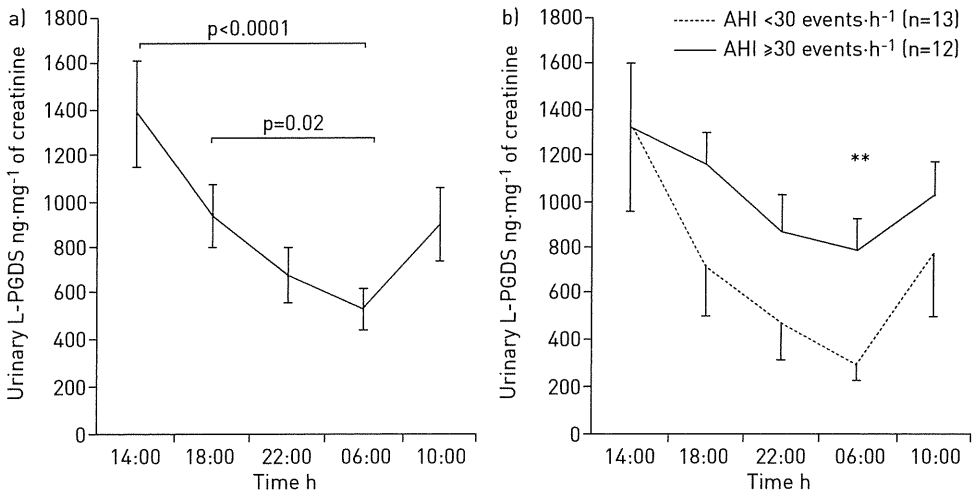

FIGURE 5 a) Urinary lipocalin-type prostaglandin D synthase (L-PGDS) concentrations in 25 study patients at 4-h intervals within a day (except midnight). b) shows the comparison of circadian variations in urinary L-PGDS between patients with apnoea/hypopnoea index (AHI) $\geq$30 events·h^{-1} (n=12) and patients with AHI <30 events·h^{-1} (n=13). Data are shown as mean±SE. **: p<0.01 between patients with AHI $\geq$30 events·h^{-1} and those with AHI <30 events·h^{-1}.

L-PGDS played a role in lipid transport. Therefore, HDL-C might be a significant factor along with the arousal index or the AHI in determining the urinary L-PGDS levels.

The present study is the first to demonstrate the circadian variations in urinary L-PGDS concentrations in OSA. Urinary L-PGDS values in both severe and non-to-moderate OSA were highest and at the same levels at 14:00 h, with the lowest values at 06:00 h in both groups. However, only 06:00 h urinary L-PGDS values correlated significantly with the AHI and were significantly higher in those with severe OSA compared with subjects with AHI <30 events·h^{-1}. In the current study, we showed that morning urinary L-PGDS concentrations were positively correlated with the AHI, 3% ODI and arousal index. Through the circadian change in L-PGDS, we propose that L-PGDS might be a stress marker that increases during daytime and decreases during sleep, whereas the decrease in L-PGDS levels in OSA, especially severe OSA, during sleep is attenuated because intermittent or sustained hypoxia, sleep fragmentation and arousals caused by OSA lead to increased stress, including oxidative stress and sympathetic activation during sleep [39]. Additionally, occurrences of CVDs peak from morning to noon, while OSA patients have an increased risk of myocardial infarction between 00:00 h and 06:00 h compared with non-OSA patients [43]. These circadian rhythms mimic the pattern of urinary L-PGDS in this study (fig. 4a).

In the current study, we did not detect a significant relationship between plasma L-PGDS and the AHI, whereas morning urinary L-PGDS levels were significantly positively correlated with the AHI. Although the reason for this discrepancy is unclear, HIRAWA *et al.* [44] reported that urinary protein excretion in the early stage of DM was correlated with urinary L-PGDS excretion, but not with plasma L-PGDS levels. In addition, serum L-PGDS levels were not shown to be associated with the AHI [25]. The influence of OSA, such as intermittent hypoxaemia, might have a significant effect on the renal system, which induced the differences in values between plasma and urinary L-PGDS. The differences in L-PGDS levels between plasma and urine should be studied in animal models. Furthermore, we did not collect peripheral blood samples at 22:00 h in the current study. The night plasma L-PGDS concentrations or the differences between morning and night plasma L-PGDS may contribute to elucidating the relation between plasma L-PGDS and OSA.

This study had some limitations. First, the sample size was small. However, the differences in the urinary L-PGDS values between control, moderate and severe OSA patients were large and, therefore, the results could be considered significant and definitive. Secondly, it is unclear whether the effect of CPAP on the PGD_2 system would persist over the long term. A long-term prospective study is needed to clarify this issue. Thirdly, we could not conduct a comparison between CPAP users and sham CPAP users. A future study that makes comparisons between CPAP users and sham CPAP users is warranted. Fourthly, we used spot urine samples for measurement of L-PGDS. There is a possibility that several factors, such as reabsorption at tubules and physical activity, influenced the urinary L-PGDS concentrations. However, use of overnight spot urine for measurement of L-PGDS has been validated because of the correlation between L-PGDS

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values of overnight urine and urine collected over 24 h [44]. Therefore, we believe overnight spot urine sampling is sufficient to evaluate the role of L-PGDS in OSA.

In conclusion, based on our results, in addition to circadian data, urinary L-PGDS might be a moderately useful marker for severe OSA. From these preliminary data, urine L-PGDS measurement may be a simple and cost-effective method to screen for and manage severe OSA. This method should be tested in unselected samples in the future because it is often difficult, costly and time consuming to find patients with OSA, while the number of OSA patients who should be treated is large.

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A mouse model mimicking human first night effect for the evaluation of hypnotics

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ABSTRACT

In humans, a first night effect (FNE) is characterized by increased sleep latency and decreased total sleep time in an unfamiliar environment, but the mechanism and treatment options for this universally experienced acute insomnia are unclear. We continuously recorded electroencephalography (EEG) and electromyogram (EMG) and measured plasma corticosterone levels to develop a mouse FNE model by inducing acute insomnia in mice that have been placed in unfamiliar cage environments. The sleep latency of mice 'moved to clean cages' (MCC) was longer than that for mice 'moved to dirty ones' (MDC). As compared to MDC mice, MCC mice showed stronger decreases in the amount of non-rapid eye movement (non-REM, NREM) and REM sleep, with a lower power density of NREM sleep, increased fragmentation and decreased stage transitions from NREM sleep to wake, and higher variation in plasma corticosterone levels. Treatment of MCC mice with zolpidem, diazepam, raclopride, pyrilamine, except SCH23390 shortened NREM sleep latency. In addition, zolpidem significantly increased NREM and REM sleep with the increase in slow wave activity (1.00–2.75 Hz), while raclopride significantly increased NREM and REM sleep without changing the EEG power density in MCC mice, whereas diazepam increased sleep with a drastic decrease in power density of the frequency band between 1.00 and 4.00 Hz, diazepam also increased the frequency band between 9.75 and 24.75 Hz during NREM sleep. These results indicate that a MCC mouse can mimic a FNE phenotype of humans and that zolpidem and raclopride may be useful drugs to prevent acute insomnia, including FNE.

1. Introduction

Insomnia is a highly prevalent condition ranging from inability to fall asleep promptly to a total lack of sleep. Acute insomnia occurs very frequently when people are in a new and unfamiliar environment where they experience significant difficulties with sleep initiation. First-night effect (FNE) is often observed in unfamiliar environments and considered to result from a person's lack of adaptation to the new sleeping environment (Rechtschaffen and Verdone, 1964). In basic human sleep research, FNE has often been used to study acute insomnia (Kitaoka et al., 2009; Suetsugi et al., 2007). Although acute insomnia is usually transient, it can progress to long-term, chronic insomnia in one third of those affected (Riemann et al., 2009), and 10–15% of patients are eventually diagnosed with moderate to severe stages of the disorder (Morphy et al., 2007). Effective treatments for acute insomnia, including FNE, are still unclear, one reason being the lack of a suitable animal model for drug development and evaluation.

Laboratory animals routinely undergo cage cleaning as part of normal husbandry, an intervention that produces significant alterations in their behaviors, including sleep disturbances (Cano et al., 2008; Tang et al., 2005). In the present study, we sought to determine whether sleep loss in rodents after cage change, as a response induced by an unfamiliar environment and/or the result of transient stress, may provide an animal model for mimicking human FNE. We found that mice moved to a clean cage (MCC) increased sleep latency more than mice moved to a dirty cage (MDC). We then investigated the ability of hypnotics including zolpidem, diazepam, as well as other sleep-inducing substances, pyrilamine, SCH23390, and raclopride, to improve sleep disturbances in MCC mice. Our results indicated that zolpidem and raclopride are highly effective in preventing FNE in MCC mice.

Abbreviations: BZ, benzodiazepine; D_1R, dopamine D_1 receptor; D_2R, dopamine D_2 receptor; EEG, electroencephalography; EMG, electromyogram; FNE, first night effect; H_1R, histamine H1 receptor; KO, knock out; MCC, mice moved to clean cages; MDC, mice moved to dirty cages; NREM, non-rapid eye movement; REM, rapid eye movement.

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2. Materials and methods

2.1. Animals

Male inbred C57BL/6J mice (Experimental Animal Center, Fudan University), 10 weeks old and weighing 24 ± 2 g, were used at the beginning of the experiments. Ambient room temperature was maintained at a constant temperature (23 ± 1 °C) and relative humidity (60 ± 5%) on an automatically controlled 12 h/12 h light/dark cycle (lights on at 08:00). Water and food were available ad libitum. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Fudan University Committee on Animal Care and Use. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.2. Drugs

Zolpidem, pyrilamine maleate, SCH-23390, and raclopride were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and diazepam from Wako Pure Chemical Industries (Osaka, Japan). All drugs were freshly prepared prior to use, and an injection volume (20 ml/kg) was kept constant through the experiments. The dosage selections, route of drug administration, and injection time of different compounds were based on preliminary experiments and pharmacokinetic considerations. Zolpidem and diazepam were dissolved in saline containing 0.3% Tween 80 and all other drugs were dissolved in saline.

2.3. Polygraphic recording and behavioral state analysis

The implant surgery was performed 10 days after the mice arrived from the supplier and they were allowed a post-surgery recovery period of 10 days. Following the recovery period after the surgery, the mice were housed individually in transparent barrels and habituated to the recording cable for 4 days before polygraphic recordings. Then the polygraphic recordings were recorded continuously for 48 h in freely moving mice.

Cortical EEG and EMG signals were first amplified and filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz) and then digitized at a sampling rate of 128 Hz and recorded by using SleepSign® (Kissei Comtec, Japan) as described earlier (Huang et al., 2005, 2006). When completed, polygraphic recordings were automatically scored off-line by 4 s epochs as wakefulness, NREM, and REM sleep according to standard criteria (Huang et al., 2001; Yan et al., 2011). As a final step, defined sleep–wake stages were examined visually, and corrected, if necessary.

2.4. Cage change procedure and drug treatment

After 24 h uninterrupted baseline sleep recording, cage change was performed at 10:00 AM (2 h after lights on, when the sleep pressure is high) (Vyazovskiy et al., 2008) on the experimental day. Each mouse was moved either into a clean cage with fresh paper-chip bedding, or a dirty cage previously occupied by another mouse for 5 days to find out the best condition for establishing the FNE mouse model. In addition, mice that only received tail handling and put back to the recording cage served as control. For evaluating effects of drugs on FNE mice, zolpidem (1.25, 2.5, or 5 mg/kg), raclopride (0.5, 1, or 2 mg/kg), diazepam (3 mg/kg), SCH-23390 (30 μg/kg), pyrilamine (5 mg/kg) or vehicle control was injected intraperitoneally, and the mouse then placed immediately into a clean cage with fresh paper-chip bedding at 10:00 AM on the experimental day, following the baseline sleep recording. Afterwards, sleep recordings were obtained for another 24 h after the drug treatment and cage change. All mice were used only once.

2.5. Blood sampling and corticosterone assay

To test the degree to which the hypothalamus–pituitary–adrenal axis was involved in the insomnia observed after placing the mice in a new cage environment, we measured the levels of circulating corticosterone. Blood sampling was performed by cardiac puncture under deep anesthesia immediately after moving mice to another cage and at 30, and 120 min after the mice were placed in a clean or dirty cage. Every blood sampling was conducted within 2 min, which is rapid enough to ensure that the stress imposed in the blood-sampling procedure did not affect corticosterone levels in plasma (Riley, 1960). To obtain the basal corticosterone levels, mouse blood were collected from their home cages at the corresponding time point, where they were undisturbed. All the mice here were used only once and all of the blood samples were collected in the EDTA-coated tubes on ice and immediately centrifuged at 15,000 rpm for 5 min, 4 °C. Plasma samples were collected into the sterilized tubes and frozen at −80 °C until assay. Plasma corticosterone was measured with specific enzyme immunoassay kits (Enzo life science, USA), following manufacturers' protocol.

2.6. Statistical analysis

All results were expressed as means ± SEM (n = 5–8). Time course changes in the amounts of sleep–wake, sleep latency, number and duration of sleep/wake bouts in light/dark phases were compared among groups by using a one-way ANOVA followed by the post hoc Tukey test or non-paired, two-tailed student's *t* tests. In all cases, $p < 0.05$ was taken as the level of significance.

3. Results

3.1. Increased sleep latency in MCC and MDC mice

As shown in Fig. 1A–C, typical examples of EEG/EMG and hypnogram showed a long continued period of wakefulness when a mouse was moved to a clean or dirty cage, or after tail handling in its host cage. The latency to sleep onset, defined as the time from the mouse being moved into another cage to the appearance of the first NREM or REM sleep episode of 20 s or more (Liu et al., 2012; Qiu et al., 2009), was significantly increased in MCC and MDC mice. As compared with the tail-handled control mice, the latency to NREM ($F_{2, 15} = 130.63$, $p < 0.01$) and REM ($F_{2, 15} = 31.08$, $p < 0.01$) sleep was increased in both MCC and MDC mice (Fig. 1D). In addition, MCC mice exhibited a longer latency to both NREM (91.8 ± 1.6 min vs. 69.4 ± 2.8 min, $p < 0.01$) and REM (113.5 ± 6.6 min vs. 91.6 ± 4.1 min, $p < 0.01$) sleep than MDC mice.

3.2. Severe sleep disturbance in MCC mice

Under basal conditions, all mice exhibited a clear circadian sleep–wake rhythm with more sleep during the light period than during the dark period (Fig. 2A). Tail handling at 10:00 (2 h after lights on) induced wakefulness that lasted for little more than 30 min. By contrast, MCC and MDC mice showed the absence of both NREM and REM sleep for more than 1 h after the cage change (Fig. 2B). Interestingly, when compared to MCC mice, MDC mice showed an increment in NREM ($t_{1, 12} = 3.81$, $p = 0.02$) and REM ($t_{1, 12} = 2.83$, $p = 0.02$) sleep during the fourth hour after cage change (Fig. 2B). The total amount of NREM ($F_{3, 20} = 13.08$, $p < 0.01$), and REM ($F_{3, 20} = 31.15$, $p < 0.01$, Fig. 2C) sleep decreased between the second hour after cage change, and the following 3 h, as compared to the baseline. When compared to tail handled mice, only the total amount of NREM sleep of the MCC mice significantly decreased ($F_{3, 20} = 13.08$, $p = 0.02$, Fig. 2C). The mean duration of NREM and REM sleep during 4 h increased 1.16-fold and 1.27-fold, respectively, in the MDC mice, while the mean duration of REM sleep decreased to 79.21% in MCC mice, as compared to tail-handled mice (Fig. 2D). The number of episodes of

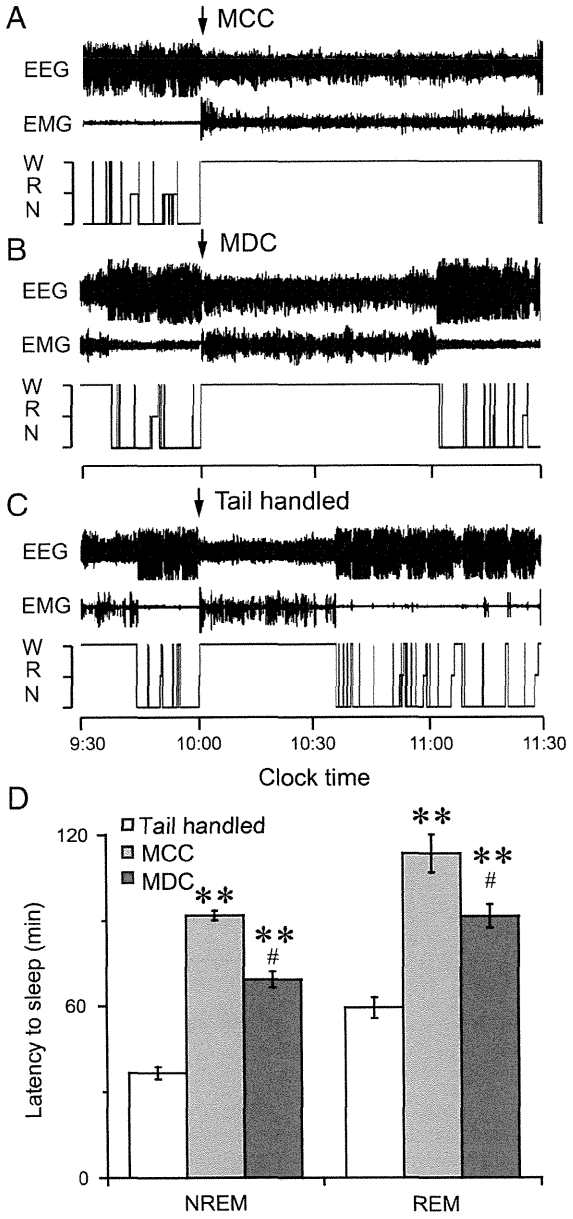

Fig. 1. Moving the mouse to a clean or dirty cage increased wakefulness. Typical examples of polygraphic recordings and corresponding hypnograms illustrating the effects of changing sleep environment on sleep of a mouse moving to a clean cage (MCC, A), moving to a dirty cage (MDC, B) or with tail handled in the same cage (C), W, Wakefulness; R, REM sleep; N, NREM sleep. (D) Average latency to NREM and REM sleep in mice moved to new cages. Open, gray and dark gray filled bars show sleep latency of tail handled, MCC or MDC mice, respectively. Values are the means ± SEM (n = 7). **p < 0.01 compared with the tail-handled mice, #p < 0.05 compared with MCC mice, assessed by one-way ANOVA followed by the Tukey test.

NREM ($F_{3, 16} = 4.36$, $p = 0.01$) and REM ($F_{3, 16} = 1.38$, $p < 0.05$) sleep was also significantly decreased in MCC and MDC mice (Fig. 2F). MCC and MDC mice showed a decrease in stage transitions between wake, NREM and REM sleep, except the stage transition from NREM sleep to wake in MDC mice, as compared with baseline or tail-handled control mice ($p < 0.01$, Fig. 2E). These findings indicate that MCC mice had more severe sleep disturbances than MDC animals.

To better understand the effects of cage change on sleep characteristics, we calculated relative power density of the EEG in 0.25 Hz bins from 0 to 24.75 Hz during 4 h after cage change. As shown in Fig. 2G, EEG power density of NREM sleep of MCC (pink line) and MDC (green line) mice increased in the frequency band of slow wave activity (0.75–3.00 Hz), as compared to that of the tail handled (blue line) mice ($F_{3, 22} = 5.14$, $p < 0.01$). The NREM sleep power density of MCC mice decreased in the frequency bands of 0.75–1.50 Hz, as compared to MDC mice ($F_{3, 22} = 5.43$, $p < 0.05$).

3.3. Sleep disturbance is independent of the plasma corticosterone levels

We measured plasma corticosterone levels to evaluate whether hypothalamus–pituitary–adrenal axis activation was involved in the wakefulness after cage change in mice. At 0, 30, and 120 min after tail handling or cage change, the plasma corticosterone levels were tested by ELISA (all mice were used only once). As shown in Fig. 2H, the plasma corticosterone level was significantly increased in the tail-handled, MCC and MDC mice at 30 min after the treatment ($F_{3, 16} = 231.54$, $p < 0.01$). After 120 min, the plasma corticosterone level of the tail-handled mice returned to the basal level. Interestingly, the plasma corticosterone level of MCC mice decreased while that of MDC mice dramatically increased ($p < 0.01$), despite the fact that the mice mostly slept during this period. Therefore, we postulated that there was no correlation between the degree of sleep disturbance under different cage change conditions and plasma corticosterone levels.

3.4. Hypnotics attenuated NREM sleep latency in FNE mouse model

The MCC mice had more severe sleep disturbances than MDC mice and therefore, we employed MCC mice as a model for the FNE to evaluate beneficial effects of hypnotics on FNE. As shown in Fig. 3A and B, MCC mice that received an intraperitoneal injection of zolpidem at a dose of 5 mg/kg immediately before the cage change, showed shortened sleep latency (average 4.8 min), while vehicle-treated MCC mice were awake for an average of 96.3 min. In addition, zolpidem-treated MCC mice spent more time in sleep than those treated with vehicle ($F_{5, 28} = 490.70$, $p < 0.01$). Similar changes were observed in MCC mice treated with raclopride, diazepam or pyrilamine, which decreased the latency to NREM sleep to 12.1, 20.2, and 43.7 min, respectively, and were significantly shorter than that of 96.3 min in vehicle-injected mice ($F_{5, 28} = 490.70$, $p < 0.01$). However, there was no significant difference in REM sleep latency among MCC mice treated with hypnotics or vehicle (Fig. 3B). All MCC mice treated with SCH23390 had no shortened latency to NREM or REM sleep, as compared to the vehicle-treated MCC mice. These findings clearly indicate that zolpidem, raclopride and diazepam suppressed cage change-induced wakefulness and accelerated the recovery of NREM sleep.

3.5. Hypnotics increased NREM and REM sleep in FNE mouse model

Fig. 4 summarizes the time-courses of the hourly amounts of NREM and REM sleep in MCC mice treated with zolpidem, raclopride, or diazepam (Fig. 4A–C) and their cumulative amounts of NREM and REM sleep for 4 h after cage change (Fig. 4D–F). As compared with the vehicle, zolpidem at 5 mg/kg markedly increased the amount of NREM sleep in the first hour ($t_{1, 10} = 14.91$, $p < 0.01$) and REM sleep in the third hour ($t_{1, 10} = 3.23$, $p < 0.01$) after the cage change. The increase in NREM sleep and decrease in wakefulness appeared 1 h after the i.p. injection (Fig. 4A). Similar changes were observed in MCC mice treated with raclopride (2 mg/kg) or diazepam (3 mg/kg) (Fig. 4B and C). However, during the dark period, the MCC mice treated with diazepam showed increases of NREM sleep, despite the fact that mice should have spent more time in wakefulness (Fig. 4C). This observation is similar to diazepam-caused daytime sleepiness in humans (Lader et al., 2009). These data indicate that zolpidem, raclopride and diazepam may suppress FNE and promote sleep. Next, we calculated the total time spent in NREM and REM sleep during 4 h after the drug treatment and cage change. Zolpidem ($F_{3, 22} = 41.23$, $p = 0.02$) and raclopride ($F_{3, 22} = 28.45$, $p = 0.04$) increased NREM sleep dose-dependently, but raclopride decreased REM sleep time ($F_{3, 22} = 18.05$, $p < 0.01$)

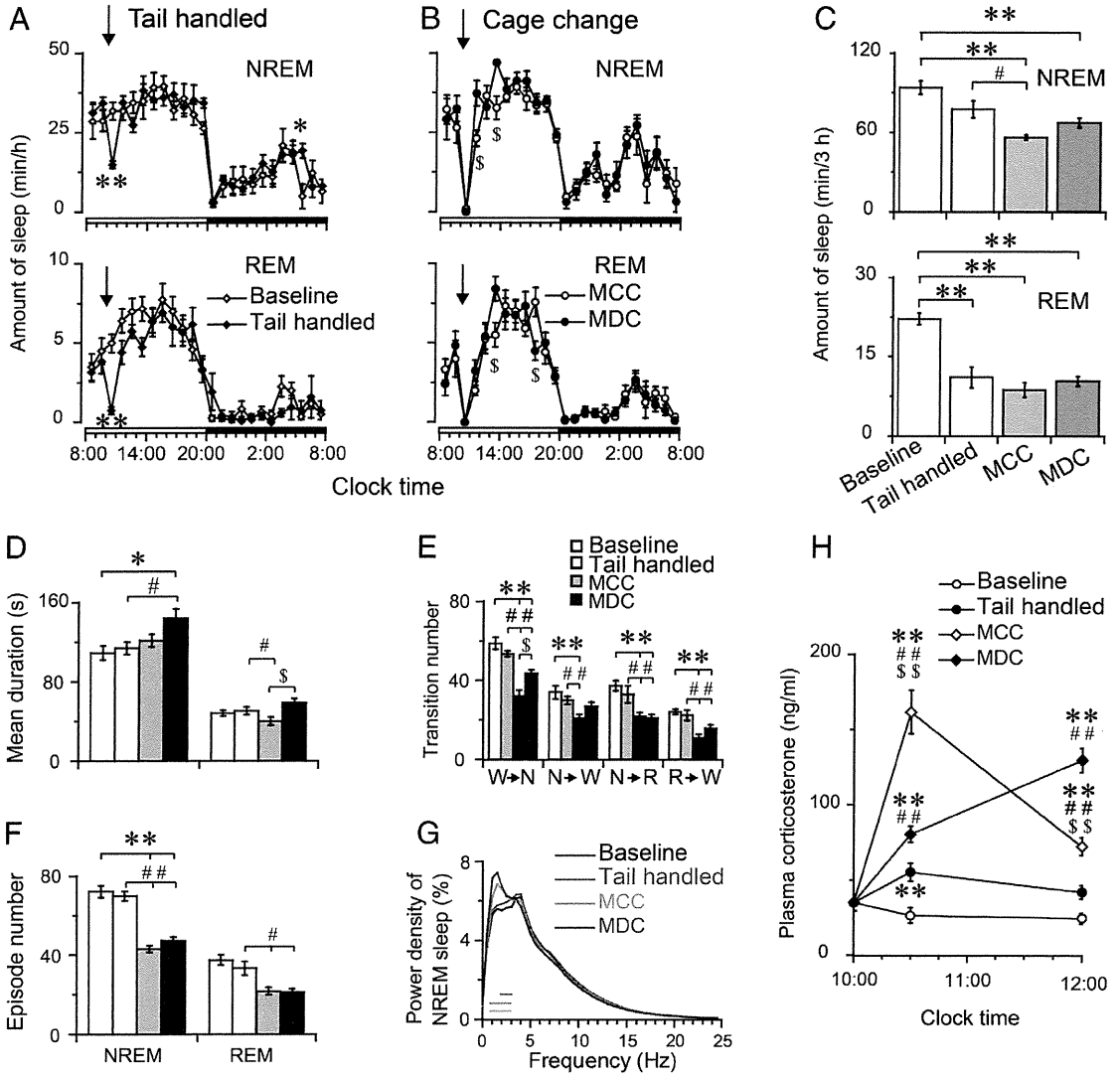

Fig. 2. Changing of cages induced sleep disturbance (A–G) and increased plasma corticosterone level (H) in mice. (A, B) Time course changes in NREM and REM sleep of the mice subjected to tail handled (A), moved to clean (MCC) or a dirty cages (MDC, B). Each circle represents the hourly mean amount of NREM and REM sleep. Open and closed rhombus and circles stand for the sleep profiles of mice under baseline conditions and after tail handled (A), as well as MCC or MDC mice (B), respectively. The horizontal open and filled bars on the χ-axes indicate the 12 h light and 12 h dark periods, respectively. (C) Total time spent in NREM, and REM sleep during the 3 h from the second hour after cage change. Mean duration (D), stage transitions (E) and episode number (F) during 4 h after the treatment. Open, light gray, gray, and dark gray filled bars show the profiles of mice under baseline, tail handled, MCC and MDC conditions, respectively. W, Wakefulness; R, REM sleep; N, NREM sleep. (G) EEG power density of NREM sleep during 4 h after cage change. The curves represent logarithmic mean values of relative power densities. The horizontal bars indicate where there is a statistical difference, compared to tail handled ($p < 0.05$). H, Plasma corticosterone levels change during the 2 h after treatment. Values are the means ± SEM ($n = 5$–7). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with baseline, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, compared with MCC mice, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with tail handled mice, assessed by two-tailed unpaired student's *t* test in (A and B), and one-way ANOVA followed by the Tukey test in (C–H). (For interpretation of the references to color, the reader is referred to the web version of this article.)

after cage change (Fig. 4D and E). However, the MCC mice treated with pyrilamine at 5 mg/kg or SCH23390 at 30 μg/kg did not increase sleep after cage change (Fig. 4F). Therefore, we did not test lower dosages of pyrilamine and SCH23390, because the dosages used are sufficient for pharmacological effects of pyrilamine (Huang et al., 2006) or SCH23390 on wakefulness (Qu et al., 2008).

3.6. Hypnotics changed the number of episodes, mean duration, stage transition, and power density of NREM sleep in MCC mice

To address sleep efficiency of tested hypnotics in MCC mice, we determined the NREM and REM sleep bout distribution as a function of bout or episode between the second hour after cage change and the 3 h that follow, because vehicle-injected FNE mice were completely awake during the first hour after the injection. Compared with the vehicle control, zolpidem and raclopride did not change the number of NREM sleep episodes and the mean duration. Only diazepam-treated MCC mice showed sleep fragmentation with increased numbers of episodes and shorter mean duration ($F_{3,16} = 15.64$, $p = 0.01$). On the other hand, the REM sleep episodes in raclopride-treated MCC mice decreased ($F_{3,16} = 20.00$, $p < 0.05$) and in diazepam-treated MCC mice increased ($F_{3,16} = 20.00$, $p < 0.01$) without alteration of their mean duration (Fig. 5A and B). In addition, zolpidem (5 mg/kg), raclopride (2 mg/kg), and diazepam (3 mg/kg) increased the number of stage transitions from wakefulness to NREM sleep and subsequently from NREM sleep to wakefulness during 3 h (11:00–14:00; Fig. 5C, $p < 0.01$). Neither a change in the number of transitions from NREM to REM sleep nor REM sleep to wakefulness was found in zolpidem-treated MCC mice. Raclopride decreased the number of stage transitions from NREM to REM sleep and REM sleep to wakefulness, whereas diazepam only increased stage transitions from REM sleep to wakefulness.

We then determined the EEG power spectra for NREM sleep for 3 h from 11:00 to 14:00, because mice in the vehicle control showed completely awake during the first hour after cage change, so that the

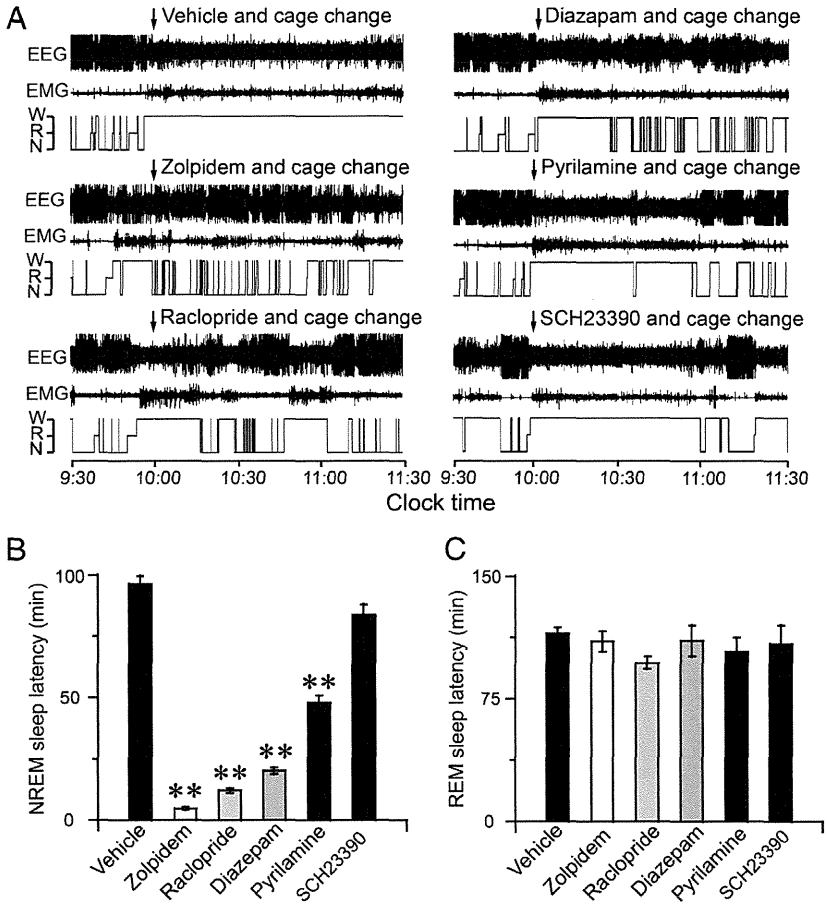

Fig. 3. Hypnotics shortened the latency to NREM sleep after moving the mice to clean cages. (A) Typical examples of polygraphic recordings and corresponding hypnograms illustrating the effects of hypnotics treatment in mice moved into clean cages. W, Wakefulness; R, REM sleep; N, NREM sleep. (B, C) Hypnotic drugs shortened the NREM (B) not REM (C) sleep latency after experimental manipulation. Values are the means ± SEM (n = 7). **p < 0.01 compared with vehicle control, assessed by one-way ANOVA followed by the Tukey test.

power density of sleep could not be calculated. The power of each 0.25 Hz-bin was first averaged across the sleep stages individually and then normalized as a group by calculating the percentage of each bin form the total power (0–24.75 Hz) of the individual animal (Yan et al., 2011). As shown in Fig. 5D, compared to the baseline, vehicle and zolpidem-treated MCC mice exhibited a drastic increase of power density in the frequency ranges of 1.00–3.25 Hz (black line in Fig. 5D, $F_{4,16} = 19.35$, $p < 0.05$, presumably because of the circadian drive and the increased homeostatic pressure caused by the sleep loss in the first hour after vehicle treatment) and 1.00–2.75 Hz (pink line in Fig. 5D, $F_{4,16} = 16.17$, $p < 0.05$), respectively, while diazepam decreased NREM power density in the frequency ranges of 1.00–4.00 Hz (green line in Fig. 5D, $F_{4,16} = 22.38$, $p < 0.05$) and increased it in the frequency range of 9.75–24.75 Hz ($F_{4,16} = 12.69$, $p = 0.05$). However, raclopride did not change the power spectral (blue line) in MCC mice. These data indicated that zolpidem increased, whereas diazepam decreased the power density in the delta range, on the other hand, diazepam increased the fast range of EEG spectra drastically. Raclopride did not change the NREM sleep power density in FNE mice.

4. Discussion

We reported the development of a mouse model for FNE based on the exposure of a mouse to a cage that is different from its host cage during the sleep phase under two different conditions. In the new and unfamiliar environment, the mice showed longer insomnia in clean cages than in dirty ones. After a long duration of continued wakefulness, MDC mice went to sleep with less sleep fragmentation characterized by a decreased episode number and longer mean duration. By contrast, MCC and MDC mice showed decreased number of stage transitions and increased EEG power density in the low frequency bands, the latter of which may be due to the circadian drive or caused by increased homeostatic pressure due to sleep loss in the first 2 h after cage change. The MCC paradigm produced an initial period of acute sleep disturbance similar to the main characteristic of FNE in humans with longer sleep latency (Rechtschaffen and Verdone, 1964). This finding suggests that the MCC procedure represents a suitable animal model for the FNE. Moreover, the use of our MCC model to evaluate potential hypnotics may be an efficient tool to predict their use under clinical conditions, as widely used hypnotics, such as diazepam, given at a dose corresponding to human dosage, have no significant effect on NREM sleep in normal mice (Kopp et al., 2004).

In our study, mice that were moved to dirty cages occupied by other mice for more than 5 days showed shortened latency to sleep than mice that were moved to clean cages. A possible explanation for this finding may be that the smell of another mouse is not a stressor per se, because the mice are housed in adjacent cages in the animal isolators and continually exposed to each other's smell. Mice use their sense of smell to detect food, predators, sexual receptivity, and many other aspects and to quickly familiarize with their environment (Beauchamp and Yamazaki, 2003), whereby mice are known to show prolonged activity in a novel environment without any social cues (Tang et al., 2005). In fact, for behavioral tests in neuroscience laboratories, many researchers wipe the facilities gently with a damp paper towel and/or only remove urine and feces, based on the assumption that a constant layer of olfactory cues will be less stressful for test animals than a very clean environment (Baumans, 2005). The mice employed in our study faced to two different stressors, i.e., clean and dirty cages, causing the circulating

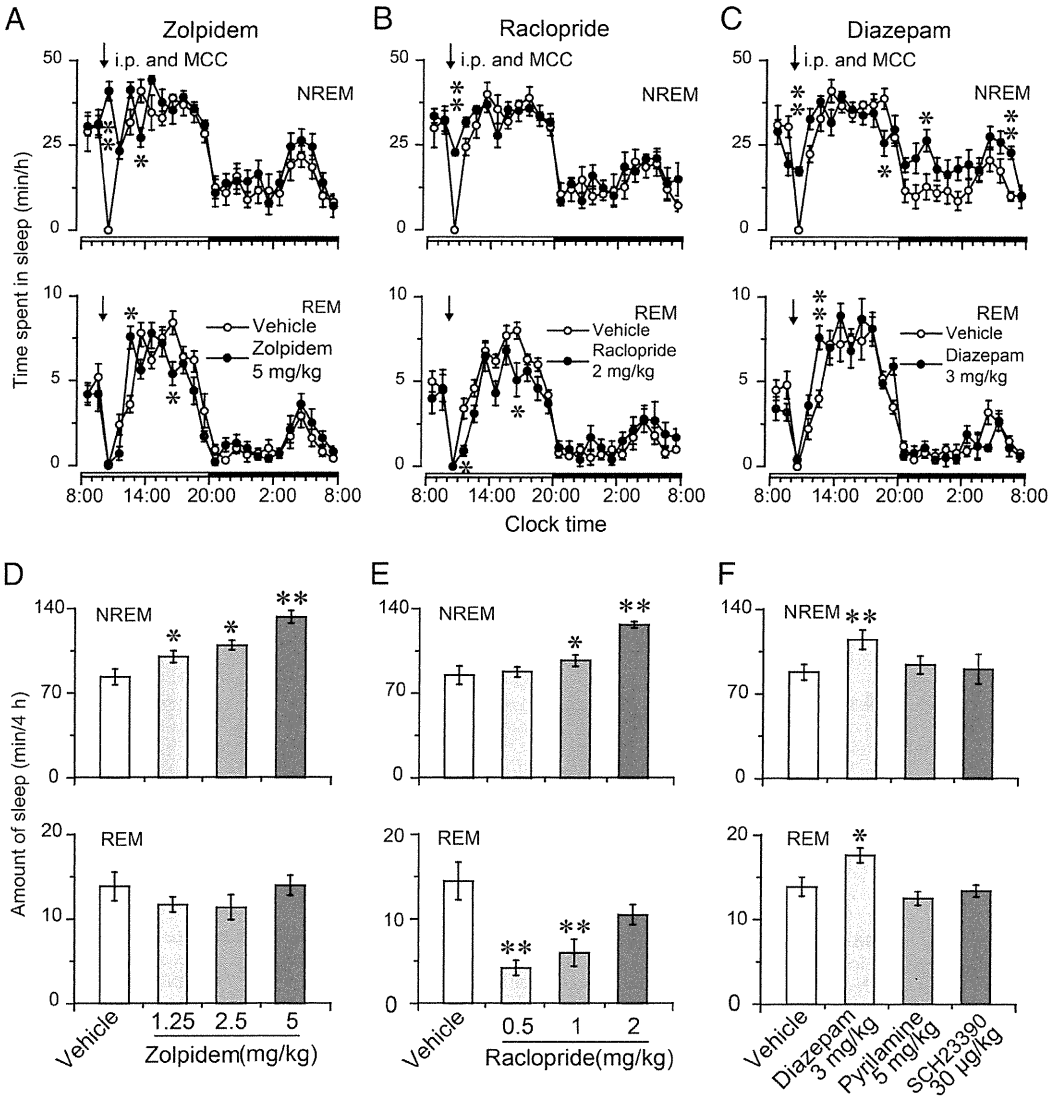

Fig. 4. Hypnotics increased the sleep time of the mice after 'moved to clean cages' (MCC). Effects of zolpidem (A), raclopride (B), diazepam (C) on sleep profiles of MCC mice. Each circle represents the hourly mean amount of each stage. The horizontal open and filled bars on the χ-axes indicate the 12 h light and 12 h dark periods, respectively. Zolpidem (D) and raclopride (E) increased total sleep time during 4 h in a dose-dependence manner. Pyrilamine and SCH23390 had no significant effect on sleep time (F). Drugs were given at 10:00, and followed by changing the mice's cages immediately on the experimental day. Values are the means ± SEM (n = 6–8). *p < 0.05, **p < 0.01 compared with vehicle control, assessed by two-tailed unpaired student's *t* test in (A–C), and one-way ANOVA followed by the Tukey test in (D–F).

corticosterone level to increase. Short mild stress usually involves a sleep rebound, while the long and intense stress periods induce sleep disturbance (Marinesco et al., 1999). In the present study we found that the plasma corticosterone levels dramatically increased in clean or dirty cage conditions, suggesting that mice under both conditions have severe sleep disturbances. However, mice slept 2 h after the cage change, despite higher corticosterone level. These results indicated the corticosterone is not the primary cause of wakefulness in our experiments.

A previous study in rats showed that the dirty cage condition is more efficient to induce acute insomnia than the clean cage approach, as well as, after being moved to dirty cages, the rats developed two phases of sleep disturbance with an acute phase after the cage change and a second sleep disturbance phase 4–6 h later (Cano et al., 2008). We found, however, that mice showed dramatically longer sleep latency when moved to clean cages (91.8 ± 1.6 min), whereby the sleep latency was not only longer than for mice moved to a dirty cage (69.4 ± 2.8 min), but also significantly longer than rats moved to a dirty cage in the previous study (58.7 ± 7.4 min). We did not observe any second/delayed phase of sleep disturbances neither under the MCC nor MDC conditions, which may be due to species differences. For example, rats are known to be more territorial after exposure to olfactory and visual cues of a competitor (Whishaw et al., 2001), whereas mice seem to be more sensitive to a new environment or novel objects (Tang et al., 2004; Whishaw et al., 2001) and are usually more actively engaged in natural behaviors, like nest building (Tang et al., 2005). Our findings indicated that FNE/MCC mice are more suitable to evaluate the effects of hypnotic substances: sleep latencies of mice or rats after vehicle injections are around 40 min (Kopp et al., 2003; Utsu et al., 2007) so that it is difficult to observe a clear difference when the rat model is employed in pharmacological experiments due to the rats' only slightly longer sleep latency when moved to another cage than the one after the vehicle injection (Cano et al., 2008).

Benzodiazepines (BZs) such as diazepam and non-BZ hypnotics such as zolpidem are first line drugs used for the therapy of insomnia, despite the fact that hypnotics such as diazepam only reduce sleep latency slightly (Pick et al., 2005; Winsky-Sommerer, 2009). We employed our new FNE model based on MCC mice to evaluate hypnotics and found that zolpidem significantly reduces sleep latency and increases total sleep time in a novel sleep environment, but lacks the typical daytime sleepiness of diazepam, the latter of which may cause clinically

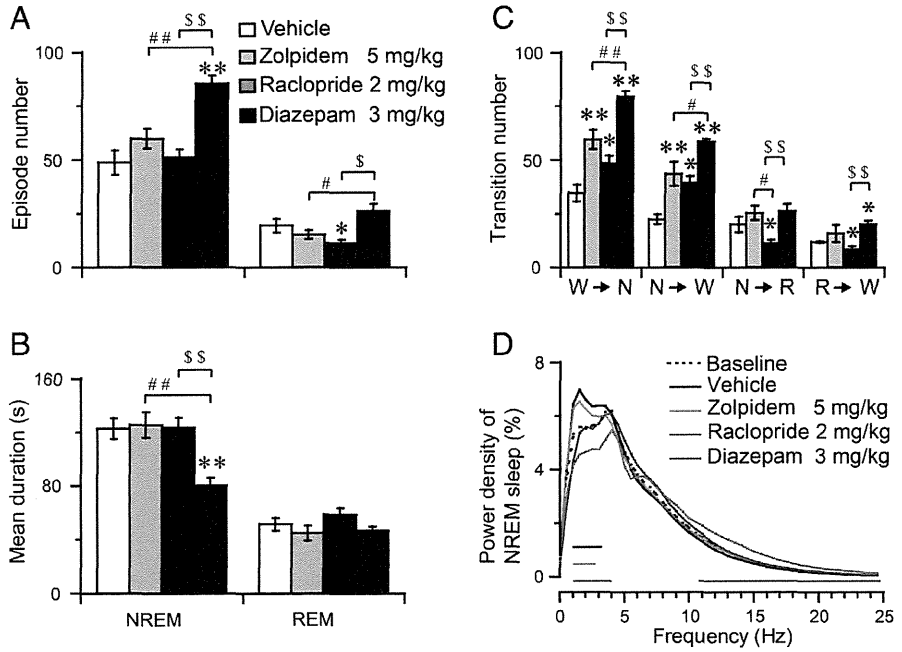

Fig. 5. Hypnotics improved the sleep disturbance in mice 'moved to clean cages' (MCC). Episode number (A), mean duration (B), and stage transitions (C) during 3 h (11:00–14:00) after drug treatment in MCC mice. (D) EEG power density from 11:00 to 14:00 after hypnotic treatment. The curves represent logarithmic mean values of relative EEG power densities. The horizontal bars indicate where there is a statistical difference, compared to baseline ($p < 0.05$). Values are the means ± SEM ($n = 6–8$). *$p < 0.05$, **$p < 0.01$ compared with vehicle control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with zolpidem, $^{\$\$}p < 0.01$ compared with raclopride, assessed by one-way ANOVA followed by the Tukey test. W, Wakefulness; R, REM sleep; N, NREM sleep. (For interpretation of the references to color, the reader is referred to the web version of this article.)

significant daytime distress and functional impairment (Dundar et al., 2004; Winsky-Sommerer, 2009). Zolpidem has pharmacological profiles distinct from those of the classical BZs such as diazepam, in that it is highly selective for α1βγ2 receptors in the CNS, thereby producing a strong sedative and hypnotic profile that predominates over the anticonvulsant and anxiolytic activity of diazepam (Koester et al., 2013), which is mediated primarily by GABA receptors composed of α2βγ2 subunits (Low et al., 2000). Although at higher concentrations zolpidem will modulate receptors containing α2 or α3 subunits (Cope et al., 2004), we did not use so higher dosage of zolpidem in the current study. Furthermore, zolpidem may shorten sleep latency in MCC mice in part of inhibiting the histamine neurons through its synaptic $GABA_A$ receptors (Zecharia et al., 2012). It was demonstrated that GABA receptors composed of α1βγ2 receptors mediate the sedative, anterograde amnestic, defensive behavioral, and in part the anticonvulsant actions of diazepam (da Cruz et al., 2013; Koester et al., 2013; Olsen and Sieghart, 2009), since diazepam predominantly interacts with GABA receptors composed of α1βγ2, α2βγ2, α3βγ2, or α5βγ2, which mediate the different clinical effects through different receptor subtypes (Olsen and Sieghart, 2009).

In addition, we previously found that D_2R knockout (KO) mice exhibited a significant decrease in wakefulness (Qu et al., 2010) and that L-stepholidine, the first compound known to have mixed dopamine D_1R agonist/D_2R antagonist properties, may potentially be used for the treatment of insomnia (Qiu et al., 2009). Here, we evaluated the hypnotic effects of the D_2R antagonist raclopride and the D_1R antagonist SCH23390 in MCC mice, and found that only raclopride significantly reduces NREM sleep latency of MCC mice, resulting in longer sleep times and more transitions from wakefulness to NREM sleep. Mice treated with raclopride also showed a power density of NREM sleep that was similar to physiological sleep under basal conditions. Our results suggest that the dopamine/D_2R system is involved in enhanced arousal in a novel environment. It is known that mice lacking dopamine (Qu et al., 2010) or histamine (Ohtsu and Watanabe, 2003) can sleep faster after a mild stress, including cage change. Dopaminergic neurons send excitatory projections to the histaminergic tuberomammillary nucleus, the norepinephrinergic locus coeruleus, and other arousal regions (Huang et al., 2007; Parmentier et al., 2002), suggesting that dopaminergic and histaminergic systems are involved in arousal. In fact, H_1R KO mice have previously been demonstrated to have fewer incidents of brief awakening under baseline conditions (Haas and Panula, 2003; Huang et al., 2006). However, mice under MCC conditions that were treated with the histamine H_1R antagonist pyrilamine had a shortened NREM sleep latency, but lacked any effect on the mean duration, episode number of each stage and stage transition as compared to untreated MCC mice (data not shown). Pyrilamine used at the dosage as described above may not be strong enough to improve acute insomnia in MCC mice with a high degree of wakefulness for more than 90 min. Long half-lives and peripheral side effects, however, prevent the clinical usage of pyrilamine at higher dosages and thus, limit its use in controlling insomnia (Tiligada et al., 2011).

5. Conclusions

We developed a mouse model for FNE characterized by prolonged sleep latency when mice are moved to a clean cage. We found that zolpidem and raclopride almost completely antagonized the FNE.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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ORIGINAL ARTICLE

Cytomegalovirus infection during immunosuppressive therapy for diffuse parenchymal lung disease

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ABSTRACT

Background and objective: **Cytomegalovirus (CMV) infection is a life-threatening condition in patients with diffuse parenchymal lung diseases (DPLDs), who are receiving immunosuppressive therapy. The aim of this study was to describe the clinical features of CMV infection and to propose a strategy for managing CMV infection in patients with DPLD who are receiving immunosuppressive therapy.**

Methods: **A retrospective longitudinal observational study was performed on 69 patients with DPLDs (39 with acute/subacute onset, 30 with chronic onset) who were receiving immunosuppressive therapy and were positive for CMV pp65 antigen (CMV-pp65Ag) in peripheral blood leukocytes (PBLs).**

Results: **Clinical CMV disease and subclinical CMV antigenaemia developed in 23 and 46 patients, respectively. The cut-off level of CMV-pp65Ag indicating clinical CMV disease, as determined by receiver operator characteristic curve analysis, was 7.5 cells per 5×10^4 PBLs. Multivariate analysis revealed that early CMV infection was associated with acute/subacute onset of underlying DPLD and with respiratory dysfunction at the commencement of immunosuppressive therapy. Multivariate analysis also suggested that the acute/subacute onset of underlying DPLD, a CMV-pp65Ag titre of >7.5 cells per 5×10^4 PBLs, and C-reactive protein levels ≥10 mg/L indicated a poor prognosis.**

Conclusions: **We recommend that CMV-pp65Ag antigenaemia of >7.5 cells per 5×10^4 PBLs in patients with DPLD should be treated with ganciclovir. Patients with lower levels of CMV-pp65Ag antigenaemia should be closely monitored or treated with ganciclovir if the clinical findings suggest a poor prognosis.**

SUMMARY AT A GLANCE

The clinical features of cytomegalovirus (CMV) infection during immunosuppressive treatment of diffuse parenchymal lung disease and the clinical importance of CMV antigenaemia as a prognostic factor were characterized. Diagnosis and treatment of these patients should be based on the levels of CMV pp65 antigen in peripheral blood leukocytes.

Key words: collagen vascular disease, immunodeficiency, interstitial lung disease, viral infection.

Abbreviations: BMT, bone marrow transplantation; CMV, cytomegalovirus; CRP, C-reactive protein; CVD-LD, collagen vascular disease-related lung diseases; DPLD, diffuse parenchymal lung diseases; GCV, ganciclovir; IIPs, interstitial pneumonias; IPF, idiopathic pulmonary fibrosis; PBL, peripheral blood leukocytes.

INTRODUCTION

Cytomegalovirus (CMV), a member of the herpesviridae family, commonly infects immunocompromised patients. CMV infection is often seen in patients with human immunodeficiency virus (HIV) infection, and bone marrow or solid-organ transplantation patients, in whom it may cause serious disease.[1] Various strategies have been developed for the rapid diagnosis of CMV disease.[1] The CMV pp65 antigen (CMV-pp65Ag) assay[2] is a standard method for detecting CMV lower matrix phosphoprotein pp65 in peripheral blood leukocytes (PBLs). A cut-off value of CMV-pp65Ag titre before commencement of ganciclovir (GCV) treatment has been determined in clinical trials targeting patients undergoing solid organ transplantation,[3,4] allogeneic bone marrow transplantation (BMT)[5,6] and allogeneic haematopoietic stem cell transplantation,[7] as well as patients infected with HIV.[8]

The National Hospital Organization Kinki-Chuo Chest Medical Center (NHO-KCCMC) has

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encountered CMV antigenaemia and CMV-related disease in patients with diffuse parenchymal lung diseases (DPLDs), including idiopathic interstitial pneumonias (IIPs) and collagen vascular disease-related lung diseases (CVD-LDs), which are commonly treated with corticosteroids and immunosuppressants. However, the frequency and type of clinical CMV disease and the cut-off level of CMV-pp65Ag antigenaemia indicating a need for treatment with antiviral drugs have not yet been investigated sufficiently. It is also unclear how CMV infection should be managed in patients receiving immunosuppressive therapy for DPLDs, taking into consideration various clinical factors.

To ascertain the clinical features and prognosis of CMV infection and to formulate a strategy for managing CMV infection in patients with DPLD who are treated with immunosuppressants, we retrospectively summarized the clinical data and determined the cut-off value of CMV-pp65Ag for diagnosing clinical CMV disease. The factors associated with the occurrence of CMV infection and the prognosis from time of onset were also identified.

METHODS

Patients

Between August 2004 and August 2008, a total of 267 patients with DPLDs (65 with idiopathic pulmonary fibrosis (IPF), 121 with IIPs other than IPF (non-IPF) and 81 with CVD-LDs) who were admitted to the NHO-KCCMC, received corticosteroids and immunosuppressants (Table S1 in the online supporting information). The PBL CMV-pp65Ag titre was evaluated in 154 of the 267 patients to exclude CMV infection when PBL counts and/or immunoglobulin G (IgG) levels decreased progressively, or when C-reactive protein (CRP) levels remained above the cut-off level. CMV-pp65Ag was not detected in 85 patients (Table S1) and other aetiological factors (e.g. bacterial infection, fungal infection, etc.) were identified through the clinical course. A total of 69 patients who were positive for CMV-pp65Ag were included in this retrospective trial. Of these, 46 patients with IIPs were diagnosed according to the international multidisciplinary consensus classification:[9] 17 patients with IPF and 29 non-IPF. The remaining 23 patients were diagnosed with CVD-LDs as follows: seven with polymyositis/dermatomyositis, six with rheumatoid arthritis, four with microscopic polyangiitis, three with idiopathic pulmonary haemorrhage, two with Sjögren syndrome and one with systemic sclerosis. Treatment of the underlying DPLDs is described in detail in the footnote to Table 1.

Patients with underlying DPLDs were classified into two groups based on the onset of DPLD before treatment commenced. Acute/subacute onset DPLDs were defined as underlying DPLDs that had developed in less than three months, or chronic onset DPLDs that progressed rapidly within one month directly before treatment. Chronic onset DPLDs were defined as underlying DPLDs that had developed more than three months before treatment began and that had progressed gradually. There were 30 patients with acute/subacute onset DPLDs and 39 patients with chronic onset DPLDs. There was no significant difference in the nature of the underlying DPLD between patients with acute/subacute onset DPLD and those with chronic onset DPLD (Table S2 in the online supporting information).

CMV-pp65Ag was not detected at the commencement of immunosuppressive therapy in 42 of the 69 patients in this trial. In the remaining 27 patients, CMV-pp65Ag was not evaluated before the commencement of immunosuppressive therapy.

All data were retrospectively obtained from the patients' medical records. The institutional review board at NHO-KCCMC approved the study.

Definition of CMV infection, its onset and CMV diseases

The definition of CMV infection included both subclinical CMV-pp65Ag antigenaemia without accompanying symptoms, abnormal laboratory findings associated with CMV infection and clinical CMV disease. The onset of CMV infection was defined as the first detection of CMV-pp65Ag, or the first occurrence of clinical symptoms and/or abnormal laboratory findings associated with CMV disease, which sometimes precedes detection of CMV-pp65Ag. The diagnosis of CMV disease was based on the modified criteria of Ljungman *et al.*.[10] In clinical CMV diseases, clinical signs and viral replication were observed. However, histological examination to detect tissue invasion could not be performed because of respiratory failure or thrombocytopenia. Thrombocytopenia was defined as a platelet count less than 100×10^9/L.

CMV-pp65Ag assay

PBLs were extracted from 3 mL of EDTA-treated whole blood. CMV-pp65Ag in PBLs was detected by the modified methods described in detail previously,[2] using a commercially available CMV-pp65Ag detection kit (TFB Inc., Tokyo, Japan), which had high sensitivity, specificity and reproducibility.[2,11] CMV antigenaemia was expressed as the number of CMV-pp65Ag positive cells per 5×10^4 PBLs. Inter-observer variability of the CMV-pp65Ag assay has been reported to be 1.8–6.0% and intra-assay variability, as evaluated by the coefficient of variation, was 9% and 22% for >50 and 5–50 CMV-pp65Ag positive cells per 5×10^4 PBLs, respectively.[11]

Statistical analysis

Statistical methods are described in detail in the legends to each table and figure. All statistical analyses were performed using JMP version 8.0.2 for Macintosh software (SAS Institute Inc., Cary, NC, USA). The statistical significance level was set at 0.05.

RESULTS

Clinical features of CMV infection

At the first detection of CMV-pp65Ag in PBLs, 20 patients were diagnosed with clinical CMV disease

Table 1 Details of the patients with diffuse parenchymal lung diseases who were positive for cytomegalovirus-pp65 antigen

	Total ($n = 69$)	Subclinical ($n = 46$)	Clinical ($n = 23$)	*P* value
Baseline data				
Gender (males)‡	61% (42)	59% (27)	65% (15)	0.7940
Underlying DPLDs (IIPs)‡	67% (46)	67% (31)	65% (15)	0.8567
Onset of DPLDs (acute/subacute)‡	57% (39)	50% (23)	70% (16)	0.1973
Smoking status (NS/ES/CS)‡	35/43/22% (24/35/15)	37/43/20% (17/20/9)	30/43/27% (7/10/6)	0.7837
At the start of initial treatment of lung disease				
Age, years†	70 (29–86)	70 (29–85)	71 (42–86)	0.6695
PSL, mg/kg†	0.75 (0.10–1.17)	0.60 (0.24–1.09)	0.85 (0.10–1.17)	0.1554
Immunosuppressive drugs¶ (YES)‡	45% (31)	41% (19)	52% (12)	0.4477
Steroid pulse therapy§ (YES)‡	67% (46)	65% (30)	70% (16)	0.7914
Neutrophils, $\times 10^9$/L†	5.5 (1.8–20.9)	5.4 (1.8–20.9)	7.3 (2.2–17.7)	0.1966
Lymphocytes, $\times 10^9$/L†	1.4 (0.2–4.3)	1.4 (0.2–4.1)	1.5 (0.54–4.3)	0.5879
Platelets, $\times 10^9$/L†	267.5 (55–483)	269 (69–483)	238 (55–464)	0.875
CRP (mg/L)†	40.9 (0.4–249)	29.3 (0.4–249)	55 (1–230)	0.2178
IgG (mg/L)†	16110 (4930–28410)	16220 (4930–26750)	15690 (8060–28410)	0.5593
LDH (IU/L)†	301 (154–716)	272 (154–612)	352 (158–716)	0.0757
DM (YES)†	30% (21)	30% (14)	30% (7)	1.0
$AaDO_2$ (mm Hg)†	57 (10–609)	56 (10–569)	57 (12–609)	0.4629
Onset of CMV infection				
Age, years†	71 (29–86)	70 (29–85)	71 (42–86)	0.5492
PSL, mg/kg†	0.62 (0.19–1.08)	0.58 (0.20–1.05)	0.75 (0.19–1.08)	0.1632
Immunosuppressive drugs (YES)‡	65% (45)	63% (29)	70% (16)	0.7891
CMV pp65Ag, per $5x10^4$ leukocytes†	3.33 (0.56–883)	1.73 (0.56–42.3)	50 (7.57–883)	<0.0001
Neutrophils, x 10^9/L†	6.4 (1–20.6)	6.35 (2.7–20.6)	6.7 (1–18.1)	0.6512
Lymphocytes, 10^9/L†	0.9 (0.2–4.9)	0.9 (0.2–4.9)	0.6 (0.2–1.8)	0.0098
Platelets, x 10^9/L†	171 (23–486)	182 (23–486)	147 (36–252)	0.043
CRP (mg/L)†	11 (0.1–274.4)	7 (0.1–236.1)	28.2 (0.4–274.4)	0.045
IgG (mg/L)†	8080 (3450–20910)	8280 (3450–20910)	7940 (4180–20270)	0.8324
LDH (IU/L)†	334 (154–745)	334 (154–703)	293 (198–745)	0.5906
DM (YES)‡	64% (44)	61% (28)	70% (16)	0.5981

Data are presented as median (range) or percentage (number of patients).

Differences between patients with subclinical antigenaemia and those with clinical CMV disease were compared by the non-parametric †Wilcoxon test or ‡chi-square test.

§ Steroid pulse therapy consisted of intravenous high-dose methylprednisolone (500–1000 mg daily) for three successive days.

¶ Immunosuppressive drugs (azathiopurine in 10 patients, cyclophosphamide in 7 patients, and cyclosporine in 14 patients) were administered at the time of initial treatment. The dose of azathiopurine was 50–75 mg/day. Cyclophosphamide was given orally in doses of 50–150 mg/day in four patients and by intravenous pulse therapy at 660–750 mg per patient in three patients. Cyclosporine was given at 50–150 mg/day and trough levels in the 14 patients ranged from 59 to 220 ng/mL, with a median value of 97 ng/mL.

Subclinical = subclinical CMV antigenaemia; Clinical = clinical CMV disease.

$AaDO_2$, alveolar-arterial oxygen tension difference; CMV, cytomegalovirus; CRP, C-reactive protein; CS, current smoker; DM, diabetes mellitus; DPLDs, diffuse parenchymal lung diseases; ES, ex-smoker; IgG, immunoglobulin G; IIPs, idiopathic interstitial pneumonias; LDH, lactate dehydrogenase; NS, non-smoker; PSL, prednisolone.

and 49 diagnosed with subclinical CMV-pp65Ag antigenaemia. Among the three patients with subclinical antigenaemia, two developed thrombocytopenia and one developed enterocolitis. Among the 23 patients diagnosed with clinical CMV disease during this study, there were 12 cases of thrombocytopenia, four of neutropenia, two of anaemia, six of pneumonitis, five of hepatitis, two of haemophagocytic syndrome (HPS) and one of enterocolitis.

The differences in clinical findings between patients with clinical CMV disease ($n = 23$) and patients with subclinical CMV-pp65Ag antigenaemia ($n = 46$), at the start of initial treatment of the underlying DPLDs, and at the onset of CMV infection are presented in Table 1. There were no differences at the start of initial treatment of the underlying DPLDs. At the onset of CMV infection, CMV-pp65Ag levels were significantly greater in patients with clinical CMV disease, and PBL counts were significantly lower in patients with clinical CMV disease than in patients with subclinical CMV-pp65Ag antigenaemia.

The median interval from the commencement of corticosteroid therapy to the initial detection of CMV infection was 47 days. CMV infection was detected significantly earlier in patients with acute/subacute onset of DPLDs (median 34 days) than in those with

Table 2 Multivariate analysis of clinical parameters at the start of immunosuppressive treatment to determine the time between onset of CMV infection and the start of therapy†

		Risk ratio	95% CI	*P* value
Onset of underlying DPLDs	Acute/subacute	3.357	1.497–7.539	0.0032
	Chronic	1	—	—
$AaDO_2$, mm Hg	≥60	1.993	1.064–3.779	0.029
	<60	1	—	—
Lymphocytes, x 10^9/L	≥1.4	1.038	0.581–1.858	0.9286
	<1.4	1	—	—
Immunosuppressants	Yes	1.057	0.61–1.817	0.7444
	No	1	—	—
CRP, mg/L	<40	1.39	0.688–2.759	0.3429
	≥40	1	—	—
Steroid pulse therapy†	Yes	1.042	0.569–1.936	0.8957
	No	1	—	—

Cox proportional hazard analysis was performed. The factors selected were those for which the *P* values were <0.05 by univariate analysis using the Wilcoxon test (Table S4 in the online supporting information). Univariate analysis showed that the underlying DPLD, gender, age, smoking status, lactate dehydrogenase levels, and initial prednisolone dose were not indicative of when CMV infection would occur (Table S4). Steroid pulse therapy consisted of high dose intravenous methylprednisolone administered on three successive days †($n = 69$).

$AaDO_2$, alveolar-arterial oxygen tension difference; CI, confidence interval; CMV, cytomegalovirus; CRP, C-reactive protein; DPLDs, diffuse parenchymal lung diseases.

chronic onset of DPLDs (median 137 days). CRP levels (median 83.1 mg/L vs 4.7 mg/L) and the alveolar-arterial oxygen tension difference ($AaDO_2$) (median 67.2 mm Hg vs 39.8 mm Hg) were significantly greater in patients with acute/subacute onset of DPLDs than in patients with chronic onset of DPLDs.

Comparison of patients without CMV-pp65Ag and those with CMV infection

The clinical findings in patients with CMV infection ($n = 69$) and those without CMV-pp65Ag ($n = 85$) are compared in Table S3 in the online supporting information. Lymphocyte counts and IgG levels were significantly lower in patients with CMV infection than in those without CMV-pp65Ag, at the time of CMV measurement after the introduction of immunosuppressive therapy.

Factors determining the time from start of prednisolone and immunosuppressive therapy to onset of CMV infection

Multivariate analysis revealed that patients with acute/subacute onset of DPLDs and a high $AaDO_2$ (≥60 mm Hg) had an early onset of CMV infection (Table 2).

Cut-off value of CMV-pp65Ag titre for the diagnosis of clinical CMV disease for treatment with ganciclovir (GCV)

A cut-off value of >7.5 cells per 5×10^4 PBLs was determined for the diagnosis of clinical CMV disease, based on the receiver operating characteristic (ROC) curve analysis shown in Figure 1. With this cut-off value, the positive likelihood ratio was 9.20. Thus, the CMV-pp65Ag titre is an important diagnostic indicator of whether to treat patients with GCV.

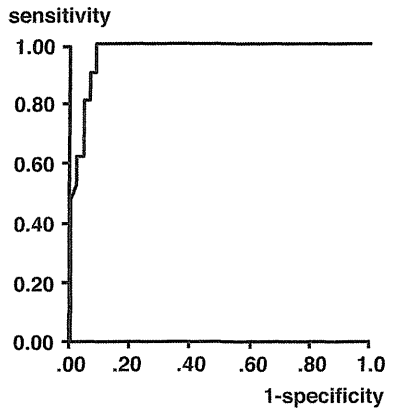

Figure 1 Receiver operating characteristic (ROC) curve analysis for the diagnosis of clinical cytomegalovirus (CMV) disease using a threshold level for CMV pp65 antigen (CMV-pp65Ag) in patients with CMV infection. The analysis included patients with clinical CMV disease ($n = 23$) and those with subclinical CMV antigenaemia ($n = 46$). A cut-off value of >7.5 cells per 5×10^4 peripheral blood leukocytes is recommended for the diagnosis of clinical CMV disease based on this ROC curve analysis. With this cutoff, the sensitivity was 1.0, the specificity was 0.891, the area under the curve was 0.972, the positive predictive value was 0.821 and the negative predictive value was 1.0.

Longitudinal observation of the clinical course in patients with low levels of CMV-pp65Ag at initial examination

Among all the CMV-pp65Ag-positive patients ($n = 69$), 44 showed low levels of CMV-pp65Ag (≤7.5

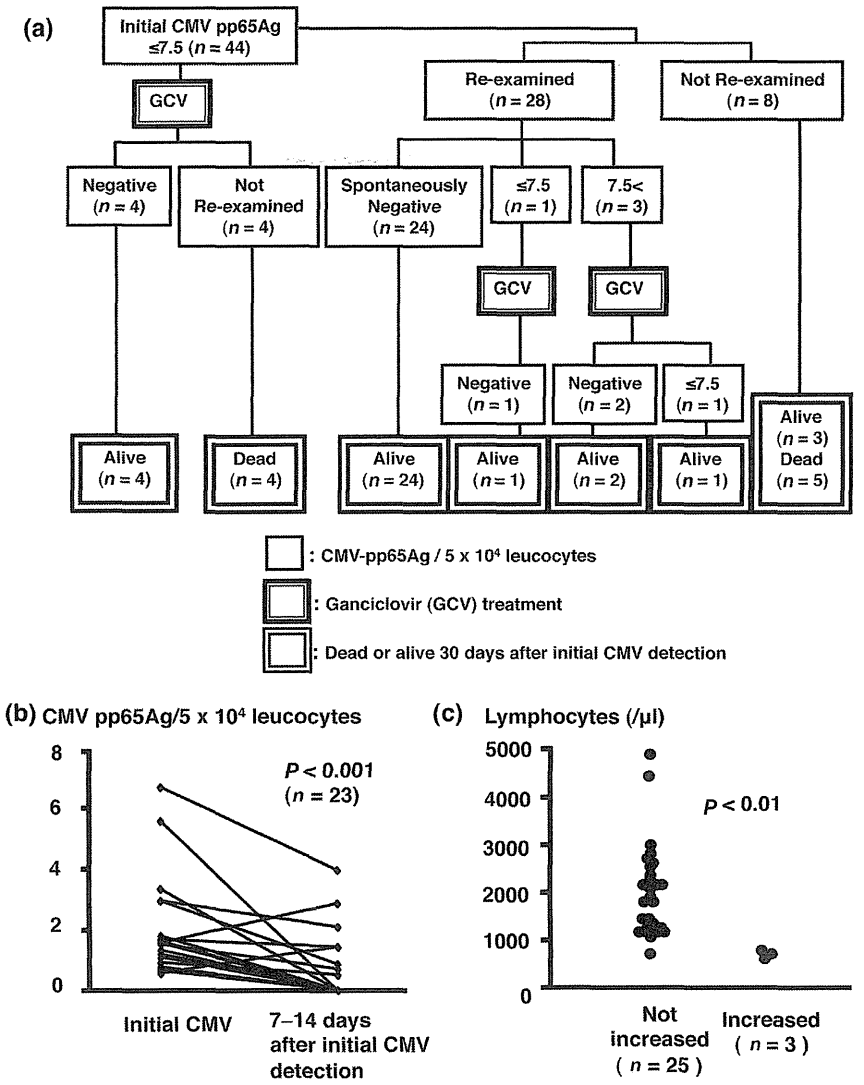

Figure 2 Longitudinal observation of the clinical course in patients with low levels of cytomegalovirus (CMV) pp65 antigen (CMV-pp65Ag) (≤7.5 cells per 5×10^4 leukocytes) at initial detection of CMV. Among all CMV pp65Ag-positive patients, 44 showed low levels of CMV-pp65Ag (a). In eight patients, ganciclovir (GCV) was administered immediately after the detection of CMV-pp65Ag. In 36 patients, GCV therapy was not commenced, and CMV-pp65Ag levels were re-examined in 28 of these 36 patients. Twenty-four of the 28 patients spontaneously became CMV-pp65Ag-negative. In 23 of the 24 patients, CMV-pp65Ag decreased significantly after 7–14 days (Wilcoxon signed-rank test), although CMV-pp65Ag levels increased slightly in two patients but still remained below the lower limit (b). In three patients, CMV-pp65Ag increased to levels of 474, 188 and 33.6 cells per 5×10^4 leukocytes, more than 3 weeks after the first detection of antigen (the patients had complications of thrombocytopenia in two instances and enterocolitis in one); In two of the three patients, corticosteroid therapy was increased after detection of CMV-pp65Ag due to deterioration of their DPLD. Peripheral blood lymphocyte counts in these three patients at the time of first detection of CMV-pp65Ag were 200/μL, 200/μL and 300/μL, which were significantly lower than the counts in the other 25 patients exhibiting low levels of CMV-pp65Ag (non-parametric Wilcoxon test (c).

cells per 5×10^4 PBLs) (Fig. 2a). CMV-pp65Ag levels were re-evaluated in 28 patients in whom GCV therapy had not been initiated, and were found to have decreased spontaneously to undetectable levels in 24 patients. In 23 of these 24 patients, CMV-pp65Ag levels decreased significantly after 7–14 days (Fig. 2b).

In three of the 28 patients not treated with GCV, and in whom CMV-pp65Ag was re-evaluated, the development of clinical CMV disease was observed, accompanied by substantial increases in CMV-pp65Ag levels. The PBL counts in these patients at the time of first detection of CMV-pp65Ag were significantly lower than the PBL counts in the other 25 patients (Fig. 2c).

Longitudinal observation of the clinical course in patients with high levels of CMV-pp65Ag at initial examination

Of the CMV-pp65Ag-positive patients, 25 showed high CMV-pp65Ag levels (>7.5 cells per 5×10^4 PBLs) (Fig. S1 in the online supporting information). In 12 of

the 22 patients treated with GCV, CMV-pp65Ag decreased to undetectable levels. Seven patients treated with GCV died within 1 month before the disappearance of CMV-pp65Ag.

Treatment of CMV-pp65Ag antigenaemia

Thirty-four patients with CMV-pp65Ag antigenaemia received GCV for a median of 11 days (interquartile range (IQR) 7–14 days) until CMV-pp65Ag could not be detected in general. GCV therapy was tolerable except for three patients, complicated with myelosuppressive and hepatotoxic effects and received foscarnet instead. Sixteen patients received immunoglobulin supplementation. Granulocyte colony-stimulating factor was administered to two patients with HPS. CMV-pp65Ag decreased to undetectable levels in 19 patients after the various therapeutic approaches described previously. In five of the 19 patients, CMV-pp65Ag antigenaemia recurred,and GCV treatment more than twice was needed. In 12 patients with clinical CMV disease, thrombocytopenia improved significantly after GCV therapy (Fig. 3).

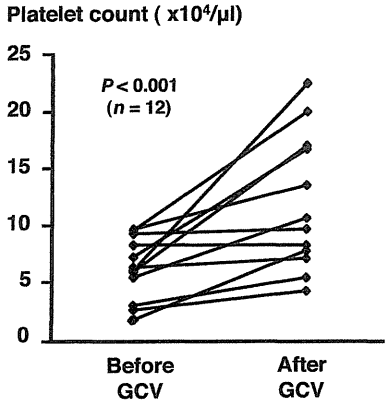

Figure 3 Thrombocytopenia in patients with clinical cytomegalovirus (CMV) disease improved significantly after ganciclovir (GCV) therapy. Platelet counts in peripheral blood before and after GCV therapy were compared using the Wilcoxon signed-rank test.

The prognosis of patients with DPLDs after diagnosis of CMV infection depended on CMV-pp65Ag levels

Multivariate analysis revealed that immunosuppressive drugs, CMV-pp65Ag and CRP levels were significantly associated with patient survival (Table 3).

DISCUSSION

CMV infection, including both clinical CMV disease and subclinical antigenaemia, occurred in 25.8% of patients with DPLDs in the present study and in about 40% of patients with collagen vascular diseases (CVDs) treated with immunosuppressants.[12,13] Thus, CMV infection is quite common in patients with DPLD and CVD who are being treated with immunosuppressants. The incidence of clinical CMV disease among patients with CMV-pp65Ag-positive PBLs was 33.3–70% in the present study and other reports on patients with CVD.[12–14] Not all subclinical antigenaemia deteriorates into clinical CMV disease; spontaneous resolution of antigenaemia may occur.[15]

Table 3 Multivariate analysis of clinical parameters at the onset of CMV infection to determine mortality after the onset of CMV infection

		Risk ratio	95% CI	*P* value
CRP, mg/L	≥10	2.703	1.387–5.504	0.0032
	<10	1	—	—
Immunosuppressants	No	2.660	1.278–5.51	0.0093
	Yes	1	—	—
CMV pp65Ag, per 5 x 10^4 leukocytes	>7.5 (high)	2.225	1.161–4.293	0.0162
	≤7.5 (low)	1	—	—
Underlying DPLDs	IIPs	2.023	0.997–4.414	0.7444
	CVD-LDs	1	—	—
Lymphocytes, x 10^9/L	≤0.9	1.874	0.981–3.694	0.3429
	>0.9	1	—	—
Gender	Male	1.525	0.763–3.148	0.8957
	Female	1	—	—
DM	Yes	1.168	0.587–2.429	0.664
	No	1	—	—

The risk ratio for survival after the onset of CMV infection was estimated by multivariate Cox proportional hazard analysis using seven factors for which the *P* values were <0.05, as determined by univariate Wilcoxon test on data for all patients ($n = 69$) (Table S5 in the online supporting information). Univariate analysis showed that age, smoking status, prednisolone dose at the onset of CMV infection, lactate dehydrogenase levels and rate of onset of DPLDs were not significant (Table S5).

CMV, cytomegalovirus; CI, confidence interval; CRP, C-reactive protein; DM, diabetes mellitus; CVD-LDs, collagen vascular disease-related lung diseases; DPLDs, diffuse parenchymal lung diseases; IIPs, idiopathic interstitial pneumonias.

We have encountered 23 cases of DPLD complicated by clinical CMV disease, most frequently accompanied by thrombocytopenia. Haematological abnormalities have been most frequently reported in patients with CVD receiving immunosuppressants who exhibited CMV antigenaemia.[12]

A decrease in lymphocyte numbers following immunosuppressive therapy was important in assessing the occurrence of subclinical CMV-pp65Ag antigenaemia and its deterioration into clinical CMV disease in the present study, consistent with other reports.[1,16]

Serum IgG is important in conferring protection from viruses, including CMV-related disease.[1] Serum IgG levels were lower in patients with CMV infection than in those without CMV-pp65Ag. However, serum concentrations of IgG were similar in patients with subclinical CMV antigenaemia and those with clinical CMV disease, which is in agreement with the findings of Takizawa *et al.*.[14] These results may reflect the fact that the effectiveness of immunoglobulin infusions for prevention of CMV disease is still unclear.[17–19]

In the present study, acute/subacute onset of DPLD and severe respiratory failure ($AaDO_2 \geq 60$ mm Hg) at the start of therapy were associated with a significantly increased likelihood of early onset CMV infection. This finding is in keeping with previous reports that serious CMV infection was often complicated by severe respiratory failure[20] and inflammatory disease.[21–24] There are two possible explanations for this finding; one is that severe inflammatory processes may disturb immunological reactions; the other is that tumour necrosis factor (TNF)-α, a central mediator of inflammation, which is often elevated in the serum of critically ill patients[25,26] and in acute fibrotic lung disease,[27] accompanied by high serum CRP levels, may induce replication of CMV by activating nuclear factor-κB-mediated early enhancer/promoter regions of the CMV genome.[25,26]

Based on our clinical findings, we suggest the following strategies for managing CMV infection during immunosuppressive therapy in patients with DPLDs. We recommend that DPLD accompanied by high CMV-pp65Ag levels (>7.5 cells per 5×10^4 PBLs) should be treated with GCV or other antiviral drugs. DPLD accompanied by low CMV-pp65Ag levels (≤7.5 cells per 5×10^4 PBLs) may not require antiviral treatment, provided CMV-pp65Ag levels are carefully monitored every 7–14 days; however, pre-emptive treatment with GCV before the onset of clinical CMV disease is preferable in patients with severe lymphocytopenia (≤0.3 x 10^9/L), elevated CRP levels (≥10 mg/L), acute/subacute onset of underlying DPLD, severe respiratory failure and/or augmentation of immunosuppressive therapy directly after detection of CMV-pp65Ag. The usefulness and appropriate frequency of routine monitoring of CMV-pp65Ag should be investigated in another trial. At least monthly monitoring during the 4 to 6 months after the initiation of immunosuppressive therapy may be beneficial, considering the median interval from initiation of therapy to the onset of CMV infection.

This study had some limitations. First, CMV seropositivity of our cases before initiation of immunosuppressive therapy, which is a very important factor in determining the onset of active CMV infection,[28] is not included in our analysis. Second, this was a retrospective study, and laboratory investigations were not routinely performed for each patient. Third, the CMV-pp65Ag assay was not sufficiently well standardized, although it was reported to have high sensitivity, specificity and reproducibility.[2,11] Therefore, the cut-off value used in the present study should be verified in another trial.

In conclusion, we recommend that patients with DPLDs and CMV-pp65Ag antigenaemia of >7.5 cells per 5×10^4 PBLs should be treated with GCV. Patients with DPLDs accompanied by lower levels of CMV-pp65Ag should be carefully monitored or treated with GCV if onset of DPLD is acute/subacute or if there are other clinical indicators of a poor prognosis. Future prospective studies should focus on verifying the validity of this strategy.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Longitudinal observation of the clinical course of patients with high levels of cytomegalovirus (CMV) pp65 antigen (CMV-pp65Ag) (>7.5 cells per 5×10^4 peripheral blood leucocytes) at the initial CMV detection. Of all the CMV-pp65Ag-positive cases, 25 patients showed high levels of CMV-pp65Ag. The CMV-pp65Ag became negative in 12 out of the 22 patients treated with ganciclovir (GCV). However, three out of the 12 patients died within one month. Three out if the four patients, whose CMV-pp65Ag levels remained high in spite of GCV therapy, died within one month. Four patients died within one month after the start of GCV therapy before reevaluation of CMV-pp65Ag. Three patients died before the diagnosis of CMV infection and before GCV therapy could be initiated.
Table S1 Underlying DPLDs and CMV-pp65Ag evaluation
Table S2 Onset of underlying DPLDs
Table S3 Patient Details of CMV-pp65Ag detected and non-detected cases
Table S4 Clinical parameters at the start of immunosuppressive treatment and the interval leading to the onset of CMV infection (univariate analysis)*
Table S5 Clinical parameters at the onset of CMV infection and survival from the CMV infection (univariate analysis)*

Sleep Breath
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ORIGINAL ARTICLE

Breathing irregularity during wakefulness associates with CPAP acceptance in sleep apnea

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Abstract
Purpose Individuals have different breathing patterns at rest, during wakefulness, and during sleep, and patients with sleep apnea are no different. The hypothesis for this study was that breathing irregularity during wakefulness associates with CPAP acceptance in obstructive sleep apnea (OSA).
Methods From a 2007–2010-database of patients with a diagnostic polysomnography (PSG) and prescribed CPAP (n=380), retrospectively, 66 patients who quit CPAP treatment at 6 months were identified. Among them, 27 OSA patients quit despite having no side effects for discontinuing CPAP (Group A) and were compared to a matched group (age, body mass index, and apnea–hypopnea index) with good 6-month CPAP adherence (Group B; n=21). Five minutes of respiratory signal during wakefulness at the initial PSG were extracted from respiratory inductance plethysmography recordings, and measured in a blinded fashion. The coefficients of variation (CV) for the breath-to-breath inspiration time (T_i), expiration time (T_e), T_i+T_e (T_{tot}), and relative tidal volume, as well as an independent information theory-based metric of signal pattern variability (mutual information) were compared between groups.

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