neurons in the medullary raphe increased the ventilatory response to hypercapnia, whereas systemic fluoxetine treatment did not might prove to be complex. We suggest two possible explanations for this: 1) different concentrations of fluoxetine reaching serotonin transporter located within serotonergic cell groups in the rat brain during focal or systemic administration and 2) the functional consequences of chronic blockade of serotonin reuptake only in the medullary raphe as opposed to throughout the entire brain.

For the microdialysis studies, we chose the initial concentration of 250 μM fluoxetine as a reasonable starting point, on the basis of a study that used reverse microdialysis delivery (59), and also a second larger concentration of 500 μM. The higher concentration of fluoxetine microdialyzed did result in a clearer increase in hypercapnic ventilation. In one study that measured concentrations of glutamate in brain tissue 1.5 mm away from the point of microdialysis probe delivery, there was a 10- to 100-fold drop in drug concentration from the probe tip (10). Thus we could expect the concentration of fluoxetine in a 1.5-mm radius to fall from 250 and 500 μM at the probe tip to 2.5–25 and 5.0–50 μM, respectively. This would also largely depend on the diffusion properties of fluoxetine through brain tissue. Diffusion of a cationic molecule through the extracellular space within brain tissue from a point source, such as a microdialysis probe, depends upon tortuosity (the pathlength a particle must navigate in a complex medium) and volume fraction (the amount of fluid found in the extracellular space) (44). However, the diffusion of fluoxetine through brain tissue may be even more complex because it is highly lipophilic, it is taken up by glia and neurons, and it cannot specifically bind to different subcellular compartments (37). The ability of fluoxetine to bind to non-specific proteins and lipids within neuronal cells may suggest in our microdialysis experiments that it could be present at bioactive concentrations for a period of time after microdialysis is stopped because fluoxetine has a relatively low IC₅₀ of 6.8 nM (51). Acute microdialysis of 100 μM fluoxetine into the frontal parietal cortex of rats (an area that has less serotonergic transporter density than serotonergic raphe nuclei) can lead to a 6- to 10-fold increase in basal 5-HT concentration after 140 min of dialysis (16). It is logical to suppose that, in our protocol, which used shorter daily 30-min microdialysis treatments, a higher concentration of fluoxetine was required to increase extracellular serotonin transporter serotonin to a point where long-term changes in serotonergic transmission and could take place and alter the ventilatory response to hypercapnia.

In contrast to local brain tissue fluoxetine concentrations that may approach 25–50 μM during reverse microdialysis, systemic 10 mg·kg⁻¹·day⁻¹ fluoxetine treatment in rats via an intraperitoneally placed osmotic minipump leads to maximal fluoxetine and norfluoxetine concentrations of −0.06 and 0.23 μM, respectively, in cerebrospinal fluid after 2 days (9). Although these concentrations are smaller than those we approximated during focal microdialysis, they remain constant so long as the minipump functions. This dose given systemically is able to alter rat performance in behavioral paradigms suggestive of anxiolytic effects (18, 58), and also it results in a significant decrease in dorsal raphe serotonergic neuron firing rates after 3 days (due to 5-HT₁A autoreceptor activity), followed by a recovery to baseline firing rates at 14 days (9). The magnitude and temporal aspects of the effect on serotonergic neuron activity are not different when the fluoxetine doses are decreased to 5 or increased to 20 mg·kg⁻¹·day⁻¹. Therefore, the 10 mg·kg⁻¹·day⁻¹ dose we chose for our study can be considered physiologically appropriate from a behavioral perspective. Taking into account that the systemic dose used in our study leads to documented functional changes in firing rates of rat serotonergic neurons, we believe the lack of ventilation effect in systemically treated rats is probably not due to dose or route of administration but is due to differences
in effecting serotonin reuptake in one subset of serotonergic neurons vs. serotonin reuptake throughout the entire brain.

If the effects of fluoxetine on serotonergic neurotransmission differ depending on whether it is applied locally or systemically, it is not easy to speculate what these differences may be, especially because it is not completely understood how SSRIs affect serotonergic neurons. Desensitization of somatodendritic 5-HT_1A receptors located on chemosensitive neurons in the medullary raphe could result in increased serotonergic tone at postsynaptic premotor sites involved in increasing the ventilatory response, although our studies have not addressed this at the present time. In comparison, chronic systemic fluoxetine administration might potentially lead to desensitization of 5-HT_1A receptors located both pre- and postsynaptically on serotonergic and on nonserotonergic neurons, affecting other brain regions and neuronal circuits that can modulate ventilation, perhaps counteractively. The nucleus tractus solitarius is one such region involved in ventilatory control where input from the rostral nucleus raphe magnus (which primarily contains serotonergic neurons) has an inhibitory, not excitatory, effect on ventilation in rats during hypoxia (13, 48). Thus the results observed in the present study in rats treated with fluoxetine locally opposed to systemically might indicate that serotonergic influence on ventilation might serve different functional roles, depending on the neuronal circuits exposed to chronic SSRI treatment.

**Fluoxetine, arousal state, and the ventilatory response to CO_2**. In the present study, we found that fluoxetine microdialysis into the medullary raphe over a period of 15 days led to similar increases in the response to 7% CO_2 during NREM sleep and wakefulness (Figs. 2 and 3). However, not all studies involving brain stem chemosensitive regions show similar ventilatory effects across arousal states. In the retrotrapezoid nucleus, 25% CO_2 microdialysis increased ventilation in wakefulness only, due entirely to an increase in V_t (29). In the nucleus tractus solitarius, 25% CO_2 microdialysis increased ventilation in wakefulness and in NREM and REM sleep due to an increase in both frequency and in V_t (41). Within the medullary raphe region, 25% CO_2 microdialysis increased ventilation only in NREM and REM sleep as a result of increased frequency, whereas in an unanesthetized goat model, 25 or 80% CO_2 microdialysis increased ventilation only during quiet wakefulness (20, 40). These results suggest that each brain stem region that acts as a central chemoreceptor site has a particular function which is also dependent on the arousal/behavioral state of the animal, or the animal model used (for reviews, see Refs. 19, 39).

Our results after focal or systemic chronic fluoxetine administration did not suggest that any drastic changes in sleep cycling occurred as a result of treatment, because there were no differences found in the percent time spent in wakefulness or in NREM sleep over the course of the experiments (Tables 1 and 2). However, the fact that we did not see any significant differences in percent time spent in any arousal state with either focal fluoxetine microdialysis or systemic administration can most likely be attributed to the short duration of our ventilation experiments. This study was not designed to accurately study sleep cycling, because much longer recordings over the course of the photoperiod are required to do so, especially to observe periods of REM sleep in the rat. Therefore, we realize it is possible that either focal microdialysis into the medullary raphe or systemic administration of fluoxetine might have altered sleep cycling during the 15-day experiments.

**Conclusions.** Serotonergic neurons located within the medullary raphe (and those located parapyramidally along the ventral surface of the brain stem) modulate a variety of functions such as repeated motor activity (23), pain processing and arousal (31), cardiovascular control (7), thermoregulation (36), ventilatory control (27), and chemosensitivity (56). These neurons have the ability to specifically respond to CO_2 in vivo (4, 20, 40) and in vitro (57), are located adjacent to large medullary arteries (6), project to brain stem respiratory groups (52), and project to spinal motoneurons effecting phrenic nerve output (50). Of clinical relevance, anatomic abnormalities in serotonergic medullary raphe neurons have been found in victims of the sudden infant death syndrome (25).

A central hypothesis of our laboratory and of others has been that disrupting serotonergic activity within the medullary raphe and extra-raphe cell groups results in a decreased ventilatory response to hypercapnia. The data in the present study, which show an increased ventilatory response to hypercapnia after chronic microdialysis treatments with the SSRI fluoxetine, are the first to suggest that the opposite might occur. The increase in the ventilatory response to hypercapnia due to repeated fluoxetine microdialysis occurred during both NREM sleep and quiet wakefulness, suggesting it is not sleep-state dependent as other aspects of ventilation may be. Systemic fluoxetine administration did not alter the ventilatory response to moderate hypercapnia in our study, suggesting that chronic serotonin transporter blockade throughout the brain may not have a cumulative effect on ventilation during CO_2 stress. Nevertheless, further studies are needed to demonstrate a direct relationship between the cellular mechanisms of medullary raphe serotonergic neurons and the ventilatory response to hypercapnia during focal SSRI administration in vivo.

### Table 2. Percent experiment time in QW or NREM states during 30 min of 7% CO_2 recording

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day 0</th>
<th>Day 1 or 3</th>
<th>Day 7</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QW</td>
<td>NREM</td>
<td>QW</td>
<td>NREM</td>
</tr>
<tr>
<td>wCSF microdialysis</td>
<td>73.1±4.7</td>
<td>25.8±5.2</td>
<td>62.8±3.6</td>
<td>37.2±5.2</td>
</tr>
<tr>
<td>250 µM fluoxetine microdialysis</td>
<td>74.2±6.5</td>
<td>23.8±5.4</td>
<td>75.8±8.8</td>
<td>24.2±7.7</td>
</tr>
<tr>
<td>500 µM fluoxetine microdialysis</td>
<td>73.7±3.3</td>
<td>29.0±4.4</td>
<td>76.0±7.7</td>
<td>22.5±7.0</td>
</tr>
<tr>
<td>Systemic vehicle</td>
<td>70.4±9.4</td>
<td>20.1±4.7</td>
<td>74.8±6.6</td>
<td>24.6±6.1</td>
</tr>
<tr>
<td>Systemic fluoxetine (10 mg/kg ‘day ’)</td>
<td>74.4±3.2</td>
<td>24.4±3.2</td>
<td>67.5±8.4</td>
<td>36.9±8.4</td>
</tr>
</tbody>
</table>

Values are means ± SE given in %.
ACKNOWLEDGMENTS

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