Inhibition of serotonergic medullary raphe obscurus neurons suppresses genioglossus and diaphragm activities in anesthetized but not conscious rats

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OBSTRUCTIVE SLEEP APNEA (OSA) is a common disorder affecting up to 4% of adults (99) and is caused by suppression of pharyngeal muscle activity in sleep and upper airway closure (79). Exogenous serotonin [5-hydroxytryptamine (5-HT)] applied to pharyngeal motoneurons increases pharyngeal muscle activity (2, 4, 6, 42, 49, 85). Based on these observations, modulation of endogenous 5-HT has been attempted as a pharmacological treatment for OSA (35, 84, 91, 92), but selective 5-HT reuptake inhibitors have little beneficial effects (7, 23, 46). It is not known whether the lack of clinically relevant effects is due to an inability to target pharyngeal motoneurons effectively via systemic drug delivery or whether endogenous 5-HT actually plays a minimal role in modulating pharyngeal muscle tone. Determining the functional role of endogenous 5-HT in the control of pharyngeal motoneurons is relevant because medullary raphe neurons have highest activity in wakefulness, slightly reduced activity in non-rapid eye movement (non-REM) sleep, and minimal activity in REM sleep (39). Accordingly, raphe neurons are positioned to provide a tonic drive to the respiratory system, with this influence varying across sleep-wake states.

We have developed an animal model for manipulation of neurotransmission in the caudal medulla of freely behaving rats (42) to determine the neural mechanisms modulating genioglossus (GG) muscle activity across natural sleep-wake states. Whereas application of exogenous 5-HT to the hypoglossal motor nucleus (HMN) increases GG activity in wakefulness and sleep (42), 5-HT receptor antagonism at the HMN has minimal effects on GG activity, suggesting a minimal endogenous 5-HT drive (86). At first glance, this result seemed to contradict several previous studies in anesthetized and decerebrate animals in which an endogenous 5-HT drive to the HMN was first demonstrated (13, 15, 48, 49, 98), including our laboratory’s own study in anesthetized rats (85) that used the exact same methodology as the study in conscious rats (86). However, those previous studies demonstrating an endogenous 5-HT drive to the HMN were all performed in the presence of vagotomy (13, 15, 48, 49, 85, 98), which was subsequently shown to augment the role of 5-HT at the HMN (86).

Because medullary raphe neurons are the sole source of 5-HT inputs to the HMN (54), these results in both conscious and anesthetized rats with the vagus nerves intact (86) implied that raphe neurons per se also likely exert a minimal influence on respiratory motor activity across natural sleep-wake states. However, that specific conclusion could not be supported from previous data because medullary raphe neurons also release other excitatory neurotransmitters onto respiratory motoneurons in addition to serotonin, such as thyrotropin-releasing hormone, substance P, and glutamate (10, 26, 32, 43, 44), which are also excitatory (3, 10, 77, 78). The present study tests the hypothesis that, if serotonergic medullary raphe neurons provide a tonic drive to the respiratory system, with this influence varying across sleep-wake states.

Raphe neurons in the caudal medulla include the nucleus raphe obscurus (NRO), pallidus, magnus, and parapyramidal region (39). In addition to potential effects at the HMN, these raphe neurons are also positioned to influence respiration via projections to dorsal and ventral respiratory group neurons (11, 80, 95) and the phrenic motor nucleus (31, 37, 50). Some

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Interventions were performed during anesthetized rats. The numbers used for each study are listed below. All procedures were in accordance with the recommendations of the Canadian Council on Animal Care, and the University of Toronto Animal Care Committee approved the protocols.

Methods

Studies were performed on adult male Wistar rats (Charles River). The numbers used for each study are listed below. All procedures were in accordance with the recommendations of the Canadian Council on Animal Care, and the University of Toronto Animal Care Committee approved the protocols.

Studies in Acutely Anesthetized Rats

Surgical preparation. Ten rats [mean body weight 321.5 ± 13.0 (SE) g, range 263–370 g] were anesthetized with intraperitoneal urethane (1 g/kg). Rats were also given atropine (1 mg/kg) and dexamethasone (0.2 mg) to minimize airway secretions and brain edema, respectively. After the onset of surgical anesthesia, the rats were tracheotomized, the femoral artery and vein were cannulated, and cervical vagotomy was performed bilaterally. The rats were vagotomized because no significant 5-HT input to the HCN occurs in vagus nerve intact rats (86), whereas a measurable 5-HT drive from the raphe neurons present in vagotomized rats (13, 15, 48, 49, 85, 98). The rats spontaneously breathed a 50:50 mixture of room air and O2 with any additional anesthesia (halothane, typically 0.2–1%) administered by inhalation. Once halothane was initiated within an animal typically no, or only minor, adjustments were necessary across the experiment to maintain stable electroencephalogram (EEG) and respiratory muscle activities. Core body temperature was monitored with a rectal probe and maintained between 36 and 38°C with a water pump and heating pad (T/Pump-Heat Therapy System, Gaymar, NY). The rats received continuous intravenous fluid (0.4 ml/h) containing 7.6 ml saline, 2 ml of 5% dextrose, and 0.4 ml of 1 M NaHCO3. Bipolar electrodes were inserted into the GG and costal diaphragm for electromyogram (EMG) recordings (63). The rats were then placed in a stereotoxic apparatus (model 962, Kopf, Tujunga, CA). To record the cortical EEG, two stainless steel screws attached to insulated wire were implanted in the skull over the frontal-parietal cortex (63).

Microdialysis. Microdialysis probes (CMA/11 14/01, CSC, St. Laurent, PQ, Canada) were targeted into the NRO to infuse either artificial cerebrospinal fluid (ACSF) or 8-OH-DPAT (8-OH-DPAT hydrobromide, FW: 328.83, Sigma). The probes were targeted to the NRO and were placed 12.60 ± 0.05 mm posterior to bregma, 0.02 mm lateral to the midline (range 0–0.02 mm), and 10.53 ± 0.11 mm ventral to bregma. The rats stabilized for at least 30 min before any interventions were performed.

The microdialysis probes were 240 μm in diameter with a 1-mm cuprophane membrane and a 6,000-Da cutoff. The probes were connected to FEP Teflon tubing (inside diameter 0.12 mm) in turn connected to 1.0-ml syringes via a zero-dead space switch (Uniswitch, BAS, West Lafayette, IN). The probes were continually flushed with at a flow rate of 2.1 μl/min using a syringe pump and controller (MD-1001 and MD-1020, BAS). The composition of ACSF (in mM) was 125 NaCl, 3 KCl, 1 KH2PO4, 2 CaCl2, 1 MgSO4, 25 NaHCO3, and 30 d-glucose.

Recording. The electrical signals were amplified and filtered (Super-Z head-stage amplifiers and BMA-400 amplifiers/filters, CWE, Ardmore, PA). The EEG was filtered between 1 and 100 Hz and the EMG signals between 100 and 1,000 Hz. The electrocardiogram was removed from the diaphragm signal using an oscilloscope and an electronic blanket (SB-1, CWE). The moving-time averages of the GG and diaphragm signals were also obtained (time constant = 200 ms, Coulbourn 576–01, Lehigh Valley, PA). Each signal, along with blood pressure (DT-X transducer, Ohmeda, Madison, WI, and VM-1000 Amplifier, CWE) and inspired CO2 (CapStar-100, CWE) were recorded on computer (Spike 2 software, 1401 interface, CED, Cambridge, UK). The moving-time averages of the EMG signals were digitized at 50 Hz and the EEG at 500 Hz. The raw EMGs were sampled by computer at 250 Hz because these signals were only used for display purposes with all the EMG analyses performed on the moving averages.

Protocol and data analyses. Interventions were performed during steady-state periods with predominantly high-voltage and low-frequency EEG activity. The microdialysis probes were perfused with ACSF for at least 30 min followed by 0.01, 0.1, and 1 mM 8-OH-DPAT each for at least 1 h. During perfusion of ACSF or 8-OH-DPAT, responses to steady-state (≥6 min) application of 7.5% inspired CO2 were also recorded (51, 52, 63, 85). GG and diaphragm responses were measured from the moving average signal above electrical zero, as previously described (51, 52, 63, 85). The GG signal was quantified as mean tonic activity (i.e., basal activity in expiration) and respiratory-related activity (i.e., peak inspiratory activity – tonic activity). Mean blood pressure and EEG power spectra in the range 0.5–30 Hz were also analyzed as previously described (51, 52, 63, 85). Measurements were taken over 1-min periods immediately before CO2 application and in the last minute of CO2.

Studies in Conscious Rats

Surgical preparation. Ten rats (mean body weight 286.2 ± 11.4 g, range 238–320 g) were anesthetized with intraperitoneal ketamine (85 mg/kg) and xylazine (15 mg/kg). In addition, the rats were given saline (3 ml, 0.9% ip) for fluid loading, atropine sulfate (1 mg/kg ip) to reduce airway secretions and buprenorphine (0.03 mg/kg ip) to reduce potential postoperative pain. Anesthesia mask (17) was placed over the snout, and the rats spontaneously breathed a 50:50 mixture of room air and O2 with any additional anesthesia (isoflurane, typically 0.2–2%) administered as necessary, by inhalation. As previously described (36, 42, 61, 62), under sterile conditions the rats were implanted with EEG and neck EMG electrodes to record sleep-wake states and with GG and diaphragm electrodes for respiratory muscle recordings. Tests for the accurate placement of the GG electrodes, and their function throughout the experiments, have also been previously described (36, 42, 61, 62). In eight rats, a radiofrequency donor (TA-F40, Data Sciences International, St. Paul, MN) was also implanted into the peritoneal cavity to record core body temperature (22). Before implantation, each transmitter was individually calibrated in a range from 35 to 41°C using a water bath and thermometer. During surgery, microdialysis guides were targeted 3 mm above the NRO at 12.82 ± 0.06 mm posterior to bregma, 0.02 ± 0.01 mm lateral to the midline, and 7.37 ± 0.44 mm ventral to bregma. A dummy cannula was placed inside the microdialysis guide to keep it patent until the day of the experiment. At the end of surgery, all the...
electrodes were connected to pins and inserted into a miniature plug (model STC-89P-220ABS, Carleton University, Ottawa, ON, Canada). The plug and microdialysis guides were affixed to the skull with dental acrylic and anchor screws. The rats recovered for ~7 days before the studies (36, 42, 61, 62).

Recordings. For habituation, the rats were placed in the recording chamber (MD-1500, BAS) with fresh bedding, food, and water the day before the experiments. The chamber was placed inside an electrically insulated, sound-attenuated cubicle (EPC-010, BRS/LVE, Laurel, MD). For recordings, a lightweight electrically shielded cable was connected to the plug on the rat's head and attached to a counterbalanced swivel that permitted free movement. A video camera allowed continuous visual monitoring without disrupting the rat.

The signals were amplified and filtered as described above for the anesthetized experiments. In addition to the moving time averages of GG and diaphragm signals, the moving time average of the neck EMG was also obtained (model MA-821, CWE, time constant = 200 ms). The telemetered body temperature signal was detected by a receiver (model RPC-1, Data Sciences International) under the rat’s cage. Each signal was recorded on computer (Spike 2 software, 1401 interface, CED, Cambridge, U.K.) as described above.

Protocol and microdialysis. All experiments were performed during the day when the rats normally sleep. The rats were lightly restrained while the internal cannula was removed from the guide, and the microdialysis probe was inserted into the NRO. Measurements of sleep-wake states and respiratory muscle activities were then made during both room air and CO2-stimulated breathing using 7.5% inspired CO2 (36, 61, 62, 86). The probes were flushed at a flow rate of 2.1 µl/min with ACSF or 0.1 mM 8-OH-DPAT. This dose was chosen because 8-OH-DPAT delivered by microdialysis perfusion at an even lower dose of 0.01 mM is capable of inhibiting raphe neuronal activity in vivo via stimulation of the inhibitory 5-HT1A autoreceptor (76), with such inhibition also occurring at caudal raphe neurons (98). Importantly, from the results in anesthetized rats, this dose of 8-OH-DPAT was also sufficient to significantly modulate respiratory muscle activity while avoiding the severe effects on blood pressure observed with 1 mM 8-OH-DPAT (see RESULTS). After at least two full sleep cycles (i.e., periods containing wakefulness, non-REM sleep, and REM sleep) had been obtained with ACSF at the NRO during room air and, two further full sleep cycles had been obtained during CO2, the perfusion medium was then switched to 8-OH-DPAT where the same protocol was followed. Data were analyzed at least 60 min after a switch between drugs.

Data analysis. Each rat served as its own control with all interventions being performed in one experiment, therefore allowing for consistent effects of experimental condition (e.g., 8-OH-DPAT) to be observed across sleep-wake states within and between rats. Sleep-wake states, respiratory muscle activities and body temperature were analyzed as previously described (22, 36, 61, 62). As described above for the anesthetized rats, the GG signal was quantified as mean tonic activity and respiratory-related activity. Only those epochs comprising at least 30 s of uninterrupted sleep without arousal, or wakefulness without drowsiness, were included in the analysis. To minimize bias in selecting periods, the EEG and neck EMG were scored for sleep-wake states without reference to the GG or diaphragm signals. As in previous studies (61, 62, 86), measurements were made for periods of active wakefulness, quiet wakefulness, non-REM sleep, and periods of REM sleep without transient GG twitches. Two to five periods of each sleep-wake state were analyzed for each experimental condition in each rat. Data were complete except that measurements for quiet wake during room air breathing could not be obtained in one rat while ACSF was being microdialyzed into the NRO because the animal spent most of the time asleep, and when awake it was engaged in active behaviors. Also, one rat had minimal REM sleep during most of the experimental conditions, and these data were therefore not included.

Tests of GG function. At the end of the experiments the rats were reanesthetized with ketamine and xylazine, and the GG electrodes were stimulated to confirm tongue movements. Stimulation of the GG electrodes at the end of the experiment showed that the voltages required to cause tongue movements were not different from at the time of surgery (0.63 ± 0.05 vs. 0.69 ± 0.04 V), showing that the electrodes were in place and functional throughout the study.

Histology

At the end of the studies, a microdialysis probe with the membrane cut at the tip was inserted into the guide cannula and a 1% solution of potassium permanganate was microinjected at 2.1 µl/min for 10 min to mark the microdialysis sites (68, 87). The rats were then euthanized with pentobarbital sodium (325 mg), and 25 ml of 0.9% saline were perfused through the ascending aorta followed by 40 ml of 10% formalin. The brains were removed and fixed in 10% formalin. The medullary regions were blocked, transferred to 30% sucrose and cut in 50-µm coronal sections with a cryostat (model CM 1850, Leica, Nussloch, Germany). Each section around the lesion site was mounted and stained with neutral red. Microdialysis sites were localized from the stained sections and marked on standard brain maps (71).

Statistical Analysis

The analyses performed for each statistical test are included in the text where appropriate. For all comparisons, differences were considered significant if the null hypothesis was rejected at P < 0.05 using a two-tailed test. Where post hoc comparisons were performed after analysis of variance with repeated measures (ANOVA-RM), the Bonferroni corrected P value was used to infer statistical significance. Analyses were performed using Sigmastat (SPSS, Chicago, IL). Data are presented as means ± SE unless otherwise indicated.

RESULTS

Sites of Microdialysis

Figure 1A shows an example of a lesion site made by the microdialysis probe in the NRO from one experiment. Figure 1A also shows the sites of microdialysis from each of the 10 anesthetized and 10 conscious rats (Fig. 1, B and C, respectively). In all the experiments, the microdialysis sites were within or immediately adjacent to the NRO.

Studies in Acutely Anesthetized Rats

Responses to 8-OH-DPAT. Figure 2 shows an example of the effects of microdialysis perfusion of 8-OH-DPAT into the NRO on physiological parameters. Note the clear decline in GG activity with 8-OH-DPAT, compared with ACSF, with lesser suppression of diaphragm activity. Note also the decline in blood pressure that was especially apparent at 1 mM 8-OH-DPAT. The example in Fig. 3 also shows that compared with ACSF, 0.1 mM 8-OH-DPAT at the NRO decreased GG and diaphragm responses to CO2 stimulation.

GG RESPONSES. The group data in Fig. 4A show that respiratory-related GG activity was significantly decreased by 8-OH-DPAT at the NRO (F3,24 = 17.28, P < 0.001 from 2-way ANOVA-RM) with significant effects first observed at 0.1 mM (P < 0.001, post hoc Bonferroni t-test vs. ACSF control). The effect with 0.01 mM 8-OH-DPAT was of borderline statistical significance (P = 0.079). Compared with ACSF, respiratory-related GG activity decreased by 30.6 ± 8.0, 60.7 ± 9.0, and 87.9 ± 4.5% for 0.01, 0.1, and 1 mM 8-OH-DPAT, respectively. There was minimal tonic GG activity in the anesthetized
DIAPHRAGM RESPONSES. Figure 4B shows that diaphragm activity was also decreased by 8-OH-DPAT at the NRO ($F_{3,24} = 12.60, P < 0.001$ from 2-way ANOVA-RM) with significant effects first observed at 0.1 mM ($P = 0.009$, post hoc Bonferroni $t$-test vs. ACSF controls). Compared with ACSF, diaphragm activity decreased by 2.1 ± 2.2, 13.3 ± 3.4, and 20.4 ± 4.1% for 0.01, 0.1, and 1 mM 8-OH-DPAT, respectively. Statistical analysis confirmed that the percent changes in GG activity with 8-OH-DPAT were larger than the percent changes in diaphragm activity ($F_{1,8} = 79.40, P < 0.001$ from 2-way ANOVA-RM) with this larger effect observed at each dose ($P < 0.001$, post hoc Bonferroni $t$-tests). In contrast to diaphragm amplitude, respiratory rate was not altered by 8-OH-DPAT at the NRO ($F_{3,24} = 1.55, P = 0.228$ from 2-way ANOVA-RM, Fig. 4C).

BLOOD PRESSURE. Figure 4D shows that blood pressure was also significantly decreased by 8-OH-DPAT at the NRO ($F_{3,15} = 15.27, P < 0.001$ from 2-way ANOVA-RM) with significant effects first observed at 0.1 mM ($P = 0.020$, post hoc Bonferroni $t$-test vs. ACSF control). Compared with ACSF, blood pressure decreased by 3.1 ± 2.9, 12.8 ± 3.7, and 26.9 ± 5.8 mmHg for 0.01, 0.1, and 1 mM 8-OH-DPAT, respectively.

EEG ACTIVITY. There was no effect of 8-OH-DPAT at the NRO on EEG power in the $\delta$ (0.5–2 Hz)-, $\theta$ (4–7.5 Hz)-, $\alpha$ (7.5–13.5 Hz)-, $\beta_1$ (13.5–20 Hz)-, and $\beta_2$ (20–30 Hz)-frequency bands (all $F_{3,24} < 1.75, P > 0.184$ from 2-way ANOVA-RM).

Responses to CO2 stimulation. GG RESPONSES. As previously observed (36), individual animals varied in their GG responses to CO2, with some rats showing clear increases in GG activity during CO2-stimulated breathing (e.g., Fig. 3) and others not. Figure 4 shows that in the group as a whole the level of CO2 that was sufficient to increase diaphragm activity (see below) was insufficient to significantly increase GG activity ($F_{1,8} = 0.62, P = 0.455$ from 2-way ANOVA-RM), and this effect did not depend on the level of 8-OH-DPAT ($F_{3,24} = 0.23, P = 0.873$). Nevertheless, it was apparent that, in those rats show-
ing increased GG activity in response to CO₂, this effect was reduced by 8-OH-DPAT at the NRO (Fig. 3). Tonic GG activity was increased by hypercapnia (F₁,₈ = 6.60, P = 0.033 from 2-way ANOVA-RM), but this effect was not affected by 8-OH-DPAT at the NRO (F₃,₂₄ = 0.411, P = 0.747).

DIAPHRAGM RESPONSES. The group data showed that although diaphragm activity was increased by CO₂ stimulation (Fig. 4 B; F₁,₈ = 18.59, P = 0.003 from 2-way ANOVA-RM) the stimulatory effect of hypercapnia depended on the level of 8-OH-DPAT (F₃,₂₄ = 6.17, P = 0.003). Further analysis confirmed that the increase in diaphragm activity with CO₂ stimulation was significantly reduced at 0.1 and 1 mM 8-OH-DPAT compared with ACSF (Fig. 4 B; P = 0.014 and 0.001, respectively, post hoc Bonferroni t-tests). Respiratory rate was not altered by CO₂ stimulation (Fig. 4 C; F₁,₈ = 0.72, P = 0.422).

BLOOD PRESSURE AND EEG ACTIVITY. Blood pressure was not altered by CO₂ stimulation (F₂,₁₀ = 0.16, P = 0.854 from 2-way ANOVA-RM), and this effect did not depend on the level of 8-OH-DPAT (F₆,₃₀ = 0.957, P = 0.471). EEG activity in the θ (4–7.5 Hz) range was increased in hypercapnia (F₁,₈ = 9.68, P = 0.014 from 2-way ANOVA-RM), consistent with an arousing stimulus, but this effect did not depend on the level of 8-OH-DPAT (F₃,₂₄ = 0.83, P = 0.491). There was no effect of hypercapnia on EEG activity in the other frequency bands (all F₁,₈ < 2.41, P > 0.159).

Responses to 8-OH-DPAT. Figure 5 shows an example of the effects of microdialysis perfusion of 0.1 mM 8-OH-DPAT into the NRO on GG and diaphragm activities across sleep-wake states during CO₂-stimulated breathing. Note the presence of tonic and respiratory-related GG activity during wakefulness, i.e., typical of previous studies (36, 61, 62). Also note that GG activity decreased from wakefulness to non-REM sleep, and it is effectively abolished in those periods of REM.

Studies in Conscious Rats
Experiments in conscious rats were performed using exactly the same methodology as those in anesthetized rats. A dose of 0.1 mM 8-OH-DPAT was chosen because it produced significant and robust reductions in GG and diaphragm activities in the anesthetized rats (60.7 ± 9.0 and 13.3 ± 3.4%, respectively, compared with ACSF; Fig. 4, A and B). Although 0.1 mM 8-OH-DPAT also decreased blood pressure by 12.8 ± 3.7 mmHg, this change was less severe than the 26.9 ± 5.8-mmHg decrease (31.9 ± 6.8%) observed with 1 mM 8-OH-DPAT (Fig. 4 D). Given this latter change in blood pressure, a dose of 1 mM 8-OH-DPAT was considered too severe for testing in the conscious rats. Indeed, such significant changes in blood pressure can elicit strong behavioral responses in conscious animals (33, 34), and higher doses of 8-OH-DPAT (1–30 mM) at medullary raphe neurons in other studies causes reduced sleep and altered behavior patterns (59, 88).
sleep without the transient GG muscle twitches typical of that state (36, 61, 62). This figure illustrates that 8-OH-DPAT had no effect on GG and diaphragm activities across sleep-wake states.

Figures 6 and 7 show group data for the effects of 8-OH-DPAT vs. ACSF on physiological variables during room air (A) and CO₂-stimulated breathing (B). The independent effects of sleep-wake states on respiratory variables (e.g., changes in

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**Fig. 3.** Example showing effects of 8-OH-DPAT at the NRO on respiratory motor responses to systemic hypercapnia in an anesthetized rat. Traces show the effects of 8-OH-DPAT on respiratory motor activities and BP while breathing 7.5% CO₂ and room air both before and after the CO₂ stimulus. Note the diminution of respiratory motor responses to hypercapnia with 8-OH-DPAT at the NRO. Abbreviations are as for Fig. 2.

**Fig. 4.** Group responses to 8-OH-DPAT at the NRO in anesthetized rats. Group data showing responses of respiratory-related GG activity (A), diaphragm amplitude (B), respiratory rate (C), and blood pressure (D) to microdialysis perfusion of 8-OH-DPAT into the NRO during room air (open bars) and CO₂-stimulated breathing (solid bars). Values are means ± SE. Arb, arbitrary. Brackets denote concentration. *Significant decrease with 8-OH-DPAT compared with ACSF controls, P < 0.05. #Significant reduction in the response to CO₂ at 0.1 and 1.0 mM 8-OH-DPAT compared with the response with ACSF, P < 0.05.
diaphragm amplitude and GG activity across states, and respiratory rate in REM sleep) and sleep variables (e.g., changes in neck EMG and EEG frequencies) were typical and are as previously described for room air and hypercapnia (36, 42, 61, 62, 86). However, although significant changes were observed in these physiological variables across sleep-wake states, there were no independent effects of 8-OH-DPAT at the NRO on these variables in conscious animals, i.e., in contrast to the effects in anesthetized animals described above.

**Room air breathing.** Figure 6A shows that in room air there was no significant effect of 8-OH-DPAT on respiratory-related or tonic GG activities, diaphragm amplitude, respiratory rate, or neck EMG (all $F_{1,9} < 1.24, P > 0.293$ from 2-way ANOVA-RM), and no drug effect was dependent on the prevailing sleep-wake state (all $F_{3,25} < 1.97, P > 0.143$). Similarly, there were no significant effects of 8-OH-DPAT on EEG power or EEG activities in the $\delta_2$, $\delta_1$, $\theta$, $\beta_1$, and $\beta_2$-frequency bands (all $F_{1,9} < 3.09, P > 0.112$). The only effect was reduced activity in the $\alpha$-frequency band with 8-OH-DPAT (F1,9 = 10.99, P = 0.009) but only in REM sleep ($P < 0.001$, post hoc Bonferroni t-test, mean change 8.9%). Nevertheless, there were no obvious behavioral changes or alterations in sleep-wake states with 8-OH-DPAT. Group mean EEG activities in the $\delta_2$ and $\alpha$-frequency bands, and body temperature, with 8-OH-DPAT across sleep-wake states are shown in Fig. 7A.

**CO2-stimulated breathing.** Figure 6B shows that during hypercapnia there was also no significant effect of 8-OH-DPAT on respiratory-related or tonic GG activities, diaphragm amplitude, respiratory rate, or neck EMG (all $F_{1,8} < 3.43, P > 0.101$ from 2-way ANOVA-RM). No drug effect was dependent on the prevailing sleep-wake state (all $F_{3,24} < 2.34, P > 0.099$) except that respiratory rate was increased by 8-OH-DPAT in a state-specific manner ($F_{3,24} = 7.37, P = 0.001$), with the increased respiratory rates only observed in non-REM and REM sleep ($P = 0.045$ and 0.006, respectively, post hoc Bonferroni t-test, mean changes = 7.4 and 10.7 breaths/min).

There were also no effects of 8-OH-DPAT on EEG power or activities in the $\delta_2$, $\delta_1$, $\theta$, and $\beta_2$-frequency bands (all $F_{1,8} < 1.70, P > 0.229$; Fig. 7B). The only effects observed with 8-OH-DPAT were reduced $\alpha$- and increased $\beta_2$-activities (both $F_{1,8} > 5.70, P < 0.045$), effects that were not dependent on the prevailing sleep-wake states (both $F_{3,24} > 1.38, P < 0.273$). There were no obvious behavioral changes or alterations in sleep-wake states with 8-OH-DPAT. Group mean EEG activities in the $\delta_2$ and $\alpha$-frequency bands, and body temperature, with 8-OH-DPAT across sleep-wake states are shown in Fig. 7B.

**Effects on body temperature.** Application of 8-OH-DPAT to the NRO had a statistically significant effect on body temperature in active wakefulness ($F_{1,7} = 6.38, P = 0.032$ from 2-way ANOVA-RM) but not in quiet wakefulness, non-REM sleep, or REM sleep (all $F < 1.50, all P > 0.260$). However, this effect on body temperature in active wakefulness depended on the level of inspired CO$_2$ ($F_{1,7} = 8.42, P = 0.018$) with decreased temperature observed during room air breathing only with 8-OH-DPAT compared with ACSF controls (36.6 ± 0.2 vs. 36.1 ± 0.3°C; $P = 0.002$, post hoc t-test).

**Responses to CO2 stimulation.** Further analyses were performed to specifically address the potential effects of 8-OH-DPAT at the NRO on the responses of the GG, diaphragm, and diaphragm minute activity to CO2 stimulation within each individual state of wakefulness and sleep. These results confirmed the results described above across sleep-wake states and showed that, within each individual state of active wakefulness, quiet wakefulness, non-REM sleep, and REM sleep, there...
Fig. 6. Group respiratory motor responses to 8-OH-DPAT at the NRO in conscious rats. Shown are group data of responses of respiratory-related and tonic GG activities, DIA amplitude, respiratory rate, and neck EMG activity to microdialysis perfusion of ACSF (○) and 0.1 mM 8-OH-DPAT (●) into the nucleus raphe obscurus during room air (A) and CO₂-stimulated breathing (B). Data are shown for active wakefulness (AW), quiet wakefulness (QW), non-REM sleep, and REM sleep. Values are means ± SE. See text for further details.
was no independent effect of 8-OH-DPAT on any of these respiratory variables (all $P > 0.160$ from 2-way ANOVA-RM), and there was no effect of 8-OH-DPAT that depended on the level of CO$_2$ stimulation (all $P > 0.165$ from 2-way ANOVA-RM). Figure 8 shows the lack of effect of 8-OH-DPAT on diaphragm minute activity, an index of neural ventilation, in response to CO$_2$ for individual states of wakefulness and sleep.

Physiological variables with and without the microdialysis probe. Further analyses were performed to determine whether insertion of the microdialysis probe caused any adverse influences on the physiological variables. Accordingly, measurements across sleep-wake states were made before insertion of the microdialysis probe and compared with those after insertion and perfusion with ACSF. During room air breathing, there was no effect of the microdialysis probe on respiratory-related or tonic GG activities, diaphragm amplitude, respiratory rate, neck EMG, body temperature, EEG power, and EEG activities in the $\delta_2$-, $\delta_1$-, $\theta$-, $\beta_1$-, and $\beta_2$-frequency bands (all $P > 0.064$ from 2-way ANOVA-RM). The only statistically significant change was a small increase in $\alpha$-EEG activity in non-REM and REM sleep ($P = 0.049$ and <0.001, respectively, post hoc Bonferroni $t$-tests, mean changes = 2.6 and 5.6%).

During hypercapnia there was an effect of the microdialysis probe on respiratory rate ($F_{1,8} = 8.21$, $P = 0.020$ from 2-way ANOVA-RM) with increased rates in the presence of the probe but only in active wakefulness and REM sleep ($P = 0.011$ and 0.022, respectively, post hoc Bonferroni $t$-tests). Otherwise, the presence of the microdialysis probe had no effects on diaphragm amplitude, diaphragm minute activity, respiratory-related or tonic GG activities, neck EMG, body temperature, EEG power, and EEG activities in the $\delta_2$-, $\delta_1$-, $\theta$-, $\alpha$-, $\beta_1$-, and $\beta_2$-frequency bands (all $P > 0.085$ from 2-way ANOVA-RM).

Taken together, these data obtained during room air and hypercapnia show that the presence of the microdialysis probe in the NRO had minimal effect on the physiological variables under study and no effects on respiratory motor activities and neural ventilation as assessed by diaphragm minute activity. This result is consistent with a previous study showing a lack of effect on physiological variables of the presence of the microdialysis probe in the medial medulla (42).

**DISCUSSION**

The present study shows that inhibition of serotonergic medullary raphe neurons at the NRO does not alter GG or diaphragm activities across natural sleep-wake states in freely behaving rats. The parallel studies in anesthetized and vagotomized rats using the exact same methodology served as positive controls for the potential of the interventions at the NRO to modulate respiratory activity, as would be expected.
given the anatomic projections of these neurons (11, 31, 37, 50, 55, 80, 95) and because the influence of serotonergic raphe neurons is enhanced by vagotomy, at least at the HMN (86). Indeed, most medullary raphe neurons that project to motoneurons are inhibited by vagal afferents (8, 14). Overall, the present results in intact conscious animals support previous data showing that endogenous 5-HT at the HMN plays a minimal role in modulating GG activity in awake and sleeping rats (86) but extends these findings to the serotonergic medullary raphe neurons that provide the 5-HT inputs. However, these results do not conflict with the notion that activation of medullary raphe neurons per se can influence respiratory activity via projections to respiratory neurons and motoneurons (28, 29, 68), potentially by release of 5-HT or other excitatory neurotransmitters such as thyrotropin-releasing hormone, substance P and glutamate (10, 26, 32, 43, 44). Indeed, in vitro evidence suggests that glutamate may importantly contribute to hypoglossal motor responses after raphe neuronal activation (10).

Responses in Anesthetized and Vagotomized Animals

GG and diaphragm activities. The experiments in anesthetized and vagotomized rats demonstrate that 8-OH-DPAT at the NRO produced dose-dependent decreases in GG and diaphragm activities, with larger GG suppression compared with the diaphragm across the range of doses tested. This result shows the efficiency of the 8-OH-DPAT interventions at the NRO, despite the potential concern that 8-OH-DPAT may be less effective in inhibiting raphe neuronal activity in medullary compared with rostral groups (41, 89). In addition, this result is in keeping with previous studies showing that suppression of medullary raphe neuronal activity, e.g., in the carbachol model of REM sleep in decerebrate and vagotomized cats (47), also leads to larger decreases in hypoglossal compared with phrenic nerve activity (45). The larger suppression of GG activity is also consistent with effects on hypoglossal and phrenic activities after nonspecific ablation or inhibition of medullary raphe neurons in anesthetized or decerebrate piglets (13). Lesions of medullary raphe neurons also suppress respiratory motor activities in vitro (72).

In contrast to the observed effects on respiratory-related GG and diaphragm activities, 8-OH-DPAT at the NRO had no effects on respiratory rate. Although this lack of effect contrasts with the respiratory slowing observed after ablation of NRO neurons in vitro (72), our results are in agreement with other in vivo experiments where 8-OH-DPAT at medullary raphe neurons also does not alter respiratory rate (59, 88). Nevertheless, the lack of a change in respiratory rate with inhibition of raphe neurons cannot be taken as evidence for a lack of influence of raphe inputs on centers controlling respiratory rhythm, and it does not conflict with an influence of 5-HT and coreleased neurotransmitters at these sites if raphe activity increases (2, 58, 73).

Diaphragm responses to CO2. The results also showed that inhibition of serotonergic medullary raphe neurons at the NRO decreased diaphragm responses to CO2 at 0.1 and 1 mM OH-DPAT. This result is in keeping with the suppressed phrenic motor responses to CO2 after nonspecific ablation or inhibition of medullary raphe neurons in anesthetized or decerebrate piglets (13). Overall, these observations are in keeping with the concept of CO2-mediated excitation of medullary raphe 5-HT neurons (93, 96, 97) being able to contribute to the ventilatory responses to CO2 (67, 82).

Effects on blood pressure and other variables. Significant decreases in blood pressure were also observed in the anesthetized rats, with effects most pronounced with 1 mM 8-OH-
DPAT where blood pressure decreased by \(\sim 32\%\) compared with control perfusion of ACSF. The effects of manipulation of medullary raphe neurons on the cardiovascular system are complex with responses affected by the site of manipulation and whether serotonergic or nonserotonergic neurons are involved (5, 12, 64). Nevertheless, serotonergic medullary raphe neurons project to sympathetic preganglionic neurons (12, 20, 53) where 5-HT depolarizes those neurons and can increase blood pressure (56, 74, 75). Accordingly, effects on blood pressure were expected in this study, and the decreases after inhibition of serotonergic medullary raphe neurons with 8-OH-DPAT may be best explained by inhibition of this sympathoexcitatory drive. Time control experiments in this preparation have repeatedly shown that physiological variables such as respiratory muscle activities, respiratory rate, and blood pressure are stable over time during continuous delivery of ACSF into the medial medulla, particularly over the time taken to apply only three drug doses as in this study (51, 52, 85). The absence of a change in EEG activity after 8-OH-DPAT in these animals is also consistent with minimal alterations in arousal state or nonspecific changes in the physiological status of the animal as being responsible for the observed changes in respiratory motor activities and blood pressure after 8-OH-DPAT. The maintenance of respiratory rate also attests to the stability of the preparation over time.

A potential concern of the anesthetized preparation, however, is that halothane was used to supplement the urethane. We routinely use this anesthesia regimen because it provides highly reliable preparations with stable respiratory and EEG activities over the course of the experiments (51, 52, 63, 85). In contrast, periodic bolus doses of supplemental urethane can suppress GG activity and alter the EEG either transiently or for prolonged periods. Nevertheless, halothane itself can suppress respiratory-related hypoglossal activity (38, 70) such that it is of potential concern that the observed responses to 8-OH-DPAT may have been influenced by adjustments of halothane over the course of the experiment. After initiation of halothane, however, if any further adjustments were required within an animal to maintain stable anesthesia these were only minor (typically 0.1%). It has been shown previously in cats that increases in halothane from 1 to 3% decreases GG activity, and that increments as high as 0.5% are required to reduce GG activity by \(\sim 50\%\) within an animal (70). Thus any potential adjustments of halothane in our study were small by comparison and, as demonstrated previously, were unlikely to have altered GG activity over the course of the experiment (51, 52). Moreover, decreases in hypoglossal and phrenic nerve activities also occur after suppression of raphe neuron activity in experiments performed without anesthesia, i.e., in decerebrate animals (13, 45, 47) and in vitro preparations (72).

**Responses in Conscious Animals**

**GG and diaphragm activities.** The robust suppression of GG and diaphragm activities in anesthetized rats after 8-OH-DPAT was in marked contrast to the lack of effect observed in conscious rats awake or asleep, despite using the exact same methodology and procedures. Indeed, the effects observed in the anesthetized rats served as positive controls for the potential of the interventions at the NRO to modulate respiratory activity. Although previous experiments in anesthetized (or decerebrate) vagotomized animals have convincingly demonstrated a role of medullary raphe neurons and 5-HT in modulating GG activity (13, 15, 48, 49, 85, 98), no previous study has directly addressed the physiological role of raphe neurons in modulating GG activity in freely behaving animals in vivo. The present results in conscious and intact animals show a minimal endogenous drive from serotonergic medullary raphe neurons influencing GG activity, a result that supports previous data showing a minimal role of endogenous 5-HT at the HMN (86). The present results also demonstrate a minimal endogenous role of serotonergic medullary raphe neurons in modulating diaphragm activity across sleep-wake states. These data are also in agreement with recent studies addressing the endogenous role of medullary raphe neurons in ventilatory control in behaving animals. Lesions targeted toward serotonergic medullary raphe neurons using saporin conjugated to an antibody of the 5-HT transporter also has no effect on room air breathing during wakefulness or sleep in rats (69). Microdialysis perfusion of 1 and 10 mM 8-OH-DPAT into medullary raphe nuclei of rats also does not change tidal volume or respiratory frequency during room air breathing, and although tidal volume was reduced after 30 mM 8-OH-DPAT overall ventilation was unchanged (88). Furthermore, neurotoxic lesions of medullary raphe neurons do not alter baseline ventilation in awake or sleeping goats (30). Taken together, these studies support the present results and agree with the concept of a minimal involvement of endogenous serotonergic raphe neuron activity in modulating respiratory motor activity during room air breathing across sleep-wake states.

With regard to the effects across sleep-wake states, it is established that raphe neurons typically discharge at their highest rates in wakefulness and at intermediate levels in non-REM sleep and show minimal activity in REM sleep (39). This generality, however, obscures the observation that the degree of sleep-state-dependent activity is different for those dorsal raphe neurons located in rostral brain regions that are involved with control of sleep-wake states, compared with medullary raphe neurons involved in motor and autonomic functions. Indeed, dorsal raphe neurons show marked declines in activity from wakefulness to non-REM and REM sleep, whereas medullary raphe neurons are only weakly modulated from wakefulness to non-REM sleep, although there remains a large suppression of activity in REM sleep (27). Rather, serotonergic medullary raphe neurons are thought to be activated more during rhythmic motor tasks associated with behavioral arousal and activities such as chewing, licking, and grooming (40, 81) but have a lesser role in modulating respiratory activity during basal breathing (83, 86). This behavior of raphe neurons may explain why 8-OH-DPAT decreased body temperature in these experiments, in keeping with previous studies (88), although this was only observed in active wakefulness when the raphe neurons are most active. It is important to note, however, that the concept of a minimal endogenous drive from raphe neurons influencing respiratory motor activity does not conflict with the notion that activation of raphe neurons can influence breathing. Stimulation of medullary raphe neurons with focal acidification increases ventilation in intact sleeping rats (68) and inspiratory airflow in awake goats (28, 29), showing functional connections to the respiratory system.

In anesthetized rats and cats, the discharge rates of identified serotonergic medullary raphe neurons, and neurons presumed
to be serotonergic based on their action potential shape, range from 1 to 3.5 Hz (18, 19, 57). Discharge rates may be higher in vagotomized animals because some medullary raphe neurons that project to motoneurons are inhibited by vagal afferents (8, 14). In decerebrate and vagotomized cats the discharge rates of 8-OH-DPAT-sensitive medullary raphe neurons was 0.1–2.6 Hz, although these were selected on the basis of <3-Hz discharge (98). In conscious rats, the discharge rates of presumed serotonergic medullary raphe neurons averages 3.7 ± 0.8 (SD) Hz in wakefulness, 2.8 ± 0.8 Hz in non-REM sleep, and 0.1 ± 0.2 Hz in REM sleep (81), i.e., overlapping the range observed in anesthetized animals. Similarly, the discharge rates of identified medullary serotonergic raphe neurons in cats also encompasses this range; 3.2–5.5 Hz in wakefulness, 1.8–3.2 Hz in non-REM sleep and 0.2–0.9 Hz in REM sleep (39, 93). Overall, the presence of robust serotonergic medullary raphe neuronal discharge under anesthesia would explain the suppression effects of 8-OH-DPAT on GG and diaphragm activities. In contrast, the lack of changes in respiratory motor activity in the conscious animals after 8-OH-DPAT, despite similarly robust raphe neuronal activity, suggests that different mechanisms may be operative in behavior. Indeed, the serotonergic control of respiratory motoneurons may not be as straightforward as first presumed; in vitro evidence suggests that a major component of the raphe pallidus inputs to hypoglossal motoneurons are glutamatergic with 5-HT actually inhibiting the release of this excitatory neurotransmitter via presynaptic effects (10).

The neural mechanisms responsible for the periods of GG motor suppression in sleep, especially REM sleep, remain to be determined, but we have shown that postsynaptic inhibition mediated via glycine and γ-aminobutyric acid, and disinhibition via 5-HT, play only a minor role in intact animals (61, 62, 86). A recent study using the carbachol model of REM sleep in anesthetized rats implicates an important role of disfacilitation in suppression of hypoglossal motor activity via withdrawal of norepinephrine (16), and this needs to be tested in natural sleep.

**Diaphragm and ventilatory responses to CO2.** The physiological role of serotonergic medullary raphe neurons in modulating the ventilatory responses to CO2 in vivo is a source of current interest and debate (21, 65, 67, 82). The results of the present study in conscious rats showed that 8-OH-DPAT at the medullary raphe nuclei did not alter baseline respiratory activities, a result in agreement with other studies using 8-OH-DPAT or neurotoxic lesions (30, 69, 88). However, our observations of a similar lack of effect of 8-OH-DPAT on the ventilatory response to inhaled CO2 does not agree with these previous studies (30, 69, 88). Application of 1, 10, and 30 mM 8-OH-DPAT to the medullary raphe nuclei decreased ventilation in the presence of hypercapnia, a result that was taken to support a role for raphe neurons in the manifestation of chemoreceptor responses (88). These high doses of 8-OH-DPAT were chosen for that study because preliminary experiments in conscious rats showed that they were necessary to significantly suppress the ventilatory responses to CO2, whereas lesser doses (0.1–0.5 mM) were ineffective, i.e., similar to the results of our experiments. However, although their initial screening to find a dose of 8-OH-DPAT that agreed with the authors’ hypothesis was effective (88), it did not take into account the possible influences of such high doses of 8-OH-DPAT on other physiological variables. Indeed, microdialysis perfusion of lower doses such as 0.01 mM 8-OH-DPAT are sufficient to inhibit raphe neuronal activity in vivo (76), i.e., in keeping with the dose range in our experiments.

One of the concerns raised by the present study is that the high doses of 8-OH-DPAT at medullary raphe neurons used in previous experiments (88) may be expected to significantly affect other physiological variables, not the least of which blood pressure and body temperature. Such changes may have influenced the behavioral state of the animal and/or produced nonspecific effects on the ventilatory response to CO2. With respect to blood pressure, our results showed that 1 mM 8-OH-DPAT at medullary raphe neurons lowered blood pressure by ~32% in anesthetized rats, in agreement with other reports (24, 25, 90), although significant increases have been observed in conscious piglets with the same interventions (59). In our experiments, this major effect on blood pressure was a significant reason for choosing 0.1 mM OH-DPAT for subsequent testing in the conscious rats because lesser decreases in blood pressure were observed with this dose and yet robust suppression of GG and diaphragm activities, and diaphragm responses to CO2, still occurred. Either lowered (33) or raised blood pressure (34) elicits significant behavioral responses in conscious animals, and this may account, at least in part, for the reduced or absent sleep observed with 10 and 30 mM 8-OH-DPAT at medullary raphe neurons in conscious rats and piglets in previous experiments (59, 88). Further evidence of behavioral or nonrespiratory effects after 10 and 30 mM 8-OH-DPAT at medullary raphe nuclei in rats include documentations of “visible increases in animal activity” with animals appearing “highly alert” and maintaining “an upright posture throughout the experiment” (88). It is unknown whether these overt behavioral changes or alterations in other physiological variables with high doses of 8-OH-DPAT may have contributed to the diminution of the ventilatory responses to CO2 (88). In the present study, the observed increase in EEG activity in the high (β2)-frequency band after 8-OH-DPAT in the conscious animals, even with the lower dose of 0.1 mM, suggests the possibility of behavioral activation, even though this was not manifest as overt behaviors and sleep states were not obviously affected in our experiments.

It is also possible that the lack of effect of 8-OH-DPAT on respiratory motor activities and responses to hypercapnia in our conscious animals may have been because only one raphe neuronal group was targeted by the interventions, and that this was insufficient to produce a physiological response in the conscious as opposed to the anesthetized rats. This suggestion is possible given that local stimulation of medullary raphe neurons with focal acidification increases inspiratory airflow in awake and chronically instrumented goats, but effects are more readily apparent with focal acidification at multiple medullary raphe sites compared with a single site alone (29). It is conceivable, therefore, that significant effects of 8-OH-DPAT on the ventilatory responses to hypercapnia may have been observed with manipulation of multiple raphe sites in the conscious animal rather than with interventions at the NRO alone. Although the coordinated involvement of the ensemble of medullary raphe neuronal groups in the control of breathing and responses to hypercapnia cannot be fully discounted, this possibility may be unlikely because our laboratory has previously shown in freely behaving rats that there is minimal
involvement of endogenous 5-HT at the hypoglossal motor nucleus in modulating GG respiratory motor activity during room air and CO₂-stimulated breathing (86), a relevant result given that the source of 5-HT innervation of the HMN is from the ensemble of medullary raphe neurons (54).

In conclusion, the results support the concept that serotonergic medullary raphe neurons play a minimal role in modulating respiratory motor activity across natural sleep-wake states in freely behaving rodents. The implications of this finding are that unless the endogenous role of serotonergic medullary raphe neurons in modulating pharyngeal muscle activity is augmented in certain conditions, e.g., as animal studies suggest by upper airway narrowing and OSA (66, 94), then pharmacological strategies aiming to increase endogenous 5-HT at pharyngeal motoneurons in humans may not be particularly effective in increasing upper airway muscle activity if a similar lack of endogenous 5-HT drive is present. Preliminary evidence in OSA patients (7, 23, 46) suggests this may be the case. Nevertheless, the contribution of endogenous 5-HT to the control of GG activity may be increased in certain conditions involving activation of raphe neurons. For example, intermittent hypoxia causes long-term facilitation of respiratory motor activity by 5-HT-dependent mechanisms in animal preparations (9, 60), and this may be relevant to upper airway function in humans (1).

The present results, however, do not rule out the potential in OSA for pharmacological interventions using agents to increase raphe neuronal activity per se or 5-HT agonists or precursors, because these do not depend on the intrinsic serotonergic activity of neurons innervating pharyngeal motoneurons. Nevertheless, the present data suggest that endogenously active serotonergic medullary raphe neurons are not principally involved in activating GG muscle in a sleep-state-dependent manner. Recent evidence from the carbachol model of REM sleep in anesthetized rats suggests that noradrenergic inputs to the HMN may be important in the suppression of hypoglossal motor activity in sleep. This postulate remains to be tested in intact conscious animals and humans.

GRANTS

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