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·Original Article·

Orexin A attenuates the sleep-promoting effect of adenosine in the lateral hypothalamus of rats

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ABSTRACT

Orexin neurons within the lateral hypothalamus play a crucial role in the promotion and maintenance of arousal. Studies have strongly suggested that orexin neurons are an important target in endogenous adenosine-regulated sleep homeostasis. Orexin A induces a robust increase in the firing activity of orexin neurons, while adenosine has an inhibitory effect. Whether the excitatory action of orexins in the lateral hypothalamus actually promotes wakefulness and reverses the sleep-producing effect of adenosine *in vivo* is less clear. In this study, electroencephalographic and electromyographic recordings were used to investigate the effects of orexin A and adenosine on sleep and wakefulness in rats. We found that microinjection of orexin A into the lateral hypothalamus increased wakefulness with a concomitant reduction of sleep during the first 3 h of post-injection recording, and this was completely blocked by a selective antagonist for orexin receptor 1, SB 334867. The enhancement of wakefulness also occurred after application of the excitatory neurotransmitter glutamate in the first 3 h post-injection. However, in the presence of the NMDA receptor antagonist APV, orexin A did not induce any change of sleep and wakefulness in the first 3 h. Further, exogenous application of adenosine into the lateral hypothalamus induced a marked increase of sleep in the first 3-h post-injection. No significant change in sleep and wakefulness was detected after adenosine application followed by orexin A administration into the same brain area. These findings suggest that the sleep-promoting action of adenosine can be reversed by orexin A applied to the lateral hypothalamus, perhaps by exciting glutamatergic input to orexin neurons *via* the action of orexin receptor 1.

Keywords: sleep; wakefulness; orexin; adenosine; lateral hypothalamus

INTRODUCTION

The neuropeptides orexin A and orexin B, produced by hypothalamic neurons, play a crucial role in the promotion and maintenance of wakefulness[1-4]. This arousal-promoting effect of orexins may be realized by excitatory actions on multiple subcortical arousal systems and the cerebral cortex[5-8]. Two G protein-coupled receptors (orexin receptor 1 and 2) are involved in maintaining a long, consolidated awake period[5, 9, 10]. Loss of orexin neurons impairs wakefulness[11], whereas their stimulation facilitates wakefulness[3]. Thus, modulation of orexin neurons is critical in the regulation of sleep and wakefulness[12-14]. *In vitro* electrophysiological studies have demonstrated that orexin A directly excites local glutamatergic neurons in the lateral hypothalamus, inducing a substantial depolarization and a robust increase in the firing of orexin neurons[15, 16]. Whether the excitatory actions of orexins on orexin neurons in the hypothalamus actually promote and maintain wakefulness *in vivo* is still unknown.

In addition, abundant evidence has suggested that orexin neurons are a potential target in the regulation of sleep homeostasis by endogenous adenosine. *In vivo*, local perfusion of an adenosine receptor agonist into the

lateral hypothalamus elicits sleep[17, 18], while microinjection of adenosine receptor-specific antagonists in the same area increases wakefulness[17-19]. Our previous study with patch clamp recordings in acute slices showed that under sustained or strong excitatory transmission, endogenous adenosine is released into the lateral hypothalamus and reduces the excitatory glutamatergic input to orexin neurons[16]. We therefore speculated that adenosine, a product of cellular energy metabolism, accumulates in the lateral hypothalamus during wakefulness and eventually promotes sleep by exerting an inhibitory effect on orexin neurons. Whether orexin A reverses the sleep-promoting effect of adenosine in this brain area has been less investigated.

The aim of the present study was to explore the possible actions of exogenous orexin A applied to the lateral hypothalamus on the sleep-wakefulness cycle and the increase of sleep induced by adenosine using electroencephalographic (EEG) and electromyographic (EMG) recordings *in vivo*.

MATERIALS AND METHODS

Animals

A total of 51 adult male Sprague-Dawley rats weighing 250–300 g were housed in a temperature-controlled room (22°C) and maintained on a 12-h light/12-h dark cycle with food and water available *ad libitum*. The animal use and all experimental protocols were approved by the Third Military Medical University Animal Care Committee.

Reagents

Reagents unless specified otherwise were from Sigma (St. Louis, MO). Orexin A, glutamate, adenosine, and D-2-amino-5-phosphonovaleric acid (APV) were dissolved in artificial cerebral spinal fluid (ACSF) containing (in mmol/L): 126 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 glucose, pH 7.3–7.4. SB 334867 (Tocris Bioscience, Bristol, UK), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 3,7-dimethyl-1-propargylxanthine (DMPX) were dissolved in ACSF containing 10% dimethylsulfoxide (DMSO).

Surgery

Under deep pentobarbital anesthesia (50 mg/kg, i.p.), rats were implanted with cortical EEG and EMG recording electrodes as described previously[20, 21]. Briefly, two stainless-steel electrodes for EEG recording were screwed through the skull onto the dura over the frontal cortex (anterior: −3 mm; lateral: 3.5 mm) and the parietal cortex (anterior: −4 mm; lateral: −3 mm)[22]. Two insulated stainless-steel, Teflon-coated wires for monitoring EMG signals were placed bilaterally into the trapezius muscles. In addition, at the stereotaxic coordinates anterior, −3.3 mm; lateral, 1.5 mm; dorsoventral, 8.5 mm, a guide cannula (O.D. 0.67 mm; RWD Life Technology Co., Ltd., Shenzhen, China) for unilateral microinjection was inserted vertically from the skull surface and blocked with stylets. The tip of this guide cannula was 2 mm above the lateral hypothalamus. Each rat was allowed at least 7 days for recovery from surgery, and was habituated individually to the experimental conditions for 3–4 days, where it was connected to the EEG/EMG recording system by a flexible cable.

Microinjection Procedure

All microinjections were unilateral and performed between 09:30 and 10:00 as described previously[19]. Then the rats were reconnected and recorded from 10:00. Behavioral states were recorded continuously for 4 days, except during microinjections. At the time of infusion, the rats were gently restrained in a towel and an injection cannula was inserted into the guide cannula for microinjection of a volume of 0.2 μL, at a rate of 0.1 μL/min. A constant injection rate was maintained using a syringe pump (CMA 402, CMA/Microdialysis, Solna, Sweden). The injection cannula was kept in place for 1–2 min to allow diffusion from the cannula tip.

Procedure 1: Effect of orexin A administration on spontaneous sleep and wakefulness Seven rats were used in this experiment. The experimental protocol is outlined in Table 1. On day 1, EEG and EMG signals without any injection were recorded as baseline. On the second day, either ACSF or orexin A (40 pmol) was microinjected into the lateral hypothalamus[19]. After recovery on day 3, these animals were given either ACSF or 40 pmol orexin A (whichever had not been injected on the second day) injected into the lateral hypothalamus on day 4. One week after completion of this session, another session using 10 pmol orexin A was initiated. Rats were

Table 1. The protocol for the microinjection procedures

Procedure	Batch	Day 1	Day 2	Day 3	Day 4
Procedure 1	Batch 1 (n = 4)	—	ACSF	—	Orexin A (40 pmol)
	Batch 2 (n = 3)	—	Orexin A (40)	—	ACSF
	Batch 3 (n = 4)	—	ACSF	—	Orexin A (10 pmol)
	Batch 4 (n = 3)	—	Orexin A (10)	—	ACSF
Procedure 2	Batch 1 (n = 3)	—	DMSO+ACSF	—	SB334867+ACSF
	Batch 2 (n = 3)	—	SB334867+ACSF	—	DMSO+ACSF
	Batch 3 (n = 3)	—	DMSO+orexin A (10)	—	SB334867+orexin A (10)
	Batch 4 (n = 3)	—	SB334867+orexin A (10)	—	DMSO+orexin A (10)
Procedure 3	Batch 1 (n = 3)	ACSF	Glutamate (40 ng)	Glutamate (80 ng)	ACSF
	Batch 2 (n = 3)	Glutamate (80)	ACSF	Glutamate (40)	ACSF
Procedure 4	Batch 1 (n = 3)	—	ACSF+ACSF	—	APV (10 nmol)+ACSF
	Batch 2 (n = 3)	—	APV (10)+ACSF	—	ACSF+ACSF
	Batch 3 (n = 3)	—	ACSF+orexin A (10)	—	APV (10)+orexin A (10)
	Batch 4 (n = 3)	—	APV (10)+orexin A (10)	—	ACSF+orexin A (10)
Procedure 5	Batch 1 (n = 3)	ACSF	Adenosine (1 nmol)	Adenosine (10 nmol)	ACSF
	Batch 2 (n = 4)	Adenosine (10)	ACSF	Adenosine (1)	ACSF
	Batch 3 (n = 7)	ACSF	Adenosine (20 nmol)	ACSF	Adenosine (20)
Procedure 6	Batch 1 (n = 3)	—	Adenosine (10)+ACSF	—	Adenosine (10)+orexin A (10)
	Batch 2 (n = 4)	—	Adenosine (10)+orexin A (10)	—	Adenosine (10)+ACSF

—, no injection.

randomly assigned to receive ACSF or orexin A.

Procedure 2: Effect of orexin receptor 1 antagonist application on spontaneous sleep and wakefulness A total of twelve rats were used. On day 2, the rats (n = 6) randomly received unilateral microinjection of ACSF containing 10% dimethylsulfoxide (DMSO) or SB 334867 (0.5 μg) followed by ACSF application at the same site 5–10 min later (Table 1). After one-day recovery, the animals were microinjected with ACSF following DMSO or SB 334857 (0.5 μg) (whichever was not applied on the second day) on day 4. Another group of animals (n = 6) received DMSO or SB 334867 (0.5 μg) (whichever was not applied on the second day) followed by orexin A (10 pmol) application on day 2 or 4 randomly.

Procedure 3: Effect of glutamate administration on spontaneous sleep and wakefulness Two delivery models and six rats were used in this experiment. Three rats received ACSF on days 1 and 4, and were microinjected with 40 ng glutamate on day 2 and 80 ng glutamate on day 3. The other three rats were given 80 ng and 40 ng glutamate on days 1 and 3, respectively.

Procedure 4: Effect of the glutamatergic receptor antagonist APV on orexin A-induced alteration of sleep and wakefulness Two groups of rats were used in this experiment (n = 12). On day 2, six rats were randomly microinjected with either ACSF or D-2-amino-5-phosphonovaleric acid (APV) (10 nmol) followed by injection of ACSF 5–10 min later. On day 4, these rats were microinjected with ACSF following APV (10 nmol) or ACSF (whichever was not applied on the second day). The other six rats received 10 pmol orexin A following ACSF or APV (10 nmol) microinjection on days 2 and 4 (Table 1).

Procedure 5: Effect of adenosine on sleep and wakefulness A total of seven rats were used in this experiment. Three rats were randomly selected to be microinjected with 1 nmol and 10 nmol adenosine on days 2 and 3, and the other four were given 10 nmol adenosine on day 1 and 1 nmol adenosine on day 3. One week after completion of the experiments, all seven animals received microinjection of 20 nmol adenosine into the lateral hypothalamus on days 2 and 4, and ACSF administration on days 1 and 3 (Table 1).

Procedure 6: Effect of orexin A on adenosine-induced changes of sleep and wakefulness Seven rats were used in this experiment (Table 1). After basal recording without any treatment on day 1, the rats received unilateral microinjection of adenosine (10 nmol) followed by ACSF or orexin A (10 pmol) 5–10 min later on day 2 or 4.

Acquisition and Analysis of Sleep-wakefulness Data

Amplified and filtered (EEG, 0.5-30 Hz; EMG, 20-200 Hz) on a multichannel data acquisition and processing system (Grass Model 15LT polygraph, model 15A54 amplifiers), the cortical EEG and EMG signals were acquired and digitized at a sampling rate of 128 Hz for on-line viewing (data acquisition program SLEEPSIGN). Continuous data files were saved on a PC for off-line analysis. EEG and EMG recordings were scored by 10-s epochs for sleep-wakefulness state as either wakefulness, non-rapid eye movement (NREM), or rapid eye movement (REM) sleep by SLEEPSIGN software, according to the methods described previously[23-25].

Histological Localization of Microinjection Site

After completion of the experiments, all rats were killed under deep pentobarbital anesthesia and perfused with cold heparinized 0.9% saline followed by perfusion of 4% paraformaldehyde. The brain was isolated, blocked, and processed for orexin A immunohistochemistry[20], to illustrate the locations of orexin neurons relative to the track of the drug injector cannula.

Statistical Analysis

All data are presented as mean ± SEM. Data analyses were conducted with Statistical Product and Service Solutions (SPSS) version 13 software. The time of wakefulness, REM sleep, and NREM sleep after application of drugs was normalized to the baseline without any treatment. Statistical analysis was carried out using one-way repeated-measures analysis of variance with the Kolmogorov-Smirnov test for normal distribution followed by *post-hoc* multiple comparisons with Newman-Keuls tests where appropriate. P <0.05 was considered statistically significant.

RESULTS

Identification of the Site of Drug Delivery

Three continuous coronal schematics of rat brain through lateral hypothalamus including orexin neurons demonstrated that all microinjection sites were localized in the perifornical lateral hypothalamus and adjoining areas between AP −3.1 and −3.6 mm (Fig. 1A)[22], consistent with previous reports[17]. Based on the location of microinjection sites, the areas of perfused drugs were confirmed to include the orexin neurons in the lateral hypothalamus.

Arousal-promoting Effect of Orexin A in the Lateral Hypothalamus

Compared to the ACSF treatment, application of 40 pmol orexin A markedly increased the time of wakefulness during the first 3 h of recording post-injection (Newman-Keuls, P < 0.01), accompanied by reduced REM (Newman-Keuls test, P <0.05) and NREM sleep (Newman-Keuls, P <0.01) (Fig. 1B). Similar changes were found for 10 pmol orexin A in the time spent in wakefulness (Newman-Keuls test, P <0.01) and sleep (Newman-Keuls test, REM: P <0.05; NREM: P <0.01) during the first 3 h of post-injection recordings. However, no significant difference was detected in the time spent in wakefulness or sleep between 40 pmol and 10 pmol orexin A (Newman-Keuls test).

Orexin A Promotes Arousal through Activation of Orexin Receptor 1

A preliminary study has demonstrated that a selective antagonist for orexin receptor 1, SB 334867, reverses the arousal-promoting action of orexin A in anesthetized animals[26]. In this study, we further tested the effect of SB 334867 on spontaneous sleep and wakefulness in conscious rats. Orexin A (10 pmol) increased the time spent in wakefulness and reduced REM and NREM sleep correspondingly (Newman-Keuls test, P <0.01), while pretreatment with SB 334867 (0.5 μg) blocked

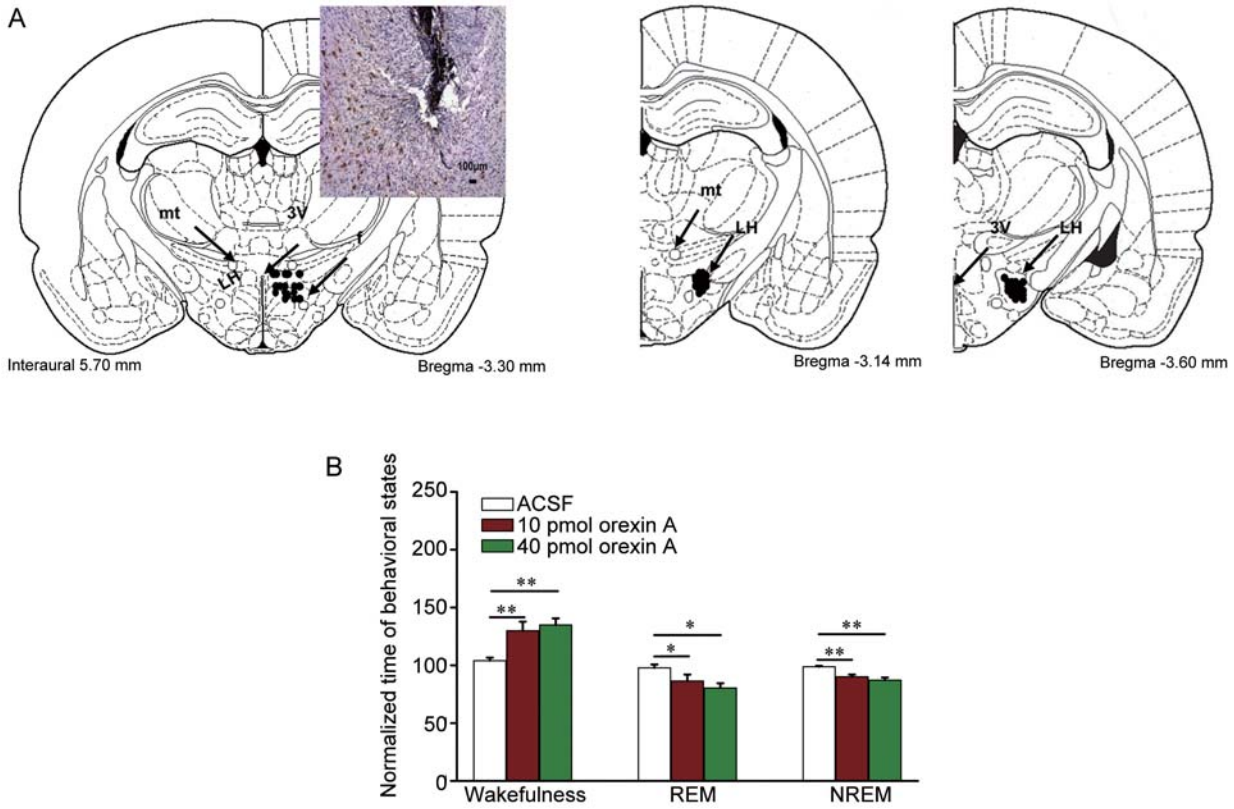

Fig. 1. Microinjection sites and the actions of orexin A on the sleep-wakefulness profile. A: Reconstruction of coronal sections through the perifornical and lateral hypothalamus showing the outlines and locations of the microinjection probes used for the unilateral delivery of drugs. Inset is a photomicrograph of a horizontal section showing the track of the microinjection probe localized in the orexin neuronal field (yellow staining). 3V, third ventricle; f: fornix; LH: lateral hypothalamus; mt, mammillothalamic tract. B: Normalized time spent in wakefulness, NREM, and REM sleep for the first 3 h after microinjection of ACSF, 40 pmol and 10 pmol orexin A. One-way ANOVA, wakefulness: $F_{(2,18)}$ = 8.50, P <0.01; REM sleep: $F_{(2,18)}$ = 4.16, P <0.05; NREM sleep: $F_{(2,18)}$ = 10.12, P <0.01. *P <0.05, **P <0.01, Newman-Keuls test.

these effects (Newman-Keuls test, P >0.05). In addition, compared to vehicle (DMSO) followed by ACSF injection, administration of SB 334867 (0.5 μg) with ACSF into the lateral hypothalamus had no effect on the three behavioral states during the first 3-h session (Newman-Keuls test, P >0.05) (Fig. 2).

Involvement of Glutamatergic Synaptic Transmission in Orexin A-induced Promotion of Wakefulness

In vitro electrophysiological experiments have demonstrated that the excitatory action of orexin A on orexin neurons in the lateral hypothalamus is achieved by increasing the excitatory glutamatergic synaptic input to these neurons[15, 16]. Thus, we further explored the role of the glutamatergic system in the orexin A-induced arousal-promoting effect *in vivo*. Compared to ACSF treatment, administration of 80 ng but not 40 ng glutamate elevated the time in wakefulness in the first 3 h post-injection (Newman-Keuls test, 80 ng: P <0.05; 40 ng: P >0.05) (Fig. 3A). Concomitantly, compared with the recordings of ACSF treatment in the same rats, application of 80 ng but not 40 ng glutamate decreased the time of NREM sleep (Newman-Keuls test, P <0.01). And a significant difference between 80 ng and 40 ng glutamate was detected in the time spent in wakefulness and NREM sleep (Newman-Keuls test, P <0.05) but not in REM sleep.

Compared to the two administrations (at a 5–10 min interval) of ACSF, administration of the NMDA receptor antagonist APV (10 nmol) followed by ACSF significantly

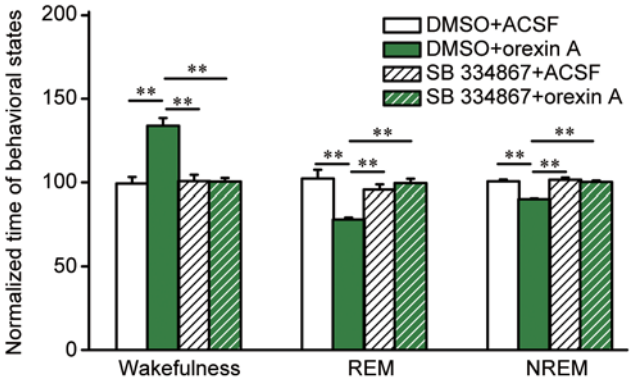

Fig. 2. Effect of orexin receptor 1 antagonist SB 334867 on sleep and wakefulness. Pooled data showing the normalized time in behavioral states during the first 3 h after application of 0.5 μg SB 334867 and 10 pmol orexin A. One-way ANOVA, wakefulness: $F_{(3,20)}$ = 19.88, P <0.01; REM sleep: $F_{(3,20)}$ = 10.17, P <0.01; NREM sleep: $F_{(3,20)}$ = 26.79, P <0.01. **P <0.01, Newman-Keuls test.

decreased the time in wakefulness in rats during the first 3 h post-injection (Newman-Keuls test, P <0.01), accompanied by increased time spent in REM and NREM sleep (Newman-Keuls test, P <0.01) (Fig. 3B). In contrast, compared to the two administrations of ACSF, application of ACSF + orexin A (10 pmol) induced an elevation of wakefulness accompanied by a significant reduction of NREM sleep but not of REM sleep (Newman-Keuls test, wakefulness and NREM sleep: P <0.01; REM sleep: P >0.05). And the differences in the three behavioral states were significant between APV (10 nmol) + ACSF administration and ACSF + orexin A treatment (Newman-Keuls test, P <0.01). In the presence of APV (10 nmol), application of orexin A (10 pmol) partially blocked the inhibitory action of APV on wakefulness by increasing the time spent in wakefulness and decreasing REM and NREM sleep (Newman-Keuls test, wakefulness and NREM sleep: P <0.01; REM sleep: P <0.05 *versus* APV followed by ACSF treatment), but there was no significant difference between ACSF + ACSF administration and APV + orexin A in the first 3 h post-injection (Newman-Keuls test, P >0.05). And significant differences in the time in wakefulness, REM and NREM sleep during the first 3 h-session were found between ACSF and APV followed by orexin A (Newman-Keuls test, P <0.01).

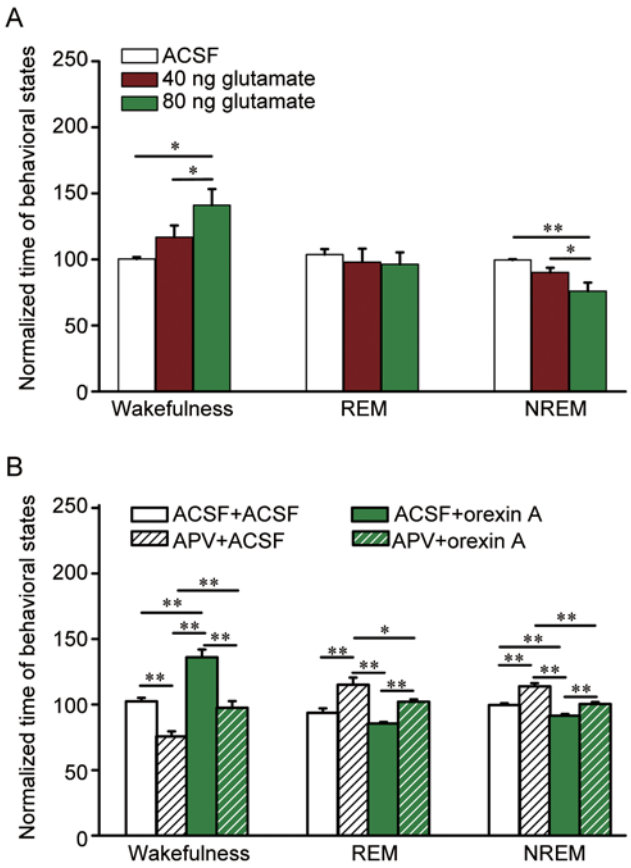

Fig. 3. Effects of glutamate and NMDA receptor antagonist APV on sleep and wakefulness. A: Normalized time spent in wakefulness, NREM, and REM sleep for the first 3 h after microinjection of ACSF, 40 ng or 80 ng glutamate. One-way ANOVA, wakefulness: $F_{(2,15)}$ = 5.55, P <0.05; NREM sleep: $F_{(2,15)}$ = 7.98, P <0.01; REM sleep: one-way ANOVA, $F_{(2,15)}$ = 3.39, P = 0.06. *P <0.05, **P <0.01, Newman-Keuls test. B: Pooled data showing the normalized time in behavioral states during the first 3 h after application of the NMDA receptor antagonist APV (10 nmol) and orexin A (10 pmol). One-way ANOVA, wakefulness: $F_{(3,20)}$ = 29.99, P <0.01; REM sleep: $F_{(3,20)}$ = 13.75, P <0.01; NREM sleep: $F_{(3,20)}$ = 31.10, P <0.01. *P <0.05, **P <0.01, Newman-Keuls test.

Effect of Orexin A on Adenosine-induced Changes of Sleep and Wakefulness

Recent behavioral experiments with adenosine antagonists *in vivo* have suggested that orexin neurons are another important target involved in the hypnotic action of adenosine[16, 17, 19, 27]. In this study, we further explored the role of exogenous adenosine in the regulation of sleep and wakefulness in the lateral hypothalamus. Analysis of the

EEG and EMG signals during the first 3 h post-injection of drugs revealed that, compared to the ACSF treatment, both 20 nmol and 10 nmol adenosine increased the time in NREM sleep but not in REM sleep with a concomitant reduction in the time in wakefulness (Newman-Keuls test, $P < 0.01$), whereas no evident changes in the three behavioral states were observed after application of 1 nmol adenosine (Newman-Keuls test, $P > 0.05$) (Fig. 4A).

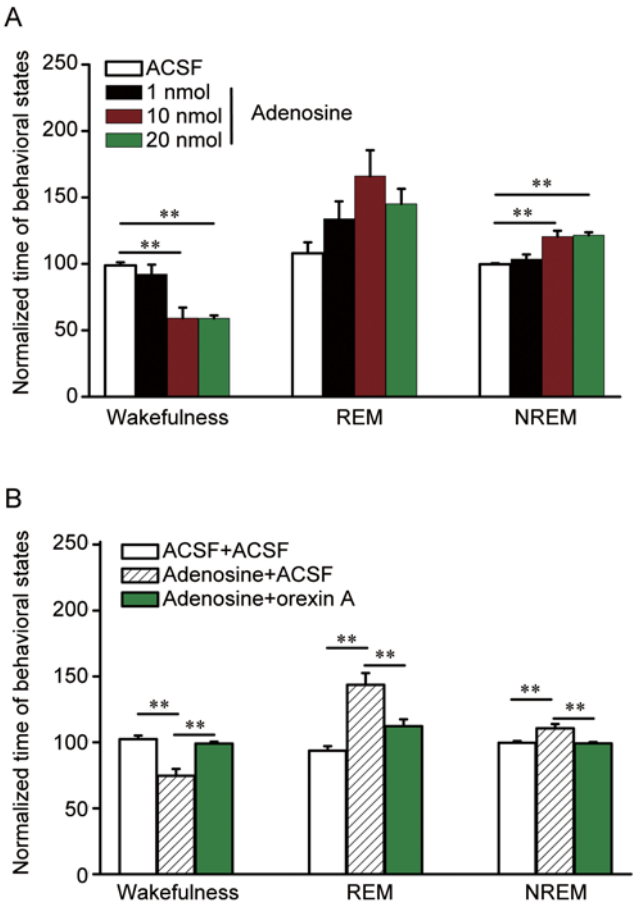

Fig. 4. Effect of orexin A on sleep-promoting action of adenosine injected into the lateral hypothalamus. A: Pooled data showing the normalized time in behavioral states during the first 3 h after application of adenosine and ACSF. One-way ANOVA, wakefulness: $F_{(3,24)} = 14.76$, $P < 0.01$; NREM sleep: $F_{(3,24)} = 13.71$, $P < 0.01$; REM sleep: $F_{(3,24)} = 1.47$, $P = 0.25$. **$P < 0.01$, Newman-Keuls test. B: Pooled data showing the comparison of normalized time in behavioral states during the first 3 h after application of 10 nmol adenosine and 10 pmol orexin A. One-way ANOVA: wakefulness, $F_{(2,17)} = 18.82$, $P < 0.01$; REM sleep, $F_{(2,17)} = 14.59$, $P < 0.01$; NREM sleep, $F_{(2,17)} = 9.17$, $P < 0.01$. **$P < 0.01$, Newman-Keuls test.

Whether the excitatory effect of orexin A on these orexin neurons could reverse the sleep propensity induced by adenosine was little known. During the first 3 h post-injection, compared to ACSF microinjection, adenosine (10 nmol) administration induced an increase in REM and NREM sleep with a concomitant reduction of wakefulness (Newman-Keuls test, $P < 0.01$). However, no significant changes in behavioral state were found after application of 10 nmol adenosine followed by 10 pmol orexin A during the first 3 h post-injection (Newman-Keuls test, $P > 0.05$) (Fig. 4B).

DISCUSSION

The main finding of this study was that exogenous application of orexin A into the lateral hypothalamus *in vivo* exerted an arousal-promoting effect through activation of orexin receptor 1. This excitatory action of orexin A may be realized by increasing glutamatergic transmission in the lateral hypothalamus. Furthermore, the excitatory effect of orexin A reversed the sleep-promoting effect of exogenous adenosine in the lateral hypothalamus.

The arousal-promoting action of the orexin system in the central nervous system (CNS) by activation of multiple subcortical arousal systems including the basal forebrain[28], tuberomammillary nucleus[23, 29] and locus coeruleus[30] has been well documented. In this study, we found that enhancement of wakefulness in rats was induced by microinjection of orexin A into the lateral hypothalamus where these orexin neurons are located. This suggests that orexins exert a strong excitatory effect in the lateral hypothalamus, and this is mediated by orexin receptor 1, in that pretreatment with a selective orexin receptor 1 antagonist completely blocked the arousal-promoting action of orexin A. *In vitro* electrophysiological studies have demonstrated that the exogenous application of orexin increases the firing rates of orexin neurons through the activation of glutamatergic input to these neurons[15, 16]. Consistently in this study, the arousal-promoting effect was not detected after blockade of glutamatergic transmission to these neurons using an NMDA receptor antagonist, strongly suggesting that the arousal-promoting action of orexin A is realized by directly exciting glutamatergic input to orexin neurons through the activation of orexin receptor 1. Thus, these findings demonstrate that the positive feedback

from excitatory glutamatergic input to orexin neurons may function in keeping orexin neurons firing continuously in wakefulness, which is essential for orexins to excite subcortical systems to promote and maintain wakefulness.

Consistent with the previous reports[31, 32], we also found that administration of glutamate into the lateral hypothalamus increased wakefulness in a dose-dependent manner. It should be noted that application of the NMDA receptor antagonist alone reduced wakefulness and increased sleep, whereas no changes were found after orexin receptor 1 antagonist administration alone. These findings suggest that *in vivo*, endogenous orexins are mainly released into other subcortical arousal systems including the basal forebrain, tuberomammillary nucleus, and locus coeruleus, where orexin could excite these neurons directly and eventually promote wakefulness; and the lateral hypothalamus, where orexin neurons are located is not the major target in the arousal-promoting action of these neuropeptides. We therefore speculated that the activation of orexin neurons needs other excitatory synaptic inputs in the CNS. Our data strongly support the idea that the excitatory effects of glutamatergic synaptic transmission on orexin neurons play a key role in exciting these neurons to promote wakefulness *in vivo*. In addition, the decreased time in wakefulness caused by the NMDA receptor antagonist nearly recovered after application of orexin A, indicating that besides NMDA receptors, AMPA receptors may also contribute to the excitatory action of glutamate on orexin neurons induced by orexin A. In fact, a previous experiment with glutamate receptor antagonists showed that microinjection of AMPA into the perifornical lateral hypothalamus of rats increases the number of orexin neurons immunoreactive for c-Fos[32].

In addition to the key role in promoting wakefulness, abundant evidence has demonstrated that orexin neurons are a potential target in the sleep-producing effect of endogenous adenosine, in which blockade of adenosine A1 receptors in the lateral hypothalamus induces a significant increase in wakefulness with a concomitant reduction in sleep[17, 19]. Consistently, we found that the exogenous application of adenosine into the lateral hypothalamus enhanced sleep. These data confirmed the involvement of orexin neurons in the regulation of sleep homeostasis by endogenous adenosine. Moreover, a study by Liu *et al.* revealed that the mechanism underlying this adenosine-induced suppression may be through inhibition of excitatory glutamatergic synaptic input to orexin neurons *via* adenosine A1 receptors[27].

Furthermore, we found no significant difference in sleep and wakefulness between control and orexin A following adenosine injection, which suggests that this sleep-promoting effect of exogenous adenosine can be blocked by application of orexin A into the same area. This suggests that the excitatory effect of orexin A on orexin neurons in the lateral hypothalamus reverses the sleep propensity induced by adenosine. Neuronal activity in wakefulness is associated with increased neuronal metabolism. As a product of cell metabolism and is ubiquity in the CNS, adenosine accumulates during wakefulness and progressively increases the propensity to sleep[33,34]. Indeed, our previous studies have provided evidence that under sustained or stronger synaptic transmission in orexin neurons, endogenous adenosine is generated and inhibits the excitability of orexin neurons *via* inhibiting glutamatergic transmission in acute hypothalamic slices[16]. Moreover, the previous study *in vitro* showed that orexin excites the orexin neurons *via* local glutamatergic transmission[15]. Thus, the indirect excitatory effect of orexin might offset the inhibitory action of adenosine on orexin neurons and consequently reverse the sleep-promoting effect of adenosine. The modulation of orexin neurons plays an important role in regulating sleep and wakefulness, especially under prolonged wakefulness in which sleep pressure is enhanced rapidly due to the large release of endogenous adenosine[35]. Caffeine, a nonselective adenosine receptor antagonist, is a widely used stimulant that effectively reverses the depressant effects of increasing sleep propensity[36], although the side-effect of drug-dependence has restricted its therapeutic use as an arousal-promoting agent[37]. It is therefore necessary to find other available drugs or measures to abolish the increase of sleep propensity. Our findings of the reversal of the sleep-promoting effect by orexin A provides a possibility that orexins may be a potential candidate to reduce the sleep propensity induced by adenosine, independent of the blockade of adenosine receptors.

In conclusion, we speculate that pharmacological manipulation of the orexin system including orexin neurons

and orexins may open up a new avenue for the regulation of sleep and wakefulness, especially under the condition of sleep propensity induced by adenosine.

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