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ORIGINAL ARTICLE

Effects of orexin-A on propofol anesthesia in rats

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Abstract
Purpose An active sleep homeostatic process is present during propofol anesthesia. Activation of the orexin system induces wakefulness, and inhibition of the orexin system causes narcolepsy. We hypothesized that orexin would affect propofol anesthesia.
Methods The effects of an intracerebroventricular (i.c.v.) injection of orexin-A (OXA) or an orexin-1 (OX-1) receptor antagonist, SB-334867, on the times to the loss and return of the righting reflex induced by propofol were examined in Wistar rats. The effects of propofol or OXA on norepinephrine (NE) and dopamine (DA) release from the prefrontal cortex (PFC) were examined using in vivo microdialysis.
Results An i.c.v. injection of OXA (1 nmol) decreased the time to emergence from propofol anesthesia mediated by the OX-1 receptor without changing anesthetic induction ($n = 8$). An i.c.v. injection of SB-334867 (5 and 50 nmol) increased the time to emergence from propofol anesthesia without changing anesthetic induction ($n = 8$). Intravenous infusion of propofol decreased NE ($48 \pm 8\%$; $n = 8$) and DA ($61.2 \pm 11\%$; $n = 8$) release from PFC mediated by the $GABA_A$ receptor. An i.c.v. injection of OXA reversed the decreases in NE and DA release induced by propofol mediated by the OX-1 receptor ($n = 8$).
Conclusion These results indicate that the orexin system may accelerate the emergence from propofol anesthesia associated with increases in the central noradrenergic and dopaminergic activity.

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Introduction

Orexinergic neurons play a critical role in the promotion and maintenance of wakefulness [1]. Orexinergic neurons are distinguished by the presence of two neuropeptides, orexin-A (OXA) and orexin-B, which are processed from a single transcript [2]. Both peptides stimulate wakefulness, and genetic and pharmacologic blockage of orexin-mediated signaling impairs arousal [3, 4]. This evidence suggests that orexin signaling can modify the anesthetic state, with orexin agonists decreasing anesthetic duration and an orexin-1 receptor (OX-1) antagonist, SB-334867, increasing anesthetic duration.

Brain noradrenergic and dopaminergic neuronal activity is related to the modulation of sleep, waking, and anesthesia [5–7]. The locus coeruleus (LC) plays a role in the sleep–wake cycle [5]. LC neurons receive dense direct orexin innervations and express orexin-1 receptors [8]. A local injection of OXA in the LC increases neuronal activity and induces wakefulness, which is supposed to be mediated by a noradrenergic neuron in the LC [9]. The noradrenergic neurons in the PFC are mainly derived from the LC [10]. Dopamine (DA)-containing neurons, which are involved in the regulation of sleep and wakefulness, arise in the ventral tegmental area, in which cells have efferent and afferent connections with the LC and prefrontal cortex (PFC) [9].

An intravenous anesthetic, propofol (2,6-diisopropylphenol), has been widely used in clinical anesthesia and sedation in the intensive care unit because of its clinical merits: rapid onset and clear emergence [11]. Propofol

modulates neuronal activity mediated by $GABA_A$ receptors in the central nervous system [12]. The hypnotic component of anesthesia is suggested to be mediated, at least in part, by $GABA_A$ receptors in an endogenous sleep pathway [13]. Sleep deprivation significantly potentiated the ability of propofol to induce a loss of the righting reflex [14]. Although these results suggest that orexinergic neurons may play an important role in propofol anesthesia via an interaction with noradrenergic and DA neurons, data on these matters are limited.

We examined the effects of OXA and SB-334867 on propofol anesthesia and propofol-induced changes in norepinephrine (NE) and DA release from the PFC using an in vivo microdialysis technique.

Materials and methods

The experimental procedures were approved by the Ethics Committee on Animal Care of the Faculty of Medicine at the University of Miyazaki and conducted in accordance with international guidelines on the ethical use of animals in a laboratory. Male Wistar rats weighing 320–350 g each were housed individually under a constant temperature of 23 ± 1°C with a 12-h light/dark cycle environment with lights on at 07:00 hours. Animals had access to food and water ad libitum except on the day of the experiment. All experiments were performed between 11:00 and 17:00 hours to control for the diurnal rhythm of sleep–wakefulness.

Loss and return of the righting reflex

Rats were implanted with a right lateral cerebroventricular cannula while under anesthesia by intraperitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg), as previously described [15]. A 24-gauge stainless-steel guide cannula (length: 19 mm) was positioned 2.5 mm from the cortex surface and 1 mm above the right lateral cerebroventricle through a burr hole located stereotaxically 0.8 mm posterior and 1.5 mm lateral to the bregma. The guide cannula was fixed to the skull with three screws and dental cement. Approximately 5 days later, the cannulated rats were given pentobarbital anesthesia (50 mg/kg i.p.). The internal jugular vein was cannulated using a PE-50 catheter for the intravenous infusion of drugs. The venous catheter was tunneled under the skin to exteriorize at the scalp. Rats were placed in a handmade plastic box in which they could move freely. After the 3-day recovery period, 4 μl of vehicle (physiological saline solution) or vehicle containing OXA (0.3, 1.0, 5 nmol) or SB-334867 (0.5, 5.0, 50 nmol) were injected intracerebroventricularly into conscious rats through an injection cannula (30-gauge stainless-steel tubing) connected to a 50 μl microsyringe by an automatic injector (LMS, Tokyo, Japan) at a rate of 1 μl/min for 4 min. This injection was made by inserting the injection cannula 1 mm beyond the tip of the guide cannula. In all experiments, the i.c.v. injection volume and speed were 4 μl and 1 μl/min, respectively.

Propofol intravenous (i.v.) infusion was begun at a rate of 800 μg/kg/min 20 min after an i.c.v. injection through a venous cannula (PE-50) connected to a 3 ml syringe by an automatic injector (LMS). The infusion rate was based on previous reports [14]. Rats were then gently prodded or positioned on their back or side to determine their vigilance state at 30 s intervals. Time to loss of the righting reflex was counted from the start of propofol infusion. A rat was considered to have lost the righting reflex if it did not turn itself prone onto all four limbs within 1 min. Intravenous infusion of propofol was continued for 20 min after the loss of the righting reflex was identified. We measured the time from the end of propofol infusion to the return of the righting reflex. Induction of and emergence from propofol anesthesia were defined behaviorally as the respective loss and return of the righting reflex.

Microdialysis

Rats were implanted with a lateral cerebroventricular cannula and a guide cannula for the microdialysis probe under pentobarbital (50 mg/kg) anesthesia. The right lateral cerebroventricle cannula was implanted as noted above. A guide cannula (AG-4, Eicom, Kyoto, Japan) for the penetration of a microdialysis probe was stereotaxically implanted 2.0 mm above the left medial PFC. The stereotaxic coordinates of the guide cannula for the left medial PFC were: anteroposterior, 3.3 mm from bregma; lateral, 0.4 mm from midline, and height, −2.0 mm from the cortical surface. The two guide cannulae were fixed to the skull with three screws and dental cement. Approximately 5 days later, the internal jugular vein was cannulated as noted above. Rats were placed in a handmade plastic box in which they could move freely. One day before the experimental day, the rats were lightly anesthetized with sevoflurane, and the dummy cannula was replaced with a microdialysis probe (A-I-4-02, Eicom). After the 24 h recovery period, microdialysis was carried out under free-moving conditions. Rats received propofol i.v. infusion for 60 min at a rate of 800 μg/kg/min or co-i.v. infusion of propofol and a $GABA_A$ receptor antagonist, GABAzine (5 mg/kg/h). Under similar experimental conditions, the effects of an i.c.v. injection of OXA (1.0 nmol) or a co-injection of OXA (1.0 nmol) and SB-334867 (5.0 nmol) on NE and DA changes in PFC induced by propofol were studied. OXA or OXA plus SB-334867 was injected

30 min later at the start of propofol infusion. Propofol was infused at a rate of 800 μg/kg/min for 60 min.

To measure the NE and DA release in the PFC, the probe was perfused at a flow rate of 1 μl/min with artificial cerebrospinal fluid of the following composition (in mM): NaCl 147, KCl 4, $CaCl_2$ 2.3. The NE and DA in the samples was determined by high-performance liquid chromatography (HPLC) with electrochemical detection (HTEC-500, Eicom) using a CA-5ODS column (4.6 mm I.D., 150 mm long; Eicom). The graphite electrode was set to +400 mV. The composition of the mobile phase was a 0.1 M phosphate buffer (pH 6.0), 1% methanol, 500 mg/l sodium decanesulfonate, and 50 mg/l EDTA. The perfusate from the PFC was injected into the HPLC every 5 min by an automatic injector. After a 3 h stabilization period, six consecutive dialysate samples were corrected to measure the baseline NE and DA release.

At the end of each experiment, the rats were killed with an overdose of pentobarbital sodium, and the brains were fixed in 10% neutral buffered formalin. The placement of the microdialysis probe was verified histologically in 40-μm coronal sections after cresyl violet staining.

Rats were randomly divided into eight groups ($n = 8$ in each group). There were four groups for measuring the induction and emergence time of propofol anesthesia: i.c.v. injection of saline, OXA, SB-334867, and OXA plus SB-334867. There were also four groups for measuring the time course of NE and DA release induced by propofol: propofol alone, GABAzine infusion, i.c.v. injection of OXA, and i.c.v. injection of OXA plus SB-334867.

To evaluate the dose-dependent responses of the loss and return of the righting reflex, each rat was tested with each concentration once a day. In these cases, the concentration of the applied drug was selected at random, and subsequent drug applications were made on a different day. In the microdialysis study, each implanted rat was challenged with a single dose of the test drug only once.

Drug preparation

Propofol was purchased from Maruishi (Osaka, Japan). OXA was purchased from Sigma Chemicals (St. Louis, MO, USA), and SB-334867, from Wako Chemicals (Osaka, Japan). The physiological saline solution was from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan).

Statistical analysis

All data are expressed as the mean ± SEM. The times to the loss and return of the righting reflex data per dose were compared with those of the corresponding control groups using the Bonferroni multiple comparison test. Changes in the concentrations of NE and DA were expressed as a percentage of the basal level calculated from the last six samples before propofol administration, and were evaluated by analysis of variance (ANOVA) for repeated measures followed by the Fisher's PLSD test. $P < 0.05$ was considered statistically significant.

Results

Effects of OXA and SB-334867 on the induction of and emergence from propofol anesthesia

To explore the effects of an i.c.v. injection of OXA on the induction of and emergence from propofol anesthesia, rats were treated with vehicle (saline) or OXA before propofol infusion. Although an i.c.v. injection of OXA failed to alter the induction of propofol anesthesia, it dose-dependently shortened emergence from anesthesia (control versus 300 pmol, $P = 0.0037$; control versus 1.0 nmol, $P < 0.0001$; control versus 5.0 nmol, $P < 0.0001$) (Fig. 1). The effect of OXA (1.0 nmol) was blocked by a co-injection of an orexin-1 receptor antagonist, SB-334867 (5.0 nmol). To explore the disruption of orexin signaling on the induction

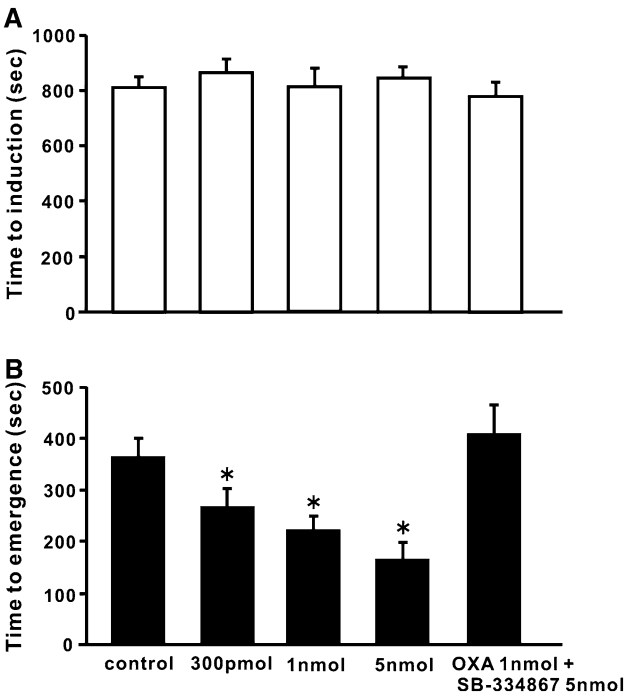

Fig. 1 Effects of orexin A (OXA) on the induction of **a** and emergence from **b** propofol anesthesia. An intracerebroventricular (i.c.v.) injection of OXA decreased the emergence time from propofol anesthesia in a dose-dependent manner. An i.c.v. injection of 5 nmol of an OX1 receptor antagonist, SB-334867, reversed the OXA (1 nmol)-induced decrease in the emergence time from propofol anesthesia. All data are the mean ± SEM; $n = 8$ in each group. *$P < 0.05$ versus control (saline)

of and emergence from propofol anesthesia, rats were treated with vehicle or SB-334867. Although no differences in the time to the induction of anesthesia were detected at all doses of SB-334867, SB-334867 delayed emergence from anesthesia in a dose-dependent manner (control versus 5.0 nmol, $P = 0.018$; control versus 50 nmol, $P = 0.002$) (Fig. 2).

Effects of OXA on propofol-induced decreases in NE and DA release

Intravenous infusion of propofol decreased NE and DA release, reaching a minimum of 48 ± 8 ($P < 0.0001$) and 61.2 ± 11% ($P = 0.002$), respectively (Fig. 3a). NE and DA release returned to their baseline values at about 150 and 140 min from the start of propofol infusion, respectively. Co-infusions of a $GABA_A$ receptor antagonist, GABAzine, blocked the effects of propofol on NE and DA release. Although the rats infused with propofol became unable to move, the rats co-infused with GABAzine and propofol were able to move by standing on four limbs during the recording time ($n = 8$). An i.c.v. injection of OXA (1.0 nmol) increased NE and DA release, reaching maxima of 153.3 ± 7 ($P = 0.001$) and 137 ± 8.6% ($P = 0.006$), respectively, in spite of propofol infusion (Fig. 3b). NE and DA release returned to their baseline values at about 130 and 120 min from the start of propofol infusion, respectively. A co-injection of SB-334867 (5.0 nmol) blocked increases in NE and DA release induced by OXA (1.0 nmol) in the PFC (Fig. 3b).

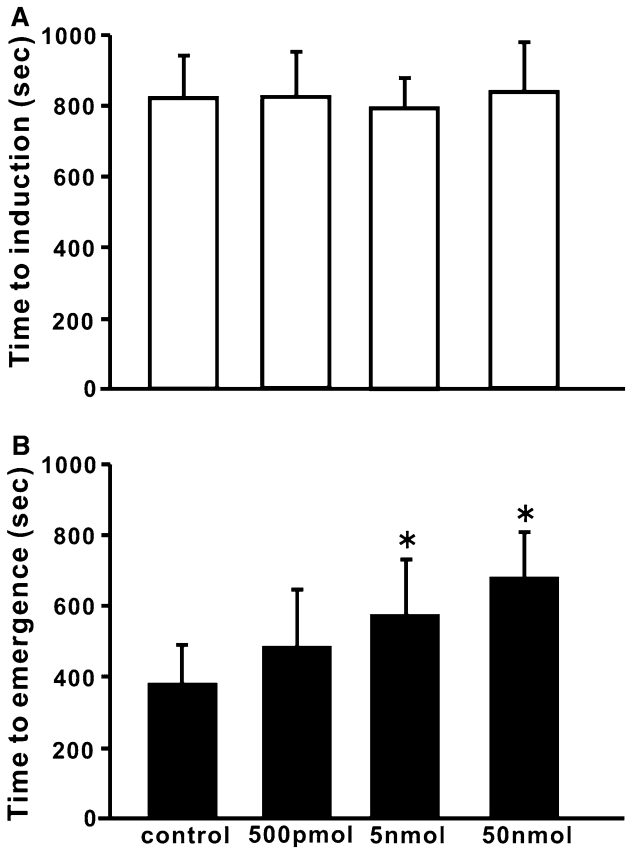

Fig. 2 Effects of an OX1 receptor antagonist, SB-334867, on the induction of **a** and emergence from **b** propofol anesthesia. An i.c.v. injection of SB-334867 increased the emergence time from propofol anesthesia in a dose-dependent manner. All data are the mean ± SEM; $n = 8$ in each group. *$P < 0.05$ versus control (saline)

Discussion

This is the first study demonstrating that OXA modifies emergence from propofol anesthesia without affecting induction and reverses the inhibition of NE and DA release in the rat PFC induced by propofol.

The orexin system has been reported to affect the anesthesia times of several anesthetics, including sevoflurane, barbiturate, and ketamine [3, 4, 16]. In this study, an i.c.v. injection of OXA shortened the emergence time from propofol anesthesia without changing anesthetic induction, and an i.c.v. injection of an OX-1 receptor antagonist, SB-334867, delayed the emergence time from propofol anesthesia without changing anesthetic induction. Our results were consistent with a study in which orexin/ataxin-3 mice with impaired orexin signaling showed delayed emergence from sevoflurane or isoflurane anesthesia without changes in anesthetic induction [3]. These results indicate that activation of the orexin system promotes emergence from anesthesia and inhibition of the orexin system delays emergence from anesthesia. However, the mechanism of action of orexin is not thoroughly understood. The reason that OXA affected emergence from propofol anesthesia but not the induction of it is unknown. Waking up from anesthesia requires the use of neural substrates and neural circuits distinct from those that are needed to become anesthetized [17]. Although the wake-promoting actions of OXA may be ineffective during sleep-promoting conditions, such as induction of anesthesia, they may be effective during wake-promoting conditions, such as emergence from anesthesia. In a word, the orexin system may be wake-promoting and -stabilizing neural circuitry that selectively contributes to emergence from anesthesia. These expectations are supported by the evidence that the plasma OXA level increases after emergence from anesthesia rather than pre- and during anesthesia [18].

We demonstrated that i.v. infusion of propofol decreased NE and DA release from the rat PFC mediated by a $GABA_A$ receptor. A decrease in DA release was also observed in parallel with that of NE, as shown in Fig. 3a. The major source of DA neurons in the PFC is a ventral tegmental area (VTA) [19], which contains $GABA_A$

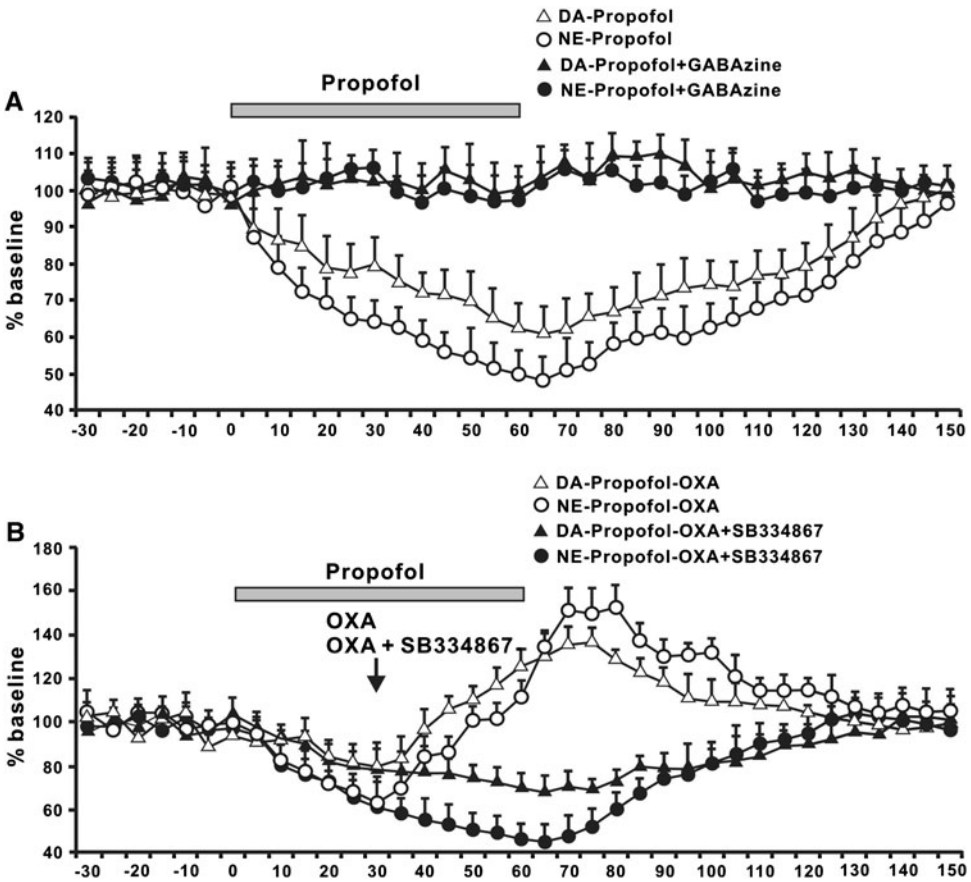

Fig. 3 **a** Effects of propofol on NE and DA release in the PFC. Intravenous propofol infusion decreased NE and DA release in the PFC. Co-infusion of a $GABA_A$ receptor antagonist, GABAzine, reversed the propofol-induced decreases in NE and DA release in the PFC. **b** Effects of OXA on changes in NE and DA release induced by propofol in the PFC. An i.c.v. injection of OXA (1 nmol) reversed the decreases in NE and DA release induced by propofol. A co-injection of an orexin-1 receptor (OX1) antagonist, SB-334867 (5 nmol), blocked increases in NE and DA release induced by OXA in the PFC. All data are the mean ± SEM; $n = 8$ in each group

receptors [20]. The inhibition of DA release induced by propofol may be mediated by $GABA_A$ receptors in the VTA. The DA concentration in the dialysate from the PFC, an area densely innervated by NA and DA neurons, was only 37% higher than that obtained from the occipital cortices that received dense NA and scarce DA projections [21]. The co-release of NE and DA from noradrenergic neurons in the cerebral cortex elicited by stimulating the locus coeruleus (LC) has been reported [22]. These results suggest that the dialysate DA may reflect not only that from DA neurons but also that from noradrenergic neurons.

The noradrenergic neurons in the PFC are mainly derived from the LC, which plays a role in the circadian regulation of the sleep–wake cycle, and receive dense, direct orexin innervation, express OX-1 receptors, and are excited by orexin [8, 10]. Thus, it is possible that the orexin system modulates noradrenergic neurons in the LC and controls physiological functions, such as the wakefulness mechanism. Sleep deprivation potentiates propofol-induced loss of the righting reflex, and propofol reduced sleep latency and increased non-rapid eye movement and total sleep times when administered into the medial preoptic area of the hypothalamus known to regulate sleep [14, 23]. In addition, a recovery process from sleep deprivation similar to that which occurs during naturally occurring sleep also takes place during propofol anesthesia [24]. These results suggest that sleep and propofol anesthesia likely share some regulatory elements. It is possible that i.v. infusion of propofol decreases NE release in the PFC mediated by the inhibition of LC neuronal activity. This is supported by the fact that propofol directly inhibits LC neuronal activity mediated by $GABA_A$ receptors [25].

An i.c.v. injection of OXA reversed the inhibition of NE and DA release induced by propofol and increased NE and DA from their baseline levels (Fig. 3b). These effects of OXA were blocked by an orexin-1 receptor antagonist, SB-334867, which is reported to antagonize OXA-mediated excitation in the LC [26]. These results suggest that the increase in activity of noradrenergic neurons by OXA counterbalanced the decrease induced by propofol anesthesia. OXA has also been reported to induce NE release from rat cerebrocortical slices and PFC [27, 28]. Our results were supported by the fact that SB-334867 blocked OXA-induced NE release in the cerebrocortical slices [27]. These results indicate that OXA facilitates NE release from PFC mediated by the OX-1 receptor.

Activation of LC neurons promotes wakefulness and behavioral arousal. Orexin increases the firing rate of the LC and, hence, the state of arousal [9]. In our previous study, an i.c.v. injection of OXA increased sympathetic

tone and locomotor activity [29]. Although DA contributes to the regulation of wakefulness through mechanisms that have not yet been elucidated, the basal level of DA in the PFC is greater during the dark phase (when rats are active) than during the light phase (when rats spend more time sleeping) [30]. Systemic or i.c.v. administration of the DA receptor agonist enhanced wakefulness in rats [31, 32]. These results suggest that the wake-promoting actions of OXA likely involve the activation of DA neurons.

Noradrenergic neurons of the LC are supposed to be involved in the production of the anesthetic state [33]. Propofol may exert its effects mediated by $GABA_A$ receptors in the LC. These results suggest that there is interaction between the orexin system and the GABAergic system in the LC.

A limitation of our study is related to the fact that we could not exclude the possibility of the effect of propofol on other brain regions and neurotransmitters. GABA-mediated transmission in the medial preoptic area of the hypothalamus also modulates arousal [23]. The tuberomammillary nucleus (TMN) and the perifornical area (Pef), which is involved in the hypothalamic sleep pathway, have been demonstrated to be an important target of propofol rather than LC in a $GABA_A$ receptor β_3N265M knock-in mouse study [34]. The possibility that the $GABA_A$ receptor in the hypothalamic sleep pathway is also involved in the hypnotic action of propofol in this study cannot be ruled out.

Not only noradrenaline but also multiple neurotransmitters have been shown to interact with GABAergic systems controlling the arousal state. For example, propofol administration to rats decreases cortical acetylcholine release, and, in human volunteers, propofol-induced unconsciousness can be reversed with physostigmine [35, 36]. The hypnotic effects of adenosine injected into the medial preoptic area are blocked by flumazenil [37]. These neurotransmitters may also play an important role in the hypnotic action of propofol.

In a clinical study, Kushikata et al. [18] demonstrated that plasma OXA and NE increase during emergence from propofol–fentanyl anesthesia. They also indicated that there is a significant correlation between plasma OXA and NE. Both plasma OXA and NE were inversely correlated with the plasma propofol concentration. Our laboratory study was supported by their clinical study. It is possible that OXA release during emergence from propofol anesthesia induces NE release and promotes wakefulness. Hence, OXA may become an antagonist of anesthetics. The agents that promote emergence could be useful clinically for promoting recovery from anesthesia. The emphasis in recovery from anesthesia in the current medical climate is on home readiness, to reduce medical expenses [38]. Patients recovering from general anesthesia can significantly benefit from orexin.

In conclusion, the present study demonstrated that the orexin system promotes wakefulness under propofol anesthesia mediated, at least in part, by cerebrocortical noradrenergic and dopaminergic neurotransmission.

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