Compensatory airway dilation and additive ventilatory augmentation mediated by dorsomedial medullary 5-hydroxytryptamine 2 receptor activity and hypercapnia

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1 mm membrane length; 0.24 mm diameter; 6000 Da cutoff; Carnegie Medicin, Stockholm Sweden) was inserted to a depth of 1 mm from the dorsal brain surface 0.45 mm lateral to the midline and 0.8 mm rostral to the obex. The cranial bone was cleaned with 10% H2O2, and the inserted probe was fixed to the cranial bone with dental cement. The skin incision was closed with sterilized 5-0 silk sutures. Each mouse was placed in a double-chamber plethysmograph and was given ~3.5 h to recover from anesthesia and become acclimatized to the chamber. The head and body chambers were provided with two continuous airflows of 150 ml/min with a vacuum pump. Dialysate (artificial cerebrospinal fluid [121.1 mM NaCl, 5 mM KCl, 24 mM NaHCO3, and 1.5 mM CaCl2 adjusted to pH 7.4 with 95% O2 and 5% CO2]) was collected at a rate of 1.2 µl/min every 25 min into a vial containing 10 µl 0.02 M acetic acid.

5-HT release was analyzed with an HPLC system equipped with an electrochemical detector (BMA-300; Eicom, Kyoto, Japan) and an EicomPAK column (CA-5ODS, 2.1 mm ID × 150 mm; Eicom). The mobile phase was composed of 0.1 M sodium phosphate buffer (pH 6.0) containing 5% methanol, 50 mg/l EDTA-2Na, and 100 mg/l sodium pentanesulfonate. The flow rate was 0.23 ml/min. The column temperature was maintained at 25°C. 5-HT was oxidized at 400 mV on a graphite electrode relative to an Ag-AgCl reference electrode. Thirty-five microliters of each 40-µl sample was injected into the HPLC apparatus with the use of an autosampler (NASPSPACE SI-2; Shiseido, Tokyo, Japan). Chromatographs were recorded and analyzed with a PowerChrom system (EPC-300; Eicom). The detection limit of the HPLC system for 5-HT was 3.4 fmol/35 µl (signal-to-noise ratio = 3).

Two concomitant curves for respiratory flow from the head and body chambers were obtained with pneumotachographs (TV-241T; Nihon Kohden, Tokyo, Japan), and pressure transducers (TR-602T; Nihon Kohden) recorded at a 10-kHz sampling rate and were later analyzed with PowerLab (ADI Instruments, NSW, Australia) (15, 47). Ventilatory volume was calculated from the respiratory flow curve for the head and body chamber flows (33). Rectal temperature was maintained at 37°C by a heating lamp throughout the entire experimental period. Probe placement sites were verified in 50-µm-thick coronal sections.

CO2 inhalation. 5-HT release in the DMM was increased by fluoxetine (Sigma-Aldrich, St. Louis, MO) perfusion or 10−5 M fluoxetine plus 10−5 M fluoxetine plus LY-53857 (Sigma-Aldrich) coperfusion in the DMM for 75 min. Airway resistance was increased concomitantly by 10−5 M fluoxetine and LY-53857 coperfusion. After confirming the increase in 5-HT release with 10−5 M fluoxetine or the increase in sRaw with 10−5 M LY-53857 or both, all mice were exposed to 100% O2 for 25 min and then to stepwise CO2 inhalation (5%, 7%, and 9% CO2 in O2) at intervals of 8 min. The flow rate of each gas to the inlet of the head chamber was 2 l/min with overflow.

Data analysis. Respiratory variables were analyzed for 5 s at 3.5 and 7.5 min after each gas exposure and were averaged over two measurements. Changes in sRaw relative to inspired CO2 concentration were expressed as means ± SE of percentages of the minimum value of sRaw (% of minimum sRaw) and evaluated with Dunnett’s test. The difference in sRaw during 100% O2 inhalation between fluoxetine perfusion and fluoxetine plus LY-53857 coperfusion was evaluated by Student’s t-test. Tidal volume (VT)-inspiratory time (TI) curves were evaluated by inverse regression curve analysis, slope changes, and shifts (3) were evaluated by covariance analysis. Other data were expressed as means ± SE and evaluated by two-way ANOVA (SPSS Japan, Tokyo, Japan). P < 0.05 was considered statistically significant.

RESULTS

Body temperature was maintained at ~37°C; there was no difference between the fluoxetine-perfused group and the fluoxetine plus LY-53857-coperfused group (Fig. 1). After recovery from anesthesia, respiratory curves showed irregular waves elicited by movement and apnea in addition to regular waves. In those cases, respiratory variables were analyzed for 5 s during periods of regular respiratory waves just before the occurrence of artifacts (Fig. 2).

5-HT release in the DMM during CO2 inhalation. Under the baseline condition during 100% O2 inhalation for 25 min, 5-HT release was 11.0 ± 3.2 fmol/35 µl with fluoxetine perfusion (n = 3) and 12.0 ± 1.4 fmol/35 µl with fluoxetine plus LY-53857 coperfusion (n = 5). 5-HT release elicited by inhalation of 5%, 7%, and 9% CO2 in O2 at 8-min intervals was 26.8 ± 6.2 fmol/35 µl with fluoxetine perfusion (n = 3) and 30.7 ± 6.0 fmol/35 µl with fluoxetine plus LY-53857 coperfusion (n = 5). 5-HT release was increased up to 2.4-fold that of basal release during fluoxetine perfusion and 2.6-fold that of basal release during fluoxetine plus LY-53857 coperfusion. The increase in 5-HT release in the DMM did not differ significantly between the two groups (Fig. 3A).

The sites of microdialysis probe placement in the DMM were in the nXII, the nTS, and the dorsal motor nucleus of the vagus. The distribution was similar between the fluoxetine-perfused group (Fig. 3B) and the fluoxetine plus LY-53857-coperfused group (Fig. 3C).

Effects of 5-HT2 receptors in the DMM on airway and respiratory responses during CO2 inhalation. Values of sRaw were minimized by 9% CO2 inhalation in the fluoxetine-perfused group (n = 3) and the fluoxetine plus LY-53857-coperfused group (n = 5). The minimum values of sRaw did not differ significantly between the two groups, as shown in Fig. 4A, inset. The relation between the percentage of the minimum value of sRaw (% of minimum sRaw) and the inspired CO2 fraction expressed as a percentage [FICO2 (%)] was significantly greater during 100% O2 inhalation than during 9% CO2 inhalation in each group (P < 0.05). During 100% O2 inhalation, the % of minimum sRaw was also significantly greater in the fluoxetine plus LY-53857-coperfused group than
in the fluoxetine-perfused group (P < 0.05). The highest value of the % of minimum sRaw was observed in the period of 100% O2 inhalation of the fluoxetine plus LY-53857-coperfused group, which was gradually decreased with increasing inspired-CO2 concentration (Fig. 4A).

Minute ventilation (V˙E) during fluoxetine perfusion was significantly and dose-dependently increased from 13.2 ± 1.1 ml/10 g body wt during 100% O2 inhalation to 31.4 ± 4.7 ml/10 g body wt at the end of the series of CO2 inhalations (2.4-fold increase in the value during 100% O2 inhalation, P < 0.05, n = 3). V˙E during fluoxetine plus LY-53857 coperfusion increased from 8.7 ± 0.4 ml/10 g body wt during 100% O2 inhalation to 22.9 ± 3.2 ml/10 g body wt at the end of the series of CO2 inhalations (2.6-fold increase in the value during 100% O2 inhalation, P < 0.05, n = 5). The change in V˙E did not differ significantly between groups. However, the absolute values of V˙E at each CO2 concentration in the fluoxetine plus LY-53857-coperfused group were significantly lower than those in the fluoxetine-perfused group (Fig. 4B, P < 0.05).

The respiratory rate (RR) in the fluoxetine-perfused group was significantly increased from 172 ± 8/min at baseline to 239 ± 4/min during 9% CO2 inhalation (n = 3, P < 0.05), and that in the fluoxetine plus LY-53857-coperfused group increased from 174 ± 7/min at baseline to 228 ± 8/min at the highest CO2 concentration of the series (n = 5). The increase in RR in the fluoxetine plus LY-53857-coperfused group did not differ significantly from that in the fluoxetine-perfused group (Fig. 5A).

V$_T$ during fluoxetine perfusion was significantly and dose dependently increased from 0.076 ± 0.004 ml/10 g body wt during 100% O2 inhalation to 0.132 ± 0.021 ml/10 g body wt at the end of the series of CO2 inhalations (1.7-fold increase in the value during 100% O2 inhalation, P < 0.05, n = 3). V$_T$ during fluoxetine plus LY-53857 coperfusion increased from 0.051 ± 0.004 ml/10 g body wt during 100% O2 inhalation to 0.099 ± 0.011 ml/10 g body wt at the end of the series of CO2 inhalations (1.4-fold increase in the values during 100% O2 inhalation, n = 5). The change in V$_T$ did not differ significantly between groups. However, the absolute values of V$_T$ at each CO2 concentration in the fluoxetine plus LY-53857-coperfused group were significantly lower than those in the fluoxetine-perfused group (Fig. 5B).

In response to step changes in inhaled CO2 concentration, the relation between VT and TI was analyzed as a hyperbolic regression curve (Fig. 3C) (3). The relation during fluoxetine plus LY-53857 coperfusion in the DMM shifted downward with unchanged slope compared with that during fluoxetine perfusion. The isopleths for V/TI, as shown by the dotted lines, were lower during fluoxetine plus LY-53857 coperfusion than during fluoxetine perfusion.

**DISCUSSION**

In the present study, the effects of 5-HT release on 5-HT2 receptors in the DMM and CO2 on ventilatory responses of airway resistance and respiratory variables were investigated in mice with microdialysis and double-chamber plethysmography. The sRaw during 5-HT2 receptor antagonism (LY-53857) plus SSRI (fluoxetine) in the DMM was higher than that during SSRI perfusion alone. The increase in sRaw was largely reversed by CO2 inhalation. Ventilatory volume increased in mice with microdialysis and double-chamber plethysmography. The sRaw during 5-HT2 receptor antagonism (LY-53857) plus SSRI (fluoxetine) in the DMM was higher than that during SSRI perfusion alone. The increase in sRaw was largely reversed by CO2 inhalation. Ventilatory volume increased in mice with microdialysis and double-chamber plethysmography.
volume, and that interaction between 5-HT2 receptor activity in the DMM and CO2 drive may cause a cycle of hyperventilation with airway dilation and hypoventilation with airway narrowing.

Local anesthesia in addition to general anesthesia (preemptive analgesia) decreases postoperative pain. Peripheral neural blockade prevents nociceptive impulses from entering the central nervous system (6, 44). Analgesics are likely to affect central function (4, 25, 49). In the present study, 2% xylocaine was injected locally after induction of general anesthesia with pentobarbital sodium to affect preemptive analgesia.

Somatic nociceptive afferents represent effective stimuli for increases in RR (5). By double-chamber plethysmography, the RR of mice after surgery in the present study was similar to that of mice without any surgery in a previous study (15). Resting values for RR, VT, and V˙E in mice in the present study were comparable with those of nonrestrained mice in a single chamber (16).

A previous study showed that C57BL/6 mice exposed to 5% CO2 in O2 for 10 min show decreased pH from 7.4 to 7.3 and increased arterial partial pressure of CO2 (Paco2) from 43 mmHg to 56 mmHg (16). Mice in the present study were exposed to step changes in inhaled CO2 concentration (5%, 7%, and 9% CO2 in O2) at 8-min intervals and were likely hypercapnic and experiencing respiratory acidosis. The RR response to CO2 inhalation was not significantly different between the fluoxetine perfusion and the fluoxetine plus LY-53857 coperfusion groups. Therefore, the arterial blood acid-base status was likely similar between the groups.

In the present study, 5-HT2 receptor activity in the DMM decreased airway resistance. 5-HT2 receptor activity in the nXII induces genioglossus muscle activity (7, 19), which causes airway dilation. In the present study, hypercapnia also increased airway resistance. Hypercapnia also elicits genioglossal muscle activity (31). We found that airway narrowing induced by 5-HT2 receptor antagonism in the DMM was largely reversed by hypercapnia. Thus, hypercapnia-induced airway dilation was not mediated via 5-HT2 receptors in the DMM. There are reports questioning the role of 5-HT in the increase of genioglossus muscle activity in response to hypercapnia in rats (39, 40). The facts suggest that hypercapnia and 5-HT2 receptor activity in the DMM compensate each other for airway dilation through distinct pathways.

5-HT2 receptor activity in the DMM increased the basal level of V˙E, dependent on tidal volume change, but did not affect the V˙E CO2 response gain [ΔV˙E/ΔFicO2 (%)], although both V˙E and serotonergic neuron activity in the caudal raphe, which serves as a source of 5-HT in the DMM, increase as each function of inspired CO2 (45). Our results suggest that 5-HT release acting on 5-HT2 receptors in the nXII and nTS, rather than dose-dependent CO2 responses, mediates increased basal levels of airway dilation and V˙E.

sRaw and V˙E responses were affected by four different conditions elicited by interaction between 5-HT2 receptor activity in the DMM and CO2 drive (Fig. 4). Condition 1 (arrow 1) was without 5-HT2 receptor activity in the DMM and with gradual increase in CO2 drive. sRaw decreased, and V˙E increased. Condition 2 was with increased 5-HT2 receptor activity in the DMM and increased CO2 drive. sRaw decreased to the minimum level, and V˙E increased to the maximum level. Condition 3 was with 5-HT2 receptor activity in the DMM and gradual decrease in CO2 drive. sRaw increased slightly, and V˙E
decreased. Condition 4 was decreased with 5-HT2 receptor activity in the DMM and decreased CO2 drive. sRaw increased to the maximum level, and V˙E decreased to the minimum level. 5-HT in the nXII increases sleeping genioglossus muscle activity to normal waking levels (19), and serotonergic caudal raphe neurons, which serve as a source of 5-HT in the DMM, are associated with locomotion, hypercapnia, and feeding, in addition to sleep-awake states (45). Therefore, condition 1 is interpreted as representing the sleep state, without specific motor activity, condition 3 is interpreted as representing the arousal state, with the specific motor activity, and conditions 2 and 4 are interpreted as representing the transitional state between sleep and arousal or between on and off states of specific motor activity.

With respect to the inspiratory off-switch mechanism of the respiratory central pattern generator, CO2 inputs have no systemic effect on volume threshold curves (1, 48). In Fig. 5C, the V_T/T1 relation was shifted downward by 5-HT2 receptor antagonism in the DMM. The isopleths for V_T/T1 (dotted lines), which indicate central inspiratory activity, were decreased by 5-HT2 receptor antagonism (arrows). However, with the same chemical drive, inspiration was terminated at a lower volume threshold. These results suggest that 5-HT acting on 5-HT2 receptors in the DMM increases both central inspiratory activity and the volume threshold in the inspiratory off-switch mechanism. Neurons from the ventrolateral nTS project bilaterally to the phrenic nucleus (36). Thus, 5-HT acting on 5-HT2 receptors in the DMM influences the inspiratory off-switch mechanism of the respiratory central pattern generator in the nTS and increases ventilatory volume.

With respect to the roles of the brain 5-HT system, there are two major hypotheses. Jacobs and Fornal (17) hypothesized that the serotonergic system in the brain facilitates activity of motor and premotor neurons. Richerson (35) hypothesized that 5-HT neurons in the medulla oblongata sense of CO2 level and pH. Neurons in the nTS also act as central CO2 chemoreceptors.
In the present study, 5-HT2 receptor activity in the DMM increased basal levels of airway dilation and ventilatory volume, which was dependent on increased central inspiratory activity and volume threshold of the inspiratory off-switch mechanism. Hypercapnic responses of airway dilation and ventilatory augmentation were not suppressed by 5-HT2 receptor antagonism in the DMM. These results suggest that 5-HT release acting on 5-HT2 receptors in the DMM contributes to facilitation of respiratory motor and premotor neuron activity rather than to facilitation of sensitivity to CO2 and pH.

5-HT afferents to the nXII originate from the raphe pallidus and obscurus nuclei (26), and those to the nTS are derived from the raphe magnus, raphe pallidus, raphe obscurus, and dorsal raphe nuclei (37, 43) and from the nodose ganglia (29). 5-HT concentration in the dorsal vagal complex, including the nTS, is increased by electrical stimulation of the raphe obscurus nucleus (2). c-Fos expression induced by CO2 is observed in serotonergic cells in the raphe pallidus nucleus (B1 group), its lateral extension (parapyramidal serotonergic cells), the raphe obscurus nucleus (B2 group), the raphe magna nucleus (B3 group), and the dorsal raphe nucleus (B7 group) (13). In the present study, CO2 inhalation may have stimulated caudal raphe neurons and increased 5-HT release in the DMM. Further studies are necessary to determine the relation between 5-HT receptors and neuronal activity in the DMM.

In this study, the nXII, the dorsal motor nucleus of the vagus, and the nTS were included in the DMM, into which microdialysis probes were inserted. The mouse DMM extends ~1 mm to each side. The microdialysis probe membrane, with a diameter of 0.24 mm and a length of 1 mm, was inserted near the center of the left DMM. Lesions produced by the microdialysis probes were located in the left DMM and included the nTS and the nXII. Fluorescent tracer perfused through a microdialysis probe on one side was reported to be centered largely within the nXII on both sides, due to extensive bilateral distribution of the dendrites of adult hypoglossal motoneurons in rats (27). In the present study, fluoxetine and LY-53857 perfused through the same kind of microdialysis probe may have spread to the nXII on both sides.

In vagotomized rats, hypoglossal nerve activity is reduced by 35% to 81% by serotonergic and/or noradrenergic antagonists applied to the nXII (8). Airway resistance is modified by many airway muscles, such as the tensor and levator velipalatine muscles, pharyngeal constrictors, and others (22). It is possible that the hypercapnia-induced decrease in airway resistance may involve noradrenergic receptors in the nXII and other airway dilator muscles in addition to serotonergic receptors in the nXII and the genioglossus muscle.

Serotonergic receptors in the brain are up-regulated or sensitized in patients with OSA (14). Eucapnic patients show an increase in inspiratory VT and a decrease in end-tidal CO2 are accompanied by increases in VT (50). Passive collapsibility and flow demand, depending on supine position and body mass index, determine the severity of OSA (51). In OSA patients, arousal during sleep accompanied by airway reopening promotes ventilatory instability and likely exacerbates OSA (52). We speculate that in OSA patients, hypercapnia during sleep and enhanced 5-HT2 receptor activity in the DMM during brief arousal cause greater airway dilation and hyperventilation. Subsequent hypocapnia and decreased 5-HT2 activity in the DMM during the return to sleep elicit airway narrowing and hypoventilation. Enhanced periodic breathing may be an exacerbating factor in periodic OSA.

In conclusion, 5-HT2 receptor activity in the DMM increases the basal levels of airway dilation and VT in mice, due to increases in central inspiratory activity and the volume threshold of the inspiratory off-switch mechanism, resulting in a facilitation of ventilation. Even though the effects of 5-HT2 receptor activity in the DMM on the airway and ventilatory volume are low, hypercapnia facilitates ventilation due to compensatory airway dilation and additive ventilatory volume augmentation. Interaction between 5-HT2 receptor activity in the DMM and CO2 drive may elicit a cycle of hyperventilation with airway dilation and hypoventilation with airway narrowing, which may be a physiological mechanism to optimize ventilation in sleep-awake states or in states of special motor activity and may underlie the pathogenesis of periodic breathing and periodic OSA.

REFERENCES