Orexins Increase Cortical Acetylcholine Release and Electroencephalographic Activation through Orexin-1 Receptor in the Rat Basal Forebrain during Isoflurane Anesthesia

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Background: Cholinergic arousal system plays an important role in the maintenance of consciousness. The authors investigated whether the intrabasalis injection of orexin-A or orexin-B and the electrically stimulated pedunculopontine tegmentum nucleus (PPTg: the origin of cholinergic ascending pathways) may alter acetylcholine efflux and electroencephalographic activity in the somatosensory cortex in relation to the orexergic system in isoflurane-anesthetized rats.

Methods: Either orexin-A (10, 30, or 100 pmol) or orexin-B (10, 30, or 100 pmol) (n = 6 each) was injected into the basal forebrain while the electroencephalogram was measured during 1.0 minimum alveolar concentration (1.2%) isoflurane anesthesia. Injection of Ringer's solution was used as a control. The PPTg was electrically stimulated twice with the following conditions: 1-s stimulus train (0.2 ms, 100 Hz, 400 μA) per min for 20 min. Twenty minutes before the second PPTg stimulation, Ringer's solution or 20 μg SB334867, an orexin-1 receptor antagonist (n = 5 each) was injected into the basal forebrain.

Results: Injection of orexin-A (30 and 100 pmol) and orexin-B (100 pmol) significantly increased the acetylcholine efflux in the somatosensory cortex (P < 0.05). Injection of orexin-A (10, 30, 100 pmol) and orexin-B (30, 100 pmol) changed the burst and suppression patterns to arousal electroencephalogram. Compared with orexin-B, injection of a lower dose of orexin-A induced increase in the acetylcholine efflux and arousal electroencephalogram. SB334867 significantly attenuated the increases in the acetylcholine efflux and electroencephalographic activation evoked by PPTg stimulation.

Conclusion: The authors demonstrated that orexin-A was more potent than orexin-B in producing alteration of cholinergic basal forebrain neuronal activity and that the cortical activation induced by the PPTg stimulation against isoflurane anesthesia may be mediated through the orexin-1 receptors in the basal forebrain.

THE neuropeptide orexins (orexin-A and orexin-B) were originally described to be important mediators of food intake. Apart from their role in appetite regulation, the orexins have also been reported to play a role in sleep and arousal. Our previous study demonstrated that orexin-A induced electroencephalographic arousal in the isoflurane-anesthetized rat, and it has been suggested that the orexergic system may be an important target for barbiturate anesthesia. Therefore, the orexins seem to take part in the regulation of anesthetic level.

The cholinergic ascending arousal system is one of the important cortical activating systems among the various arousal systems in the brain. The system originates from the pedunculopontine tegmentum (PPTg) nucleus, which is innervated and activated by the reticular formation and projects to the somatosensory cortex (S1BF). There are two pathways in the cholinergic ascending arousal system: a dorsal pathway from the PPTg to the S1BF through the thalamus and a ventral pathway through the subthalamus and/or posterior hypothalamus toward the basal forebrain (BF). Of the two systems, the ventral ascending activating system has shown to have a predominant role on cortical activation. In fact, the cholinergic ventral ascending activating system, which is composed of the posterior hypothalamus, the BF, and the S1BF, regulates the state of consciousness during natural sleep–wake cycle. The BF neurons are activated by glutamate released from the brainstem reticular formation, noradrenaline from the locus coeruleus, and orexins and histamine from the posterior hypothalamus. Among these neuronal systems, which relay input to the BF, the role of orexins on the maintenance of awaking remains to be well defined. It has been demonstrated that bilateral orexin-A infusion into the basal forebrain elicited consistent increases in total time spent awake. In addition, the infusion of orexin-A into the BF elicited an increase in the cortical acetylcholine release in the awake condition of the rat. However, it remains unclear whether orexins change the acetylcholine efflux and electroencephalogram in the S1BF and which subtype of orexin has a predominant influence on the regulation of arousal during isoflurane anesthesia. Therefore, in the current study, we examined whether intrabasalis injection of orexin-A or orexin-B might influence the acetylcholine efflux and electroencephalogram in the S1BF during isoflurane anesthesia in the rat. In addition, it is unclear whether orexin might be involved in the cholinergic ventral ascending system and particularly in the BF after stimulation of the PPTg. Therefore, we examined whether intrabasalis injection of SB334867, an orexin-1 receptor...
right lateral 0.8 mm). The leads were connected to a reference electrode placed in the frontal bone (posterior 7.4 mm from bregma, lateral 5.0 mm). They were housed at a constant temperature (24° ± 0.5°C) with a relative humidity (60 ± 2%) on a light-controlled schedule (light on between 6:00 AM and 6:00 PM) and had free access to food and water. The experimental protocols were approved by the Animal Care Committee of University of Fukui (Fukui, Japan).

Materials and Methods

Animals
Male Wistar rats, weighing 270-320 g, were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were housed at a constant temperature (24° ± 0.5°C) with a relative humidity (60 ± 2%) on a light-controlled schedule (light on between 6:00 AM and 6:00 PM) and had free access to food and water. The experimental protocols were approved by the Animal Care Committee of University of Fukui (Fukui, Japan).

Surgical Operations
During pentobarbital anesthesia (50 mg/kg, intraperitoneal), rats underwent surgery for implantation of electrodes for electroencephalographic recording and placement of a guide cannula for the microdialysis probe and injection needle. A guide cannula (OD, 0.6 mm) for the microinjection needle (14 mm) and a guide cannula (OD, 0.5 mm) for the microdialysis probe (membrane length, 2 mm; OD, 0.35 mm; cutoff size, 20 kd; Eicom, Kyoto, Japan) were directed stereotaxically at the BF, the S1BF, or both. The coordinates of the microinjection needle and microdialysis probe tip according to the atlas of Paxinos and Watson18 were as follows: BF (nucleus basalis, substantia innominata, and magnocellular preoptic nucleus): anteroposterior −1.4 mm from bregma, lateral 2.5 mm, dorsoventral −8.5 mm; S1BF: anteroposterior −1.4 mm from bregma, lateral 5.0 mm, dorsoventral −2.8 mm, inserting at a 15° angle from the dura. Before the start of the microdialysis perfusion, the stylet of the microdialysis probe was removed. The injection needle for the BF was 2 mm longer than the guide cannula.

In the second experiments, a concentric bipolar stimulating electrode (250-μm tip diameter) made by ourselves was placed into the PPTg (anteroposterior −8.4 mm from bregma, lateral 2.0 mm, dorsoventral −6.6 to −6.8 mm) for stimulation and the microdialysis probe for the S1BF and the microinjection cannula into the BF were inserted at the same coordinates as the first experiments and fixed with dental cement 5-7 days before the experiment.

Five stainless steel screws for epidural electroencephalogram recording were implanted. The EEG screws were placed bilaterally over the frontal (anterior 3.9 mm from bregma, lateral ±2.0 mm) and the occipital cortex (posterior 7.4 mm from bregma, lateral ±5.0 mm). The reference electrode was placed in the frontal bone rostral to the frontal cortex (anteroposterior 5.5 mm from bregma, right lateral 0.8 mm). The leads were connected to a socket, which was fixed to the skull together with the electrodes and the microdialysis guide cannula using dental cement.

Microdialysis and Orexin Microinjection
The rats were anesthetized again with 1.8% isoflurane 1 day before the experiments. A heparin saline-filled polyethylene catheter (MRE-040; Eicom) was inserted into the femoral artery for measurement of arterial blood pressure. The tip of the arterial catheter was positioned in the abdominal aorta. The catheter was exteriorized on the back of the rat. After 5-7 days of recovery from the surgical operations for implantation of electroencephalogram electrodes and insertion of the microinjection and microdialysis cannula, the rats were transferred from their own home cage to a cylindrical cage (internal diameter, 25 cm; height, 50 cm; volume, 25 l) for multichannel infusions and electrical recordings (Neijiren, Osaka Microsystems, Japan). They were connected to the electroencephalographic recording cable for adaptation to the experimental conditions. The twisting of liquid lines was avoided by an automated floor rotating system controlled by a twist degree detector, allowing free movement of the animal.19 During measurement, the stylet of the microdialysis guide cannula was replaced by a microdialysis probe. The probe was continuously perfused with Ringer’s solution (140 mM NaCl, 3 mM KCl, 1.3 mM CaCl2; pH 7.4) containing eserine (10−7 M) at a rate of 1.0 μl/min. One minimum alveolar concentration (MAC, 1.2%) isoflurane was added to the gas mixture for 1 h after a control period. The concentration reached a plateau within 1-2 min and became stable, because the outlet of the gas tubing was placed at the bottom of the cylindrical cage and a high flow rate (3 l/min) of oxygen containing isoflurane immediately filled with the atmosphere around the rat. The concentration of isoflurane was continuously measured by a side-stream capnometer (Capnox; Colin, Aichi, Japan). The 1.2% isoflurane was considered to be 1.0 MAC as reported previously.20 According to the solutions that were injected into the BF, the animals were divided into two groups. In the orexin-A group (n = 6), 0.3 μl of 10, 30, or 100 pmol orexin-A or Ringer’s solution (control) was injected into the BF at a random sequence during 1.0 MAC isoflurane anesthesia. In the orexin-B group (n = 6), 10, 30, or 100 pmol orexin-B or Ringer’s solution (control) was injected into the BF using the same procedures as in the orexin-A group. The interval between injections of the solutions was 100 min. After each injection, five postinjection samples were collected for acetylcholine measurements. The samples were collected every 20 min, and the last sample collected before microinjection of another dose of orexin or Ringer’s solution was set as a control. Microinjections were performed with a Hamilton syringe (1 μl; Hamilton, Reno, NV) connected to polyethylene...
OREXIN-INDUCED AROUSAL DURING ISOFLURANE ANESTHESIA

Orexin-induced arousal during isoflurane anesthesia

Baseline, a 1-s stimulus train (0.2 ms, 100 Hz, 400 μA) was collected over a 20-min period as described previously. Each dialysis sample for the measurement of acetylcholine was inserted into the femoral artery and vein for the measurements of mean arterial pressure, heart rate, blood gas, and electroencephalogram were monitored during the experiment.

PPTg Electrical Stimulation

Ten rats were assigned to the Ringer’s solution (control) group and SB334867 group. On the day of the experiment, the rats were tracheally intubated and ventilated via a respirator (Rodent Ventilator, model 683; Harvard Apparatus, Holliston, MA). Polyethylene catheters filled with heparin containing saline solution were inserted into the femoral artery and vein for the measurements of heart rate, blood gas, and infusion of drugs during 1.5 MAC isoflurane. Then, the inspired isoflurane concentration was maintained at 1.2% (1.0 MAC) for 120 min. Muscle paralysis was induced by a bolus injection of 1 mg/kg vecuronium bromide and then maintained with a continuous infusion of this drug through a venous catheter inserted into the femoral vein at a rate of 2 mg · kg⁻¹ · h⁻¹. Sample collections started after a 120-min equilibration period. Each dialysis sample for the measurement of acetylcholine was collected over a 20-min period as described above. Three baseline samples were collected to establish basal acetylcholine levels. After stabilization of the baseline, a 1-s stimulus train (0.2 ms, 100 Hz, 400 μA) per min was applied to the PPTg for 20 min with an electrical stimulator (SEN3101; Nihon-Koden Kogyo, Tokyo, Japan), and the amount of acetylcholine efflux was obtained during and after the stimulation every 20 min. As the cortical acetylcholine efflux returned to baseline levels, 0.5 μL Ringer’s solution (n = 5) or 20 μg (0.5 μL) SB334867, a selective orexin-1 receptor antagonist, dissolved in the Ringer’s solution (n = 5) was injected into the BF through the injection needle 20 min before the second PPTg stimulation. Then, the second PPTg stimulation was applied with the same conditions used in the first stimulation, and the amount of acetylcholine efflux was obtained every 20 min. The mean arterial pressure, heart rate, blood gas, and electroencephalogram were monitored during the experiments.

Acetylcholine Assay

The acetylcholine in the dialysates was measured using the on-line high-performance liquid chromatography system (HITEC-500; Eicom) containing an immobilized enzyme column and electrochemical detector. The pump delivered the mobile phase (50 mM Na₂HPO₄, 0.1 mM EDTA, 0.03% SDS; pH adjusted to 8.2 with phosphate acylate) at a rate of 150 μL/min.

The inlet of the dialysis probe was connected to the perfusion pump, and the outlet of the probe was connected to the automated sample injector with polyethylene tubing. The internal standard, isopropylhomocholine (50 nmol), delivered by the perfusion pump at a flow rate of 0.25 μL/min, was connected to the perfusate tube just proximal to the injection valve through a three-way joint. Data were collected and analyzed using Powerchrome software (Castle Hill, New South Wales, Australia). The retention times of isopropylhomocholine and acetylcholine were approximately 9.5 min and 11 min. The system was calibrated for each experiment using standard solutions containing 0.1, 0.5, and 1 pmol acetylcholine before and after the sample measurements. The detection limit was approximately 10 fmol at a signal-to-noise ratio of 3. Quantification of the collected acetylcholine was evaluated with the internal standard peak area.

In microdialysis studies, the experimental stability for the measurement of acetylcholine was confirmed by the fact that the coefficient of variance of these data during the mean initial 60-min dialysate collections was within 10% range. When the stability of the measurement was confirmed, we used this value as a basal value. The recovery rate for the microdialysis probes for acetylcholine was approximately 29.6 ± 5.8%.

Electroencephalographic Recording and Analysis

The electroencephalogram was measured continuously before and after orexin-A or orexin-B microinjection in experiment 1 and the PPTg stimulation in the experiment 2.

The electroencephalographic signal was digitized online at a sampling rate of 200 Hz and subjected to off-line spectral analysis. Power spectra were computed for consecutive 2.56-s epochs and 0.39-Hz frequency bins by using the SLEEPSIGN® analysis system (version 2; Kissei Comtec Co., Nagano, Japan). In this study, the electroencephalographic global frequency band (0–30 Hz) was divided into four frequency bands: 6 (0.5–4 Hz), θ (4–8 Hz), α (8–13 Hz), and β (13–30 Hz) bands. If the electroencephalogram was exhibiting a burst suppression pattern, the burst suppression ratio (BSR: percentage of total sum of isoelectric electroencephalographic time for 60 s) was measured. In the experiment 1, the time of electroencephalographic arousal was measured after intrabasalis microinjection of orexin-A or orexin-B. Electroencephalographic arousal was defined as the arousal pattern that is characterized by low-amplitude, high-frequency signals. In experiment 2, a 5-min electroencephalographic sample after the PPTg stimulation was collected for the analysis of the power bands (20 min after the start of the PPTg stimulation).

Histologic Verification

Correct placement of the tip of the microdialysis probe, the guide cannula for the microinjection, and the stimulating electrode in the nucleus was confirmed by histologic examination in each animal. At
the end of the experiment, Evans blue (0.3 or 0.5 μL) was injected into the BF to verify the site of orexin administration through the microinjection needle. Then, the animals were further anesthetized with excess sodium pentobarbital and perfused transcardially with the saline followed by 10% formalin in 0.1 m phosphate buffer (pH 7.4). Fifty-micrometer-thick sections were collected on a cryostat microtome. Sections containing the track of the microdialysis probe were stained with neutral red and visually inspected for evidence of a dialysis probe-induced lesion. Digitized sections were compared with coronal plates from a rat brain atlas to determine the stereotaxic coordinates for each dialysis perfusion, microinjection, and stimulating electrode sites. The photographs of the representative brain sections showing the guide cannula track, microinjection location, and electrode track are shown in figure 1.

**Statistical Analysis**

All of the data for acetylcholine were expressed as percent control of the basal values and represent mean ± SEM. The significance of differences between mean values of acetylcholine efflux induced by orexin-A or orexin-B was determined by analysis of variance (ANOVA) with repeated measures, followed by the Scheffé F test for multiple comparisons. The sum of all of the values of acetylcholine efflux (percent control) for the various time points (area under the curve [AUC]) for 80 min among orexin-A, orexin-B, and Ringer’s solution groups was compared by one-way ANOVA. One-way ANOVA was used for the comparisons of BSR in electroencephalograms and the time of electroencephalographic arousal.

In the PPTg experiments, the changes in relative power in each of the bands between the groups were assessed using one-way ANOVA. The acetylcholine effluxes after the first and the second PPTg stimulations were compared by ANOVA with repeated measures. If there were significant differences of acetylcholine effluxes between the first and second stimulations in the control or SB334867 group using ANOVA with repeated measures, we used an unpaired t test for comparison of the acetylcholine effluxes at each sample number after the two electrical stimulations. The AUCs after the first and second electrical stimulations for 100 min in the control and SB334867 groups were compared by unpaired t test. All statistical analyses were undertaken using the SPSS 11.0 program (SPSS, Chicago, IL), and a 5% probability of type I errors was used to determine statistical significance. In all cases, \( P < 0.05 \) was taken as the level of significance.

**Results**

**Effects of Intrabasalis Microinjection of Orexins on Acetylcholine Efflux and Electroencephalogram in the S1BF during Isoflurane Anesthesia**

The mean basal amount of acetylcholine in the cortex was 0.17 ± 0.03 \( \times 10^{-7} \) m in the orexin-A group (n = 6) and 0.16 ± 0.05 \( \times 10^{-7} \) m in the orexin-B group (n = 6) during 1.0 MAC isoflurane anesthesia. The coefficient of variance of acetylcholine measurement in the basal level was 4.18 ± 0.96% in the orexin-A group and 3.81 ± 0.45% in the orexin-B group. After each injection, the acetylcholine efflux returned to basal levels within 100 min. Therefore, the last 20-min sample (100 min after microinjection) was considered as a control for the response to the next injection. The effects of orexin-A on acetylcholine release are shown in figure 2A. Intrabasalis microinjection of orexin-A (30 and 100 pmol) resulted in significant increases in the acetylcholine efflux in the S1BF. The maximal increase in acetylcholine efflux was 147.8 ± 4.5% after 30 pmol orexin-A microinjection and 175.0 ± 10.7% after 100 pmol orexin-A microinjection. After orexin A microinjection, the AUCs of acetylcholine efflux (532.5 ± 35.2 in 100-pmol group and 463.0 ± 10.7 in 30-pmol group) were significantly greater than that in the Ringer’s group (364.6 ± 6.4) (\( P < 0.001 \) and \( P = 0.003 \), respectively). The AUC in the 10 pmol orexin-A microinjection group (402.8 ± 15.9) was not significantly different from that in the Ringer’s group.

The effects of orexin-B on acetylcholine release are shown in figure 2B. No significant change in acetylcholine efflux in the S1BF was observed after 10 and 30 pmol orexin-B microinjections. Only 100 pmol orexin-B resulted in a significant increase in the acetylcholine efflux in the S1BF. The maximal increase in the acetylcholine efflux was 164.5 ± 11.2% after 100 pmol
Orexin-B microinjection. After orexin-B microinjection, the AUC in the 100-pmol group (507.6 ± 31.8) was greater than that in the Ringer’s group (355.5 ± 18.4) (P < 0.001). The AUCs in the 10-pmol group (362.4 ± 22.6) and the 30-pmol group (391.0 ± 11.4) were not significantly different from that in the Ringer’s group.

The changes in BSR, number of electroencephalographically aroused animals, and time of electroencephalographic arousal (in minutes) induced by intrabasalis microinjection of orexins are shown in tables 1 and 2. Before injection of orexins, burst and suppression patterns in the electroencephalograms were observed in all animals during 1.0 MAC isoflurane anesthesia. An representative illustration of the electroencephalographic changes after microinjection of 100 pmol orexin-A or orexin-B is shown in figures 3A and B. Microinjection of 100 pmol orexin-A resulted in a change in electroencephalogram from the burst and suppression patterns to the arousal patterns in all six animals used in the group (fig. 3A), whereas in two of six animals, the same dose of orexin-B did not influence the electroencephalogram (fig. 3B). No significant difference in BSR was found between the basal condition and the conditions after microinjection of Ringer’s solutions. The BSR after 10 pmol orexin-A microinjection was significantly smaller than that in the basal condition (P < 0.05; table 1). Three of six animals after 10-pmol microinjections, five of the six animals after 30-pmol microinjection, and all six animals after 100 pmol orexin-A microinjection showed the arousal pattern changes in the electroencephalogram during 1.0 MAC isoflurane anesthesia. The time of electroencephalographic arousal after intrabasalis microinjection of 30 and 100 pmol orexin-A were significantly longer than that observed after microinjection of 10 pmol orexin-A (P < 0.05; table 1).

No significant difference in BSR was observed for the basal conditions or after microinjection of Ringer’s solution and 10 pmol orexin-B into the BF. The BSRs after intrabasalis microinjection of 30 and 100 pmol orexin-B were significantly smaller than those in the basal condition (P < 0.05; table 2). Microinjection of 30 and 100 pmol orexin-B changed the burst and suppression patterns to an arousal electroencephalographic pattern during 1.0 MAC isoflurane anesthesia in three of six and four of six animals, respectively (table 2). The duration of

![Fig. 2. Effect of intrabasalis administration of orexin-A or orexin-B on cortical acetylcholine effluxes.](A) Microinjections of orexin-A (30 or 100 pmol) into the basal forebrain increased cortical acetylcholine efflux. However, microinjection of Ringer’s solution (control) and orexin-A (10 pmol) into the basal forebrain did not significantly increase the cortical acetylcholine efflux. (B) Microinjections of orexin-B (100 pmol) into the basal forebrain increased the cortical acetylcholine efflux, whereas those of the Ringer’s solution and orexin-B (10 or 30 pmol) into the basal forebrain did not significantly increase cortical acetylcholine efflux. **P < 0.001, *P < 0.05 versus control.

Table 1. Electroencephalographic Changes Induced by Intrabasalis Microinjection of Orexin-A during 1.0 MAC Isoflurane

<table>
<thead>
<tr>
<th>Conditions</th>
<th>BSR</th>
<th>Number of Electroencephalographically Aroused Animals</th>
<th>Time of Electroencephalographic Arousal, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal condition</td>
<td>78.0 ± 1.13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>After microinjection of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ringer’s solution</td>
<td>76.1 ± 0.73</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 pmol orexin-A</td>
<td>67.8 ± 2.01*</td>
<td>3</td>
<td>7.4 ± 5.20</td>
</tr>
<tr>
<td>30 pmol orexin-A</td>
<td>67.7</td>
<td>5</td>
<td>23.1 ± 4.28†</td>
</tr>
<tr>
<td>100 pmol orexin-A</td>
<td>N</td>
<td>6</td>
<td>27.4 ± 6.26†</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. basal condition. † P < 0.05 vs. 10 pmol orexin-A microinjection.

BSR = burst suppression ratio; MAC = minimum alveolar concentration; N = no animal was shown to have burst suppression pattern in electroencephalogram after microinjection.
electroencephalographic arousal after 100 pmol orexin-B microinjection was significantly longer than that after 30 pmol orexin-B microinjection ($P < 0.05$).

No significant difference in mean arterial pressure, heart rate, or blood gas was observed after each dose of orexin-A, orexin-B, and Ringer’s solution.

After microinjection of orexins, we did not observe any movements of the rat.

**Effects of Intrabasalis Microinjection of Sb334867, an Orexin-1 Receptor Antagonist, on the Evoked Cortical Acetylcholine Efflux Elicited by PPTg Electrical Stimulation**

The mean basal amount of acetylcholine efflux in the cortex before the first PPTg electrical stimulation was $0.32 \pm 0.04 \times 10^{-7}$ μm in the control group (n = 5) and $0.32 \pm 0.02 \times 10^{-7}$ μm in the SB334867 group (n = 5). The coefficient of variance of acetylcholine measurement in the basal condition was $4.58 \pm 0.89$% in the control group and $4.09 \pm 0.74$% in the SB334867 group. After the first PPTg stimulation, the maximal amount of acetylcholine efflux from the cortex increased significantly ($P < 0.001$) to an average of $0.48 \pm 0.03 \times 10^{-7}$ μm in the control group and compared with $0.53 \pm 0.06 \times 10^{-7}$ μm in the SB334867 group. The second PPTg stimulation–evoked acetylcholine effluxes at the first 20-min samples were significantly attenuated by microinjection of SB334867 into the BF ($P < 0.05$; fig. 4). There was no significant difference between the AUC of acetylcholine efflux after the second stimulation (535.2 ± 23.7) and that after the first stimulation (509.5 ± 39.2) in the control group. The AUC after the second stimulation (411.4 ± 6.1) was significantly less than that after the first stimulation (523.0 ± 41.1) in the SB334867 group ($P = 0.028$).

Stimulation of the PPTg changed the electroencephalogram of all of the control animals from burst suppression patterns to arousal patterns immediately, but the changes in the electroencephalogram did not remain for a long time for the first several stimulations. However, the sustained arousal electroencephalogram could be obtained within 15 min after the successive PPTg stimulations. The percentages of each arousal electroencephalogram power band in the control group showed no significant difference between the first and second PPTg stimulations (fig. 5A). Intrabasalis injection of SB334867 or Ringer’s solution before the second PPTg stimulation did not change the electroencephalographic burst suppression patterns or the BSR. SB334867 caused arousal electroencephalographic patterns in four of five animals after the second PPTg stimulation. Hence, the percentage of electroencephalographic power bands in the first and the second PPTg stimulations in four animals showed a significant difference between two cycles of PPTg stimulation in SB334867-treated group (fig. 5B). Delta power was significantly increased by second PPTg stimulation ($P < 0.05$, vs. the first PPTg stimulation). The α and β power bands were significantly decreased by the second PPTg stimulations ($P < 0.05$, vs. the first PPTg stimulation). Theta power did not show a significant difference between the two stimulations.

**Discussion**

The main finding of our study was that microinjection of orexin-A and orexin-B into the BF induced the isoflu-
Orexin-induced anesthetic arousal in terms of the increases in the cortical acetylcholine efflux and electroencephalographic activation. Microinjection of orexin-A in the BF was more potent than orexin-B in producing a greater acetylcholine efflux in the S1BF correlated with an arousal electroencephalographic shift. We demonstrated that intrabasalis microinjection of orexin-1 receptor antagonist (SB334867) inhibits the PPTg stimulation-induced acetylcholine efflux in the S1BF and electroencephalographic activation. These findings suggest the involvement of an orexinergic pathway in anesthetic-arousal regulation of the cholinergic ventral ascending arousal system originating from the PPTg.

Previous studies have demonstrated that orexin and orexin neurons are important regulators of sleep and arousal. However, the effect of orexin and orexin neurons on the anesthesia-arousal regulation remained unclear. Previously, we demonstrated that intracerebroventricular injection of orexin-A elicited an arousal electroencephalogram during isoflurane anesthesia. The current study also supports the role of orexinergic system in the anesthetic-arousal regulation during isoflurane anesthesia. The cholinergic ascending pathway is the most powerful cortical activating system that releases acetylcholine in the cortex and is made up of two cholinergic ascending cortical activation pathways. The ventral ascending activating sys-
tem, comprising the posterior hypothalamus, the BF, and the S1BF, has been known to regulate the state of consciousness during the natural sleep–wake cycle.\textsuperscript{14,15} and contains orexin fibers.\textsuperscript{24} Among these nuclei, the BF is believed to play the most dominant role.\textsuperscript{13,14} In the current study, we observed that intrabasalis microinjection of orexins significantly increased acetylcholine efflux in the S1BF and activated electroencephalogram during isoflurane anesthesia. The findings suggest that the orexins may be one of the important regulators of the cholinergic ventral ascending activating system.

In addition, intrabasalis orexin-A microinjection induced a significant and dose-dependent increase in acetylcholine efflux in the S1BF during isoflurane anesthesia. Orexin-A also induced arousal electroencephalographic patterns during 1.0 MAC isoflurane anesthesia. However, microinjection of low doses of orexin-B into the intrabasalis did not induce significant increased efflux of acetylcholine in the S1BF during isoflurane anesthesia. The arousal electroencephalographic patterns could be observed just after 30 or 100 pmol orexin-B intrabasalis microinjection. These findings suggest that isoflurane anesthesia can be partially reversed by microinjection of orexins into the BF.

In the current study, we observed the dissociation of the increases in the acetylcholine efflux with electroencephalographic activation and no movement of the rat. The first possibility of this dissociation is that microinjection of orexins into the BF might induce paradoxical sleep (rapid eye movement [REM] sleep), activation of the electroencephalogram, and muscle atonia. It has been reported that electroencephalographic activation in REM sleep is similar to that of wakefulness and that cortical acetylcholine release is greater during wakefulness and REM sleep than during non-REM sleep.\textsuperscript{25} The difference between REM sleep and wakefulness is such that during REM sleep, the postural muscles are actively inhibited, whereas they are active during wakefulness.\textsuperscript{26} The second possibility is that cortical acetylcholine increases and electroencephalographic activation induced by microinjection of orexins might not reach the level of the awake state. If the concentration of isoflurane is lower, injection of orexins might induce movement of the rat. The third possibility is that isoflurane may act on the mesopontine tegmental anesthesia area in the brainstem region and induce atonia, even if microinjection of orexins into the BF activated on the BF cholinergic neurons and then increased the acetylcholine efflux and the activity of the electroencephalogram, because recent literature showed that microinjection of γ-aminobutyric acid receptor (GABA) type A agonist anesthetics into the mesopontine tegmental anesthesia area in the brainstem region induce loss of the righting reflex, atonia, nonresponsiveness to noxious stimuli, and apparent loss of consciousness.\textsuperscript{27,28} The GABA type A receptor is the target of a structurally diverse group of sedative, hypnotic, and anesthetic drugs, including isoflurane.\textsuperscript{29} Further studies are necessary to clarify the dissociation observed in this study.

In our study, all of the animals did not necessarily induce the arousal pattern from the burst and suppression pattern in the electroencephalogram at each dose of orexin as shown in tables 1 and 2. The possibility of the dislocation of the tip of the microinjection tube is excluded because we checked every animal after each experiment by histologic verification. From our study, we cannot explain why the responses of the animals to orexins are different even at the same dosage. It is noteworthy that single nucleotide genetic polymorphism can lead to different sensitivity to some drugs, such as opioids, benzodiazepines, muscle relaxants, and local anesthetics, in each animal.\textsuperscript{30} Further study is necessary to clarify the reason of the different responses to the same dose of orexins in each animal.

The existence of hypothalamic projections to the BF has been demonstrated.\textsuperscript{31,32} Anatomical studies suggest that orexin neurons contribute to this hypothalamobasal forebrain pathway.\textsuperscript{33} Espana et al.\textsuperscript{16} found that intracebroventricular injection of orexin-A induced more potent effects than orexin-B on behavioral and electrophysiologic measures of arousal. In addition to these findings, intrabasalis administration of orexin-A dose-dependently increased acetylcholine release within the prefrontal cortex in awake condition.\textsuperscript{17} The results of these experiments performed in unanesthetized animals were consistent with our current study performed in isoflurane-anesthetized animals in the aspects that both orexins activated the cortical activation and that orexin-A induced more potent influences than orexin-B. Two receptor types have been identified for orexin-A and orexin-B: orexin-1 and orexin-2 receptors.\textsuperscript{5} Orexin-A has higher affinity for orexin-1 receptor than orexin-B, whereas both orexin-A and orexin-B have almost equal affinities for orexin-2 receptor.\textsuperscript{34} In the current study, orexin-A was more potent than orexin-B in causing acetylcholine efflux in the S1BF and electroencephalographic activation, suggesting that the orexin-1 receptor may have major contribution to the activity at the BF neurons during isoflurane anesthesia. It has been reported that SB334867 has a 20-fold higher affinity for the orexin-1 receptor compared with the orexin-2 receptor,\textsuperscript{35} and the current demonstration of intrabasalis SB334867–induced attenuation of the acetylcholine efflux in the S1BF and electroencephalographic activation after the second PPTg stimulation suggest that orexin-1 receptor may be a significant mediator of neurotransmission between the BF and the PPTg. However, further study is necessary to elucidate how the orexinergic system is involved in the cholinergic ventral ascending pathways.

The absence of a change in the basal amounts of acetylcholine efflux between the first and second PPTg
OREXIN-INDUCED AROUSAL DURING ISOFLURANE ANESTHESIA

stimulations suggests that repeated exposure to electrical stimulation neither damaged the PPTg neurons nor altered the acetylcholine release machinery of the basal forebrain neurons. Therefore, any observed changes to the two cycles of electrical stimulations in the SB334867 group might be due to local effects of this drug. It has been reported that acetylcholine-containing neurons of the pontomesencephalic tegmentum including the PPTg also project, in parallel with neurons of the reticular formation, to the BF through to the ventral extrathalamic pathway. It has also been shown that the major excitatory input to the cholinergic cell compartment of the PPTg arises from glutamatergic neurons in the pontine reticular formation. Therefore, the PPTg can be considered as an essential component of the ascending reticular activating systems, and then the BF can be regarded as a rostral extension of the ascending reticular activating system. It has been demonstrated that majorafferent fibers to the PPTg originate from the various nuclei including the pontine and medullary reticular field, which is activated by noxious stimuli in the peripheral nerves. The increased acetylcholine efflux in the S1BF and electroencephalographic activation resulting from stimulation of the PPTg during isoflurane anesthesia suggests that the isoflurane-induced anesthetic state might be partially reversed by the increased activity of the ascending reticular formation evoked by noxious stimuli.

Nelson et al. reported that systemic administration of GABA-mediated agents such as propofol and pentobarbital increased the expression of c-Fos, a marker of neuronal activation, in the ventral preoptic area. The ventral preoptic area neurons project to and inhibit the orexin neurons around the perifornical area in the hypothalamus through releasing GABA at the nerve terminals. In the current study, we demonstrated that intrabasal injection of orexins increased the acetylcholine efflux and activated the electroencephalogram during isoflurane anesthesia, and suggested that orexinergic neurons projected to the basal forebrain through the cholinergic ventral ascending pathway from the study of the effect of SB334867 on PPTg stimulation. From these data, we suggest that anesthetic state may be modulated, at least in part, by the flip-flop interaction between the orexinergic system and the GABA-mediated system, as proposed by Saper et al. in natural sleep-wake interaction. It is noteworthy that intraventricular injection of orexin-A significantly decreased barbiturate-induced anesthesia time, and SB334867 reversed the barbiturate-induced anesthesia time. According to these findings, we speculate that microinjection of orexins into the basal forebrain might increase the acetylcholine efflux and activate electroencephalogram under GABA-mediated agent-induced anesthesia such as propofol.

In summary, we demonstrated that intrabasal administration of orexin-A and orexin-B elicited anesthetic arousal during isoflurane anesthesia in terms of electroencephalographic pattern and increased acetylcholine efflux in the cortex. Orexin-A is more potent in the cholinergic BF neurons than is orexin-B. The effect of orexin might be mediated mainly through the orexin-1 receptor in the BF. Taken together, the activation of cholinergic ventral ascending pathways may contribute, at least in part, to the arousal during isoflurane anesthesia through the orexinergic system.

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