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Serotonergic neurons in the dorsal raphe nucleus mediate the arousal-promoting effect of orexin during isoflurane anesthesia in male rats

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ABSTRACT

Previous studies have demonstrated that the activation of orexinergic neurons facilitates the recovery of animals from general anesthesia. Moreover, serotonergic neurons that receive projections from orexin neurons have also been shown to participate in sleep-wakefulness regulation. In the present study, we aimed to explore whether orexinergic neurons facilitate emergence from isoflurane anesthesia in rats by activating serotonergic neurons. Orexin A (30 or 100 pmol), orexin B (30 or 100 pmol), and their respective antagonists SB-334867 and TCS-OX2–29 (5 or 20 μg) were microinjected into the dorsal raphe nucleus (DRN) of rats, and their effects on induction and emergence times were analyzed. Electroencephalogram (EEG) changes were also recorded and analyzed to illuminate the effect of orexin injection into the DRN on cortical excitability under isoflurane anesthesia. Activation of serotonergic neurons was detected via immunohistochemical analysis of c-Fos expression following orexin administration. Our results indicated that injection of neither orexins nor orexin antagonists into the rat DRN exerted an impact on induction time, whereas orexin-A injection (100 pmol) enhanced arousal when compared with the saline group. In contrast, administration of orexin receptor type 1 antagonist SB-334867 (20 μg) prolonged emergence time from isoflurane anesthesia. Microinjection of orexin-A induced an arousal pattern on EEG, and decreased the burst suppression ratio under isoflurane anesthesia. Isoflurane anesthesia inhibited the activity of serotonergic neurons, as shown by decrease in the number of c-Fos-immunoreactive serotonergic neurons when compared with the sham group. This inhibitory effect was partially reversed by administration of orexin-A. Taken together, our findings suggest that orexinergic signals facilitate emergence from isoflurane anesthesia, at least partially, by reversing the effects of isoflurane on serotonergic neurons of the DRN.

1. Introduction

Orexin-A (OX-A) and orexin-B (OX-B) are neuropeptides synthesized by neurons located mainly in the lateral and posterior hypothalamus, especially in the perifornical region (Sakurai et al., 2010; Sakurai et al., 1999). There are approximately 70,000 orexinergic neurons in the rodent brain that project throughout the central nervous system, with especially dense projections to monoaminergic nuclei, such as the noradrenergic locus coeruleus (LC), histaminergic tuberomammillary nucleus (TMN), and serotonergic dorsal raphe nucleus (DRN) (Peyron et al., 1998). Orexins bind to orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R). Orexins have been implicated in the regulation of multiple cerebral functions, including appetite, reward conditioning, sleep, and arousal. Accumulating evidence has indicated that orexinergic neurons are essential in promoting arousal, and that genetic and pharmacologic blockade of the orexinergic signaling pathways impairs arousal (Sakurai et al., 1998; Sakurai et al., 2010; Sakurai et al., 1999). Other studies have shown that administering orexins into the basal forebrain augments the release of cortical acetylcholine during isoflurane anesthesia and facilitates emergence from sevoflurane anesthesia (Dong et al., 2009; Dong et al., 2006). These studies also show that administration of orexin-A induces electroencephalographic (EEG) arousal during sevoflurane anesthesia (Dong et al., 2006). Kelz et al. reported that orexins play a vital role in arousal from general anesthesia (Kelz et al., 2008). Although these findings (Kelz et al., 2008; Wang et al., 2014; Zhang et al., 2016) demonstrate that the orexinergic pathway is important for emergence from general anesthesia, the precise downstream mechanism utilized by the orexinergic neural pathway

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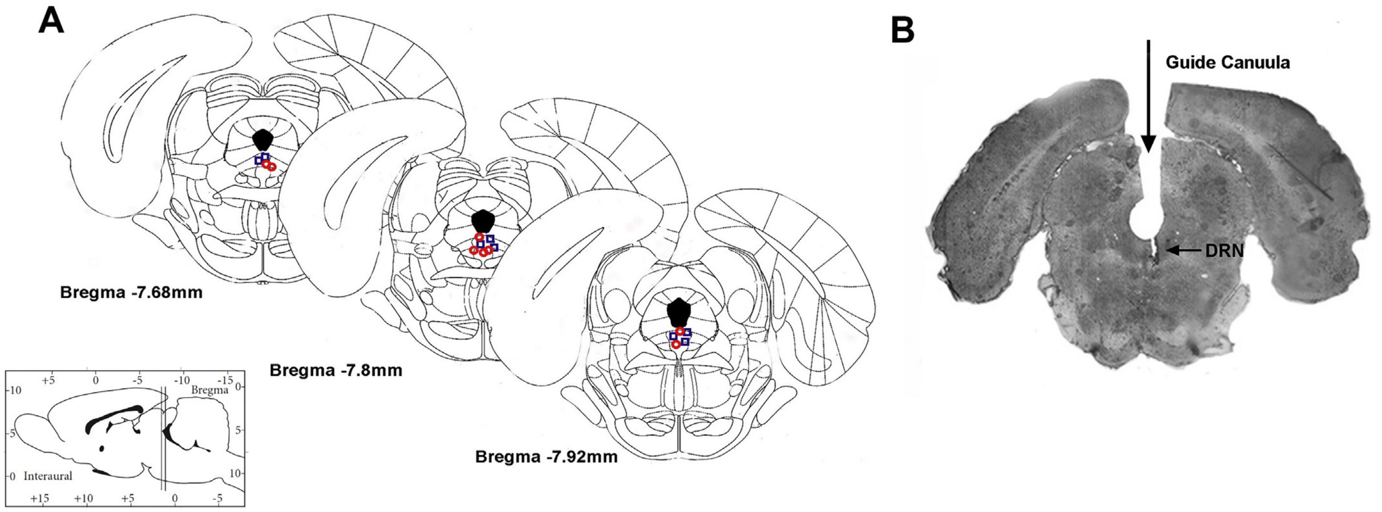

Fig. 1. Histologic verification of the location of microinjection in the dorsal raphe nucleus (DRN). A. Representative coronal sections show the location of the tip of the microinjection needles in the 100-pmol orexin-A group (n = 8, blue squares) and saline group (n = 8, red circles). Numbers below each section indicates the distance (mm) posterior to the bregma. The lower left sagittal graph shows the anterior to posterior range of the coronal sections. B. Representative histological photomicrograph showing the location of injection site in the DRN. The vertical black arrow represents the track of guide cannula, while the lower horizontal arrow indicates the microinjection site of DRN. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in regulating arousal from anesthesia remains unknown.

Serotonin (5-hydroxytryptamine, 5-HT) is the dominant monoamine neurotransmitter in the DRN (Berger et al., 2009), playing key roles in the regulation of mood, appetite, sleep/arousal, memory, and learning. Some evidence suggests that serotonin is involved in general anesthesia. Michael et al. demonstrated that destroying serotonergic brainstem areas (Roizen et al., 1978) decreases the minimum alveolar concentration (MAC) of halothane. Using in vivo microdialysis and ethological techniques, Mukaida et al. (2007) revealed that increasing the concentration of isoflurane anesthesia (0.1–1.5 MAC) decreases the release of 5-HT in the rat frontal cortex to 21–44% of that during the waking state. Furthermore, dramatically higher isoflurane concentrations were required to achieve unconsciousness (reflected by loss of the righting reflex) in rats treated with the selective 5-HT reuptake inhibitor fluoxetine, indicating that serotonin is involved in the anesthesia-arousal cycle (Mukaida et al., 2007). In addition, previous authors have reported that orexinergic neurons project to DRN (Peyron et al., 1998). Therefore, we hypothesized that there is a relationship between orexinergic and serotonergic neurons during isoflurane anesthesia. We used neuropharmacological, EEG, and immunohistochemical techniques to determine whether orexin facilitates emergence from isoflurane anesthesia via the excitation of serotonergic neurons in the DRN.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (body weight: 250–300 g; age: 49–56 d) were acquired from the Animal Center of the Fourth Military Medical University (Xi'an, China). The rats were housed under temperature- and humidity-controlled conditions (temperature: 23 ± 1 °C; relative humidity: 50 ± 10%) and an artificial light-controlled schedule (lights on: 07:00–19:00). Animals have free access to food and water. The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation and was conducted in accordance with the Guidelines for Animal Experimentation of the Fourth Military Medical University (Xi'an, China). All efforts were made to minimize the number of animals used and their suffering.

2.2. Surgical procedure for implantation of the guide cannula and electrodes

Under pentobarbital anesthesia (50 mg/kg, intraperitoneal), rats were placed on a stereotaxic apparatus, and a guide cannula (O.D., 0.48; I.D., 0.34; RWD, Inc., Shenzhen, China) was stereotaxically directed to the DRN or lateral ventricles. The tip of the microinjection needle (O.D., 0.30; I.D., 0.14; RWD, Inc., Shenzhen, China) was 2 mm longer than that of the guide cannula. The cannula was fixed by dental cement. The animals were allowed to rest for 5–7 days after surgery. The coordinates of the guide cannula tip, according to the Paxinos and Watson rat brain atlas, were as follows: DRN: AP = -7.8 mm, L = 0.0 mm, DV = -6.5 mm (Fiske et al., 2008; Paxinos and Watson, 1986); lateral ventricles: AP = − 0.8 mm; L = 1.5 mm; DV = − 3.5 mm.

Five stainless steel screws were fixed into the skull to record the epidural EEG in accordance with previously described methods (Dong et al., 2006). All the screws were affixed to the skull with dental cement.

2.3. Examination of the injection site

To confirm the validity of the injection site following behavioral experiments, rats were transcardially perfused with physiological saline (100 mL) followed by 4% paraformaldehyde (500 mL; BDH) in 0.1 M phosphate buffer solution. The brains were removed, and brain sections containing the DRN were cut into 40-μm thick slices using a cryostat. The location of the guide cannula was examined under a microscope. On the sections, the DRN is located in the ventral part of periaqueductal gray, dorsal and medial to the medial longitudinal fasciculus. If the position of the guide cannula was incorrect, the rat was excluded from the study. Seventeen rats with incorrect positioning of the guide cannula were excluded from the study, and only rats whose injection sites could be verified via histological examination were included. The position of the guide cannula in the DRN of each rat in the 100-pmol orexin-A group (n = 8, blue squares) and saline group (n = 8, red circles) is shown in Fig. 1 as an example.

2.4. Estimation of induction and emergence time

In the present study, we used a heating pad to sustain body temperature at 37 °C during anesthesia. Induction and emergence from isoflurane anesthesia were defined on loss and return of the righting reflex, respectively. Rats were placed in a transparent cylindrical chamber and anesthetized via inhalation of isoflurane. The concentration of isoflurane was monitored using a gas monitor (Philips IntelliVue G5-M1019A, Philips Co., Germany). The cylindrical chamber was rotated 180° at 15-s intervals during induction or emergence. Rats that did not return to the sternal position were considered to have lost the righting reflex. The time from the start of isoflurane inhalation to loss of the righting reflex was recorded as the induction time. Return of the righting reflex was defined by the rat's ability to return to the sternal position after isoflurane inhalation had been terminated, and this duration was recorded as the emergence time.

2.5. EEG signal recording and analysis

After connecting the electrodes, the EEG signals were recorded using a Powerlab Acquisition System (ADInstruments Pty Ltd., NSW, Australia). Data were filtered between 0.1 and 50 Hz, and no line filter was used. We selected a sampling rate of 400 Hz. Spectral analysis was performed using MATLAB2011 (Mathworks, Natick, MA, USA). The spectrograms were computed using the multitaper method (window lengths of $T = 4$ s with 0 s overlap, time-bandwidth product TW = 3, number of tapers K = 5, filter pass 0.1–30 Hz). In this study, MATLAB was used to calculate the burst suppression ratio (BSR), which was defined as the percentage of the total sum of isoelectric EEG signals over 1 min. Suppression patterns were determined based on subthreshold activity for 0.5 s, while burst patterns were determined based on supra-threshold activity for 0.5 s. We implemented a binary signal for the burst suppression ratio (BSR) identification and calculation, with the value being 1 if EEG amplitude was outside the defined voltage, which was manually estimated for each rat individually, and 0 if it was within the threshold. By calculation of BSR within a time window of 60 s with a 59-s overlap, we created a table of pre- and post-treatment BSRs (Table 1).

2.6. Immunofluorescence staining

Rats were intracardially perfused with physiological saline (100 mL), followed by 4% paraformaldehyde (500 mL) in 0.1 M phosphate buffer solution. After post-fixation in 4% paraformaldehyde for 24 h, the brains were immersed in 20%, 30% sucrose sequentially. Rat DRN tissue was directly placed in optimal cutting temperature compound (Tissue-Tek®, Sakura Finetek USA Inc., Torrance, CA, USA). The DRN was cut into 40-μm slices using a cryostat, following which we performed double immunofluorescence for staining of neurons expressing serotonin and c-Fos. Slices were incubated with a rabbit polyclonal antibody against c-Fos (1:200; Cell Signaling, Technology, Inc., Danvers, MA, USA) and a goat monoclonal antibody against serotonin (1:1000; Abcam, Cambridge, UK) for 24 h at 4 °C. The slices were then incubated in secondary fluorescent antibodies, which were Alexa Flour 488-labeled donkey anti-rabbit antibody (1:200; Invitrogen, California, USA) for c-Fos and Alexa Flour 594-labeled donkey anti-goat antibody (1:200; Invitrogen, California, USA) for serotonin. Following incubation with fluorescent secondary antibodies, the slices were then washed three times with phosphate-buffered saline (PBS).

The fluorescence images were acquired under a fluorescence microscope. A researcher blinded to the experimental grouping counted the single-labeled serotonin neurons and double-labeled c-Fos/serotonin neurons. We observed the target DRN sites from 7.3–8.3 mm rostral to the bregma, according to the rat brain atlas by Paxinos and Watson, taking one sections in every three sections. Eight to nine sections per animal were used for counting the immunohistochemically stained cells. The number of positive cells per animal is expressed as the average number per section (6 animals in each group).

2.7. Experimental protocols

To observe the effects of intra-DRN administration of orexin-A on arousal time from isoflurane anesthesia, the rats were randomly divided into three groups (n = 8 per group): 30 pmol orexin-A (Tocris Bioscience, Bristol, UK), 100 pmol orexin-A and saline control. A similar experimental design was used to examine the influence of orexin-B on emergence time, with rats divided into the following three groups (n = 8 per group): 30 pmol orexin-B group (Tocris Bioscience, Bristol, UK), 100 pmol orexin-B group, and saline group. The saline group was considered as the control in each experiment.

To investigate the influence of the OX1R antagonist SB-334867 on emergence from isoflurane anesthesia, rats were randomly assigned to three groups (n = 8 per group): 5 μg SB-334867 (Tocris Bioscience, Bristol, UK), 20 μg SB-334867 and its vehicle solution (dimethylsulfoxide, DMSO). To assess the effect of the OX2R antagonist TCS-OX2–29 on the emergence, the rats were also randomly assigned into three groups (n = 8 per group): 5 μg of TCS-OX2–29 (Tocris Bioscience, Bristol, UK), 20 μg of TCS-OX2–29, and DMSO. The same vehicle solution (or 5% DMSO diluted in saline) was used in each experiment. All drugs (volume, 0.3 μL) were manually injected into the DRN over 5 min by the experimenters. Drugs were administered 15 min prior to the cessation of 1.4% (1 MAC) isoflurane anesthesia (oxygen: 1.5 L/min). Emergence times were recorded. Drug dosages were determined based on the findings of several previous studies. (Dong et al., 2009; Dong et al., 2006; Zhang et al., 2012; Zhang et al., 2016)

The same protocol was used to investigate the effect of intra-DRN administration of orexins and their receptor antagonists on isoflurane anesthesia induction time, except that the drugs were administered 15 min prior to the induction of anesthesia. Two percent isoflurane was administered in oxygen (1.5 L/min). Induction time was recorded as the interval between initiating the inhalation of isoflurane and the loss of the righting reflex.

To assess the impact of intra-DRN microinjection of orexins on the BSR during isoflurane anesthesia, rats were divided into three groups (n = 6 per group). Animals were anesthetized with 1.4% (1 MAC) isoflurane in oxygen (1.5 L/min). Orexin-A (100 pmol, 0.3 μL), orexin-B (100 pmol, 0.3 μL), or saline (0.3 μL) was injected into the DRN 30 min after the induction of anesthesia (n = 6 per group). Two-hour continuous EEG signal composed of1-h free-moving time (30 min before anesthesia, and 30 min after the anesthesia) and 1-h anesthesia time. Twenty minutes of EEG signal were analyzed (10 min before and 10 min after drug administration).

To investigate the effects of the intra-DRN microinjection of orexin-A and saline on the EEG band power during isoflurane anesthesia, rats were assigned into three groups (n = 6 per group). Orexin-A (30 or 100 pmol, 0.3 μL) or saline (0.3 μL) was injected into DRN 30-min point of 1% (0.75 MAC) isoflurane inhalation during a total anesthesia time of 1-h. The EEG global frequency band (0–30 Hz) was segmented to

Table 1
Electroencephalographic Changes Induced by Intra-DRN Administration of Orexins During Isoflurane Anesthesia (n = 6).

Drugs	BSR before administration (ratio ± SEM)	BSR after administration (ratio ± SEM)	Number of aroused rats
Saline	55.39 ± 2.66	61.81 ± 0.82	0
Orexin-A	57.89 ± 1.89	40.79 ± 5.11*	0
Orexin-B	53.61 ± 0.55	55.94 ± 0.91	0

delta (0.5–4 Hz), theta (4–8 Hz), beta (13–30 Hz), and alpha (8–13 Hz). Band power was analyzed based on 20-min EEG samples (from 10 min before until 10 min after drug administration).

To evaluate the influence of orexin-A on the activity of serotonergic neurons, c-Fos expression in DRN neurons was examined using double immunofluorescence staining. Rats were divided into three groups (n = 6 per group). In the orexin-A and saline groups, rats were anesthetized with 1.4% (1 MAC) isoflurane in oxygen (1.5 L/min). After 15 min of isoflurane anesthesia, we microinjected orexin-A (3 nmol, 2 μL) or saline (2 μL) into the lateral ventricle. Rats were sacrificed 60 min after injection. The rats in the control group (n = 6) inhaled 100% oxygen for 75 min prior to sacrifice via an intracardiac injection of 10% KCl. Larger dosages of orexin-A were injected into the cerebral ventricle rather than into the DRN to avoid structural damage during intra-nuclear injection, which may hamper observation and counting of immune stained cells. The numbers of serotonin-immunore active (serotonin-IR) neurons, and double-labeled c-Fos/serotonin-IR neurons were counted and analyzed.

2.8. Statistical analysis

Data are expressed as means ± SEM. Changes in relative power in each band before and after drug administration were assessed with paired *t*-test. Other data were analyzed by one-way analyses of variance (ANOVA) with post hoc Bonferroni multiple-comparison tests when appropriate. All statistical analyses were performed with SPSS 17.0 software (SPSS, Chicago, IL, USA), and graphs were developed on GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA).In all cases, $P < .05$ was considered statistically significant.

3. Results

3.1. Microinjection of orexin-a into the DRN facilitated emergence from isoflurane anesthesia but did not influence induction

When compared with saline group (13.91 ± 0.90 min), intra-DRN injection of 100 pmol orexin-A (10.05 ± 0.36 min) and 30 pmol orexin-A (11.6 ± 0.36 min) significantly reduced emergence time ($P < .05$, 100 pmol orexin-A vs. saline; $P < .05$, 30 pmol orexin-A vs saline, $F(2,21) = 10.48$, Fig. 2A). Meanwhile, microinjection of 30 pmol (11.94 ± 0.57 min) and 100 pmol orexin-B (11.29 ± 0.40 min) into the DRN exerted no significant impact on arousal when compared with saline injection (12.83 ± 0.64 min; $P > .05$, 30 pmol orexin-B vs. saline; $P > .05$, 100 pmol orexin-B vs. saline, $F(2,21) = 1.981$, Fig. 2B). Neither orexin-A ($P > .05$, vs. saline) nor orexin-B ($P > .05$ vs. saline) had any impact on induction time (Fig. 2C and D).

3.2. Orexin receptor type 1 antagonist postponed arousal from isoflurane anesthesia

Intra-DRN injection of 20 μg of the OX1R antagonist SB-334867 (14.78 ± 0.74 min) significantly prolonged emergence time, compared with the control (11.9 ± 0.68 min; $P < .05$, 20 μg SB-334867 vs. control, $F(2,21) = 4.86$, Fig. 3A), while injection of 5 μg SB-334867 had no impact on emergence time (12.48 ± 0.63 min; $P > .05$, 5 μg SB-334867 vs. control). Administration of either 20 μg (13.39 ± 0.98 min) or 5 μg (13.43 ± 0.81 min) of the OX2R antagonist TCS-OX2–29 had no impact on arousal ($P > .05$, 20 μg TCS-OX2–29 vs. control; $P > .05$, 5 μg TCS-OX2–29 vs. control, $F(2,21) = 0.20$, Fig. 3B). Neither receptor antagonists affected induction time of anesthesia ($P > .05$, SB-334867 vs. control; $P > .05$ TCS-OX2–29 vs. control, Fig. 3C and D).

3.3. Administration of orexin-a into the DRN decreased the BSR and induced an arousal pattern on EEG

The arousal pattern of EEG consists of low amplitude and high frequency waves (Fig. 4A). Burst and suppression waves usually appear alternately under 1.4% (1 MAC) of isoflurane anesthesia (Fig. 4B). Microinjection of orexin-A into the DRN increased the EEG frequency and attenuated the burst-suppression pattern (Fig. 4C).

Fig. 5 shows the spectrograms computed from the EEG recordings during 1.4% isoflurane anesthesia. No significant differences in BSR were observed among the three groups prior to drug administration. Orexin-A significantly decreased the BSR compared with that before the drug administration ($P < .05$, before orexin-A vs. after orexin-A administration, Fig. 5B). Microinjection of orexin-B ($P > .05$, before orexin-B vs. after orexin-B administration, Fig. 5C) and saline ($P > .05$, before saline vs. after saline administration, Fig. 5A) had no effect on the BSR (Table 1).

Microinjection of saline or orexin-B had no impact on BSR. Microinjection of 100 pmol orexin-A decreased the BSR. $^*P < 0.05$, before vs. after orexin-A administration. BSR, burst suppression ratio; ChinaSEM, standard error of the mean.

Fig. 6 shows representative individual spectrograms computed from EEG recordings during 1% (0.75 MAC) isoflurane anesthesia. A spectrogram computed from a rat inhaling 1% isoflurane indicates the predominance of delta band power (0.5–4 Hz). Microinjection of 100pmolorexin-A significantly decreased the delta band power ($P < .05$, before orexin-A vs. after orexin-A administration, Fig. 6C). However, administration of 30 pmol orexin-A ($P > .05$, before microinjection of 30 pmol orexin-A vs. after microinjection of orexin-A, Fig. 6B) and saline ($P > .05$, before vs. after saline, Fig. 6A) induced no significant changes in the power spectrum.

3.4. Administration of orexin-a into the cerebral ventricle increased the activity of serotonergic neurons

Expression of the immediate early gene c-Fos is a valid index of neuronal activity(Bullitt, 1990). The number of serotonergic neurons in the DRN did not change during the isoflurane anesthesia-arousal cycle (Fig. 7C). Isoflurane anesthesia, however, dramatically reduced the quantity of c-Fos positive serotonergic neurons. Furthermore, the percentage of c-Fos-positive serotonergic neurons was higher in the orexin-A group than in the saline group (12.54 ± 1.45% vs. 7.20 ± 0.78%; $P < .05$, orexin-A vs. saline)(Fig. 7A and B) under isoflurane anesthesia. No such difference in the percentage of c-Fos-positive serotonergic neurons was detected between the awaken group (13.24 ± 1.64%) and the orexin-A group (12.54 ± 1.45%; $P > .05$,orexin-A vs. awake, Fig. 7B).

4. Discussion

In the current study, we demonstrated that administration of orexin-A into the DRN facilitates emergence of the rat from isoflurane anesthesia, whereas administration of an OX1R antagonist delays emergence. Our findings also indicate that orexin-A administration reduces the BSR and the delta band power of EEG under isoflurane anesthesia. Moreover, our results suggest that isoflurane inhalation inhibits serotonergic neuronal activity , as demonstrated by decreases of c-Fos expression, and such inhibition can be partly restored with administration of orexin-A. These findings suggest that orexinergic signaling facilitates emergence from isoflurane anesthesia by exciting serotonergic neurons in the DRN.

Previous studies have demonstrated that activation of orexinergic neurons facilitates the recovery of animals from both inhalation anesthesia (e.g., sevoflurane) (Dong et al., 2009) and intravenous anesthesia (e.g., propofol) (Zhang et al., 2012). Furthermore, intra-basalis microinjection of orexin-An under isoflurane anesthesia elicits an

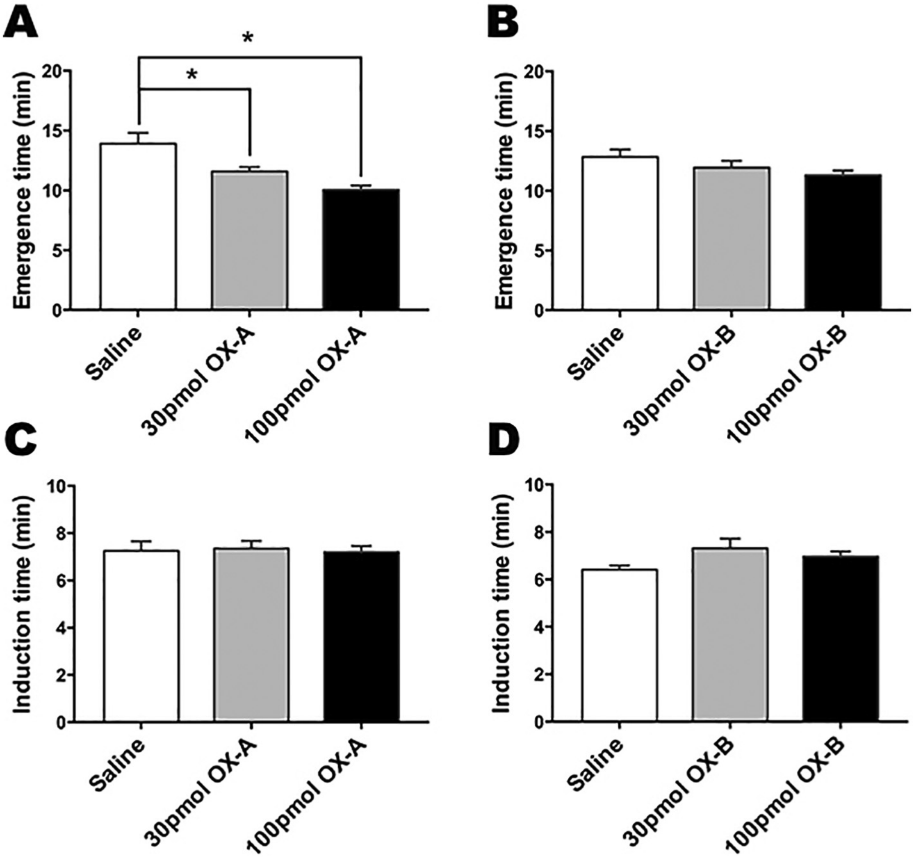

Fig. 2. The effect of different doses of orexins on induction and emergence time during isoflurane anesthesia (n = 8 per group). Microinjection of different doses of orexin-A (A) accelerated arousal (*P < .05 vs. saline group), whereas orexin-B did not influence emergence time (B). (C)(D) Neither orexin-A nor orexin-B had any impact on the induction time. OX-A, orexin-A; OX-B, orexin-B.

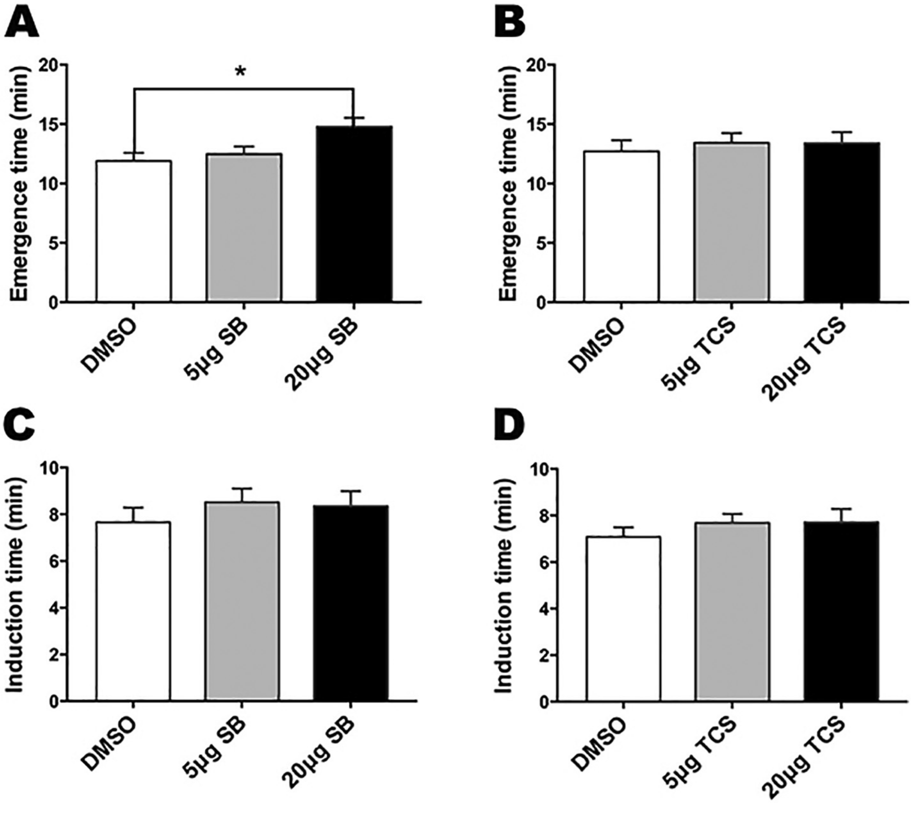

Fig. 3. Induction and emergence time following intra-DRN microinjection of different doses of orexins antagonists (n = 8 per group). (A) The orexin receptor type 1 antagonist SB334867 (20 μg) markedly delayed emergence (*P < 0.05vs. the DMSO group). (B) Neither doses of orexins receptor type 2 antagonist TCS-OX2–29 had no effect on arousal. (C, D) Neither SB-334867 nor TCS-OX2–29 influenced induction time. SB, SB-334867; DMSO, dimethylsulfoxide; TCS, TCS-OX2–29.

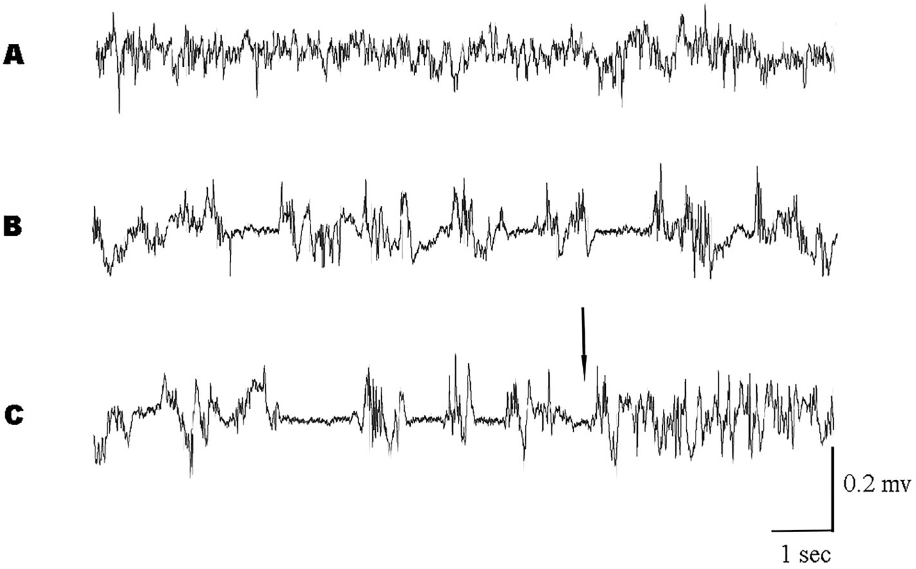

Fig. 4. Representative traces of electroencephalogram (EEG) recordings in different conditions. (A) EEG recording obtained during the waking state.(B) EEG recording obtained during isoflurane anesthesia. Consistent, alternating burst-suppression patterns were noted.(C) EEG recording during isoflurane anesthesia with a microinjection of orexin-A(100 pmol, 0.3 μL). The arrow indicates the time of microinjection of orexin-A.

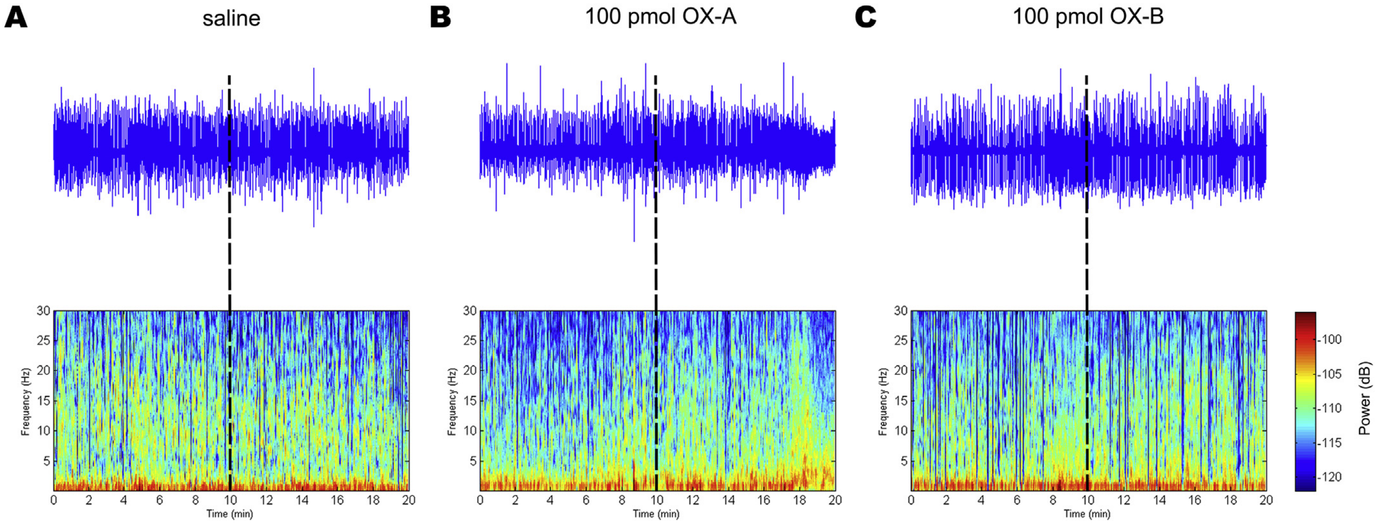

Fig. 5. Representative EEG recordings and spectrograms during 1.4% isoflurane anesthesia. The black line represents the administration of different drugs. The upper graph represents the EEG signal tracing; the lower graph represents the corresponding spectrum. Burst suppression in the spectrogram is shown as periods of blue (frequency components with lower power) interspersed with periods of red-yellow (frequency components with higher power). (A) A representative spectrogram computed from a rat during inhaling of 1.4% isoflurane revealed that burst suppression remain unchanged before and after microinjection of saline. However, administration of 100 pmol orexin-A promptly decreased burst suppression (B). No significant differences in BSR were observed before and after administration of 100 pmol orexin-B during isoflurane anesthesia (C). BSR, burst-suppression ratio. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

arousal pattern in EEG (Dong et al., 2006). Based on these findings, we deduce that orexinergic neurons can trigger emergence from anesthesia.

Serotonergic neurons are the most abundant neurons in the DRN (Descarries et al., 1982; Jacobs and Azmitia, 1992; Steinbusch et al., 1980), playing crucial roles in sleep-wakefulness regulation (Jacobs and Azmitia, 1992). Some studies have demonstrated that serotonergic neurons are involved in general anesthesia (Mukaida et al., 2007; Roizen et al., 1978). Marcrus J.N et al. (Marcus et al., 2001) demonstrated that DRN contains both OX1R and OX2R mRNA, although that for OX1R was more prevalent medially, while that for OX2R was more prevalent laterally in location. Although recent studies have suggested that orexin can modulate the activity of serotonergic neurons (Brown et al., 2001; Tao et al., 2006) and Liu et al. (2002) using intracellular and whole-cell recordings in rat brain slices revealed that administration of orexin regulates the activity of 5-HT neurons, the precise relationships between orexin and serotonin during general anesthesia remain poorly understood.

Our findings suggest that intra-DRN administration of orexin-A enhances emergence from isoflurane anesthesia, without impact on anesthesia induction with isoflurane. Similarly, Kelz et al. reported that orexinergic signaling affects emergence from anesthesia induced by inhalation of volatile substances without affecting induction (Kelz et al., 2008). We speculate that the mechanisms of induction and emergence are different, and may involve disparate neuronal circuits. Our findings indicate that orexin-A plays a vital role in the arousal, and that the DRN may be involved in this pathway. Furthermore, our results show that an OX1R antagonist, rather than an OX2R antagonist, can delay emergence, indicating that the regulatory effect of orexins in the DRN on anesthesia-arousal is mainly mediated by OX1R.

The notion of using EEG readings to detect the depth of anesthesia was established in 1930 (Gibbs et al., 1937). Burst suppression has been regarded as a state of cortical hypersensitivity (Kroeger and Amzica, 2007) and deep anesthesia (Amzica, 2009; Chemali et al., 2011; Ching

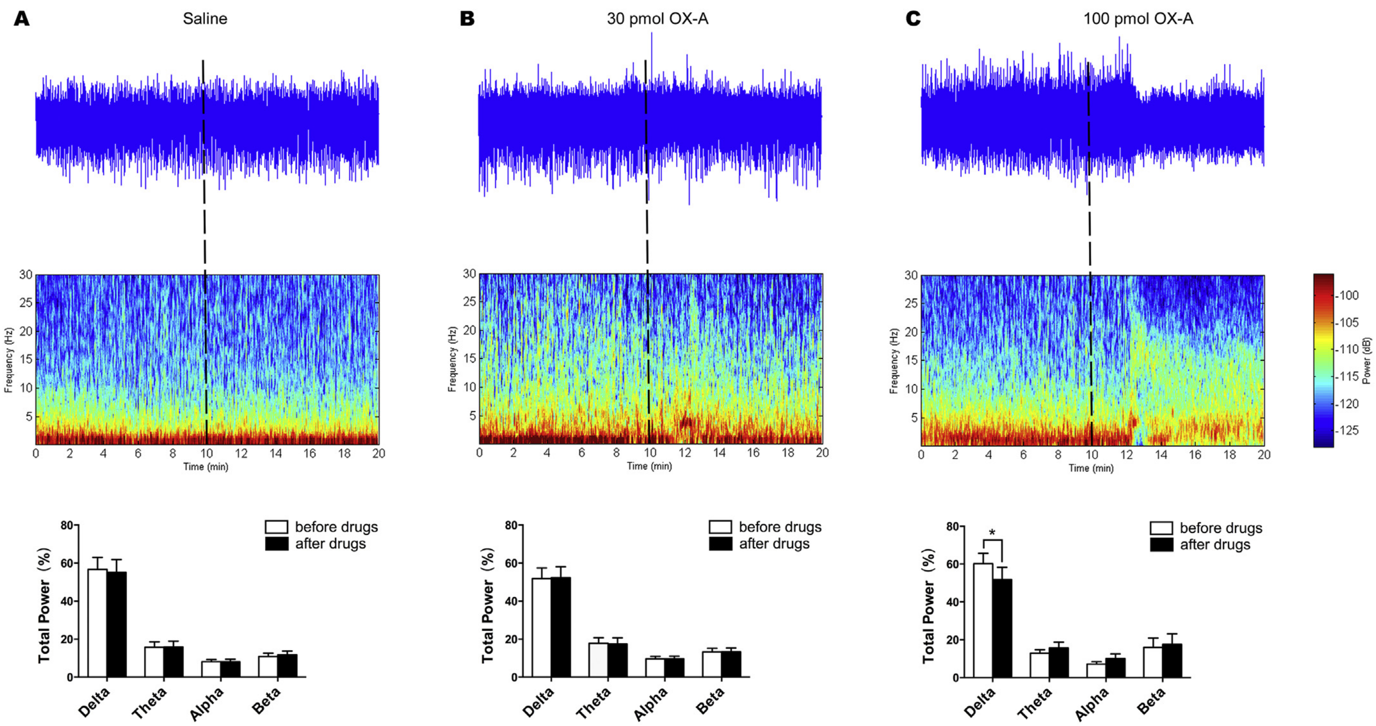

Fig. 6. Representative EEG spectrograms during 1.0% isoflurane anesthesia. The black line represents drug administration into the DRN. The uppermost graph represents the EEG, the middle graph represents the corresponding spectrum, and the lower graph shows the ratio of different waves. (A) A representative spectrogram recorded from a rat inhaling 1% isoflurane shows the predominance of delta band power (0.5–4 Hz) before and after the administration of physiological saline. Ratios of delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–13 Hz), and beta (13–30 Hz) powers remained unchanged. However, (B) administration of 100 pmol orexin-A promptly decreased the delta band power. (C) A representative spectrogram computed from a rat that received 30 pmol orexin-A. Note that the spectrogram is similar to those obtained for saline-treated rats: Delta band power was dominant during inhalation of 1% isoflurane. *P < .05, before orexin-A vs. after orexin-A (n = 6 per group).

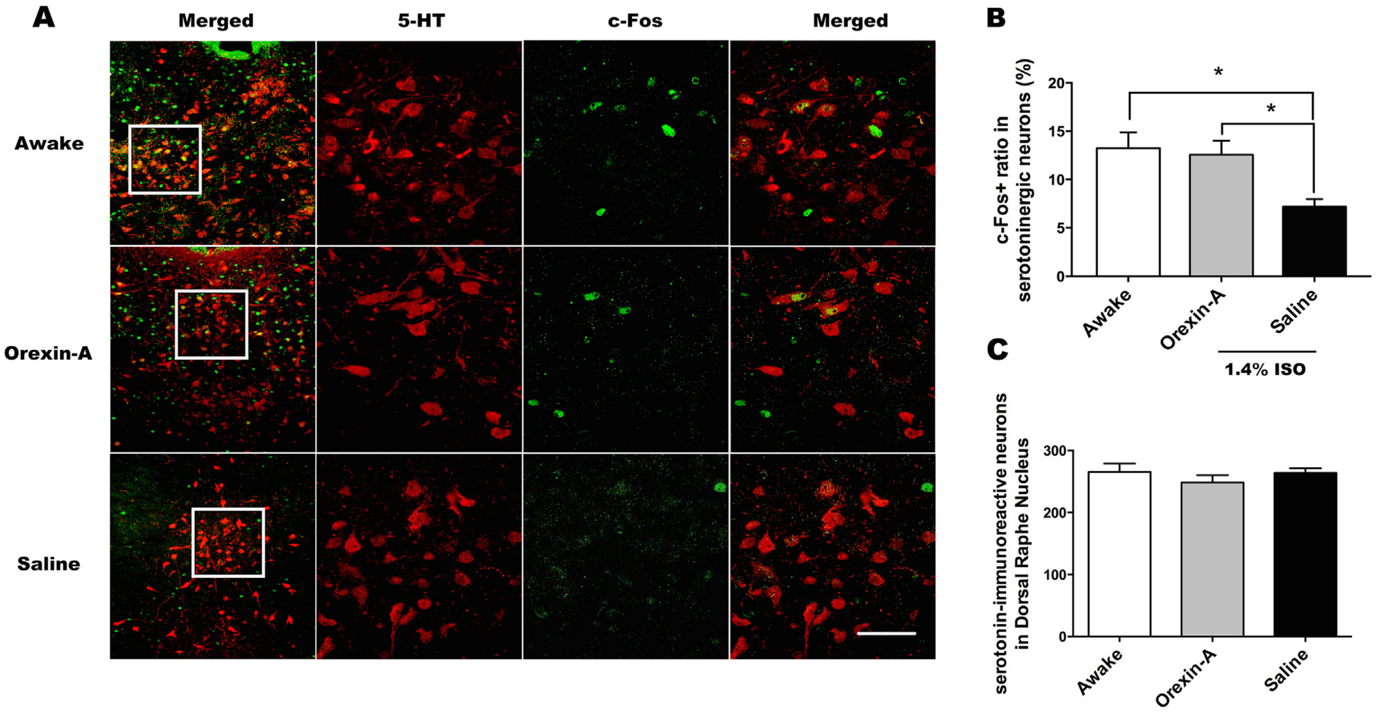

Fig. 7. Representative photomicrographs of serotonin (red) and c-Fos (green) immunofluorescence staining in the DRN. (A) Pictures in the far-left column are of low magnifications (20×). Pictures in the other 3 columns are higher magnified versions (60×) of the framed areas. The images show serotonin staining, c-Fos staining, and the merged image, respectively. (B) Compared to saline, microinjection of orexin-A increased the number of double-labeled neurons. Number of double-labeled neurons were similar in awake and orexin-A treated animals. (C) The isoflurane anesthesia-arousal cycle did not change the number of serotoninergic neurons in the DRN.* P < .05 vs. saline group. Scar bar = 50 μm (n = 6 per group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2012). Our findings indicate that administration of orexin-A into the DRN under isoflurane anesthesia can dramatically reduce the BSR. The delta band power is another indicator of the depth of anesthesia (Davis et al., 2011);(Hashemi et al., 2015; Kenny et al., 2014). Alkire et al. (2000) proposed that low concentrations of anesthetics can decrease excitation, causing the firing of some neurons to shift from a tonic pattern to a burst pattern, thereby producing delta activity. In our study, the delta band power was reduced by intra-DRN administration of 100 pmol orexin-A. Similar findings were reported by Yasuda et al. (2003), who demonstrated that intracerebroventricular injection of orexin-An under isoflurane anesthesia promotes EEG arousal. We speculate that this arousal effect may be due to direct stimulation of OX1R receptors in the DRN by orexin-A.

Since non-serotonergic neurons are also present in the DRN, we examined changes in the number of serotonin/c-Fos co-expressing neurons following isoflurane inhalation and injection of orexin-A into the DRN to clarify whether serotonergic neuronal activity is indeed affected. Our results indicated that the percentage of c-Fos-positive serotonergic neurons decreased during isoflurane inhalation; and that this decrease was significantly attenuated by administration of orexin-A into the DRN. Such findings suggest that the activity of serotonergic neurons is inhibited by isoflurane anesthesia, and that orexin-A can partially restore this serotonergic activity. Our findings also suggest that orexinergic signals exert an excitatory effect on serotonergic neurons, causing them to retain their tonic activity and contribute to anesthesia-arousal regulation. Liu et al. reported that orexins directly activate serotonergic neurons mainly through a tetrodotoxin-insensitive Na^+/K^+ nonselective cation current (Liu et al., 2002). Taken together, the accumulated evidence suggests that the arousal effects of orexin-A are partially mediated by serotonergic neurons in the DRN.

The main limitation of the current study is that administration of exogenous orexins into the DRN cannot entirely mimic the effects of endogenous orexins. Previous studies have shown that endogenous orexins may also influence other brain regions, such as the LC (Hagan et al., 1999) and TMN (Eriksson et al., 2001), which also play roles in arousal. Therefore, we cannot exclude the impact of the orexinergic pathway on other neural circuits. Further studies are required to more fully elucidate the direct interaction between serotonergic and orexinergic pathways.

In summary, the main findings of this study suggest that activation of orexinergic signals in the DRN facilitates emergence from isoflurane anesthesia. Furthermore, blockade of orexinergic signals in serotonergic neurons by an OX1R antagonist can delay emergence from isoflurane anesthesia. The present findings sustain the notion that the serotonergic system contributes to the arousal-promoting effect of orexin during isoflurane anesthesia.

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Disclosure statement

The authors have no conflicts of interest to declare.

Declarations of interest

None.

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