Association between Plasma Neutrophil Gelatinase Associated Lipocalin Level and Obstructive Sleep Apnea or Nocturnal Intermittent Hypoxia

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Abstract

Background: Both obstructive sleep apnea (OSA) and a novel lipocalin, neutrophil gelatinase associated lipocalin (Ngal), have been reported to be closely linked with cardiovascular disease and loss of kidney function through chronic inflammation. However, the relationship between OSA and Ngal has never been investigated.

Objectives: To evaluate the relationship between Ngal and OSA in clinical practice.

Methods: In 102 patients, polysomnography was performed to diagnose OSA and plasma Ngal levels were measured. The correlations between Ngal levels and OSA severity and other clinical variables were evaluated. Of the 46 patients who began treatment with continuous positive airway pressure (CPAP), Ngal levels were reevaluated after three months of treatment in 25 patients.

Results: The Ngal level correlated significantly with OSA severity as determined by the apnea hypopnea index ($r = 0.24$, $p = 0.01$) and 4% oxygen desaturation index (ODI) ($r = 0.26$, $p = 0.01$). Multiple regression analysis showed that the Ngal level was associated with 4%ODI independently of other clinical variables. Compliance was good in 13 of the 25 patients who used CPAP. Although the OSA (4%ODI: 33.1 ± 16.7 to 1.1 ± 1.9/h, $p < 0.01$) had significantly improved in those with good compliance, the Ngal levels were not significantly changed (60.5 ± 18.1 before CPAP vs 64.2 ± 13.9 ng/ml after CPAP, $p = 0.27$).

Conclusions: Plasma Ngal levels were positively associated with the severity of OSA. However, the contribution rate of OSA to systemic Ngal secretion was small and changes in Ngal levels appeared to be influenced largely by other confounding factors. Therefore, it does not seem reasonable to use the Ngal level as a specific biomarker of OSA in clinical practice.

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Introduction

Neutrophil gelatinase associated lipocalin (Ngal), also known as lipocalin 2, is a 25-kDa secretory glycoprotein that was originally identified in human neutrophils. This protein was originally known as an innate immunity antibacterial factor released by activated neutrophils. [1,2] It has also become known to be produced by renal tubular cells in response to different types of injury. [3] Based on experimental and clinical findings, Ngal is widely considered as an excellent indicator of acute and chronic kidney injury.[3–7] Moreover, because this protein is also released by endothelial cells and failing myocardium, a close relationship between blood Ngal levels and heart failure or cardiovascular diseases has been suggested.[8–10].

Obstructive sleep apnea (OSA) is a highly prevalent disorder, affecting about 4–20% of adults and is characterized by repetitive episodes of partial or complete obstruction of the upper airway during sleep associated with transient oxygen desaturation.[11–13] Accumulating clinical evidence suggests that OSA is an independent risk factor for cardiovascular disease and loss of kidney function through nocturnal hypoxia and chronic inflammation.[14–17] From an in vitro model of OSA, it was suggested that the pro-inflammatory transcription factor, nuclear factor-kappa B (NF-κB), plays an important role in the inflammatory

process of a cell's reaction to intermittent hypoxia/reoxygenation. [18] Meanwhile, it has been reported that several inflammatory stimuli, such as interleukin 1β, stimulate systemic Ngal expression and secretion. NF-κB also has been shown to transactivate Ngal expression, suggesting that Ngal might be involved in inflammatory responses. [19,20].

Therefore, a positive correlation between OSA severity and systemic Ngal secretion through chronic inflammation seems possible. However, this relationship has never been investigated. Thus, we hypothesized that blood Ngal levels are elevated in patients with OSA and that its levels are modified by the treatment of OSA with continuous positive airway pressure (CPAP). In the present study, we measured plasma Ngal levels in patients with OSA and evaluated its utility in clinical practice.

Methods

Subjects

Study patients were consecutively recruited from the Sleep Unit of Kyoto University Hospital between January 2009 and May 2012. All had been referred to our sleep unit under suspicion of OSA with symptoms such as habitual snoring or daytime sleepiness. None had been previously diagnosed with or treated for OSA. Patients with overt renal failure (serum creatinine >1.3 mg/dl) or with any history of cardiovascular diseases, heart failure or arrhythmia were excluded because severe renal and/or heart failure can directly affect plasma Ngal levels. Also excluded were patients with pulmonary diseases, chronic infection, history of cancer or collagen disease. Since a consensus about the relationship between Ngal levels and metabolic syndrome has not yet been formed, we aimed to evaluate the correlations between risk factors for metabolic syndrome and plasma Ngal levels in actual clinical practice. We did not exclude patients with components of metabolic syndrome such as hypertension, diabetes and dyslipidemia even if they were under treatment for these comorbidities.[21–23] This study was approved by Kyoto University Graduate School and Faculty of Medicine Ethics Committee, and written informed consent was obtained from all patients.

Polysomnography and CPAP Implementation

The diagnosis of OSA was confirmed by polysomnography (SomnoStar pro, Cardinal Health, Dublin, OH, USA or Alice 4, Philips Respironics, Inc., Murrysville, PA, USA), which was started at 22:00 and ended at 6:00 the following morning. Surface electrodes were attached using standard techniques to obtain an electrooculogram, electromyogram of the chin and 12-lead electroencephalograph. Sleep stages were defined according to the criteria of Rechtchaffen and Kales. [24] Ventilation was monitored by inductive plethysmography (Respitrace QDC, Viasys Healthcare, Palm Springs, CA, USA). Airflow was monitored by a nasal pressure transducer and supplemented by an oronasal thermal sensor. Arterial oxygen saturation (SpO_2) was monitored continuously with a pulse oximeter.

Apnea was defined as the continuous cessation of airflow for more than 10 seconds and hypopnea was defined as a reduction in airflow of 30% or more lasting for 10 seconds or more accompanied by a decrease in SpO_2 of at least 4%. [25] Apnea-hypopnea index (AHI) values were calculated as the number of episodes of apnea and hypopnea per hour over the total sleep time. 4% oxygen desaturation index (ODI) values were defined as the

Table 1. Baseline characteristics and data on metabolic syndrome and its components in study patients.

	non OSA (n = 15)	mild OSA (n = 37)	moderate OSA (n = 24)	severe OSA (n = 26)	p
Age (y)	48.2±17.2	55.1±13.3	57.3±14.5	58.6±11.5	0.12
Sex (male), n(%)	8 (53.3)	24 (64.9)	18 (75)	18 (69.2)	0.56
Smoking status never/ex/current, n	2/3/10	5/13/19	3/5/16	3/8/15	0.88
Body mass index (kg/m^2)	23.5±3.7	25.1±4.6	24.8±4.1	29.2±7.8^{a,b,c}	<0.01
Neck circumference (cm)	36.2±3.1	37.9±3.7	37.8±3.6	39.8±4.0^{a}	0.03
Waist circumference (cm)	84.3±11.5	90.1±13.1	89.7±11.0	98.6±14.4^{a,b}	0.01
Hip circumference (cm)	90.3±8.8	94.6±10.6	92.1±9.3	101.3±15.3^{a,c}	0.01
Waist-to-hip ratio	0.93±0.07	0.95±0.05	0.97±0.04	0.97±0.03	0.04
SBP (mmHg)	119.8±16.1	124.2±16.3	127.3±12.9	127.4±16.0	0.40
DBP (mmHg)	72.7±11.9	76.7±11.5	78.6±11.2	76.2±12.0	0.50
Percentages of patients with metabolic syndrome or components of metabolic syndrome					
Hypertension, n (%)	7 (46.7)	20 (54.1)	15 (62.5)	19 (73.1)	0.16
Hyperglycemia, n (%)	5 (33.3)	7 (18.9)	4 (16.7)	5 (19.2)	0.56
Dyslipidemia, n(%)	6 (40.0)	16 (43.2)	11 (45.8)	13 (50.0)	0.98
Visceral fat accumulation, n(%)	7 (46.7)	22 (59.5)	18 (75.0)	23 (88.5)	0.01
Metabolic syndrome, n(%)	5 (33.3)	13 (35.1)	8 (33.3)	11 (42.3)	0.90
Percentages of patients under treatment for components of metabolic syndrome					
Hypertension, n (%)	2 (13.3)	14 (37.8)	9 (37.5)	12 (46.2)	0.16
Diabetes, n (%)	2 (13.3)	3 (8.1)	2 (8.3)	5 (19.2)	0.56
Dyslipidemia, n(%)	4 (26.7)	8 (21.6)	5 (20.8)	6 (23.1)	0.98

Data are expressed in mean ± SD or n (%).
OSA: obstructive sleep apnea; SBP: systolic blood pressure; DBP: diastolic blood pressure;
$^{a}p<0.05$ vs non OSA; $^{b}p<0.05$ vs mild OSA; $^{c}p<0.05$ vs moderate OSA.
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Table 2. OSA parameters and laboratory profiles.

	non OSA (n = 15)	mild OSA (n = 37)	moderate OSA (n = 24)	severe OSA (n = 26)	p
Parameters of OSA					
Apnea hypopnea index/h	2.2±1.6	9.4±2.6	22.2±5.0[a,b]	50.5±19.8[a,b,c]	<0.01
4%ODI/h	1.8±1.6	8.2±3.3	20.7±6.0[a,b]	49.6±20.2[a,b,c]	<0.01
Minimum SpO_2 (%)	90.3±4.5	84.9±4.3	78.4±8.6[a,b]	71.3±12.2[a,b,c]	<0.01
Arousal index/h	24.3±12.1	22.8±9.6	30.7±13.2	43.4±18.3[a,b,c]	<0.01
Length of time SpO_2<90% (m)	3.2±5.4	11.4±17.4	37.3±52.2	121.1±107.8[a,b,c]	<0.01
Laboratory profiles					
FPG (mg/dl)	107.7±39.9	95.4±20.1	96.4±24.3	102.5±20.2	0.35
HbA1c (%)	5.80±1.22	5.49±0.65	5.45±0.91	5.74±0.86	0.45
Total cholesterol (mg/dl)	185.5±32.3	195.2±35.4	203.0±44.0	201.5±44.0	0.53
LDL cholesterol (mg/dl)	103.2±27.9	116.2±27.8	118.5±36.8	109.5±31.8	0.40
HDL cholesterol (mg/dl)	55.2±12.9	52.8±13.5	53.0±16.1	51.9±13.9	0.91
Triglycerides (mg/dl)	125.1±86.5	119.8±58.0	139.1±79.1	168.0±185.2	0.39
BNP (pg/ml)	14.4±8.7	20.6±24.4	22.4±31.8	21.5±18.7	0.73
Creatinine (mg/dl)	0.72±0.19	0.74±0.15	0.80±0.17	0.78±0.21	0.45
Ngal (ng/ml)	46.9±6.0	48.9±10.9	51.3±15.2	55.4±16.7	0.16

Data are expressed in mean ± SD or n (%).

OSA: obstructive sleep apnea; ODI: oxygen desaturation index; SpO_2: saturation of oxygen; FPG: fasting plasma glucose; LDL: low density lipoprotein; HDL: high density lipoprotein; BNP: brain natriuretic peptide; Ngal: neutrophil gelatinase associated lipocalin.

[a]p<0.05 vs non OSA; [b]p<0.05 vs mild OSA; [c]p<0.05 vs moderate OSA.

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number of desaturations ≥4% per hour of sleep. The length of time SpO_2<90% during sleep was calculated in each patient. Patients with central sleep apnea were excluded. OSA severity was defined by the AHI as follows: non OSA (AHI<5), mild OSA (5≤AHI<15), moderate OSA (15≤AHI<30) and severe OSA (30≤AHI).

Patients with an AHI ≥15 were candidates for nasal CPAP. Those who agreed with CPAP implementation underwent a second polysomnography with CPAP titration. We implemented CPAP with the auto adjusting positive airway pressure (PAP) function for all patients. Based on the second sleep study, minimum and maximum PAP were determined to abolish all respiratory events, arousal and desaturation events.

Follow-Up

At the three-month follow-up, we urged the patients to undergo a third sleep study to confirm whether an adjustment of the CPAP setting was necessary. To investigate the effect of CPAP treatment on plasma Ngal levels, at the third sleep study blood samples were collected in the same way as at the first sleep study. We also checked use time of the CPAP machine by reading the time counter on the CPAP machines. Similar to prior studies, we defined 'good compliance' as the use of CPAP for >4 h per night on >70% of nights and categorized the patients into two groups, those with 'good compliance' or 'poor compliance'. [26] We analyzed the data separately for each group and compared clinical variables before and after CPAP treatment.

Blood Sampling and Measurement of Plasma Ngal Level

Blood samples were drawn at 7:00 in the morning after the subjects had fasted beginning at 20:00 the previous night. Blood samples were centrifuged immediately at 3,000 rpm at 4°C for 10 min. The separated samples were stored −80°C until assay. Plasma Ngal concentrations were determined by an ELISA kit provided by Bioporto Diagnostics, Gentofte, Denmark. Intra- and inter-assay coefficients of variation for Ngal were 1.2–4.0% and 2.2–11.2%, respectively.

Definition of Metabolic Syndrome

In classifying patients based on the components of metabolic syndrome, we utilized Japanese criteria. [27] Waist circumference (WC) was measured at the level of the navel with the patient standing, and visceral fat accumulation was determined to be positive at WC ≥85 cm for men and ≥90 cm for women. A diagnosis of metabolic syndrome required the subject to have visceral fat accumulation and 2 or 3 of the following: (a) dyslipidemia (triglycerides ≥150 mg/dL and/or high-density lipoprotein cholesterol level <40 mg/dL, or specific treatment for these lipid abnormalities); (b) hypertension (systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg, or treatment of previously diagnosed hypertension); and (c) hyperglycemia (fasting plasma glucose ≥110 mg/dL or specific treatment for diabetes mellitus). Anthropometric parameters and blood pressure were measured immediately after polysomnography recording ended.

Statistical Analysis

In the analysis of data, we classified the patients depending on the severity of OSA and compared their clinical backgrounds. We also compared plasma Ngal levels between patients with and without each component of metabolic syndrome to investigate the relationships between plasma Ngal levels and metabolic syndrome. Data were expressed as means ± standard deviation. The significance of intergroup differences based on the severity of OSA was determined by an analysis of variance. When a significant difference was found, we used the Tukey's honestly

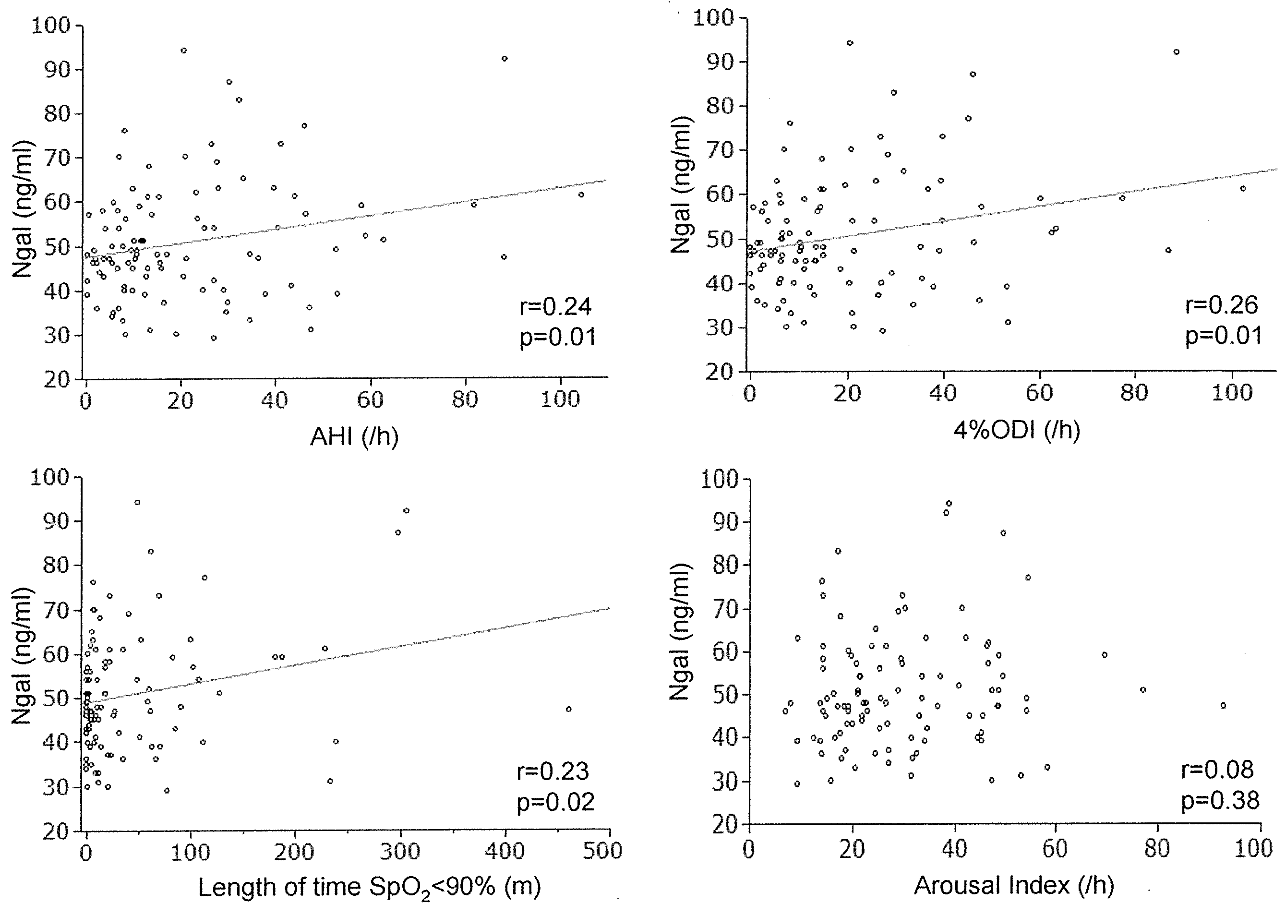

Figure 1. Simple correlations between plasma neutrophil gelatinase associated lipocalin (Ngal) levels and parameters of obstructive sleep apnea. AHI: apnea hypopnea index; ODI: oxygen desaturation index; SpO_2: saturation of oxygen.
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significant difference procedure to identify where the difference was significant. A chi-square test and the Mann-Whitney U test were used to compare categorical and continuous variables, respectively. We used Pearson's coefficient tests to evaluate the relationship between the plasma Ngal level and other continuous variables. Based on the results of this analysis, multiple regression analyses were performed to clarify the contribution rate of OSA and other comorbidities to systemic Ngal secretion. Wilcoxon signed rank test was used to compare clinical variables before and after CPAP treatment. Two-tailed p-values <0.05 were considered statistically significant. All statistical analyses were performed using JMP 7.0.2 statistical software (SAS Institute Inc., Cary, NC, USA).

Results

Baseline Characteristics of Study Patients

A total of 102 patients were studied, and their baseline characteristics are shown in table 1.Those with severe OSA were characterized by a significantly higher body mass index (BMI) than in the other three groups. The percentages of patients who fulfilled the criterion for visceral fat accumulation increased as the severity of OSA increased. Other anthropometric parameters with significant differences among the groups are also shown in table 1. With the exception of the parameters for OSA, there were no significant differences among the four groups in other clinical background factors. (Tables 1 and 2).

Plasma Ngal Levels in Patients at Diagnosis and follow up

Table 2 shows baseline plasma Ngal levels in the four groups, with no statistically significant differences found among them. However, simple linear regression analysis showed significant correlations of the plasma Ngal level with the following parameters of OSA: AHI ($r = 0.24$, $p = 0.01$), 4%ODI ($r = 0.26$, $p = 0.01$) and time of $SpO_2<90\%$ ($r = 0.23$, $p = 0.02$). (Figure 1) The plasma Ngal level was also correlated with values for serum low density lipoprotein (LDL) cholesterol ($r = -0.31$, $p<0.01$), triglycerides ($r = 0.24$, $p = 0.01$) and creatinine ($r = 0.34$, $p<0.01$). On the other hand, none of anthropometric parameters and parameters associated with diabetes such as fasting plasma glucose and HbA1c levels showed significant correlations with Ngal levels.(Table 3) Furthermore, in the present cohort, significant differences were not found in plasma Ngal levels between patients with and without each of the components of metabolic syndrome. (Table 4).

For the multiple regression analysis, we chose 4%ODI as the representative variable for OSA severity as it had the best correlation with the Ngal level among OSA parameters in the simple correlation analysis. The analysis demonstrated that 4%ODI was associated with the Ngal level independently of creatinine and LDL-cholesterol levels. The contribution rate of 4% ODI to the Ngal level was 6.2% (Table 5).

Table 3. Simple correlations between plasma neutrophil gelatinase associated (Ngal) levels and clinical variables.

	r	p
Age (y)	0.04	0.62
Body mass index (kg/m^2)	0.13	0.17
Neck circumference (cm)	0.00	0.84
Waist circumference (cm)	0.00	0.91
Hip circumference (cm)	0.00	0.99
Waist-to-hip ratio	0.03	0.76
SBP (mmHg)	0.00	0.96
DBP (mmHg)	−0.13	0.17
FPG(mg/dl)	−0.08	0.39
HbA1c (%)	−0.06	0.52
Total cholesterol (mg/dl)	0.12	0.21
LDL-cholesterol (mg/dl)	−0.31	<0.01
HDL-cholesterol (mg/dl)	−0.18	0.06
Triglycerides (mg/dl)	0.24	0.01
BNP (pg/ml)	0.00	0.74
Creatinine (mg/dl)	0.34	<0.01

r: correlation coefficient; SBP: systolic blood pressure; DBP: diastolic blood pressure; FPG: fasting plasma glucose; LDL: low density lipoprotein; HDL: high density lipoprotein; BNP: brain natriuretic peptide.
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CPAP was implemented for 46 of the 50 patients with moderate or severe OSA. Of the 46 patients, 27 agreed to a follow-up sleep study. Just before the reevaluation, cardiac medicine was prescribed for one patient and an upper airway infection was found in another patient. These two patients were excluded from the analysis, and the remaining 25 patients were reevaluated. Thirteen were categorized into the good compliance group and the other 12 patients into the poor compliance group. Those in the good compliance group were significantly older than patients in the poor compliance group. The determined maximum and minimum PAP did not differ between the two groups.

After CPAP implementation, OSA was significantly improved in both groups. In the good compliance group, despite improvements in OSA, no significant change was noted in plasma Ngal levels from values before CPAP use. Furthermore, in the poor compliance group, Ngal levels were significantly elevated after CPAP implementation. There were not significant differences in the other confounding factors before and after CPAP treatment (Table 6).

Discussion

In this cross sectional evaluation, although significant differences in plasma Ngal levels were not found among groups classified according to the severity of OSA, parameters of OSA, such as 4%ODI and AHI per se, correlated with plasma Ngal levels in regression analysis. This suggests that OSA contributes, although weakly, to elevated plasma Ngal levels through nocturnal hypoxia. Because it has been reported that hypoxia induces an elevation in plasma Ngal levels in an experimental animal model, it is possible that OSA induces Ngal elevation through nocturnal intermittent hypoxia. [28] To the best of our knowledge, this is the first report to evaluate the relationship between the Ngal protein level and OSA severity in clinical practice.

Table 4. Plasma Ngal levels in patients with and without each component of metabolic syndrome.

	Plasma Ngal levels (ng/ml)		
	Comorbidity(+)	Comorbidity(−)	p
Hypertension	52.7±1.7	48.0±2.1	0.17
	(n = 61)	(n = 41)	
Hyperglycemia	48.4±14.5	51.4±13.0	0.27
	(n = 21)	(n = 81)	
Dyslipidemia	51.9±14.7	49.9±12.2	0.49
	(n = 46)	(n = 56)	
Visceral fat accumulation	50.9±14.1	50.5±11.6	0.92
	(n = 70)	(n = 32)	
Metabolic syndrome	51.8±16.7	50.2±11.1	0.93
	(n = 37)	(n = 65)	

Data are expressed in mean ± SD.
Ngal: neutrophil gelatinase associated lipocalin.
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The relationship between another protein in the lipocalin family and OSA has been investigated. Makino et al and Nena et al, respectively, investigated the relationship between the plasma level of retinol binding protein 4 (RBP-4), which also belongs to the lipocalin protein family, and OSA. [29,30] However, neither study found a correlation between RBP-4 levels and apnea-related indices. Although both Ngal and RBP-4 belong to the lipocalin family and share a common tertiary structure, these two proteins appear to have different patterns of regulation in response to inflammatory mediators. [21,23].

Our results also demonstrated a significant inverse correlation between Ngal and LDL cholesterol levels. Wallenius et al also reported such an inverse correlation in their epidemiological study. [21] However, in other studies, correlations between these two variables were not found. [23,31,32] Although this inverse correlation is possible, the results seem to vary depending on the clinical characteristics of the examined cohorts. Furthermore, the mechanisms of this correlation remain utterly unknown.

The relationship between Ngal and metabolic syndrome is quite controversial. Whereas Wang et al and Yan et al reported a close

Table 5. Multiple regression analyses using plasma neutrophil gelatinase associated lipocalin (Ngal) level as a dependent variable.

	p	β	r	R^2(%)
Body mass index (kg/m^2)	0.39	–		
4%ODI/h	<0.01	0.24	0.26	6.2
LDL-cholesterol (mg/dl)	<0.01	−0.29	−0.31	9.0
HDL-cholesterol (mg/dl)	0.71	–		
Triglycerides (mg/dl)	0.31	–		
Creatinine (mg/dl)	<0.01	0.28	0.34	9.5
Cumulative R^2				24.7

β: standard regression coefficient; r: correlation efficient; R^2: contribution rate; ODI: oxygen desaturation index; LDL: low density lipoprotein; HDL: high density lipoprotein.
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Table 6. Changes in clinical variables from baseline to after CPAP implementation.

	CPAP good compliance (n = 13)			CPAP poor compliance (n = 12)			
	before CPAP	after CPAP	p*	before CPAP	after CPAP	p*	p#
Ngal (ng/ml)	60.5±18.1	64.2±13.9	0.27	52.8±16.8	63.1±14.2	<0.01	-
4%ODI (/h)	33.1±16.7	1.1±1.9	<0.01	41.5±22.5	1.5±2.3	<0.01	-
Creatinine (mg/dl)	0.85±0.20	0.88±0.19	0.13	0.77±0.21	0.79±0.20	0.25	-
LDL cholesterol (mg/dL)	107.5±32.7	103.8±30.8	0.70	115.2±28.2	121.0±28.0	0.58	-
Body mass index (kg/m^2)	23.9±2.0	23.9±2.2	0.85	28.2±6.6	28.6±6.6	0.14	
Age (y)	67.5±8.3		-	54.5±12.2		-	0.01
Days with CPAP use >4 h (%)	85.8±9.6		-	43.3±20.7		-	<0.01
Maximum PAP (cmH$_2$O)	9.9±2.8		-	10.8±2.5		-	0.62
Minimum PAP (cmH$_2$O)	4.5±0.9		-	4.7±0.8		-	0.35

Data are expressed in mean±SD.
CPAP: continuous positive airway pressure; Ngal: neutrophil gelatinase associated lipocalin; ODI: oxygen desaturation index; LDL: low density lipoprotein; PAP: positive airway pressure; p*: p value for comparison with values before and after CPAP treatment; p# : p value for comparison between CPAP good compliance and CPAP poor compliance groups.
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association between Ngal and obesity or insulin resistance, Wallenius et al found no correlation between these risk factors. [21,22,31] Our results did not show any significant correlation between Ngal levels and obesity or diabetic indices. Also, in the present cohort there were no significant differences in Ngal levels between patients with and without metabolic syndrome. These results also seem to depend on the clinical characteristics of the cohorts. Specifically, we did not exclude patients under treatment for metabolic syndrome to investigate the utility of the plasma Ngal level in actual clinical practice. Because it was reported that the pharmaceutical treatment of diabetes and dyslipidemia can change plasma Ngal levels, treatment of metabolic syndrome in our cohort possibly affected the results. [31,33] Furthermore, in most of these studies, renal function was not taken into account as an explanatory variable of the Ngal level. Risk factors for metabolic syndrome induce latent renal function impairment and even a subtle change in renal function is known to affect blood and urinary Ngal levels. [34] Giaginis et al reported that plasma Ngal levels were higher in patients with than without hypertension and they speculated that the association between elevated Ngal levels and hypertension is secondary to the confounding effect of renal impairment. [35] In our study, even though we did not include patients with overt renal failure, the plasma Ngal levels correlated significantly with serum creatinine levels. Therefore, in evaluating the direct link between metabolic syndrome and Ngal levels, statistical correction for renal function seem to be necessary. In fact, Liu et al reported that the significant correlation between Ngal and insulin resistance detected in their study cohort disappeared after adjustment for serum creatinine values. [32].

Contrary to our expectation, plasma Ngal levels did not change even with the appropriate use of CPAP that effected an improvement in OSA. We could not confirm a direct causality between Ngal levels and OSA. Although we based the three-month treatment observation period on experiences in previous studies, we might need an extended period to find more remarkable changes in Ngal levels in the present cohort. [23,31].

Ngal levels were elevated after CPAP implementation in patients with poor compliance with CPAP. Because we did not include an actual control group that did not use CPAP, we could not judge whether the change in Ngal levels was caused by incomplete CPAP use or other reasons. Although we took every conceivable confounding factor into account, other determinants that we were not aware of might have influenced the results. In the cross sectional studies, the contribution rate of 4%ODI to Ngal levels was not large (6.2%). Therefore, it seems quite possible that other determinants negated the influence of the improvement in OSA. The presence of certain components of metabolic syndrome and their treatment in the present cohort might be among these determinants.

We recognize several limitations in the present study. First, the sample size was small, so it is not reasonable to extrapolate our data to the general population. In addition, the results of this study might have been influenced by the small sample size. Second, as we previously noted, we did not exclude patients with comorbidities such as hypertension and diabetes even if they were under treatment. It is possible that these comorbidities and their treatment affected the results. Third, as mentioned above, we did not have a planned control group without CPAP use. Therefore, we could not judge precisely whether changes in Ngal levels after CPAP implementation were caused by CPAP use or other reasons. Lastly, we did not measure C reactive protein (CRP) levels. Because inflammation has an influence on Ngal, results of measurement of high sensitive CRP would have been a good reflection of the inflammation status of patients and would have been helpful to achieve a more comprehensive understanding of the relationship between OSA and Ngal.

In summary, the present study provides the first clinical evidence demonstrating that plasma Ngal levels were positively but weakly associated with the severity of OSA. Plasma Ngal levels did not change after improvement in OSA, so we could not testify to the causality between Ngal levels and OSA severity. Because Ngal levels were just weakly correlated with the severity of OSA, changes in those levels appear to be influenced largely by other confounding factors. Thus, it would be difficult to use the Ngal level as a specific biomarker representing nocturnal hypoxia in OSA. The links between Ngal levels and metabolic syndrome remain controversial, and unrecognized determinants of plasma Ngal levels are likely to be present. Further studies are warranted to more comprehensively understand the regulation of Ngal in relation to OSA and metabolic syndrome.

Author Contributions

Conceived and designed the experiments: K. Murase K. Mori KC. Performed the experiments: K. Murase CY KA YC MA YH. Analyzed the data: K. Murase K. Mori. Contributed reagents/materials/analysis tools: YT KT T. Handa T. Hitomi TO MM. Wrote the paper: K. Murase K. Mori KC.

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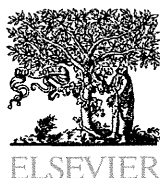

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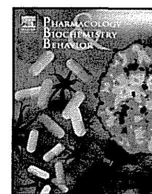

A mouse model mimicking human first night effect for the evaluation of hypnotics

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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.

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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

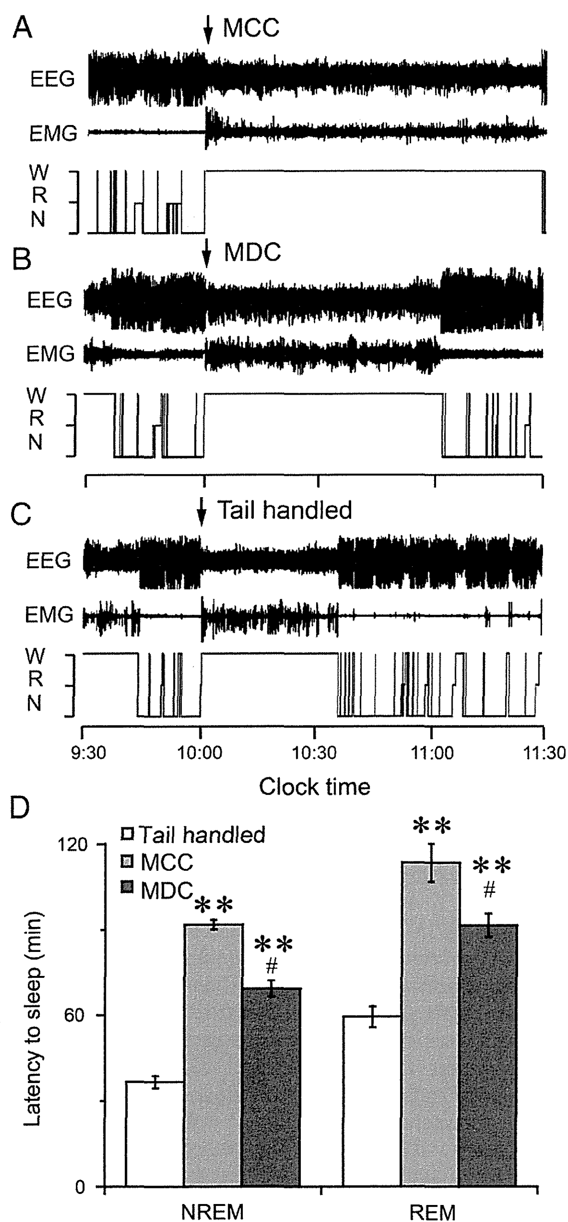

Fig. 1. Moving the mouse to a clean or dirty cage increased wakefulness. Typical examples of polygraphic recordings and corresponding hypnograms illustrating the effects of changing sleep environment on sleep of a mouse moving to a clean cage (MCC, A), moving to a dirty cage (MDC, B) or with tail handled 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 ($F_{3,22} = 18.05$, $p < 0.01$)

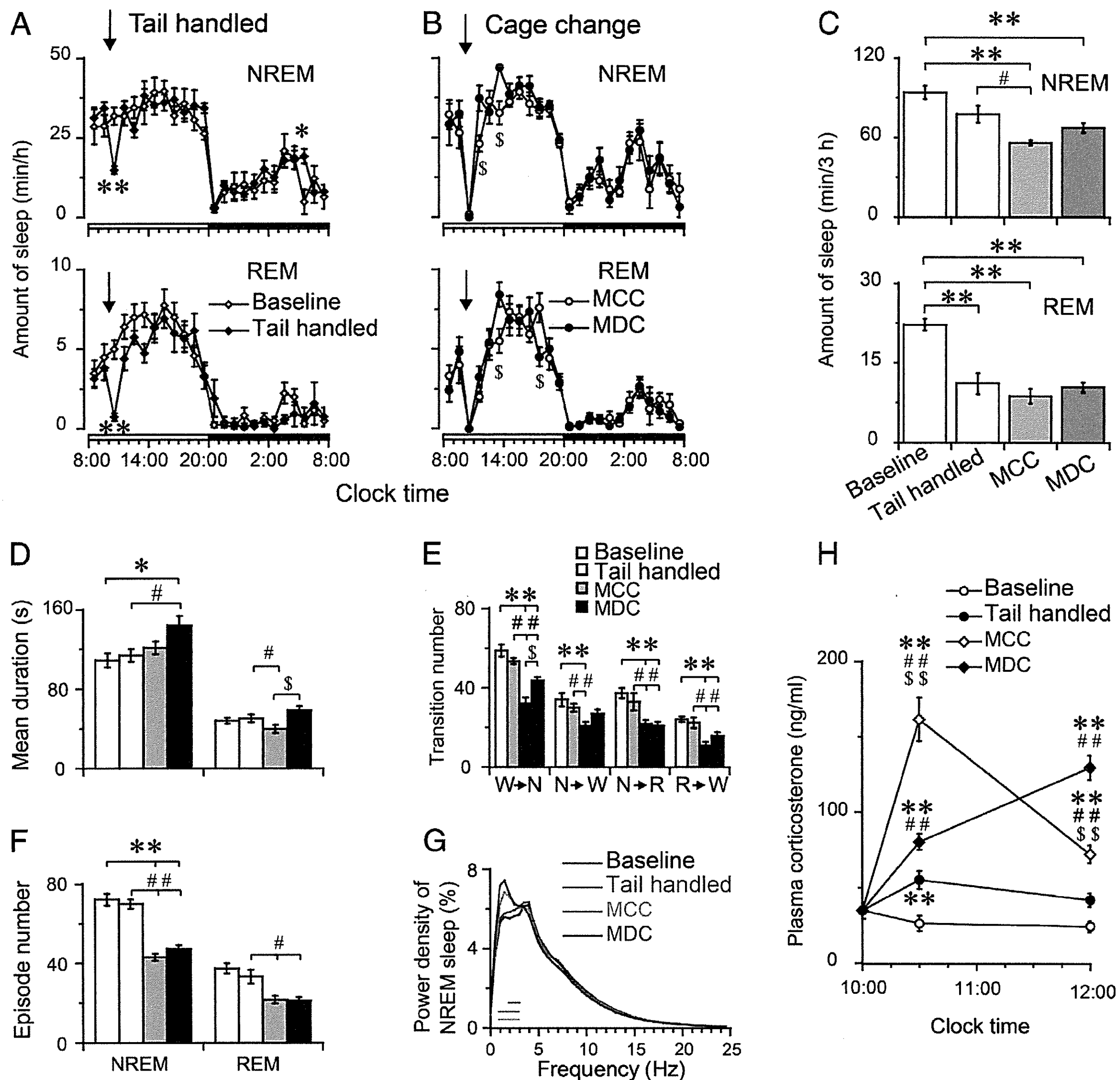

Fig. 2. Changing of cages induced sleep disturbance (A–G) and increased plasma corticosterone level (H) in mice. (A, B) Time course changes in NREM and REM sleep of the mice subjected to tail handled (A), moved to clean (MCC) or a dirty cages (MDC, B). Each circle represents the hourly mean amount of 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

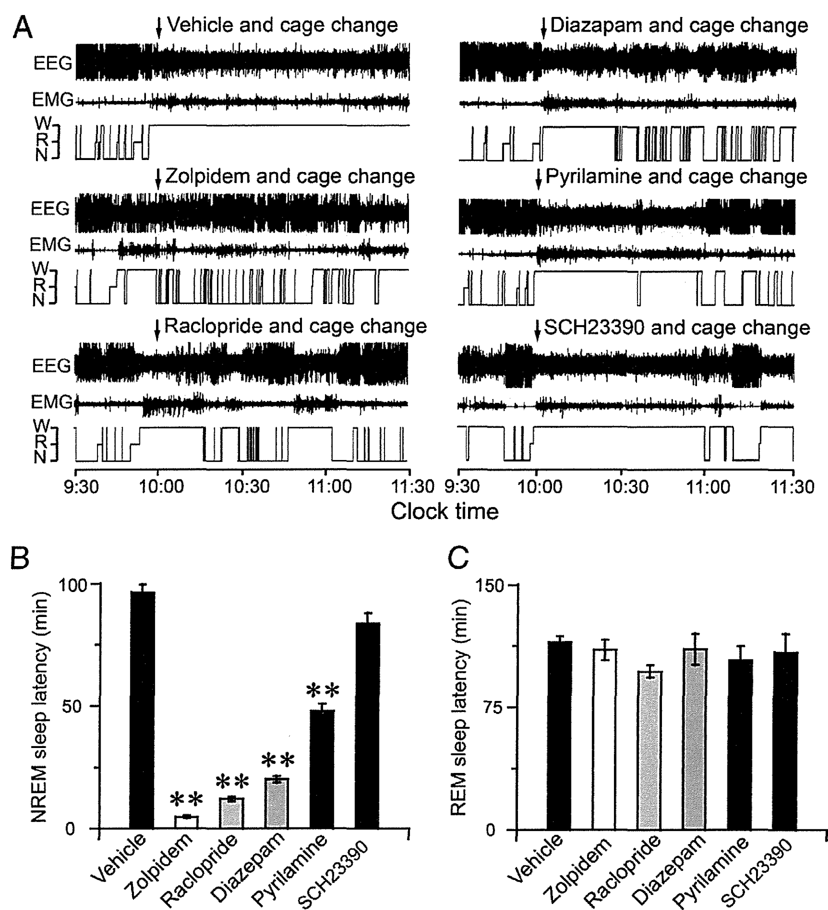

Fig. 3. Hypnotics shortened the latency to NREM sleep after moving the mice to clean cages. (A) Typical examples of polygraphic recordings and corresponding hypnograms illustrating the effects of hypnotics treatment in mice moved into clean cages. W, Wakefulness; R, REM sleep; N, NREM sleep. (B, C) Hypnotic 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

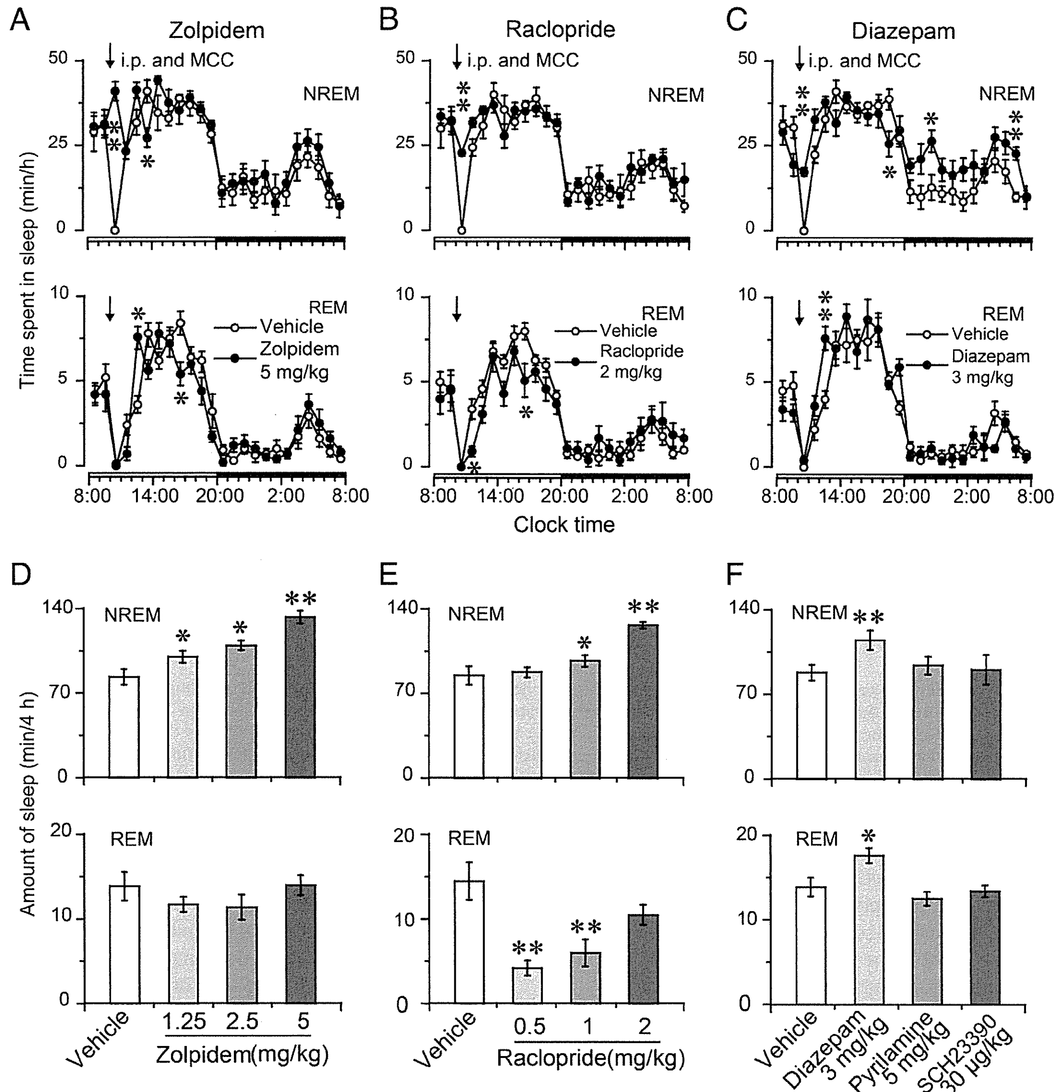

Fig. 4. Hypnotics increased the sleep time of the mice after 'moved to clean cages' (MCC). Effects of zolpidem (A), raclopride (B), diazepam (C) on sleep profiles of MCC mice. Each circle represents the hourly mean amount of each stage. The horizontal open and filled bars on the χ-axes indicate the 12 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

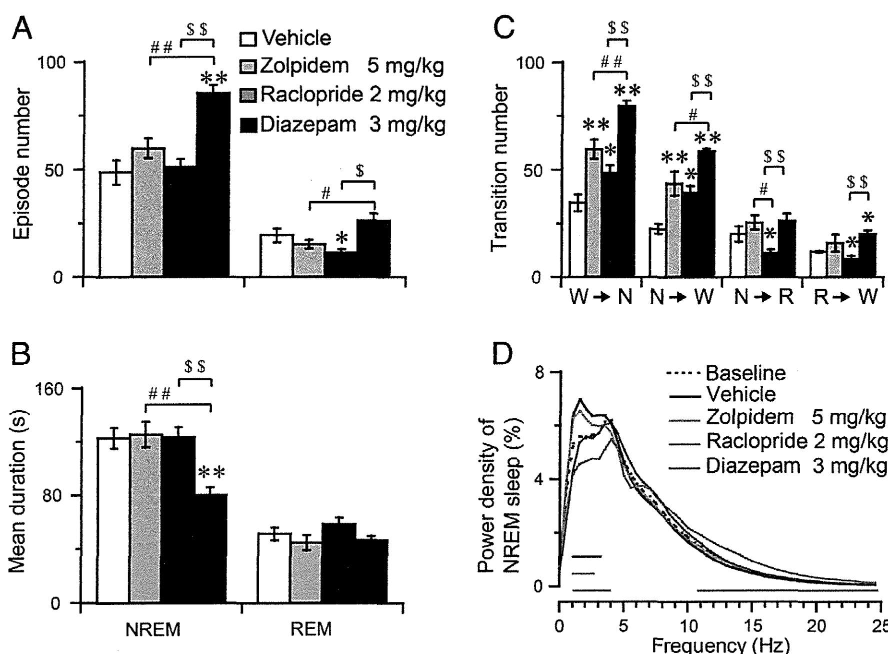

Fig. 5. Hypnotics improved the sleep disturbance in mice 'moved to clean cages' (MCC). Episode number (A), mean duration (B), and stage transitions (C) during 3 h (11:00–14:00) after drug treatment in MCC mice. (D) EEG power density from 11:00 to 14:00 after hypnotic treatment. The curves represent logarithmic 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.**

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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

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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 5×10^4 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

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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.

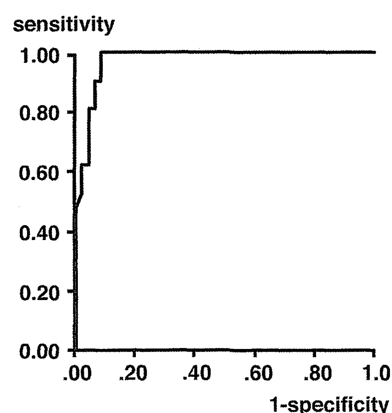

Figure 1 Receiver operating characteristic (ROC) curve analysis for the diagnosis of clinical cytomegalovirus (CMV) disease using a threshold level for CMV pp65 antigen (CMV-pp65Ag) in patients with CMV infection. The analysis included patients with clinical CMV disease ($n = 23$) and those with subclinical 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

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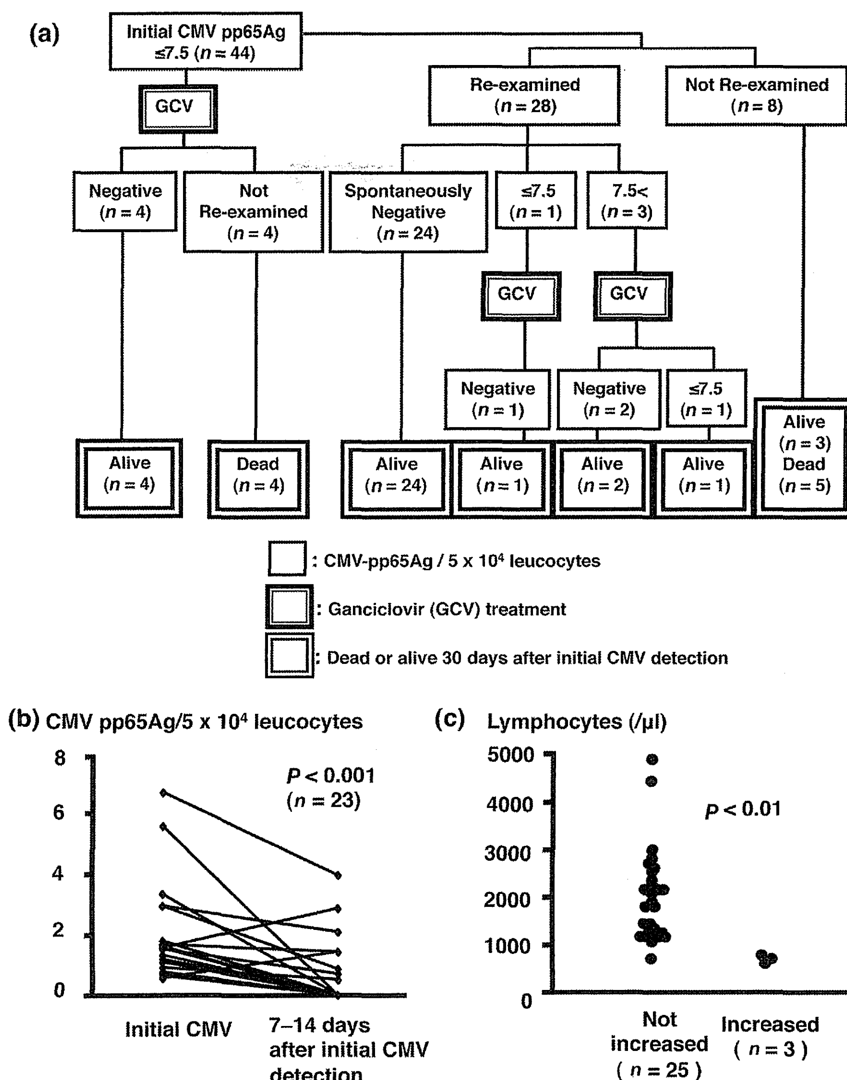

Figure 2 Longitudinal observation of the clinical course in patients with low levels of cytomegalovirus (CMV) pp65 antigen (CMV-pp65Ag) (≤7.5 cells per 5×10^4 leukocytes) at initial detection of CMV. Among all CMV pp65Ag-positive patients, 44 showed low levels of CMV-pp65Ag (a). In eight patients, ganciclovir (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

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