

Original Article

# Adenosine A<sub>2A</sub> receptor deficiency attenuates the somnogenic effect of prostaglandin D<sub>2</sub> in mice

Bin-jia ZHANG<sup>1</sup>, Zhi-li HUANG<sup>1</sup>, Jiang-fan CHEN<sup>2</sup>, Yoshihiro URADE<sup>3,\*</sup>, Wei-min QU<sup>1,\*</sup>

<sup>1</sup>Department of Pharmacology and Shanghai Key Laboratory of Bioactive Small Molecules, School of Basic Medical Sciences, State Key Laboratory of Medical Neurobiology, Institutes of Brain Science and Collaborative Innovation Center for Brain Science, Fudan University, Shanghai 200032, China; <sup>2</sup>Department of Neurology, Boston University School of Medicine, Boston, MA 02118, USA; <sup>3</sup>International Institute for Integrative Sleep Medicine (WPI-IIS), University of Tsukuba, Tsukuba, Ibaraki 305–8575, Japan

## Abstract

Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) is one of the most potent endogenous sleep promoting substances. PGD<sub>2</sub> activates the PGD<sub>2</sub> receptor (DPR) and increases the extracellular level of adenosine in wild-type (WT) mice but not DPR knockout (KO) mice, suggesting that PGD<sub>2</sub>-induced sleep is DPR-dependent, and adenosine may be the signaling molecule that mediates the somnogenic effect of PGD<sub>2</sub>. The aim of this study was to determine the involvement of the adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) in PGD<sub>2</sub>-induced sleep. We infused PGD<sub>2</sub> into the lateral ventricle of WT and A<sub>2A</sub>R KO mice between 20:00 and 2:00 for 6 h, and electroencephalograms and electromyograms were simultaneously recorded. In WT mice, PGD<sub>2</sub> infusion dose-dependently increased non-rapid eye movement (non-REM, NREM) sleep, which was 139.1%, 145.0% and 202.7% as large as that of vehicle-treated mice at doses of 10, 20 and 50 pmol/min, respectively. PGD<sub>2</sub> infusion at doses of 20 and 50 pmol/min also increased REM sleep during the 6-h PGD<sub>2</sub> infusion and 4-h post-dosing periods in WT mice to 148.9% and 166.7%, respectively. In A<sub>2A</sub>R KO mice, however, PGD<sub>2</sub> infusion at 10 pmol/min did not change the sleep profile, whereas higher doses at 20 and 50 pmol/min increased the NREM sleep during the 6-h PGD<sub>2</sub> infusion to 117.5% and 155.6%, respectively, but did not change the sleep in the post-dosing period. Moreover, PGD<sub>2</sub> infusion at 50 pmol/min significantly increased the episode number in both genotypes but only enhanced the episode duration in WT mice. The results demonstrate that PGD<sub>2</sub>-induced sleep in mice is mediated by both adenosine A<sub>2A</sub>R-dependent and -independent systems.

**Keywords:** PGD<sub>2</sub>; adenosine A<sub>2A</sub> receptor; A<sub>2A</sub>R KO mice; NREM sleep; REM sleep; electroencephalogram

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## Introduction

Prostaglandin (PG) D<sub>2</sub> is the most abundant prostanoid produced in the central nervous system of mammals<sup>[1]</sup> and one of the most potent sleep-inducing substances<sup>[2–5]</sup>. Clinical observations show that excessive endogenous production of PGD<sub>2</sub> is responsible for sleep in humans under certain pathological conditions such as systemic mastocytosis<sup>[6]</sup> and African sleeping sickness<sup>[7]</sup>. Under physiological conditions, the PGD<sub>2</sub> concentration in the cerebrospinal fluid (CSF) of rats exhibits a circadian fluctuation coupled with the sleep-wake cycle in which it is increased in the light period when rodents mainly sleep<sup>[8]</sup>. The infusion of PGD<sub>2</sub> through a microdialysis probe showed that PGD<sub>2</sub> did not induce sleep in most parts of the

brain parenchyma but effectively promoted sleep when it was infused into the subarachnoid space underlying the rostral basal forebrain of rats<sup>[9,10]</sup> where the DP receptor (DPR) is predominantly localized<sup>[11]</sup>. PGD<sub>2</sub> promoted sleep and increased the extracellular level of adenosine in wild-type (WT) mice but not in DPR knockout (KO) mice<sup>[11]</sup>, indicating that PGD<sub>2</sub>-induced sleep is DPR-dependent, and adenosine may be a signaling molecule that mediates the somnogenic effect of PGD<sub>2</sub>.

Adenosine is a modulator of the sleepiness associated with prolonged wakefulness<sup>[12]</sup>. During prolonged wakefulness, extracellular adenosine accumulates selectively in the basal forebrain and cortex and promotes the transition from wakefulness to non-rapid eye movement (non-REM, NREM) sleep by inhibiting cholinergic and non-cholinergic wakefulness-promoting neurons of the basal forebrain through the adenosine A<sub>1</sub> receptor (A<sub>1</sub>R)<sup>[2,3,13]</sup>. Moreover, Satoh *et al* found that the adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) agonist could mimic the somnogenic activity of PGD<sub>2</sub>. Administration of CGS21680, a

\*To whom correspondence should be addressed.

E-mail quweimin@fudan.edu.cn (Wei-min QU);

urade.yoshihiro.ft@u.tsukuba.ac.jp (Yoshihiro URADE)

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selective  $A_{2A}R$  agonist, into the subarachnoid space induced NREM and REM sleep<sup>[14–16]</sup>, and PGD<sub>2</sub>-induced sleep was completely inhibited by pretreatment with KF17837, an  $A_{2A}R$ -selective antagonist in rats<sup>[14]</sup>. In contrast, an  $A_1R$  agonist did not produce any significant increases in the total amount of either type of sleep when administered into the lateral ventricle or subarachnoid space in mice and rats<sup>[15,17]</sup>, suggesting that PGD<sub>2</sub>-induced sleep may be mediated by adenosine through the  $A_{2A}R$  system.  $A_{2A}R$  KO mice generated by Chen *et al*<sup>[18]</sup> offer complete and specific deletion of the  $A_{2A}R$  system *in vivo* and thereby avoid the problems of non-specific and partial  $A_{2A}R$  antagonists. Therefore, mice lacking the  $A_{2A}R$  provided a unique model to elucidate the impact of  $A_{2A}R$  deficiency on the sleep-wake regulation of PGD<sub>2</sub> through the combination of microinfusion of PGD<sub>2</sub> into the lateral ventricle of mice and electroencephalogram (EEG) and electromyogram (EMG) recordings.

In the present study, we investigated the sleep-wake profiles after microinfusion of PGD<sub>2</sub> into the lateral ventricle of the brain in WT and  $A_{2A}R$  KO mice. The results showed that PGD<sub>2</sub> increased sleep in both WT and  $A_{2A}R$  KO mice in a dose-dependent manner, yet the somnogenic effect was observed in  $A_{2A}R$  KO mice when much higher doses of PGD<sub>2</sub> were given. The increase in PGD<sub>2</sub>-induced sleep in  $A_{2A}R$  KO mice was much less than that in WT mice, indicating that PGD<sub>2</sub>-induced sleep is mediated by both adenosine  $A_{2A}R$ -dependent and -independent systems.

## Materials and methods

### Animals and chemicals

Male WT and  $A_{2A}R$  KO mice of the inbred C57BL/6 strain<sup>[18]</sup> (weighing 23–27 g, 11–13 weeks old) were maintained at Oriental Bioservice Ltd (Kyoto, Japan) and used in these experiments. They were housed at a constant temperature (24±0.5°C) with a relative humidity of 60%±2% on an automatically controlled 12 h:12 h light/dark cycle (lights on at 8:00 AM) and had free access to food and water. The experimental protocols were approved by the Animal Care Committee of Osaka Bioscience Institute and were in complete compliance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. All attempts were made to minimize the number of animals used in the study and their suffering.

PGD<sub>2</sub> (Cayman Chemical Co, Ann Arbor, MI, USA) was dissolved in sterile artificial CSF (aCSF) containing (in mmol/L, pH 7.4) 140 NaCl, 3 KCl, 1.0 MgCl<sub>2</sub>, 1.3 CaCl<sub>2</sub>, 2 Na<sub>2</sub>HPO<sub>4</sub>, and 0.2 NaH<sub>2</sub>PO<sub>4</sub>, stored in aliquots at -20°C, and diluted to the final concentration immediately before use.

### Implantation of electrodes for EEG/EMG recordings and cannulae for PGD<sub>2</sub> microinfusion

Mice were anesthetized with sodium pentobarbital (50 mg/kg, ip) and then mounted on the stereotaxic instrument (SR-5R, Narishige, Tokyo, Japan). The incisor bar was adjusted to achieve equal heights of lambda and bregma. EEG and EMG electrodes for polysomnographic recordings were implanted

as previously described<sup>[19,20]</sup>. Briefly, for monitoring EEG signals, two stainless steel EEG recording screws were positioned 1 mm anterior to bregma or lambda and 1.5 mm lateral to the midline. EMG activity was monitored by stainless steel, Teflon-coated wires bilaterally placed into both trapezius muscles.

For continuous infusion of solution, one 33-gauge stainless-steel cannula (Plastics One, Roanoke, VA, USA) was implanted stereotaxically into the left lateral ventricle 2 mm lateral from bregma and 2.2 mm ventral from the surface of the cortex at an angle of 25° to the midsagittal plane according to the brain atlas of Franklin and Paxinos<sup>[21]</sup>. A dummy cannula, protruding 0.1 mm below the tip of the cannula, was placed to prevent clogging before microinfusion. The cannula and electrodes were fixed to the skull with dental cement. When an experiment was complete, mice were euthanized with an overdose of pentobarbital sodium. The positions of the cannulae were histologically verified.

### EEG and EMG recordings with microinfusion of PGD<sub>2</sub> into the lateral ventricle of the mouse brain

After a 10-day recovery period, the mice were transferred to an experimental system for adaptation. The dummy cannulae were removed. The cannula was connected to a 100-μL microsyringe (Hamilton, Reno, Nevada, USA) with PE20 polyethylene tubing (Becton Dickinson, Sparks, MD, USA). The chronic microinjection into the lateral ventricle of the mouse was operated by a micropump (Harvard apparatus, South Natick, MT, USA) at a speed of 1 μL/h. PGD<sub>2</sub> infusion and EEG/EMG recordings were carried out via a swivel and slip-ring designed so that behavioral movement of the mouse was not restricted.

After an acclimation period of 4 days, sleep-wakefulness states were monitored for 48 h, which comprised control and experimental days. The EEG/EMG signals were amplified, filtered (EEG: 0.5–30 Hz, EMG: 20–200 Hz), and then digitized at a sampling rate of 128 Hz and recorded using SleepSign (Kissei Comtec, Nagoya, Japan) as previously described<sup>[22–24]</sup>. The EEG power spectrum was computed for 4-s epochs by fast Fourier transform within the frequency range of 0–24.75 Hz. Baseline recordings with continuous aCSF infusion were taken in each animal for 24 h, beginning at 20:00, which served as the within-animal control. On the next day, PGD<sub>2</sub> (5, 10, 20, or 50 pmol/min) was infused into the lateral ventricle of mice for 6 h between 20:00 and 2:00, respectively. Each animal received only 1 dose of PGD<sub>2</sub>.

### Vigilance state analysis

The vigilance states were automatically classified off-line in 4-s epochs into 3 stages of wakefulness, NREM and REM sleep by SleepSign, according to the standard criteria<sup>[23,24]</sup>. Then, defined sleep-wake stages were examined visually and corrected by hand.

### Statistical analysis

The data are presented as the mean±SEM. The animal number

is  $n=5-7$  for each group. The number of the different sleep-wake states was expressed in minutes for the vigilance studies. Comparisons between vehicle and PGD<sub>2</sub> infusion were conducted with the paired Student's *t*-test. The time-course data were analyzed by the repeated measures analysis of variance (ANOVA) followed by Tukey's test. The changes in sleep for 10 h after PGD<sub>2</sub> infusion in WT and A<sub>2A</sub>R KO mice were analyzed by one-way ANOVA followed by Fisher's PLSD test. In all cases,  $P<0.05$  was taken as the level of significance.

## Results

### Sleep-wake profiles induced by PGD<sub>2</sub> in A<sub>2A</sub>R KO mice

Under basal conditions, both WT and A<sub>2A</sub>R KO mice had the following clear circadian variations on EEG: decreased sleep during the dark period and increased during the light period. Quantitative analysis of the time spent in NREM and REM sleep within 24 h showed that there was no difference between WT mice and A<sub>2A</sub>R KO mice. However, during the first 6 h of the light-off period, A<sub>2A</sub>R KO mice spent  $91.9\pm 9.2$  min in NREM sleep, the duration of which was 1.5-fold of that in WT mice ( $61.0\pm 8.2$  min), indicating that the genetic deficiency of A<sub>2A</sub>R resulted in an increase in the total amount of NREM sleep during the first 6 h of the light-off period.

To investigate the role of adenosine A<sub>2A</sub>R in the somnogenic effect of PGD<sub>2</sub>, PGD<sub>2</sub> was infused into the lateral ventricle of WT and A<sub>2A</sub>R KO mice during 20:00–2:00 for 6 h. As shown in Figure 1A–C, PGD<sub>2</sub> increased sleep in WT mice. PGD<sub>2</sub> at 20 pmol/min significantly increased NREM sleep during the 12-h dark period ( $F_{1,50}=6.94$ ,  $P<0.05$ ,  $n=6$ ). During the first, second and third 2-h periods of PGD<sub>2</sub> infusion, NREM sleep was increased to 227.6% (vehicle,  $11.6\pm 1.9$  min; PGD<sub>2</sub>,  $26.4\pm 5.0$  min,  $n=6$ ,  $P<0.01$ ), 181.5% (vehicle,  $23.3\pm 5.1$  min; PGD<sub>2</sub>,  $42.3\pm 4.6$  min,  $n=6$ ,  $P<0.05$ ), and 216.2% (vehicle,  $23.4\pm 5.2$  min; PGD<sub>2</sub>,  $50.6\pm 4.4$  min,  $n=6$ ,  $P<0.01$ ) of the duration of NREM sleep in vehicle control, respectively. In contrast, there was no significant difference in REM sleep during infusion of PGD<sub>2</sub>. This enhancement of NREM sleep was concomitant with a decrease in wakefulness. PGD<sub>2</sub> decreased the wakefulness by 13.8% (vehicle,  $108.0\pm 1.9$  min; PGD<sub>2</sub>,  $93.1\pm 4.9$  min,  $n=6$ ,  $P<0.05$ ), 20.7% (vehicle,  $96.3\pm 5.1$  min; PGD<sub>2</sub>,  $76.4\pm 4.6$  min,  $n=6$ ,  $P<0.05$ ), and 29.4% (vehicle,  $95.2\pm 4.7$  min; PGD<sub>2</sub>,  $67.2\pm 4.4$  min,  $n=6$ ,  $P<0.01$ ) during the first, second and third 2-h periods of infusion, respectively. There was no further disruption in the sleep architecture during the subsequent period. Similar time course profiles were observed with a low dose of PGD<sub>2</sub> (10 pmol/min, Figure 1A) ( $F_{1,50}=5.412$ ,  $P<0.05$ ,  $n=6$ ). PGD<sub>2</sub> at 5 pmol/min did not affect the sleep-wake distribution (data not shown). However, A<sub>2A</sub>R KO mice displayed a tendency to show an increase in NREM sleep during the 6-h infusion of PGD<sub>2</sub> (10 or 20 pmol/min) (Figure 1D, 1E). There was no significant difference between the NREM sleep duration of each 2-h period during the experimental day and that during the baseline day.

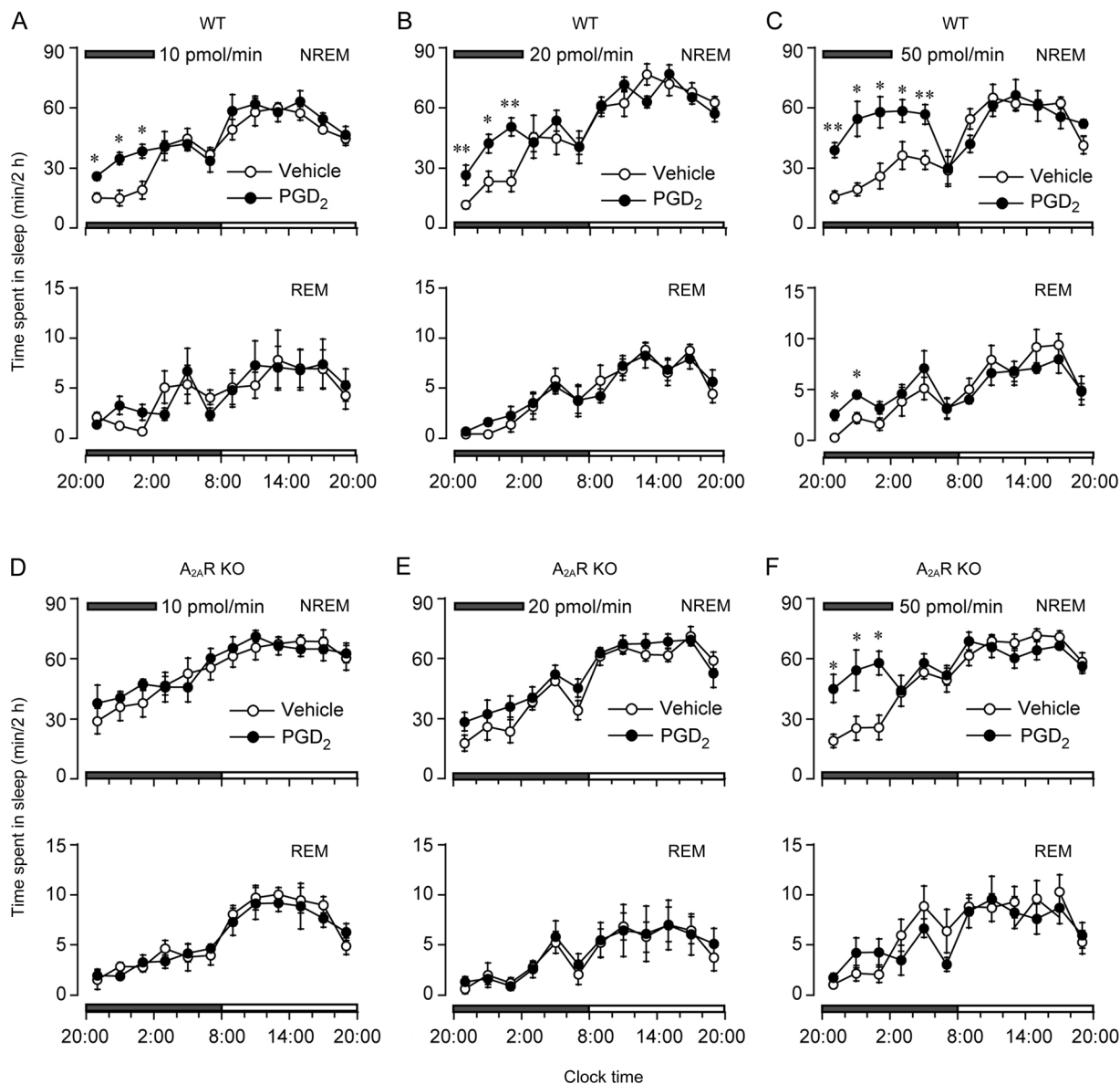
When the dosage of PGD<sub>2</sub> was increased to 50 pmol/min, NREM sleep was significantly increased in WT mice during the 6-h infusion and even during the 4 h after the infusion

ended (Figure 1C,  $F_{1,50}=14.923$ ,  $P<0.05$ ,  $n=6$ ). The amount of NREM sleep during these 10 h was almost comparable to that observed during the day time, which is an inactive period for mice. PGD<sub>2</sub> also increased REM sleep during the first and second 2-h periods after PGD<sub>2</sub> infusion. On the other hand, PGD<sub>2</sub> (50 pmol/min) increased NREM sleep in A<sub>2A</sub>R KO mice during the first, second and third 2-h periods to 237.4% (vehicle,  $19.0\pm 3.4$  min; PGD<sub>2</sub>,  $45.1\pm 7.0$  min,  $n=7$ ,  $P<0.05$ ), 213.8% (vehicle,  $25.4\pm 5.9$  min; PGD<sub>2</sub>,  $54.3\pm 10.0$  min,  $n=7$ ,  $P<0.05$ ) and 225.3% (vehicle,  $25.7\pm 6.2$  min; PGD<sub>2</sub>,  $57.9\pm 5.8$  min,  $n=7$ ,  $P<0.05$ ) of that in vehicle control, respectively (Figure 1F,  $F_{1,60}=10.165$ ,  $P<0.05$ ,  $n=7$ ). However, PGD<sub>2</sub> did not increase the NREM sleep during the 4 h post-dosing.

We calculated the total increase in NREM and REM sleep for 10 h, during the 6-h PGD<sub>2</sub> infusion and 4-h post-dosing periods, as the value during an experimental day minus that of the baseline day. In WT mice, PGD<sub>2</sub> given at 5 pmol/min had little effect on sleep-wake profiles (Figure 2). PGD<sub>2</sub> at doses of 10, 20, and 50 pmol/min significantly increased NREM sleep to 139.1% (vehicle,  $134.9\pm 7.5$  min; PGD<sub>2</sub>,  $187.7\pm 7.8$  min,  $n=6$ ,  $P<0.05$ ), 145.0% (vehicle,  $148.9\pm 25.2$  min; PGD<sub>2</sub>,  $215.9\pm 11.4$  min,  $n=6$ ,  $P<0.05$ ), and 202.7% (vehicle,  $135.4\pm 14.7$  min; PGD<sub>2</sub>,  $274.5\pm 24.6$  min,  $n=6$ ,  $P<0.01$ ) ( $F_{3,20}=7.405$ ,  $P<0.01$ ,  $n=6$  in each group) of that in vehicle control, respectively. PGD<sub>2</sub> at doses of 20 and 50 pmol/min also increased REM sleep to 148.9% (vehicle,  $8.8\pm 2.1$  min; PGD<sub>2</sub>,  $13.1\pm 1.5$  min,  $n=6$ ,  $P<0.05$ ) and 166.7% (vehicle,  $13.2\pm 2.4$ ; PGD<sub>2</sub>,  $22.0\pm 2.4$  min,  $n=6$ ,  $P<0.05$ ) ( $F_{3,20}=4.552$ ,  $P<0.05$ ,  $n=6$  in each group), respectively. However, PGD<sub>2</sub> at 5 and 10 pmol/min did not change the sleep duration in A<sub>2A</sub>R KO mice (Figure 2). PGD<sub>2</sub> infusion at 20 and 50 pmol/min increased NREM sleep in A<sub>2A</sub>R KO mice to 117.5% (vehicle,  $154.7\pm 17.1$  min; PGD<sub>2</sub>,  $181.8\pm 11.4$  min,  $n=5$ ,  $P<0.05$ ) and 155.6% (vehicle,  $166.7\pm 11.0$  min; PGD<sub>2</sub>,  $259.4\pm 26.7$  min,  $n=7$ ,  $P<0.01$ ) of that in vehicle control mice, respectively ( $F_{3,18}=5.942$ ,  $P<0.01$ ,  $n=5-7$ ). The increases in NREM sleep in A<sub>2A</sub>R KO mice were significantly less than those of WT mice with administration of PGD<sub>2</sub> at 10, 20, and 50 pmol/min by 78.7% (WT,  $49.7\pm 9.2$  min,  $n=6$ ; A<sub>2A</sub>R KO,  $10.6\pm 13.1$  min,  $n=5$ ,  $P<0.05$ ), 47.0% (WT,  $67.0\pm 21.1$  min,  $n=6$ ; A<sub>2A</sub>R KO,  $27.1\pm 11.6$  min,  $n=5$ ,  $P<0.05$ ), and 41.0% (WT,  $139.1\pm 20.2$  min,  $n=6$ ; A<sub>2A</sub>R KO,  $92.7\pm 24.6$  min,  $n=7$ ,  $P<0.05$ ), respectively. The somnogenic effect of PGD<sub>2</sub> at 10 pmol/min was abolished in A<sub>2A</sub>R KO mice, and the increase in sleep induced by PGD<sub>2</sub> at higher doses was significantly lower in A<sub>2A</sub>R KO mice than in WT mice, indicating that PGD<sub>2</sub>-induced NREM sleep is mediated by both A<sub>2A</sub>R-dependent and -independent mechanisms.

### Analysis of power spectra and episode numbers and durations

Under basal conditions, the EEG power density of NREM sleep in frequencies 1–3.75 Hz was significantly lower in A<sub>2A</sub>R KO mice than in WT mice during the first 6 h in the dark period. The peak values appeared in the 2-Hz frequency, in which the EEG power of NREM sleep was 5.0% in WT mice and 3.8% in A<sub>2A</sub>R KO mice (Figure 3A, upper). The EEG power density of REM sleep was also smaller in A<sub>2A</sub>R KO mice at 7.25–7.75 Hz during the first 6 h in the dark period than that



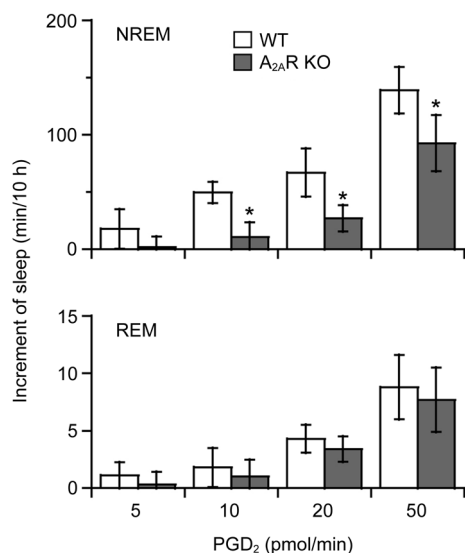
**Figure 1.** Sleep-stage distribution produced by PGD<sub>2</sub> (10, 20, and 50 pmol/min) infusion into the lateral ventricle in WT mice (A–C) and A<sub>2A</sub>R KO mice (D–F) between 20:00 and 2:00 indicated by horizontal shaded bars on the experimental day. Each circle represents 2-h amounts of NREM and REM sleep. Open and closed circles signify the vehicle control and PGD<sub>2</sub> treatment, respectively. Values are expressed as the mean±SEM (*n*=5–7). Time course changes in hourly amounts of each stage between vehicle and PGD<sub>2</sub> infusion were compared with repeated measures ANOVA followed by Tukey's test and paired *t*-test. \**P*<0.05, \*\**P*<0.01.

in the WT mice (Figure 3A, lower). There was no significant change in EEG power densities of NREM or REM sleep during the other periods. These results indicate that A<sub>2A</sub>R is essential for increases in NREM and REM sleep components in those EEG ranges during the first half of the active period of mice.

When PGD<sub>2</sub> was given at 50 pmol/min, it did not change the EEG power density of NREM or REM sleep during the 6-h infusion in WT and A<sub>2A</sub>R KO mice (Figure 3B and 3C), suggesting that PGD<sub>2</sub> did not change the EEG components in WT or A<sub>2A</sub>R KO mice and that it did not disturb their physiological sleep.

We then analyzed the episode number and duration of NREM and REM sleep in WT and A<sub>2A</sub>R KO mice during the 6-h PGD<sub>2</sub> infusion and 4-h post-doing periods (Figure 4). PGD<sub>2</sub> given at 20 pmol/min only increased NREM episode numbers to 132.3% (vehicle, 91.7±11.0, PGD<sub>2</sub>, 121.3±15.1, *n*=6, *P*<0.05) in WT mice (Figure 4A, upper), while PGD<sub>2</sub> given at 50 pmol/min increased NREM episode numbers in WT to 153.8% (vehicle, 98.2±17.4; PGD<sub>2</sub>, 151.0±25.7, *n*=6, *P*<0.05) and those in A<sub>2A</sub>R KO mice to 143.4% (vehicle, 108.8±10.5; PGD<sub>2</sub>, 156.0±18.2, *n*=7, *P*<0.05) (Figure 4A and B, upper). With respect to REM sleep, the number of REM episodes was increased with





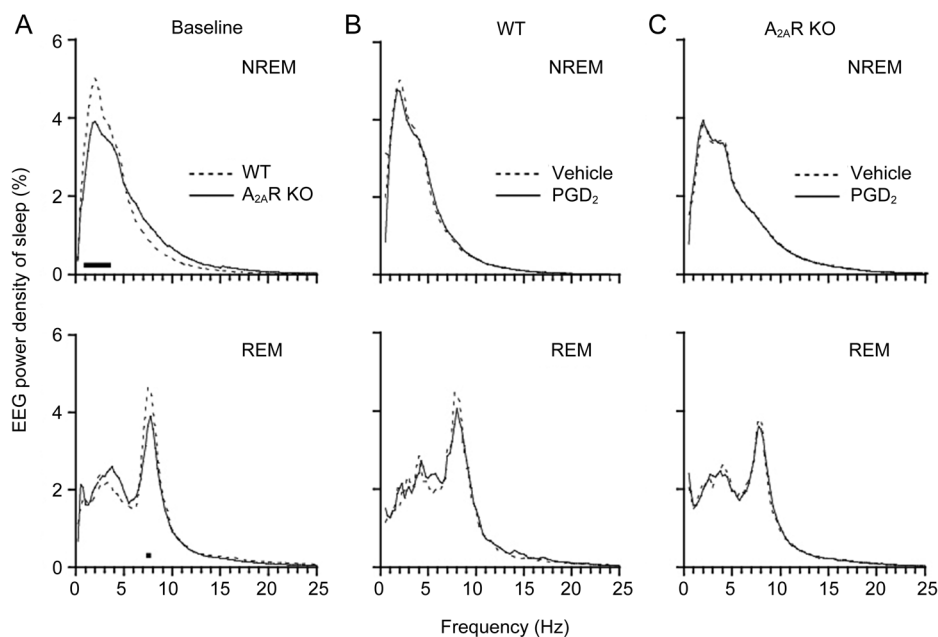
**Figure 2.** Increase in sleep for 10 h during PGD<sub>2</sub> 5, 10, 20, and 50 pmol/min infusion for 6 h and the 4-h period post-dosing. Open and shaded columns show the profiles in WT and A<sub>2A</sub>R KO mice, respectively. Values are expressed as the mean±SEM (n=5–7). \*P<0.05 by one-way ANOVA followed by Fisher's PLSD test and unpaired t-test.

50 pmol/min of PGD<sub>2</sub> to 195.0% (vehicle, 28.2±5.1; PGD<sub>2</sub>, 55.0±8.6, n=6, P<0.05) in WT mice (Figure 4A, lower), but there was no increase observed in A<sub>2A</sub>R KO mice (Figure 4B, lower). In addition, WT mice also displayed a significant increase in episode duration of NREM sleep with 50 pmol/min of PGD<sub>2</sub>

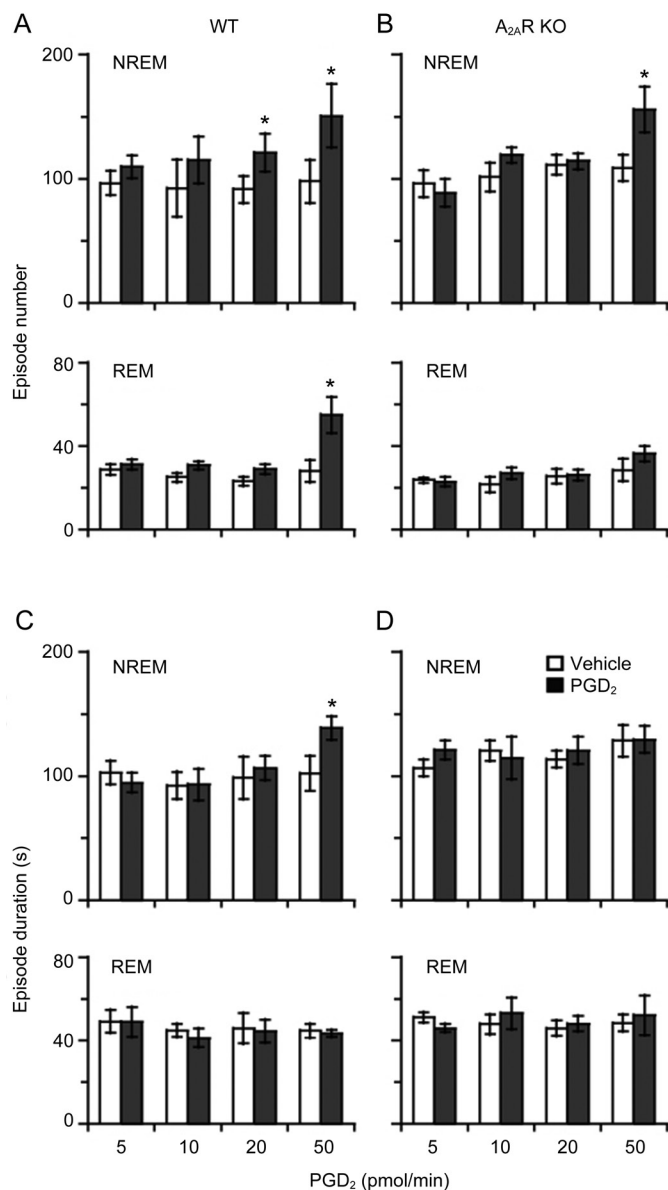
to 135.5% (vehicle, 102.4±18.7; PGD<sub>2</sub>, 138.8±9.4, n=6, P<0.05) (Figure 4C, upper), but A<sub>2A</sub>R KO mice did not (Figure 4D, upper). These changes resulted in more NREM sleep in WT than in A<sub>2A</sub>R KO mice after administration of PGD<sub>2</sub> at 50 pmol/min. When the concentration of PGD<sub>2</sub> was decreased to 10 and 5 pmol/min, PGD<sub>2</sub> did not affect the episode number or duration in both genotypes. These analyses indicated that A<sub>2A</sub>R is crucial for the increase in episode numbers of NREM sleep by low doses of PGD<sub>2</sub> and the increase in episode numbers of REM sleep and the elongation of NREM episodes by high doses of PGD<sub>2</sub>.

### Discussion

In this study, we demonstrated that PGD<sub>2</sub> induces sleep by a combination of A<sub>2A</sub>R-dependent and -independent mechanisms using A<sub>2A</sub>R KO mice. We previously demonstrated that intracerebroventricular infusion of PGD<sub>2</sub> induces sleep and increases the extracellular levels of adenosine in the brain of rats and mice by activation of DPR, as these two effects were completely absent in DPR KO mice<sup>[11]</sup>. Adenosine receptors are pharmacologically and structurally classified into A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> subtypes<sup>[25]</sup>. Both the A<sub>1</sub>R and A<sub>2A</sub>R subtypes are reported to be involved in sleep regulation (see review)<sup>[26, 27]</sup>. Therefore, we predicted that released adenosine is involved in PGD<sub>2</sub>-induced sleep by activating these receptors. Because A<sub>2A</sub>R deficiency attenuated the somnogenic effect of PGD<sub>2</sub> in mice, as shown in the present study, it appeared that the adenosine A<sub>2A</sub>R system is, at least in part, involved in PGD<sub>2</sub>-induced sleep. However, PGD<sub>2</sub>-induced sleep was also observed in A<sub>2A</sub>R KO mice to various extents depending on



**Figure 3.** Mean percentage power density in the basal condition (A) and PGD<sub>2</sub> (50 pmol/min) infusion for the first 6 h in WT (B) and A<sub>2A</sub>R KO (C) mice. Mean percentage power density calculated as the mean power (in square microvolts) in each 0.25 Hz frequency bin divided by the total power (0–24.75 Hz) in the same epoch. The horizontal bands indicate the frequency bins that significantly differed between A<sub>2A</sub>R KO and WT mice (P<0.05) by unpaired t-test in the baseline group and paired t-test in the other groups. n=5–7.



**Figure 4.** Episode number (A, B) and episode duration (C, D) for 10 h during PGD<sub>2</sub> 5, 10, 20, and 50 pmol/min infusion for 6 h and the 4-h period post-dosing in WT and A<sub>2A</sub>R KO mice. Open and shaded columns show the vehicle control and PGD<sub>2</sub> treatment, respectively. Values are expressed as the mean ± SEM. *n* = 5–7. \**P* < 0.05 by paired *t*-test between vehicle and PGD<sub>2</sub> infusion.

the dose of PGD<sub>2</sub>, indicating that a part of PGD<sub>2</sub>-induced sleep is independent of A<sub>2A</sub>R.

When PGD<sub>2</sub> was infused into the subarachnoid space just anterior to the ventrolateral preoptic area (VLPO), which has been identified as a distinct nucleus that contains a cluster of sleep-active neurons<sup>[28]</sup>, it promotes sleep more effectively than injections in a variety of other sites within the brain of rats<sup>[9]</sup>. In addition, PGD<sub>2</sub> and an adenosine A<sub>2A</sub>R agonist induce Fos-immunoreactivity in the VLPO that is proportional to the production of sleep<sup>[10, 29]</sup>, thereby suggesting that PGD<sub>2</sub> and

an adenosine A<sub>2A</sub>R agonist may stimulate sleep-active VLPO neurons through the same pathway. These sleep-active cells are both galaninergic and GABAergic and send inhibitory projections to waking-related neurons in the tuberomammillary nucleus (TMN)<sup>[30]</sup>, the sole source of histaminergic innervation of the mammalian brain, as well as the locus coeruleus and raphe nuclei<sup>[31]</sup>. It is known that both galanin and GABA inhibit neurons in the TMN and locus coeruleus<sup>[32, 33]</sup>, implying that the descending projection from the VLPO is inhibitory in nature (see review)<sup>[34]</sup>. However, the VLPO receives reciprocal inputs from several monoaminergic systems, including the arousal-related histaminergic projections from the TMN, noradrenergic inputs from the locus coeruleus, and serotonergic inputs from the midbrain raphe nuclei<sup>[35]</sup>. VLPO neurons from acute hypothalamic slices are inhibited by noradrenaline, 5-hydroxytryptamine<sup>[36]</sup> and histamine<sup>[37]</sup>, and TMN neurons also contain GABA and galanin, which may inhibit the VLPO<sup>[37]</sup>. The reciprocity of projections of the sleep- and wake-promoting brain regions, the VLPO and TMN, respectively, may work like a “flip-flop” circuit in electrical engineering terms. Briefly stated, when VLPO neurons are active during sleep and firing rapidly, they inhibit the monoaminergic cell groups, allowing for their own disinhibition and reinforced firing. Conversely, during wakefulness, monoaminergic neurons fire at a high rate, thus inhibiting the VLPO and resulting in the disinhibition of their own firing.

Although Ribeiro reported the importance of adenosine A<sub>1</sub>R on neurotransmitter release and the existence of a balance between adenosine activation at A<sub>1</sub>R and A<sub>2A</sub>R at many synapses<sup>[38]</sup>, A<sub>1</sub>R expression was indistinguishable in the cerebral cortex and striatum between WT and A<sub>2A</sub>R KO mice<sup>[39]</sup>. N6-Cyclopentyladenosine, a highly selective A<sub>1</sub>R agonist, did not induce sleep in WT and A<sub>2A</sub>R KO mice<sup>[17]</sup>, suggesting that the A<sub>1</sub>R system may not compensate for the genetic A<sub>2A</sub>R deficiency. Moreover, total sleep deprivation for 6 h induced an increase in NREM and REM sleep during the recovery period in WT mice but not in A<sub>2A</sub>R KO mice<sup>[17]</sup>, whereas sleep deprivation induced a strong NREM rebound in WT, heterozygotes and A<sub>1</sub>R KO mice<sup>[40]</sup>, suggesting that A<sub>2A</sub>R but not A<sub>1</sub>R plays an important role in the homeostatic regulation of sleep.

Activation of adenosine A<sub>2A</sub>R has been shown to promote sleep. Therefore, mice that lack A<sub>2A</sub>R should be expected to spend more time in wakefulness. However, quantitative analysis of sleep profiles during the light and dark periods showed increased spontaneous sleep and, concomitantly, decreased wakefulness during the dark period in A<sub>2A</sub>R KO mice (Figure 1). A<sub>2A</sub>Rs are particularly abundant in the striatum, although they are also expressed at a much lower level in other brain areas<sup>[41]</sup>. A<sub>2A</sub>R is colocalized with dopamine D<sub>2</sub> receptors (D<sub>2</sub>R) in GABAergic striatopallidal neurons. Formation of functional A<sub>2A</sub>R-D<sub>2</sub>R heteromeric complexes was proposed as a morphological framework for the existence of an A<sub>2A</sub>R-D<sub>2</sub>R interaction at the biochemical level<sup>[42–44]</sup>. Dopamine has been implicated in inducing wakefulness<sup>[45]</sup>. We reported that D<sub>2</sub>R KO mice displayed an increase in NREM sleep and a decrease in wakefulness<sup>[23]</sup>. In addition, microdialysis in the striatum revealed

a 45% reduction in extracellular dopamine concentration in  $A_{2A}R$  KO mice<sup>[39]</sup>. Therefore, the increase in NREM sleep (Figure 1) coupled to the decrease in the delta wave (1–3.75 Hz) component of NREM sleep (Figure 3A) is probably due to functional striatal hypodopaminergic activity in  $A_{2A}R$  KO mice.

PGD<sub>2</sub>-induced sleep has been reported to be completely inhibited by an  $A_{2A}R$  antagonist in rats<sup>[14]</sup>. However, we found that PGD<sub>2</sub> at a high dose also increased NREM sleep in  $A_{2A}R$  KO mice. The difference in the PGD<sub>2</sub>-induced sleep between pharmacological  $A_{2A}R$  antagonist treatment and receptor KO mice remains to be clarified. Koyama and Hayaishi<sup>[46]</sup> used head-restrained unanesthetized rats to examine the effects of PGD<sub>2</sub> on the activity of neurons in the preoptic/anterior hypothalamic areas and found that PGD<sub>2</sub> had an excitatory effect on approximately one-third of the NREM related-neurons examined, thereby suggesting that another possibility may be that PGD<sub>2</sub> directly stimulates sleep-active neurons to induce sleep. Those neurons may be involved in  $A_{2A}R$ -independent PGD<sub>2</sub>-induced sleep. On the other hand, Matsumura<sup>[9]</sup> et al discovered that the PGD<sub>2</sub>-sensitive sleep promoting zone was in the ventral surface of the rostral basal forebrain; thus, region-specific microinfusion of PGD<sub>2</sub> should be in the sub-arachnoid space underlying the rostral basal forebrain instead of the lateral ventricle. These differences may result in the lack of an effect of PGD<sub>2</sub> at low doses in  $A_{2A}R$  KO mice.

In the present study, PGD<sub>2</sub> given at doses of 10 and 20 pmol/min induced sleep only in WT mice but not in  $A_{2A}R$  KO mice. With an increase in dosage, PGD<sub>2</sub> also promoted sleep in  $A_{2A}R$  KO mice, but the amounts of sleep were much lower than that in the WT mice, suggesting that  $A_{2A}R$  signal transduction is one of the pathways for PGD<sub>2</sub> somnogenic effects. In summary, PGD<sub>2</sub> promotes sleep in a manner partially dependent on  $A_{2A}R$ , and adenosine  $A_{2A}R$  deficiency attenuates PGD<sub>2</sub>-induced sleep.

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### Author contribution

Wei-min QU, Zhi-li HUANG, and Yoshihiro URADE designed this study; Bin-jia ZHANG and Wei-min QU performed the experiments and analyzed the data; Jiang-fan CHEN provided the  $A_{2A}R$  KO mice, reviewed the manuscript and gave suggestions. All authors prepared the manuscript and approved the final version.

### Abbreviations

$A_{2A}R$ , adenosine  $A_{2A}$  receptor; aCSF, artificial cerebrospinal fluid;  $A_1R$ , adenosine  $A_1$  receptor; DPR, PGD<sub>2</sub> receptor; EEG, electroencephalogram; EMG, electromyogram; KO, knockout;

PGD<sub>2</sub>, prostaglandin D<sub>2</sub>; REM, rapid eye movement; NREM, non-REM; VLPO, ventrolateral preoptic area; TMN, tuberomammillary nucleus; WT, wild-type.

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