Title: Orexin-B antagonized respiratory depression induced by sevoflurane, propofol, and remifentanil in isolated brainstem-spinal cords of neonatal rats

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Abstract

Orexins (hypocretins) play a crucial role in arousal, feeding, and endocrine function. We previously reported that orexin-B activated respiratory neurons in isolated brainstem-spinal cords of neonatal rats. We here determined whether orexin-B antagonized respiratory depression induced by sevoflurane, propofol, or remifentanil. We recorded C4 nerve bursts as an index of inspiratory activity in the brainstem-spinal cord preparation. The preparation was superfused with a solution equilibrated with 3% sevoflurane alone for 10 min and the superfusate was then switched to a solution containing sevoflurane plus orexin-B. Sevoflurane decreased the C4 burst rate and integrated the C4 amplitude. The C4 burst rate and amplitude were reversed by 0.5 μM orexin-B, but not by 0.1 μM orexin-B. The decrease induced in the C4 burst rate by 10 μM propofol or 0.01 μM remifentanil was significantly antagonized by 0.1 μM orexin-B. Respiratory depression induced by a higher concentration (0.1 μM) of remifentanil was not restored by 0.1 μM orexin-B. These results demonstrated that orexin-B antagonized respiratory depression induced by sevoflurane, propofol, or remifentanil.

Keywords:

orexin, respiration, anesthetics, isolated brainstem, neontal rat

1. Introduction

Endogenous neuropeptide orexins (hypocretins) play an important role in the regulation of feeding, drinking, endocrine function, and sleep/wakefulness (Maleszka A et al., 2013; Sthiuchi T et al., 2009; de Lecea L et al., 1998; Sakurai T, 2007). Previous studies reported using immunohistochemistry or *in situ* hybridization that orexin-producing neurons (orexinergic neurons) were exclusively localized in the perifornical area and lateral and posterior hypothalamic area in the rat brain (Peyron C et al., 1998; Date Y et al., 1999; Nambu T et al., 1999). The main projection sites in the brain were the paraventricular thalamic nucleus, arcuate nucleus of the hypothalamus, pedunculopontine tegmental nucleus, raphe nuclei, tuberomammillary nucleus, and locus coeruleus.

Orexin A and orexin-B are processed from a common precursor, prepro-orexin, and stimulate target cells via two G-protein-coupled receptors, orexin receptor-1 (OX1R) and orexin receptor-2 (OX2R). Orexin-A binds to both receptors, whereas orexin-B binds selectively to OX2R (Sakurai T et al., 1998). Orexinergic neuronal terminals and both orexin receptors are located in the autonomic centers of the hypothalamus and brainstem, including the periaqueductal gray, parabrachial nucleus, nucleus solitary tract, rostral ventrolateral medulla, and medullary raphe. These areas have been linked to the central control sites of cardiovascular and respiratory function. Nakamura (Nakamura A et al., 2007) demonstrated that orexins modulated the central CO_2 response of ventilation via OX1R during wakefulness in the rodent. We previously examined the effects of the orexinergic system on central respiratory control by adding orexin-B to a superfusion medium in an isolated brain stem-spinal cord of the neonatal rat (Sugita T et al., 2014). We showed that the application of orexin-B enhanced respiratory activity by depolarizing the inspiratory and pre-inspiratory neurons of the medulla.

On the other hand, the anesthetics using in the clinical practice depressed respiration as a side effect at anesthetic doses. Several studies showed that orexin-A facilitated emergence from propofol anesthesia (Shirasaka T et al., 2011; Zhang LN et al., 2012) and the emergence time from isoflurane anesthesia was prolonged in orexin-neuron-ablated mice (Kuroki C et al., 2013). We hypothesized that antagonism between orexin-B and anesthetics presented in respiratory control system. To examine this hypothesis, we used two anesthetics and one sadative, that is, sevoflurane, propofol and remifentanil, which supposed to be different mechanisms of anesthetic or sedative action and were widely used in the clinical practice. Sevoflurane is well-known for a volatile anesthetic. In a previous study using a brainstem-spinal cord preparation of the neonatal rat, we showed that sevoflurane decreased both the C4 burst rate and amplitude. (Kuribayashi J et al., 2008). A separate perfusion of sevoflurane to the medulla and the spinal cord decreased the C4 burst rate and amplitude, respectively. The $GABA_A$ receptor antagonists, picrotoxin and bicuculline, attenuated the reduction observed in the C4 burst rate, but not in the amplitude. Thus, we concluded that $GABA_A$ receptors were involved in sevoflurane-induced respiratory depression within the medulla, but not within the spinal cord. Intravenous anesthetic propofol mainly decreased the C4 burst rate in the brainstem-spinal cords of neonatal rats, and this could be reversed by the administration of bicuculline (Kashiwagi M et al., 2004). The depressive effects of sevoflurane and propofol were, at least partly, mediated by agonistic actions on $GABA_A$ receptors (Garcia PS et al., 2010). Remifentanil is a synthetic □-opioid agonist that is a potent analgesic and sedative and also induces respiratory depression. Remifentanil is rapidly broken down by esterases in the blood and tissue; therefore, remifentanil has recently been used in clinical practice as a short-acting anesthetic agent (Egan TD et al., 1993; Westmoreland CL et al., 1993). In the present study, therefore, we determined whether orexin-B antagonized

respiratory depression induced by sevoflurane, propofol, or remifentanil in the brainstem-spinal cords of neonatal rats.

2. Methods

2.1. Preparation

All procedures were conducted in accordance with the guidelines of the Uekusa Gakuen University Laboratory Animal Care and Use Committee. Data were obtained from 60 neonatal Wistar rats (2-3 days old). The isolated brainstem-spinal cord preparation has been described in detail elsewhere (Kuwana S et al., 1998). In brief, rats were deeply anesthetized with diethyl ether, and the brainstem 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 4ml/min) with control ACSF equilibrated with a control gas mixture (5 % CO_2 in O_2; pH7.4). Its temperature was maintained at 25-26 °C. The composition of the ACSF was (in mM): 126 NaCl, 5 KCl, 1.25 NaH_2PO_4, 1.5 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, and 30 glucose. C4 ventral root activity was recorded using a glass suction electrode, amplified with a conventional AC amplifier (AVH 11, Nihon Kohden, Tokyo, Japan), and integrated (time constant: 100 ms). We measured the C4 burst rate as an index of the respiratory rate (Murakoshi T et al., 1985) and the integrated amplitude as an index of tidal volume (Eldridge FL, 1971). All amplitude data were normalized to the values obtained during a drug-free control state, which was assigned a value of 100%.

2.2. Drug administration

Sevoflurane was applied according to the method described by Matute (Matute E et al., 2004). Sevoflurane (Maruishi Pharmaceutical Co. Ltd, Osaka, Japan) was mixed with the control gas by a vaporizer (Sevotec 3, Ohmeda, Steeton, West Yorkshire, UK) at 3 vol%. The mixed gas was

bubbled into the ACSF for 30 min. A stock solution of 1 M propofol (Aldridge, Germany) were prepared in dimethylsulfoxide (Wako Pure Chemical, Japan) and diluted with control ACSF to give 10 μM. Remifentanil (Janssen Pharma., Japan) was diluted with control ACSF to give a final concentrations of 0.01 μM, 0.05 μM, and 0.1 μM. Orexin-B (Rat, Mouse) was obtained from the Peptide Institute Inc. (Japan). We administered ~~0.01~~ **0.1** μM or 0.5 μM orexin-B dissolved in ACSF containing sevoflurane, propofol, or remifentanil.

2.3. Protocols

After the preparation was superfused with control ACSF (CO_2 fraction 5 %) for at least 30 min and C4 activity reached a steady state, the superfusate bathing the preparation was replaced as follows: ACSF containing anesthetics for 10 min, ACSF containing anesthetics in the presence of ~~0.01~~ **0.1** μM or 0.5 μM of orexin-B for 10 min, and control ACSF for a 10-min washout.

2.4. Data Analysis

All signals were fed into a personal computer after A/D conversion (Power Lab/4sp, ADInstruments, Castle Hill, Australia) and recorded for subsequent analyses (Chart version 7, ADInstruments, Castle Hill, Australia). An analysis of the respiratory parameters was performed off-line. Respiratory parameters obtained before the superfusion of ACSF containing drugs were defined as control values. Changes in the C4 burst rate and amplitude were compared using a one-way analysis of variance, followed by the Tukey-Kramer test. Differences between two groups were compared using Welch's *t*-test. All statistical analyses were conducted using Statcel (OMS publisher, Japan). All values were reported as the mean ± SE and all P values < 0.05 were considered significant.

3. Results

3.1. Antagonism of orexin-B on respiratory depression induced by sevoflurane

Figure 1 shows a representative recording of C4 activity during the superfusion of 3 % sevoflurane with and without 0.5 μM orexin-B. The superfusion of the brainstem-spinal cord with 3 % sevoflurane for 10 min reduced the C4 burst rate and integrated C4 amplitude. By switching the superfusate from sevoflurane to sevoflurane with orexin-B, both the C4 burst rate and integrated C4 amplitude increased. Tonic firing of non-respiratory activity was observed during the superfusion of 3 % sevoflurane with 0.5 μM orexin-B.

Table 1 shows a summary of the antagonism of 0.1 μM and 0.5 μM orexin-B on respiratory depression induced by 3 % sevoflurane. The C4 burst rate was significantly reduced during the superfusion of 3 % sevoflurane, while the reduction observed in the integrated C4 amplitude was not significant. Neither the C4 burst rate nor integrated C4 amplitude increased during the superfusion of 3 % sevoflurane with 0.1 μM orexin-B. However, both the C4 burst rate and integrated C4 amplitude were significantly increased during the superfusion of 3 % sevoflurane with 0.5 μM orexin-B. These values were larger than those of the control. These results demonstrated that respiratory depression by 3 % sevoflurane was antagonized by 0.5 μM, but not 0.1 μM orexin-B.

3.2. Antagonism of orexin-B on respiratory depression induced by propofol

Figure 2 shows a representative recording of C4 activity during the superfusion of 10 μM propofol with and without 0.1 μM orexin-B. The superfusion of 10 μM propofol for 10 min reduced the C4 burst rate, but not the integrated C4 amplitude. The C4 burst rate increased when the superfusate was switched from propofol to propofol with 0.1 μM orexin-B.

Table 2 shows a summary of the antagonism of 0.1 μM orexin-B on respiratory depression induced by 10 μM propofol. The superfusion of 10 μM propofol led to a significant reduction in the C4 burst rate, but not in the integrated C4 burst rate. The C4 burst rate increased during the superfusion of 10 μM propofol with 0.1 μM orexin-B. The integrated C4 amplitude increased slightly during the superfusion of 10 μM propofol with 0.1 μM orexin-B. These results showed that the depression induced in the C4 burst rate by 10 μM propofol was antagonized by 0.1 μM orexin-B. The integrated C4 amplitude was unaffected by the superfusion of 10 μM propofol or 10 μM propofol plus 0.1 μM orexin-B.

3.3. Antagonism of orexin-B on respiratory depression induced by remifentanil

Figure 3 shows a representative recording of C4 activity during the superfusion of 0.01 μM remifentanil with and without 0.1 μM orexin-B. The superfusion of 0.01 μM remifentanil for 10 min reduced the C4 burst rate, but not the integrated C4 amplitude. An increase was observed in the C4 burst rate when the superfusate was switched from remifentanil to remifentanil plus 0.1 μM orexin-B.

Table 3 shows a summary of the antagonism of 0.1 μM orexin-B on respiratory depression induced by 0.01 μM, 0.05 μM, or 0.1 μM remifentanil. The superfusion of any concentration of remifentanil induced significantly decreases in the C4 burst rate, and reduced the integrated C4 amplitude. Regarding the C4 burst rate, 0.1μM orexin-B antagonized the respiratory depression induced by 0.01μM remifentanil. However, the decrease observed in the respiratory rate by 0.05μM or 0.1μM remifentanil was not restored by the superfusion of 0.1μM orexin-B. The integrated C4 amplitude did not change during the superfusion of 0.01μM remifentanil. The superfusion of 0.01 μM remifentanil plus 0.1 μM orexin-B increased the integrated C4 amplitude.

No significant changes were observed in the integrated C4 amplitude during the superfusion of a higher concentration of remifentanil (0.05 μM or 0.1 μM). Furthermore, the additional superfusion of orexin-B was ineffective on the integrated C4 amplitude. These results demonstrated that respiratory depression induced by 0.01 μM remifentanil was antagonized by 0.1 μM orexin-B, whereas that induced by a higher concentration of remifentanil was not antagonized.

4. Discussion

In the present study, we showed that orexin-B antagonized the respiratory depression induced by sevoflurane, propofol, and remifentanil, which are widely used in clinical practice. This result suggests that orexin-B derivatives may be used as an emergence drug for anesthesia.

The result that orexin-B antagonized the respiratory depression induced by different types of anesthetics suggested that targeting sites or receptors of orexin-B and anesthetics may not be common. Accumulating evidence has indicated that sevoflurane and propofol enhanced the function of $GABA_A$ receptors, the most abundant fast inhibitory neurotransmitter receptor in the central nervous system (Garcia PS et al., 2010; Stucke AG et al. 2005; Yip GMS et al. 2013). We previously confirmed that the depressive effects of sevoflurane and propofol were, at least partly, mediated by agonistic actions on $GABA_A$ receptors in the respiratory neurons (Kashiwagi M et al., 2004; Kuribayashi J et al., 2008). The depressive effects of remifentanil were shown to be mediated by the activation of μ-opioid receptors (Egan TD et al., 1993; Westmoreland CL et al., 1993). Thus, the antagonistic effects of orexin-B in respiratory depression induced by the anesthetics used in the present study may not have occurred through a specific common receptor of the neuromodulator or neurotransmitter.

Orexins are strongly neuroexcitatory, as demonstrated in an initial study (de Lecea L et al., 1998) and subsequent studies on different brain regions mainly through the use of electrophysiological techniques on isolated preparations. The excitatory effects of orexins have been attributed to pre- and postsynaptic mechanisms, i.e., enhanced transmitter release and the facilitation of depolarization, respectively. Two classical mechanisms appear to contribute to postsynaptic depolarization: the inhibition of K^+ channels and activation of nonselective cation

channels. We previously reported that the application of orexin-B induced depolarization and decreased membrane resistance in inspiratory and pre-inspiratory neurons. These results suggested that the depolarization of inspiratory and pre-inspiratory neurons was induced by the influx of cations (Yang and Ferguson 2002). Thus, we suggest that the excitation of medullary respiratory neurons by orexin-B may have contributed to its antagonistic effects on respiratory depression induced by general anesthetics.

A pre-synaptic mechanism for neural excitation should also be considered in the present study. Kuwaki *et al.* (2010) reported that orexins activated the central chemoreceptors of the respiratory control system (Kuwaki T et al., 2010). Multiple sites have been suggested for central chemoreceptors in the lower brain stem, including the retrotrapezoid nucleus (RTN), nucleus tractus solitarii (NTS), medullary raphe nucleus, locus coeruleus, and parabrachial nucleus (Nattie E., 2000; Guynet PG et al., 2013). Histochemical studies demonstrated the presence of orexin-immunoreactive axons and orexin receptors within the RTN, NTS, raphe nucleus, and locus coeruleus (Yang and Ferguson 2002; Marcus JN et al., 2001). Therefore, we suggested that the orexin pre-synaptically activated these chemosensitive neurons and induced the excitation of medullary respiratory neurons (Kuwaki T et al., 2010).

On the other hand, Shirasaka *et al.* (2011) showed that an intracerebroventricular injection of orexin A decreased the time emergence from propofol anesthesia and reversed the decrease in noradrenaline and dopamine release induced by propofol (Shirasaka T et al., 2011). They concluded that the orexin system may facilitate emergence from propofol anesthesia by increasing central noradrenergic and dopaminergic activities. This catecholaminergic system was shown to modulate respiratory activity in the brainstem-spinal cords of neonatal rats (Fujii M et al., 2004; Ito Y et al., 2009). Thus, in the present study, the possibility that orexin-B may have

reversed the decrease in noradrenaline and dopamine release induced by anesthesia cannot be ruled out.

In conclusion, orexin-B had antagonistic effects on central respiratory depression induced by sevoflurane, propofol, and remifentanil in the brainstem-spinal cord preparations from neonatal rats. However, the mechanisms underlying the antagonistic effects of orexin-B on respiratory depression induced by the anesthetics and opioids remains unclear. Further studies are warranted to evaluate these mechanisms. However, respiratory depression is a severe side effect of general anesthesia in clinical practice. Therefore, orexin-B derivatives may be used for emergence from general anesthesia.

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Figure Legends

Figure 1. Antagonistic effects of orexin-B on respiratory depression induced by sevoflurane. The rate of C4 bursts and integrated C4 amplitude decreased with the superfusion of ACSF equilibrated with 3% sevoflurane. The superfusion of ACSF containing 0.5 μM orexin-B and equilibrated with 3% sevoflurane reversed the decrease in the rate of C4 bursts and integrated C4 amplitude.

Figure 2. Antagonistic effects of orexin-B on respiratory depression induced by propofol. The rate of C4 bursts and integrated C4 amplitude decreased with the superfusion of ACSF containing 10 μM propofol. The superfusion of ACF containing 0.5 μM orexin-B and 10 μM propofol reversed the decrease in the rate of C4 bursts and integrated C4 amplitude.

Figure 3. Antagonistic effects of orexin-B on respiratory depression induced by remifentanil. The rate of C4 bursts and integrated C4 amplitude was decreased by the superfusion of ACSF containing 0.01 μM remifentanil. The superfusion of ACSF containing 0.1 μM orexin-B and 0.01 μM remifentanil reversed the decrease in the rate of C4 bursts and integrated C4 amplitude.

Tables

Table 1. Antagonism of orexin-B for respiratory depression by sevoflurane

	Concentration	n	C4 **burst** rate(min^{-1})			Integ. C4 **amplitude** (% of control)	
			control	3%Sevoflurane	3%Sevoflurane+Orexin-B	3%Sevoflurane	3%Sevoflurane+Orexin-B
Orexin-B	0.1μM	14	6.5±0.7	3.5±0.5*	3.9±0.6$_{NS}$ *	94.4±1.3**	97.7±1.3$_{NS}$ *
	0.5μM	14	5.4±0.7	3.6±0.6*	8.2±1.2**	96.9±2.0$_{NS}$	134±15.8*

Data are shown as mean±S.E.

n number of preparations; * $p<0.05$; ** $p<0.01$; NS not significant

Table 2. Antagonism of orexin-B for respiratory depression by propofol

	Concentration	n	C4 **burst** rate(min^{-1})			Integ. C4 **amplitude** (% of control)	
			Control	10μM propofol	10μM propofol +Orexin-B	10μM propofol	10μM propofol +Orexin-B
Orexin-B	0.1μM	8	4.2±0.4	2.7±0.4*	5.4±0.4**	119.9±17.8	157±30.4$_{NS}$

Data are shown as mean±S.E.

n number of preparations; * $p<0.05$; ** $p<0.01$; NS not significant

Table 3. Antagonism of orexin-B for respiratory depression by remifentanil

	Concentration	n	C4 **burst** rate(min^{-1})			Integ. C4 **amplitude** (% of control)	
			Control	Remifentanil	Remifentanil +0.1μM Orexin-B	Remifentanil	Remifentanil +0.1μM Orexin-B
Remifentanil	0.01μM	7	5.6±0.7	3.1±0.6*	6.1±0.5* *	101±1.7	167±18** *
	0.05μM	6	6.4±0.9	2.9±1.0*	3.9±0.8NS	100±1.0	123±14NS
	0.1μM	9	5.2±0.7	2.8±0.7*	3.3±0.6NS	102±2.6	118±4.5NS

Data are shown as mean±S.E.

n number of preparations; * $p<0.05$; ** $p<0.01$; NS not significant

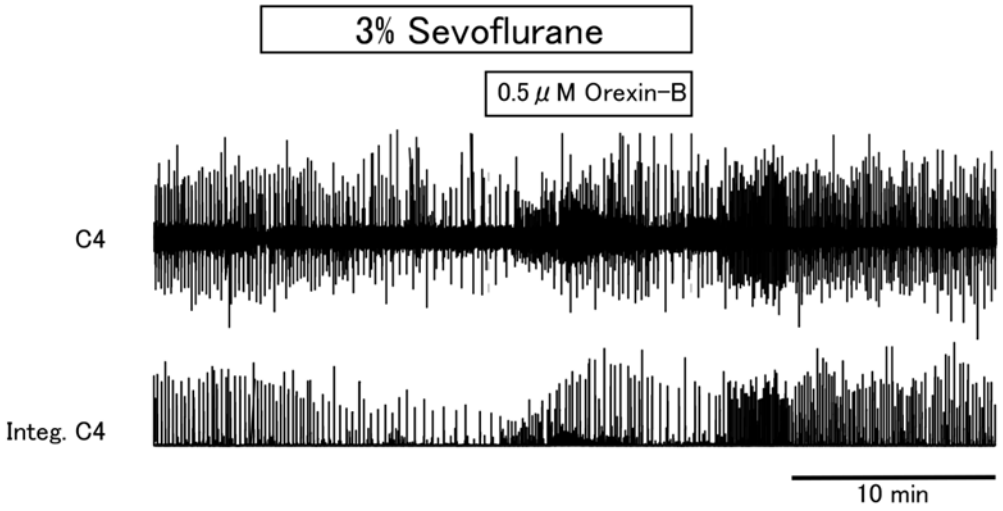
3% Sevoflurane
0.5 μ M Orexin-B
C4
Integ. C4
10 min

Fig. 1

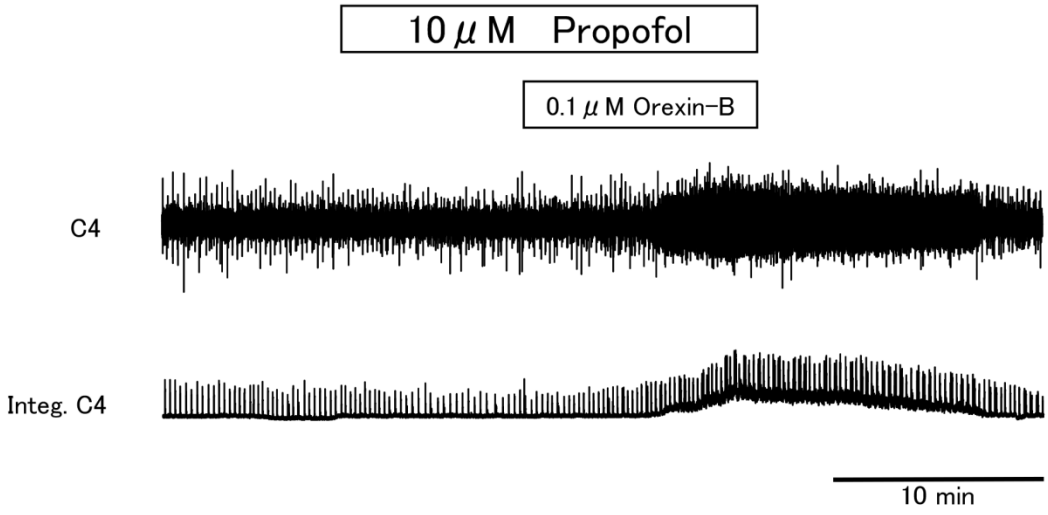
10 μ M Propofol
0.1 μ M Orexin-B
C4
Integ. C4
10 min

Fig. 2

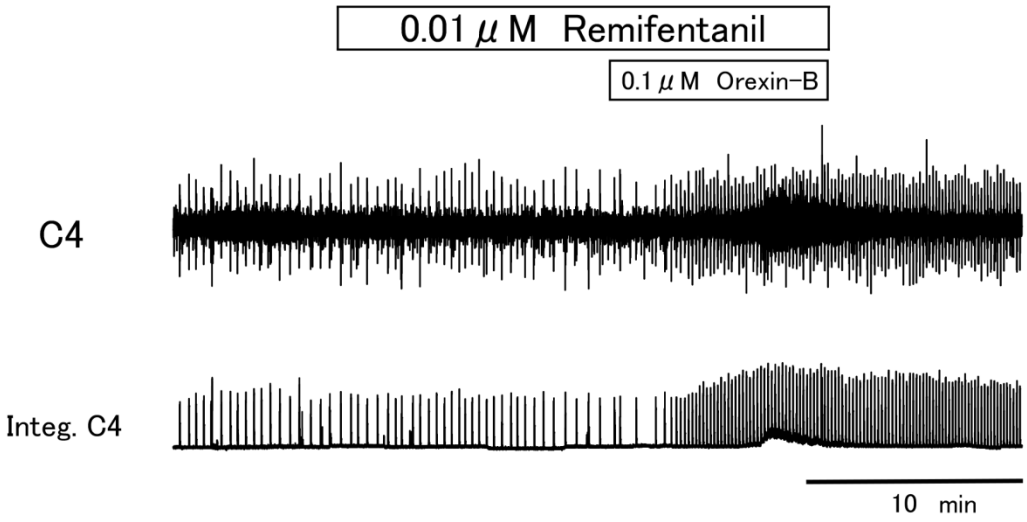

Fig.3