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Neural Mechanisms of Sevoflurane-induced Respiratory Depression in Newborn Rats

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Background: **Sevoflurane-induced respiratory depression has been reported to be due to the action on medullary respiratory and phrenic motor neurons. These results were obtained from extracellular recordings of the neurons. Here, the authors made intracellular recordings of respiratory neurons and analyzed their membrane properties during sevoflurane application. Furthermore, they clarified the role of γ-aminobutyric acid type A receptors in sevoflurane-induced respiratory depression.**

Methods: **In the isolated brainstem–spinal cord of newborn rat, the authors recorded the C4 nerve burst as an index of inspiratory activity. The preparation was superfused with a solution containing sevoflurane alone or sevoflurane plus the γ-aminobutyric acid type A receptor antagonist picrotoxin or bicuculline. Neuronal activities were also recorded using patch clamp techniques.**

Results: **Sevoflurane decreased C4 burst rate and amplitude. Separate perfusion of sevoflurane to the medulla and to the spinal cord decreased C4 burst rate and amplitude, respectively. Both picrotoxin and bicuculline attenuated the reduction of C4 burst rate. Sevoflurane reduced both intraburst firing frequency and membrane resistance of respiratory neurons except for inspiratory neurons.**

Conclusion: **Under the influence of sevoflurane, the region containing inspiratory neurons, *i.e.*, the pre-Bötzinger complex, may determine the inspiratory rhythm, because reduced C4 bursts were still synchronized with the bursts of inspiratory neurons within the pre-Bötzinger complex. In contrast, the sevoflurane-induced decrease in C4 burst amplitude is mediated through the inhibition of phrenic motor neurons. γ-Aminobutyric acid type A receptors may be involved in the sevoflurane-induced respiratory depression within the medulla, but not within the spinal cord.**

SEVOFLURANE is a volatile anesthetic that is widely used in clinical practice; it depresses respiration as a side effect at anesthetic doses.[1,2] *In vivo* studies using extracellular recordings of respiratory neurons in decerebrate, vagotomized, and peripherally deafferented canines have suggested that this effect may be due to the anesthetic's action on medullary premotor neurons mediated by γ-aminobutyric acid type A ($GABA_A$) transmission.[3–6]

Based on findings in *in vitro* preparations of neonatal rodents, the ventrolateral medullary region of the brainstem is thought to be a critically important site for respiratory rhythm generation.[7–10] Specifically, the pre-Bötzinger complex and the more rostral parafacial respiratory group presumably constitute a dual respiratory center.[7,11,12] Among the two candidates, it is hypothesized that the pre-Bötzinger complex generates the inspiratory rhythm and the parafacial respiratory group generates the expiratory rhythm, although these groups are functionally coupled.[11] Medullary respiratory neurons in the brainstem–spinal cord preparation have been classified into inspiratory (which are further divided into three subtypes, I–III), preinspiratory, and expiratory neurons on the basis of the temporal correlation of their firing pattern with inspiratory-related C4 ventral root activity.[7] Considering its respiratory function, the medulla is probably one of the targets of the respiratory depression induced by sevoflurane. The spinal cord, containing phrenic motor neurons, may be another target because sevoflurane has been reported to depress the excitability of nonrespiratory spinal motor neurons[13] and the activity of hypoglossal motor neurons,[14] which are primarily active in the inspiratory phase in the *en bloc* preparation.[9]

Using the isolated brainstem–spinal cord preparation of neonatal rat, Otsuka[15] recorded the neuronal activities of respiratory neurons to investigate the effect of halothane on fictive breathing. In his study, neurons were recorded extracellularly but not intracellularly. Recently, several *in vitro* studies have made intracellular recordings of respiratory neurons using the perforated patch clamp technique to examine the effect on the respiratory neural network of other agents generally used by anesthesiologists, such as propofol[16,17] and opioids.[10] Intracellular recordings have made possible the analysis of membrane properties and synaptic input, which cannot be examined through extracellular recordings. Therefore, *in vitro* perforated patch clamp recordings using the isolated brainstem–spinal cord preparation of neonatal rat seem to be suitable for detailed investigation of the intracellular mechanisms of sevoflurane-induced respiratory depression.

After reviewing the previous data,[3–6,15] we hypothesized that the effect of sevoflurane may differ between the types of respiratory neurons. In the current study, we analyzed the response of medullary respiratory and phrenic motor

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neurons to sevoflurane, and the role of $GABA_A$ receptors in sevoflurane-induced respiratory depression.

Materials and Methods

Preparation

All procedures were conducted in accordance with the guidelines of the Keio University Laboratory Animal Care and Use Committee (Tokyo, Japan), Kitasato Institute Animal Care and Use Committee (Tokyo, Japan), and Teikyo University Laboratory Animal Care and Use Committee (Tokyo, Japan). Data were obtained from 64 neonatal Wistar rats (aged 0–4 days). Generation of the isolated brainstem–spinal cord preparation has been described in detail elsewhere.[18] In brief, the rats were anesthetized with diethyl ether, and after the paw withdrawal reflex disappeared, the brainstem caudal to the caudal cerebellar artery and cervical spinal cord were isolated in a chamber filled with oxygenated artificial cerebrospinal fluid (ACSF). The cerebellum and pons were ablated. Each preparation was placed ventral side up in a recording chamber (volume, 2 ml) and superfused (flow, 4 ml/min) with control ACSF equilibrated with a control gas mixture (5% CO_2 in oxygen, pH7.4). Its temperature was maintained at 25°–26°C. The composition of the ACSF was 126 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, and 30 mM glucose. C4 ventral root activity was recorded using a glass suction electrode, amplified with a conventional alternating current amplifier (AVH 11; Nihon Kohden, Tokyo, Japan), and integrated (time constant, 100 ms). We measured C4 burst rate as an index of the inspiratory rate[19] and the integrated amplitude as an index of the tidal volume.[20] All amplitude data were normalized to the values obtained during 10–15 min of the anesthetic-free control state, which was assigned a value of 100%.

Drug Administration

Sevoflurane was applied according to the method described by Matute *et al.*[13] Sevoflurane (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) was mixed with the control gas mixture (5% CO_2 in oxygen, pH 7.4) by a vaporizer (Sevotec 3; Ohmeda, Steeton, West Yorkshire, United Kingdom) at known volume percentages (1, 3, and 5%). The ACSF was gassed with this mixture for 30 min. In the preliminary experiment, in which the same perfusion system was used without the brainstem–spinal cord, we measured the concentration of sevoflurane in the ACSF in the recording chamber using gas chromatography (GC-2014; Shimadzu, Kyoto, Japan). The concentration of sevoflurane in the ACSF was estimated from the vaporizer readings (r^2 = 0.972; slope, 0.25 ± 0.01; y-intercept, −0.03 ± 0.06; fig. 1). The sevoflurane percentages of 1, 3, and 5% in gas corresponded to approximately 0.23, 0.74, and 1.25 mM, respectively. Sevoflurane EC_{50} equivalent to 1 minimum alveolar concentration (MAC) in clinical anesthesia corresponds to an aqueous concentration of 0.35 mM sevoflurane.[21] In the current study, we present the estimated aqueous concentration and MAC (in parentheses).

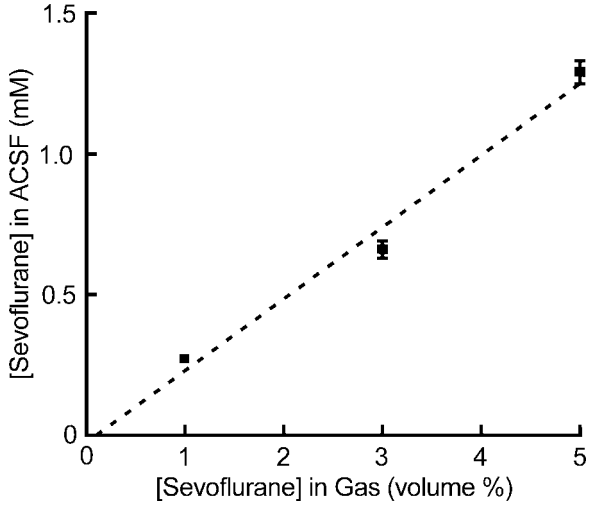

Fig. 1. Simple regression of vaporizer readings (volume %) and molar concentrations of sevoflurane in artificial cerebrospinal fluid (ACSF). Three observations were performed at each vaporizer reading (1, 3, and 5%).

The $GABA_A$ receptor antagonist picrotoxin or bicuculline methiodide (both Sigma Chemical Co., St. Louis, MO) was dissolved in ACSF containing 1.25 mM (3.57 MAC) sevoflurane at 10 μM.

Experiment 1: Effect of Sevoflurane on C4 Respiratory Activity

We analyzed the effect of sevoflurane on C4 burst rate and amplitude. Twenty preparations were randomly allocated to one of four groups to for exposure to sevoflurane at 0, 0.23, 0.74, or 1.25 mM (0, 0.77, 2.11, or 3.57 MAC); each group contained five preparations. Preparation were superfused after a control period of 10–15 min with a solution containing sevoflurane at the specified concentration for 30 min, followed by a washout period using control ACSF for 30 min.

Experiment 2: Separate Perfusion of Sevoflurane to the Spinal Cord or to the Medulla

We used a separate perfusion system[16,19,22,23] to permit selective medullary or spinal application of drugs. The chamber was partitioned at the spinomedullary junction to permit the selective application of drugs. The partition was made using two thin acrylic plates placed parallel to each other with woven nylon packed between them. Each partitioned section had a capacity of 2 ml and was superfused continuously at a flow rate of 4 ml/min. Five preparations each were randomly allocated either to a medulla perfusion group or a spinal cord perfusion group. After C4 activity stabilized, the super-

fusate was replaced with ACSF containing 0.74 mM (2.11 MAC) of sevoflurane for 30 min, followed by a washout period using the control ACSF for 30 min.

Experiment 3: Effect of Sevoflurane on C4 Respiratory Activity in the Presence of a $GABA_A$ Receptor Antagonist

Effects of 10 μM picrotoxin were studied on sevoflurane-induced reduction on C4 burst rate and amplitude. Experiments were performed in five preparations. After a control period of 10–15 min, the superfusate was replaced with ACSF containing 1.25 mM (3.57 MAC) sevoflurane in the presence of 10 μM picrotoxin for 30 min, followed by a washout period using the control ACSF for 30 min. To assess the effect of picrotoxin, these values were compared with those obtained using the same concentration of sevoflurane alone. Using 10 μM bicuculline in place of picrotoxin, the same experiments were repeated in another five preparations.

Experiment 4: Effect of Sevoflurane on Respiratory Neurons

We analyzed the effect of sevoflurane on the intracellular activity of respiratory neurons using the perforated patch clamp technique.[18] Respiratory neurons were identified and classified on the basis of their firing patterns and the temporal correlation of their activity with the respiratory cycle of C4 respiratory activity.[7] Inspiratory neurons discharged action potentials during the inspiratory phase (C4 burst activity phase) and were further categorized into three subtypes.[7,10] Type I inspiratory neurons are depolarized by periods of summated excitatory postsynaptic potentials before the onset of and after termination of C4 bursts (*i.e.*, the preinspiratory and postinspiratory phases). Type II inspiratory neurons show excitatory postsynaptic potentials during only the inspiratory phase. Type III inspiratory neurons are hyperpolarized by periods of summated inhibitory postsynaptic potentials in the preinspiratory and postinspiratory phases. Preinspiratory neurons were characterized by preinspiratory and postinspiratory action potential discharges and hyperpolarization during the inspiratory phase; they send excitatory and inhibitory inputs to type I and III inspiratory neurons, respectively.[7,10] Expiratory neurons are first characterized by tonic discharge of action potentials in the interval between the inspiratory phases and are categorized into two subtypes based on the following criterion.[7] In Exp-i neurons, inspiratory-related action potential discharge is blocked due to summating hyperpolarizing inhibitory postsynaptic potentials. In contrast, Exp-p-i neurons are subjected to a longer period of hyperpolarization covering the preinspiratory, inspiratory, and postinspiratory phases. Inspiratory neurons were searched for in the pre-Bötzinger complex, which covered presumably from the rostrocaudal extension root of the most rostral root of the hypoglossal nerve.[12] Preinspiratory neurons were searched for in the main area of the parafacial respiratory group, which extended presumably from the caudal cerebellar artery to approximately 0.25 mm caudal to the caudal end of the facial motor nucleus.[12] Expiratory neurons were searched for in the Bötzinger complex, which was the area between pre-Bötzinger complex and the caudal end of the vagal nerve root.[12]

Membrane potentials were recorded under current clamp mode with a single-electrode voltage clamp amplifier (CEZ 3100; Nihon Kohden). A glass pipette (GC100-TF-10; Clark, Reading, United Kingdom) was horizontally pulled (PA-91; Narishige, Tokyo, Japan) to a tip size of approximately 2 μm, with very gradual tapering. Electrode resistance ranged from 5 to 20 MΩ when the pipette was filled with a solution containing 140 mM K-gluconate, 3 mM KCl, 10 mM EGTA, 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and nystatin (100 μg/ml). pH was maintained at 7.2–7.3 using potassium hydroxide. The micropipette was inserted into the rostral ventrolateral medulla using a manual hydraulic micromanipulator. During tracking for the neurons, positive pressure (10–20 cm H_2O) was applied inside the pipette. After a gigaohm seal was obtained, the recorded membrane potential became negative and stabilized in approximately 10 min. After we made the control recording, the control ACSF was changed to ACSF containing 1.25 mM (3.57 MAC) sevoflurane for 3–6 min, followed by a 10- to 20-min washout period using control ACSF. In this experiment, the brief application of sevoflurane at high concentration enabled us to obtain high-quality intracellular neuronal recordings because the effect then appears more quickly.[16]

To assess the intracellular activity of respiratory neurons, we focused on three parameters. *Intraburst firing frequency* of inspiratory neurons was calculated as the mean firing frequency during the inspiratory phase, whereas that of preinspiratory or expiratory neurons is represented by the mean firing frequency during the expiratory phase.[16] *Membrane potential* was presented without correcting for the liquid junction potential. *Membrane resistance* was determined from the change in voltage in response to direct current pulses (300 ms, −50 pA) during the silent phase of the respiratory cycle.

Perforated patch recording of phrenic motor neurons has been described elsewhere.[24] The spinal cord of the preparation was carefully transected with a vibratome between C4 and C5 ventral roots and was bent so that the cut edge, rostral to the section, was placed horizontally just below the surface of the ACSF. Phrenic motor neurons were identified on the basis of their firing pattern and location. They discharged action potentials during the inspiratory phase (C4 burst activity phase) and were located in C4 ventral horn.[25] To search for them, the micropipette was inserted into C4 ventral horn. The methods of perforated patch clamp techniques were

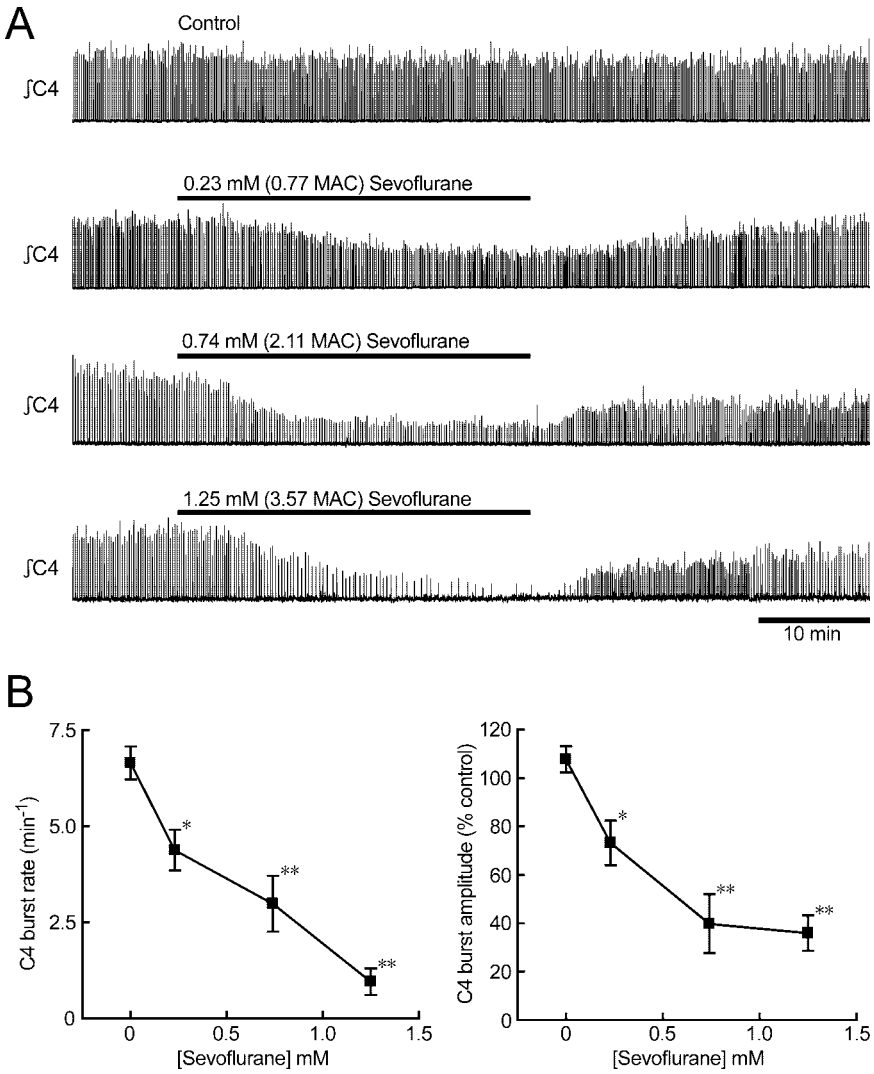

Fig. 2. (*A*) A representative recording of the integrated C4 activity (∫C4) before, during, and after superfusion with 0, 0.23, 0.74, and 1.25 mM (0, 0.77, 2.11, and 3.57 minimum alveolar concentration [MAC], respectively) sevoflurane solution. The *horizontal bars* indicate the duration of superfusion with sevoflurane. (*B*) Concentration-dependent effect of sevoflurane on C4 burst rate (*left*) and amplitude (*right*). Higher concentrations of sevoflurane tended to produce a greater decrease in C4 burst rate and amplitude. * $P < 0.05$ *versus* control. ** $P < 0.01$ *versus* control.

identical throughout to those described for medullary respiratory neurons.

Data Analysis

All signals were recorded on a thermal array recorder (WS-682G; Hihon Kohden) and fed into a personal computer after analog–digital conversion (Power Lab/4sp; AD-Instruments, Castle Hill, Australia) for subsequent analysis (Chart version 5; ADInstruments). Analysis of the respiratory parameters was performed off-line. Respiratory parameters obtained before the superfusion of the ACSF-containing drugs were defined as control values.

All variables were evaluated using a Kolmogorov-Smirnov test, which revealed that it was appropriate to make parametric assumptions about our data. In figure 1, the molar concentration of sevoflurane in the ACSF was practically estimated from the vaporizer readings by using simple regression. In experiment 1, changes in C4 burst rate and amplitude were compared using a one-way analysis of variance, followed by a Dunnett test. In experiments 2, 3 and 4, the data before and during the application of the drugs were analyzed using a paired *t* test. The significance of the difference between two groups was assessed using an unpaired *t* test. All statistical analyses were conducted using Graph-Pad Prism 3.0 software (Graph-Pad Software Inc., San Diego, CA). All values were reported as mean ± SE, and all *P* values less than 0.05 were considered significant.

Results

Effect of Sevoflurane on C4 Respiratory Activity

Representative recordings of integrated C4 activity before and during superfusion with sevoflurane-containing ACSF are shown in figure 2A. C4 burst rate and amplitude were both decreased by sevoflurane in a concentration-dependent manner (fig. 2B). C4 burst rate during the application of 0, 0.23, 0.74, or 1.25 mM (0, 0.77, 2.11, or 3.57 MAC) sevoflurane was 6.65 ± 0.43, 4.37 ± 0.52, 2.98 ± 0.72, and 0.954 ± 0.347 min^{-1} (98.2 ± 3.1, 71.6 ± 6.9, 42.4 ± 7.6, and 15.3 ± 4.7% of the control rate), respectively. C4 burst amplitude during the application of 0, 0.23,

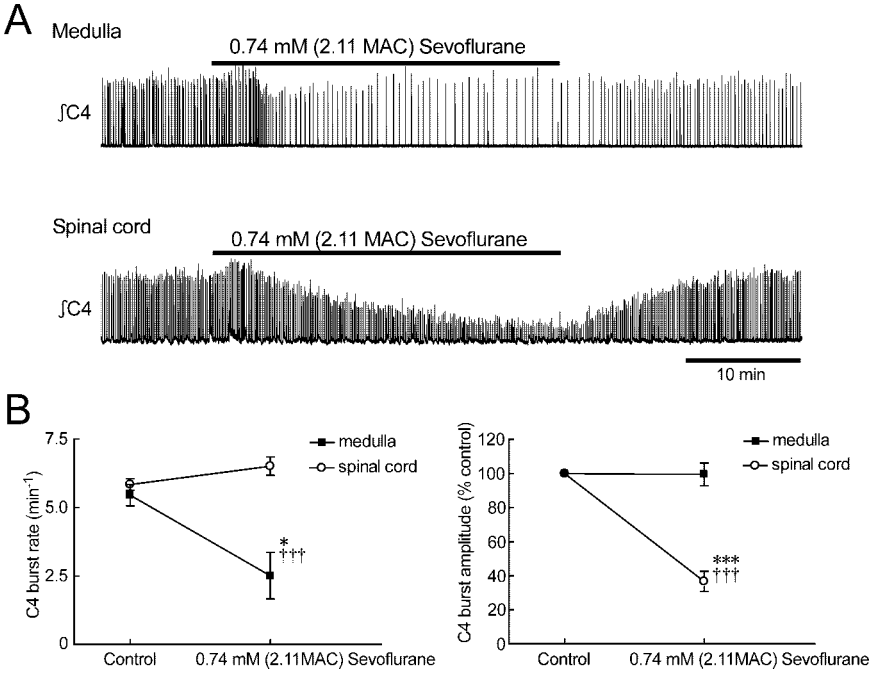

Fig. 3. (*A*) A representative recording of the integrated C4 activity (∫C4) before, during, and after separate superfusion with sevoflurane solution (*upper trace*: medulla; *lower trace*: spinal cord). The *horizontal bars* indicate the duration of superfusion with sevoflurane. (*B*) The effect of separate perfusion of 0.74 mM (2.11 minimum alveolar concentration [MAC]) sevoflurane on C4 burst rate (*left*) and amplitude (*right*). C4 burst amplitude decreased significantly upon application of sevoflurane to the spinal cord. On the other hand, C4 burst rate decreased significantly when sevoflurane was applied to the medulla. * $P < 0.05$ *versus* control. *** $P < 0.005$ *versus* control. ††† $P < 0.005$, medulla perfusion group *versus* spinal cord perfusion group.

0.74, or 1.25 mM (0, 0.77, 2.11, or 3.57 MAC) sevoflurane changed to 107.8 ± 5.4, 73.2 ± 9.1, 39.7 ± 12.1, and 35.9 ± 7.3% of the control amplitude, respectively.

Separate Perfusion of Sevoflurane to the Spinal Cord and to the Medulla

Representative recordings of C4 activity before and during application of 0.74 mM (2.11 MAC) sevoflurane to the medulla or the spinal cord are shown in figure 3A. During the administration of sevoflurane to the medulla, C4 burst rate decreased, but C4 burst amplitude did not change significantly. In contrast, when applied to the spinal cord, the application of 0.74 mM (2.11 MAC) sevoflurane decreased C4 burst amplitude only (figs. 3A and B). C4 burst rate and amplitude during the application of 0.74 mM (2.11 MAC) sevoflurane to the medulla were 2.51 ± 0.85 min^{-1} (46.1 ± 14.3% of the control rate) and 99.5 ± 6.6% of the control rate, respectively. C4 burst rate and amplitude during the application of 0.74 mM (2.11 MAC) sevoflurane to the spinal cord were 6.51 ± 0.33 min^{-1} (111.8 ± 5.8% of the control rate) and 36.6 ± 5.9% of the control amplitude, respectively.

Effect of Sevoflurane in the Presence of a $GABA_A$ Antagonist on C4 Respiratory Activity

Representative recordings of C4 activity before and during superfusion with ACSF containing 1.25 mM (3.57 MAC) sevoflurane in the presence of 10 μM picrotoxin or bicuculline are shown in figure 4A. A recording of the application of 1.25 mM (3.57 MAC) sevoflurane alone also is provided. Sevoflurane still reduced C4 burst amplitude in the presence of picrotoxin. However, the reduction of C4 burst rate was attenuated significantly compared with the application of 1.25 mM (3.57 MAC) sevoflurane alone ($P < 0.005$) (figs. 4A and B). C4 burst rate and amplitude in response to 1.29 mM (3.57 MAC) sevoflurane with 10 μM picrotoxin were 7.49 ± 0.86 min^{-1} (117.3 ± 10.1% of the control rate) and 38.6 ± 8.6% of the control amplitude, respectively. Application of sevoflurane with bicuculline still reduced C4 burst amplitude. Bicuculline, like picrotoxin, significantly attenuated only the reduction of C4 burst rate ($P < 0.005$ *vs.* application of 1.29 mM [3.57 MAC] sevoflurane alone; figs. 4A and C). C4 burst rate and amplitude during the application of 1.29 mM (3.57 MAC) sevoflurane with 10 μM bicuculline were 3.36 ± 0.42 min^{-1} (64.7 ± 5.0% of the control rate) and 52.6 ± 10.6% of the control amplitude, respectively.

Effect of Sevoflurane on Respiratory Neurons

Twenty-three medullary respiratory and phrenic motor neurons were recorded intracellularly to examine the effects of 1.25 mM (3.57 MAC) sevoflurane. From eight inspiratory neurons, presumably located in the pre-Bötzinger complex, three cells were classified as type I, and the remaining five neurons were classified as type II. In both types of inspiratory neurons, 1.25 mM (3.57 MAC) sevoflurane decreased the rate of inspiratory-related rhythmic drive potentials and concomitant action potential discharge (fig. 5). However, intraburst firing frequency, resting membrane potential, and membrane resistance remained unchanged ($P > 0.05$) during sevoflurane-induced respiratory depression (table 1).

In six preinspiratory neurons presumably located in the main area of the parafacial respiratory group, the depolarization of both preinspiratory and postinspira-

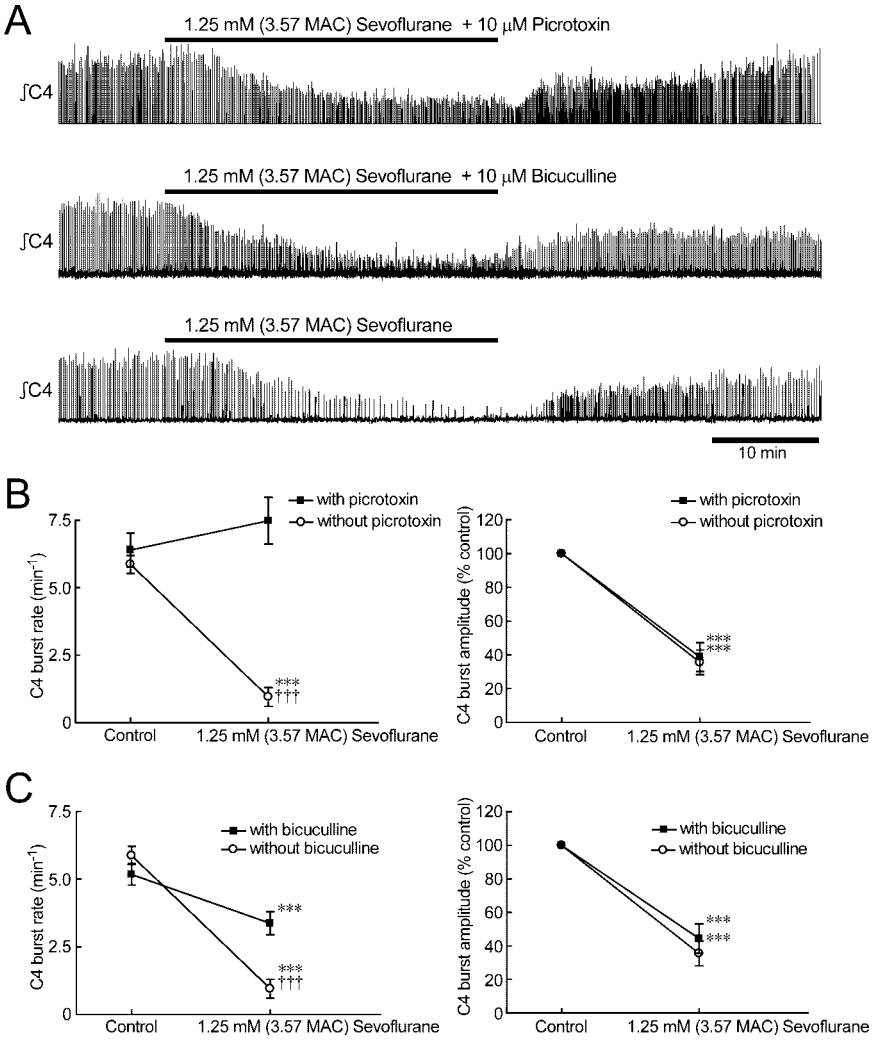

Fig. 4. (*A*) A representative recording of the integrated C4 activity (∫C4) before, during, and after superfusion with 1.25 mM (3.57 minimum alveolar concentration [MAC]) sevoflurane plus 10 μM picrotoxin (*upper trace*) or plus 10 μM bicuculline (*middle trace*). A recording of the superfusion with 1.25 mM (3.57 MAC) sevoflurane alone is also provided (*lower trace*). The *horizontal bars* indicate the duration of superfusion with drugs. (*B*) The effect of sevoflurane on C4 burst rate (*left*) and amplitude (*right*) with and without picrotoxin. C4 burst amplitude decreased significantly in both groups. In the sevoflurane plus picrotoxin group, C4 burst rate did not change significantly in comparison to the control rate, *i.e.*, the depressant effect on C4 burst rate was attenuated. (*C*) The effect of sevoflurane on C4 burst rate (*left*) and amplitude (*right*) with and without bicuculline. The amplitude decreased significantly in both groups. The respiratory rate decreased significantly in both groups but the depressant effect was weaker in the sevoflurane plus bicuculline group. *** $P < 0.005$ *versus* control. ††† $P < 0.005$, application of sevoflurane plus picrotoxin or bicuculline *versus* application of sevoflurane alone.

tory phases became smaller, and seemed to be phase locked during superfusion with 1.25 mM (3.57 MAC) sevoflurane, although C4 burst was maintained (fig. 5). In four of the six preinspiratory neurons, the inhibitory postsynaptic potential decreased, whereas that of the remaining two preinspiratory neurons did not change. A 1.25 mM (3.57 MAC) amount of sevoflurane decreased the intraburst firing frequency and membrane resistance significantly (4% of control value [$P < 0.05$] and 76% of control value [$P < 0.01$], respectively), although it did not change the resting membrane potential (table 1).

In five Exp-i neurons (no Exp-p-i neurons were recorded), presumably located in the Bötzinger complex, tonic discharge disappeared and the inhibitory postsynaptic potential decreased during superfusion of 1.25 mM (3.57 MAC) sevoflurane, although C4 burst was maintained (fig. 5). In these cells, 1.25 mM (3.57 MAC) sevoflurane significantly decreased the intraburst firing frequency and membrane resistance (29% of control value and 78% of control value [$P < 0.05$], respectively), although it did not change the resting membrane potential significantly (table 1).

In six phrenic motor neurons from C4 ventral horn, 1.25 mM (3.57 MAC) sevoflurane decreased the rate of inspiratory-related rhythmic drive potentials and concomitant action potential discharge (fig. 5). Sevoflurane significantly decreased the intraburst firing frequency and membrane resistance, and the cells hyperpolarized (78% of control value [$P < 0.01$], −5 mV from control value [$P < 0.01$], and 81% of control value [$P < 0.05$], respectively; table 1).

Discussion

The current investigation shows that sevoflurane decreases inspiratory-related C4 burst rate and intraburst firing frequency of preinspiratory and expiratory neurons, but not inspiratory neurons in the medulla. Furthermore, the agent decreased C4 burst amplitude (*i.e.*, tidal volume) by inhibiting the activity of phrenic motor neurons in the spinal cord. Our results suggest that the effect of GABAergic transmission contributes to the mechanism of this decreased C4 burst rate.

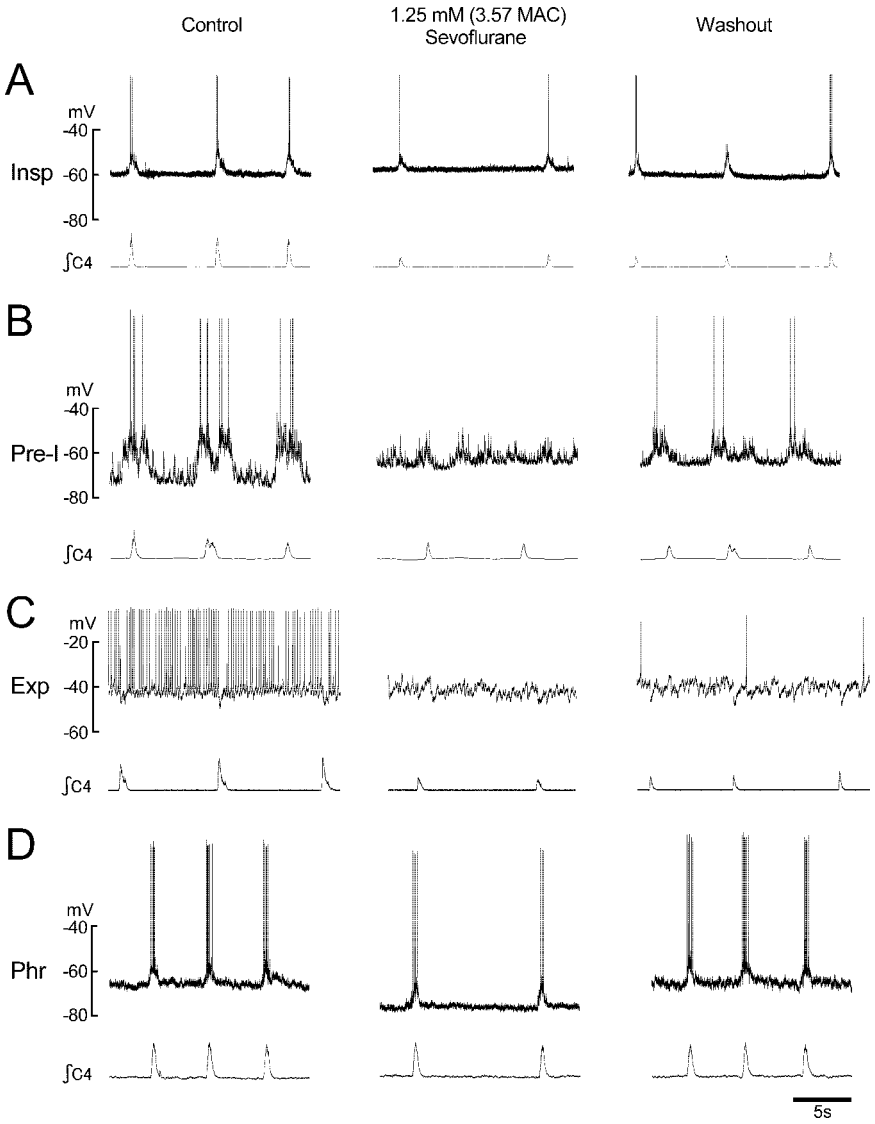

Fig. 5. The effect of approximately 1.25 mM (3.57 minimum alveolar concentration [MAC]) sevoflurane on the activities of inspiratory (Insp; *A*), preinspiratory (Pre-I; *B*), expiratory (Exp; *C*), and phrenic motor neurons (Phr; *D*). Simultaneous recordings of the integrated C4 activity (∫C4) are also provided. After the control recording, the control artificial cerebrospinal fluid was changed to artificial cerebrospinal fluid containing approximately 1.25 mM (3.57 MAC) sevoflurane for approximately 3–6 min, followed by a 10- to 20-min washout period using control artificial cerebrospinal fluid. In inspiratory neurons, sevoflurane did not affect the intraburst firing frequency or the resting membrane potential. The depolarizing cycle rate reduced by sevoflurane was synchronized with C4 burst rate. In preinspiratory and expiratory neurons, sevoflurane induced a decrease in the intraburst firing frequencies but did not affect the resting membrane potentials. In preinspiratory neurons, the depolarization of both preinspiratory and postinspiratory phases became smaller and seemed to be phase locked during superfusion of sevoflurane, although C4 burst was maintained. In phrenic motor neurons, sevoflurane induced hyperpolarization of the resting membrane potential and decreased the intraburst firing frequency.

Effect of Sevoflurane on C4 Burst Rate and Respiratory Neurons

Sevoflurane reduced C4 burst rate in a dose-dependent fashion, and this effect appeared when it was applied exclusively to the medulla. Halothane, isoflurane, and enflurane also have been shown to reduce C4 burst rate in this *en bloc* brainstem model.[15] Therefore, in this preparation, volatile anesthetics may have general rate-reducing properties. Contrary, in human studies,[1,2] sevoflurane at anesthetic doses increased the respiratory rate *via* a chemoreflex loop. In one *in vivo* study using decerebrate, vagotomized, and peripherally deafferented dogs,[3] sevoflurane also increased the respiratory rate. The discrepancy between the current results and the results of these *in vivo* and human studies may be explained as follows. The pons has a strong modulatory effect on respiratory rate, and its ablation increases C4 burst rate.[26] In addition, it was found[27] that the noradrenergic A_5 area in the pons exerts an inhibitory effect on the respiratory rate. Adenosine 5′-triphosphate–induced inward currents of the locus ceruleus neurons in the pons are reduced by sevoflurane,[28] suggesting that neural excitation is reduced. From these studies, it seems that sevoflurane may increase the respiratory rate by inducing disinhibition of the pontine respiratory neural circuits. Therefore, the finding that the sevoflurane decreased the C4 burst rate might be due to the absence of the pons.

Inspiratory medullary neurons exhibited a constant intraburst firing frequency, resting membrane potential, and membrane resistance during application of sevoflurane, despite the decrease of their burst rate that occurred in a 1:1 fashion with slowing of C4 burst rate. In *in vivo* canine experiments, however, sevoflurane reduced the discharge frequency of premotor inspiratory neurons.[5,6] The lack of agreement between these experiments and the current experiments may be due to differences in the subtypes of inspiratory neurons. Inspiratory neurons are categorized into several subtypes based on their location and function. The inspiratory

Table 1. Effect of Sevoflurane on Respiratory Neurons

	n	Control	1.25 mM (3.57 MAC) Sevoflurane
Inspiratory neurons			
Intraburst firing frequency, spike/s	7	5.2 ± 1.5	4.9 ± 1.5
Resting membrane potential, mV	7	−58.7 ± 8.6	−58.0 ± 8.4
Membrane resistance, MΩ	4	372.2 ± 53.4	341.5 ± 76.1
Preinspiratory neurons			
Intraburst firing frequency, spike/s	6	0.7 ± 0.2	0.03 ± 0.02*
Resting membrane potential, mV	6	−51.6 ± 4.8	−51.7 ± 3.0
Membrane resistance, MΩ	6	406.2 ± 68.1	309.0 ± 61.0†
Expiratory neurons			
Intraburst firing frequency, spike/s	5	2.4 ± 0.5	0.7 ± 0.4*
Resting membrane potential, mV	5	−42.5 ± 2.4	−41.5 ± 3.1
Membrane resistance, MΩ	4	417.0 ± 33.4	328.0 ± 21.2*
Phrenic motor neurons			
Intraburst firing frequency, spike/s	6	22.5 ± 6.1	17.5 ± 6.5†
Resting membrane potential, mV	6	−62.1 ± 5.7	−68.0 ± 6.6†
Membrane resistance, MΩ	3	612.0 ± 103.8	489.8 ± 97.5*

* $P < 0.05$ and † $P < 0.01$ indicate statistically significant differences from control values.

MAC = minimum alveolar concentration.

neurons in the current study were presumably located in the pre-Bötzinger complex, which is considered to be an inspiratory rhythm–generating region.[11] The inspiratory neurons recorded by Stucke *et al.*[5,6] were located in the caudal ventral respiratory group and are categorized as bulbospinal premotor neurons. In the same preparations as used here, halothane at high (but not low) concentration only slightly reduced the intraburst firing frequency of inspiratory neurons, *i.e.*, these neurons resisted the effect of halothane.[15] Therefore, inspiratory neurons in the pre-Bötzinger complex seem to resist the depressing effect of volatile anesthetics in brainstem–spinal cord preparations. Sevoflurane at concentrations higher than those used in clinical use may reduce the intraburst firing frequency of these inspiratory neurons.

Preinspiratory neurons showed a reduced intraburst firing frequency (the firing disappeared in most of these neurons although the respiratory rhythm of the C4 activity was maintained) as well as membrane resistance during application of sevoflurane. Preinspiratory neurons located in the parafacial respiratory group that may overlap the retrotrapezoid nucleus[11,29,30] receive inhibitory synaptic connections from inspiratory neurons, whereas they send excitatory and inhibitory synaptic input to inspiratory neurons.[7] It has been hypothesized[30] that the parafacial respiratory group (composed of preinspiratory neurons) is critical for respiratory rhythm generation. This is not in accord with the hypothesis that the pre-Bötzinger complex (which is composed of inspiratory neurons) is the primary site of respiratory rhythm generation.[9,11] For a unifying hypothesis, the functionally inspiratory pre-Bötzinger complex and the functionally expiratory parafacial respiratory group may constitute a dual respiratory center.[31] Recently, it was reported that the region containing the parafacial respiratory group (rostral to the pre-Bötzinger complex) seem to control expiratory abdominal muscles[12,32,33] and to contribute the longevity of inspiratory rhythm.[12] Several agents in general use by anesthesiologists, such as propofol[16,17] and opioids,[10] affected these two generators differently. Halothane as well as sevoflurane also reduced the intraburst firing frequency of the preinspiratory neurons in the brainstem–spinal cord preparation.[15] From these results, we suggest that inhibition of preinspiratory neurons by volatile anesthetics may result in the inhibition of active expiratory rhythm and that the inspiratory rhythm of pre-Bötzinger complex may be maintained during application of volatile anesthetics. Ruangkittisakul *et al.*[12] reported that transverse slices containing only a minor amount of brainstem tissue rostral to the pre-Bötzinger complex slices and *en bloc* medullas without major aspect of the parafacial respiratory group show stable inspiratory rhythm at physiologic calcium and potassium concentrations. During application of sevoflurane, the pre-Bötzinger complex containing inspiratory neurons may determine the respiratory rhythm, because C4 burst was preserved after the firing of preinspiratory neurons disappeared and the rhythm was always synchronized with the burst of inspiratory neurons.

Sevoflurane decreased intraburst firing frequency and membrane resistance of medullary expiratory neurons. The area in which we recorded the activity of the expiratory neurons corresponds likely to that of the Bötzinger complex in adult mammals, where expiratory neurons function as interneurons[11] and are found at high densities.[8] In the *in vivo* canine experiment, sevoflurane also reduced the firing of premotor expiratory neurons located in the caudal ventral medulla.[3,4] In the same preparation as ours, halothane also reduced the intraburst firing frequency of expiratory neurons.[15] Expiratory-related neurons, unlike inspiratory neurons, thus seem to be inhibited by volatile anesthetics across differences in location and function.

Blockade of $GABA_A$ receptors attenuated the sevoflurane-induced reduction in C4 burst rate. Because separate perfusion of sevoflurane to the medulla decreased the C4 burst rate, sevoflurane should act on $GABA_A$ergic transmission in the medulla. A previous study reported that sevoflurane seemed to enhance inhibitory synaptic transmission by promoting the function of the α_1 and α_2 subunits of the $GABA_A$ receptor, resulting in the activation of chloride channels.[34] Our finding that the membrane resistance of preinspiratory and expiratory neu-

rons is decreased by sevoflurane may be explained by the activation of these $GABA_A$ receptors. In addition, both preinspiratory and expiratory neurons are subject to $GABA_A$ergic inhibition, which causes the inhibitory postsynaptic potential during the inspiratory phase.[35,36] In fact, the current study suggests that activation of $GABA_A$ receptors on preinspiratory and expiratory neurons by sevoflurane inhibits the expiratory component of respiratory rhythm. However, the resting membrane potential of preinspiratory neurons and expiratory neurons did not change even when $GABA_A$ receptors may have been activated. Therefore, we cannot explain the unchanged resting membrane potential by pointing to $GABA_A$eric transmission only.

On the other hand, neurons in the pre-Bötzinger complex contained intense neurokinin-1 receptor immunoreactivity, and some of these neurons are presumed to be essential for inspiratory rhythm generation.[37] GABAergic synapses onto neurokinin-1 receptor–immunoreactive neurons in the pre-Bötzinger complex have been identified morphologically.[38] Mechanism of sevoflurane-induced depression of C4 burst rate may include the activation of $GABA_A$ receptors in the pre-Bötzinger complex. However, the electrophysiologic properties of inspiratory neurons did not change significantly. One such mechanism could be presynaptic inhibition within the "emerging" interneuronal network in the pre-Bötzinger complex as indicated by a lack of postsynaptic effects on putative inspiratory neurons in the pre-Bötzinger complex.[11,33]

Effect of Sevoflurane on C4 Burst Amplitude and Phrenic Motor Neurons

Sevoflurane reduced C4 burst amplitude in a dose-dependent fashion. This result agrees with human studies[1,2] in which sevoflurane at anesthetic doses decreased the tidal volume, and also with an *in vivo* canine study[3] in which sevoflurane decreased phrenic nerve activity. Because the reduced C4 burst amplitude appeared only when applied to the spinal cord, this effect of sevoflurane seems to target spinal respiratory neural circuits. This is supported by the current findings that sevoflurane does not affect the activity of the inspiratory neurons presumably located in the pre-Bötzinger complex, which has been thought to be an inspiratory rhythm generator.[39] Therefore, the amplitude depression seems to result from the effect of sevoflurane on the spinal cord.

In accord with this view, sevoflurane hyperpolarized phrenic motor neurons and decreased both their intraburst firing frequency and membrane resistance. The finding that blockade of $GABA_A$ receptors by picrotoxin or bicuculline did not attenuate the reduction of C4 burst amplitude suggests that sevoflurane does not reduce C4 burst amplitude by acting on $GABA_A$ergic transmission in the spinal cord. It has been reported that sevoflurane has multiple molecular targets at the spinal cord level. Matute and Lopez-Garcia[40] have shown that the depolarizations in motor neurons induced by α-amino-3-hydroxy-5-methylisoxazole-4-propionate and *N*-methyl-D-aspartate were reduced by sevoflurane. Two-pore-domain acid-sensitive K^+-1 channels, which have sensitivity to sevoflurane,[14] are reported to be densely expressed in the spinal cord.[41] Moreover, Grasshoff and Antkowiak[42] found that blocking glycine receptors reversed the depression of spontaneous firing elicited by sevoflurane on the spinal cord. Among these molecular targets, the current study suggests that the two-pore-domain acid-sensitive K^+-1 channel is involved. In slices containing the pre-Bötzinger complex, hypoglossal motor neurons generate endogenous respiratory-related motor output.[9] Recently, these neurons have been reported to express the two-pore-domain acid-sensitive K^+-1 channels and to be sensitive to sevoflurane.[14] Considering our data and these previous studies, it seems that in phrenic motor neurons, activation of the two-pore-domain acid-sensitive K^+-1 channel by sevoflurane may induce an increase in K^+ conductance (*i.e.*, K^+ leakage), resulting in membrane hyperpolarization and a reduction in intraburst firing frequency. However, further studies will be required to confirm the acid sensitivity of phrenic motor neurons during application of sevoflurane.

In summary, during application of sevoflurane, the pre-Bötzinger complex containing inspiratory neurons may determine the respiratory rhythm. Contrary, sevoflurane-induced depression of C4 burst amplitude seems to occur at the level of phrenic motor neurons. Our findings also suggest that $GABA_A$ receptors in the pre-Bötzinger complex plays an essential role in sevoflurane-induced depression of C4 burst rate, and that $GABA_A$ergic transmission in the spinal cord is not involved in sevoflurane-induced depression of C4 burst amplitude.

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References

1. Doi M, Ikeda K: Respiratory effects of sevoflurane. Anesth Analg 1987; 66:241–4

2. Pandit JJ, Manning-Fox J, Dorrington KL, Robbins PA: Effects of subanaesthetic sevoflurane on ventilation, 1: Response to acute and sustained hypercapnia in humans. Br J Anaesth 1999; 83:204–9

3. Stucke AG, Stuth EA, Tonkovic-Capin V, Tonkovic-Capin M, Hopp FA, Kampine JP, Zuperku EJ: Effects of sevoflurane on excitatory neurotransmission to medullary expiratory neurons and on phrenic nerve activity in a decerebrate dog model. ANESTHESIOLOGY 2001; 95:485–91

4. Stucke AG, Stuth EA, Tonkovic-Capin V, Tonkovic-Capin M, Hopp FA, Kampine JP, Zuperku EJ: Effects of halothane and sevoflurane on inhibitory neurotransmission to medullary expiratory neurons in a decerebrate dog model. ANESTHESIOLOGY 2002; 96:955–62

5. Stucke AG, Zuperku EJ, Krolo M, Brandes IF, Hopp FA, Kampine JP, Stuth EA: Sevoflurane enhances γ-aminobutyric acid type A receptor function and overall inhibition of inspiratory premotor neurons in a decerebrate dog model. ANESTHESIOLOGY 2005; 103:57–64

6. Stucke AG, Zuperku EJ, Tonkovic-Capin V, Krolo M, Hopp FA, Kampine JP, Stuth EA: Sevoflurane depresses glutamatergic neurotransmission to brainstem inspiratory premotor neurons but not postsynaptic receptor function in a decerebrate dog model. ANESTHESIOLOGY 2005; 103:50–6

7. Onimaru H, Arata A, Homma I: Neuronal mechanisms of respiratory rhythm generation: An approach using *in vitro* preparation. Jpn J Physiol 1997; 47:385–403

8. Rekling JC, Feldman JL: PreBotzinger complex and pacemaker neurons: Hypothesized site and kernel for respiratory rhythm generation. Annu Rev Physiol 1998; 60:385–405

9. Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL: Pre-Botzinger complex: A brainstem region that may generate respiratory rhythm in mammals. Science 1991; 254:726–9

10. Takeda S, Eriksson LI, Yamamoto Y, Joensen H, Onimaru H, Lindahl SG: Opioid action on respiratory neuron activity of the isolated respiratory network in newborn rats. ANESTHESIOLOGY 2001; 95:740–9

11. Feldman JL, Del Negro CA: Looking for inspiration: New perspectives on respiratory rhythm. Nat Rev Neurosci 2006; 7:232–42

12. Ruangkittisakul A, Secchia L, Bornes TD, Palathinkal DM, Ballanyi K: Dependence on extracellular Ca2+/K+ antagonism of inspiratory centre rhythms in slices and en bloc preparations of newborn rat brainstem. J Physiol 2007; 584:489–508

13. Matute E, Rivera-Arconada I, Lopez-Garcia JA: Effects of propofol and sevoflurane on the excitability of rat spinal motoneurones and nociceptive reflexes *in vitro*. Br J Anaesth 2004; 93:422–7

14. Sirois JE, Lei Q, Talley EM, Lynch C III, Bayliss DA: The TASK-1 two-pore domain K+ channel is a molecular substrate for neuronal effects of inhalation anesthetics. J Neurosci 2000; 20:6347–54

15. Otsuka H: Effects of volatile anesthetics on respiratory activity and chemosensitivity in the isolated brainstem-spinal cord of the newborn rat. Hokkaido Igaku Zasshi 1998; 73:117–36

16. Kashiwagi M, Okada Y, Kuwana S, Sakuraba S, Ochiai R, Takeda J: A neuronal mechanism of propofol-induced central respiratory depression in newborn rats. Anesth Analg 2004; 99:49–55

17. Kashiwagi M, Okada Y, Kuwana S, Sakuraba S, Ochiai R, Takeda J: Mechanism of propofol-induced central respiratory depression in neonatal rats anatomical sites and receptor types of action. Adv Exp Med Biol 2004; 551:221–6

18. Kuwana S, Okada Y, Natsui T: Effects of extracellular calcium and magnesium on central respiratory control in the brainstem-spinal cord of neonatal rat. Brain Res 1998; 786:194–204

19. Murakoshi T, Suzue T, Tamai S: A pharmacological study on respiratory rhythm in the isolated brainstem-spinal cord preparation of the newborn rat. Br J Pharmacol 1985; 86:95–104

20. Eldridge FL: Relationship between phrenic nerve activity and ventilation. Am J Physiol 1971; 221:535–43

21. Franks NP, Lieb WR: Temperature dependence of the potency of volatile general anesthetics: Implications for *in vitro* experiments. ANESTHESIOLOGY 1996; 84:716–20

22. Arata A, Onimaru H, Homma I: The adrenergic modulation of firings of respiratory rhythm-generating neurons in medulla-spinal cord preparation from newborn rat. Exp Brain Res 1998; 119:399–408

23. Sakuraba S, Kuwana S, Ochiai R, Okada Y, Kashiwagi M, Hatori E, Takeda J: Effects of neuromuscular blocking agents on central respiratory control in the isolated brainstem-spinal cord of neonatal rat. Neurosci Res 2003; 47:289–98

24. Di Pasquale E, Tell F, Monteau R, Hilaire G: Perinatal developmental changes in respiratory activity of medullary and spinal neurons: An *in vitro* study on fetal and newborn rats. Brain Res Dev Brain Res 1996; 91:121–30

25. Robinson D, Ellenberger H: Distribution of N-methyl-D-aspartate and non-N-methyl-D-aspartate glutamate receptor subunits on respiratory motor and premotor neurons in the rat. J Comp Neurol 1997; 389:94–116

26. Okada Y, Kawai A, Muckenhoff K, Scheid P: Role of the pons in hypoxic respiratory depression in the neonatal rat. Respir Physiol 1998; 111:55–63

27. Hilaire G, Monteau R, Errchidi S: Possible modulation of the medullary respiratory rhythm generator by the noradrenergic A5 area: An *in vitro* study in the newborn rat. Brain Res 1989; 485:325–32

28. Masaki E, Kawamura M, Kato F: Reduction by sevoflurane of adenosine 5′-triphosphate-activated inward current of locus coeruleus neurons in pontine slices of rats. Brain Res 2001; 921:226–32

29. Guyenet PG, Mulkey DK, Stornetta RL, Bayliss DA: Regulation of ventral surface chemoreceptors by the central respiratory pattern generator. J Neurosci 2005; 25:8938–47

30. Onimaru H, Homma I: A novel functional neuron group for respiratory rhythm generation in the ventral medulla. J Neurosci 2003; 23:1478–86

31. Mellen NM, Janczewski WA, Bocchiaro CM, Feldman JL: Opioid-induced quantal slowing reveals dual networks for respiratory rhythm generation. Neuron 2003; 37:821–6

32. Janczewski WA, Feldman JL: Distinct rhythm generators for inspiration and expiration in the juvenile rat. J Physiol 2006; 570:407–20

33. Ruangkittisakul A, Schwarzacher SW, Secchia L, Poon BY, Ma Y, Funk GD, Ballanyi K: High sensitivity to neuromodulator-activated signaling pathways at physiological [K+] of confocally imaged respiratory center neurons in on-line-calibrated newborn rat brainstem slices. J Neurosci 2006; 26:11870–80

34. Nishikawa K, Harrison NL: The actions of sevoflurane and desflurane on the γ-aminobutyric acid receptor type A: Effects of TM2 mutations in the alpha and beta subunits. ANESTHESIOLOGY 2003; 99:678–84

35. Brockhaus J, Ballanyi K: Synaptic inhibition in the isolated respiratory network of neonatal rats. Eur J Neurosci 1998; 10:3823–39

36. Onimaru H, Arata A, Homma I: Inhibitory synaptic inputs to the respiratory rhythm generator in the medulla isolated from newborn rats. Pflugers Arch 1990; 417:425–32

37. Gray PA, Rekling JC, Bocchiaro CM, Feldman JL: Modulation of respiratory frequency by peptidergic input to rhythmogenic neurons in the preBotzinger complex. Science 1999; 286:1566–8

38. Liu YY, Wong-Riley MT, Liu JP, Jia Y, Liu HL, Jiao XY, Ju G: GABAergic and glycinergic synapses onto neurokinin-1 receptor-immunoreactive neurons in the pre-Botzinger complex of rats: Light and electron microscopic studies. Eur J Neurosci 2002; 16:1058–66

39. Feldman JL, Janczewski WA: Point:Counterpoint: The parafacial respiratory group (pFRG)/pre-Botzinger complex (preBotC) is the primary site of respiratory rhythm generation in the mammal. Counterpoint: The preBotC is the primary site of respiratory rhythm generation in the mammal. J Appl Physiol 2006; 100:2096–7

40. Matute E, Lopez-Garcia JA: Characterisation of sevoflurane effects on spinal somato-motor nociceptive and non-nociceptive transmission in neonatal rat spinal cord: An electrophysiological study *in vitro*. Neuropharmacology 2003; 44:811–6

41. Talley EM, Lei Q, Sirois JE, Bayliss DA: TASK-1, a two-pore domain K+ channel, is modulated by multiple neurotransmitters in motoneurons. Neuron 2000; 25:399–410

42. Grasshoff C, Antkowiak B: Propofol and sevoflurane depress spinal neurons *in vitro via* different molecular targets. ANESTHESIOLOGY 2004; 101: 1167–76