BIOCHEMICAL PHARMACOLOGY 75 (2008) 1616–1622

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Modulatory effects of 5-fluorouracil on the rhythmic expression of circadian clock genes: A possible mechanism of chemotherapy-induced circadian rhythm disturbances

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ARTICLE INFO

Article history:
Received 31 December 2007
Accepted 24 January 2008

Keywords:
Chemotherapy
5-Fluorouracil
Circadian rhythm
Clock genes
Suprachiasmatic nuclei

ABSTRACT

The circadian clock system is necessary to adapt endogenous physiological functions to daily variations in environmental conditions. Abnormality in circadian rhythms, such as the sleep–wake cycle and the timing of hormonal secretions, is implicated in various physiological and psychiatrical disorders. Recent molecular studies have revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of 24 h cycles of physiology and behavior. It has been noticed that patients receiving chemotherapeutic agents experience disturbances in their behavioral and physical performances, including circadian rhythms. To explore the underlying mechanism of chemotherapeutic agent-induced disturbance of these rhythms, we investigated the influence of 5-fluorouracil (5-FU), one of the most widely used chemotherapeutic agents for the treatment of cancers, on the expression of clock genes. Treatment of cultured NIH3T3 cells with 5-FU for 48 h resulted in a significant reduction of mRNA levels of *Period1* (*Per1*) and *Period2* (*Per2*) without affecting cell viability; however, treatment with the same amount of uracil, a structural analog of 5-FU, had little effect on the expression of clock genes. Consistent with its inhibitory actions, continuous administration of 5-FU (2 mg/kg/h) to mice attenuated the oscillation in the expressions of *Per1* and *Per2* in the liver and suprachiasmatic nuclei, the center of the mammalian circadian clock. These results reveal a possible pharmacological action by the chemotherapeutic agent 5-FU on the circadian clock mechanism, which is the underlying cause of its adverse effects on 24-h rhythms of physiology and behavior.

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Abbreviations: 5-FU, 5-fluorouracil; ATP, adenosine triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SCN, suprachiasmatic nucleus.

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doi:10.1016/j.bcp.2008.01.011

1. Introduction

Anticancer chemotherapeutic medication in either curative or palliative settings is associated with various undesirable side effects, and the patient's quality of life is remarkably decreased [1]. These side effects may include acute and delayed nausea, vomiting, and anorexia [2–4]. Decreased whole body weight, disrupted gastrointestinal tract function, including dyspepsia and diarrhea, and also fatigue are accompanied in many cases [5,6].

Cancer chemotherapeutic drug-associated fatigue has subjective (self-reported) and objective (reduced physical activity or capacity to undertake physical and mental tasks) dimensions. It differs from body tiredness and the feeling of fatigue by sleep deprivation or excessive physical exercise in that this fatigue is not relieved by rest or sleep [5]. Measuring the amount of physical activity using ''Actigraphy'' in patients receiving chemotherapeutic drugs and comparing with the results of normal people showed that physical activity is not only decreased but also that there is a disturbance of the circadian rhythm [7]. However, the detailed mechanism of this disturbance of the circadian rhythm has not been clarified.

The mammalian circadian system is hierarchically organized by central and peripheral oscillators. An ensemble of coupled oscillators in the suprachiasmatic nucleus (SCN) of the hypothalamus is entrained to a 24-h period by daily light input from the visual system. Neural and humoral output signals from the SCN coordinate the phase of independent circadian oscillators in peripheral tissues throughout the organism [8,9]. Self-sustaining circadian oscillators in the SCN use a molecular mechanism similar to that used in subsidiary oscillators present in all cell types in the organism [10]. Recent molecular dissection of the circadian biological clock system has revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of circadian rhythms. Gene products of *Clock* and *Bmal1* form a heterodimer that activates the transcription of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. Once PER and CRY proteins have reached a critical concentration, they attenuate CLOCK/BMAL1 transactivation, thereby generating circadian oscillation in their own transcription [11,12]. The clock genes, consisting of the core oscillation loop, control downstream events by regulating the rhythmic expression of clock-controlled genes [13–15].

To address the mechanism underlying chemotherapeutic agent-induced circadian rhythm disruption, we investigated whether oscillation in the expression of clock genes was influenced by chronic treatment with 5-fluorouracil (5-FU), one of the most frequently used chemotherapeutic agents for the treatment of cancers. Furthermore, we also explored how treatment with 5-FU affected the SCN oscillatory function and locomotor activity rhythm in mice.

2. Materials and methods

2.1. Materials

The following materials were commercially obtained: 5-fluorouracil, uracil and dexamethasone (Dex) from Wako Pure Chemical Industries Ltd. (Tokyo, Japan); 2-deoxy-[^{3}H]-glucose ([^{3}H]-2-DG) and [^{14}C]-Leucine ([^{14}C]-Leu) from GE Healthcare (Chalfont St. Giles, UK); Dulbecco's modified Eagle's medium (DMEM; product #D6046) and fetal bovine serum (FBS), sometimes called fetal calf serum (FCS) from Sigma–Aldrich (St. Louis, MO).

2.2. Cells and treatment

Mouse NIH3T3 fibroblasts were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Period2-Luciferase (Per2-Luc)-expressing C6 cells were constructed as described previously [16]. Confluent culture of NIH3T3 cells and Per2-Luc C6 cells were cultured under 5% CO_2 environment at 37 °C in DMEM medium contain 10% of FCS. NIH3T3 were treated with the indicated concentrations (1.0–10.0 μM) of 5-FU or 10.0 μM uracil for 48 h. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's procedure. Oscillations of reporter luciferase bioluminescence driven by the mouse Per2 promoter in Per2-Leu C6 cells were triggered by 2-h treatment of 10 nM Dex, and Dex-treated cells were subsequently incubated in serum-free DMEM in the presence or absence of 10 μM of 5-FU. Bioluminescence from Per2-Leu C6 cells was measured by photomultiplier tube detector assemblies (Lumicycle, Neuroscience). Cell viability in culture was determined by measuring the level of ATP in cells during treatment of 5-FU (Cell Viability Luminescent Assay; Promega, Madison, WI).

2.3. Animals and treatment

Male ICR mice (5 weeks old) were purchased from Charles River Co. (Kanagawa, Japan). They were housed under a standardized 12-h light–dark cycle (light, 7:00–19:00) at room temperature of 24 °C and humidity of 60 ± 10% with food and water *ad libitum*. The animals were adapted to the light–dark cycle for 2 weeks before the experiments. An osmotic minipump (model 2001, ALZET; Alza Corp., Palo Alto, CA) was implanted under the dorsal hypodermic region of mice, and was used for continuous administration of 5-FU (2 mg/kg/h) or saline. The mRNA levels of the clock gene (*Per1* and *Per2*) in the SCN and liver were measured six times, at 09:00, 13:00, 17:00, 21:00, 01:00, and 05:00 on day 7 after the initiation of treatment with 5-FU or saline. The brain and liver were removed at each of the above-mentioned six times, and coronal brain slices (500 μm) were prepared using rodent brain matrix (RBM-2000C). The SCN was punched out of bilateral brain slices. RNA was extracted by using Trizol reagent (Invitrogen) from the SCN and liver. SCN oscillatory function was evaluated by measuring the amount of uptake of [^{3}H]-2-DG and [^{14}C]-Leu at 7:00, 13:00, 19:00, and 01:00 on day 7 after the initiation of treatment with 5-FU or saline. Locomotor activity was measured after the initiation of treatment with 5-FU or saline.

2.4. Quantitative RT-PCR

The cDNA of mouse *Per1*, *Per2*, and *GAPDH* gene was synthesized and amplified by using Superscript One-step RT-PCR system (Invitrogen). Electrophoresis of the reaction product using 2% agarose gel, and then ultraviolet rays were

irradiated after staining with ethidium bromide. The photograph was digitalized with a scanner, and the fluorescent strength of each band was expressed numerically with software (NIH Image 1.63, National Institute of Health) for image analysis.

2.5. Measurement of [^{3}H]-2-DG and [^{14}C]-Leu uptake in the SCN

Coronal brain slices, including SCN, were prepared as described above, and pre-incubated for 30 min with 1 ml of Krebs-Ringel buffer (128.9 mM NaCl/4.2 mM KCl/1.5 mM $CaCl_2$/22.4 mM $NaHCO_3$/1.2 mM KH_2PO_4/1.3 mM $MgSO_4$/10 mM D-glucose) warmed to 37 °C and equilibrated with 95% O_2 and 5% CO_2. After pre-incubation, [^{3}H]-2-DG and [^{14}C]-Leu solution were added to the incubation buffer to give final concentrations of 1 and 5 μCi/ml of each isotope reagent. The uptake of each reagent by brain slices was carried out for 60 min at 37 °C. Uptake was terminated by aspirating the incubation buffer, and rinsing the slice with 2 ml of ice-cold gassed Krebs-Ringel buffer at 3 times. The SCN was punched out of the brain slice and homogenized in 100 μl phosphate buffer. The radioactivity of the homogenate was determined by liquid scintillation counter (LSC-1000, Aloka LC1000 Co., Mitaka, Tokyo, Japan) after sufficient solubilization. The amounts of each reagent uptake in SCN were expressed as dpm/μg protein.

2.6. Measurement of locomotor activity

Locomotor activity data were recorded continuously on a PC (Chronobiology Kit; Stanford Software Systems, Santa Cruz, CA). The breeding cage was put into the infrared ray area sensor, and the amount of the action was measured every hour.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used for multiple comparison and the Bonferroni test was used for specific comparison between the two groups. A significance level of 5% or less was assumed to be significant.

3. Results

3.1. Influence of 5-FU on mRNA levels of clock gene in NIH3T3 cells

To test the possibility that chemotherapeutic agents affect the expression of clock genes, we investigated the influence of 5-FU on mRNA levels of *Per1* and *Per2* in NIH3T3 cells. Treatment of confluent cultured cells with 5-FU for 48 h resulted in a reduction of mRNA levels of *Per1* and *Per2* in a dose-dependent manner (Fig. 1A). The most significant reduction was seen at a concentration of 10 μM ($P < 0.05$); however, the same concentration of uracil had little effect on mRNA levels of these clock genes. Consistent with these observations, exposing NIH3T3 cells to 10 μM 5-FU gradually decreased the mRNA levels of *Per1* and *Per2*, but the same concentration of uracil did not show a significant influence on the expression of these genes (Fig. 1B). During these experiments, there were no significant influences of 10 μM 5-FU on cell viability, although treatment with 100 μM 5-FU significantly decreased the viability of cells ($P < 0.05$; Fig. 1C).

3.2. Influence of 5-FU on the oscillation of reporter luciferase bioluminescence in Per2::Leu C6 cells

We next examined whether chronic treatment with 5-FU affected rhythmicity in the expression of clock genes. Following treatment with 10 nM Dex for 2 h, Per2-Luc C6 cells showed bioluminescence oscillation over a period close to 24 h. This circadian oscillation was generally sustained for more than 5 days (Fig. 2 upper panel). On the other hand, no obvious bioluminescence rhythm was observed for Per2-Luc C6 cells treated with 10 μM of 5-FU (Fig. 2 lower panel). The amplitude of the rhythm was decreased by treatment. These results suggest that 5-FU has the ability to inhibit oscillation in the expression of clock genes.

3.3. Influence of 5-FU on rhythmicity in the expression of clock genes in the SCN and liver

In mammals, a master circadian pacemaker regulating the rhythmicity of physiology and behavior resides in the SCN of the anterior hypothalamus [8,9]. We thus examined whether 5-FU affected rhythmicity in the expression of clock genes in the SCN of mice. The mRNA levels of *Per1* and *Per2* in the SCN of saline-treated mice showed significant circadian oscillations ($P < 0.05$). Their mRNA levels increased during the light phase and decreased during the dark period (Fig. 3A). On the other hand, continuous administration of 5-FU severely decreased the amplitude of the rhythms in the expression of *Per1* and *Per2* in the SCN, although their mRNA levels still exhibited circadian oscillations. Similar decreased amplitude of the rhythms was also observed in the expression of *Per1* and *Per2* in the liver of mice continuously administered 5-FU (Fig. 3B).

3.4. Influence of 5-FU on the SCN oscillatory function and locomotor activity rhythm

Because the expressions of clock genes in the SCN and liver were altered by continuous administration of 5-FU, we examined how 5-FU affected overt rhythms in physiology and behavior. The amounts of [^{3}H]-2-DG uptake, an index of energy metabolism, into the SCN of saline-treated mice showed a significant circadian oscillation, with a high value during the light phase (13:00) and a low value during the dark phase (Fig. 4A, $P < 0.01$). In marked contrast, the continuous administration of 5-FU completely abolished the rhythm of [^{3}H]-2-DG uptake in SCN: the amount of [^{3}H]-2-DG remained at trough levels of the control. In addition, the amount of [^{14}C]-Leu uptake, an index of protein synthesis, into the SCN of the saline-treated group was high in the early light phase, while the nadir was observed during the dark phase (Fig. 4B). In contrast, continuous administration of 5-FU induced an altered rhythm of [^{14}C]-Leu uptake into the SCN.

In the behavioral study, control mice clearly exhibited an entrained locomotor activity rhythm, and thus hyperactivity

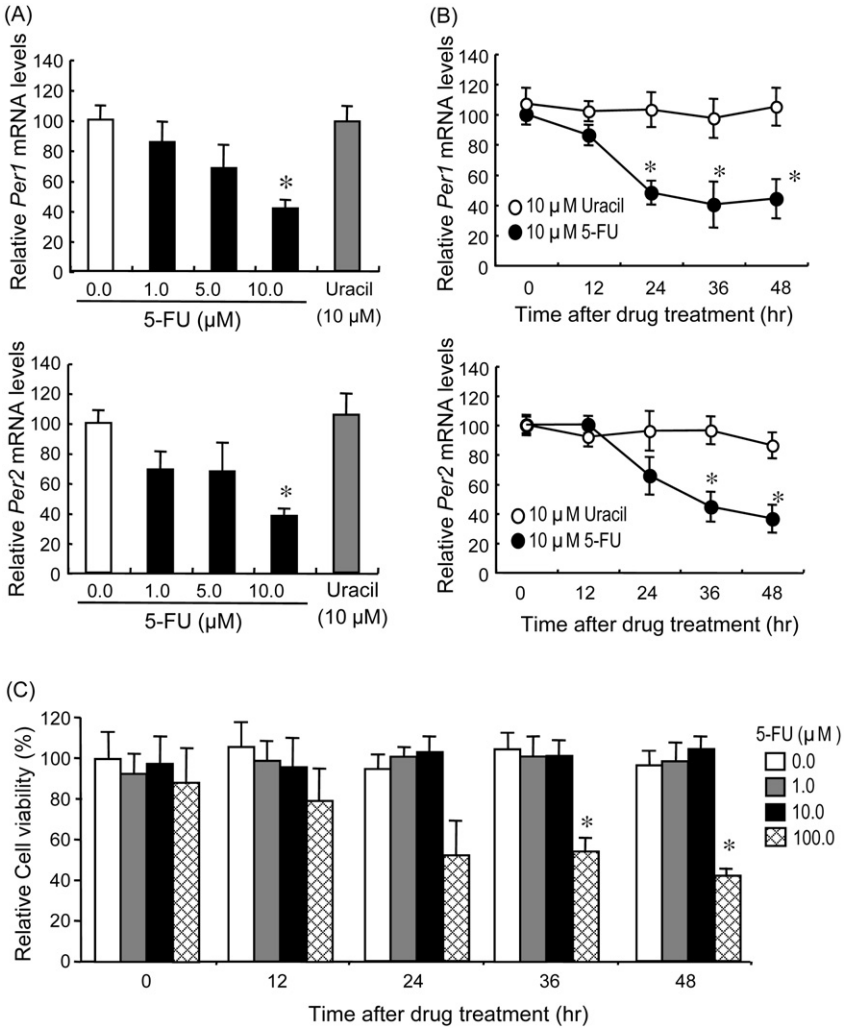

Fig. 1 – Influence of 5-FU on mRNA levels of *Per1* and *Per2* in NIH3T3 cells. (A) Cells were treated with indicated concentrations of 5-FU or Uracil for 48 h. The mRNA levels of *Per1* and *Per2* were determined by RT-PCR. Each value represents the mean ± S.E. ($n = 3$). $^{*}P < 0.05$, compared with the non-treated group (0 μM). (B) Time course of mRNA levels of *Per1* and *Per2* in NIH3T3 cells during treatment with 10 μM 5-FU or 10 μM Uracil. Each value represents the mean ± S.E. ($n = 3$). $^{*}P < 0.05$, compared with the initiation of drug treatment (0 h). (C) Time course of viability of NIH3T3 cells during treatment with indicated concentrations of 5-FU. Each value represents the mean ± S.E. ($n = 3$). $^{*}P < 0.05$, compared with control groups (0 μM).

was observed during the dark phase (Fig. 4B). In contrast, continuous administration of 5-FU substantially decreased the amplitude of locomotor rhythm. Locomotor activities during the dark phase were significantly decreased, resulting in a reduction of total daily activity.

4. Discussion

In this study, we showed that chronic treatment with a chemotherapeutic agent, 5-FU, modulated circadian clock function at the molecular level. Treatment of cultured mouse NIH3T3 cells with 5-FU resulted in a significant reduction of mRNA levels of *Per1* and *Per2* expression, and also attenuated the oscillation of reporter luciferase bioluminescence driven by the mouse *Per2* promoter in rat C6 glioma cells. 5-FU is widely used for several types of cancer. The principal mechanism of 5-FU cytotoxicity is the inhibition of thymidylate synthase (TS), which provides the only *de novo* source of thymidylate for DNA synthesis [17]. TS is inactivated rapidly in the presence of the 5-FU metabolite, 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), and 5, 10-methylenetetrahydrofolate (CH_2FH_4) by the formation of an enzyme: FdUMP:CH_2FH_4 covalently bonded ternary complex [17]. Furthermore, 5-FU is incorporated into RNA as well as DNA, and then inhibits RNA synthesis [17]. This may account for the reduction of mRNA levels of clock genes induced by 5-FU. Consistent with the inhibitory effect of 5-FU on the expression of clock genes in cultured cells, continuous administration of 5-FU into mice also prevented oscillation in the expressions of *Per1* and *Per2* in the SCN and liver. 5-FU can distribute not only to systemic tissue but also to the brain, and affects the whole body [18];

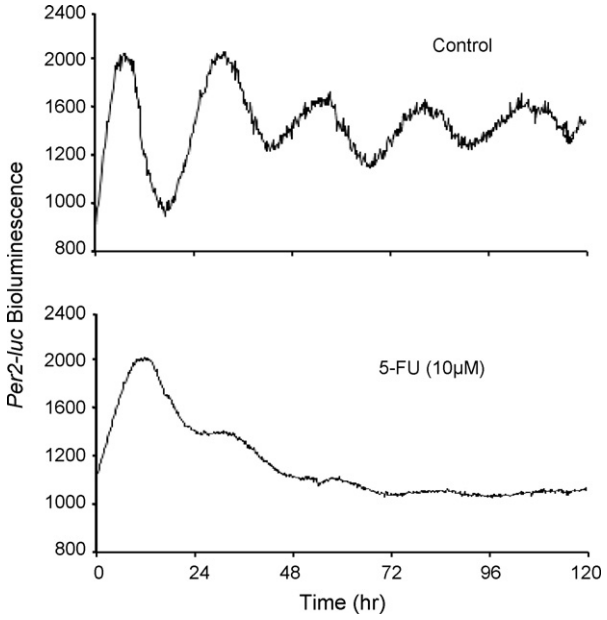

Fig. 2 – Bioluminescence profiles of *Per2* promoter-driven luciferase reporter (*Per2-Luc*) activity in C6 cells. Rat C6 glioma cells stably expressing *Per2-Luc* were stimulated with DEX in the absence (control) or presence of 10 μM 5-FU.

therefore, these factors seem to contribute to repress oscillation in the expression of clock genes in SCN as well as peripheral tissues.

In the current model, the mammalian circadian clock system is hierarchically organized: the master pacemaker in the SCN governs subsidiary oscillators in other brain regions and many peripheral tissues [8,9]. These subsidiary oscillators coordinate a variety of biological processes, producing overt rhythms in physiology and behavior. Continuous administration of 5-FU into mice affected the oscillations of energy metabolism ([^{3}H]-2-DG uptake) and protein synthesis ([^{14}C]-Leu) in the SCN, and also modulated locomotor activity rhythm. Endogenous circadian rhythms of [^{3}H]-2-DG and/or [^{14}C]-Leu uptake into the SCN have been reported in vivo and in vitro, which appears to be direct evidence of the circadian pacemaker function of the brain nucleus [19,20]. The circadian rhythm of [^{14}C]-Leu uptake into the rat SCN under tissue culture conditions is altered by various protein synthesis inhibitors [20,22]. Furthermore, inhibitors of protein synthesis, such as anisomycin or cycloheximide, disturb the circadian rhythm of locomotor activity in mammals [19,21]. Since 5-FU affected both the SCN and periphery, it is difficult to clarify whether the effects of 5-FU on SCN clock genes are secondarily related to those on the rhythmicity of locomotor activity. However, 5-FU directly acts on the hypothalamus neurons (supplemental data) and actually alters the rhythmicity of the expression of clock genes in the SCN. Therefore, the modulatory effects of 5-FU on clock-gene function in the SCN may be responsible for not all but some of the adverse behavioral effects.

Abnormal circadian physiological rhythms, such as body temperature and pituitary–adrenal functions, have been frequently observed in patients with affective illness [23]. On the other hand, intentional failure of physiological rhythms such as shift work or jet lag causes various malaise symptoms, suggesting a close relationship between the biological clock system and affective illness. Consequently,

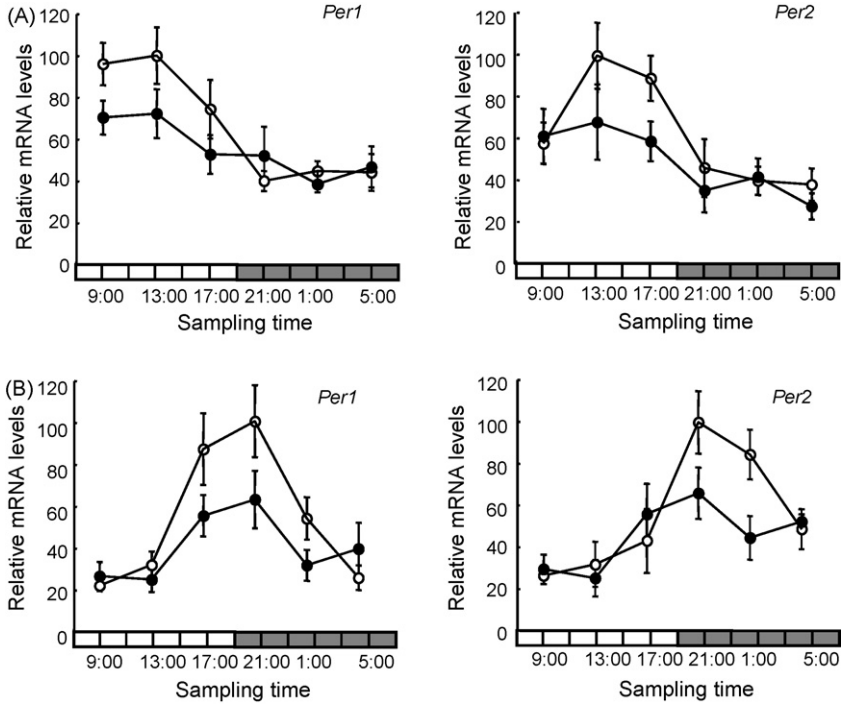

Fig. 3 – Influence of 5-FU continuous administration on mRNA levels of Per1 and Per2 in the SCN (A) and liver (B) of mice. Mice were administered 5-FU (closed circle: 2.0 mg/kg/h, s.c.) or saline (open circle) using osmotic minipumps for 7 days. Each value represents the mean ± S.E. of 3 mice.

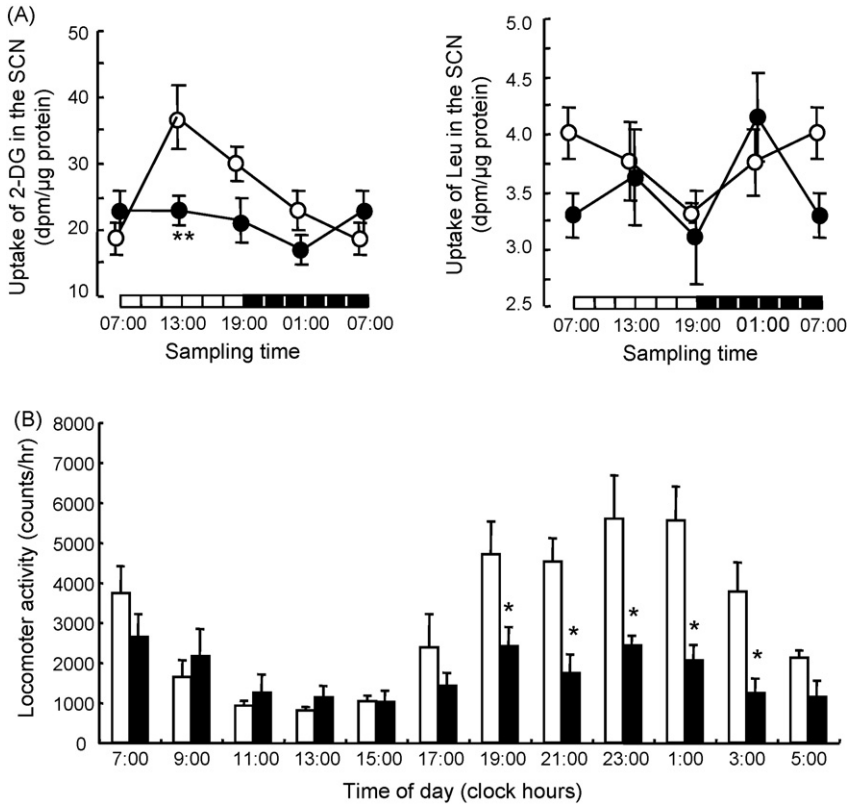

Fig. 4 – Influence of 5-FU continuous administration on SCN oscillatory function (A) and locomotor activity rhythm (B) in mice. Mice were administered 5-FU (closed symbol: 2.0 mg/kg/h, s.c.) or saline (open symbol) using osmotic minipumps for 7 days. Each value represents the mean ± S.E. of 3 mice. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with saline-treated group.

disturbance of the biological clock system is considered to be implicated in the etiology of affective illness [23]. Since 5-FU administration leads to a decrease of the amplitude of rhythms in clock-gene expression, causing abnormal SCN oscillatory function and locomotor rhythm, such abnormalities may contribute to the malaise symptoms observed in patients receiving 5-FU therapy. We should pay attention to the alteration of clock-gene expression and consider it an adverse effect.

The present findings in this animal model will provide a clue to clarify the mechanism underlying 24-h rhythm disturbances induced by chemotherapeutic agents and will help to understand the causes of illness-related 24-h rhythm disorders in certain experimental and clinical situations.

5. Acknowledgments

This study was partially supported by a Grant-in-Aid for Scientific Research on Priority Areas 'Cancer' (S.O., 18014020) from the Ministry of Education, Culture, Sports, Science and Technology, a Grant-in-Aid for Scientific Research (B) (S.O., 18390050) and a Grant-in-Aid for the Encouragement of Young Scientists (S.K., 18790692) from the Japan Society for the Promotion of Science.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.01.011.

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