the second edition of the International Classification of Sleep Disorders (ICSD-2),[13] diagnoses of RBD were made by at least two sleep disorder expert neurologists or neuropsychiatrists based on results of both PSG findings and through clinical interviews with the patients or their bed partners. Patients with any abnormal neurological finding suggestive of PD, any other neurological or psychiatric disorder, moderate or severe sleep apnea (apnea-hypopnea index $\geq$ 15), or habitual use of any medication known to modify EEG activity or muscle tone were excluded.

To detect cognitive impairment, the Montreal Cognitive Assessment (MoCA)[14,15] and Mini-Mental State Examination (MMSE)[16] were administered to all participants before starting treatment. These neuropsychological tests were conducted during 01:00 to 03:00 on the day of Sniffin' Sticks Testing. Sleep disorder expert neurologists and neuropsychiatrists carefully reviewed patients' cognitive status. MCI was diagnosed according to the following criteria: subjective cognitive complaint, cognitive decline on neuropsychological testing, and preserved daily life activities.[8] The cognitive decline was judged by the MoCA and MMSE. Simultaneously, the Japanese version of RBD questionnaire (RBDQ-JP)[17] was self-checked by all participants to measure the severity of RBD symptoms.

PSG Recording and Scoring

A standard system (Alice 5; Respironics Inc., Murrysville, PA, USA) with video monitoring of patient behavior, diagnostic n-PSG recordings, and measurements including four channels of the scalp EEG (C3/A2, C4/A1, O1/A2, O2/A1), two electrooculographs (EOG), submental electromyograph (EMG), electrocardiograph, nasal/oral airflow measured using both thermistor and nasal pressure sensor, an oximetry sensor for saturation of peripheral oxygen (SpO_2) recording, a microphone for snoring sounds, chest/abdominal respiratory effort, and anterior tibialis electromyographs for leg movements (bipolar derivations with two electrodes placed 3 cm apart on the belly of the anterior tibialis muscle of right and left legs) was used.

PSG data were scored according to the current criteria set for scoring of sleep and associated events by the American Academy of Sleep Medicine (AASM)[18] with allowance to score slow wave sleep (SWS) activity in the central region. Sleep variables were scored as follows: wake after sleep onset (WASO), stage N1, N2, N3, and REM (% sleep period time), arousal index, and periodic leg movements during sleep (PLMS) index. Because patients with RBD are likely to show a lack of muscle atonia, REM sleep was scored without considering submental EMG atonia.[19] The onset and the termination of REM sleep were determined according to the method reported by Lapierre and Montplaisir.[19] Tonic EMG activity in submentalis muscle and phasic EMG activity in submentalis as well as anterior tibialis muscles were calculated according to the criteria set by AASM.[18]

Quantitative EEG Evaluation

The EEG samples during wake were recorded before starting the PSG recording. Technicians instructed the patients to lie awake in bed keeping the eyes closed. The EEG samples during REM sleep were selected from all sections of clear REM sleep between two REMs. The EEG samples during nonrapid eye movement (NREM) sleep were selected from N2 and N3 periods.[10] For all PSG data, recording artifacts were eliminated carefully by a board-certified sleep technician. Quantitative EEG evaluation was performed blindly using fast Fourier transform (FFT) on the artifact-free periods during wake, REM sleep, and NREM sleep.[11] The FFT on 4-sec epochs with a Hamming window yielding 0.25 Hz of spectral resolution was performed on C3/A2, C4/A1, O1/A2, and O2/A1 derivations using a computer program (CSA play analysis; Norpro Light Systems, Tokyo, Japan).

Statistical Analysis

To test the normality and equality of variances of the outcome parameters, the Shapiro-Wilk test and Levene test were conducted. After checking the normality and equality of variances with a P value greater than 0.05, the following analyses were conducted. Analysis of variance followed by Bonferroni *post hoc* test was used to compare clinical RBD variables and sleep variables among the iRBD groups (younger and older group) and healthy control patients. The Student *t*-test was used to compare the EEG spectral power between the younger iRBD group and healthy control patients. To investigate factors associated with deteriorated MoCA score, at first partial correlation analysis controlled by age was conducted on the clinical RBD variables, sleep variables, and EEG power of all patients with iRBD. Then, a multiple regression equation was used to identify an actual influence of the factors with stepwise selection of variables that appeared to be significant on the partial correlation analysis. The multiple regression analysis instead of the analysis of the groups stratified by age was conducted because the correlations between age and the EEG variables or clinical RBD variables were significant but weak. In the multiple regression analysis, the significance level was set as $P > 0.05$ to verify the significance of the multivariate model. The values of R (multiple correlation coefficient) and R^2 (the coefficient of determination) were also expected to be > 0.05 and to be > 0.26 to verify the goodness of fit of the model. Log-transformed EEG powers were used in the multiple regression equation. These statistical analyses were conducted using R version 2.15.1, or SPSS version 17.0 J (IBM, Tokyo, Japan) when possible. Significance was inferred for $P < 0.05$.

RESULTS

Clinical RBD-Related Variables and PSG Findings

As for clinical RBD-related variables, significant differences were found in RBDSQ-J ($F_{(2, 47)} = 100.9$, $P < 0.01$) and RBDQ-JP ($F_{(2, 47)} = 75.7$, $P < 0.01$) among the patient groups. *Post hoc* testing revealed that either the younger iRBD group or older iRBD group showed higher scores of RBDSQ-J and RBDQ-JP than the healthy control patients ($P < 0.01$).

As for PSG variables, significant differences were found in N3 ($F_{(2, 47)} = 2.2$, $P < 0.01$), stage REM ($F_{(2, 47)} = 9.8$, $P < 0.01$), and the PLMS index ($F_{(2, 47)} = 6.0$, $P < 0.01$) among the three groups. The older iRBD group showed significantly higher proportion of N3 than either the younger iRBD group or the control patients ($P < 0.01$). In addition, the older iRBD group

Table 1—Clinical, polysomnographic and neuropsychological findings

	Younger patients with iRBD (younger than 70 y) (1)	Older patients with iRBD (70 y or older) (2)	Control patients (3)	
Number of patients	17	14	17	
Age (y)	60.5 ± 5.3	73.6 ± 3.7	59.5 ± 5.6	1 < 2*, 2 > 3*
RBDSQ-J	9.9 ± 0.4	8.6 ± 2.1	2.3 ± 1.4	1 > 3*, 2 > 3*
RBDQ-JP	48.1 ± 8.4	42.6 ± 15.6	8.2 ± 4.7	1 > 3*, 2 > 3*
Morbidity (y)	6.7 ± 5.3	4.1 ± 2.5	–	n.s.
MoCA	24.6 ± 1.6	22.6 ± 2.0	26.9 ± 1.2	1 > 2*, 1 < 3*, 2 < 3*
MMSE	27.3 ± 2.2	26.1 ± 1.7	29.1 ± 1.1	1 < 3*, 2 < 3*
TDI score on Sniffin' Sticks Test	19.8 ± 6.2	17.2 ± 6.4	–	n.s.
Epworth Sleepiness Scale	7.7 ± 3.1	9.1 ± 6.0	8.1 ± 2.5	n.s.
Polysomnographic measures				
Phasic RWA (%)	23.9 ± 16.5	34.2 ± 39.1	–	n.s.
Tonic RWA (%)	8.8 ± 8.9	8.6 ± 7.9	–	n.s.
WASO (%)	12.8 ± 11.2	18.6 ± 9.2	10.8 ± 6.2	n.s.
N1 (%)	10.2 ± 4.3	8.8 ± 3.4	15.4 ± 7.8	n.s.
N2 (%)	49.8 ± 8.4	48.8 ± 9.6	49.1 ± 12.8	n.s.
N3 (%)	1.8 ± 2.4	4.6 ± 5.4	2.3 ± 4.8	1 < 2*, 2 > 3*
Stage REM (%)	16.4 ± 7.4	10.7 ± 6.1	20.6 ± 4.6	2 < 3*
Arousal index (/h)	19.9 ± 11.0	18.8 ± 5.7	17.7 ± 6.9	n.s.
PLMS index (/h)	26.1 ± 30.4	35.5 ± 37.3	3.2 ± 8.7	1 > 3*, 2 > 3*
AHI (/h)	3.8 ± 1.2	5.3 ± 4.0	7.4 ± 2.6	n.s.

*P < 0.05 for Student *t*-test, and P < 0.017 for Bonferroni *post hoc* test following analysis of variance. Data are expressed as mean ± standard deviation. AHI, apnea-hypopnea index; MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; PLMS, periodic leg movements during sleep; RBDQ-JP, Japanese version of REM sleep behavior disorder questionnaire; RBDSQ-J, Japanese version of REM sleep behavior disorder screening questionnaire; RWA, REM sleep without atonia; SPT, sleep period time; TDI, threshold-discrimination-identification; WASO, wake after sleep onset.

showed a lower proportion of stage REM compared with the control patients ($P < 0.01$). Either the younger iRBD group or older iRBD group had a higher PLMS index than the control patients ($P < 0.01$) (Table 1).

Cognitive Function Measures

Significant differences in the scores of MoCA ($F_{(2, 47)} = 28.6$, $P < 0.01$) and MMSE ($F_{(2, 47)} = 11.6$, $P < 0.01$) were found among the patient groups (Table 1). The younger iRBD group showed significantly lower scores on MoCA and MMSE than the age-matched control patients ($P < 0.05$). The older iRBD group showed a significantly lower score than the younger group did only on the MoCA test ($P < 0.05$).

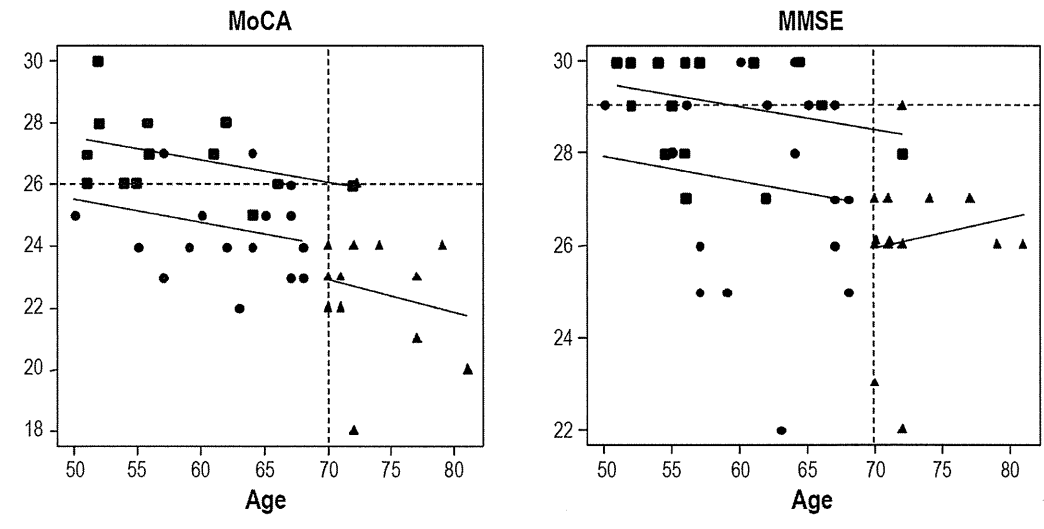

Figure 1—Distribution of the MoCA and MMSE scores among ages. Circles signify the younger RBD group (younger than 70 y), triangles signify the older RBD group (70 y or older), and squares signify the control group for the younger RBD group. MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; RBD, rapid eye movement sleep behavior disorder.

Figure 1 presents the distribution of the scores of MoCA and MMSE. The MoCA score was lower than the cutoff (25 of 26) for detecting MCI[20,21] in 13 of 17 (76.5%) of the younger iRBD group, 13 of 14 (92.9%) of the older iRBD group, and 1 of 17 (5.9%) of control patients. The MMSE score was lower than the cutoff (29 of 30) for detecting MCI[21] in 10 of 17 (58.8%) of

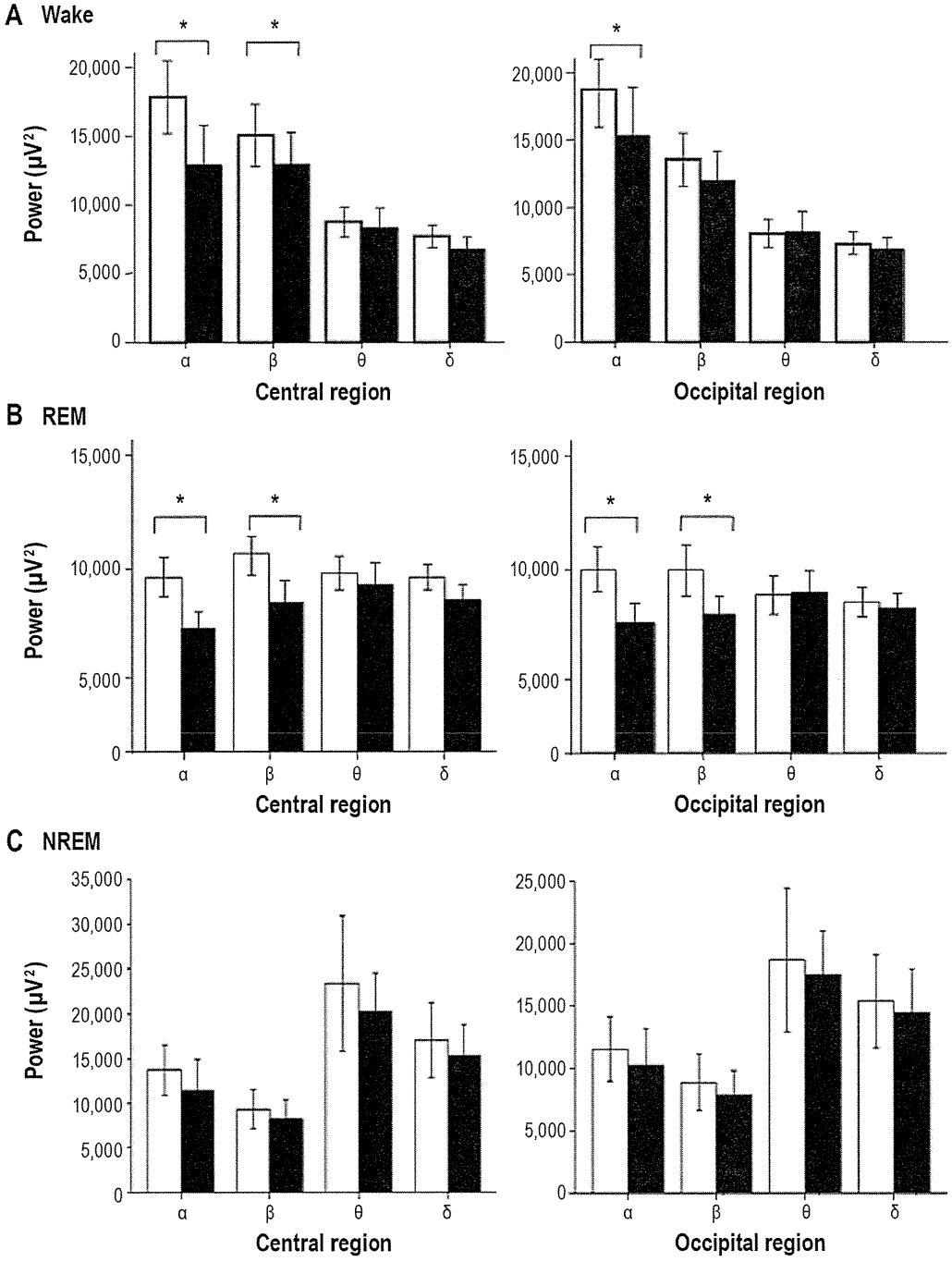

Figure 2—Comparison of electroencephalographic spectral power during wake, rapid eye movement sleep, and non-rapid eye movement sleep in patients with idiopathic rapid eye movement sleep behavior disorder and control patients. Black bars show data for the younger rapid eye movement sleep behavior disorder group. White bars show data for the age-matched control group. *P < 0.05, values are expressed as mean ± standard deviation.

the younger iRBD group, 13 of 14 (92.9%) of the older iRBD group, and 4 of 17 (23.5%) of control patients.

Comparison of EEG Spectral Power Between the Younger iRBD Group and Control Patients

During Wake

The younger iRBD group showed significantly lower alpha power in central regions ($t_{(32)}$ = -2.488, P < 0.05, Cohen d = 0.77) and occipital regions ($t_{(32)}$ = -2.347, P < 0.05, Cohen d = 0.43) compared with the control patients. This group also showed significantly lower beta power in the central region than that in the control patients ($t_{(32)}$ = -2.265, P < 0.05, Cohen d = 0.51) (Figure 2A).

During REM Sleep

The younger iRBD group showed significantly lower alpha power ($t_{(32)}$ = -2.476, P < 0.05, Cohen d = 0.58) and beta power ($t_{(32)}$ = -2.354, P < 0.05, Cohen d = 0.45) in the central region than did the control patients. They also showed significantly lower alpha power ($t_{(32)}$ = -2.277, P < 0.05, Cohen d = 0.48) and beta power ($t_{(32)}$ = -2.398, P < 0.05, Cohen d = 0.45) in the occipital region than did the control patients (Figure 2B).

During NREM Sleep

No significant differences in EEG spectral powers in each frequency band were found between the younger iRBD group and the control patients (Figure 2C).

Partial Correlations of the MoCA Score to Clinical RBD-Related Variables or PSG Findings

Among all the patients with iRBD, significant correlation was found between age and each of the following variables: MoCA (r = -0.388, P < 0.05), RBDSQ-J (r = -0.421, P < 0.05), RBDQ-JP (r = -0.388, P < 0.05), beta power in the central region during wake (r = -0.399, P < 0.05), alpha and beta power in the occipital region during wake (r = -0.384, r = -0.425, P < 0.05), and beta power in the central and occipital region during REM sleep (r = -0.391, r = -421, P < 0.05). No EEG variable during NREM sleep showed significant correlation with the MoCA score. To eliminate the influence of age, partial correlation analyses controlled by age were conducted between the MoCA score and the clinical RBD-related variables or EEG spectral power in respective areas during wake, REM sleep, and NREM sleep.

Table 2—Partial correlation coefficients of the Montreal Cognitive Assessment score to clinical rapid eye movement sleep disorder-related variables or polysomnographic variables

	Partial correlation coefficient	P
RBD morbidity	0.092	n.s.
RBDQ-JP	-0.090	n.s.
TDI score on Sniffin' Sticks Test	0.423	< 0.05
Phasic EMG activity (%)	-0.070	n.s.
Tonic EMG activity (%)	0.201	n.s.
SWS (% SPT)	-0.410	< 0.05
REM sleep (% SPT)	0.065	n.s.

Partial correlation analyses controlled for age were conducted on all subject patients with iRBD (n = 31). EMG, electromyogram; n.s., not significant; RBD, rapid eye movement sleep disorder; RBDQ-JP, Japanese version of REM sleep behavior disorder questionnaire; SPT, sleep period time; SWS, slow wave sleep; TDI, threshold–discrimination–identification.

Table 2 shows age-controlled partial correlation coefficients of the MoCA score to the morbidity length of RBD, score of RBDQ-JP, threshold-discrimination-identification (TDI) score on Sniffin' Sticks Test, phasic EMG activity, tonic EMG activity, and proportion of N3 as well as REM sleep. Among these variables, the TDI score was positively correlated with the MoCA score, although the proportion of SWS was correlated negatively with the score (r = -0.410, r = 0.423, P < 0.05).

Partial Correlation Between the MoCA Score and EEG Spectral Powers in Each Frequency Band

During Wake

Age-controlled partial correlation analyses revealed that delta EEG power in the central region showed negative correlation with the MoCA score (r = -0.459, P < 0.05). In the occipital region, beta, theta, and delta EEG powers showed negative correlation with the score (r = -0.495, r = -0.500, r = -0.517, P < 0.05) (Table 3).

During REM Sleep

Theta and delta power in either the central region or occipital region showed negative correlation to the MoCA score (r = -0.490, r = -0.536, P < 0.05) (Table 3).

During NREM Sleep

All EEG variables during NREM sleep showed significant negative correlation with the MoCA score with the significance level set as P < 0.05; central region: alpha, r = -0.505; beta, r = -0.392; theta, r = -0.503; and delta, r = -0.383; occipital region: alpha, r = -0.514; beta, r = -0.398; theta, r = -0.500; and delta, r = -0.476 (Table 3).

Multiple Regression Analysis

To investigate factors associated with the decreased MoCA score, multiple regression analysis was conducted with the variables, which showed significant partial correlations to the MoCA score. Results indicated the following multiple regression equation: MoCA score = 50.871-0.116*age-5.307*log (δ power during REM sleep in occipital regions) + 0.086*TDI score. For this equation, the R value was 0.773, R^2 was 0.598, and the regression coefficients were -0.558 for age, -0.468 for δ power, and 0.357 for TDI score (F = 9.900, P < 0.001).

Table 3—Partial correlation between scores of Montreal Cognitive Assessment and electroencephalogram spectral powers in respective frequency bands

	Region	Frequency band	Coefficient	P
Wake	Central	α	-0.265	n.s.
		β	-0.386	n.s.
		θ	-0.348	n.s.
		δ	-0.459	< 0.05
	Occipital	α	-0.353	n.s.
		β	-0.495	< 0.05
		θ	-0.500	< 0.05
		δ	-0.517	< 0.05
REM sleep	Central	α	-0.461	n.s.
		β	-0.431	n.s.
		θ	-0.490	< 0.05
		δ	-0.536	< 0.05
	Occipital	α	-0.400	n.s.
		β	-0.417	n.s.
		θ	-0.511	< 0.05
		δ	-0.549	< 0.05
NREM sleep	Central	α	-0.505	< 0.05
		β	-0.392	< 0.05
		θ	-0.503	< 0.05
		δ	-0.383	< 0.05
	Occipital	α	-0.514	< 0.05
		β	-0.398	< 0.05
		θ	-0.500	< 0.05
		δ	-0.476	< 0.05

Partial correlation analyses controlled by age for all patients with rapid eye movement sleep behavior disorder (n = 31). NREM, nonrapid eye movement; n.s., not significant; REM, rapid eye movement.

DISCUSSION

This study revealed MCI in patients with iRBD and the relation between MCI and delta power during REM sleep or olfactory dysfunction in iRBD. The younger iRBD group showed lower scores in both MoCA test and MMSE compared with the age-matched control patients. The older iRBD group showed a lower score only on the MoCA test compared with the younger iRBD group. The distribution of the score of cognitive function tests demonstrated that 76.5% of the younger patients with iRBD and 92.9% of the older patients with the disorder showed the lower MoCA score than the cutoff value for MCI. In contrast, no greater percentage than 58.8% of the younger patients with iRBD showed a lower score of MMSE than the cutoff value for MCI. These results confirmed that the MoCA is more sensitive to MCI even in younger patients with iRBD, as consistent with the previous studies of MCI in patients with PD.[20-22]

The younger iRBD group showed significantly lower EEG spectral power, especially in fast frequency bands during both wake and REM sleep, compared with age-matched control patients. However, neither EEG power in low-frequency bands nor the percentage of N3 differed between the younger iRBD group and age-matched control patients. In previous studies, the increased percentage of SWS characterized PSG findings of patients with iRBD.[23-25] Massicotte-Marquez et al.[10] first reported that patients with iRBD had a greater percentage of SWS and delta power during total NREM sleep compared with control patients. However, some previous reports have described negative results for this finding.[9,26] Latreille et al.[26] have reported that no differences exist in spectral power in delta band or slow wave characteristics during NREM sleep between patients with iRBD and control patients. Moreover, they described that cognitive deficits observed in iRBD patients were associated with neither slow wave characteristics nor delta spectral power.[26] Consistent with the study by Latreille et al.,[26] the current study showed no significant difference either in the percentage of SWS or in the EEG spectral power in the delta band during NREM sleep in the younger iRBD group, in which patients' ages were similar to their study,[26] and the age-matched control patients.

During the past decade, several studies using EEG spectral analysis demonstrated increased EEG power in delta and theta bands during both wake and REM sleep in patients with RBD.[9-11,27] Fantini et al.[9] reported increased theta power and decreased beta power during wake and REM sleep in frontal, temporal, and occipital regions. This EEG finding has been regarded as representing a modest cognitive decline in the early stages of dementia.[28] Iranzo et al.[11] reported that patients with RBD who developed MCI shortly later (2.5 ± 1.5 y) had shown increased delta and theta power during wake and REM sleep in central and occipital regions at the baseline. Considering the previous result for increased delta power during NREM sleep in patients with iRBD,[10] EEG slowing during NREM sleep as well as that during wake or REM sleep is regarded as related with the cognitive decline. In the current study, the younger iRBD group showed decreased EEG power in alpha or beta bands in central and occipital regions during wake or REM sleep. However, the group showed no increased EEG power in low-frequency bands during each sleep status compared with the age-matched control patients in this study. The reason for this inconsistency was unclear. However, considering that the patients in the younger iRBD group in this study were younger than the iRBD patients investigated in previous studies,[9-11] the inconsistency is inferred to result from a difference not only in the sample size or in EEG montage but also in the patients' age. If this is the case, then it is possible that EEG slowing, which might be an early marker of development of MCI associated with RBD,[11] is likely to be detectable only in elderly patients with RBD.

To investigate factors associated with an early stage of cognitive impairment in RBD, we conducted partial correlation analysis controlled by age and subsequent multiple regression analysis of all patients with iRBD because the frequency of EEG slowing has been known to increase with normal aging.[29,30] Results show that among the clinical RBD-related variables, the MoCA score showed negative correlation with percentage of SWS and positive correlation with TDI score on the Sniffin' Sticks Test. Regarding the values of EEG spectral power, the MoCA score showed negative correlation, especially to theta and delta power during wake and REM sleep, and to EEG powers in all frequency bands during NREM sleep in either central or occipital regions. Reportedly, the quantitative EEG measure is a biomarker associated with cognitive status not only in patients with Alzheimer disease (AD)[31] but also in those with PD with dementia or DLB.[32,33] In addition, the increased EEG power in delta and theta bands during wake has been reported to differentiate DLB from AD.[33] Taking these findings into consideration, the quantitative EEG slowing in the subject iRBD patients might reflect an early stage of cognitive impairment in this disorder.

It is particularly interesting that the multiple regression equation revealed that increased delta power during REM sleep in the occipital region and olfactory dysfunction were associated with MCI in iRBD. As a possible reason for this finding, cholinergic dysfunction could be considered. Acetylcholine is an excitatory neurotransmitter that is well known to be crucial for cognitive processes. In addition, cholinergic activity is widely accepted as promoting REM sleep,[34] and is known to be associated with cortical activity.[35] In this regard, decreased acetylcholinesterase (AchE) activity in the cerebral cortex (especially in the occipital cortex) is observed even in the early stage of PD, although the decrease is more widespread and profound in PD dementia (PDD) or DLB.[36,37] In DLB, increased EEG spectral power has reportedly been observed in theta or delta bands.[33] One recent report has described that the presence of RBD symptoms might signal cholinergic system degeneration in the brainstem.[38] Moreover, cholinergic denervation of the limbic cortex is a more robust determinant of hyposmia. It can indicate a risk of cognitive impairment in patients with PD.[39] Taking these previous results into consideration, the intimate association between the EEG slowing, especially during REM sleep or olfactory dysfunction, and MCI can be explained partially by cholinergic dysfunction occurring in RBD as a prodrome of α-synucleinopathies. However, further research must be undertaken to verify this hypothesis.

This study and its results are burdened by some limitations. First, EEG activities in frontal, temporal, and parietal regions were not evaluated.[9] Considering that slow wave activity is likely to appear in the frontal region,[40] not only sleep scoring but also spectral analysis should be conducted in the frontal region as well as the central or occipital regions to clarify whether pathological EEG slowing shows electroencephalographic localization. Second, no comprehensive neuropsychological test battery was performed. Third, the sample size was small, and there is an important lack of follow-up data for PSG and neuropsychological tests. Fourth is the lack of control patients for the older iRBD group is a factor. Considering the change over time in the frequency of EEG with normal aging,[29,30] the association between degrees of EEG slowing and cognitive impairment compared with that of control patients should be investigated in both younger and older patients with iRBD. Nevertheless, this study revealed physiological findings that reflect an early stage of MCI in idiopathic RBD. In future studies, association between characteristics or overtime changes in the EEG and cognitive dysfunction should be investigated to clarify the process of MCI progression.

In conclusion, patients with iRBD showed MCI and EEG slowing during wake and REM sleep. The EEG slowing especially during REM sleep rather than that during NREM sleep and olfactory dysfunction are thought to reflect a subsequent early change of cognitive function with the disorder.

DISCLOSURE STATEMENT

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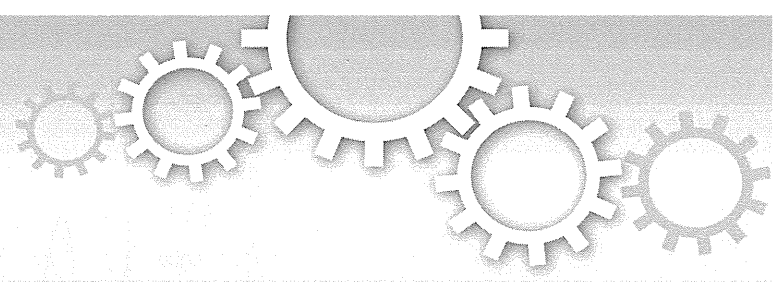

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In vitro circadian period is associated with circadian/sleep preference

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Evaluation of circadian phenotypes is crucial for understanding the pathophysiology of diseases associated with disturbed biological rhythms such as circadian rhythm sleep disorders (CRSDs). We measured clock gene expression in fibroblasts from individual subjects and observed circadian rhythms in the cells (*in vitro* rhythms). Period length of the *in vitro* rhythm (*in vitro* period) was compared with the intrinsic circadian period, τ, measured under a forced desynchrony protocol (*in vivo* period) and circadian/sleep parameters evaluated by questionnaires, sleep log, and actigraphy. Although no significant correlation was observed between the *in vitro* and *in vivo* periods, the *in vitro* period was correlated with chronotype, habitual sleep time, and preferred sleep time. Our data demonstrate that the *in vitro* period is significantly correlated with circadian/sleep preference. The findings suggest that fibroblasts from individual patients can be utilized for *in vitro* screening of therapeutic agents to provide personalized therapeutic regimens for CRSD patients.

Behavioral and physiological processes such as sleep/wakefulness and hormone secretion exhibit circadian rhythms[1]. Individual differences in daily activity/sleep time, known as the diurnal preference/chronotype, are commonly assessed using the conventional self-reported Horne-Östberg Morningness-Eveningness Questionnaire (MEQ)[2] and/or the recently developed online self-reported Munich ChronoType Questionnaire (MCTQ)[3]. The morning (early) chronotype manifests earlier timings for sleep and physiological rhythms such as core body temperature and melatonin secretion than the intermediate chronotype, and still earlier than the evening (late) chronotype[4–6]. The various daily behavioral and physiological rhythms are regulated by a system of self-sustained clocks and are entrained to environmental cues, such as light exposure, food intake, and work schedules, enabling us to adapt to changes in the external environment[7,8]. In mammals, the circadian clock system is hierarchically organized such that the central oscillator in the suprachiasmatic nuclei (SCN) of the hypothalamus integrates environmental information and synchronizes the phase of oscillators in peripheral cells, tissues, and organs[9,10]. The molecular mechanism of the circadian clock system involves a complex set of transcription-translation negative feedback loops that regulate multiple clock genes including *Bmal1*, *Clock*, *Cry*, *Per*, *Ror*, and *Rev-Erb*[11,12].

Circadian rhythm sleep disorders (CRSDs) are characterized by the inability to fall asleep and awaken at a desired time[13]. There are several subtypes of CRSDs: advanced sleep phase type (ASPT), delayed sleep phase type (DSPT), and free-running type (FRT). ASPT patients show extremely advanced involuntary timing of sleep and wake, DSPT patients show significantly delayed sleep onset and wake times, and FRT patients have sleep times that occur with ~1-h delay each day and are not able to adapt to the external 24-h day. CRSDs are attributed etiologically to malfunction and/or maladaptation of the circadian clock system[14–17], and therefore evaluation of circadian phenotypes is crucial for understanding the pathophysiology of CRSDs. The intrinsic circadian period, τ (the free-running period of circadian rhythms in the absence of external cues), is considered to be a critical factor in the pathophysiology of CRSDs. Indeed, we recently demonstrated that τ was significantly prolonged in FRT

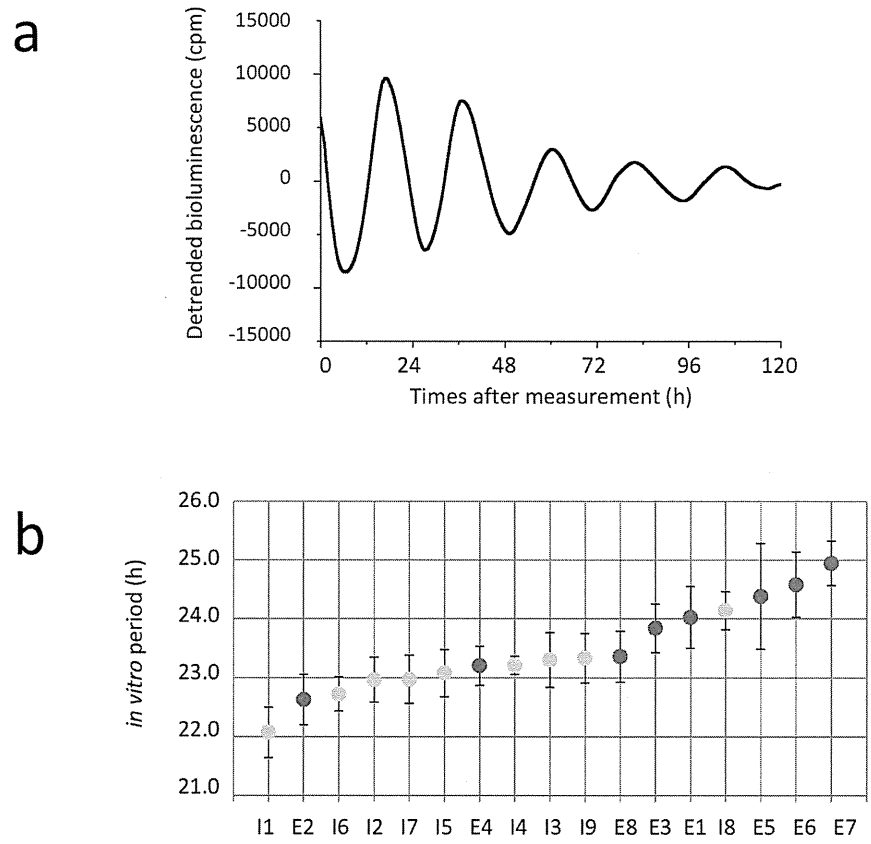

Figure 1 | An individual's circadian rhythm *in vitro* and the period length of *in vitro* rhythms. (a) Representative detrended data of *Bmal1-luc* rhythm in cultured fibroblasts from subject I4. Primary fibroblasts were obtained from a skin biopsy sample and were transfected with the circadian reporter *Bmal1-luc* utilizing an electroporation system. After the cultured cells were synchronized by treatment with dexamethasone for 2 h, bioluminescence rhythms from the cells were continuously measured for 5 cycles. (b) *In vitro* period length of *Bmal1-luc* rhythms in 9 intermediate type subjects (green circles) and 8 evening type subjects (blue circles). Data are presented as mean value ± standard error of the mean.

patients under a strict forced desynchrony (FD) protocol compared to healthy subjects with the intermediate chronotype[18]. However, although the FD protocol is regarded as the most reliable and valid method for the assessment of τ in humans, it is laborious and costly to perform in clinical settings[19,20]. A more convenient evaluation of circadian phenotypes is therefore required both to reduce burden on the subjects and to increase the feasibility of examination.

To this end, Brown et al. developed a luminescence rhythm assay system using biopsy samples to evaluate an individual's circadian phenotype[21]. In this system, the biopsy-derived fibroblasts are transfected with a circadian reporter, the *Bmal1* promoter-driven *luciferase* gene (*Bmal1-luc*), using a lentiviral system. Luciferase activity under the control of the *Bmal1* promoter was found to show daily rhythms in cultured fibroblasts (*in vitro* rhythm). Moreover, by monitoring the luciferase activity level for 4–6 cycles and evaluating the rhythmic characteristics of luminescence expression in these fibroblasts, Brown et al. found that cultured fibroblasts from morning-type subjects had a shorter period length than those from evening-type subjects[22]. Additionally, the period length of the *in vitro* rhythm is proportionally related to that of the physiological rhythm as assessed under a constant routine (CR), multiple nap, or nearly dark protocol[23]. On the other hand, Hasan et al. recently reported that neither chronotype nor τ (the period of melatonin rhythm assessed under a 9-day FD protocol) were significantly correlated with *in vitro* period length[24]. It is unclear then whether surrogate measurements using cultured fibroblasts derived from an individual's biopsy samples are in fact useful for assessing circadian phenotype. Furthermore, exactly what *in vitro* rhythms represent is not yet fully understood.

To address these issues, in this study we measured clock gene expression in primary fibroblasts derived from subjects' skin biopsy samples using a non-viral luminescence assay system, and compared the period of *in vitro* rhythms with τ measured under a strict FD protocol and circadian/sleep parameters evaluated by questionnaires, sleep log, and actigraphy in a real-life setting.

Results

Circadian rhythms were sustained in cultured cells for several cycles, as indicated by luminescence levels (Fig. 1a). The *in vitro* period length of *Bmal1-luc* rhythm (*in vitro* period) varied among fibroblasts from different individuals (Fig. 1b, Table 1). The *in vivo* period length of melatonin rhythm (*in vivo* period) had been determined for each subject in our previous study[18]. The average *in vitro* period was significantly shorter than the average *in vivo* period in our subjects (23.46 ± 0.76 h vs. 24.17 ± 0.20 h; $t = -3.80$, $df = 16$, $P = 0.002$).

Next, a comparison of the *in vitro* and *in vivo* periods for each subject (9 intermediate types and 8 evening types) revealed a significant correlation between the two periods in intermediate types ($R = 0.750$, $P = 0.020$) but not in evening types ($R = -0.336$, $P = 0.416$) or in all subjects ($R = 0.093$, $P = 0.723$) (Fig. 2). The *in vivo* period did not differ between intermediate and evening types (24.12 ± 0.12 h vs. 24.22 ± 0.27 h; $t = -0.98$, $df = 9.31$, $P = 0.353$). By contrast, the *in vitro* period did differ significantly between the two types (23.09 ± 0.55 h vs. 23.87 ± 0.77 h; $t = -2.42$, $df = 15$, $P = 0.028$).

MEQ scores indicate morningness-eveningness preference (chronotype). As anticipated, the *in vitro* period was significantly correlated with individual MEQ score ($R = -0.570$, $P = 0.017$) (Fig. 3a).

Table 1 | Period length of *in vitro* and *in vivo* rhythms

Subject (number)	Age (years)	*in vitro* rhythm period length (h)	±SD	*in vivo* rhythm period length (h)
Intermediate type (9)				
I1	19	22.07	1.29	23.95
I2	23	22.97	1.00	24.03
I3	21	23.30	1.23	24.17
I4	22	23.21	0.45	24.10
I5	22	23.08	1.27	24.00
I6	20	22.72	0.92	24.10
I7	24	22.97	1.30	24.26
I8	22	24.14	1.02	24.31
I9	39	23.33	1.34	24.14
mean	23.56	23.09		24.12
SD	5.98	0.55		0.12
Evening type (8)				
E1	22	24.03	1.49	24.36
E2	22	22.63	1.14	24.23
E3	20	23.84	1.09	23.89
E4	22	23.21	0.99	24.68
E5	20	24.38	2.20	24.44
E6	23	24.58	1.36	23.93
E7	21	24.95	1.12	24.08
E8	22	23.36	1.36	24.14
mean	21.50	23.87		24.22
SD	1.07	0.77		0.27
Total (17)				
mean	22.59	23.46		24.17
SD	4.42	0.76		0.20

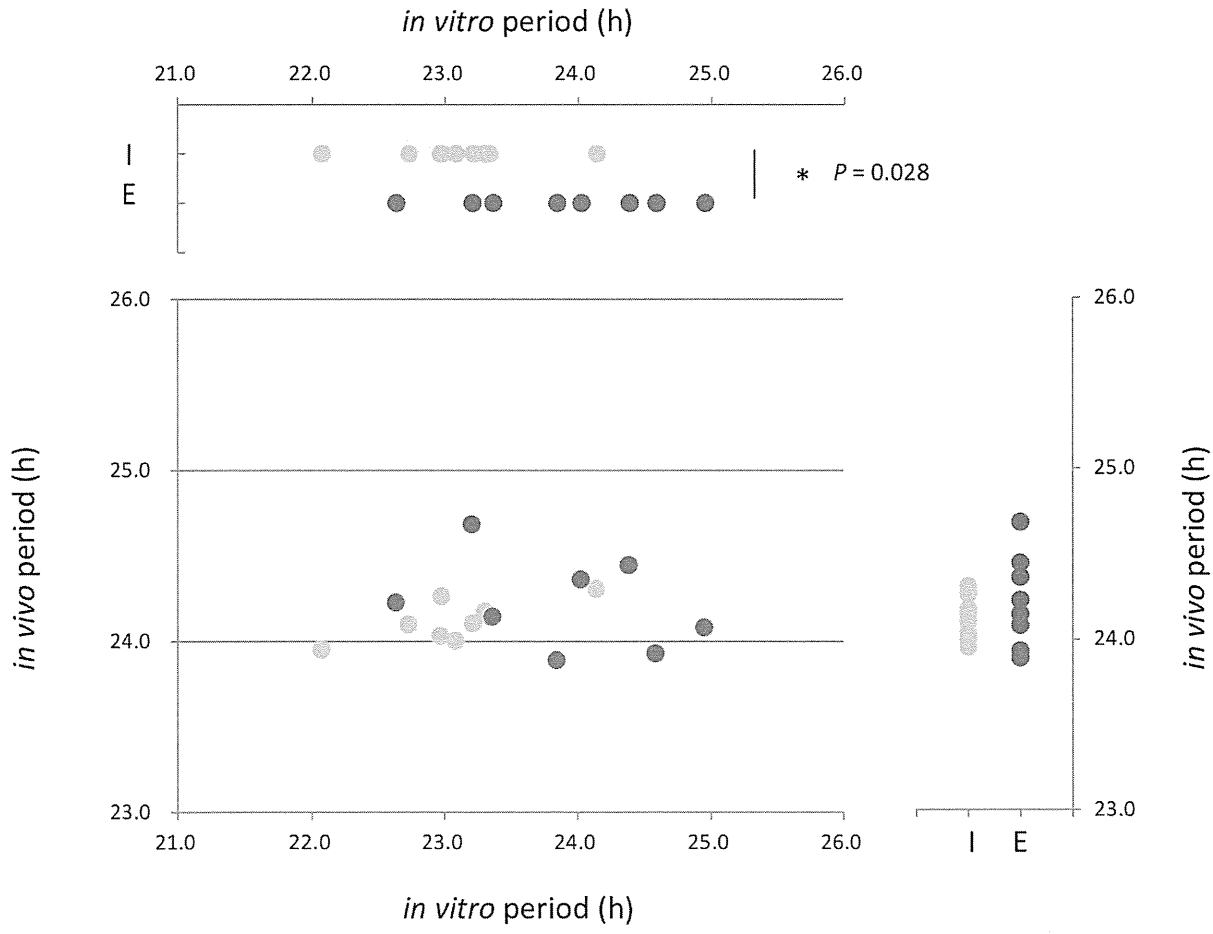

Figure 2 | Comparison of *in vitro* and *in vivo* rhythms between intermediate (I, green circles) and evening (E, blue circles) types. Dots represent the period length of the *in vitro* (horizontal axis) or *in vivo* (vertical axis) rhythm for each subject. No significant correlation was found between *in vitro* and *in vivo* periods when all subjects were examined. A longer *in vitro* period was observed in evening types compared to intermediate types ($P = 0.028$).

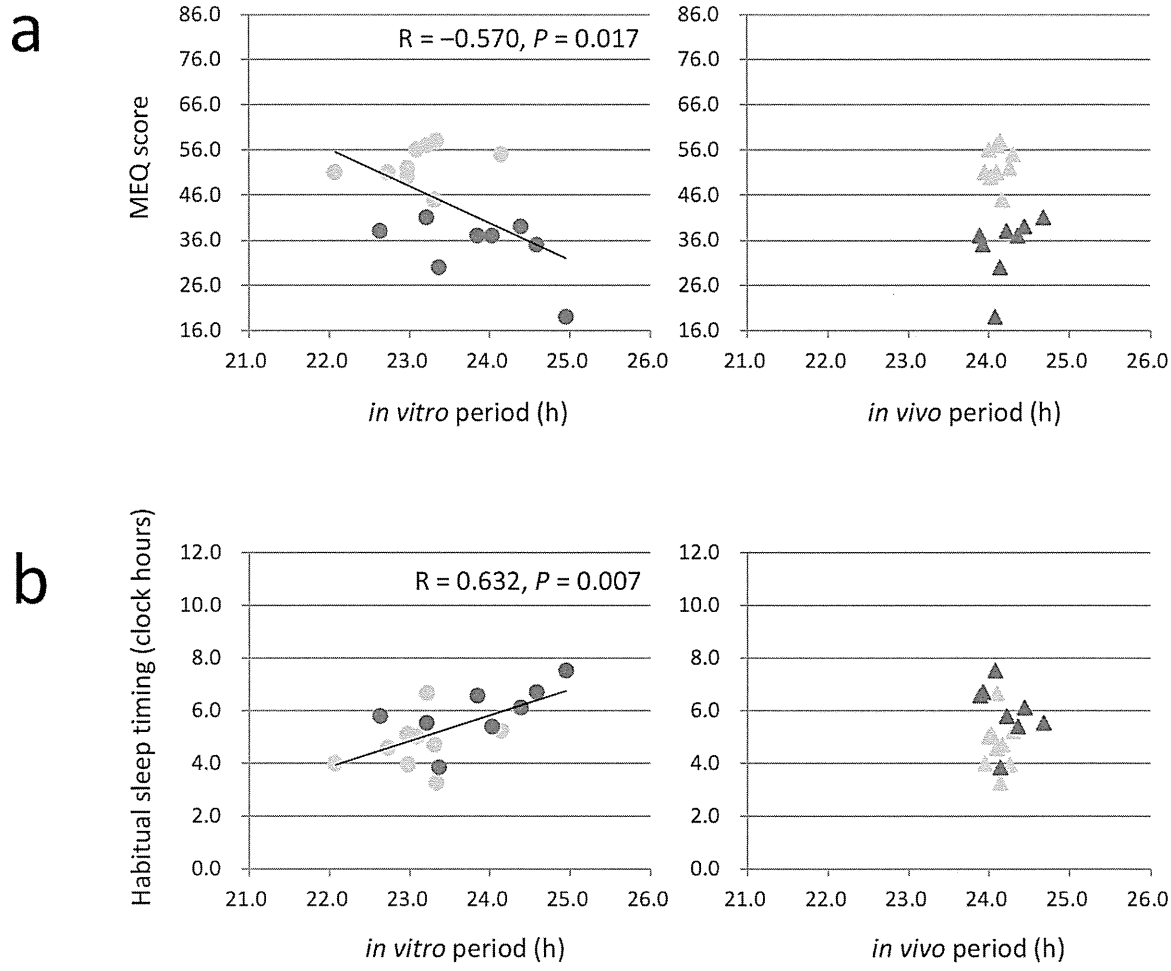

Figure 3 | Correlations between the *in vitro* (circles) or *in vivo* (triangles) period and (a) MEQ score or (b) habitual sleep timing. A strong correlation was seen between *in vitro* period and MEQ score (R = −0.570, $P = 0.017$) and habitual sleep time (R = 0.632, $P = 0.007$) when all subjects (9 intermediate types denoted by green circles and 8 evening types, blue circles) were examined.

Additionally, there was a significant correlation between the *in vitro* period and habitual sleep time (R = 0.632, $P = 0.007$) (Fig. 3b). By contrast, the *in vivo* period was not associated with MEQ score (R = −0.046, $P = 0.860$) (Fig. 3a) or habitual sleep time (R = −0.060, $P = 0.819$) (Fig. 3b). Correlations between the *in vitro* or *in vivo* period and circadian/sleep parameters were assessed using mid-sleep timings on work days (MSW), mid-sleep timings on free days (MSF), and sleep-corrected MSF (MSFsc; another indicator of chronotype) obtained by the MCTQ. No significant correlation was found between MSW and the *in vitro* period (R = 0.343, $P = 0.178$) or the *in vivo* period (R = −0.249, $P = 0.336$) (Fig. 4a). By contrast, MSF, which represents the preferred sleep timing free of social constraints, was strongly correlated with the *in vitro* period (R = 0.617, $P = 0.008$) (Fig. 4b), as was MSFsc, which represents chronotype (R = 0.592, $P = 0.012$) (Fig. 4c). Evening preference was associated with a longer *in vitro* period. There was no association between MSF (R = −0.037, $P = 0.889$) or MSFsc (R = −0.108, $P = 0.680$) and the *in vivo* period (Fig. 4b and 4c).

Discussion

Despite the fact that only a limited number of subjects were assessed in this study, the results demonstrate that an individual's *in vitro* circadian period is significantly correlated with circadian/sleep preference.

Consistent with previous reports[21,22,24], primary fibroblast cells derived from individuals showed rhythmic expression of the circadian reporter *Bmal1-luc*. However, despite Hasan et al. finding that *in vitro* periods were longer than *in vivo* periods[24], we observed that *in vitro* periods were in fact shorter. There are a number of differences in the experimental conditions between the present study and previous studies, such as the reporter constructs utilized, transfection methods, and recording media. Serum factors, pH levels and Ca^{2+} concentration are known to alter the circadian characteristics of *in vitro* rhythms[25–27]. Consequently, the differences in experimental conditions between the present and past studies might account for the differences observed in *in vitro* period length.

When *in vitro* and *in vivo* circadian rhythms were compared, a moderate but significant correlation between the two rhythms was observed in intermediate types, but not in evening types or in all subjects. Additionally, Hasan et al. found no correlation between *in vitro* and *in vivo* periods in their subjects[24]. Pagani et al. reported that the *in vitro* period was proportional to the *in vivo* period in their subjects, although they did not observe a longer *in vitro* period in blind subjects who are known to have a significantly longer *in vivo* period than sighted subjects[23]. *In vivo* rhythms such as core body temperature and melatonin secretion are known to be affected by the after-effects of entrainment[28]. Long-term effects of previous long sleep-wake cycles might cause the long *in vivo* period in blind individuals. These data suggest that *in vitro* rhythms are not strongly correlated with *in vivo* rhythms.

The relationship between the central and peripheral oscillators has been studied by measuring luminescence rhythms in cultured SCN cells and peripheral tissues explanted from circadian reporter transgenic animals[9,29,30]. The period and the phase of SCN rhythm are

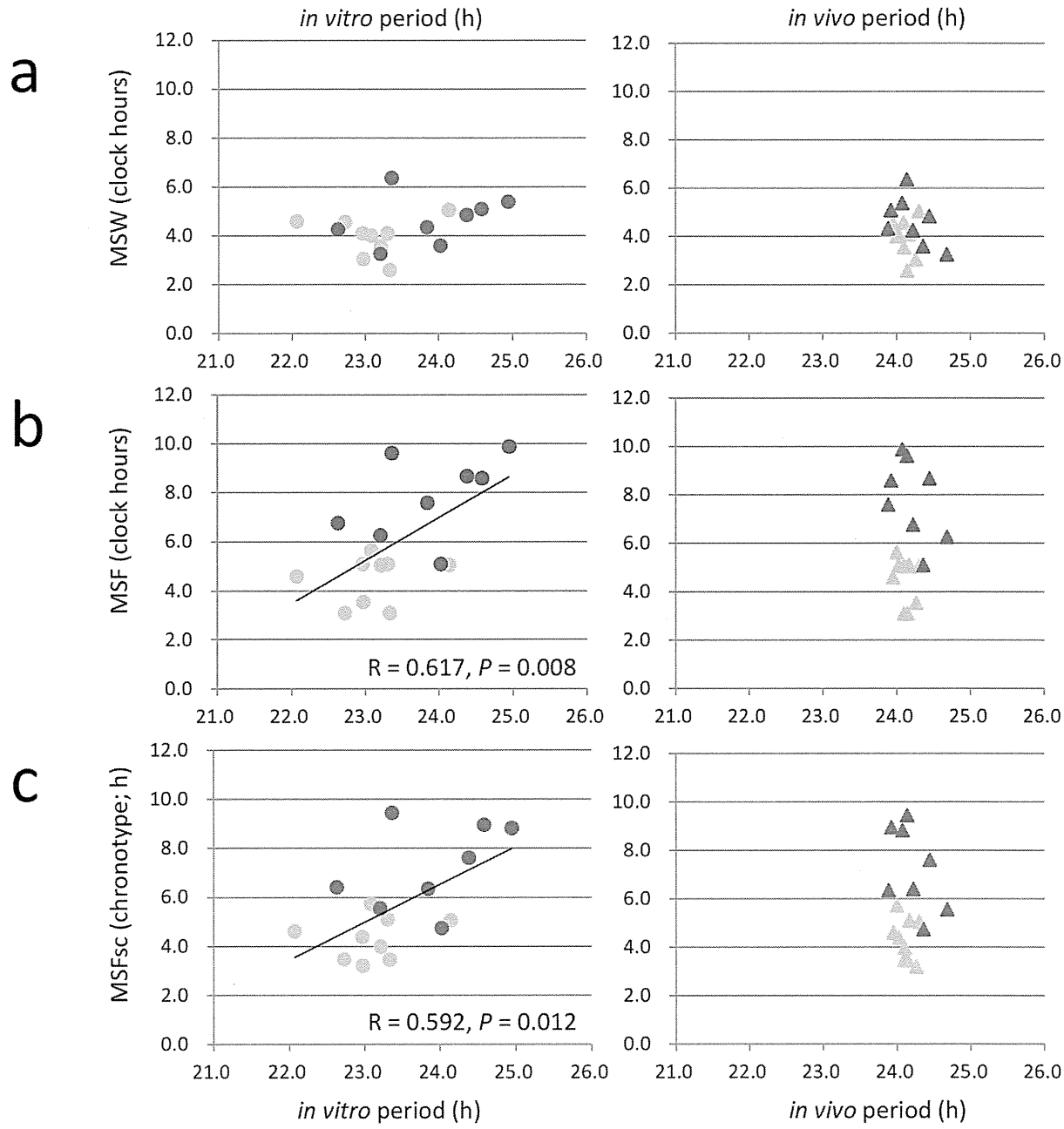

Figure 4 | Correlations between the *in vitro* (circles) or *in vivo* (triangles) period and (a) MSW, (b) MSF, or (c) MSFsc. Strong correlations were observed between the *in vitro* period and MSF (R = 0.617, $P = 0.008$) or MSFsc (R = 0.592, $P = 0.012$) when all subjects (9 intermediate types denoted by green circles and 8 evening types, blue circles) were examined.

different from those of peripheral rhythms even under the same condition (i.e., in organotypic slice culture)[31,32]. This implies that individual tissues show distinct circadian characteristics under conditions in which the tissues are dissociated. Primary fibroblasts used in this study are a group of dissociated cells established from skin biopsy samples. Unlike tissues *in vivo*, cultured cells do not receive environmental information or any circadian signals from other tissues (SCN and periphery). On the contrary, almost all of the *in vivo* tissues are co-regulated or are interdependent even when masking effects are minimized. It was recently reported that age-related differences are observed for numerous characteristics of behavioral and physiological rhythms but not in the molecular machinery of peripheral circadian clocks[25]. These findings imply that *in vitro* rhythms reflect the molecular mechanism of circadian clock components in peripheral cells, whereas *in vivo* rhythms reflect the physiological mechanism of the circadian clock system of an individual.

The longer *in vitro* period observed in evening types compared to intermediate types in this study is in agreement with a previous report that extreme evening types had a longer *in vitro* period than extreme morning types[22]. Furthermore, *in vitro* period length, but not *in vivo* period length, was significantly correlated with MEQ score (chronotype), habitual sleep time, MSF (preferred sleep time), and MSFsc (chronotype). Our data strongly support the notion that the period length of circadian rhythms in fibroblasts from individuals represents their circadian/sleep preference. By contrast, the *in vivo* period was not correlated with any of these parameters. The *in vivo* period converges to a nearly 24-h period[20,33] and does not vary greatly among individuals. This characteristic of *in vivo* rhythms

might weaken the correlation between the *in vivo* period and circadian/sleep preference. Given that genetic factors (individual traits) have a significant effect on the determination of circadian/sleep preference[34], these findings suggest that the properties of *in vitro* rhythms might reflect individual differences in circadian clock traits better than those of *in vivo* rhythms. Evaluating rhythmic expression of clock genes in isolated fibroblast cells might therefore be an appropriate method to assess an individual's circadian clock phenotype.

Hasan et al. reported that MEQ score is correlated with the *in vivo* period but not with the *in vitro* period[24], which is inconsistent with our findings. Most of their subjects were intermediate types, whereas 8 of our 17 subjects were evening types. In the present study, *in vitro* period length varied greatly between intermediate and evening types and a significant correlation was observed between the *in vitro* period and chronotype. These differences in subjects might explain the discrepancy between the two sets of results. Further validation using larger cohorts should be performed to accurately determine whether the period length of the *in vitro* rhythm can predict an individual's circadian clock phenotype.

In the age of personalized medicine, one of our goals is to tailor therapies to individuals based upon their specific disorders. However, sleep disorders, like many of the conditions for which therapeutic intervention would be useful, are extremely complex genetically. Even if genomic or single nucleotide polymorphism analysis were to be performed in patients with these conditions, the data obtained would not provide sufficient information to test effective new pharmaceutical agents, let alone prescribe them for treatment. To overcome this, effective *in vitro* screens to test therapeutic agents are required. To this end, we have now shown that fibroblasts in culture have circadian periods correlated with the circadian clock phenotype of the individual. Thus, we believe that these isolated fibroblasts could be utilized for *in vitro* screening of therapeutic agents to modify circadian disruption (e.g. altered period, phase, and amplitude of circadian rhythms) to develop personalized therapies for patients with CRSDs.

Methods

Subjects. Subjects were 17 healthy males aged 19–39 years (mean age ± standard deviation (SD), 22.6 ± 4.4 years) who participated in our previous study[18]. None had sleep disorders (as assessed by clinical polysomnography and the Pittsburgh Sleep Quality Index questionnaire), psychiatric disorders (assessed by a semi-structured interview with a psychiatrist and the Center for Epidemiology Studies Depression Scale questionnaire) or severe physical diseases. None had traveled across time zones or had been on any medication over the past 6 months. MEQ score was used to determine each subject's chronotype, where a score of 16–41 denoted evening type and that of 42–58 denoted intermediate type. Accordingly, 9 subjects were classed as intermediate type (subjects I1, I2, I3, I4, I5, I6, I7, I8, and I9) and 8 as evening type (subjects E1, E2, E3, E4, E5, E6, E7, and E8).

The protocol was approved by the Institutional Ethics Committee of the National Center of Neurology and Psychiatry, and written informed consent was obtained from all subjects.

***In vivo* rhythm assay.** A total of 17 subjects participated in a 13-day FD protocol in a sleep laboratory free from external time cues in our previous study[18]. Briefly, the FD protocol was composed of 3 experiments: 1) initial assessment of circadian phase under CR[35] (1st CR); 2) a 28-h sleep-wake schedule (9.33 h of sleep and 18.67 h of wakefulness) for 7 days; and 3) a second assessment of circadian phase under CR (2nd CR). Throughout the experiments, lights were maintained at a low intensity (<15 lx) during the wake period and turned off (0 lx) during the sleep period. Ambient temperature and humidity in the laboratory were maintained at 25 ± 0.5°C and 50 ± 5% relative humidity, respectively. During the periods of CR, subjects were required to lie on a reclining chair in a semi-recumbent position and stay awake for 34 h. Water was available at all times and a 200-kcal meal was provided every 2 h. Blood samples were collected every hour using an intravenous catheter placed in a forearm vein. Plasma was immediately separated by centrifugation (15 min at 1600 × g and 4°C) and stored at −80°C until analysis. Concentrations of plasma melatonin were measured by radioimmunoassay. Dim light melatonin onset (DLMO) time was defined as the time when plasma melatonin concentration rose from a low background level to above 10 pg/mL[36]. To calculate intrinsic circadian period, τ_{DLMO}, the difference in the DLMO time measured during the 1st CR and 2nd CR conducted at the beginning and end of the FD protocol, respectively, was divided by the number of experimental days. τ_{DLMO} was used as the period length of *in vivo* rhythm (*in vivo* period) in this study (Table 1).

Habitual sleep time. For 7 days prior to laboratory admission, subjects maintained their daily routines and slept at a regular time every night at home under a dim light condition. Their regular sleep-wake routine was verified by sleep log and actigraphy. Wrist activity was monitored with an Actiwatch (Philips Respironics) around the non-dominant wrist. Activity data were analyzed using computer-calculated sleep-wake determinations[37]. Average sleep onset and wake times during these 7 days were used as the habitual sleep onset time and wake time, respectively. Habitual sleep time was designated as the midpoint between habitual sleep onset time and wake time.

Munich chronotype questionnaire[3]. The MCTQ was administered on the admission day to assess the subjects' sleep onset and wake times separately on work days or free days. MSW and MSF were calculated as the midpoint between sleep onset time and wake time on work days and free days, respectively. MSFsc was used as another indicator of chronotype.

Skin biopsy, cell culture, and *in vitro* rhythm assay. A skin biopsy of the dorsal region was performed using a biopsy punch (2 mm in diameter and 7 mm in length) with a plunger system (Kai Industries) on the first day of the 28-h sleep–wake schedule (i.e., Day 4 of the FD protocol). Primary fibroblast cultures, derived from the skin biopsy samples, were established by culturing in DMEM/F12 (GIBCO/Life Technologies) supplemented with 20% FBS (NICHIREI BIOSCIENCES), 1% FUNGIZONE (GIBCO, Life Technologies), 0.5 μg/mL MC-210 (DS Pharma Biomedical Co., Ltd.), and 1% penicillin/streptomycin (GIBCO/Life Technologies) at 37°C and 5% CO_2. For each measurement, 3×10^5 primary cells were transfected with 3 μg of the *Bmal1-luc* construct Bp/527-LUC[38] using Neon (Life Technologies) and were plated in a 35-mm dish containing DMEM/F12 supplemented with 20% FBS without penicillin/streptomycin (Day 0). After 3 days (Day 3), the medium was changed to DMEM/F12 supplemented with 5% FBS and 1% penicillin/streptomycin, and on Day 10 was changed to fresh medium (DMEM/F12 supplemented with 5% FBS and 1% penicillin/streptomycin). On Day 17, 0.1 μM dexamethasone (Sigma-Aldrich) was applied and the cells were incubated for 2 h to synchronize rhythms in the fibroblasts. Luminescence from the cells was measured for at least 5 cycles in recording medium (DMEM #D-2902; Sigma-Aldrich) supplemented with 19.4 mM glucose (final concentration 25 mM), 10 mM HEPES (Sigma-Aldrich), 0.25% penicillin/streptomycin, and 0.1 mM beetle luciferin potassium salt (Promega), using photomultiplier tubes (Hamamatsu) in a dark box at 37°C as previously described[39]. The data were detrended by subtracting the 24-h running average from the raw data and then smoothed with a 2-h running average using Origin7.0 (OriginLab) as previously described[40]. The period length of the *Bmal1-luc* rhythm (*in vitro* period) was determined by regression analysis using the second to fourth peak times of the luminescence rhythm. The *in vitro* period for each subject is presented as the mean of 6 to 10 independent measurements ± SD (Table 1).

Statistical analysis. Kolmogorov–Smirnov tests were performed and frequencies for the parameters tested in this study were normally distributed. Paired t-tests were used to compare *in vivo* and *in vitro* period length and unpaired t-tests were used to compare the *in vivo* and *in vitro* periods between the intermediate and evening types. Correlations between the parameters were assessed by Pearson's correlation analysis. Unpaired Student's t-tests were used to compare the physiological *in vivo* period and the fibroblast *in vitro* period between the intermediate and evening types. $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS ver. 11 (SPSS Japan Inc.). Data are presented as mean ± SD.

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Author contributions

A.H., S.K. and K.M. designed research. A.H., S.K., Y.O., M.E., Y.K., Yu, M., Yo, M., K.N., M.W., S.A., S.H., M.K., Y.K. and K.M. performed research. A.H., S.K., Y.O. and M.E. analyzed the data. S.Y., Y.G. and M.I. contributed reagents/materials/analysis tools. A.H., S.K. and K.M. wrote the paper.

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Association between melanopsin gene polymorphism (I394T) and pupillary light reflex is dependent on light wavelength

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Abstract

Background

Our aim was to determine the association between melanopsin gene polymorphism and pupillary light reflex under diverse photic conditions, including different intensities andwavelengths.

Methods

A total of 195 visually corrected subjects volunteered for investigation of the melanopsin gene of single nucleotide polymorphism (SNP) of rs1079610 (I394T). The genotype groups were TT (n = 126), TC (n = 55), and CC (n = 8), and 75 of the subjects, including subjects with TT (n = 34), TC (n = 33), and CC (n = 8) participated in our experiment. Three

monochromatic lights with peak wavelengths of 465 nm (blue), 536 nm (green), and 632 nm (red) were prepared, and each light was projected to the subjects with five intensities, 12, 13, 14, 14.5 and 15 log photons/(cm^2 s), for one minute. The pupil size of the left eye was measured under each light condition after a 1-minute adaptation.

Results

The pupils of the TC + CC genotypes (n = 38) were significantly smaller than those of the TT genotype (n = 31) under a blue (463 nm) light condition with 15 log photons/(cm^2 s) (P < 0.05). In contrast, there were no significant differences under green (536 nm) and red (632 nm) light conditions. Conversely, relative pupil constrictions of the TC + CC genotypes were greater than those of the TT genotype under both blue and green conditions with high intensities (14.5 and 15 log photons/(cm^2 s)). In contrast, there were no significant differences between genotype groups in pupil size and relative pupilloconstriction under the red light conditions.

Conclusions

Our findings suggest that the melanopsin gene polymorphism (I394T) functionally interacts with pupillary light reflex, depending on light intensity and, particularly, wavelength, and that under a light condition fulfilling both high intensity and short wavelength, the pupillary light response of subjects with the C allele (TC + CC) is more sensitive to light than that of subjects with the TT genotype.

Keywords

Genotype, Human, Intrinsically photosensitive retinal ganglion cells, Melanopsin gene (*OPN4*), Non-image-forming responses, Pupillary light reflex, Single nucleotide polymorphism, Steady-state pupil response, Monochromatic light

Background

In mammals, a small subset of retinal ganglion cells express the photopigment melanopsin, and they are intrinsically photosensitive (hence, intrinsically photosensitive retinal ganglion cells, ipRGCs) [1-3]. Parallel studies have been carried out to identify the functional roles of melanopsin or ipRGCs using transgenic mice, such as mice lacking rods or cones [4,5] and mice lacking melanopsin [6,7]. We now know that ipRGCs transmit photic irradiance information to the suprachiasmatic nuclei, intergeniculate leaflet, and olivary pretectal nucleus (OPN) through the retinohypothalamic tract [8,9] and that they play important roles in non-image-forming responses, including circadian photoentrainment [6,10], pupillary light reflex (PLR) [11,12], and other behavioral and physiological functions [13-15].

In human beings, there are large inter-individual phenotypic variations in the non-image-forming effects of light [16-18]. For instance, it is known that dark- and light-adapted pupil sizes in normal healthy subjects have large inter-individual differences [17,19,20]. What causes these inter-individual variations? According to the database of the International HapMap Project, in human beings, there are some genetic variations in the melanopsin (*OPN4*) gene. Higuchi *et al.* [21] attempted to demonstrate the functional differences of

OPN4 gene polymorphism (I394T) by measuring steady-state pupil response during exposure to light, and they revealed that there is a functional connection between *OPN4* gene polymorphism and pupillary light response. However, the spectral sensitivity of melanopsin was not considered in their study. Melanopsin is a vitamin A-based opsin, and all of the opsin and vitamin A-based photoreceptors have characteristic spectral sensitivities [22]. Parallel studies have shown the peak sensitivity of melanopsin in other mammals to be around 480 nm [23,24], unlike rods (λ_{max} 498 nm) and short, medium, and long-wavelength cones (λ_{max} 420, 534, and 563 nm) [25], and consistent with human melanopsin λ_{max} [11,26]. Thus, we hypothesized, first, that functional differences of the *OPN4* gene polymorphism in PLR would be apparent at a short-wavelength light but not at a long-wavelength light related to M- and L-cone excitation and, second, that these differences depend on light intensity, as suggested by our previous study [21]. Hence, the aim of this study was to determine whether *OPN4* gene polymorphism in a young Japanese population is associated with steady-state pupil size during exposure to light of various wavelengths and intensities.

Methods

Subjects

Prior to the experiment, 195 Japanese university students were gathered to investigate *OPN4* gene polymorphism. This recruitment was totally different from that in our previous study [21]. All subjects had normal color vision, as tested by the Ishihara color vision test. Scalp hairs were used to extract genomic DNA samples, to genotype the single nucleotide polymorphism (SNP) of rs1079610 (I394T) located in the coding region. Genomic DNA was extracted from a hair using a DNA Extractor FM Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The genotype groups were classified as TT, TC, or CC, and the numbers of subjects in those groups were 126, 55, and 8, respectively (six being undetermined). The T and C allele frequencies of I394T were 81.2% and 18.8%, respectively. Genotype frequency of I394T was consistent with the Hardy-Weinberg equilibrium ($\chi^2 = 0.40$, ns). Subjects gave written informed consent for participation in the study, which was approved by the ethical committee of Kyushu University and the ethics committee of the National Center of Neurology and Psychiatry.

Seventy-five subjects, including subjects with TT ($n = 34$, 16 men and 18 women; 20.79 ± 2.0 years old), subjects with TC ($n = 33$, 16 men and 17 women; 21.03 ± 2.3 years old), and subjects with CC ($n = 8$, one man and seven women; 21.33 ± 1.7 years old) volunteered for the study and participated in all of the experiments. Exclusion criteria included medication or drug consumption and shift work.

Experimental conditions

A total of 225 modules of a three-in-one type (red-green-blue, RGB) light-emitting diode (LED) on a square panel (350 × 350 mm) were prepared for exposure of subjects to monochromatic light. The intensity of lights was controlled by analog voltage-width modulation. A diffuser, located in front of the light device, was arranged 300 mm from subject's eyes (Figure 1). To control the range of subject's eye movement during the measurement, we marked five points forming a cross.

Figure 1 Set-up for the experiments. A total of 225 modules of a 3-in-1 type (RGB) LED on a square panel (350 × 350 mm) were prepared, and the intensity of lights was controlled by analog voltage-width modulation. The diffuser, located in front of the light device, was 300 mm from subject's eyes. Pupil diameter was measured in the left eye using an EMR-8 eye-tracking system with an infrared camera (Nac Image Technology, Inc., Japan).

The peak wavelengths of each color condition were measured by an illuminance spectroradiometer (CL-500A, KONICA MINOLTA INC., Japan) in the vertical direction from the height of the subject's eyes. The peak wavelengths were 632 nm (red), 536 nm (green), and 465 nm (blue) with half-bandwidths of 30 to 45 nm. Five intensity conditions, including 12, 13, 14, 14.5 and 15 log photons/(cm^2 s), were modulated at each color condition equally. Table 1 shows the illuminance (lx) and irradiance ($\mu W/cm^2$) levels of the respective light conditions.

Table 1 Illuminance (lux) and irradiance ($\mu W/cm^2$) levels of each light condition

	Blue		Green		Red	
Intensity (log photons/(cm^2 s))	**lux**	**$\mu W/cm^2$**	**lux**	**$\mu W/cm^2$**	**lux**	**$\mu W/cm^2$**
12	0.28	0.35	1.95	0.33	0.54	0.28
13	2.75	3.52	19.90	3.04	6.27	3.25
14	24.96	33.92	167.14	29.56	53.89	28.01
14.5	72.62	104.42	525.22	97.74	158.28	83.58
15	208.33	318.37	1391.48	285.49	505.54	272.59

Procedure

The blue, green, and red stimuli were presented in separate sessions, consisting of five intensities. Dark adaptation (20 minutes) preceded each run, and test stimuli were presented in the dark for one minute in stages from low-intensity to strong-intensity. Pupil diameter was measured in the left eye using an EMR-8 eye-tracking system with an infrared camera (Nac Image Technology, Inc., Japan). After adaptation to each test stimulus, the steady-state pupil size was measured two or three times for 5 seconds with a sample rate of 60 Hz. Test stimuli were maintained during the measurements. The head of each subject was fastened to a chin rest during the measurements.

It is suggested that PLR is modulated by an endogenous circadian clock (24 hours) [27]. To minimize these circadian effects on PLR, experiments were performed during the daytime between 10 am and 5 pm and the time of each run was counterbalanced between *OPN4* genotypes.

Data analysis

Six subjects (TT = 3, TC = 2, CC = 1) were excluded, owing to outlier or unstable data. Hence, data from a total of 69 subjects (TT = 31, TC + CC = 38) were used for statistical analysis. In our comparison between genotype groups (TT, TC, and CC), we treated TC and CC as one group, since our previous study showed that pupillary light responses of subjects who were homozygous (TT genotype) were significantly different from those of subjects who were heterozygous (TC genotype) and homozygous (CC genotype) and that there was no significant difference between TC and CC genotypes in PLR [21]. Thus, we focused on

determining what differences are caused by the C allele and verifying the reproducibility of results of our previous study.

The steady-state pupil data in all experimental conditions were analyzed using a repeated-measures three-way analysis of variance (ANOVA) (IBM© SPSS© version 21) with genotype (TT, TC + CC) as between-subject factor and light conditions (color and intensity) as independent variables. Bonferroni post-hoc comparisons were conducted when main effects or interactions were present. Also, comparisons of pupil responses in all experimental conditions between the two genotype groups were performed using a two-sided, independent-sample Student's *t* test. A *P* value less than 0.05 was considered statistically significant.

Results

OPN4 polymorphism and pupil size

Before the comparison between *OPN4* polymorphisms, it was important to confirm that the pupillary responses in this study were driven by ipRGCs. Figure 1 shows the pupil sizes of all subjects in all of the test conditions. In the analysis of pupil sizes of all subjects, there were main effects in both color ($F = 197.394$; $P < 0.001$) and intensity ($F = 1532.19$; $P < 0.001$) and there was an interaction between color and intensity ($F = 118.961$; $P < 0.01$) (Figure 2). The pupil sizes under blue conditions were significantly smaller than those under red conditions in all intensities. Similarly, the pupil sizes under green conditions were, except for 13 log photons/cm^2/s, also smaller than those under red conditions. In the comparison between blue and green conditions, the pupil sizes in blue conditions were smaller than those in green conditions for all of the intensities except for the 12 log photons/cm^2/s condition.

Figure 2 Pupil sizes (mean + standard deviation) of all subjects under five intensities of blue light (blue bars), green light (green bars), and red light (red bars). There were main effects of color ($P < 0.001$) and intensity ($P < 0.001$), and there was an interaction between color and intensity ($P < 0.001$). Pupil sizes under blue conditions were significantly smaller than those under the other color conditions. Pupil sizes under green conditions, except under the 13-green condition, were significantly smaller than those under red conditions. $^{**}P < 0.01$.

Next (Figure 3), the repeated-measures three-way ANOVA (three levels of color × five levels of intensity × two groups of genotype) showed an interaction between color, intensity, and genotype ($F = 3.702$; $P < 0.05$), although the main effects of genotype were not significant. Results of the Student's *t* test between the two groups of genotype showed that there was a significant difference only under the blue condition of 15 log photons/(cm^2 s), and the pupil size of TC + CC genotypes was significantly smaller than that of TT genotype ($P < 0.05$). There were no significant differences between genotypes under green and red conditions.

Figure 3 Comparison of pupil sizes (mean + standard deviation) between TT ($n = 31$; black bars) and TC + CC ($n = 38$; white bars) genotypes. **(a)** Blue conditions, **(b)** green conditions, **(c)** red conditions. Pupil sizes of TC + CC were significantly smaller than those of TT under the 15-blue condition, but there were no significant differences under green and red conditions. $^{*}P < 0.05$.

OPN4 polymorphism and relative pupil constriction

The relative pupil constriction was calculated as the percentage of pupil area under 13 to 15 log photons/(cm^2 s) relative to the pupil area under 12 log photons/(cm^2 s) of each color condition. In the relative pupil constriction (Figure 4), there were main effects in both color ($F = 42.171$; $P < 0.001$) and intensity ($F = 2100.906$; $P < 0.001$) and there was an interaction between color, intensity, and genotype ($F = 3.901$; $P < 0.01$). The Student's t test showed that the relative pupilloconstriction of the TC + CC genotypes was larger than that of the TT genotype under the blue conditions of 14.5 and 15 log photons/(cm^2 s) ($P < 0.05$), and the same results were obtained under the green conditions of 14.5 and 15 log photons/(cm^2 s) ($P < 0.05$). However, there were no significant differences between genotypes under the red conditions.

Figure 4 Comparison of relative pupil constrictions (mean + standard deviation), based on 12 log photons/(cm^2 s), between TT ($n = 31$; black bars) and TC + CC ($n = 38$; white bars) genotypes. (a) Blue conditions, **(b)** Green conditions, **(c)** Red conditions. There were significant differences between TT and TC + CC under both blue and green conditions, but there was no difference under red conditions. *$P < 0.05$.

Discussion

The aim of this study was to determine the functional differences of the *OPN4* polymorphism (I394T) in PLR by addressing the relationship with light intensity and particularly light wavelength. First of all, it is important to confirm that the pupillary responses to the test stimuli were driven by ipRGCs. Several studies have demonstrated that the spectral characteristics of human ipRGCs have $\lambda_{max} = 480$ nm [11,26,28-30]. It has also been reported that melanopsin, included in ipRGCs, is the primary photopigment in driving pupillary response to high-irradiance light [31,32]. Consistent with those findings, our results showed that pupil sizes became smaller as the wavelength of light became closer to blue light (465 nm) and that pupil sizes decreased continuously with increasing intensity in both blue and green conditions. In contrast, there was no notable change in pupil size under red light conditions of 13 to 15 log photons/(cm^2 s), indicating that ipRGCs were not triggered by red conditions. Hence, the experimental data obtained in the present study are considered to be a result of the excitement of ipRGCs.

In the comparison of pupil sizes between genotypes, a significant difference between TT and TC + CC genotypes was found solely under the blue light condition of 15 log photons/(cm^2 s), and the pupil size of TC + CC genotypes was smaller than that of the TT genotype. Similarly, in our previous study, we found that *OPN4* gene I394T differences in PLR were apparent under a high-illuminance light condition [21]. The results of the present study, obtained using a new sample population, strongly support the results of our previous study. A notable result, however, is that a pupil size difference between the genotype groups only appeared under blue light. This seems to be a consequence of the spectral sensitivity of ipRGCs showing a peak at a short wavelength, as mentioned. Given that an association between genotypes and pupil size was found under a condition satisfying high intensity and short wavelength, our findings strongly suggest a causal relationship between I394T and PLR.