

*Full Paper***Glucocorticoid Is Involved in Food-Entrainable Rhythm of μ -Opioid Receptor Expression in Mouse Brainstem and Analgesic Effect of Morphine**

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Abstract. The repeated manipulation of feeding schedule has a marked influence on the chronopharmacological aspects of many drugs. In this study, we investigated the role of endogenous glucocorticoid in the mechanism by which restricting the feeding time modulates the analgesic effect of morphine. Male ICR mice were housed under a light-dark cycle (light on from 07:00 to 19:00) with food and water ad libitum or under repeated time-restricted feeding (feeding time from 09:00 to 17:00) for 2 weeks before the experiment. Under the ad libitum feeding, mRNA levels of μ -opioid receptor and its binding capacity in mouse brainstem increased around the early dark phase, following the 24-h variation in circulating glucocorticoid levels. As a consequence, potent analgesic effects of morphine were observed in mice injected with the drug during the dark phase. Daily restricted feeding modulated the time-dependency of μ -opioid receptor function, accompanied by the alteration of the rhythm in circulating glucocorticoid levels. Under the time-restricted feeding, potent analgesic effects of morphine were found in mice injected with the drug during the light phase. Because the manipulation of feeding schedule was unable to produce the food-entrainable rhythm in the expression of μ -opioid receptor in the brainstem of adrenalectomized mice, endogenous rhythm of glucocorticoid secretion seems to be involved in the mechanism by which the time-restricted feeding modulates the analgesic effects of morphine.

Keywords: food-entrainable rhythm, feeding schedule, glucocorticoid, μ -opioid receptor, morphine

Introduction

Mammalian biological clocks, which generate various physiological rhythms, are known to be located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Recent molecular studies of the circadian biological clock system have revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of 24-h rhythms (1–7). The master clock located in the SCN follows a daily light/dark

cycle and, in turn, synchronizes subsidiary oscillators in other brain regions and many peripheral tissues through neural and/or hormonal signals (8, 9). These subsidiary oscillators coordinate a variety of biological processes, producing 24-h rhythms in physiology and behavior. Finally, the variations in biological function are also associated with the dosing time-dependent differences in efficacy and/or toxicity of many drugs.

The act of feeding in mammals generates powerful cues for biological rhythms so that under certain conditions, they can override the entraining signals coming from the SCN. In fact, restricting the feeding time can produce food-entrainable rhythms even in SCN-lesioned mice (10). Conceptually, the manipulation of feeding

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schedule is considered to have a marked influence on the chronopharmacological aspects of many drugs (11, 12).

The analgesic effect of morphine also varies according to the administration time (13, 14). The dosing time-dependent difference in the analgesic effect is associated with 24-h variation in μ -opioid receptor function in the brainstem which is regulated by endogenous glucocorticoid hormone (14, 15). The rhythmic change in circulating glucocorticoid levels is modulated by the repeated manipulation of feeding schedule, accompanied by the alteration of rhythm in the expression of peripheral clock genes (16). It is therefore possible that the manipulation of feeding schedule modifies the chronopharmacological aspects of morphine by changing the rhythmicity in endogenous glucocorticoid secretion. In the present study, we investigated the influence of, and the potential mechanism underlying, the manipulation of feeding schedule on the dosing time-dependency of analgesic effect of morphine.

Materials and Methods

Materials

Morphine hydrochloride was purchased from Sankyo Co., Ltd. (Tokyo) and dissolved in sterilized saline for treatment. Corticosterone was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in propylene glycol for treatment. Naloxone was purchased from Sigma-Aldrich. [D-al², N-methyl-phe⁴, glyol⁵] [tyrosyl-3,5-³H]enkephalin ([³H]-DAMGO) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, UK).

Animals and treatment

Male ICR mice (4 weeks of age) were purchased from Charles River Japan, Inc. (Kanagawa). They were housed under a standardized light/dark cycle condition (light on from 07:00–19:00) at room temperature of $24 \pm 1^\circ\text{C}$ and a humidity of $60 \pm 10\%$ with food and water ad libitum (ALF) or under a time-restricted feeding schedule (feeding time between 09:00 and 17:00) (TRF). The animals were adapted to the feeding condition for 2 weeks before the experiments. To explore the role of endogenous glucocorticoid in food-entrainable oscillation of μ -opioid receptor function, mice were bilaterally adrenalectomized as described previously (17). The adrenals were removed via a dorsal approach using aseptic technique under sodium pentobarbital anesthesia. The adrenalectomized mice were given 0.9% NaCl to drink during the duration of the experiment. Sham adrenalectomy was conducted by the same procedure to expose the adrenals without their removal. During periods referred to as darkness, dim red light was used to aid the treatment of all mice. The

experiments were carried out in compliance with the guidelines stipulated by the Animal Care and Use committee of Fukuoka University.

Determination of plasma corticosterone concentration

To determine the temporal profiles of plasma corticosterone levels, blood samples were drawn by orbital sinus collection at 09:00, 13:00, 17:00, 21:00, 01:00, and 05:00. Plasma samples were obtained after centrifugation at 3,000 rpm for 3 min. These samples were heated at 56°C for 30 min to displace the corticosterone-binding protein. The plasma corticosterone concentration was determined by radio-immunosorbent assay (Corticosterone [¹²⁵I] Assay System, Amersham Biosciences UK, Ltd.).

Quantitative RT-PCR analysis

To determine the temporal profile of μ -opioid receptor mRNA expression in the brainstem, total RNA of the brainstem was isolated at one of six different times described above. The brain was excised quickly, the cerebral cortex and cerebellum were removed, and the brainstem was isolated on an ice-cold petri-dish using the brain atlas of Franklin and Paxinos (18). Total RNA from the brainstem of individual mice was extracted separately using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA of the mouse μ -opioid receptor (Genbank accession number U19380), *mPer2* (GenBank accession No. AF035830), *Bmal1* (GenBank accession No. AB014494), and *GAPDH* (Genbank accession No. M88354) were synthesized and amplified using Superscript One-Step RT-PCR (Invitrogen). To quantify mRNAs, kinetic analysis of the amplified products, ensuring that signals were derived only from the exponential phase of amplifications, was performed in each sample as follows: after the first 24 cycles of amplification, an aliquot of $6 \mu\text{g/ml}$ was drawn for electrophoresis and the tubes were submitted to one more cycle of PCR. This procedure was repeated until 28 cycles had been performed. The PCR products were run on a 3% agarose gel. After the gel was stained with ethidium bromide, the density of each band was analyzed using Kodak 1D image analysis software. The amplification efficiencies of the *GAPDH* and clock or μ -opioid receptor genes were comparable. The amplification products were therefore collected and quantified at the 27th or 28th cycle. The ratio of the amplified target to the amplified internal control (calculated by dividing the value of each *mPer2*, *Bmal1*, μ -opioid receptor by that of *GAPDH*) was compared among groups.

Specific μ -opioid receptor binding assay

To determine the temporal variation in μ -opioid

receptor function in brainstem, the binding amounts of [^3H]-DAMGO in brainstem were assessed at 09:00 and 21:00. The brainstem was homogenized in 1 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). The homogenate was then centrifuged at 15,000 rpm for 15 min at 4°C. The obtained pellet was resuspended in 1 ml of Tris-HCl buffer (pH 7.4) and incubated at 37°C for 15 min, and the homogenate was centrifuged again. The pellet was resuspended in 3 ml of Tris-HCl buffer. The protein concentration was approximately 2 mg/ml using Lowry's method (DC Protein Assay; Bio-Rad, Hercules, CA, USA). The binding assay was performed with a reaction mixture (total volume, 200 μl) containing 100 μl of aliquot of brainstem homogenate and a saturating concentration of 5 nM [^3H]-DAMGO. Non-specific binding was determined by using 10 μM naloxone. After incubation at 37°C for 30 min, the reaction mixture, together with 100 μl of Tris-HCl buffer to wash the tube, was laid over the 300 μl ice-cold fetal bovine serum and centrifuged at 10,000 rpm for 1 min at 4°C. The supernatant was removed, and the pellet was transferred to scintillation vials with 10 ml of scintillation cocktail and counted using a liquid scintillation counter (LSC-1000; Aloka Co., Mitaka) after standing for 6 h. Specific binding is the difference between binding determined in the absence of ligand and in the presence of ligand, and it was calculated as follows: specific binding (fmol/mg protein) = [total binding (fmol/mg protein)] - [non-specific binding (fmol/mg protein)].

Determination of the analgesic effect

First, groups of 10 mice each were given a single i.p. dose of morphine hydrochloride at 7.5, 15, or 30 mg/kg or saline at the same 24-h phase (09:00) for dose finding. At 30 min after morphine injection, there was no significant difference between the latency time in mice given morphine (7.5 mg/kg) and in mice given saline. However, the latency time was significantly longer in mice given morphine (15 mg/kg) than in mice given saline. Moreover, the latency time in mice given morphine had smaller interindividual difference than that in mice given morphine (30 mg/kg). The dose of morphine hydrochloride at 15 mg/kg was accepted as the proper dose in our experiment. To determine temporal variation of the analgesic effect of morphine, mice were administered morphine (15 mg/kg, intraperitoneal) or saline at 09:00 or 21:00. A thermal technique (hot plate analgesia meter MK-350; Muromati Kikai Co., Ltd., Tokyo) was used to evaluate analgesic latency after morphine or saline injection (19). The surface of the plate was maintained at a temperature of $55 \pm 0.5^\circ\text{C}$. The analgesic latency was determined at 30 min after morphine or saline injection. Time (in

seconds) to either hind paw licking or jumping was recorded as pain response latency. To avoid heat injury, mice not responding after 120 s were removed from the hot plate. The latency of those mice was 120 s. To avoid the likelihood of habituation or tolerance of the mice to the hot plate, animals were not used repeatedly.

Statistical analyses

The significance of 24-h variation in each parameter was tested by analysis of variance (ANOVA). The statistical significance of difference was evaluated by Student's *t*-test or Bonferroni multiple comparison test. A 5% level of probability was considered significant.

Results

Influence of feeding schedule on the 24-h variation in mRNA levels of μ -opioid receptor in brainstem

A significant 24-h variation in circulating glucocorticoid levels was observed in control (sham-operated) mice under the ALF condition (Fig. 1A). The levels of corticosterone increased from the late light phase to early dark phase and decreased from the late dark phase to early light phase [$F(5, 42) = 13.884$, $P < 0.01$]. Under this feeding condition, mRNA levels of μ -opioid receptor in brainstem fluctuated following the 24-h variation in circulating glucocorticoid levels (Fig. 1A). Higher levels of μ -opioid receptor mRNA was observed during the dark phase, whereas lower levels were observed during the light phase [$F(5, 42) = 46.566$, $P < 0.01$]. The TRF had a marked influence on rhythmicity in both circulating glucocorticoid levels and expression of μ -opioid receptor mRNA (Fig. 1B). The rhythmic phase of corticosterone levels under the manipulation of feeding schedule was nearly antiphase to that under the ALF condition [$F(5, 42) = 5.760$, $P < 0.01$]. As a consequence, mRNA levels of μ -opioid receptor increased during the light phase, whereas they decreased during the dark phase [$F(5, 42) = 14.043$, $P < 0.01$]. Under the ALF condition, the mRNA levels of μ -opioid receptor in brainstem of adrenalectomized mice remained at the trough level for the control mice throughout the day (Fig. 1C). Moreover, the food-entrainable rhythm of μ -opioid receptor expression was not observed in adrenalectomized mice (Fig. 1D).

Influence of feeding schedule on the time-dependency of analgesic effect of morphine

Under the ALF condition, there was a significant time-dependent variation in the specific binding of [^3H]-DAMGO in the brainstem of control mice ($P < 0.01$, Student's *t*-test) (Fig. 2A). A large amount of binding was observed at 21:00. As a consequence, the potent

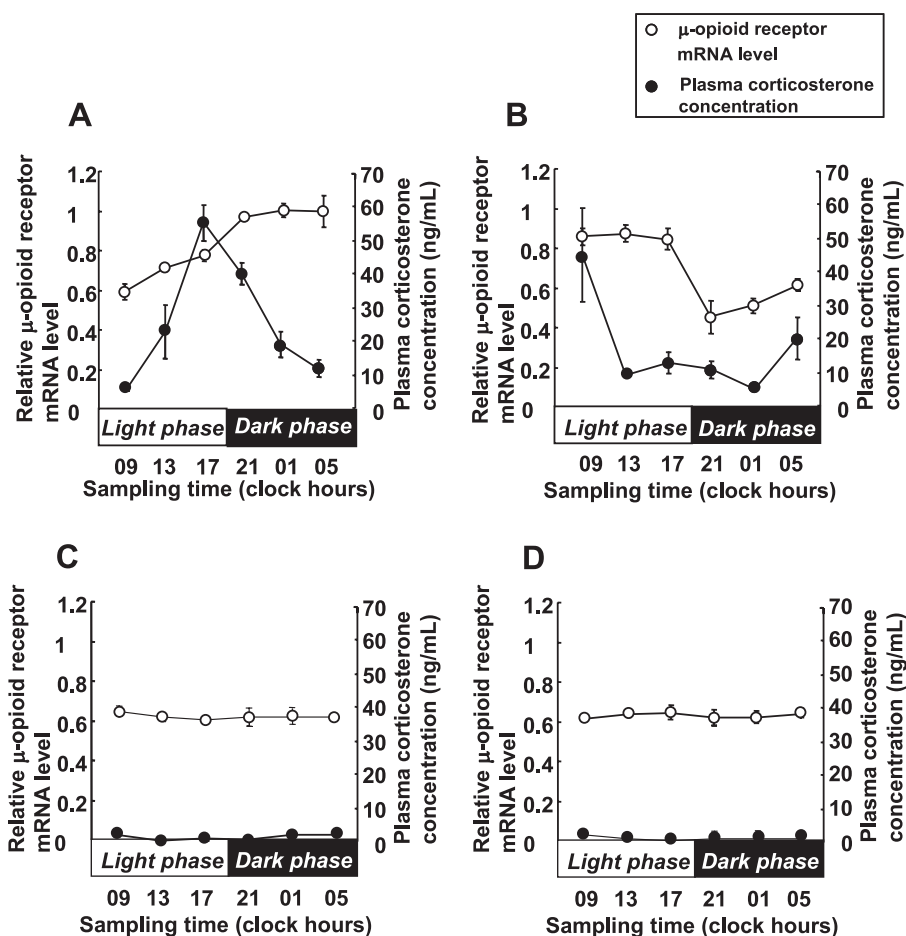


Fig. 1. Influence of the time-restricted feeding (TRF) on 24-h rhythm of plasma corticosterone level and μ -opioid receptor mRNA levels of brainstem. The relative mRNA levels set the mean value of control mice under the ad libitum feeding (ALF) at 01:00. Each value is the mean \pm S.E.M. of eight mice. A: Temporal profile of corticosterone levels in plasma and μ -opioid receptor mRNA level in brainstem of control (sham-operated) mice under the ALF condition. Both levels showed significant 24-h variation ($P < 0.01$, ANOVA). B: Temporal profile of corticosterone levels in plasma and μ -opioid receptor mRNA level in brainstem of control (sham-operated) mice under the TRF condition. Both levels showed significant 24-h variation ($P < 0.01$, ANOVA). C: Temporal profile of corticosterone levels in plasma and μ -opioid receptor mRNA level in brainstem of adrenalectomized mice under the ALF condition. Both levels showed no significant 24-h variation. D: Temporal profile of corticosterone levels in plasma and μ -opioid receptor mRNA level in brainstem of adrenalectomized mice under the TRF condition. Both levels showed no significant 24-h variation.

analgesic effect of morphine was observed at 21:00 ($P < 0.01$, Bonferroni multiple comparison test). The TRF had marked influence on time-dependency both of [3 H]-DAMGO in the brainstem and in the analgesic effect of morphine (Fig. 2B). Although, under the TRF condition, the specific binding of [3 H]-DAMGO in the brainstem of control mice showed a significant time-dependent variation, a large amount of binding was observed at 09:00 ($P < 0.01$, Student's t -test). As a consequence, the analgesic effect of morphine at 09:00 was more potent than that at 21:00 ($P < 0.01$, Bonferroni multiple comparison test). These results suggest that the food-entrainable variation in analgesic effect induced by morphine is attributable to the alteration of μ -opioid

receptor function in brainstem. However, there was no significant time-dependent variation in the amount of [3 H]-DAMGO binding in brainstem of adrenalectomized mice under the ALF condition (Fig. 2C). As a consequence, there was no significant time-dependent variation in the analgesic effect of morphine in adrenalectomized mice under the ALF condition. Moreover, the manipulation of feeding schedule was unable to produce the food-entrainable oscillation in the binding of [3 H]-DAMGO in the brainstem of adrenalectomized mice (Fig. 2D). There was no significant time-dependent variation in the amount of [3 H]-DAMGO binding in brainstem of adrenalectomized mice under the TRF condition. Consequently, the food-entrainable variation

in analgesic response induced by morphine was not observed in adrenalectomized mice (Fig. 2D).

*Influence of feeding schedule in the time-dependent variation of the *mPer2* and *Bmal1* expression in the brainstem*

Circadian oscillation in mRNA levels for the *mPer2* and *Bmal1* genes in brainstem was observed in control (sham-operated) mice under the ALF condition (Fig. 3A). *mPer2* mRNA levels peaked around the early dark phase, whereas *Bmal1* mRNA oscillations were nearly antiphase to those of the mRNA rhythms, with

peak levels from the late dark phase to the early light phase. The TRF had a marked influence on rhythmicity in both expressions of *mPer2* and *Bmal1* mRNA (Fig. 3B). Under the TRF condition, *mPer2* mRNA levels peaked around the early light phase, whereas *Bmal1* mRNA levels peaked around from the middle to the late dark phase. Circadian oscillation in mRNA levels for the *mPer2* and *Bmal1* genes in brainstem was also observed in adrenalectomized mice under the ALF condition (Fig. 3C). *mPer2* mRNA levels peaked around the early dark phase, whereas *Bmal1* mRNA oscillations were nearly antiphase to those of the mRNA rhythms, with peak levels from the late dark phase to the early light phase. The manipulation of feeding schedule was able to produce the food-entrainable variation of *mPer2*, and *Bmal1* mRNA expressions in brainstem was observed even in adrenalectomized mice (Fig. 3D). Under the TRF condition, *mPer2* mRNA levels peaked around the early light phase, whereas *Bmal1* mRNA levels peaked around from the middle to the late dark phase.

Discussion

The effectiveness and/or toxicity of many drugs vary according to their administration time (20–26) because there are time-dependent variations in biological functions such as gene expressions and protein synthesis affecting the efficacy and/or toxicity of drugs. Feeding is the most powerful environmental cue and it has a marked influence on physical rhythmic variables in mammals. For example, the TRF has been shown to alter daily behavioral and physiological rhythms. Consequently, 24-h variations in the efficacy and/or toxicity

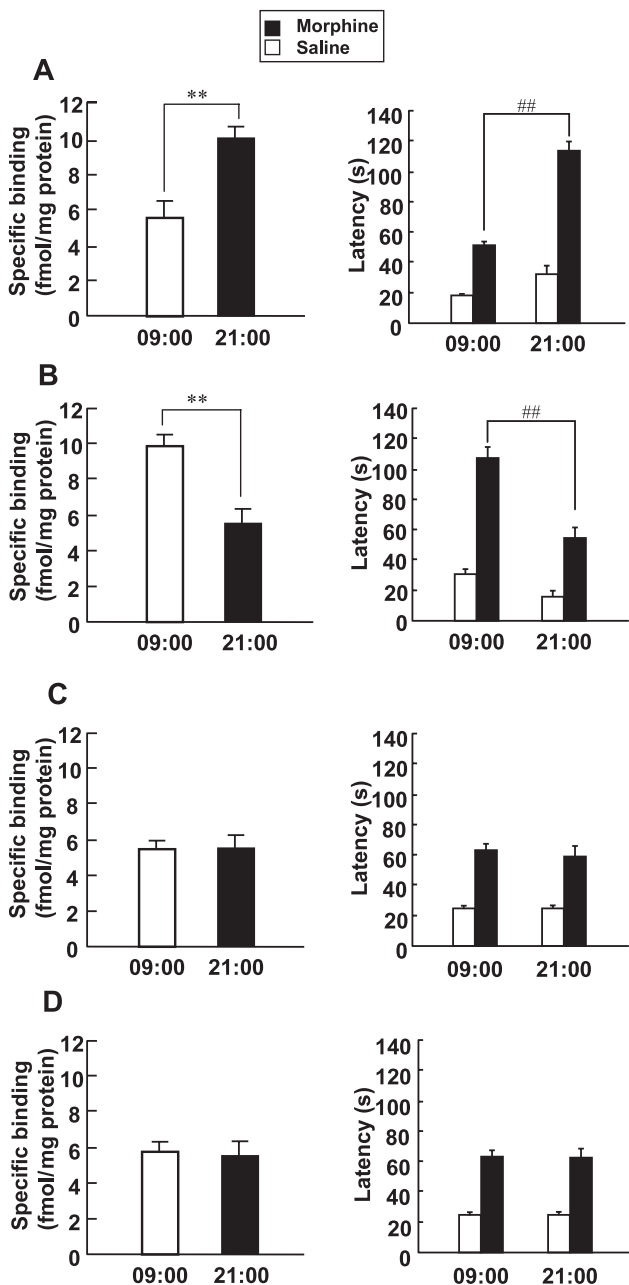


Fig. 2. Influence of the time-restricted feeding (TRF) on time-dependence of μ -opioid receptor binding capacity in brainstem and analgesic effect of morphine. Each value is the mean \pm S.E.M. of eight mice. A: Temporal profiles in the specific binding of [3 H]-DAMGO in the brainstem and the analgesic effect of morphine in control (sham-operated) mice under the ad libitum feeding (ALF). Statistical analysis in the specific binding of [3 H]-DAMGO in brainstem was performed by using Student's *t*-test (** $P < 0.01$). Statistical analysis in analgesic effect of morphine was performed by using the Bonferroni multiple comparison test (## $P < 0.01$). B: Temporal profiles in the specific binding of [3 H]-DAMGO in the brainstem and the analgesic effect of morphine in control (sham-operated) mice under the TRF condition. Statistical analysis in the specific binding of [3 H]-DAMGO in brainstem was performed by using Student's *t*-test (** $P < 0.01$). Statistical analysis in analgesic effect of morphine was performed by using the Bonferroni multiple comparison test (## $P < 0.01$). C: Temporal profiles in the specific binding of [3 H]-DAMGO in the brainstem and the analgesic effect of morphine in adrenalectomized mice under the ALF condition. D: Temporal profiles in the specific binding of [3 H]-DAMGO in the brainstem and the analgesic effect of morphine in adrenalectomized mice under the TRF condition.

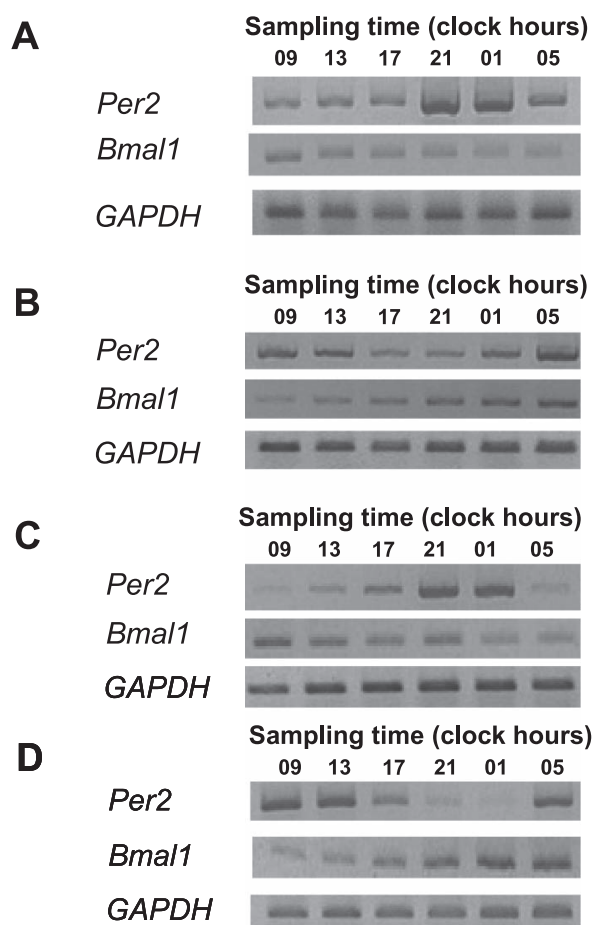


Fig. 3. Influence of the time-restricted feeding (TRF) on circadian variation in mRNA levels of clock genes in brainstem of sham-operated or adrenalectomized mice. *GAPDH* mRNA was used as an internal control for transcripts whose expression was constant throughout the day. A: Representative electrophoretic image of RT-PCR products of clock genes, *mPer2* and *Bmal1*, in the brainstem of control (sham-operated) mice under the ad libitum feeding (ALF). B: Representative electrophoretic image of RT-PCR products of clock genes, *mPer2* and *Bmal1*, in the brainstem of control (sham-operated) mice under the TRF condition. C: Representative electrophoretic image of RT-PCR products of clock genes, *mPer2* and *Bmal1*, in the brainstem of adrenalectomized mice under the ALF condition. D: Representative electrophoretic image of RT-PCR products of clock genes, *mPer2* and *Bmal1*, in the brainstem of adrenalectomized mice under the TRF condition.

of many drugs are suggested to phase-shift following alteration in the 24-h variation of biological functions (11, 12). The present study demonstrated a possible mechanism by which the manipulation of feeding schedule can modulate the chronopharmacological aspects of the analgesic effect of morphine.

There was a significant time-dependent variation of the response latency in saline-treated sham-operated mice under the ALF condition. The significantly increased response latency was observed at 21:00

compared to the latency at 09:00 ($P < 0.01$, Bonferroni multiple comparison test). The rhythmic phase of the basal latency under the TRF condition was nearly antiphase to that under the ALF condition ($P < 0.01$, Bonferroni multiple comparison test). The rhythmic pattern of basal latency is supported by the time-dependent level of opioid peptides such as endorphin, enkephalin (27), and μ -opioid receptor. Furthermore, the TRF can produce the food-entrainable rhythms of endorphin (28) and μ -opioid receptor corresponding to the food-entrainable rhythm of basal latency. Opioid peptides and μ -opioid receptor may be involved in the food-entrainable rhythm of the basal latency. Consequently, the increase of basal latency may contribute to the increase of morphine analgesia.

In control (sham-operated) mice under the ALF condition, time-dependency of the mRNA levels and binding capacity of μ -opioid receptor in brainstem were modulated by changing the rhythmicity in circulating glucocorticoid levels. The analgesic effect of morphine was significantly enhanced by administering the drug when the μ -opioid receptor function was increased. These results are consistent with those of previous studies (13, 14). The TRF can produce food-entrainable variation in μ -opioid receptor function in brainstem accompanied by changing the rhythmicity of circulating corticosterone levels. As a consequence, the time-dependent variation in analgesic effect of morphine was also modulated by the manipulation of feeding schedule.

The dosing time-dependent difference in the analgesic effect is associated with 24-h variation in μ -opioid receptor function in brainstem which is regulated by endogenous glucocorticoid hormone (15, 16). In adrenalectomized mice under the TRF condition, the food-entrainable rhythm was unable to entrain 24-h rhythm of the analgesic effect of morphine.

Circadian secretion of glucocorticoids, controlled by the hypothalamus-pituitary-adrenal (HPA) axis, acts to synchronize peripheral clocks with the central SCN pacemaker, thereby coordinating the physiological functions according to the circadian time (29). The activities of the HPA axis in rodents are known to be modified by restricted daily feeding (30, 31). Consequently, the TRF has been shown to alter circadian rhythm of glucocorticoid secretion (13).

Recent molecular dissection of the circadian biological clock system in peripheral tissue has revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of circadian rhythms. The circadian organization of molecular clockwork governs the rhythm in expression of some protein production (32). Glucocorticoid has the ability to change

the phase of circadian gene expression in peripheral tissues of mice (33). Considering these results, circadian changes of clock genes in brainstem by food-entrainable rhythm in glucocorticoid secretion may synchronize the rhythm of μ -opioid receptor expression in mouse brainstem. However, circadian clock gene expression in brainstem may not be involved in that synchronization. In fact, the circadian rhythms of clock genes in brainstem were observed in adrenalectomized mice under the ALF condition and these rhythms were phase-shifted by the TRF in spite of disappearance in 24-h rhythm of μ -opioid receptor.

The food-entrainable rhythm in the μ -opioid receptor expression was observed not only in brainstem but also in other areas such as the spinal cord. Similar to the 24-h variation of μ -opioid receptor mRNA expression in brainstem, higher levels of μ -opioid receptor mRNA in the spinal cord of mice under the ALF condition was observed during the dark phase, whereas lower levels were observed during the light phase. The TRF also had a marked influence on rhythmicity in μ -opioid receptor mRNA expression in the spinal cord. The rhythmic phase of μ -opioid receptor mRNA expression under the TRF condition was nearly antiphase to that under the ALF condition (M. Yoshida et al., unpublished data). The TRF is suggested to be able to produce the similar food-entrainable rhythm both of central and peripheral μ -opioid receptor expressions.

Our findings suggest that the TRF changed the rhythm of μ -opioid receptor expression in brainstem and analgesic effect of morphine, which synchronized food-entrainable variation of endogenous glucocorticoid secretion. As the mechanism underlying the production of food-entrainable rhythm in μ -opioid receptor expression in brainstem by the TRF, glucocorticoid may directly act on μ -opioid receptor expression in brainstem without mediation of clock genes in brainstem. A potent analgesic effect of morphine could be expected by administering the drug when μ -opioid receptor expression is increased. The present study also suggests that feeding schedule may be one of indices to establish the dosing schedule of morphine based on 24-h variation in μ -opioid receptor expression.

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