Paeoniflorin Promotes Non-rapid Eye Movement Sleep via Adenosine A₁ Receptors

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ABSTRACT

Paeoniflorin (PF, C₂₃H₂₈O₁₁), one of the principal active ingredients of Paeonia Radix, exerts depressant effects on the central nervous system. We determined whether PF could modulate sleep behaviors and the mechanisms involved. Electroencephalogram and electromyogram recordings in mice showed that intraperitoneal PF administered at a dose of 25 or 50 mg/kg significantly shortened the sleep latency and increased the amount of non-rapid eye movement (NREM). Immunohistochemical study revealed that PF decreased c-fos expression in the histaminergic tuberomammillary nucleus (TMN). The sleep-promoting effects and changes in c-fos induced by PF were reversed by 8-cyclopentyl-1,3-dimethylxanthine (CPT), an adenosine A₁ receptor antagonist, and PF-induced sleep was not observed in adenosine A₁ receptor knockout mice. Whole-cell patch clamping in mouse brain slices showed that PF significantly decreased the firing frequency of histaminergic neurons in TMN, which could be completely blocked by CPT. These results indicate that PF increased NREM sleep by inhibiting the histaminergic system via A₁ receptors.

Introduction

Insomnia is a prevalent disorder characterized by difficulties in initiating or maintaining sleep, which results in fatigue and impaired concentration, thereby seriously impacting the quality of life (Zisapel, 2012). Non-benzodiazepine drugs (Z-drugs) such as zolpidem are the first line of management for insomnia, followed by benzodiazepines (BZ) and other drugs. Multiple adverse effects hamper the application of Z-drugs and BZ (Besnard et al., 2007; Greenblatt and Roth, 2012). BZ could increase the risk of dependence and potential for abuse, so BZ are commonly used for short-term management of insomnia (Morin, 2006). Z-drugs are effective for initiating sleep but are not as effective as BZ for improving sleep quality or efficiency. Furthermore, their prolonged use produces adverse effects similar to those observed with BZ (Zammit, 2009). Therefore, there is a need for hypnotic agents that not only decrease sleep latency but also increase total sleep time and sleep efficiency without significant adverse effects.

Paeoniflorin (PF, C₂₃H₂₈O₁₁, 5-β-(benzoyloxy)methyl tetrahydro-5-hydroxy-2-methyl-2,5-methano-1H-3,4-dioxacyclobuta[cd]pentalen-1-oxyl-2H-yI-β-D-glucopyranoside; for chemical structure, see Fig. 1) (Aimi et al., 1969) is one of the principal active ingredients of Paeonia Radix, a traditional Chinese herbal medicine derived from the root of Paeonia lactiflora Pall. Paeoniflorin exerts anticonvulsant effects on experimental febrile seizures (Hino et al., 2012). This observation suggests that PF might have a sleep-promoting effect.

Accumulating evidence suggests that the effects of PF are closely associated with the adenosine A₁ receptor (RₐA₁R). PF can bind to RₐA₁R (Liu et al., 2005) and potentiate the analgesic effects of an RₐA₁R agonist N⁶-cyclopentyl adenosine (Liu et al., 2006a). PF has also been shown to reduce 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced toxicity by activating RₐA₁R (Liu et al., 2006b).

The RₐA₁Rs are widely expressed in the cortex, thalamus, hippocampus, and basal ganglia regions (Thakkar et al., 2002; Huang et al., 2007, 2011; Oishi et al., 2008; Lazarus et al., 2013). Oishi et al. (2008) reported that RₐA₁R was highly expressed in the ventrolateral preoptic area; WT, wild type; Z-drugs, non-benzodiazepine drugs.
expressed in histaminergic neurons of the tuberomammillary nucleus (TMN), which are located in the caudalateral hypothalamus. In the mammalian brain, histaminergic output from the TMN is thought to play an important role in mediating forebrain arousal (Parmentier et al., 2002; Gondard et al., 2013). We speculated that PF may promote sleep by inhibiting the histaminergic system via A1Rs.

In the present study, we characterized the sleep-wake behavior of mice by electroencephalogram (EEG) and electromyogram (EMG) recordings. Using a specific R_A1R antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) and R_A1R knockout (KO) mice to test the sleep promoting effect of PF. In addition, c-fos immunostaining and whole-cell patch-clamp recording were used to explore the mechanisms responsible for the sleep-promoting effects of PF.

Materials and Methods

Animals

Male inbred C57BL/6J mice (weighing 20–25 g, 11–13 weeks old) were obtained from the Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, People’s Republic of China). Male R_A1R KO mice and their wild-type (WT) littermate controls of the inbred C57BL/6 strains from heterozygotes were generated as previously described elsewhere (Johnsson et al., 2001). Polymerase chain reaction with the animal tail DNA was used to determine their genotypes (Goldman et al., 2010; Xiao et al., 2011). We also used glutamate decarboxylase GAD67 promoter (Tamamaki et al., 2003). The animals were housed individually at a constant temperature (24 ± 0.5°C) with ad libitum food and water and ventilation fans to mask ambient noise. Mice were entrained to a 100 lux 12-hour light/ dark cycle (lights on at 6:00 PM, lights off at 6:00 AM) with vehicle (i.p.) at 21:00. All mice were treated with vehicle (i.p.) at 21:00. On day 2, the mice were treated with PF at 21:00. The dosages of PF were selected based on previous study (Yu et al., 2007) and our preliminary data. We chose 21:00 as the injection time because the mice were most active after lights off, so the hypnotic effects were expected to be Beck.

Vigilance State Analysis

The EEG/EMG signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz), then digitized at a sampling rate of 128 Hz and recorded using SLEEPSIGN software (KISSEI COMTEC CO., LTD., Japan) as described elsewhere (Qu et al., 2008, 2010, 2012). The vigilance states were automatically classified offline in 4-second epochs into rapid eye movement (REM) sleep, NREM sleep, and wakefulness by SLEEPSIGN, according to the standard criteria (Chen et al., 2012). As a final step, defined sleep–wake stages were examined visually and corrected if necessary.

Pharmacologic Treatments

PF was dissolved in saline, and diazepam and CPT were dissolved in saline with 5% dimethylsulfoxide. PF was administered i.p. at 21:00 on the experimental day at a dose of 12.5, 25, or 50 mg/kg. Diazepam at 6 mg/kg is used as a positive control for sleep changes. All drugs were freshly prepared before use, and the injection volume (10 ml/kg) was kept constant for in vivo experiments. For baseline data, mice were injected i.p. with vehicle (10 ml/kg). To test receptor mechanisms, 30 minutes before the PF injection, the mice were pretreated with CPT i.p. at 1 or 2 mg/kg.

Immunohistochemistry

Seven groups of mice were used. One group was treated with vehicle, and three groups were injected i.p. with PF at doses of 12.5, 25, and 50 mg/kg, respectively. To test receptor mechanisms, the other three groups of mice were used, CPT 2 mg/kg + vehicle, CPT 1 mg/kg + PF 50 mg/kg and CPT 2 mg/kg + PF 50 mg/kg group, respectively. Thirty minutes before the PF injection, the mice were pretreated with CPT i.p. at 1 or 2 mg/kg. One hour after PF administration at 21:00, the animals were anesthetized with 10% chloral hydrate and perfused with saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The mice brains were then removed and immersed in 20% sucrose overnight. Thereafter, frozen sections were cut at 30 μm in coronal planes by use of a freezing microtome (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry was performed in accordance with the free-floating method described elsewhere (Chen et al., 2011, 2012; Qu et al., 2012). Sections were incubated at room temperature for 24 hours with a rabbit polyclonal antibody against c-fos at a 1:10,000 dilution in phosphate-buffered saline (PBS) containing 0.3% Triton X-100. On the second day, the sections were incubated with a 1:1000 dilution of biotinylated goat anti-c-fos antibody for 1 hour followed by a 1:10,000 dilution of avidin-biotin peroxidase for 1 hour at room temperature. Finally they were exposed for 5–10 minutes at room temperature to a solution of 0.05% 3,3′-diaminobenzidine-tetrahydrochloride containing 0.01% H2O2.

Sections were mounted, dehydrated, and cover slipped. Digital images were viewed and captured using the Olympus DP 72 microscope (Olympus America; Center Valley, PA). Figures were assembled.
and adjusted for brightness and contrast in Adobe Photoshop (Adobe Systems, San Jose, CA).

Confocal Microscopy

The sections were incubated with rabbit anti-histidine decarboxylase antibody (1:500; Euro-Diagnostica AB, Malmö, Sweden). Incubation time was 24 hours in a rotary shaker at room temperature. Sections were then washed in 0.01 M PBS and incubated in a secondary antibody containing Texas Red donkey anti-rabbit (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) antibodies prepared with 0.3% Triton X-100 in 0.01 M PBS for 2 hours in the dark on a rotary shaker and sealed with nail polish. All images were recorded by a confocal laser-scanning microscope (TCS-NT; Leica Microsystems) with excitation/emission wavelengths set to 488/520 nm for GFP and 561/620 nm for Texas Red in the sequential mode. The images were acquired at 0.5 mm steps and analyzed with Leica TCS NT/SP Scanware (version 1.6.587) (Kukko-Lukjanov and Panula, 2003).

Electrophysiology

Slice Preparation. Coronal tissue slices containing the TMN were prepared from heterozygous GAD67-GFP knock-in mice (28–42 day) housed under standard conditions. The transgenic mouse line expressing enhanced GFP under the control of the regulatory region of mouse GAD67 gene was used to identify histaminergic neurons in the TMN. Animals were anesthetized using isoflurane and killed by decapitation. The brain was quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) saturated with carbogen (95% O2/5% CO2), in which NaCl had been replaced by 207 mM sucrose for 2 minutes. TMN was identified according to the stereotaxic coordinates (Franklin and Paxinos, 1997). Coronal brain slices from the posterior hypothalamus (300 μm thick) containing TMN were cut using a Vibratome (Leica VT 1000 S) in ice-cold ACSF containing (in mM): NaCl 130, KCl 5, CaCl2 2.4, MgSO4 1.3, NaH2PO4 1.24, NaHCO3 20, and glucose 10. Slices (300 μm thick) were quickly transferred to the recording bath, where they were continuously perfused with oxygenated ACSF and allowed to equilibrate for 1 hour at 32°C before the recording began (Wang et al., 2013). Paeoniflorin was dissolved and diluted with fresh ACSF (1:1000) before use. All drugs were diluted in fresh ACSF to the final concentration immediately before the experiment.

Patch-Clamp Recordings in the Whole-Cell Configuration

Patch electrodes were pulled from borosilicate glass capillaries (1.5 mm o.d., 0.8 mm i.d.; Harvard Apparatus, Les Ulis, France) on a Brown-Flaming micropipette puller (Model P-97; Sutter Instrument, Novato, CA). The patch electrodes had a resistance of 4–6 MΩ when filled with the pipette solution containing (in mM): potassium glutamate, 130; KCl, 10; MgCl2, 2; HEPES, 10; MgATP, 2; and NaGTP, 0.3, with pH adjusted to 7.3 with KOH (Kolaj et al., 2008). The micropipettes were attached to an electric microdrive (MP-285; Sutter Instrument) and placed under visual control in contact with the soma of the selected cell. Neurons were current clamped (I output = 0 pA) to record spontaneous action potentials.

Fig. 2. Sleep-stage distributions produced by intraperitoneal administration of PF in mice. (A) Typical examples of polygraphic recordings and corresponding hypnograms in a mouse treated with vehicle or PF at a dose of 50 mg/kg. (B) Effect of PF on NREM sleep latency. Open and filled bars show the profiles for the respective baseline day (vehicle injection) and experimental day (PF and diazepam injection). (C) Time-course changes produced by the i.p. administration of PF at 50 mg/kg. Each circle represents the hourly mean ± S.E.M. of NREM and REM sleep. (D) Baseline and (•) experimental day profiles. PF was given at 21:00. The horizontal filled and open bars on the x-axes indicate the 12-hour dark and 12-hour light periods, respectively. (D) Total time spent in NREM and REM sleep for 2 hours after the PF and diazepam administration. Open and filled bars show the profiles for the respective baseline day (vehicle injection) and experimental day (PF and diazepam injection). Values are mean ± S.E.M. (n = 6–9). *P < 0.05, **P < 0.01, compared with the vehicle group, as assessed by repeated analysis of variance, followed by probable least-squares difference test.
control, as assessed by two-tailed paired t test. Data were obtained with an Axon 700B amplifier (Molecular Devices, Sunnyvale, CA), a Digilab CED1401 converter and Spike2 software (Cambridge Electronic Design, Cambridge, United Kingdom). Data were filtered at 1 kHz and sampled at 10 kHz.

TMN neurons were identified under visual guidance using infrared differential interference contrast video microscopy with a 40× water immersion objective lens. The images were detected with an infrared-sensitive charge-coupled device camera and displayed on a monitor. We only selected brightly fluorescent neurons throughout this study as GAD positive.

In whole-cell and attached-cell configurations, electrical signals during the subsequent period. Diazepam was given at 6 mg/kg as a positive control. Typical examples of EEG, EMG, and hypnograms from a mouse given vehicle or PF at a dose of 50 mg/kg are shown in Fig. 2A. PF given at 50 mg/kg increased NREM sleep for 1 hour from 22:00 to 23:00 (power = 0.994). As shown in Fig. 2B, PF significantly shortened the NREM sleep latency (time: $F_{1,20} = 31.98$, $P < 0.001$; group: $F_{3,20} = 6.15$, $P < 0.01$; time × group: $F_{3,20} = 0.88$, $P > 0.05$) compared with vehicle. NREM sleep latency is defined as the time from the saline or PF injection to the appearance of the first NREM sleep episode lasting for at least 20 seconds. The latency to NREM sleep in mice treated with PF at 25 and 50 mg/kg was 40.9 ± 3.4 and 31.4 ± 4.3 minutes, respectively, periods that were markedly and significantly shorter than the latencies of 87.3 ± 19.9 and 81.8 ± 3.5 minutes for the respective vehicle injections.

Similar changes were seen in the positive group given diazepam (6 mg/kg). The decrease in sleep latency in the PF-injected mice clearly indicates that PF accelerated the initiation of NREM sleep. However, when PF was given at the low dose of 12.5 mg/kg, the sleep latency was 81.7 ± 12.7 seconds, which was not statistically different from the latency of the vehicle-treated group ($n = 6$–7, $P > 0.05$).

Figure 2, C and D, summarizes time courses of the hourly amounts of NREM and REM sleep at 50 mg/kg and their cumulative amounts for 2 hours after the PF injection, respectively. As compared with the vehicle control, PF at 50 mg/kg markedly increased the amount of NREM sleep but did not affect REM sleep (Fig. 2C). When PF was injected at a dose of 50 mg/kg on the experimental day, the animal spent more time sleeping than on the control day. Although PF given at 50 mg/kg only increased NREM sleep for 1 hour, from 22:00 to 23:00, we still analyzed 2-hour periods after application because PF significantly increased NREM sleep for 1 hour and showed an increased tendency for an additional 1 hour after dosing. There was no further disruption of the sleep architecture during the subsequent period.

Similar time-course profiles were observed with PF given at 25 mg/kg, and the effect on sleep lasted approximately 1 hour (power = 0.994, data not shown). However, when PF was given at 12.5 mg/kg, no significant change was found in the sleep-wake profile (data not shown).

Analysis of variance analysis revealed that PF increased NREM sleep (time: $F_{1,23} = 57.4$, $P < 0.001$; group: $F_{3,20} = 10.37$, $P < 0.001$; time × group: $F_{3,20} = 8.25$, $P < 0.01$, Fig. 2D). PF given at 25 and 50 mg/kg significantly increased the total NREM sleep after the injection by 1.9- and 2.0-fold, respectively, in comparison with the vehicle injection, whereas REM sleep was not affected. PF at 12.5 mg/kg did not affect the cumulative amount of NREM sleep or that of REM after injection ($P > 0.05$). In contrast, diazepam at 6 mg/kg increased the total amount of NREM sleep by 2.95-fold compared with the vehicle control ($P < 0.01$). There was no significant difference in REM sleep after injection between diazepam and its vehicle control.

Involvement of Adenosine A1R in the Promotion of NREM Sleep by PF. To investigate the involvement of the

**Results**

**PF Increased NREM Sleep and Shortened Sleep Latency.** We examined the sleep-wake profile after i.p. injection of PF at 21:00 in mice when the animals spend most of their time in wakefulness. Diazepam was given at 6 mg/kg as a positive control. Typical examples of EEG, EMG, and hypnograms from a mouse given vehicle or PF at a dose of 50 mg/kg are shown in Fig. 2A. PF given at 50 mg/kg increased NREM sleep for 1 hour from 22:00 to 23:00 (power = 0.994). As shown in Fig. 2B, PF significantly shortened the NREM sleep latency (time: $F_{1,20} = 31.98$, $P < 0.001$; group: $F_{3,20} = 6.15$, $P < 0.01$; time × group: $F_{3,20} = 0.88$, $P > 0.05$) compared with vehicle. NREM sleep latency is defined as the time from the saline or PF injection to the appearance of the first NREM sleep episode lasting for at least 20 seconds. The latency to NREM sleep in mice treated with PF at 25 and 50 mg/kg was 40.9 ± 3.4 and 31.4 ± 4.3 minutes, respectively, periods that were markedly and significantly shorter than the latencies of 87.3 ± 19.9 and 81.8 ± 3.5 minutes for the respective vehicle injections.

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Similar time-course profiles were observed with PF given at 25 mg/kg, and the effect on sleep lasted approximately 1 hour (power = 0.994, data not shown). However, when PF was given at 12.5 mg/kg, no significant change was found in the sleep-wake profile (data not shown).

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**Involvement of Adenosine A1R in the Promotion of NREM Sleep by PF.** To investigate the involvement of the
adenosine RₐA₁R, we examined the effects of a selective aden-
osine RₐA₁R antagonist CPT on sleep-wake profile of PF.
The increase in NREM sleep lasted 1 hour after PF (50 mg/kg)
injection. The increase in NREM sleep by administration of
PF (50 mg/kg) was completely suppressed by the pretreatment
of CPT at 2 mg/kg (Fig. 3A). CPT at 2 mg/kg alone showed
no significant effects on total time spent in NREM sleep; the
increased total amount of NREM sleep induced by PF 50 mg/kg
was totally blocked by CPT at 2 mg/kg (Fig. 3B).

To better understand the sleep-wake profile caused by PF,
we determined the number and the mean duration of NREM
and wake in 2 hours in which PF increased sleep significantly.
The distribution of the episodes of NREM sleep and stage
transition number were also evaluated. As shown in Fig. 4, PF
at 50 mg/kg only increased the number of NREM bouts during
64–128 seconds, and 256–512 seconds. However, the total
number and mean duration of NREM sleep and wake episodes
did not change. The mean duration and bouts of REM sleep
were also not altered. CPT at 2 mg/kg totally blocked the
changes of the number of bouts in NREM secondary to PF,
indicating that RₐA₁R is crucial for the increased NREM sleep
caused by PF.

**Effects of PF on Characteristics of Sleep-Wake Episodész
Episodes and Power Density.** As shown in Fig. 5A, at a
dose of 50 mg/kg, PF increased the number of state transitions
from wakefulness (W) to NREM sleep (S) and from NREM
sleep to wakefulness. Neither a change in the number of
transitions from NREM sleep to REM (R) nor from REM to
wakefulness was found. State transitions from wakefulness to
NREM sleep and from NREM sleep to wakefulness for 2 hours
after administration of PF 50 mg/kg were completely blocked
by 2 mg/kg CPT, further indicating that RₐA₁R is crucial for
the increased number of state transitions between wake and
NREM by PF.

Then we determined the EEG power spectra during NREM
sleep in mice. The power of each 0.5 Hz bin was first averaged
across the sleep stages individually and then normalized as a
group by calculating the percentage of each bin from the total

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**Fig. 4.** Characteristics of sleep-wake episodes produced
by the administration of PF at 50 mg/kg, and CPT pretreat-
ment followed by PF at 50 mg/kg. (A) Total number and mean
duration of wake, NREM, and REM bouts in a 2-hour period.
(B) Changes in the numbers of NREM and REM bouts
across different ranges of episode durations over the course
of 2 hours after the administration PF at 50 mg/kg, CPT
2 mg/kg + PF 50 mg/kg. Open and filled bars show the
profiles for the respective baseline day (vehicle) and experi-
mental day (PF, CPT + PF). Values are mean ± S.E.M. (n =
5–7). *P < 0.05, **P < 0.01, two-tailed paired t test.
power (0–24.5 Hz) of the individual animal. As shown in Fig. 5C, there were no significant differences in EEG power density of NREM sleep between the PF treatment and the vehicle control. These results suggest that PF induced NREM sleep in a manner similar to physiologic NREM sleep.

**NREM Sleep Promotion Induced by PF in R, A1RW WT Mice but Not in R, A1R KO Mice.** To clarify the importance of R, A1R for the effects of PF, we used littermate WT and R, A1R KO mice. PF given at 50 mg/kg increased NREM sleep in the WT mice 1 hour after the injection as compared with the vehicle control (power = 0.996, Fig. 6A). However, R, A1R KO mice did not exhibit any significant increase in NREM sleep after administration of 50 mg/kg PF (Fig. 6B); these results clearly indicate that the R, A1R is crucial in PF-induced sleep.

**PF Increased c-fos Expression in the Ventrolateral Preoptic Area and Decreased c-fos Expression in the TMN.** The immunohistochemistry experiments were used to examine the brain regions involved in the sleep-promoting effect of PF. To study the effect of PF on the ventrolateral preoptic area (VLPO) sleep center and TMN, we counted the number of c-fos-immunoreactive neurons in the VLPO and TMN. Figure 7 shows c-fos expression of the VLPO (Fig. 7, A and B) and TMN (Fig. 7, C and D) in mice treated with vehicle or PF 50 mg/kg. There were only few fos-positive cells in the VLPO of the saline-treated group (Fig. 7A). PF significantly increased the number of c-fos-immunoreactive neurons in the VLPO (Fig. 7B; $F_{[3,20]} = 35.2, P < 0.001$). Analysis for the number of c-fos-immunoreactive nuclei showed that PF at 25 mg/kg and 50 mg/kg increased the expression of c-fos in the VLPO by 3.8- and 4.6-fold, respectively, as compared with the vehicle control (Fig. 7E). PF significantly decreased the expression of c-fos in the TMN ($F_{[3,20]} = 19.4, P < 0.001$). PF at 25 mg/kg and 50 mg/kg significantly decreased the expression of c-fos in the TMN by 57% and 68%, respectively, as compared with the vehicle control (Fig. 7F). These findings indicate that PF activated neurons in the VLPO sleep center, with inhibiting the TMN.

**PF Decreased the Firing Frequency of TMN Histaminergic Neurons via A1Rs.** To determine whether the PF could affect TMN histaminergic neurons in vitro, we used GAD67-GFP knock-in mouse brain slices to record TMN histaminergic neurons. We found a high degree of colocalization between histaminergic and GABAergic neurons in TMN of GAD67-GFP knock-in mouse (Fig. 8A). There are about 70% histidine decarboxylase-positive cells in the GFP-positive cells. Figure 8B shows the represented TMN histaminergic neurons, which are multipolar with three to four long dendrites and diameters of approximately 20–30 μm.

As originally demonstrated in TMN histaminergic neurons, cells recorded in the present study were all endowed with two important membrane rectifications (Haas and Reiner, 1988). The first was revealed by a sag during hyperpolarizing pulses (dot in Fig. 8C) and has been shown to depend on the presence of an $I_h$ current (Kamondi and Reiner, 1991). The second was
visible as a delayed return to the baseline (arrow in Fig. 8C) after hyperpolarizing pulses, and was shown to depend on transient outward $I_A$ currents (Greene et al., 1990). After a hyperpolarization, return to resting potential strongly activates the transient outward current in TMN histaminergic neurons, while in the nonhistaminergic neurons, which are rarely encountered, break spikes or bursts follow the return to resting potential from a hyperpolarization. Thus, in current-clamp recordings in the whole-cell configuration, the effects on the membrane potential of transient outward current and hyperpolarization-activated current could be distinguished as previously reported (Haas and Reiner, 1988), and these cells displayed rather broad action potentials. TMN histaminergic neurons have a broad action potential with a $Ca^{2+}$ shoulder on...
R, A1R antagonist CPT (3 μM) inhibited effect on histaminergic neurons in the presence of CPT (3 μM). Inhibition effect on histaminergic neurons in the presence of PF administered at 1000 μM, the average firing rates of TMN histaminergic neurons (F(1,39) = 55.3, P < 0.001) (Fig. 8F). When the slices were continuously perfused with PF at concentrations of 100, 300, and 1000 μM, the average firing rates of TMN neurons decreased from 2.3 ± 0.3 Hz to 1.9 ± 0.2 Hz (n = 9, P > 0.05), from 2.3 ± 0.5 Hz to 1.4 ± 0.5 Hz (n = 11, P < 0.05), and from 2.5 ± 0.4 Hz to 0.9 ± 0.2 Hz (n = 11, P < 0.01), respectively. PF administered at 1000 μM did not show the inhibition effect on histaminergic neurons in the presence of R, A1R antagonist CPT (3 μM) (Fig. 8E and F, P > 0.05, n = 9), confirming that the action of PF was mediated by A1Rs.

Discussion

The present study showed that PF shortened NREM sleep latency and increased NREM sleep. The R, A1R antagonist CPT reversed these effects, indicating that PF exerted its sleep promoting effects through activation of A1Rs. EEG delta activity is an indicator of the depth of NREM sleep (Tobler et al., 2001). In humans and rodents, BZ significantly decreases the total duration of wakefulness and increases NREM sleep, but is accompanied by typically reduced EEG delta activity in NREM sleep (Tobler et al., 2001; Kopp et al., 2004). These effects are common for agonists acting at the BZ site, irrespective of whether they are BZ or Z-drugs, such as zolpidem or zopiclone (Aeschbach et al., 1994). However, unlike BZ, PF increased the total amount of NREM sleep, but did not change the EEG power density of NREM sleep, suggesting that PF induces sleep similar to physiologic sleep.

PF significantly shortened NREM sleep latency and increased NREM sleep, but the hypnotic effect was not so lasting. The previous research reported that the mean half-life of PF is 94.16 minutes in mice (Chen et al., 1999). The short half-life for PF is consistent with its short sleep-inducing effect.

PF can quickly cross the blood–brain barrier to reach brain tissues (He et al., 2004) and bind to R, A1R (Liu et al., 2005). R, A1R has been suggested to participate in multiple biologic activities of PF, such as antihypotension (Cheng et al., 1999), neuronal protection (Liu et al., 2005), and the antivisceral...
pain effect (Zhang et al., 2009). The R.A.R antagonist CPT blocked these effects induced by PF (Liu et al., 2005; Zhang et al., 2009). The R.A.R agonist increases sleep after perfusion into the basal forebrain and TMN (Murillo-Rodríguez et al., 2004; Oishi et al., 2008). The unilateral infusion of an R.A.R-selective antagonist into the basal forebrain decreased sleep (Strecker et al., 2000). However, administration of an R.A.R agonist into the lateral preoptic area induced wakefulness (Metzppara et al., 2005). These findings indicated that the somnogenic or arousal effect via R.A.R is region dependent. This is borne out by our previous finding that sleep amounts were not changed at all when an R.A.R agonist was infused into the lateral ventricle of mice (Urada et al., 2003). Benington et al. (1995) also reported that N6-cyclopentyl adenosine can only increase EEG slow-wave activity in NREM sleep when administered systemically in the rat, and did not show potent sleep-promoting effects. Here we found that PF inhibited the histaminergic firing rate by activating R.A1R on the cell body of TMN neurons. PF also decreased c-fos protein expression significantly in the TMN. Therefore, PF may promote sleep by directly inhibiting the activity of histaminergic neurons in the TMN.

The sleep-inducing effect of PF in WT littermates of R.A1R KO mice seems to be weaker than in C57BL/6J mice. In fact, compared with their respective baseline treated with vehicle, the increase of NREM sleep caused by PF in these WT and C57BL/6J mice. These results suggest similar hypnotic effects of PF in these WT and C57BL/6J mice.

In conclusion, PF inhibited histaminergic neurons in TMN via R.A1R, increased the amount of NREM sleep, and shortened sleep latency in mice. These effects are such that a test of the usefulness of PF in modifying sleep in humans may be productive.

Authorship Contributions

Participated in research design: C.R. Chen, Qu, Huang
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Contributed new reagents or analytic tools: Zhao, J.F. Chen, Yanagawa
Performed data analysis: C.R. Chen, Sun, Luo
Wrote or contributed to the writing of the manuscript: C.R. Chen, Sun, Luo, Qu, Huang

References


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Conclusion

These findings indicate that the somnogenic or arousal effect via R.A.R is region dependent. This is borne out by our previous finding that sleep amounts were not changed at all when an R.A.R agonist was infused into the lateral ventricle of mice (Urada et al., 2003). Benington et al. (1995) also reported that N6-cyclopentyl adenosine can only increase EEG slow-wave activity in NREM sleep when administered systemically in the rat, and did not show potent sleep-promoting effects. Here we found that PF inhibited the histaminergic firing rate by activating R.A1R on the cell body of TMN neurons. PF also decreased c-fos protein expression significantly in the TMN. Therefore, PF may promote sleep by directly inhibiting the activity of histaminergic neurons in the TMN.

The sleep-inducing effect of PF in WT littermates of R.A1R KO mice seems to be weaker than in C57BL/6J mice. In fact, compared with their respective baseline treated with vehicle, the increase of NREM sleep caused by PF in these WT and C57BL/6J mice is 2.5-fold and 2.0-fold, respectively, because the baseline of NREM sleep in WT littermates of R.A1R KO mice is slightly lower than in C57BL/6J mice. These results suggest similar hypnotic effects of PF in these WT and C57BL/6J mice.

In conclusion, PF inhibited histaminergic neurons in TMN via R.A1R, increased the amount of NREM sleep, and shortened sleep latency in mice. These effects are such that a test of the usefulness of PF in modifying sleep in humans may be productive.


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