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Disruption of behavioral circadian rhythms induced by psychophysiological stress affects plasma free amino acid profiles without affecting peripheral clock gene expression in mice

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ABSTRACT

Disordered circadian rhythms are associated with various psychiatric conditions and metabolic diseases. We recently established a mouse model of a psychophysiological stress-induced chronic sleep disorder (CSD) characterized by reduced amplitude of circadian wheel-running activity and sleep–wake cycles, sleep fragmentation and hyperphagia. Here, we evaluate day–night fluctuations in plasma concentrations of free amino acids (FAA), appetite hormones and prolactin as well as the hepatic expression of circadian clock-related genes in mice with CSD (CSD mice). Nocturnal increases in wheel-running activity and circadian rhythms of plasma prolactin concentrations were significantly disrupted in CSD mice. Hyperphagia with a decreased leptin/ghrelin ratio was found in CSD mice. Day–night fluctuations in plasma FAA contents were severely disrupted without affecting total FAA levels in CSD mice. Nocturnal increases in branched-chain amino acids such as Ile, Leu, and Val were further augmented in CSD mice, while daytime increases in Gly, Ala, Ser, Thr, Lys, Arg, His, Tyr, Met, Cys, Glu, and Asn were significantly attenuated. Importantly, the circadian expression of hepatic clock genes was completely unaffected in CSD mice. These findings suggest that circadian clock gene expression does not always reflect disordered behavior and sleep rhythms and that plasma FFA profiles could serve as a potential biomarker of circadian rhythm disorders.

1. Introduction

The endogenous circadian clock in the suprachiasmatic nucleus (SCN) governs the various circadian rhythms of mammalian behavior and physiology such as sleep, body temperature, blood pressure and heart rate, as well as immune and metabolic functions. The findings of several studies at the molecular level have suggested that the periodic expression of clock genes such as *period* (*Per1*, *Per2*, and *Per3*), *cryptochrome* (*Cry1* and *Cry2*), *Clock*, *Bmal1*, and *Rev-erbα* drive the circadian oscillator in the SCN [1]. Autonomous oscillation mechanisms in various peripheral tissues such as the heart, lungs, liver, kidneys and circulating blood cells are entrained to the SCN. Measuring peripheral clock gene expression has been considered a useful method of detecting body time [2,3].

Chronic sleep disorders are associated with significant and cumulative neurobehavioral deficits and physiological changes that result in hypertension, obesity, diabetes, cardiovascular morbidity and stroke in addition to psychiatric conditions such as depression [4]. However, the underlying mechanisms have not yet been elucidated. Circadian rhythm-related sleep disorders arise from chronic changes, disruptions or misalignment of the circadian clock in response to environmental cues. We recently developed a mouse model of a chronic sleep disorder (CSD) induced by psychophysiological stress that is characterized by a reduction in the amplitude of circadian rhythms such as sleep–wake cycles, sleep fragmentation, hyperphagia and voluntary wheel-running for several weeks without adaptation [5]. Classical mouse models of CSD

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created using methods such as the disk-over-water, gentle handling, platforms (also called flower-pot) and slowly rotating wheels artificially affect circadian behavioral rhythms by providing circadian time cues, because they are based on forced daily physical sleep deprivation [6]. Our CSD model would be useful for investigating sleep disorders in humans that are associated with disrupted circadian rhythms induced by psychophysiological stress [5].

Concentrations of plasma metabolites such as sugars, fatty acids, phospholipids and amino acids fluctuate according to the time of day-dependent [7,8]. Metabolic disturbances might affect plasma free amino acid (FAA) concentrations, and plasma FAA profiles are abnormal in patients with various diseases such as obesity, diabetes, cancer, chronic kidney disease, acute and chronic liver disease, Alzheimer's disease and psychiatric disorders such as schizophrenia and depression [9–14]. The present study evaluates day–night fluctuations in plasma concentrations of FAAs, appetite hormones and prolactin, as well as the hepatic expression of circadian clock-related genes in mice with CSD (CSD mice).

2. Materials and methods

2.1. Animals and behavioral monitoring

Seven-week-old male C3H/HeN mice (Japan SLC Inc., Hamamatsu, Japan) that were individually maintained in plastic cages with running wheels (SW-15, Melquest, Toyama, Japan) were fed with a normal diet (CE-2, Clea Japan Inc., Tokyo, Japan) *ad libitum* for three weeks until daily wheel-running activity reached a plateau under a 12 h light–12 h dark cycle (LD 12:12; lights on at Zeitgeber time [ZT] 0; light [500 lux] was provided by a white fluorescent lamp placed at the cage level). The mice were then exposed to psychophysiological stress to induce CSD as described [5]. Briefly, paper-chip bedding was replaced with water to a depth of 1.5 cm depth which caused the mice to run on the wheel all day. After one week of CSD, the mice were sacrificed to obtain whole-blood samples and liver tissues at the indicated time points. Plasma samples for FAA evaluations were collected from the mice after a five-hour fast. Wheel-running activity was continuously recorded at 1-min intervals using Chronobiology Kits® (Stanford Software Systems, Stanford, CA, USA) and activity data are displayed as actograms. Animals were maintained and experiments proceeded under the approval of the Animal Care and Use Committee at the National Institute of Advanced Industrial Science and Technology (AIST) (Permission #2013-020).

2.2. RNA extraction and quantitative real-time RT-PCR

Hepatic total RNA was extracted using RNAiso Plus (Takara Bio Inc., Otsu, Japan). Single-stranded cDNA was synthesized using PrimeScript™ RT reagent kits with gDNA Eraser (Takara Bio Inc., Otsu, Japan). Real-time RT-PCR proceeded using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Otsu, Japan) and a LightCycler™ (Roche Diagnostics, Mannheim, Germany). The reaction conditions were 95 °C for 10 s followed by 45 cycles of 95 °C for 5 s, 57 °C for 10 s and 72 °C for 10 s. Supplemental Table 1 shows the sequences of the primer pairs. The amount of target mRNA was normalized relative to that of β-actin.

2.3. Amino acid analysis

Mouse blood collected in EDTA-coated tubes was immediately separated by centrifugation for 15 min at 5800×*g*. Plasma samples were deproteinized by mixing with an equal volume of 6% sulfosalicylic acid and separated by centrifugation for 15 min at 5800×*g*. The supernatants were decanted and stored at −80 °C. Plasma FAA contents were determined using a Hitachi L-8800E amino acid analyzer. Amino acids were separated by high-resolution ion-exchange chromatography and detected by spectrophotometry at 570 and 440 nm after post-column ninhydrin derivatization. The areas under the curves (AUC) were calculated using the manufacturer's software for final quantitation.

2.4. Statistical analysis

All data are expressed as means ± standard error of the means (SEM) and were statistically evaluated using the analysis of variance (ANOVA) for repeated measures followed by Tukey's test using Excel-Toukei 2010 software (Social Survey Research Information Co. Ltd., Tokyo, Japan). $P < 0.05$ indicated a statistically significant difference.

3. Results and discussion

Nighttime wheel-running by nocturnal mice exposed to psychophysiological stress-induced CSD decreased, whereas that in the morning increased as described (Fig. 1A and B) [5]. Daily food intake increased by 35%, although body weight did not significantly differ (Fig. 1C and D). The hyperphagia found in CSD mice

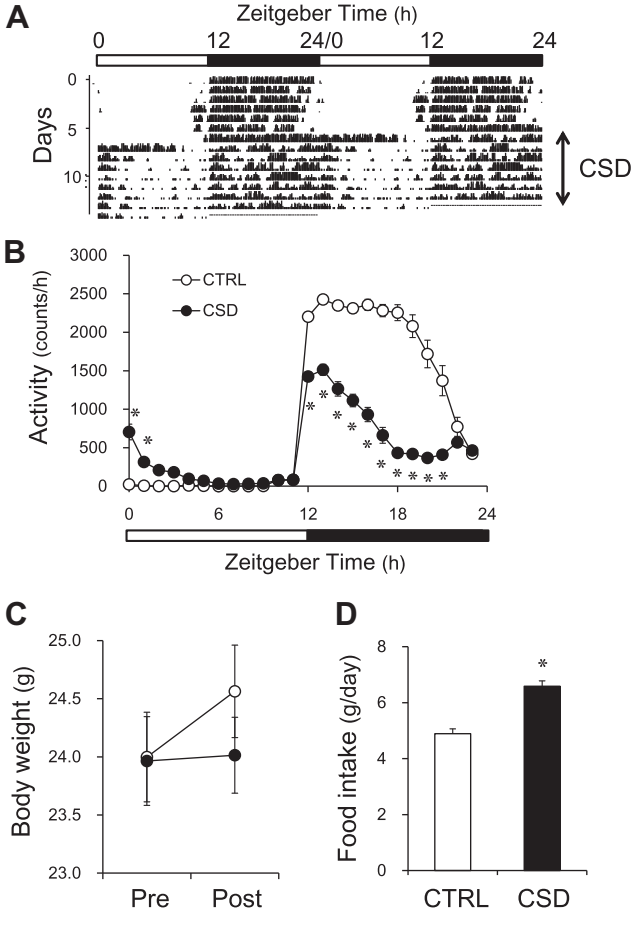

Fig. 1. Disrupted circadian wheel-running activity in a mouse model of chronic sleep disorders induced by psychophysiological stress (CSD). Mice individually housed under LD 12:12 (lights on at 0 h) were exposed to psychophysiological stress for seven days to induce CSD. (A) Representative double-plot actogram of wheel running behavior. (B) Wheel running activities of control (CTRL) and CSD mice. (C) Changes in body weight during CSD. (D) Food consumption in CTRL and CSD mice. Horizontal unfilled and solid bars indicate day and night, respectively. Open circles/bars and closed circles/bars indicate CTRL and CSD mice, respectively. *$P < 0.01$, significant differences between groups.

seemed to result from a decrease in satiety levels because the ratio of the anorexigenic hormone leptin to the orexigenic factor ghrelin was significantly lower during the morning (Supplemental Fig. 1).

Plasma concentrations of prolactin, a multifunctional pituitary hormone, increase with the onset of sleep irrespective of the time of the day that sleep occurs, although a circadian component might also be involved in prolactin secretion [15]. We found here that plasma prolactin levels in control mice increased during the light period and decreased during the night (Supplemental Fig. 1), whereas those in CSD mice were 3.6-fold higher than those in control mice at midnight. These findings could account for the physical inactivity and increased sleep during the dark phase in CSD mice [5]. Functional impairment of hypothalamic dopamine might be associated with the CSD-induced nocturnal increase in prolactin, because the regulation of prolactin secretion is mainly inhibitory and dopamine is the principle inhibitory factor [16].

We assessed temporal expression profiles of circadian clock genes (*Per1*, *Per2*, *Bmal1*, and *Dec2*) and a representative clock-controlled gene *Dbp* in the liver to determine the effect of CSD on peripheral circadian clocks (Fig. 2). Rhythmic expression of these circadian genes was unaffected, although CSD obviously disrupted the circadian profiles of wheel running activity and sleep–wake cycles.

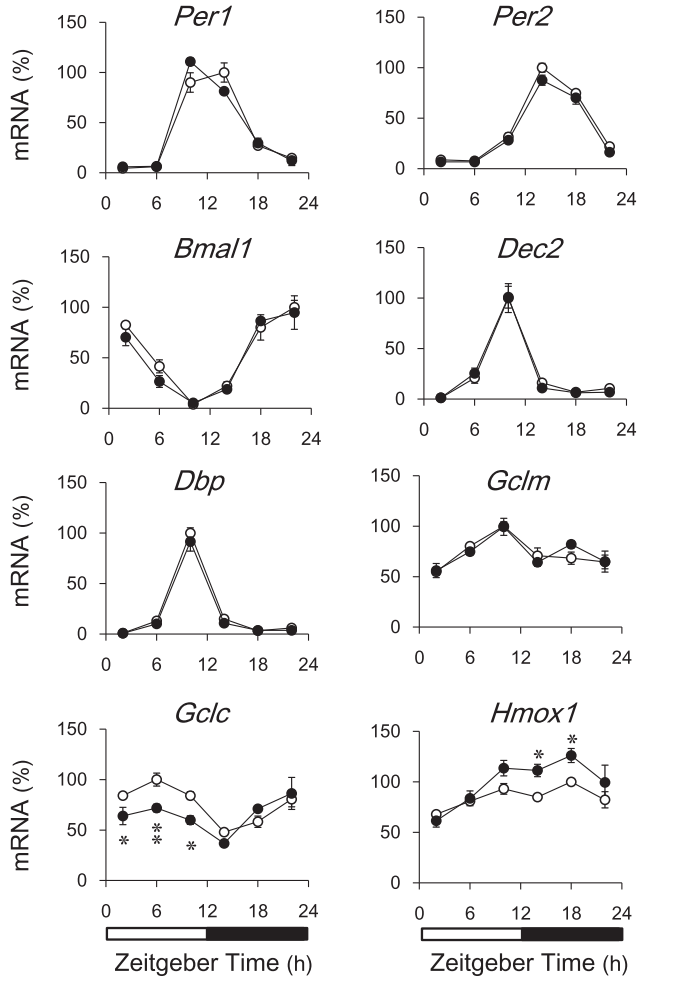

Fig. 2. Temporal mRNA expression profiles of circadian genes in the liver of CSD mice. Mice were induced CSD as described in Fig. 1. After one week of CSD, mice were sacrificed at each time point and total RNA was extracted from livers. Messenger RNA levels were measured by quantitative RT-PCR. Maximal value for control mice is expressed as 100%. Values are shown as means ± SEM ($n = 4$). Horizontal open and solid bars, day and night, respectively. Significant differences between groups are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$.

Chronic psychological stress affects anti-oxidative functions in the liver [17], although the relationship between disordered sleep and hepatic redox status is unknown. The circadian clock appears to directly or indirectly regulate physiological redox status and the transcription of redox-related genes [18,19]. We assessed the temporal mRNA expression of oxidative stress-responsive NRF2 target genes such as *Gclm*, *Gclc*, and *Hmox1* in the livers of CSD and control mice. Messenger RNA levels of *Gclc* and *Hmox1* in CSD mice significantly decreased and increased during the light and dark periods, respectively, (Fig. 2). These findings suggest that CSD affects the redox status in a circadian manner without affecting the peripheral molecular clock in the liver.

We measured plasma FAA concentrations at ZT6 and ZT18 in the CSD mice (Table 1 and Fig. 3). Plasma concentrations of Gly, Ala, Ser, Thr, Lys, Arg, His, Tyr, Met, Cys, Pro, Glu, and Asn were significantly higher at ZT6 (inactive phase) than at ZT18, although concentrations of Phe and branched-chain amino acids (BCAAs) such as Val, Ile, and Leu were significantly higher at ZT18 (active phase) in control mice. Plasma concentrations of Trp, Gln, and Asp were identical between ZT6 and ZT18. Nighttime increases in Phe and BCAA concentrations were essentially the same as those in previous reports [20]. Plasma concentrations of Thr, Pro, and Met were higher at ZT6 than at ZT18, which were in contrast to

Table 1
Plasma concentrations of amino acids.

		CTRL	CSD
Gly	ZT6	301.6 ± 23.1	214.0 ± 13.9**
	ZT18	200.2 ± 72.1††	181.8 ± 15.5
Ala	ZT6	447.4 ± 20.4	360.6 ± 22.7*
	ZT18	129.6 ± 12.8††	164.4 ± 38.9††
Ser	ZT6	175.0 ± 9.2	129.4 ± 7.7**
	ZT18	92.4 ± 5.1††	101.8 ± 6.6†
Thr	ZT6	209.6 ± 23.3	141.0 ± 12.9**
	ZT18	126.0 ± 7.3††	142.4 ± 17.2
Val	ZT6	254.0 ± 28.6	207.6 ± 13.5
	ZT18	392.0 ± 15.3††	509.0 ± 28.0**,††
Ile	ZT6	104.8 ± 11.6	88.4 ± 4.4
	ZT18	171.0 ± 7.7††	205.4 ± 8.1*,††
Leu	ZT6	157.8 ± 14.4	143.8 ± 6.1
	ZT18	280.2 ± 12.4††	345.4 ± 21.0**,††
Lys	ZT6	389.0 ± 32.8	258.8 ± 20.2**
	ZT18	196.6 ± 11.4††	250.4 ± 29.4
Arg	ZT6	167.0 ± 18.1	124.2 ± 11.1*
	ZT18	72.6 ± 2.5††	95.6 ± 6.9
His	ZT6	74.6 ± 3.7	63.2 ± 2.1**
	ZT18	58.2 ± 0.97††	58.6 ± 3.3
Tyr	ZT6	97.2 ± 10.0	70.8 ± 40.7**
	ZT18	53.4 ± 2.2††	62.8 ± 2.7
Phe	ZT6	67.4 ± 48.1	59.2 ± 16.2
	ZT18	80.8 ± 1.2††	88.8 ± 4.0†
Trp	ZT6	74.0 ± 6.7	69.2 ± 2.3
	ZT18	71.4 ± 6.8	86.0 ± 3.9†
Met	ZT6	67.4 ± 4.9	50.4 ± 3.1*
	ZT18	37.4 ± 4.0††	40.8 ± 6.0
Cys	ZT6	58.6 ± 3.0	47.8 ± 2.8**
	ZT18	50.4 ± 2.5†	41.0 ± 1.2*
Pro	ZT6	99.2 ± 9.9	77.2 ± 7.1
	ZT18	43.2 ± 6.5††	53.0 ± 13.3
Gln	ZT6	541.8 ± 37.3	509.0 ± 33.8
	ZT18	620.4 ± 17.1	607.0 ± 29.2†
Glu	ZT6	53.0 ± 4.5	40.2 ± 1.7*
	ZT18	42.6 ± 1.8†	37.4 ± 4.6
Asn	ZT6	53.8 ± 3.9	37.8 ± 3.1**
	ZT18	29.8 ± 0.97††	29.8 ± 2.0
Asp	ZT6	8.4 ± 0.81	7.6 ± 0.60
	ZT18	9.0 ± 0.45	8.8 ± 0.58

CTRL, control; CSD, chronic sleep disorder. Data are shown as means ± SEM (ng/ml).
* Significant differences compared with CTRL value are indicated as: $P < 0.05$.
** Significant differences compared with CTRL value are indicated as: $P < 0.01$.
† Significant differences compared with ZT6 value are indicated as: $P < 0.05$.
†† Significant differences compared with ZT6 value are indicated as: $P < 0.01$.

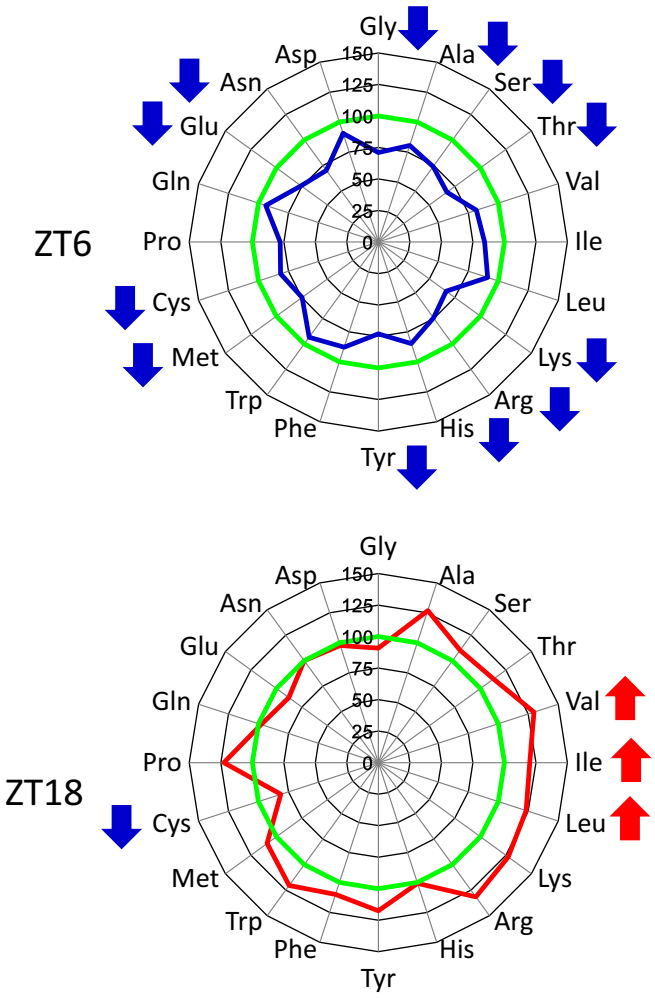

Fig. 3. The radar charts for the relative changes in plasma FAAs. Chronic sleep deprivation (CSD) was induced in mice as described in Fig. 1. Mice were sacrificed one week later at ZT6 and ZT18 after five hours of fasting. Averaged value for control mice is expressed as 100% at each time point. (Table 1 shows absolute values of each group.) Upward and downward arrows indicate significant increases and decreases, respectively, in CSD mice.

the findings of Minami et al. [20], who found that these FAA concentrations were higher at ZT18 than ZT6. These differences seemed to be associated with different experimental conditions because we starved the mice for five hours before collecting blood. Many metabolic processes [21] as well as feeding and activity rhythms appear to be involved in the circadian regulation of plasma FAA concentrations.

Only 8 of 20 FAAs showed day–night fluctuation in CSD mice, whereas 17 of 20 FAAs significantly fluctuated in control mice (Table 1). Daytime increases in plasma Gly, Thr, Lys, Arg, His, Tyr, Met, Cys, Pro, Glu, and Asn concentrations were significantly reduced whereas nighttime increases in BCAAs were significantly enhanced in CSD mice. Although the underlying mechanisms remain obscure, these findings nonetheless showed that plasma FAA profiles were time-of-day-dependently disrupted in CSD mice whereas the molecular clocks were not affected. Unlike other animal models of sleep deprivation generated by methods such as the disk-over-water, gentle handling, platforms and slowly rotating wheels [6], our method caused stress in mice for more than six weeks without complicated implementation, adaptation, or artificially affecting circadian behavioral rhythms by providing circadian time cues [5]. We found that the effect of CSD on plasma FAAs was considerably differed between day and night. Reductions in the levels of many diurnal FAAs in CSD mice were similar to the transient effects of typical stressors such as restraint [22], immobilization [23] and electric shocks to paws [24]. The day–night amplitude of plasma BCAAs was significantly augmented by increasing nighttime concentrations in CSD mice. Skeletal muscle contains the largest physiological FAA pool and physical exercise increases BCAA catabolism by activating branched-chain α-keto acid dehydrogenase [25]. Nighttime inactivity in CSD mice might have been involved in the increased levels of plasma BCAAs in the present study.

Biomarkers for sleep disorders would be of enormous practical use in sleepless societies. Body time has been widely determined based on measurements of peripheral clock gene expression [2,3]. However, environmental stress can remarkably disrupt circadian behavioral and sleep–wake rhythms without affecting clock gene expression according to the present and previous findings [5]. Circadian profiles of FAAs might be a potential biomarker for sleep disorders such as jet-lag and insomnia and for sleep-related disorders such as depression, anxiety and chronic fatigue syndrome. Relationships between sleep disorders and plasma FAA profiles in humans should be investigated in detail.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.083.

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