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Original Article

The effect of night shift work on the expression of clock genes in beard hair follicle cells

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ABSTRACT

Objective: Shift work encompasses a broad range of work time arrangements. However, how shift work affects the circadian expression of clock genes remains to be explored. The objective of this study was to evaluate the pattern of clock gene expression in shift workers in the field.
Methods: We examined clock gene expression in Japanese men who work: (1) one night shift followed by a day off (caregivers: nurses and doctors; the one-night group); (2) three or more consecutive night shifts (factory workers; the consecutive-night group); or (3) daytime only (the daytime group), using beard follicle samples. The expression of *Period3*, *Nuclear Receptor Subfamily 1 Group D Member 1* (*Nr1d1*), and *Nuclear Receptor Subfamily 1 Group D Member 2* (*Nr1d2*) was examined by real-time polymerase chain reaction.
Results: *Period3* expression in the daytime and one-night groups together with *Nr1d2* expression in the one-night group fitted a 24-h-period cosine curve better than in the consecutive-night group ($p = 0.004$, 0.012, and 0.001, respectively). The level of overall *Period3* gene expression, calibrated with that of *18S-rRNA*, was decreased in the consecutive-night group compared with that in the daytime group ($p = 0.006$). The patterns of *Period3* and *Nr1d2* expression in the daytime and one-night groups were more coherent than those in the consecutive-night group.
Conclusions: These results suggest that night shift work affects the rhythms and levels of circadian *Period3* and *Nr1d2* expression dependent on the shift schedule or type of the shift; however, there is substantial variation between individuals.

1. Introduction

More than 15% of the worldwide labor force currently works either in shift schedules or solely at night [1]. Shift work encompasses a broad range of working time arrangements; with schedule and working hours varying depending on the type of work. Shift work schedules include evening, night, morning, rotating, and irregular shifts. Sleepiness and fatigue are common complaints among shift workers. In three-shift systems, even the pattern of shift rotation is associated with sleep quality and quantity [2]. Thus, the type of shift work is closely associated with disturbances in the sleep–wake pattern [1]. Moreover, circadian misalignment affects the metabolic and cardiovascular responses, which can lead to

Abbreviations: ANOVA, analysis of variance; Cry, Cryptochrome; Ct, threshold cycle; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; MEQ, Horne–Ostberg Morningness–Eveningness Questionnaire; Nr1d1, Nuclear Receptor Subfamily 1 Group D Member 1; Nr1d2, Nuclear Receptor Subfamily 1 Group D Member 2; PCR, polymerase chain reaction; Per, Period; Per2, Period2; Per3, Period3.

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obesity and hypertension [3]. Many reports have described that shift work affects the rate of disease occurrence in diseases such as diabetes mellitus, gastrointestinal disorders, breast cancer, colorectal cancer, and prostate cancer in humans and mice [4].

Circadian mutants of *Drosophila melanogaster* were first reported in 1971 [5], and the responsible genes were identified in 1984 [6]. Furthermore, King et al., identified *Clock* as the first mammalian clock gene in 1997 [7]. Since then, many clock genes have been cloned and their functions have been thoroughly studied, mainly in mouse models. In the molecular circadian clock, CLOCK:BMAL1 heterodimers form the positive limb of a feedback circuit by initiating the transcription of a set of genes including *Period* (*Per*) and *Cryptochrome* (*Cry*); in turn, activated PER:CRY heterodimers inhibit CLOCK:BMAL1 transcriptional activity [8]. *Nuclear Receptor Subfamily 1 Group D Member 1* (*Nr1d1*; *Rev-erbα*) and *Nuclear Receptor Subfamily 1 Group D Member 2* (*Nr1d2*; *Rev-erbβ*) are necessary components for the normal regulation of circadian periodicity [9] and are also involved in lipid metabolism [10]. Clock genes also have effects on circadian period, sleep, fertility, psychology, and metabolism [8].

An evaluation of clock gene expression in human peripheral tissues was performed using oral mucosa [11], blood mononuclear cells [12–14], fibroblasts [15], hair and beard follicle cells [16], and adipose tissue [17]. Hair and beard follicle cells are the most convenient tissue source to evaluate gene expression by performing multiple sampling in the field [16,18,19], since the hair and beard can be plucked less invasively with tweezers by the subjects themselves, without help from others. Circadian fluctuations in *Period3* (*Per3*), *Nuclear Receptor Subfamily 1 Group D Member 1* (*Nr1d1*, *Rev-erbα*), and *Nr1d2* expression were clearly shown in the hair follicle cells of normal volunteers [16,20]. These three clock genes are suitable markers because changes in work time and heavy physical exercise at night delay the phase of circadian fluctuations in *Per3*, *Nr1d1*, and *Nr1d2* [16,19]. Since comprehensive information about clock gene expression is essential for understanding the circadian rhythmicity of shift workers, we sought, in this study, to explore *Per3*, *Nr1d1*, and *Nr1d2* gene expression in the peripheral tissues of shift workers, using the beard as an informative tissue source for which sampling is less invasive.

2. Subjects and methods

2.1. Participants

Five caregivers, two nurses and three medical doctors, were recruited from geriatric health service facilities and hospitals. They mostly worked a one-night shift, followed by a day off (the one-night group). Five caregivers worked from 07:30 or 10:00 for 9–10 h in the daytime shift and from 17:00 for 12–14 h in the night shift. Nurses and doctors work from 8:00 or 9:00 for 7–8.5 h on daytime duty. Night time shift starts at 16:30 or 17:00 and lasts for 13.5–14 h. Factory workers were also recruited from three printing factories. The length of each shift was typically 8 h in all three factories. Four participants worked from 8:30 for three days, and then from 19:15 for three days, followed by three days off. Five participants worked from 8:30 for two weeks, and from 19:15 for two weeks, alternately. Two participants worked from 11:30 for one week, and from 8:30 for one week, followed by a night shift starting at 21:00 for two weeks. Thus, all of the factory workers worked night shifts for three consecutive days or more (the consecutive-night group). The daytime group consisted of adult subjects who do not perform shift work. All of the participants were male (Table 1), as beard follicle cells were used as a source of RNA. Beard samples were mostly collected on the final day of consecutive night shifts. However, if there were concerns that the sampling may interfere with the execution of the participants' working duties, sampling was performed the same day that the night shift was concluded as soon as it was clear that sampling would not interfere with their duties. No participants had any serious sleep disorders including sleep apnea.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and the institutional review board of Tokyo Women's Medical University approved this prospective study. Written informed consent was obtained from all subjects. Sampling was performed without altering the subjects' usual daily life routine, without a fixed schedule for waking up, eating meals, or going to sleep. The subjects were requested to eat as usual and limit their intake of alcohol to two glasses of beer or the appropriate equivalent around the sampling days. As the participants were not strictly controlled and allowed to continue with their daily routines, this work constituted a field study.

2.2. Questionnaires

We used the Japanese version of the Horne–Ostberg Morningness–Eveningness Questionnaire (MEQ) to evaluate morning–evening tendency in daily life [21,22]. Age-adjusted MEQ scores [MEQ score + 0.3512 × (39.212 − age)] were used for classification into three groups: morning type (score 59–86), intermediate type (score 42–58), or evening type (score 16–41). The participants with insufficient answers to the questions in the questionnaires were excluded from the analysis.

2.3. RNA preparation

Beard follicle cells were used to evaluate the sequence of clock gene expression, as reported previously [16,22]. The participants were asked to collect beard follicle samples six times, preferably at 4-h intervals, over 24 h. Five or more beard hairs, including the root, were plucked with tweezers at each sampling point. The samples were immediately placed in a dissolution buffer (RNeasy Micro Kit;

Table 1
Characteristics and age-adjusted Morningness–Eveningness Questionnaire scores for the participants in whom clock gene expression was analyzed.

	Daytime	One-night	Consecutive
Age (years)	33.7 ± 9.6 (n = 11)	32.8 ± 9.2 (n = 10)	29.5 ± 6.7 (n = 11)
Duration of shift work (years)	N/A	4.4 ± 4.5 (n = 10)	9.2 ± 6.1 (n = 11)
Chronotype			
Morning	3	1	1
Intermediate	7	9	9
Evening	1	0	1
Age-adjusted MEQ Score	52.4 ± 7.9 (n = 11)	53.5 ± 4.9 (n = 10)	50.5 ± 6.0 (n = 11)
Sleep Duration Before Sampling (hours)	5.9 ± 1.1 (n = 11)	6.4 ± 1.6 (n = 9)	6.2 ± 1.6 (n = 11)
Usual Sleep Duration (hours)	6.4 ± 1.1 (n = 11)	7.0 ± 0.6 (n = 10)	7.0 ± 0.7 (n = 9)

Daytime, Daytime group; One-night, one-night group; Consecutive, consecutive-night group. Duration of shift work, duration of shift work before the sampling; MEQ Score, Horne–Ostberg Morningness–Eveningness Questionnaire score; N/A, not applicable. Data are expressed as mean ± standard deviation.

QIAGEN, Hilden, Germany) and kept in a freezer at −20 °C or below until they were used to extract RNA. Total RNA was reverse-transcribed using an Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.4. Measurement of clock gene expression

Real-time polymerase chain reaction (PCR) was performed using TaqMan MGB probes (Applied Biosystems, Foster city, CA, USA) in a 7500 Fast thermal cycler (Applied Biosystems). The sequences of the primers and probes used in this study are shown in Table 2. As a reference gene, we measured *18S-rRNA* expression. One- and five-microliter aliquots of a 100-fold dilution of cDNA were subjected to real-time PCR for *18S-rRNA* and *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), respectively. For quantitation of the other genes, 1.5-μL aliquots of cDNA were used to obtain threshold cycle (Ct) values by real-time PCR. The normalized expression levels of each gene were estimated by ΔCt, which was defined as the difference in Ct between the test gene and *18S-rRNA*. The peak time was defined as the actual beard collection time that gave the lowest ΔCt. Means of all the ΔCt values obtained for each gene were calculated to estimate clock gene expression levels for each subject. The rhythmicity of the expression time-course for each gene was evaluated by fitting the ΔCt values to a 24-h-period cosine curve using cosine curve-fitting software (Acro.exe version 3.5) [23]. Here, p values were used for individual expression profiles to assess the significance of the rhythms, and group differences were compared using the proportion of individuals in whom the expression of each of the clock genes was rhythmic. Analysis was performed when at least five out of six measurements were performed. The acrophases and amplitudes, which were determined by the cosine curve-fitting software, were analyzed only in cases with $p < 0.05$, although the peak times and ΔCt values were determined for all data.

2.5. Statistical analysis

Statistical analysis was performed using JMP (version 13.1; SAS Institute, NC, USA) and R-3.5.1. For comparison of each group, one-way analysis of variance (ANOVA), unpaired Student's t-test, Fisher's exact test, and Levene's test were performed using JMP, as appropriate. Circular statistics including the Rayleigh test were performed using R-3.5.1. A p value < 0.05 was considered to indicate statistical significance. In cases of post hoc comparison of three groups, p value < 0.017 was considered to indicate statistical significance according to Bonferroni correction. Results are presented as mean ± standard deviation.

3. Results

The expression of clock genes was examined using beard follicle samples collected in the field. Beard sampling was performed by two nurses after the night shift in the one-night group. Six samplings were achieved within an hour of the targeted sampling time in 27 out of 32 participants. All of the participants in the one-night group and the consecutive-night group had worked a similar shift schedule for more than one year before the study (one-night group 4.4 ± 4.5 years and consecutive-night group 9.2 ± 6.1 years; Table 1). The one-night group usually had 5–7 night shifts per month, while the consecutive-night group worked 9–12 nights per month. Mean age did not differ significantly among the three groups. Mean age-adjusted MEQ scores of the three groups were of the intermediate type ($p > 0.05$ among three groups, one-way ANOVA).

Real-time PCR data were fitted to a 24-h-period cosine curve. Examples of the peak times (actual sampling time with the highest expression level in a day) and acrophases (times estimated by cosine curve fitting) in the daytime group are shown in Fig. 1. *Per3* expression showed a significant fit to a 24-h-period cosine curve in the daytime group (64%) and the one-night group (50%). In sharp contrast, the proportion of individuals with *Per3* expression fitted to a 24-h-period cosine curve was less in the consecutive-night group than in the daytime group and in the one-night group (comparison among three groups, $p = 0.004$; daytime group versus consecutive-night group, $p = 0.004$; one-night group versus consecutive-night group, $p = 0.012$, two-sided Fisher's exact test; Table 3).

The proportion of individuals whose *Nr1d2* expression fitted to a 24-h-period cosine curve was greater in the one-night group than in the consecutive-night group (comparison among three groups, $p = 0.002$; one-night versus consecutive-night group, $p = 0.001$, two-sided Fisher's exact test). However, there was no significant difference in the proportion of *Nr1d1* expression among the three groups ($p > 0.05$, two-sided Fisher's exact test; Table 3). When the significantly fitting data were compared between the daytime and one-night groups, there were no significant differences in the acrophases or amplitudes of *Per3*, *Nr1d1*, or *Nr1d2* (Table 3).

Differences in mean ΔCt indicated that the overall levels of *Per3* expression in the consecutive-night group were decreased compared with those in the daytime group [comparison among three groups, $F(2,29) = 5.752$, $p = 0.008$, one-way ANOVA; daytime versus consecutive-night group, $p = 0.006$; Fig. 2]. ΔCt of *Per3* expression of four factory workers repeating a day shift, night shift, and day off every three days was not significantly different from that of seven other factory workers with consecutive night shifts for two weeks (15.78 ± 1.04 vs. 16.42 ± 1.34). *Nr1d2* expression in the one-night group was decreased compared with that in the daytime group [comparison among three groups, $F(2,29) = 5.013$, $p = 0.014$, one-way ANOVA; daytime versus one-night group, $p = 0.012$; Fig. 2]. No significant differences were detected in *Nr1d1* and *GAPDH* gene expression among the three groups [comparison among three groups, $F(2,29) = 1.021$, $p > 0.05$, and $F(2,29) = 0.148$, $p > 0.05$, respectively; one-way ANOVA].

For each subject, the actual clock time showing the highest level of gene expression was plotted as a peak time of gene expression (see Fig. 1). Most *Per3* peak times in the daytime and one-night groups were significantly directional (daytime group, mean 06:34, $p = 0.004$; one-night group, mean 05:34, $p = 0.002$,

Table 2
The sequences of primers for real-time PCR and TaqMan MGB probes.

Primer set	Forward primer	Reverse primer	TaqMan MGB probe
Per3	TCCAGCCCTACCACAGGTCT	ACGCCATAGAAAGCGGTGACT	TCGCCCACAGGGTCCTGCAGGCT
Nr1d1	GCTCAGTGCCATGTTCGACTTC	AAGTCTCCAAGGGCCGGTTC	AAGCTCAACTCCCTGGC
Nr1d2	TCCAGTACAAGAAGTGCCTGAAGAATGAAA	CACGCTTAGGAATACGACCAAACCGA	ATGTCAGCAATGTCG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC
18S-rRNA	CGCCGCTAGAGGTGAAATTC	CGAACCTCCGACTTTCGTTCT	CCGGCGCAAGACGGACCAGA

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Nr1d1, Nuclear Receptor Subfamily 1 Group D Member 1; Nr1d2, Nuclear Receptor Subfamily 1 Group D Member 2; Per3, Period3.

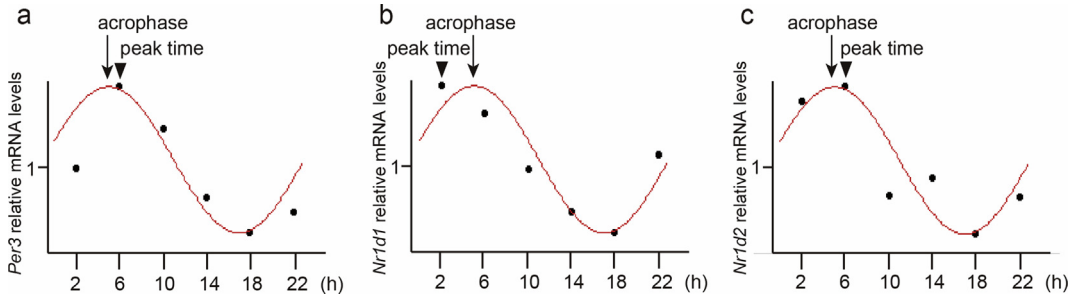

Fig. 1. Examples of 24-h-period cosine curves fitted to *Per3* (a), *Nr1d1* (b), and *Nr1d2* (c) expression data for a subject in the daytime group. Relative mRNA levels are plotted with mean mRNA levels scaled to 1. Peak times (actual time based on raw data) and acrophases (time estimated from the fitted cosine curve) are indicated by arrowheads and arrows, respectively.

Table 3
Twenty-four-hour cosine curve fitting for the daytime, one-night, and consecutive-night groups.

Gene		Daytime	One-night	Consecutive
Per3	Cosine fit	7/11	5/10	0/11 *,†
	Amplitude	0.7 ± 0.2 (n = 7)	0.9 ± 0.3 (n = 5)	(n = 0)
	Acrophase	7.7 ± 2.8 (n = 7)	5.8 ± 3.0 (n = 5)	(n = 0)
Nr1d1	Cosine fit	3/11	1/10	1/11
	Amplitude	0.5 ± 0.3 (n = 3)	0.5 (n = 1)	0.4 (n = 1)
	Acrophase	3.6 ± 1.8 (n = 3)	5.0 (n = 1)	11.5 (n = 1)
Nr1d2	Cosine fit	5/11	7/10	0/11 †
	Amplitude	0.6 ± 0.3 (n = 5)	0.5 ± 0.2 (n = 7)	(n = 0)
	Acrophase	5.0 ± 0.1 (n = 5)	5.6 ± 2.6 (n = 7)	(n = 0)

Daytime, Daytime group; One-night, One-night group; Consecutive, Consecutive-night group. Cosine fit, the numbers of participants with a significant fit to a 24-h-period cosine curve ($p < 0.05$) are shown. The acrophases and amplitudes of clock gene expression are compared between participants with a significant fit to a 24-h-period cosine curve ($p < 0.05$). Data are expressed as mean ± standard deviation. *: $p < 0.017$ versus daytime group; †: $p < 0.017$ versus one-night group (Bonferroni correction).

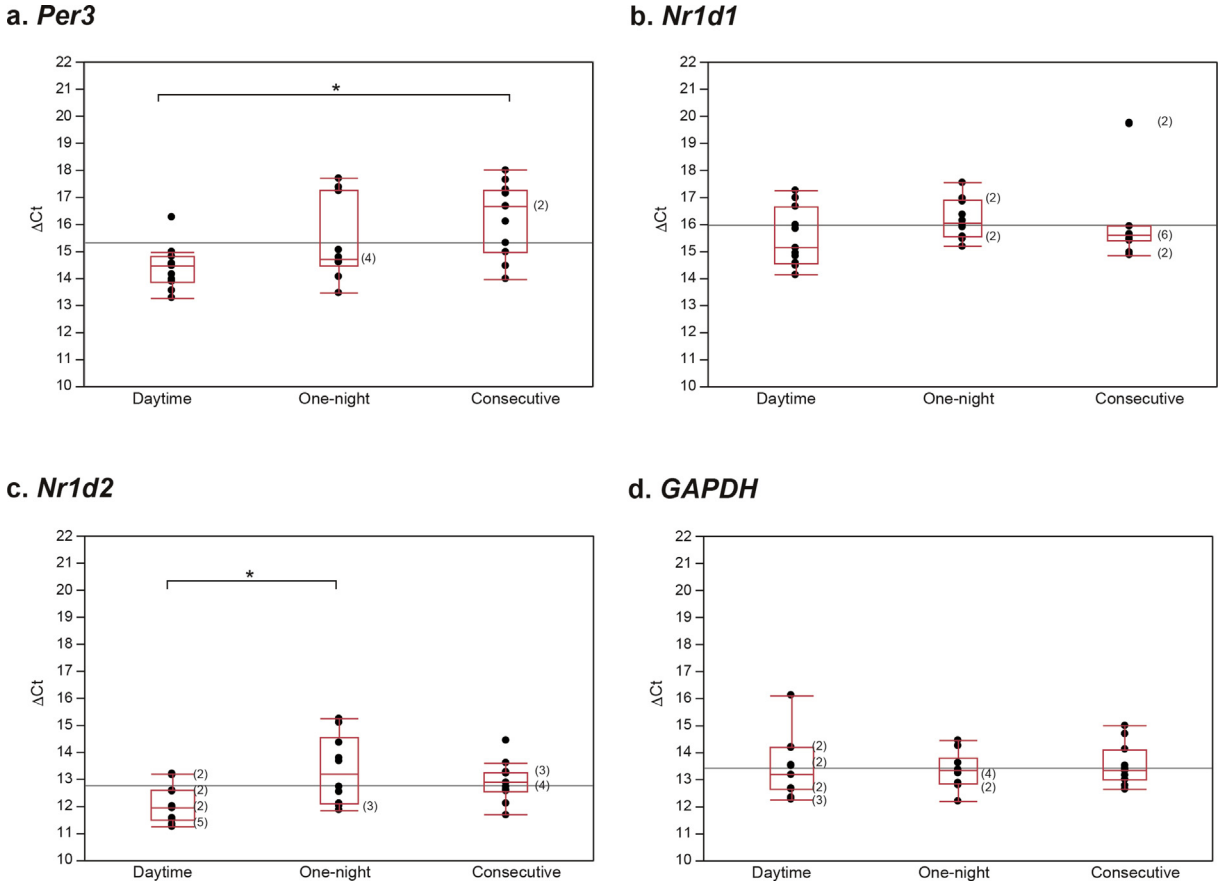

Fig. 2. Box-and-whisker plots of ΔCt values, calibrated against *18S-rRNA*, for *Per3* (a), *Nr1d1* (b), *Nr1d2* (c), and *GAPDH* (d) expression in the daytime group (Daytime), one-night group (One-night), and consecutive-night group (Consecutive). Lines indicate mean values. Where plotted data-points overlap, the numbers of points are indicated in parentheses. * denotes $p < 0.05$.

respectively, Rayleigh test; Fig. 3). In contrast, *Per3* peak times were less directional in the consecutive-night group (Fig. 3). Most *Nr1d2* peak times in the daytime and one-night groups were also significantly directional (daytime group, mean 07:33, $p = 0.008$; one-night group, mean 05:24, $p = 0.041$, Rayleigh test, respectively; Fig. 3). In contrast, *Nr1d2* peak times were less directional in the consecutive-night group ($p > 0.05$, Rayleigh test; Fig. 3). There were also no significant differences in *Nr1d1* peak times among the three groups (all three groups, $p > 0.05$; Rayleigh test).

4. Discussion

Per3 expression in the beard follicle cells is reported to be a reliable biomarker for the biological clock in men [16,20]. The present study suggested that peripheral clock gene expression in some shift workers did not follow a circadian cosine curve in usual daily life, since the expression patterns of *Per3* of most consecutive-night workers were far from a 24-h-period cosine pattern examined in the field (Table 3 and Supplementary data 1–3).

Moreover, the proportion of workers showing *Per3* and *Nr1d2* expression that fitted a 24-h-period cosine curve was significantly lower in the consecutive night-group than in the one-night group, suggesting that the effects of shift work on clock gene expression differed depending on the shift system. It is worth noting that the burden of night work for each man might have been heavier in the consecutive-night group than in the one-night group. Therefore, it might be that the frequency, rather than the type, of night shift work that played some role in clock gene expression. Of note, it was recently reported that a severe shift schedule reduced survival in mice more than a mild one [24]. The response of circadian

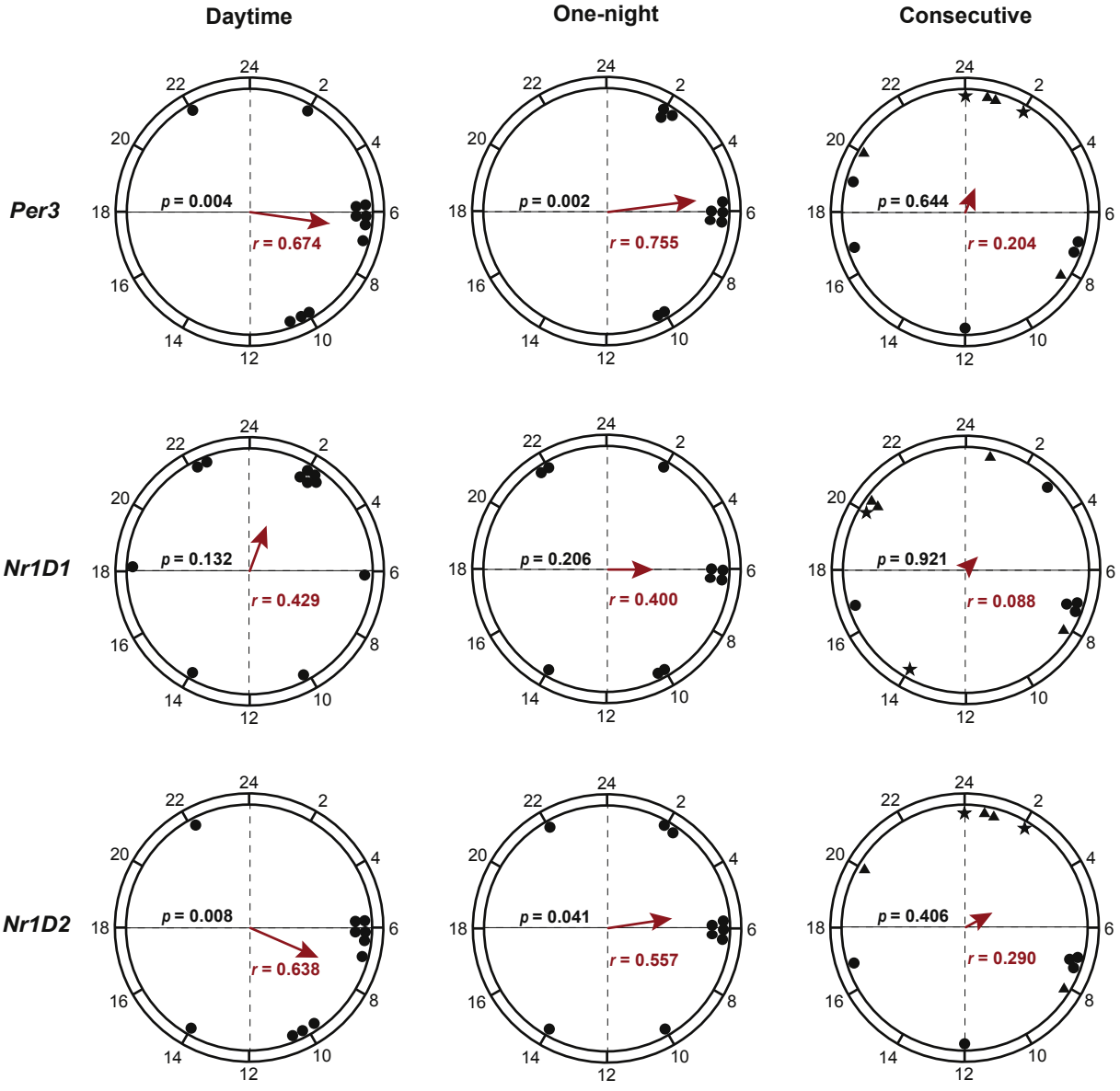

Fig. 3. Circular plots of the peak times of *Per3*, *Nr1d1*, and *Nr1d2* expression in the daytime group (Daytime), one-night group (One-night), and consecutive-night group (Consecutive). Mean peak times are indicated by red arrows. The length of the arrows reflects mean resultant lengths of the Rayleigh test, indicated as *r*, which reflect the strength of the directionality. Circles, the daytime group, the one-night group, and the workers of one factory with alternate two week night shifts; stars, the workers of the factory with the other alternate two week night shift schedule; triangles, the workers of the factory repeating day shift, night shift, and day off every three days.

gene expression to certain types of shift system might show substantial variation between individuals, since the variation of *Per3* expression was also exaggerated in consecutive-night workers.

The levels of *Per3* expression in the consecutive-night group and of *Nr1d2* expression in the one-night group were significantly reduced compared with those in the daytime group. The effect of shift work on the phase of circadian gene expression has often been discussed [16,25,26]; alternately, few reports describing the effect of shift work on the levels of clock gene expression have been published. On the one hand, the maxima of *Period2* (*Per2*) expression in pubic hair follicle cells were reported to be of lower intensity in rotating shift nurses [18]. On the other hand, the maxima and amplitudes of *Per3* and *Nr1d2* expression were both higher in rotating night shift workers than in daytime workers [16]. The reason for the inconsistent results regarding the levels of clock gene expression is not clear; however, the duration of shift work or the type of shift system might have a causal influence. The amplitudes of *Per3* and *Nr1d2* expression in the consecutive-night group could not be clearly determined in our study, since gene expression did not fit a 24-h-period cosine curve in most cases.

Disruption of *Per3* in mice does not lead to a robust phenotype, unlike disruption of *Per1* and *Per2* [27,28]. Although the physiological function of *Per3* remains elusive, *Per3* polymorphism is related to sleep structure, sleep disorders, and seasonal mood regulation [29–32], as well as vulnerability to daytime sleep disturbance in association with shift work [33]. It is also noteworthy that alteration of sleep behavior has been reported in *PER3*-deficient mice [34].

Our study has the following limitations. First, the present data represent gene expression for only one 24-h period in a small number of men. It might useful to analyze the gene expression in individuals over multiple circadian cycles to obtain a more comprehensive understanding of the sequence of clock gene expression in shift workers. It is also important to analyze clock gene expression in females to clarify whether there are sex differences. Second, circadian markers other than clock genes, such as the secretion of melatonin and cortisol, were not examined in the present study, limiting the comprehensiveness of our results. Third, we could not monitor the sleep–wake behavior of the participants because it was considered that the use of wrist actigraphy might obstruct the participants' work in the factory. In addition the working environments of the participants were markedly different, which could have contributed to between-group differences. Finally, there was incomplete information about the health of the participants and the sampling environment, such as lighting. It can thus not be ruled out that the health of the participants and their working environment influenced the results.

The occurrence of disease in association with shift work is related to both the shift system and an individual's tolerance to shift work [1]. A thorough understanding of clock gene expression profiles should be helpful for determining desirable shift work schedules.

5. Conclusion

Our data suggest that shift work has effects on the rhythmicity and levels of *Per3* and *Nr1d2* expression, depending on an individual's type of shift work. Characterizing an individual's pattern of clock-related gene expression might be helpful for individualizing their shift system in the future.

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Conflict of interest

The authors declare that they do not have anything to disclose regarding conflicts of interest with respect to this manuscript.

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: https://doi.org/10.1016/j.sleep.2019.01.005.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sleep.2019.01.005.

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