睡眠－覚醒リズムの分子基盤解明と新規睡眠薬の開発

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研究成果

ヒトの場合、時差ぼけ状態の時などに睡眠・覚醒リズムは体温リズムと乖離することがよく知られており、この２つのリズムは別々なリズム機構で支配されていることが示唆されている。夜行性動物のマウスやラットに毎日昼間の一定時刻に給餌するという制限給餌をおこなうと、動物はこのリズムに同調し、行動上あたかも昼行性動物のように見受けられる。すなわち、睡眠・覚醒リズムは餌を手がかりにした給餌性の同調刺激でリセットされるといえる。そこで、この給餌性の同調時に、中心時計の視交差上核、脳の時計として機能している大脳皮質、海馬、線条体の時計遺伝子発現を調べることとした。また、末梢時計のある肝臓での時計遺伝子発現についても調べることとした。

次に、摂食行動と睡眠・覚醒に深くかかわっていると考えられているオレキシン神経の給餌性の同調とのかかわりについて調べた。このような研究が、体内時計遺伝子と睡眠覚醒リズムのリセットを結び付ける研究に大いに役立ち、オレキシンを中心とした新規な睡眠薬の開発に寄与するものと考えられた。以下にこの２つの項目の研究成果を述べる。

（１）給餌性リズム形成における時計遺伝子発現変化。

動物に毎日一定時刻餌を与えると、動物は給餌時刻を覚え給餌時刻の 2－3 時間前から活動量が増大するいわゆる予知行動が出現してくる。この行動は視交差上核を破壊しても出現するが約 24 時間の周期の給餌でないと形成できないことから、視交差上核とは異なったある種のサーカディアンリズムであると考えられている。給餌性リズムが形成されたときの大脳皮質や肝臓での時計遺伝子ならびにその下流遺伝子発現を調べた。その結果、給餌制限を行ったマウスの肝臓の mPer1, mPer2 遺伝子発現は、対照マウスの位相が夜間であるのに比較して、昼間に移行した。また、下流遺伝子である CYP7alpha hydoxylase の遺伝子発現リズムも位相が前進することがわかった。この現象は視交差上核を破壊した動物でも観察された。また、大脳皮質、海馬、線条体いずれの脳部位でも肝臓と同様の結果が得られた。

つぎに clock ミュータントマウスに対する給餌性同調時の体内時計遺伝子発現変化を調べた。clock ミュータント動物は恒暗条件で飼育すると行動リズムの周期が延長し、やがて無周期性を示すようになる。これは視交差上核の体内時計機構が機能を失い行動のリズム性を消失するものと考えられている。そこで、このようにリズムが消失した状態でも給餌性のリズム形成が起こるか否か調べた。その結果、自由摂食の clock ミュータントマウスの心臓では mPer1, mPer2, Bmal1 のいずれの時計遺伝子発現も振幅が増大していた。ところが、給餌制限を加えると、ミュータントマウスでも心臓の時計遺伝子発現は昼間に明瞭なピークを示すようになった。また、その下流遺伝子である Pai-1 遺伝子発現でも昼間にピークが見られるようになった。すなわち、給餌性リズム形成には視交差上核の時計機構は必要でないこと、またそれ以外の場所にも発現している Clock タンパク質は給餌性リズム形成には必要でな

いこともわかった。

（２）オレキシン神経の給餌性リズム形成における役割解明。

オレキシン神経の給餌性リズム形成における役割について調べた。オレキシン神経は視床下部の外側に発現する神経ペプチドで、その欠損実験から、摂食行動とナルコレプシーなどの睡眠・覚醒にかかわっていることが明らかとなってきた。ところで動物に昼間の一定時刻にだけ給餌をおこなうと、中心時計の視交差上核以外の脳部位や末梢の肝臓などの時計遺伝子発現リズムに位相が夜型から昼型に変わる。つまり、一定の給餌制限で、睡眠・覚醒リズムとするリズムの位相を変えうることがわかったが、その神経機構についてはまったく不明である。先に述べたオレキシンは摂食と睡眠・覚醒にかかわる分子であるので、この給餌性リズム形成にオレキシンがかかわっている可能性について調べた。 １）マウスに昼間の４時間だけ餌を与える操作を６日間行い、７日目に脳を取り出し、プレプロオレキシンの mRNA 発現と、オレキシン抗体によるオレキシンタンパク質の発現リズムを調べた。その結果、正常マウスも給餌制限したマウスもいずれも、プレプロオレキシンの遺伝子、オレキシンタンパク質の発現に日内リズムは見られなかった。そこで、次に ２）オレキシン神経が支配している脳部位でのオレキシン受容体の変化を調べることとした。レキシン受容体のmRNA 分布を調べたところ、OREXR1 も OREXR2 もいずれも視床の室傍核に強い発現が見られ、OREXR2 はオレキシ視床下部腹内側核にも強い発現が見られた。しかしながらいずれの受容体でも、またいずれの脳部位でも、その発現変化にリズム性は見られなかった。給餌制限を施したマウスでも、自由摂食群と同様な結果が得られ、これらの受容体の遺伝子発現リズムに影響を及ぼさなかった。以上の結果、オレキシン神経は、ペプチドのmRNA 発現レベルでも、タンパク質発現レベルでも、またその受容体のmRNA レベルでも、日内リズム形成にかかわっていないことがわかった。

ヒトのナルコレプシー患者ではオレキシンのペプチド含有低下すのではなく、オレキシン含有神経が脱落することが原因であると考えられている。つまりオレキシン含有神経の機能と摂食、睡眠・覚醒リズム変化を調べてみる必要があることがわかった。そこで、オレキシン神経の活動性がリズム形成にかかわっている可能性を調べた。すなわち神経の活動性のマーカー蛋白質である Fos 蛋白とオレキシン蛋白の二重染色を行った。オレキシンと Fos の共発現細胞数には夜間高いという明瞭な日内リズムが見られ、昼間に給餌制限をすると、そのリズムの位相が前進して昼間に現れることもわかった。以上の結果、オレキシンそのものではなく、オレキシンを含有している神経の活動性が、摂食行動や睡眠・覚醒にかかわるのみならず、これはリズム形成にもかかわっている可能性が示唆された。オレキシン神経が選択的に破壊されたマウスが作成されており、現在このマウスを使用して、給餌性リズム形成について調べている。

以上の研究成果から、オレキシン神経の発現制御に関わる化合物を検索することで、新規な睡眠薬を開発できる可能性がある。

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Methamphetamine-induced, suprachiasmatic nucleus-independent circadian rhythms of activity and *mPer* gene expression in the striatum of the mouse

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Abstract

While the suprachiasmatic nucleus (SCN) coordinates the majority of daily rhythms, some circadian patterns of expression are controlled from outside of the SCN. These include responses to daily methamphetamine (MAP) injection, or daily restricted feeding. The mechanisms underlying these SCN-independent circadian rhythms are unknown. A circadian oscillation in the expression of *mPer1* and/or *mPer2*, mouse *period,* in the SCN is considered necessary to generate an SCN-dependent circadian rhythm. Therefore, in this experiment, we examined the association between *mPer* gene expression and the MAP-induced, SCN-independent circadian rhythm. Acute injection of MAP caused an elevation of *mPer1, mBmal1, and mNpas2* gene expression in the striatum and *mPer1* in the liver. Daily MAP injection at a fixed time for 6 days shifted the rhythmic *mPer1* and *mPer2* expression in the striatum from a nocturnal to a diurnal rhythm, but failed to affect that in the SCN. Although lesion of the SCN 'flattened' *mPer* gene oscillation in the striatum and liver, daily MAP injection caused both behavioural and *mPer* gene expression rhythms. Daily MAP injection at variable injection intervals (12–36 h) for 6 days, however, failed to produce *mPer* gene rhythm in the striatum. Daily repeated MAP signals may strengthen the oscillatory force of SCN-independent circadian behavioural and molecular rhythms. The present results suggest that daily oscillation of *mPer* genes outside the SCN is closely associated with the regulation of SCN-independent rhythms. Thus, the present experiment highlights strongly the important role of clock gene expression, in the brain, that underlies the circadian behavioural rhythm.

Introduction

The suprachiasmatic nucleus (SCN) contains a master pacemaker that regulates behavioural and physiological circadian rhythms (King & Takahashi, 2000). Destruction of the SCN abolishes the rhythms of many physiological functions. Outside of the SCN, there are at least two types of oscillations; a food-associated oscillation produced by daily restricted feeding and a methamphetamine (MAP)-associated oscillation produced by daily injection of MAP (Shibata *et al.*, 1995). Locomotor activity increases before feeding time or fixed injection time under scheduled daily restricted feeding or daily injection of MAP, respectively [(for review, see Mistlberger, 1994) Shibata *et al.*, 1994, 1995]. This food-anticipatory activity rhythm appears even if the SCN is lesioned, but does not appear in the absence of a circadian (24 h) feeding schedule (Stephan *et al.*, 1981; Mistlberger & Marchant, 1995). Therefore, this food-anticipatory activity rhythm is considered an SCN-independent circadian rhythm. Although we demonstrated previously that the MAP-anticipatory activity rhythm was attenuated by either dopamine D_1 and D_2 receptor antagonists or an NMDA receptor antagonist (Shibata *et al.*, 1995), the site of action of MAP has not yet been elucidated.

Recently, it has been established that a number of putative clock genes such as: *Per1*, *Per2*, *Per3*, *clock*, *Bmal1*, *Tim*, *Cry1* and *Cry2* are expressed in the SCN. Studies on the molecular aspects of clock genes have produced a functional model of circadian rhythms (for review, see Dunlap, 1999; Reppert & Weaver, 2001). Restricted feeding rapidly entrains the *Per* gene expression rhythm in the liver (Damiola *et al.*, 2000; Hara *et al.*, 2001; Stokkan *et al.*, 2001) as well as clocks in the cerebral cortex and hippocampus, but not the SCN (Wakamatsu *et al.*, 2001). Based on these facts, it has been suggested that SCN-independent circadian oscillators exist in the peripheral organs and those areas of the brain other than SCN. Recently, we reported that acute injection of MAP increased *mPer1* gene expression in the striatum, and this expression was augmented by repeated injection of MAP – much like sensitization. (Nikaido *et al.*, 2001). Our expectation was that results would indicate that daily injections of MAP can entrain the SCN-independent circadian oscillation of clock gene expression in the brain and peripheral organs. Therefore, we investigated whether the MAP-induced circadian rhythm was associated with circadian expression of *mPer* genes in the striatum, a representative of the brain clock, and/or the liver, a peripheral clock, using SCN-lesioned arrhythmic animals.

In addition, to better understand the mechanism of MAP-induced oscillation, we examined the acute effect of MAP on *mBmal1* and *mNpas2* gene expression in the striatum and liver. Npas2 possesses high homology to CLOCK, and coexpression of Npas2 and Bmal1 activates transcription of the *Per1*, *Per2*, and *Cry1* genes, suggesting that Npas2 actually forms part of this peripheral molecular clock in the mammalian forebrain and periphery but not in the SCN (Reick *et al.*, 2001).

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Materials and methods

Animals

Male ddY mice (Takasugi, Saitama, Japan), 4–6 weeks of age, were used in all experiments. Mice were maintained under a 12-h light : 12-h dark cycle and allowed free access to food and water.

Locomotor activity measurement

To assess locomotor activity, mice were housed individually in transparent plastic cages (31 × 20 × 13 cm). Motor activity was measured using an infrared area sensor (Omron F5B, Tokyo, Japan). All activity was measured and activity count was recorded by our own specially designed computer software. The number of movements were then stored on disk at 6-min intervals.

Sampling procedure

Mice were anaesthetized deeply with ether and perfused intracardially with ice-cold saline to remove excess blood. A sample (approximately 100 mg wet weight) was removed from the liver to measure liver clock gene expression by RT-PCR. The liver sample was frozen in liquid nitrogen and stored at –80 °C until RNA isolation. Mice were then intracardinally perfused with 0.1 M phosphate buffer (PB) (pH = 7.4) containing 4% paraformaldehyde (PFA) (total 25 mL for 10 min). Brains were removed, postfixed in 0.1 M PB containing 4% PFA for 24 h at 4 °C, and transferred into 20% sucrose in 0.1 M PB for 72 h at 4 °C. Brain slices (40 μm thick) including the striatum, temporal cortex, and the SCN were made using a cryostat (Microm, HM505E, Walldorf, Germany) and placed in 2 × standard saline citrate until processing for hybridization

In situ *hybridization*

In situ hybridization was used to determine the quantity of *mPer1*, *mPer2* or *mBmal1*, *mNpas2* mRNA expression in the various brain areas. Slices were treated with 1 μg/mL proteinase K in 10 mM Tris–HCl buffer (pH = 7.5) containing 10 mM EDTA for 10 min at 37 °C, followed by 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% NaCl for 10 min The slices were then incubated in hybridization buffer (60% formamide, 10% dextran sulphate, 10 mM Tris-HCl, pH = 7.4, 1 mM EDTA, 0.6 M NaCl, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% bovine serum albumin), 0.2 mg/mL transferRNA, 0.25% sodium dodecyl sulphate) containing ^{33}P-labelled cRNA probes for 16 h at 60 °C. Radioisotope [RI: a[^{33}P]UTP (PerkinElmer Life Sciences, Boston, MA)] -labelled antisense cRNA probes were made from restriction enzyme-linearized cDNA templates [nucleotide positions: *mPer1* (538–1752), *mPer2* (1–638), *mBmal1* (1656–2259) (kindly provided by Dr Okamura, Kobe University, Kobe, Japan), and *mNpas2* (672–1164)]. After high-stringency posthybridization wash in 2× standard saline citrate–50% formamide, slices were treated with RNaseA (10 μg/mL) for 30 min at 37 °C. Slices were then washed in 2× standard saline citrate–50% formamide, and were air-dried. Images were visualized by autoradiogram using BioMax MR film (Eastman, Kodak, Rochester, NY) and analysed with an image analysing system (MCID, Imaging Research Inc., St. Catherines, ON, Canada) after conversion into optical density by ^{14}C-autoradiographic microscales (Amersham Pharmacia Biotech, Ltd, Little Chalfont, Buckinghamshire, UK). Details of measurement are described in Nikaido *et al.* (2001). For data analysis, we subtracted the intensities of the optical density in the corpus callosum from those in the SCN and striatum of each section and regarded this value as the net intensity of these areas. The intensity values of the sections from the rostral–caudal part of the SCN and striatum (3–5 sections per mouse brain) were then summed; the sum was considered to be a measure of the amount of *mPer1*, *mPer2*, *mBmal1* and *mNpas2* mRNA in this region. The mean values were calculated for 3–7 animals from each experimental group.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the liver sample was extracted using ISOGEN Reagent (Nippon Gene, Tokyo, Japan), and the remaining DNA was removed completely by RNase-free DNase treatment. Total RNA (100 ng) was reverse transcribed and amplified using the Superscript One-Step RT-PCR System (Invitrogen, CA) and a GeneAmp PCR System 9700 (Applied Biosystems, CA) with the following specific primer pairs designed based on published data on the *mPer1*, *mPer2*, *mBmal1*, *mNpas2*, and *α-actin* genes in GenBank:

- *mPer1* [289 bp]:
5′-CAAGTGGCAATGAGTCCAACG-3′ (forward) and
5′-CGAAGTTTGAGCTCCCGAAGT-3′ (reverse);
- *mPer2* [381 bp]:
5′-CAGACTCATGATGACAGAGG-3′ (forward) and
5′-GAGATGTACAGGATCTTCCC-3′ (reverse);
- *mBmal1b* [344 bp]:
5′-CACTGACTACCAAGAAAGTATG-3′ (forward) and
5′- ATCCATCTGCTGCCCTGAGA-3′ (reverse);
- *mNpas2* [243 bp]:
5′-CTCAGTGGTCAGTTACGCAG-3′ (forward) and
5′-TGGAGGTGGGTTCTGACATG-3′ (reverse);
- *β-actin* [452 bp]:
5′-GAGGGAAATCGTGCGTGACAT-3′ (forward)
and 5′-ACATCTGCTGGAAGGTGGACA-3′ (reverse).

PCR was executed under the following conditions: cDNA synthesis at 50 °C for 30 min followed by 94 °C for 2 min, PCR amplification for 28 cycles with denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min The target clock gene cDNA was coamplified with the *β-actin* cDNA in a single PCR tube. A semiquantitative RT-PCR method was used for measuring the expression level of mRNA. All PCR products were under linear amplification from cycle 26–30, however, from the 32nd cycle there was a plateau in product levels (data not shown). Therefore, we obtained PCR products in the 28th cycle for quantification. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide and analysed by an EDAS-290 system (Eastman, Kodak, Rochester, NY). The intensity of PCR product of the target gene was normalized to the intensity of *β-actin*. The amplitude (ratio of the peak and trough) and phase determined by this method were reproducible in another experiment (Ohdo *et al.*, 2001). This reproducibility suggests that the present experimental conditions can detect a circadian change in *mPer1* and *mPer2* gene expression in the mouse liver.

Drug and injection schedule

Methamphetamine HCl (Dainippon Pharmaceutical. Co., Osaka, Japan), dissolved in saline, was injected i.p. daily at a fixed time (ZT 7, ZT, Zeitgeber time; ZT 0 is defined as the lights-on time and ZT 12 as the lights-off time) for 6 consecutive days and withdrawn on day 7 and 8. The increase in activity from ZT 6–ZT 12 on the day injection was withdrawn was identified as MAP-associated activity, as demonstrated in a previous report (Shibata *et al.*, 1995). We used intact vs SCN-lesioned mice and either saline or MAP injection to examine the effect of daily MAP injection on the day–night rhythm of *mPer* expression in the brain and liver. These animals were deeply anaesthetized with ether and killed at ZT 20 on day 6 and ZT 2, 8 and

14 on day 7. To examine the developmental time-course when striatal *mPer* gene expression produces a new diurnal rhythm, we killed animals on day 4 after 3-day injection of MAP. To examine the importance of a fixed time schedule for producing circadian oscillation, daily MAP injection was conducted randomly for 6 days, and then animals were killed at ZT 8 on day 7. In addition, for a better understanding of the mechanism under lying MAP-induced oscillation, we examined the acute effect of MAP on *mPer1*, *mPer2*, *mBmal1*, and *mNpas2* gene expression in the striatum and liver.

As a previous study demonstrated that a 2-mg/kg dose of MAP was enough to cause *mPer1* gene expression and sensitization in mice (Nikaido *et al.*, 1999, 2001) and anticipation in rats (Shibata *et al.*, 1995), this dose was selected for the present study.

SCN lesion

Bilateral thermal lesion of the SCN was performed as described previously (Wakamatsu *et al.*, 2001). Mice were deeply anaesthetized with ketamine (50 mg/kg i.p.) and xylazine (20 mg/kg i.p.), and a stainless steel electrode (0.35 mm inner diameter) was inserted into the SCN (0.5 mm posterior and 0.0 mm lateral to bregma at a depth 5.3 mm below the skull surface) using a thermal lesion device (RFG-4 A, Muromachi Medical Co, Tokyo, Japan). A lesion was made by maintaining a temperature of 55 °C for 15 s via a current path. A sham operation was performed by exposing the skull and drilling a hole on the skull surface (0.5 mm posterior and 0.0 mm lateral to the bregma), without inserting the lesion electrode. After recovery from anaesthesia, animals were moved to a locomotor activity device. One month after surgery, we selected animals with complete lesions of the SCN, once arrhythmicity was confirmed, using a chi-square periodogram (Sokolove & Bushell, 1978) in the range of 20–28 h. All SCN-lesioned animals with a confirmed loss of rhythmic activity were used in this study. The lesion sites were confirmed histologically at the end of the experiment. We excluded the data from SCN-lesioned animals exhibiting a significant ($P < 0.05$) 24 h oscillation rhythm by chi-square periodogram and incomplete SCN lesion by cresyl violet staining.

Statistics

Results are expressed as the mean ± SEM. The significance of differences between groups was determined by one-way analysis of variance followed by a Mann–Whitney *U*-test. To compare the daily clock gene expression rhythm between saline and MAP-injected groups, two-way analysis of variance was applied.

Results

Effect of daily MAP injection on locomotor activity and mPer *in the striatum and SCN*

Intact mice were injected daily with MAP (2 mg/kg, i.p.) at ZT 7 for 6 days. On day 7, locomotor activity was calculated for an entire day without MAP injection. Compared with daily saline-injected animals, these mice exhibited high locomotor activity from ZT 6 to ZT 12. Thus, an anticipatory activity rhythm resulted with daily MAP injection (Fig. 1A). There were significant differences in locomotion from ZT 6–ZT 12 between saline- and MAP-pretreated groups on day 7 (Fig. 1A). Intact mice were injected daily with MAP at ZT 7 for 6 days, then expression of *mPer1* and *mPer2* in the striatum and SCN were examined at ZT 20 on day 6 and ZT 2, 8 and 14 on day 7. In the striatum (Fig. 2A), *mPer* gene expression was high at ZT 14 in saline-treated mice and high at ZT 8 in MAP-treated mice. Thus, daily injection of MAP shifted from a nocturnal pattern of *mPer1* and *mPer2* gene expression to a diurnal pattern in the striatum ($P < 0.01$ for saline- and MAP-injected groups by two-way ANOVA), whereas *mPer* gene expression in the SCN was higher at ZT 8 than at ZT 20 in both saline- and MAP-injected groups (Fig. 2B).

In the next experiment, we examined the time frame for the establishment of MAP-induced anticipatory locomotor activity and the shift of striatal *mPer* gene expression rhythm. On day 4, after three-day injection of MAP or saline, anticipatory locomotion was increased, and the expression of *mPer1* and *mPer2* at ZT 8 significantly increased in MAP-pretreated animals (data not shown).

Effect of varied intervals of MAP injection on mPer *gene expression in the striatum and SCN*

A food anticipatory activity rhythm does not appear without a circadian (24 h) feeding schedule (Stephan *et al.*, 1981; Mistlberger & Marchant, 1995). One possibility is that anticipatory locomotion increase and *mPer* gene expression in the striatum result from daily MAP injection at a fixed time. To confirm this idea, we injected mice with MAP at variable injection intervals (12–36 h, average 24 h) (Fig. 3A). Anticipatory locomotor activity was not observed under these conditions (data not shown), and there were no significant differences in *mPer1* and *mPer2* expression in the striatum (Fig. 3B) and SCN (Fig. 3C) between saline-injected and MAP-injected groups at ZT 8.

MAP-induced anticipatory locomotion and mPer *gene expression in the striatum and liver of SCN-lesioned mice*

A behavioural study revealed that daily injection of MAP (2 mg/kg) at ZT 7 for 6 days elicited anticipatory locomotor activity around ZT 9–12 on day 7 in SCN-lesion mice with a previously verified arrhythmic behavioural rhythm (Fig. 1B). Saline injection, on the other hand, did not induce an anticipatory behavioural rhythm (Fig. 1B). Thus, daily injection of MAP at a fixed time of day is important to produce an anticipatory activity rhythm even in SCN-lesioned arrhythmic mice. Another group of SCN-lesioned mice were injected daily with saline or MAP at ZT 7 for 6 days, and *mPer* gene expression was examined at ZT 20 on day 6 and ZT 2, 8 and 14 on day 7. Daily rhythms of *mPer* gene expression disappeared in the striatum of saline-injected SCN-lesioned mice, whereas daily MAP injection caused a significant circadian rhythm of *mPer* gene expression in the striatum with a peak at ZT 8 (Fig. 4) ($P < 0.01$, 2-way ANOVA).

In the liver of intact animals, a pattern of *mPer1* and *mPer2* gene expression was observed with the peak expression occurring at ZT 14 for both genes (Fig. 5A). Daily saline injection into SCN-lesioned mice did not lead to daily expression of either the *mPer1* or *mPer2* gene in the liver (Fig. 5B) ($P > 0.05$, one-way ANOVA). On the other hand, the MAP-injected liver exhibited a significant daily rhythm of *mPer1* and *mPer2* expression with a peak at ZT 8 (Fig. 5B) ($P < 0.05$, one-way ANOVA), and significant differences between the saline- and MAP-injected groups were observed ($P < 0.05$, two-way ANOVA).

Acute effect of MAP on clock gene expression in the striatum, SCN and liver

In order to elucidate the oscillation mechanism of *mPer1* and *mPer2* gene expression in the striatum and liver, but not the SCN, the acute effect of MAP on clock gene expression was examined in these areas using intact mice. Our previous findings revealed that 5 mg/kg of MAP increased *mPer1* but not *mPer2* gene expression in the striatum 60 min but not 120 or 240 min after injection (Nikaido *et al.*, 2001).

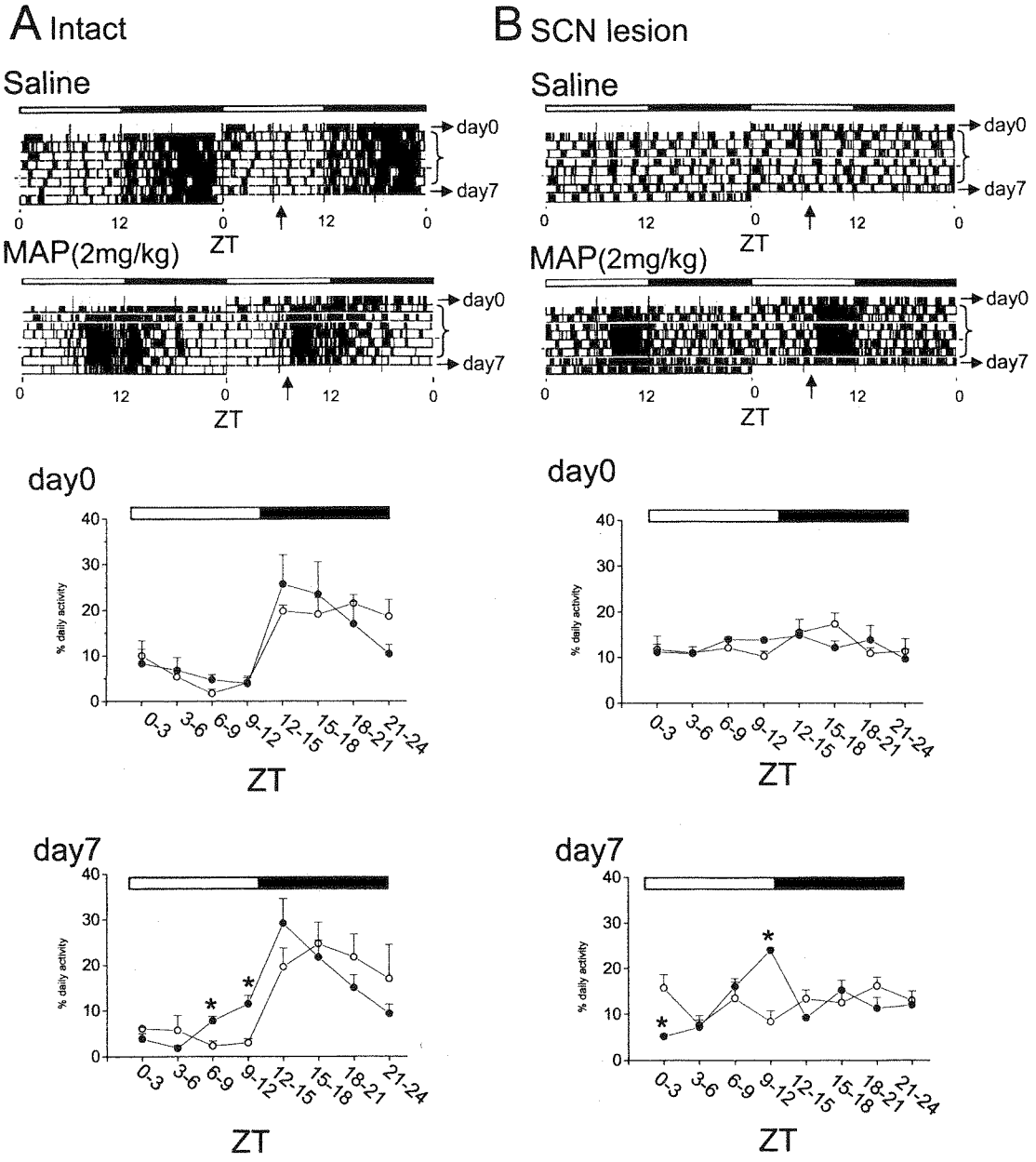


FIG. 1. Anticipatory locomotor activity in intact (A) and suprachiasmatic nucleus (SCN)- lesioned (B) mice (4–7 animals per group) after daily injection of methamphetamine (MAP). Upper panel shows double-plotted actograms of locomotor activity in saline- or MAP (2 mg/kg)-injected mice at ZT 7 for 6 days. Vertical arrows indicate the time of injection. Lower panels summarize anticipatory activity. Vertical values show the anticipatory activity (%) {[(activity counts from every 3 h)/(activity count for a full day)] × 100} the day before (day 0) or after (day 7) 2 mg/kg injections of saline (open circles) or MAP (filled circles). As SCN-lesioned mice exhibited arrhythmic activity, anticipatory activity values (%) were higher in lesioned animals than in intact mice. Before injection on day 0, there were no differences in anticipatory activity values between the saline and MAP groups of either intact or SCN-lesioned mice. On day 7, after 6-day injection of saline or MAP, anticipatory activity values for the MAP group were significantly higher than those of the saline group (*$P < 0.05$, Mann–Whitney *U*-test) in both intact and SCN-lesioned mice.

In the present experiment, we examined the effect of MAP (2 mg/kg) on *mBmal1* and *mNpas2* gene expression in the striatum and found that MAP injected at ZT 7 increased gene expression of *mBmal1* (Fig. 6A) 2 h later and *mNpas2* (Fig. 6B) 4 h later. Interestingly, in the liver, MAP injection increased the expression of *mPer1* but not *mPer2* 60 min after injection (Fig. 5C). In the case of the liver, however, MAP did not affect *mBmal1* or *mNpas2* gene expression at any time after injection (data not shown). In support of our previous results (Nikaido *et al.*, 2001), MAP did not affect any type of clock gene expression in the SCN.

Discussion

In the present study, periodic injection of MAP produced a persistent increase in locomotor activity the day after termination of MAP treatment. Thus, daily MAP injection at a fixed time resulted in an anticipatory activity rhythm in mice. Both *mPer1* and *mPer2* gene expression exhibited a nocturnal rhythm in the mouse striatum with peaks around ZT 11–ZT 15, while *mPer* expression in the SCN exhibited clear diurnal rhythms similar to those reported in previous papers (Albrecht *et al.*, 1997; Tei *et al.*, 1997; Takumi *et al.*, 1998).

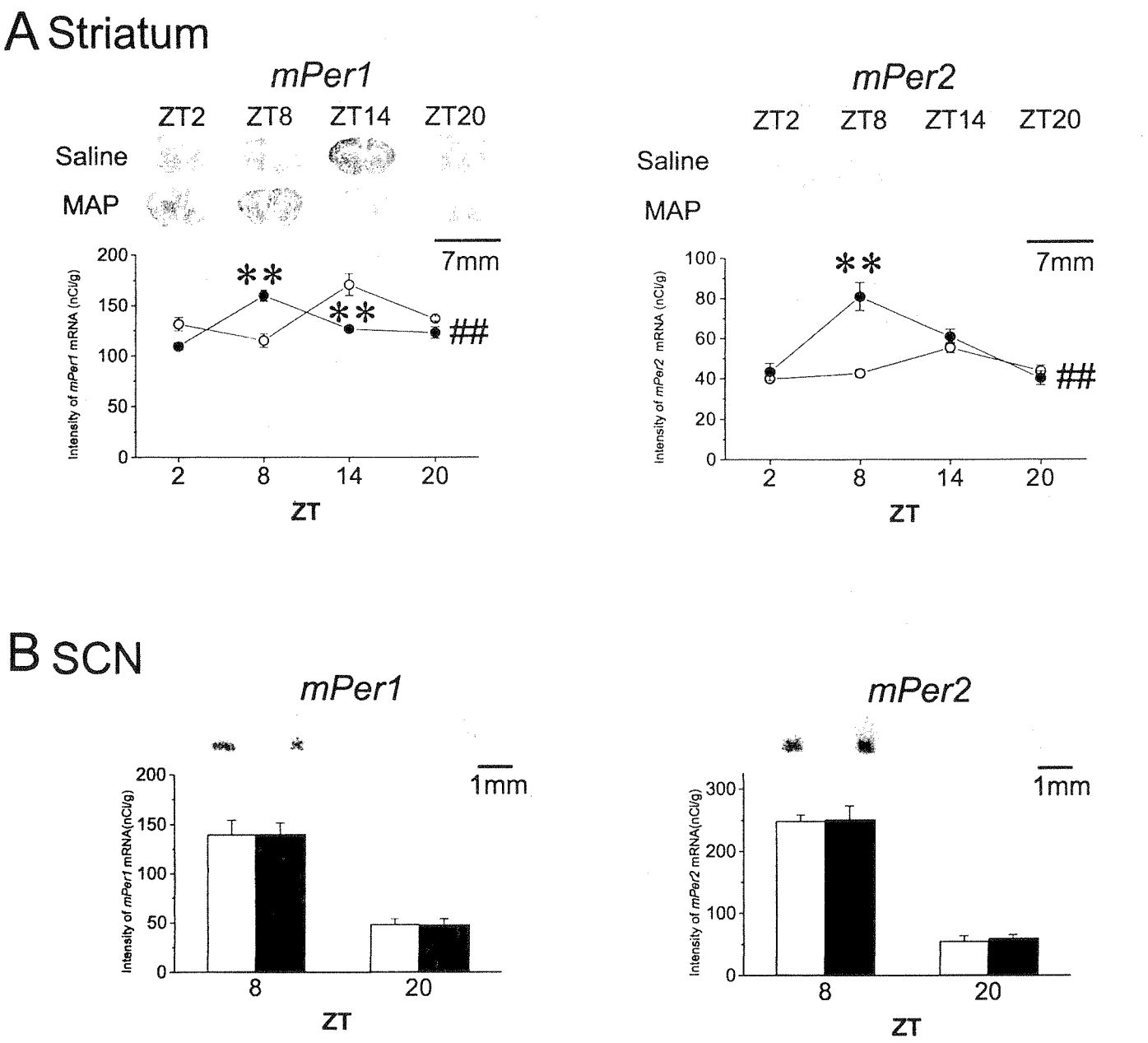


FIG. 2. Expression pattern of *mPer1* and *mPer2* in the striatum (A) and SCN (B) (6 animals per point) on day 7 after daily injection of saline [open circles (A) or open columns (B)] or MAP (2 mg/kg) [filled circles (A) or filled columns (B)] for 6 days in intact mice. The representative film autoradiograms are shown above each graph and the mRNA expression levels are plotted over the course of a full day after quantitative analysis. In the saline-injected mice, there were clear daily rhythms of *mPer1* and *mPer2* expression in the striatum, both of which peaked at ZT 14. On the other hand, a daily rhythmic expression of *mPer1* and *mPer2* with a peak at ZT 8 was observed in the striatum of MAP-injected mice. In the SCN, there were no significant differences in *mPer* gene expression patterns between the saline- and MAP-injected animals. $^{**}P < 0.01$ difference from the saline-injected group by Mann–Whitney *U*-test. $^{\#\#}P < 0.01$ difference between saline- and MAP-injected groups by two-way ANOVA. Scale bars, 7 mm (A); 1 mm, (B).

When we examined the expression pattern of *mPer1* and *mPer2* on day 7 after 6 days of MAP injection, in striatal areas, *mPer1* and *mPer2* peak expression had moved to the daytime at ZT 8. Thus, the present result demonstrated strongly that clock gene expression is initiated into at least one cycle by MAP delivery, corresponding with behavioural data. On the other hand, the expression pattern of *mPer1* and *mPer2* in the SCN was unaffected by daily MAP injection. Scheduled MAP injections did not cause additional new peaks at ZT 8 but instead moved these nocturnal peaks to ZT 8, indicating that the MAP-entrained oscillation of locomotor activity is closely associated with circadian expression of these genes in the striatum but not in the SCN.

At present, the mechanism underlying the anticipatory expression of *mPer1* and *mPer2* in the brain of the mouse injected daily with MAP remains unknown. Further experiments are required to clarify whether the elevated mRNA expression at ZT 8 is the cause or result of MAP-entrained oscillation. We demonstrated in previous studies that MAP coadministered with the D_1/D_2 receptor antagonist, haloperidol, the D_1 receptor antagonist, SCH23390, or the NMDA receptor antagonist, MK-801, inhibited the development of

MAP-induced anticipatory locomotor activity and *mPer1* gene expression in the striatum (Shibata *et al.*, 1995; Nikaido *et al.*, 2001). In addition, Honma & Honma (1995) reported that the free-

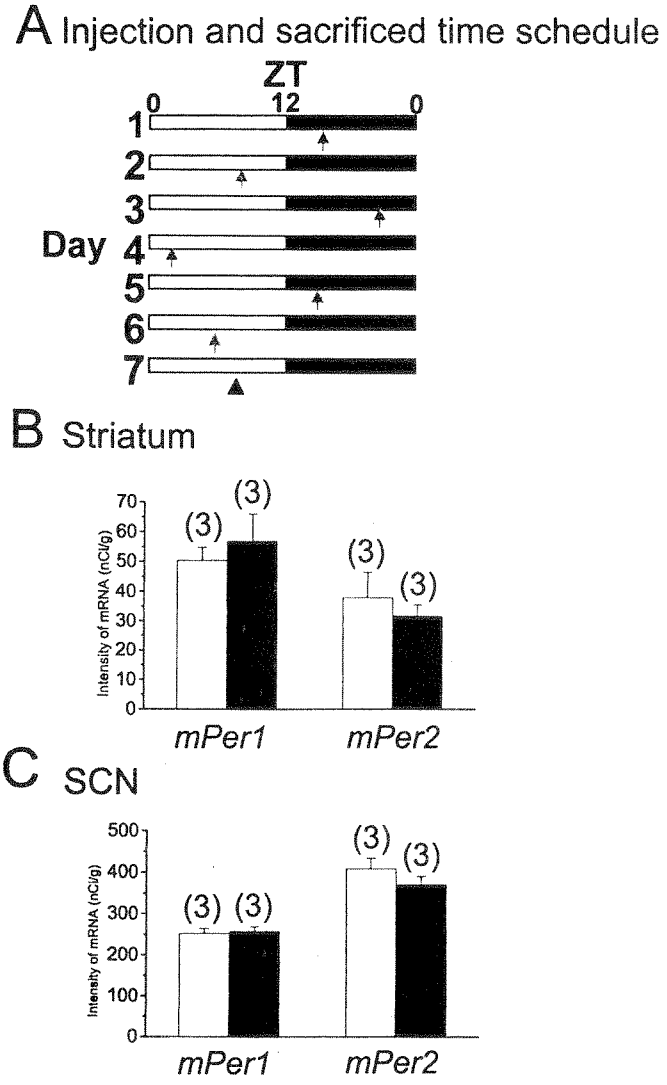


FIG. 3. Repeated MAP injection at random times failed to affect the *mPer* mRNA expression pattern in the striatum and SCN. (A) Schematic graph showing the injection and decapitation time schedule. Arrows for days 1 through 6 show the injection time and the arrowhead on day 7 when the animals were killed (ZT 8). m*Per1* and m*Per2* gene expression levels in the striatum (B) and the SCN (C)at ZT 8 on day 7 after random daily injection of saline (open columns) or MAP (2 mg/kg) (filled columns). Note that there were no significant differences in expression levels in the striatum and SCN between the two groups. Numbers in parentheses indicate the number of animals.

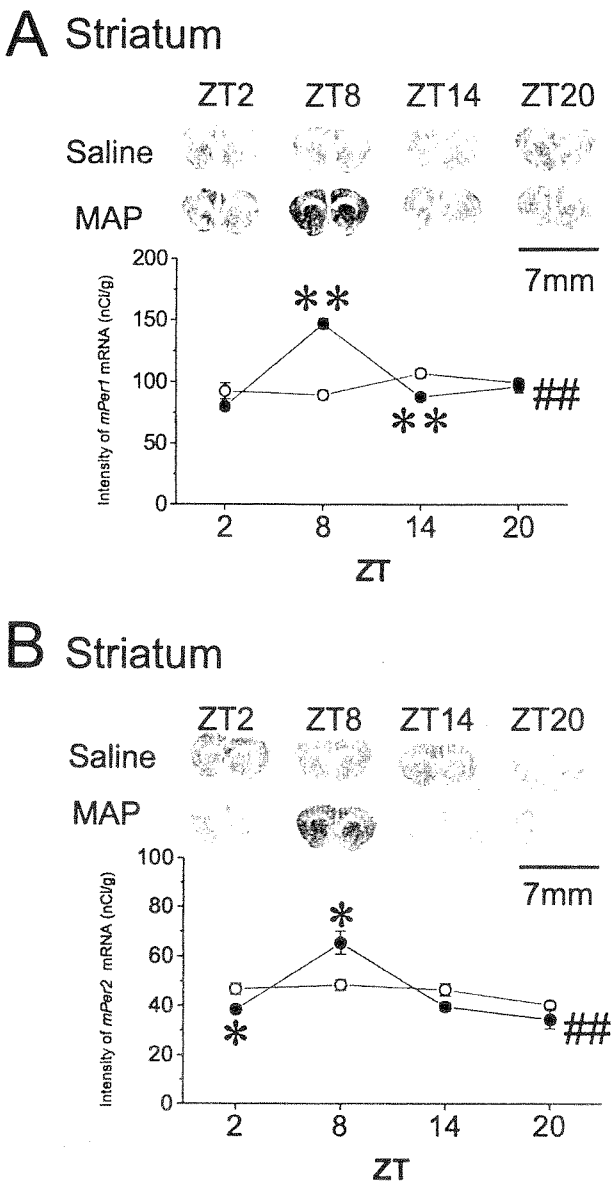
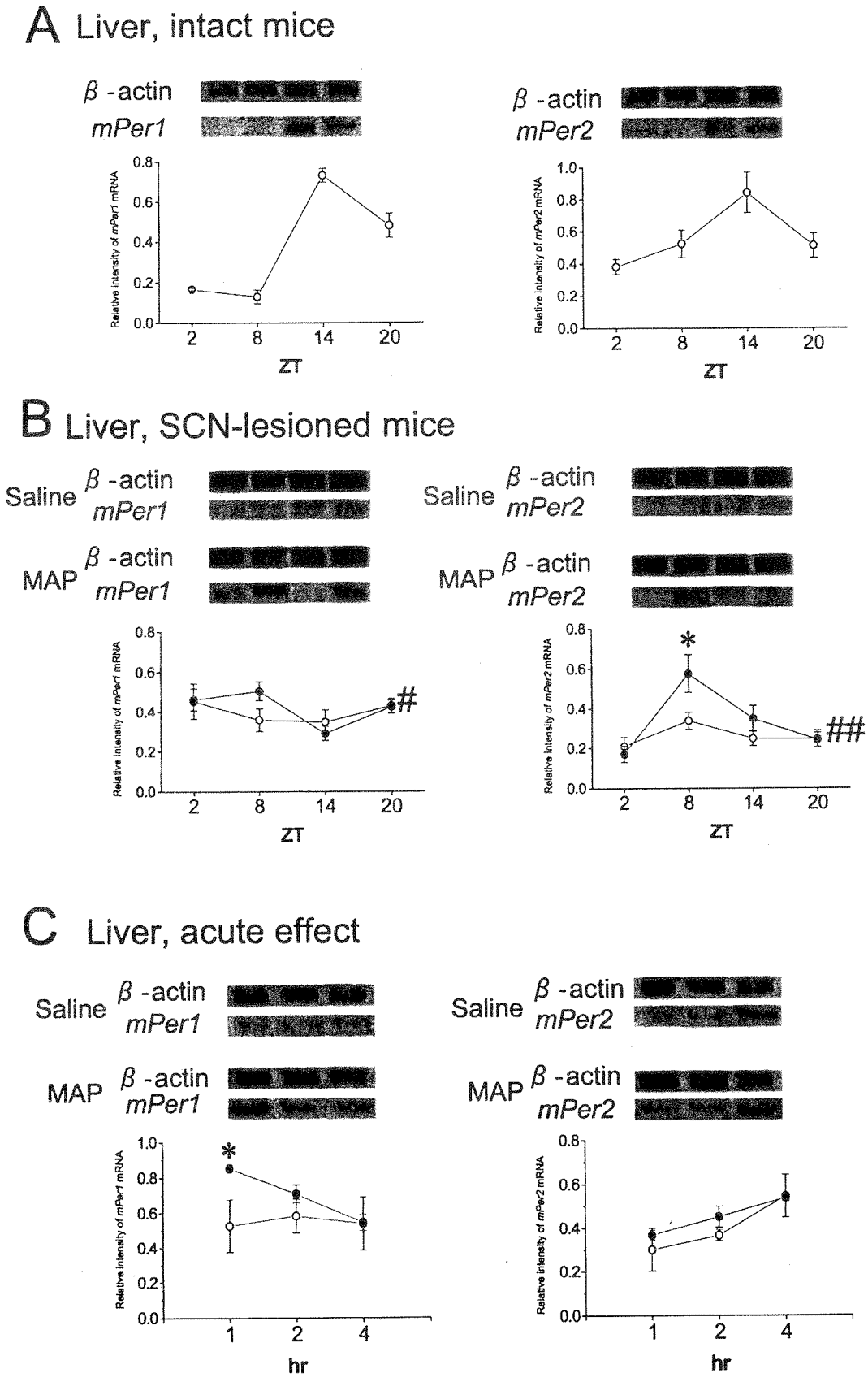


FIG. 4. Expression pattern of *mPer1* (A) and *mPer2* (B) in the striatum of SCN-lesioned mice on day 7 after daily injection of saline (open circles) or MAP (2 mg/kg) (filled circles) (7 animals per point) for 6 days. The representative film autoradiograms are shown above each graph and the mRNA expression levels are plotted over the course of a full day after quantitative analysis. In the saline-injected mice, there were no significant daily rhythms of *mPer1* and *mPer2* expression in the striatum. In contrast, daily MAP treatment produced a daily rhythm of *mPer1* and *mPer2* gene expression that peaked at ZT 8. $^{**}P < 0.01$, $^{*}P < 0.05$ difference from the saline-injected group by Mann–Whitney *U*-test. $^{\#\#}p < 0.01$ difference between the saline- and MAP-injected groups by two-way ANOVA. Scale bars, 7 mm (A and B).

FIG. 5. Effect of MAP treatment on *mPer1* and *mPer2* expression levels in the mouse liver. (A) Daily expression rhythm of *mPer1* (open circles) and *mPer2* (open triangles) (4 animals per point) mRNA in the liver of intact mice with a peak at ZT 14. (B) *mPer1* (left panel) and *mPer2* (right panel) expression pattern on day 7 in the liver of SCN-lesioned mice after daily treatment with saline (open circles) or MAP (2 mg/kg) (filled circles) (7 animals per point) at ZT 7 for six consecutive days. In the saline-injected mouse with a lesioned SCN, there is no daily rhythmic expression of *mPer1* and *mPer2* in the liver. In the MAP-injected mouse with an SCN lesion, a daily rhythmic expression of *mPer1* and *mPer2* appeared and peaked at ZT 8. $^{*}P < 0.05$ vs saline injection, Mann–Whitney *U*-test. $^{\#\#}P < 0.01$ $^{\#}P < 0.05$ vs time in MAP-injected groups by one-way ANOVA. (C) Acute effect of MAP (2 mg/kg) on *mPer1* and *mPer2* gene expression levels in the liver of intact mice. Saline (open columns) or MAP (2 mg/kg) (filled columns) (4 animals per point) was injected at ZT 8, and animals were killed 1, 2 or 4 h after injection. $^{*}P < 0.05$ vs saline injection, Mann–Whitney *U*-test. The mRNA expression (upper panel of each graph) was demonstrated by RT-PCR and signals were performed for the semiquantitative analysis.



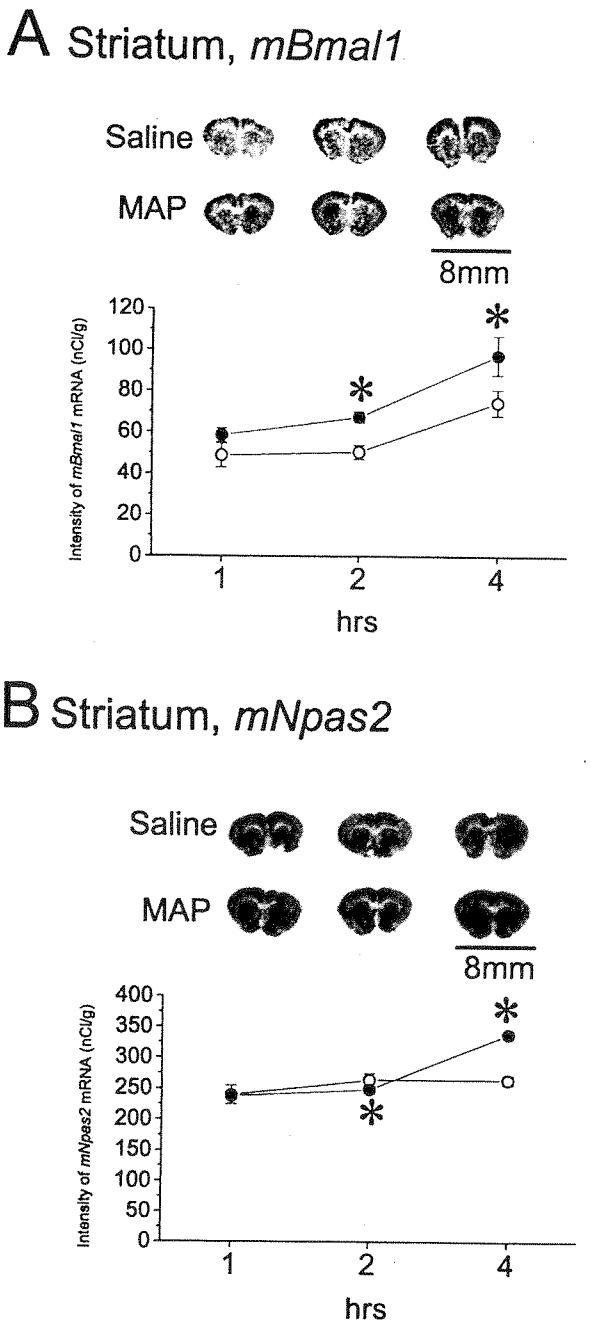


FIG. 6. Acute effect of MAP on striatal *mBmal1* (A)and *mNpas2* (B)gene expression. Representative film autoradiograms are shown above each graph and the mRNA expression levels are plotted over a designated course of time after injection. Saline (open circles) or MAP (2 mg/kg) (filled circles) (4 animals per point) was injected at ZT 7, and animals were killed 1, 2 or 4 h after injection. *$P < 0.01$ vs saline injection, by Mann–Whitney *U*-test. Scale bars, 8 mm (A and B).

running rhythm induced by ingested MAP was reset by haloperidol. Taken together, these results suggest that both D_1 and NMDA receptor subtypes are involved in the appearance of MAP-associated behavioural rhythms and *mPer* gene expression. Previous studies have demonstrated that both D_1 and NMDA receptor subtypes contribute to the behavioural sensitization induced by chronic MAP administration (Ujike *et al.*, 1989; for a review, seeSripada *et al.*, 2001). Further, one of our recent studies demonstrated the sensitization phenomenon in relation to the free-running rhythm induced by ingested MAP (Nikaido *et al.*, 1999). The amplitude of *mPer1* and *mPer2* expression in the striatum at ZT 8 increased following daily MAP injection, suggesting that the daily entrainment signals associated with MAP injection may strengthen oscillatory force. Therefore, there may be common neuronal mechanisms between sensitization and anticipation that are induced by repeated MAP injection.

Interestingly, varied intervals of MAP injection did not increase *mPer1* and *mPer2* expression in the striatum at ZT 8 on the day injection was withdrawn. Similarly, the food-anticipatory activity rhythm appeared even when the SCN was lesioned but did not appear without a circadian (24 h) feeding schedule (Stephan *et al.*, 1979a; Stephan *et al.*, 1979b; Mistlberger & Marchant, 1995). Thus, 24-h intervals of stimuli application are necessary to produce both a food-associated activity rhythm and also a MAP-associated rhythm as an SCN-independent circadian rhythm.

Little is known of the mechanistic site that mediates the generation and entrainment of this MAP-entrained oscillation, and the brain site for food-entrained oscillation remains unidentified (Choi *et al.*, 1998; Mistlberger, 1994). If compared to the brain regions examined in the present experiments, the MAP-entrained oscillation of *mPer1* and *mPer2* is closely associated with the striatum and liver and poorly associated with the SCN. Therefore, tentatively speaking, the brain and peripheral clock system may be important sites related to the MAP-entrained oscillation. This conclusion is supported by a recent report demonstrating that ingested MAP caused a free-running locomotor rhythm with a change in phase of *Per* gene expression in the cortex and striatum but not in the SCN of rats (Masubuchi *et al.*, 2000).

A core clock mechanism in the mouse SCN appears to involve a transcriptional feedback loop in which CLOCK and BMAL1 function as positive regulators and *Per* and *Cry* genes participate in negative feedback (Dunlap, 1999; Reppert & Weaver, 2001). Interestingly, acute MAP injection increased the striatal expression of *mPer1* 60 min later, and *mBmal1* and *mNpas2* 2–4 h later, whereas in the liver, MAP increased only *mPer1* gene expression. NPAS2 possesses high homology to CLOCK and coexpression of NPAS2 and BMAL1 activates transcription of the *Per1*, *Per2*, and *Cry1* genes, suggesting that NPAS2 probably operates as a function of the molecular clock in regions of the mammalian forebrain like the striatum but not the SCN (Reick *et al.*, 2001). Therefore, possibly *mPer1* and *mPer2*, or other clock genes in which the expression is regulated through the E-box, are not only controlled by CLOCK:BMAL1 but also other factors such as NPAS2:BMAL1 in the striatum and liver. A recent finding denoted that the presence of NADH and NADPH facilitates the dimerization of BMAL1:CLOCK (Rutter *et al.*, 2001). The redox state may be the main signal triggering oscillation in the striatum. Possibly the MAP-induced increase of mBMAL1 and mNPAS2 regulates *mPer1* and *mPer2* gene expression in the striatum but not the liver and SCN, and starts the oscillation of an SCN-independent rhythm.

Circadian oscillation of *mPer1* and *mPer2* gene expression in the liver after 6 days of MAP injection may be controlled secondarily by a brain clock as MAP injection never produced *mBmal1* and *mNpas2* gene expression in the liver. Although the induction mechanism underlying the induction of *mBmal1* and *mNpas2* genes in the striatum should be clarified through further experimentation, the MAP-induced increase in the expression of these genes may be an important signal that starts the oscillation of an SCN-independent rhythm.

It is well known that MAP increases cAMP response element binding protein (CREB) phosphorylation in the striatum (Muratake *et al.*, 1998). Yamaguchi *et al.* (2000) reported that the promoter for the *mPer1* gene contains four CRE sites, suggesting that the CREB–CRE transcriptional pathway has the capacity to activate *Per1* gene expression. These results suggest that the PER1 produced by MAP interacts with *Per2* promoter regions to start the oscillation of *Per2* gene expression. In fact, there is indication of interactions among PER1, PER2 and PER3 (Yagita *et al.*, 2000). This possibility, however, was not supported by a recent *mPer1* knockout experiment in which the *mPer1* gene was not essential for either circadian core oscillation in the SCN or the light-induced phase shift in behavioural rhythm (Cermakian *et al.*, 2001; Zheng *et al.*, 2001). Therefore, the *mPer1* gene expression in the striatum and liver from acute MAP injection may not be associated with oscillation of an SCN-independent rhythm. Such inconsistency with acute induction of *mPer1* and oscillation of *mPer1* and *mPer2* has already been observed using rat-1 cells (Balsalobre *et al.*, 2000).

In summary, the present results suggest strongly that daily MAP stimulation causes circadian oscillation in not only the behavioural rhythm, but also molecular rhythms in the striatum and liver, without participation of an SCN clock rhythm.

Acknowledgements

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Abbreviations

MAP, methamphetamine; NMDA, *N*-methyl-D-aspartate; PB, phosphate buffer; PFA, paraformaldehyde; SCN, suprachiasmatic nucleus; ZT, zeitgeber time.

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Gastrin-Releasing Peptide Mediates Photic Entrainable Signals to Dorsal Subsets of Suprachiasmatic Nucleus via Induction of *Period* Gene in Mice

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ABSTRACT

The suprachiasmatic nucleus (SCN), locus of the central circadian clock, consists of two neuronal populations (i.e., a light-recipient ventral SCN subpopulation directly entrained by light and a dorsal SCN subpopulation with an autonomous oscillatory function possessing an indirect or weak light response). However, the mechanism underlying the transmission of photic signals from the ventral to dorsal SCN remains unclear. Because gastrin-releasing peptide (GRP), expressed mainly in the ventral SCN, exerts phase-shifting actions, loss of the GRP receptor intuitively implies a reduction of photic information from the ventral to dorsal SCN. Therefore, using GRP receptor-deficient mice, we examined the involvement of GRP and the GRP receptor in light- and GRP-induced entrainment by the assessment of behavioral rhythm and induction of *mouse-Period* (*mPer*) gene in the SCN, which is believed to be a critical for photic entrainment. Administration of GRP during nighttime dose dependently produced a phase delay of behavior in wild-type but not GRP receptor-deficient mice. This phase-shift by GRP was closely associated with induction of *mPer1* and *mPer2* mRNA as well as c-Fos protein in the dorsal portion of the SCN, where the GRP receptor was also expressed abundantly. Both the light-induced phase shift in behavior and the induction of mPer mRNA and c-Fos protein in the dorsal SCN were attenuated in GRP receptor-deficient mice. Our present studies suggest that GRP neurons in the retinorecipient ventral area of the SCN convey the photic entrainable signals from the ventral SCN to the dorsal SCN via induction of the *mPer* gene.

Daily behavioral and physiological rhythms persist under conditions absent of environmental time cues, suggesting the existence of endogenous time-keeping systems and daily light/dark cycle entrains the self-oscillating circadian rhythms to the environmental 24-h period. The suprachiasmatic nucleus (SCN) was found to harbor the central circadian pacemaker in mammals (for review, see Ralph et al., 1990). Photic signals for entrainment reach the SCN mainly via a monosynaptic afferent from the retina, the retinohypothalamic tract (RHT), by using glutamate as a major neurotransmitter (for review, see Inouye and Shibata, 1994). In accordance with the characteristics of expressed neuropeptide or innervation, the SCN is divided into ventral and dorsal subpopulations. The dorsal SCN undergoes a strong autonomous oscillation possessing a weak and/or indirect light responsiveness, whereas the ventral, innervated by glutamatergic afferents from the RHT, plays a crucial role in photic entrainment with a weakly oscillating function (Shibata et al., 1984). In the ventral SCN, the *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptors, is thought to mediate photic entrainable signals because an NMDA receptor blockade suppressed photic induction of immediate early genes in the ventral but not in the dorsal SCN (Abe et al., 1991). However, it remains to be clarified how light for entrainment conveys signals from the ventral to dorsal subpopulation of the SCN.

Gastrin-releasing peptide (GRP) may be a possible candidate for neurotransmitters involved in transmission to the dorsal SCN based on the following reports. First, the cell somata of GRP neurons were restricted to the ventral SCN, whereas the fibers extended into the dorsal portion (Gun-

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ABBREVIATIONS: SCN, suprachiasmatic nucleus; RHT, retinohypothalamic tract; NMDA, *N*-methyl-D-aspartate; GRP, gastrin-releasing peptide; Per, Period; LD, light-dark; ZT, zeitgeber time; CT, circadian time; DD, constant darkness; PB, phosphate buffer; PFA, paraformaldehyde; PVN, hypothalamic paraventricular nucleus; PBS, phosphate-buffered saline.

dlach and Knobe, 1992; Silver et al., 1999) where they could communicate with other types of SCN neurons by using GRP as a synaptic transmitter (van den Pol and Gorcs, 1986; Mikkelsen et al., 1991; Romijn et al., 1997). Second, light-induced expression of c-Fos protein, corresponding to neuronal activation, was observed substantially within GRP neurons but moderately within other neurons of the SCN (Earnest et al., 1993; Aioun et al., 1998). Finally, GRP administration into the SCN during "subjective night" (Subjective night means the time when the animal's physiology is under nighttime condition without environmental time cue; therefore, it means active time for nocturnal mice.) could elicit a light-like phase shift in behavioral (Albers et al., 1991; Piggins et al., 1995) as well as firing rhythms in the SCN slice (McArthur et al., 2000). Therefore, we could postulate that GRP mediates the photic signal initially received in ventral portions to dorsal portions of the SCN where the core oscillating system is involved.

It is becoming abundantly clear that the core clock mechanism in the SCN involves a transcriptional and translational negative-feedback loop (for review, see Dunlap, 1999) in which the transcription of three *Period* genes [*mouse-Period* (*mPer*); *mPer1* (Shigeyoshi et al., 1997; Sun et al., 1997), *mPer2* (Shearman et al., 1997), *mPer3* (Takumi et al., 1998; Zylka et al., 1998)] are driven by the CLOCK:BMAL1 complex and negatively regulated directly by the Period proteins and the products of two *cryptochromes* genes (*Cry1* and *Cry2*) (Kume et al., 1999). In terms of photic entrainment, reportedly a transient increase in *Per1* and *Per2* mRNA in the SCN is elicited substantially in the ventral SCN via NMDA receptor activation, and moderately in the dorsal SCN upon photic stimulation during subjective night (Shearman et al., 1997; Shigeyoshi et al., 1997; Moriya et al., 2000). In addition, we demonstrated that the photic induction of *Per* genes is causally involved in photic entrainment, because an antisense oligonucleotide targeting either *mPer1* (Akiyama et al., 1999) or *mPer2* (Wakamatsu et al., 2001) mRNA inhibits the light- or glutamate-induced phase shift in behavior as well as in neuronal firing in the SCN slice preparation.

To clarify the mechanisms underlying photic entrainment in the dorsal SCN, we first examined the effects of GRP on the expression of *mPer1* and *mPer2* as well as c-Fos protein in the SCN with respect to topographical characteristics of the expression. We used both wild-type and GRP receptor-deficient mice (Wada et al., 1997) to confirm receptor specificity for actions of GRP on *mPer* and c-Fos. Finally, to elucidate the role of the GRP receptor in photic signaling within the SCN, we investigated the topographical difference in the photic induction of *mPer1*, *mPer2* mRNA, and c-Fos between wild-type and GRP receptor-deficient mice. Moreover, we examined GRP- and light-induced behavioral phase shifts in wild-type and GRP receptor-deficient mice to confirm that a change in *Per* gene expression in the SCN is associated with overt behavioral entrainment.

Experimental Procedures

Animals. Male GRP receptor-deficient mutant mice and their wild-type littermates were used for behavioral studies and quantitative analysis of *mPer1*, *mPer2* mRNA and c-Fos protein expression. The GRP receptor gene is located on chromosome X in both mice and humans (Maslen and Boyd, 1993). Therefore, hemizygous male (−/Y)(GRP receptor-deficient mice) and wild-type male (+/Y) were produced by mating heterozygous female mice (+/−) with C57BL/6J males and used for experiments. Male C57BL/6J mice were also used for the quantitative analysis of *GRP receptor* mRNA because both GRP receptor-deficient mice (−/Y) and their wild-type littermates (+/Y) were maintained on a C57BL/6J background. Mice were housed in temperature-controlled animal quarters (23 ± 2°C) under a 12:12-h light-dark (LD) cycle before use in the experiments. We used "zeitgeber time" (ZT) to reflect the time of day under LD conditions (ZT0 or ZT12 was lights-on or -off time under LD conditions, respectively). In the experiment under constant darkness condition, circadian time (CT) was defined instead of ZT, and CT12 referred to the onset of activity for nocturnal mice. Food and water were given ad libitum. Animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Materials. GRP was obtained from Peptide Institute, Inc. (Osaka, Japan) and dissolved in sterile water and stocked at −20°C until use for the experiments.

Intracerebroventricular Injection. Mice were deeply anesthetized with ketamine (50 mg/kg i.p.) and xylazine (20 mg/kg i.p.) and stereotaxically implanted with a 22-gauge stainless steel cannula (total length, 6.0 mm). Stereotaxic coordinates were as follows: 0.52 mm posterior and 1.1 mm lateral to the bregma, and 2.2 mm ventral to the skull surface. After 10 days of recovery from surgery under LD conditions, animals were anesthetized with ether for 30 s and a 27-gauge injection cannula (total length, 6.5 mm) was inserted. Drug or saline (total volume, 4 μl; injection duration, 2 min) was administered by a 10-μl Hamilton syringe under dim red illumination (<1 lux) to mice gently restrained by hand. After injection, the injection cannula was left in position for 15 s to facilitate drug diffusion.

Recording of Locomotor Activity Rhythm. Mice were housed individually in transparent plastic cages (31 × 20 × 13 cm) and their locomotor activity was measured using an area sensor (F5B; Omron, Kyoto, Japan) located 30 cm above the surface of the cage. Each area sensor was previously calibrated using the same animals for consistency. Locomotor activity was continuously recorded in 6-min epochs by personal computer.

To examine the locomotor activity rhythm under LD followed by constant darkness (DD) conditions, mice were first maintained under LD conditions for at least 2 weeks then released into DD conditions for 1 month. Light intensities during the light period and dark period were set at 50 lux and less than 0.05 lux, respectively. The period of locomotor activity rhythm under DD conditions was calculated by using a χ^2 periodogram in the range of 20 to 28 h. To evaluate the response to photic stimuli or GRP injection, mice were maintained under DD conditions for at least 10 days and either exposed to a light pulse (30 or 300 lux) for 15 min or administered an i.c.v. injection of GRP or saline at CT16. The drug and vehicle groups were crossed over and animals were given the opposite drug treatment. Each animal received no more than four i.c.v. injections. The phase shift in locomotor activity rhythm under DD conditions was calculated based on the distance between the two regression lines drawn from daily onset of locomotor activity for at least 7 days before and after light pulse.

Brain Sampling Procedure for In Situ Hybridization and Immunohistochemistry. In the experiments for gene expression in the SCN, we used a systematic and routine procedure, in which the drug injection or light pulse is given to animals 52 h after release from LD into DD conditions (2 days after releasing into DD), whereas behavioral experiments were performed at least 10 days after release into DD conditions. We used "projected ZT" as the time of treatment under DD conditions (projected ZT0 or ZT12 was lights-on or -off time before release into DD conditions); therefore, 52 h after release into DD conditions refers to projected ZT16. Our previous reports demonstrated that light pulse-induced *Per* induction in the SCN of the animals that had been kept in DD for 2 days was well associated with light-induced phase response in activity rhythm measured under DD conditions for long term (Shigeyoshi et al., 1997; Moriya et

al., 2000). Furthermore, we could not detect any significant difference in the amount of *mPer1* or *mPer2* mRNA induction in the SCN in the response to light pulse (300 lux for 15 min) or GRP injection (15 nmol) at CT16 between mice that had been kept in DD for 2 and 10 days [light pulse-elicited induction (nCi/g), (*mPer1*) 2 days: 121.46 ± 7.72 (n = 3), 10 days: 114.38 ± 0.97 (n = 3), $p > 0.05$, (*mPer2*) 2 days: 255.5 ± 9.99 (n = 3), 10 days: 285.5 ± 18.57 (n = 3), $p > 0.05$] [GRP-elicited induction (nCi/g), (*mPer1*) 2 days: 86.15 ± 1.47 (n = 3), 10 days: 92.43 ± 2.14 (n = 3), $p > 0.05$, (*mPer2*) 2 days: 94.63 ± 5.45 (n = 3), 10 days: 112.42 ± 4.44 (n = 3), $p > 0.05$]. Therefore, we believe that the duration after release into DD may not make a significant difference in the gene induction in the SCN or behavioral phase shift in response to light or the drug at least under our experimental conditions. At the appropriate time, mice were deeply anesthetized with ether and intracardially perfused with chilled saline (25 ml) followed by 0.1 M phosphate buffer (PB), pH 7.4, containing 4% paraformaldehyde (PFA; 25 ml). Brains were removed, postfixed in 0.1 M PB containing 4% PFA for 24 h at 4°C, and transferred into 20% sucrose in 0.1 M PB for 72 h at 4°C. Slices 30 μm thick, including the SCN, were made using a cryostat (HM505E; Microm, Walldorf, Germany) and divided into three equal groups from rostral to caudal parts for the measurement of *mPer1*, *mPer2* mRNA and c-Fos protein (as described below).

In Situ Hybridization with Radioisotope-Labeled cRNA Probe. In situ hybridization was executed to determine the quantity of Per and GRP receptor mRNA expression in the SCN by using *mPer1* and *mPer2* cRNA probes and mice *GRP receptor* cRNA probes, respectively [nucleotide positions: *mPer1* (538–1752), *mPer2* (1–638), *GRP receptor* (822–1700; GenBank accession no. M57922.1)]. Slices made as described above were placed in 2× standard saline citrate and were treated with 1 μg/ml proteinase K in 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM EDTA for 10 min at 37°C, followed by treatment with 0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% NaCl for 10 min. The slices were then incubated in hybridization buffer [60% formamide, 10% dextran sulfate, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.6 M NaCl, 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin), 0.2 mg/ml tRNA, and 0.25% SDS] containing ^{33}P-labeled cRNA probes for 16 h at 60°C. Antisense cRNA probes labeled with [α-^{33}P]UTP (PerkinElmer Life Sciences, Boston, MA) were made from restriction enzyme-linearized cDNA templates. After high-stringency posthybridization washes with 2× standard saline citrate/50% formamide, slices were treated with RNaseA (10 μg/ml) for 30 min at 37°C. Images were visualized by autoradiogram and Bio-Max MR film (Eastman Kodak, Rochester, NY), and analyzed using an image analyzing system (MCID; Imaging Research Inc., St. Catherines, ON, Canada) after conversion into absorbance by ^{14}C autoradiographic microscales (Amersham Pharmacia Biotech, Ltd., Little Chalfont, Buckinghamshire, UK). For data analysis, we subtracted the intensities of absorbance of the corpus callosum from those of the SCN or the hypothalamic paraventricular nucleus (PVN) in each section and regarded this value as the net intensity in the SCN or the PVN, respectively. To evaluate the mRNA expression in the "entire" SCN, the intensity values of sections from the most rostral to the most caudal part of the SCN (four sections per mouse brain) were then summed; the sum was considered to be a measure of the amount of mRNA in the entire SCN. The amount of mRNA in the PVN was also measured as same as in the SCN. To examine the subnuclear distribution of mRNA (the ventral versus the dorsal SCN) in response to light or GRP injection, we used emulsion autoradiography. Mounted slices after exposure to X-ray film were dipped into emulsion (NTB2; Eastman Kodak; diluted 1:1 with distilled water), air-dried for 3 h, and stored in light-tight slide boxes at 4°C for 3 weeks. The slides were developed with a D19 developer (Eastman Kodak) then fixed with Fujifix (Fujifilm, Tokyo, Japan) and counterstained with cresyl violet. Digital images of autoradiograms were made using an optical microscope equipped with charge-coupled device camera and the area (the number of pixels) of silver grains was analyzed by Scion Image Beta 4.02 (Scion Corporation, Frederick, MD). First, we selected one slice of the caudal SCN that exhibited the strongest mRNA intensity among the all slices of each animal. Then a digital image of the SCN area was visualized at the threshold level of 100 and the number of pixels inside the ventral or dorsal half of the SCN (defined as upper and lower halves of the SCN separated at a midpoint between the top and bottom of the SCN based on the cresyl violet counterstaining) were counted and expressed as a relative value. An SCN outline was drawn by an observer without knowledge of the treatment conditions.

TABLE 1

Period (h) of locomotor activity rhythm in wild-type mice and GRP receptor-deficient mice under LD followed by DD conditions

Photic Condition	Days for Analysis	Wild-Type Mice (n = 9)	GRP Receptor-Deficient Mice (n = 8)
LD	LD last 6 days	24.02 ± 0.02	24.02 ± 0.04
DD	DD 1–10 days	23.81 ± 0.03	23.80 ± 0.04
	DD 11–20 days	23.82 ± 0.03	23.84 ± 0.03
	DD 21–30 days	23.89 ± 0.04	23.86 ± 0.04

c-Fos Immunohistochemistry. Brain slices made as mentioned previously were incubated for 48 h with anti-Fos antibody (Ab-5; Oncogene Research Products, Cambridge, MA) diluted to 1:20000 with 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 1% normal goat serum and 0.3% Triton X-100 at 4°C. All slices were then washed three times with 0.01 M PBS (10 min each) and incu-

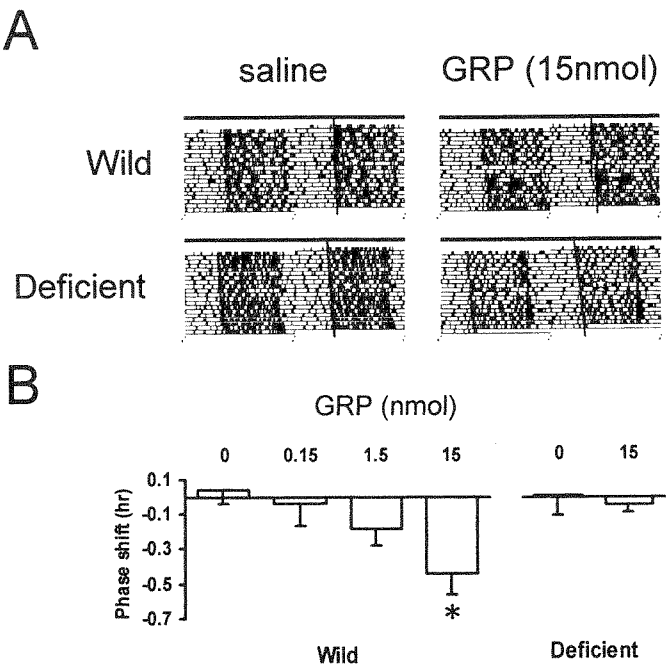

Fig. 1. Phase delays of behavioral activity rhythm by i.c.v. injection of GRP in wild-type (Wild) and GRP receptor-deficient mice (Deficient) under DD conditions. A, representative double-plotted actograms demonstrating that an i.c.v. injection of GRP (15 nmol) phase-delayed the locomotor activity rhythm in wild-type mice yet did not affect this rhythm in GRP receptor-deficient mice. Time of day is indicated horizontally and consecutive days vertically. Each white star indicates the time of GRP or saline injection. Mice were maintained under DD conditions for at least 10 days and administered an i.c.v. injection of GRP or saline at CT16. B, dose dependence for phase-shifting effects of GRP on behavioral activity rhythm. The degree of phase shift in each mouse was calculated from the distance between the two regression lines drawn from daily onset of locomotor activity for at least 7 days before and after light pulse (as drawn in Fig. 1A) and was averaged in each group. The minus values indicate the phase delay of behavioral rhythm and this value is thought to reflect the degree for resetting of circadian clock in response to drugs. n = 4 to 14; *, $p < 0.05$ versus saline group (one-way analysis of variance followed by Dunnett's test).

bated for 1 h with biotinylated anti-rabbit goat antibody (diluted to 1:200 with PBS containing 1% normal goat serum and 0.3% Triton X-100; Vectastain, Burlingame, CA). The slices were again washed three times with 0.01 M PBS and incubated for 1 h in an avidin-biotin complex solution (ABC kit; Vectastain). After three washes with 0.01 M PBS, slices were visualized with diaminobenzidine chromogen and mounted on gelatin-coated glass slides. The slices were counterstained with methyl green to identify the anatomical location of the SCN. All procedures were performed at room temperature except for the incubation with a primary antibody. The number of cells expressing Fos immunoreactivity was counted by Scion Image Beta 4.02. Briefly, a digital image of the SCN area was visualized at the threshold level of 160 and the number of particles (minimum and maximum particle sizes are 10 and 40 pixels, respectively) inside the bilateral SCN border was counted. The measurement was done in the entire SCN (four sections from the most rostral to the most caudal part of the SCN) or the ventral half and the dorsal half of the caudal SCN as described previously. Average cell numbers in the bilateral SCN per one slice were calculated.

Statistical Analysis. The values are expressed as means ± S.E.M. For statistical analysis, one-way analysis of variance followed by Dunnett's test or Student's unpaired *t* test was applied.

Results

Free-Running Rhythm of Wild-Type and GRP Receptor-Deficient Mice under DD Conditions. To gain an understanding of the basic nature of the circadian clock in GRP receptor-deficient mice, we compared the locomotor activity rhythms of wild-type and GRP receptor-deficient mice under LD and DD conditions. Both wild-type mice and GRP receptor-deficient mice showed an LD-entrained behavioral rhythm, and locomotor activities were restricted to the dark period (Table 1). Under DD conditions lasting 1 month, both types of mice exhibited a stable free-running rhythm, and there was no observably significant difference in the period of the activity rhythm during the first 10 days (days 1–10), next 10 days (days 11–20), or last 10 days (days 21–30) (Table 1). Thus, the circadian oscillatory nature seemed to be unaltered in GRP receptor-deficient mice.

GRP-Induced Phase Shifts in Behavioral Activity. In the next experiment, we tried to confirm that an i.c.v. injection of GRP could phase-shift the locomotor activity rhythm in mice via GRP receptor activation using both wild-type

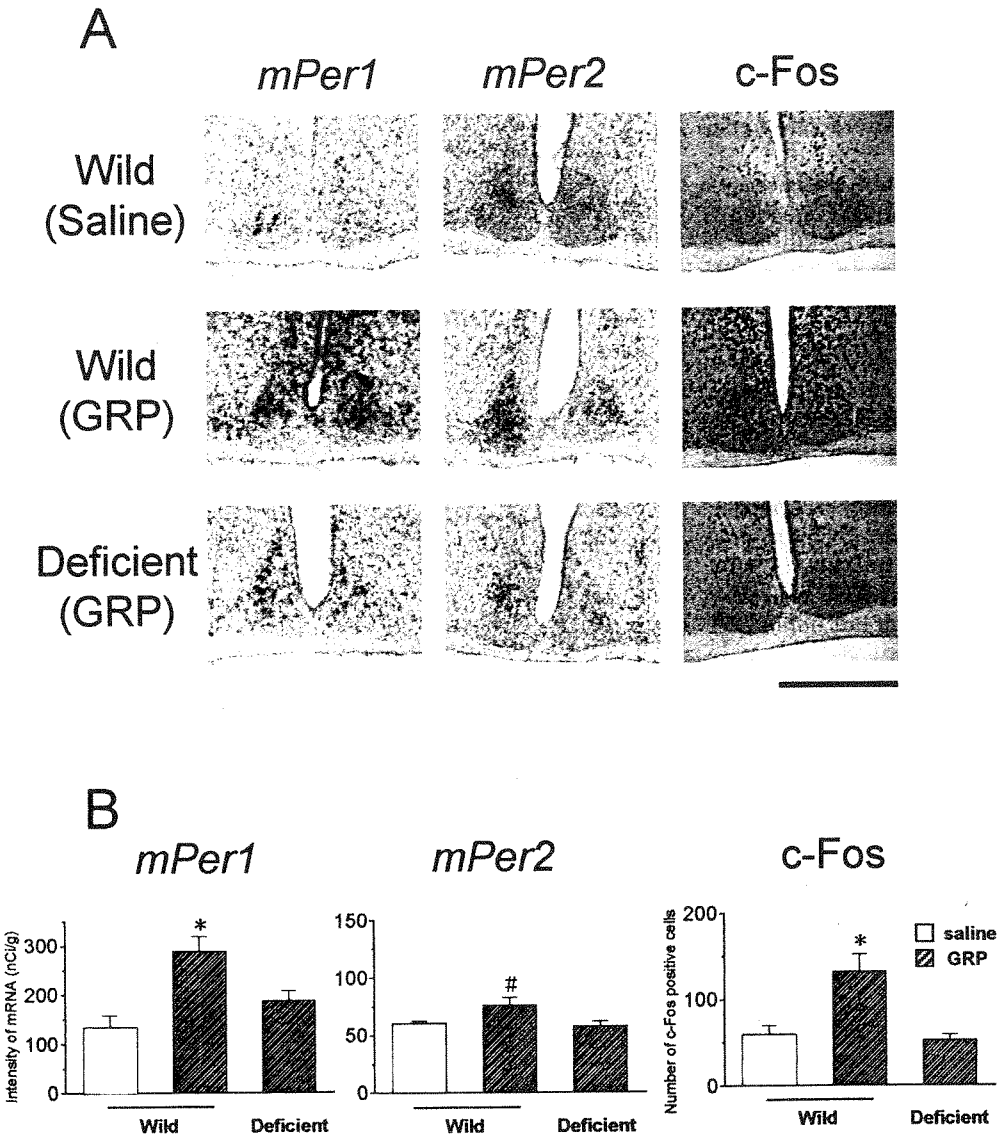

Fig. 2. Induction of *mPer1* and *mPer2* mRNA and c-Fos protein in the SCN by an i.c.v. injection of GRP in wild-type (Wild) and GRP receptor-deficient mice (Deficient). A, representative emulsion autoradiograms or photographs showing that an i.c.v. injection of GRP elicited the induction of *mPer1*, *mPer2*, and c-Fos in the dorsal portion of the SCN of wild-type, but not GRP receptor-deficient mice. After release into DD for 2 days, mice were injected with GRP (15 nmol) or saline at projected ZT16 and then perfused intracardially with 4% PFA 90 min after drug injection. Scale bar, 0.5 mm. B, quantitative analysis of *mPer* mRNA and c-Fos protein in the entire SCN. The intensities of the expression were measured in the entire SCN (sections from the most rostral to the most caudal part of the SCN) with film autoradiograms (*mPer1* and *mPer2* mRNA) or the immunohistochemical photographs (c-Fos protein). Wild, wild-type mice; Deficient GRP receptor-deficient mice. $n = 3$ to 6; *, $p < 0.05$ versus saline group (unpaired Student's *t* test); #, $p < 0.10$ versus saline group (unpaired Student's *t* test).

mice and GRP receptor-deficient mice. An i.c.v. injection with GRP (15 nmol) at CT16 produced phase delays of locomotor activity rhythm in wild-type mice maintained under DD conditions, whereas saline treatment failed to affect the phase in activity (Fig. 1A). This phase-shifting action of GRP demonstrated clear dose dependence (Fig. 1B). A 15-nmol dose of GRP could elicit sufficiently significant phase delays, at which the magnitude of the average delays was 26.7 ± 6.8 min. In contrast, injections of GRP (15 nmol) or saline did not affect the phase of activity in GRP receptor-deficient mice (Fig. 1).

Induction of *mPer* mRNA and c-Fos Protein in the SCN after i.c.v. Injection of GRP. The level of both *mPer1* and *mPer2* mRNA was low at projected ZT17.5 (1.5 h after injection of drugs at projected ZT16) in saline-injected mice (Fig. 2A). GRP injection at a dose of 15 nmol at projected ZT16 on 2 days after releasing into DD condition caused a substantial increase in the levels of *mPer1* mRNA in the SCN of wild-type mice, whereas GRP receptor-deficient mice were unaffected (Fig. 2, A and B). *mPer2* mRNA level in the SCN of wild-type mice, but not of GRP receptor-deficient mice, also increased after the i.c.v. injection of GRP (15 nmol), but this increase was insignificant ($p = 0.09$). Based on the emulsion autoradiograms of all examined slices, it seems that an i.c.v. injection of GRP caused *mPer1* and *mPer2* induction mainly in the dorsal portion of the SCN (Fig. 2A). GRP injection also induced *mPer1* and *mPer2* in the PVN, the periventricular nuclei, and cerebral cortex, but not in the supraoptic nuclei (data not shown).

As shown in Fig. 2, a number of c-Fos protein-positive cells in wild-type mice increased in the SCN after GRP injection at projected ZT16 on 2 day after releasing into DD condition. In contrast, very scattered c-Fos-positive cells were observed in the SCN of saline-treated wild-type mice or GRP receptor-deficient mice administered GRP (15 nmol). GRP-induced expression of c-Fos protein in wild-type mice was observed substantially in the dorsal portion of the caudal SCN and moderately throughout the entire rostral SCN and the ventral portion of the caudal SCN. GRP administration also increased c-Fos expression in the ependymal cells surrounding the third ventricle, whereas this induction was moderately observed in GRP-treated GRP receptor-deficient mice and wild-type mice injected with saline, but not in wild, intact animals (no injection with saline nor GRP), indicating the nonspecific actions of i.c.v. injection itself but not of GRP injection.

Topographical Expression of GRP Receptor in the SCN. Because GRP-elicited induction of *mPer1* and *mPer2* mRNA and c-Fos protein in the SCN was limited to the dorsal subpopulation of the SCN, we next investigated the topographical characteristics of the expression of GRP receptor mRNA in the SCN. Quantitative in situ hybridization analysis revealed a substantial expression of GRP receptor mRNA in the SCN during the night time (ZT16) compared with that in the PVN [SCN, 185.84 ± 13.85 (nCi/g) ($n = 3$); PVN, 75.92 ± 1.77 (nCi/g) ($n = 3$)]. Emulsion autoradiograms showed that the GRP receptor was expressed abundantly in the dorsal portion of the caudal SCN and moderately in the rostral SCN and ventral portion of the caudal SCN (Fig. 3). This distribution pattern was highly consistent with the topographical features of GRP-elicited *mPer* mRNA and c-Fos protein expression in the SCN (Fig. 2A).

Light Pulse-Induced Phase Shifts of Activity Rhythm in GRP Receptor-Deficient Mice. To clarify the involvement of GRP and its receptor in the photic resetting mechanism of the circadian clock, the phase changes of locomotor activity in response to brief light pulse were compared between wild-type and GRP receptor-deficient mice under DD conditions. In wild-type mice, 15-min light pulse at CT16 caused an apparent phase delay in activity in a light intensity-dependent manner (Fig. 4). In contrast, the degree of phase shifting by bright light pulse (300 lux) was significantly attenuated in GRP receptor-deficient mice, although we could not find any significant difference in phase delays by low intensity (30 lux) of light pulse between wild-type and GRP receptor-deficient mice. There was no difference in the degree of phase delay between low- (30 lux) and high (300 lux)-intensity light pulse in GRP receptor-deficient mice (Fig. 4).

Light Pulse-Induced Expression of mPer mRNA and c-Fos Protein in the SCN of GRP Receptor-Deficient Mice. We measured the amount of *mPer* mRNA in the SCN 90 min after light pulse onset, because previous work demonstrated that the peak time of both *mPer1* and *mPer2* induction occurred approximately 90 min after photic stimula-

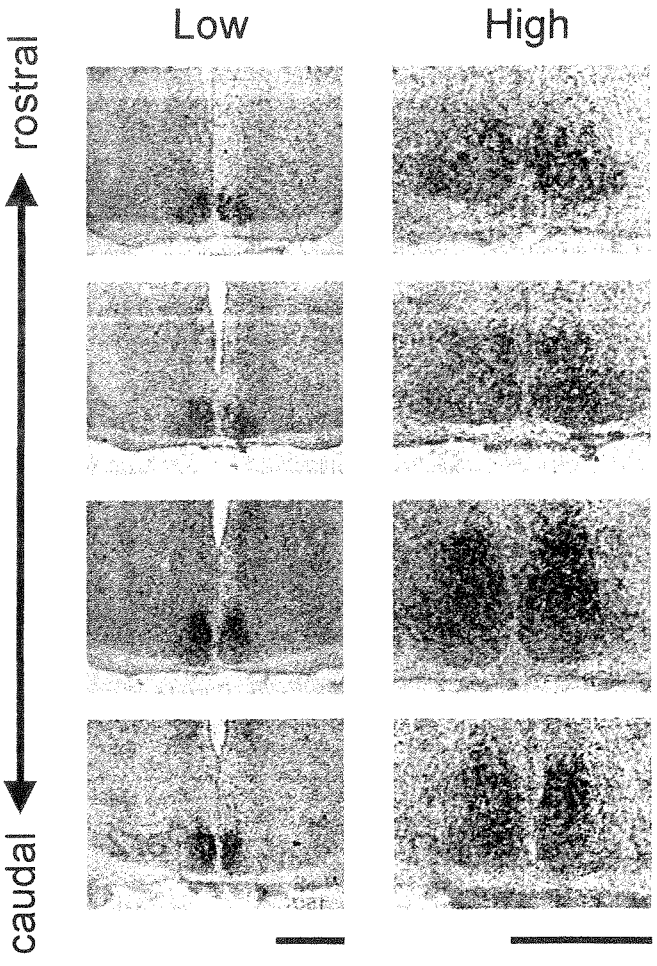

Fig. 3. Topographical analysis for GRP receptor expression in the SCN. Low-power (left) and high-power (right) emulsion autoradiograms showing the rostro-caudal distribution of GRP receptor mRNA in the SCN. C57BL/6J mice were perfused intracardially with 4% PFA at ZT16. Note that the GRP receptor signal expressed by silver grain was especially restricted to the dorsal portion of the caudal SCN. Scale bars (in both low- and high-power autoradiograms), 0.5 mm.

tion (Shigeyoshi et al., 1997). Substantial induction of *mPer1* and *mPer2* mRNA was observed in the SCN of both wild-type and GRP receptor-deficient mice receiving a brief light pulse (300 lux) for 15 min at projected ZT16 on 2 day after releasing into DD condition (Fig. 5A). Quantitative analysis in the entire SCN revealed that photic induction was significantly (*mPer2*; $p < 0.05$) and partially but insignificantly (*mPer1*; $p > 0.05$) diminished in GRP receptor-deficient mice (Fig. 5B). In emulsion autoradiograms, a reduced photic induction of both *mPer1* and *mPer2* in GRP receptor-deficient mice was observed relatively in dorsal area of the caudal SCN but not in rostral SCN and the ventral halves of the caudal SCN (Fig. 5A). Furthermore, a semiquantitative analysis using emulsion autoradiograms revealed that the diminishment in photic induction of *mPer1* and *mPer2* mRNA in GRP receptor-deficient mice was apparent in the dorsal portion in comparison with the ventral SCN (Fig. 6). Figure 5 also shows the distribution of c-Fos-positive cells in the SCN 90 min after brief light pulse (300 lux) for 15 min at projected ZT16 on 2 day after releasing into DD condition. In wild-type mice, c-Fos-positive cells were abundant in the ventral portion and evident but not abundant in the dorsal SCN. In contrast, c-Fos-positive cells were relatively limited to the ventral portion of the SCN and weakly observed in the dorsal portion of the caudal SCN of GRP receptor-deficient mice (Fig. 6).

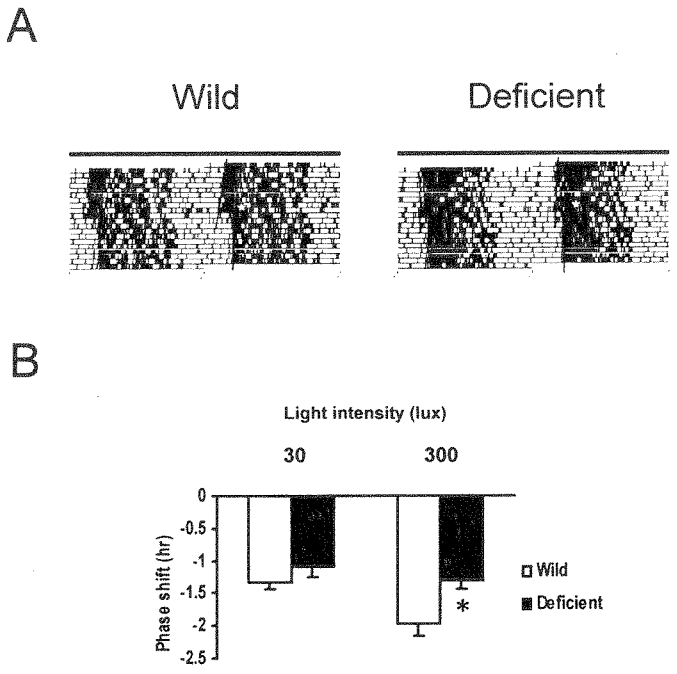

Fig. 4. Attenuated phase shift of locomotor activity rhythm in GRP receptor-deficient mice in response to brief light pulse under DD conditions. A, representative double-plotted actograms demonstrating a phase shift of locomotor activity rhythm in wild-type (Wild) and GRP receptor-deficient mice (Deficient) by bright light pulse (300 lux) at CT16. Each white star indicates the time of light pulse. Mice were maintained under DD conditions for at least 10 days and exposed to a light pulse at CT16. B, quantitative analysis of the degree of phase shifts induced by dim (30 lux) or bright (300 lux) light pulse at CT16 in wild-type (Wild) and GRP receptor-deficient mice (Deficient). The phase shifts were calculated and averaged as described in the legend for Fig. 1. $n = 16$; *, $p < 0.05$ versus wild-type mice (unpaired Student's t test).

Discussion

We previously demonstrated that transient induction of both *mPer1* and *mPer2* serves as a critical step for photic entrainment of the circadian clock in the SCN, because the suppression of *mPer1* or *mPer2* gene expression in the presence of an antisense oligonucleotide inhibits the light- or glutamate-induced phase shift of behavioral rhythm and firing rhythm in the SCN (Akiyama et al., 1999; Wakamatsu et al., 2001). In the present study, we demonstrated that central administration of GRP did elicit *mPer1* mRNA induction, especially in the dorsal SCN, as well as the relative small but significant phase shift in behavioral activity rhythm similar to light-elicited phase shift. These actions of GRP on *mPer1* mRNA and the behavioral rhythm must be caused by the transmitter-receptor interaction between GRP and the GRP receptor, because GRP failed to affect mRNA induction and the phase in behavioral rhythm in GRP receptor-deficient mice in this way. On the other hand, GRP administration also caused a weak but insignificant increase in *mPer2* mRNA. Therefore, the entrainable action of GRP may be mediated via a strong induction of *mPer1*, but not via a weak induction of *mPer2*. Similarly, the adenylate cyclase activator forskolin reportedly elicited an acute induction of *Per1*, but not *Per2*, mRNA to begin the circadian oscillation in the transcription of *Per1*, *Per2*, or an output gene such as *dbp* in cultured fibroblast cells (Yagita and Okamura, 2000). However, we cannot rule out the possibility that GRP-induced expression of *mPer2* was either so slow or fast that we could not detect the actions of GRP on *mPer2* expression at the sampling time point (90 min after injection) used in this study.

We recently reported that NMDA, which elicits a light-type phase shift in vitro (Shibata et al., 1994) and in vivo (Mintz et al., 1999), caused substantial expression of *Per1* and *Per2* mRNA in the SCN of hamsters (Moriya et al., 2000). Furthermore, Nielsen et al. (2001) demonstrated that a low concentration (1 nM) of pituitary adenylate cyclase-activating polypeptide, which caused a phase shift similar to light (Harrington et al., 1999), increased *Per1* and *Per2* mRNA in the SCN in vitro during subjective night. These reports taken together with our findings suggest that neurotransmitters or neuropeptides such as glutamate, pituitary adenylate cyclase-activating polypeptide, or GRP, capable of evoking a light-type phase-resetting, have an inductive action on the *Per* gene in SCN neurons possessing common mechanisms. This action then leads to photic entrainment of the circadian clock.

We also demonstrate that GRP receptor signaling is indeed involved in the photic resetting of the circadian clock because the phase delays elicited by high intensity (300 lux) of light pulse were significantly attenuated in GRP receptor-deficient mice. We could not exactly explain the reason of no difference in phase delays by low intensity (30 lux) of light pulse between wild-type and GRP receptor-deficient mice. Some qualitative differences were reported between low- and high-intensity light in terms of immediate-early gene induction in the SCN (Guido et al., 1999). Furthermore, several reports demonstrated that low-intensity light activated more restricted neurons in the SCN, whereas the activation of widespread neural population in the SCN was observed upon bright light stimulation (Dkhissi-Benyahya et al., 2000).

Taken together with these reports, we speculate that GRP signaling may work only when bright light entrains the circadian clock in the SCN.

Corresponding with attenuation of the light-elicited phase shift of behavior in GRP receptor-deficient mice, the present results demonstrated an attenuated photic induction of *mPer1* and *mPer2*, especially in the dorsal SCN. Therefore, we also propose that GRP and its receptor signaling play a role in light-induced *Per* mRNA expression in the SCN as well as the behavioral phase shift produced by light.

We also found that the expression pattern of c-Fos protein upon photic stimulation or GRP administration correlated well with that of *Per* mRNA in the SCN, suggesting that the induction mechanism of the *Per* and *c-fos* genes by either light stimuli or GRP uses common signaling pathways in part. Furthermore, a behavioral study with an antisense oligonucleotide against the *c-fos* and *jun-B* genes revealed that transcription of these immediate-early genes was required for photic entrainment of the circadian clock (Wollnik et al., 1995). Thus, the resetting action of GRP on the circadian clock may be mediated by the cooperative works of both *Per* and *c-fos* gene induction in SCN neurons.

In contrast to the attenuated responses of activity rhythm or *mPer1* or *mPer2* mRNA induction to light stimulation in GRP receptor-deficient mice, these mutant mice exhibited a stable activity rhythm, which did not differ from that of wild-type mice, under LD or DD conditions. Circadian oscillating nature itself is known to be cell autonomous, because a suppression of synaptic transmission by tetrodotoxin failed to affect the phase of circadian rhythm driven within the SCN neurons (Welsh et al., 1995). Taken together, GRP and its receptor are involved in the photic entrainment pathway but not in the circadian oscillating machinery in the SCN.

As described previously, the SCN consists of two neuronal subpopulations, a light-responsive ventral subpopulation with a weak oscillatory function, and a light-unresponsive dorsal subpopulation with a strong autonomous oscillatory function (Shibata et al., 1984; Yan et al., 1999). In hamsters and mice, a light pulse during subjective night causes an increase in *Per1* and *Per2* mRNA or c-Fos protein substan-

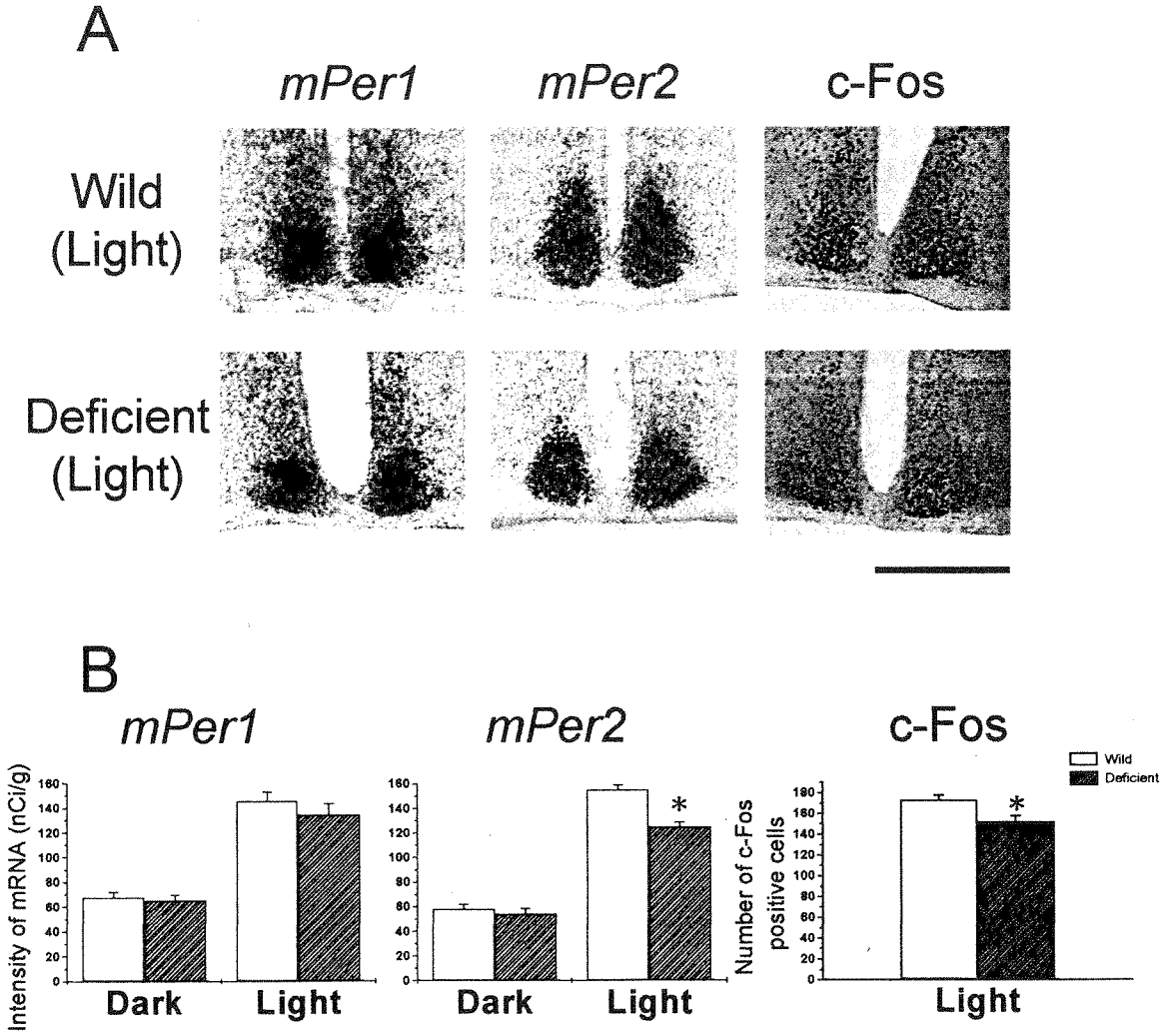

Fig. 5. Light pulse-induced *mPer1*, *mPer2* mRNA, and c-Fos protein expression were partially attenuated in GRP receptor-deficient mice. A, representative emulsion autoradiograms (*mPer1* and *mPer2*) and photographs (c-Fos protein) in wild-type (Wild) and GRP receptor-deficient mice (Deficient). After release into DD for 2 days, mice were exposed to a brief light pulse (300 lux) at projected ZT16 and then perfused intracardially with 4% PFA 90 min later. Note that both *mPer* mRNA and c-Fos protein in the dorsal SCN were diminished in GRP receptor-deficient mice. B, quantitative analysis of *mPer* mRNA and c-Fos protein in the entire SCN with film autoradiograms (*mPer1* and *mPer2* mRNA) or the immunohistochemical photographs (c-Fos protein). $n = 4$ to 9; *, $p < 0.05$ versus wild-type mice (unpaired Student's t test).

tially in the ventral subdivision, whereas the remaining dorsal SCN neurons are only moderately responsive to the light stimulus (Shigeyoshi et al., 1997; Moriya et al., 2000). We have recently shown that activation of the NMDA receptor is involved in the photic induction of *Per1* and *Per2* in the ventral portion of the hamster SCN. This involvement is supported by the finding that NMDA receptor blockade substantially suppressed the photic induction of *Per1* and *Per2* mRNA in the ventral portion of the SCN, but not in the dorsal subpopulation (Moriya et al., 2000). Thus, light signals entrain the ventral neurons in the SCN via NMDA receptor activation, which leads to acute induction of the *Per1* and *Per2* genes. On the other hand, GRP induces *Per* mRNA as well as c-Fos protein in the dorsal portion of the SCN. It should be noted that the degree of phase shift elicited by GRP is small (less than 0.5 h) by comparison with that induced by light (in the present study) or by other receptor agonists such as melatonin (Benloucif and Dubocovich, 1996), serotonin agonist (Tominaga et al., 1992), and neuropeptide Y (Albers and Ferris, 1984) (usually 0.6–2.0 h). It may account for this relative small phase shift by GRP that exogenous GRP activates neurons located only in the dorsal, but not ventral SCN and that endogenous GRP would mediate some portion of the photic signal from the ventral SCN to the dorsal SCN. We also demonstrated that photic induction of *mPer1*, *mPer2* mRNA, and c-Fos protein was attenuated, especially in the dorsal SCN, in GRP receptor-deficient mice. Furthermore, cell somata and fibers of GRP-positive neurons were abundantly expressed in the ventral and dorsal SCN, respectively. Therefore, this finding, taken together with our previous report (Moriya et al., 2000), suggests that the photic induction of *Per1* and *Per2* mRNA in the ventral SCN is mediated via the glutamate-NMDA receptor pathway and photic induction in the dorsal SCN relates to activation of the GRP receptor pathway, which is secondarily cascaded by RHT activation via a multisynaptic transmission within the SCN (Jiang et al., 1997). The present observation of the abundant expression of GRP receptor mRNA in the dorsal SCN strongly supports the above-mentioned working hypothesis.

In summary, our pharmacological analysis using GRP receptor-deficient mice elucidated that GRP and its receptor activation are certainly involved in the photic entrainment of the circadian clock, especially in the dorsal subpopulation of the SCN. This action is mediated in the SCN neurons via the induction of *Per* and *c-fos*.

Acknowledgments

We thank Dr. H. Okamura (Kobe University, Kobe, Japan) for kindly donating *mPer1* and *mPer2* probes for the in situ hybridization.

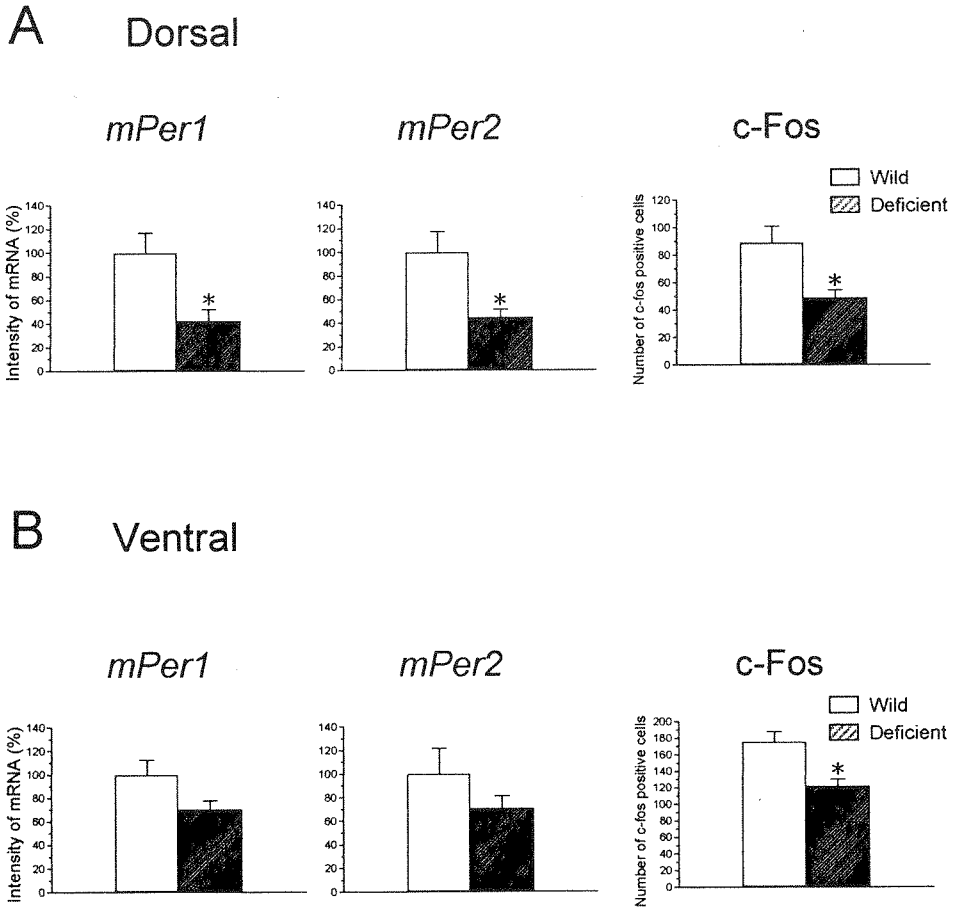

Fig. 6. Light pulse-induced *mPer1*, *mPer2* mRNA, and c-Fos protein expression were attenuated especially in the dorsal SCN of GRP receptor-deficient mice. Quantitative analysis of *mPer* mRNA and c-Fos protein in the dorsal (A) or ventral (B) portion of the caudal SCN. Emulsion autoradiograms (*mPer1* and *mPer2* mRNA) or the immunohistochemical photographs (c-Fos protein), as typical ones were shown in Fig. 5A, were quantitatively analyzed separately in the dorsal and ventral SCN as described under *Experimental Procedures*. $n = 4$ to 9; *, $p < 0.05$ versus wild-type mice (unpaired Student's t test).

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PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE PRODUCES A PHASE SHIFT ASSOCIATED WITH INDUCTION OF *mPer* EXPRESSION IN THE MOUSE SUPRACHIASMATIC NUCLEUS

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Abstract—The main mammalian circadian pacemaker is located in the suprachiasmatic nucleus of the hypothalamus. Clock genes such as the mouse *Period* gene (*mPer*) play a role in this core clock mechanism in the mouse. With brief light exposure during the subjective night, the photic information, which is conveyed directly to the suprachiasmatic nucleus via the retinohypothalamic tract, results in *mPer1* and *mPer2* expression in the suprachiasmatic nucleus.

Glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) are co-stored in the retinohypothalamic tract. Recent studies have suggested that not only glutamate but also PACAP are key players in the phase shift that occurs during subject night; however, research demonstrating a direct association between the PACAP-induced phase shift and *mPer* gene expression has yet to be conducted.

In the present study, PACAP (200 pmol) injected into the lateral ventricle during subjective night (circadian time 16; circadian time 12, onset of locomotor activity) caused a moderate phase delay associated with moderate expression of *mPer1* and only slight expression of *mPer2* in the mouse suprachiasmatic nucleus. PACAP-induced *mPer1* expression was also observed in the paraventricular nucleus and periventricular area of the hypothalamus. (+)MK-801 (0.5 mg/kg), an *N*-methyl-D-aspartate (NMDA) receptor antagonist, suppressed both the PACAP-induced phase delay and *mPer1* expression. From these results we suggest that PACAP induces phase delays in the mouse circadian rhythm in association with an increase of *mPer* expression in the suprachiasmatic nucleus via the activation of NMDA receptors. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: circadian rhythm, entrainment, i.c.v. injection, NMDA receptor, (+)MK-801.

The main mammalian circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Rusak and Zucker, 1979; Ralph et al., 1990) is entrained to the 24 h light–dark cycle. The core clock mechanism in the mouse SCN appears to involve a transcriptional feedback loop in which CLOCK and BMAL1 function as positive regulators and the three mouse *Period* genes (*mPer*) and *Cry1* and *Cry2* gene products are involved in negative feedback (Reppert and Weaver, 2001). We found that brief exposure to light during the subjective night resulted in a large and rapid induction of *mPer1* expression corresponding with light-induced phase delays of mouse behavioral rhythms (Shigeyoshi et al., 1997) and *mPer2* mRNA expression in the SCN (Shearman et al., 1997; Takumi et al., 1998). We recently demonstrated that a light-induced phase delay in locomotor activity at circadian time (CT) 16 is significantly inhibited when mice are pretreated with *mPer1* or *mPer2* antisense oligonucleotide 1 h before light exposure (Akiyama et al., 1999; Wakamatsu et al., 2001). Therefore, gating expression of the *mPer1* and *mPer2* genes is suggested as an important step in photic entrainment.

Recently, presence of the 38 amino acid neuropeptide pituitary adenylate cyclase-activating polypeptide-38 (PACAP) was demonstrated in the rat retinohypothalamic tract (RHT) (Hannibal et al., 1997, 2000). *In situ* hybridization studies have also demonstrated that mRNA of the PAC1 receptor for PACAP is expressed in the rat SCN (Hannibal et al., 1997; Cagampang et al., 1998a). Initial *in vitro* studies showed that application of PACAP reset the phase of the rodent SCN pacemaker in a manner opposite of the phase-resetting effects of light (Hannibal et al., 1997; Harrington and Hoque, 1997). These studies characterize the actions of PACAP on the rat and hamster SCN *in vitro*. More recent studies demonstrated that microinjection of PACAP into the SCN region affected hamster behavioral rhythms (Piggins et al., 2001). When administered during mid-subjective day, PACAP evokes large but transient

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Abbreviations: CaMKII/IV, Ca^{2+}/calmodulin-dependent protein kinase II/IV; CRE, cAMP response element; CREB, CRE binding protein; CRH, corticotropin-releasing hormone; CT, circadian time; DD, constant darkness; (+)MK-801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5-H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen maleate; *mPer*, mouse *Period*; NMDA, *N*-methyl-D-aspartate; PACAP, pituitary adenylate cyclase-activating polypeptide-38; Pe, periventricular area of hypothalamus; PKA, protein kinase A; PVN, paraventricular nucleus of hypothalamus; RHT, retinohypothalamic tract; SCN, suprachiasmatic nucleus; ZT, zeitgeber time.

phase advances (~60 min), followed by small, steady-state phase delays (Piggins et al., 2001). During the early subjective night, PACAP elicits small to moderate phase delays without any detectable concentration dependence.

Glutamate, thought to be the main neurotransmitter of the RHT, and glutamate receptors have been shown to play a critical role in mediating the effects of light in the rodent circadian pacemaker (Abe and Rusak, 1994; Colwell and Menaker, 1996; Ebling, 1996). As interestingly, glutamate and PACAP are colocalized in the RHT terminals (Hannibal et al., 1997, 2000). More recent studies reported that PACAP modulated the nocturnal resetting actions of glutamate in the rodent SCN in a complex dose- and phase-dependent manner (Chen et al., 1999; Harrington et al., 1999). Although PACAP application *in vitro* reportedly caused *mPer1* and *mPer2* expression in the rat SCN (Nielsen et al., 2001), this paper did not evaluate whether PACAP-induced *mPer* expression was related to the PACAP-induced phase shift. Therefore, in the present study we evaluated the association between the behavioral phase shift and *Per* gene expression in the SCN through *in vivo* PACAP application. Recently, we demonstrated that *N*-methyl-D-aspartate (NMDA) receptor antagonists such as (+)MK-801 and DL-2-amino-5-phosphonovaleric acid (APV) block light-induced behavioral phase shifts, as well as *Per1* and *Per2* but not *Per3* expression in the hamster SCN (Moriya et al., 2000). Since, the activation of NMDA receptors seems to underlie light-induced *mPer1* and *mPer2* gene expression in the SCN, we further examined whether this same activation underlies the PACAP-induced *mPer1* expression and phase shift.

EXPERIMENTAL PROCEDURES

Animals

Male *balb/c* mice (Takasugi, Saitama, Japan) were purchased 3–6 weeks postpartum and maintained under a 12 h light/dark cycle. Food and water were given *ad libitum*. Experimental animal care was conducted under the permission of the 'Experimental Animal Welfare Committee in the School of Human Sciences of Waseda University' (Permission #00-17).

Surgery

Mice were deeply anesthetized with ketamine [50 mg/kg intraperitoneally (i.p.)] and xylazine (20 mg/kg i.p.) and stainless steel guide cannulae (6 mm, 22 gauge) were implanted in the intralateral ventricle (−0.5 mm anterior to bregma, 1.1 mm lateral to midline, and 1.7 mm ventral to skull surface) using a stereotaxic frame (Narishige, Tokyo, Japan).

Sample preparation

To examine the effect of PACAP administration, mice were moved to constant darkness (DD) conditions for 2 days, and a 5 μl aliquot of PACAP or vehicle (sterilized saline) was injected into the lateral ventricle via an injection cannula at a rate of 1 μl per min using a 25 μl Hamilton syringe under ether anesthesia. We adopted a 5 μl volume for injection because peptides and oligonucleotides in this volume easily reached the SCN in our previous studies (Akiyama et al., 1999; Aida et al., 2002). To examine the interaction between PACAP and the glutamate receptor, some mice were i.p. injected with (+)MK-801, a non-competitive antagonist of the NMDA type glutamate receptor, with or without intracerebroventricular (i.c.v.) injection of PACAP. Injections were performed at either projected zeitgeber time 8 (ZT8; ZT12, lights-off time) or ZT16. Ninety minutes after injection, mice were deeply anesthetized with ether and perfused intracardially with ice-cold saline and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Brains were removed and post-fixed for 24 h at 4°C, then placed into 0.1 M phosphate buffer with 20% sucrose for 72 h at 4°C. Brain sections (40 μm thick) including the SCN, periventricular area of hypothalamus (Pe) or paraventricular nucleus of hypothalamus (PVN) were made using a cryostat (HM505E; Microm, Walldorf, Germany) and placed in 2× saline sodium citrate (SSC) (16.7 mM NaCl, 16.7 mM $C_6H_5O_7Na_3$, pH 7.0) until processing for hybridization.

In situ hybridization using radio isotope-labeled cRNA probe

The quantity of *mPer1* or *mPer2* mRNA expression in the SCN, PVN, or Pe was assessed by *in situ* hybridization as previously described (Takumi et al., 1998, Takahashi et al., 2001). Restriction enzyme-linearized cDNA templates [nucleotide positions: *mPer1* (538–1752), *mPer2* (1–638)] were kindly provided by Dr. Okamura (Kobe University, Kobe, Japan). For data analysis, we subtracted the intensities of the optical density in sections from the corpus callosum from those in the SCN, PVN, and Pe, and regarded this value as the net intensity for these areas. The intensity values of sections from the rostral to the caudal part of the SCN, PVN, and Pe (three sections per mouse brain) were then averaged. This averaged value was considered to be a measure of the amount of *mPer1* or *mPer2* mRNA in these regions.

For emulsion autoradiography, all mounted sections were dipped into emulsion (NTB2, Kodak, Rochester, NY, USA) after X-ray film exposure, air dried for 3 h, and stored in light-tight slide boxes at 4°C for 4 weeks. The slides were developed with a D19 developer (Eastman Kodak), then fixed with Fujifix (Fuji film, Tokyo, Japan) and counterstained with Cresyl Violet. Subnuclear silver grain distribution in the SCN of all sections was examined using an optical microscope.

Locomotor activity rhythm

Mice were housed individually in transparent plastic cages (31×20 ×13 cm) under DD conditions for at least 2 weeks and their locomotor activity was measured by area sensor (F5B; Omron, Tokyo, Japan) with a thermal radiation detector system. Locomotor activity was continuously recorded in 6 min epochs by personal computer. PACAP was injected i.c.v. at CT16 (CT12 is defined as onset time for locomotor activity). (+)MK-801 was administered i.p. 30 min before the injection of PACAP. To formulate comparisons on the effect of PACAP, some mice were exposed to a light pulse (70 or 300 lux) for 5 min at CT16. To evaluate the interaction between PACAP- and light-induced phase shifts, PACAP was injected at CT16 and then a light pulse (70 lux for 5 min) was given 15 min after PACAP injection.

Phase shift was calculated based on the distance between two regression lines drawn from daily onset of locomotor activity for at least 10 days before and after drug injection or light pulse. Uninformed observers drew the two regression lines.

Drugs

PACAP and (+)MK-801 were obtained from the Peptide Institute (Osaka, Japan) and Sigma-RBI (St. Louis, MO, USA), respectively. We used 75 and 200 pmol of PACAP for both behavioral and gene expression experiments.

Statistics

Results are expressed as the mean ± S.E.M. The significance of the differences between groups was determined by the Stu-

dent's *t*-test or a one-way analysis of variance following the Tukey–Kramer test using analysis software (StatView for Windows version 5.0, SAS Institute Inc., Cazy, NC, USA).

RESULTS

Effect of PACAP on mPer1 and mPer2 mRNA expression in the SCN

We studied the effect of PACAP on *mPer1* expression in the SCN using *in situ* hybridization (Fig. 1). When PACAP (200 pmol) was injected i.c.v. at ZT16, there was an obvious increase in *mPer1* mRNA expression in the SCN (260% increase comparing to vehicle treatment, $P < 0.01$). On the other hand, PACAP (200 pmol) injected i.c.v. at ZT8 did not affect *mPer1* mRNA expression in the SCN. Although PACAP injection at ZT16 induced *mPer2* mRNA expression slightly (130% increase comparing to vehicle treatment), the difference between vehicle- and PACAP-injected groups at ZT16 was not significant ($P = 0.068$, Student's *t*-test). Emulsion autoradiography demonstrated that PACAP induced *mPer1* but not *mPer2* expression in the central to dorsal

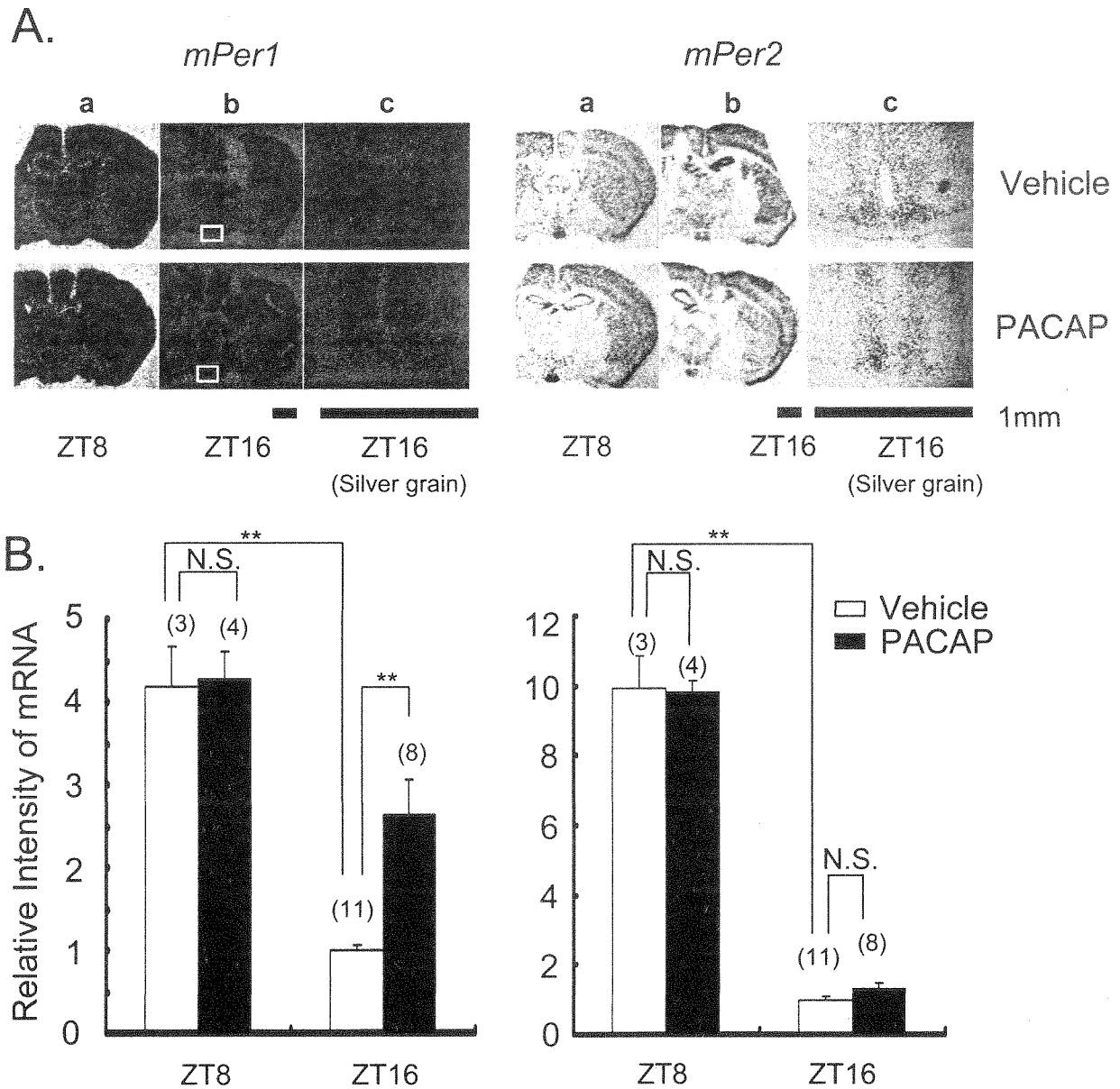

Fig. 1. PACAP-induced *mPer1* mRNA expression during early subjective night. (A) Representative *mPer1* mRNA (left panels) or *mPer2* mRNA (right panels) autoradiograms showing PACAP injection at ZT8 or at ZT16. (c) Enlargement of rectangular area of (b). Scale bars = 1 mm. (B) Quantitative data of PACAP influence on *mPer1* mRNA and *mPer2* mRNA levels in the SCN. White columns, vehicle; gray columns, PACAP injection. Value for vehicle injection at ZT16 was set to 1. $^{**}P < 0.01$ vs. vehicle injection at ZT16 (Tukey–Kramer test). N.S., no significance.

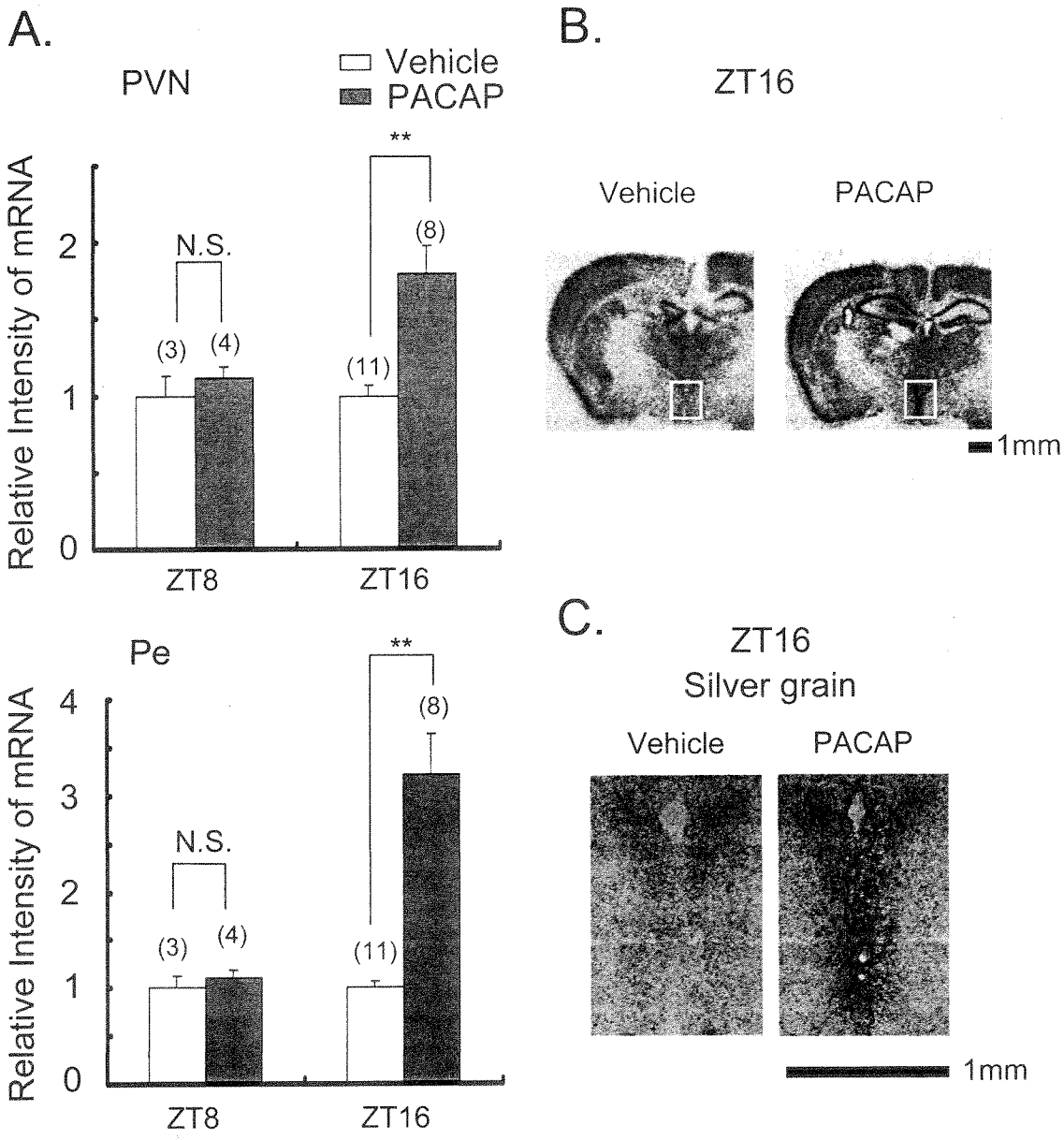

Fig. 2. PACAP-induced *mPer1* mRNA expression in the PVN and Pe. (A) Quantitative data of PACAP influence on *mPer1* mRNA level in the PVN and Pe. Values for vehicle injection at ZT8 and ZT16 are set to 1, respectively. White columns, vehicle; gray columns, PACAP injection. $^{**}P < 0.01$ vs. vehicle injection at ZT16 (Student's *t*-test). (B) Representative autoradiograms showing that PACAP injection at ZT16 strongly increased *mPer1* mRNA in the PVN and Pe. (C) Enlargement of rectangular area in (B).

area of the SCN (Fig. 1A,c). Similar to *mPer1* expression, PACAP injection at ZT8 did not affect *mPer2* mRNA expression (Fig. 1B).

PACAP injection at ZT16 also induced *mPer1* mRNA expression in another hypothalamic region, such as the Pe and PVN (Fig. 2B,C). PACAP increased *mPer1* mRNA expression in the PVN about 180% comparing to vehicle treatment and 320% in the Pe ($P < 0.01$, Fig. 2A). Emulsion autoradiography demonstrated that PACAP induced *mPer1* expression in the PVN and Pe (Fig. 2C). Similar to findings in the SCN, PACAP (200 pmol) injected i.c.v. at ZT8 did not affect *mPer1* mRNA expression in the PVN or Pe.

Differences in PACAP- and light-induced mPer expression in the SCN and phase delay in locomotor activity rhythm

We assessed the relationship between PACAP-induced *mPer1* expression and the phase delays that occurred when PACAP was injected at ZT16 or CT16 (Fig. 3). PACAP induced phase delays corresponding with *mPer1* mRNA expression in a dose-dependent manner but not with *mPer2*. When PACAP (200 pmol) was injected i.c.v. at CT16, both *mPer1* expression ($P < 0.01$) and the phase delays ($P < 0.01$) changed significantly (Fig. 3B, left panel). On the other hand, there was no

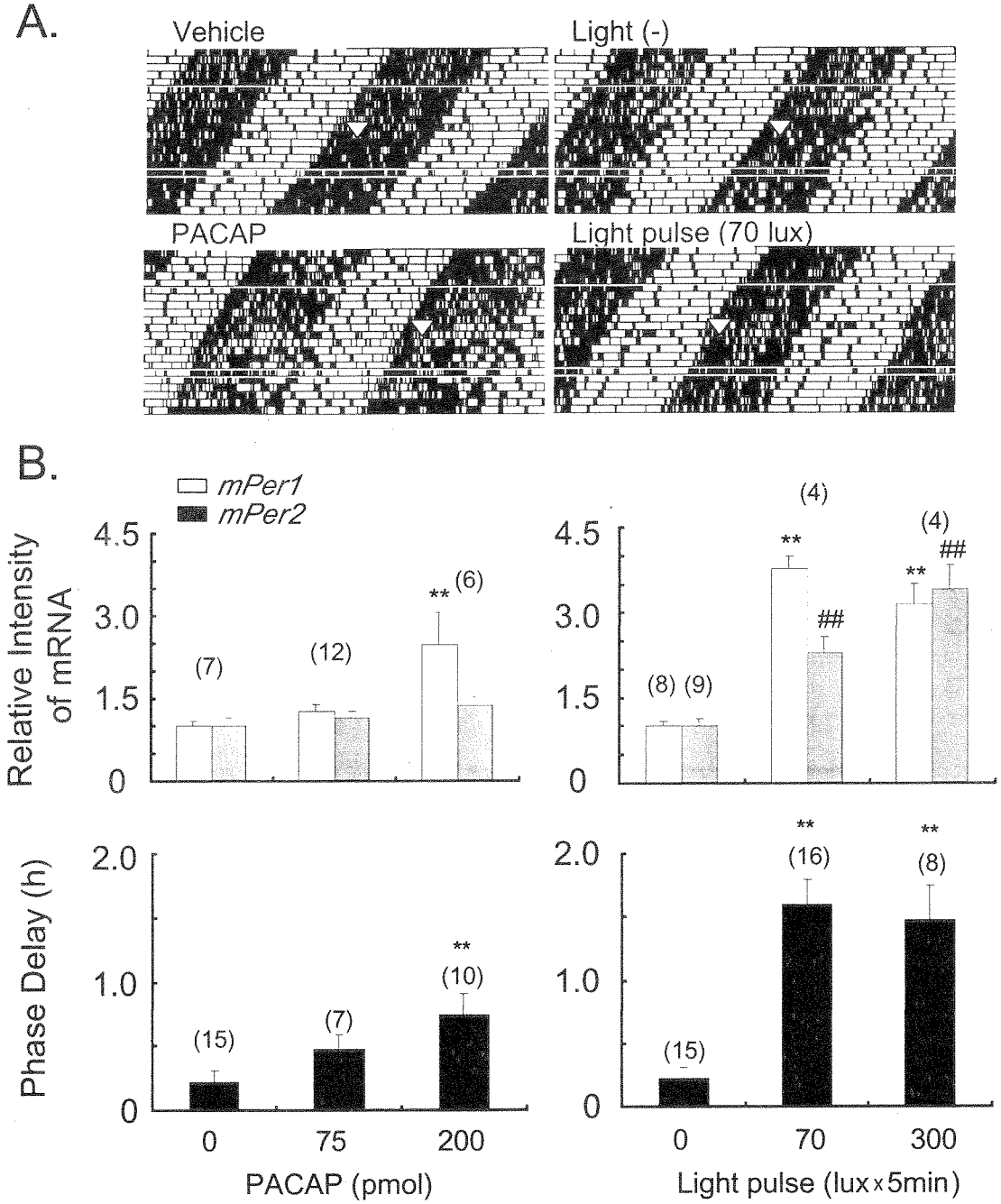

Fig. 3. PACAP- or light-induced phase delay of circadian locomotor activity in mice. (A) Representative double plot actograms of PACAP-induced (left panel) or light-induced phase delays (right panel). White arrowheads show the day of injection or light pulse. (B) Relationship between *mPer1* or *mPer2* mRNA expression and phase delays. mRNA value of vehicle injection or non-light treatment was set to 1. White columns, amount of *mPer1*; gray columns, amount of *mPer2* mRNA expression (upper panels); dark column, phase delay (lower panels). $^{**}P < 0.01$ vs. value of vehicle injection or light (−). $^{\#\#}P < 0.01$ vs. value of vehicle injection or light (−) (Tukey–Kramer test).

significant change in phase with PACAP (200 pmol) injection at CT8 (data not shown).

Light exposure (70 lux for 5 min) was of enough intensity to cause maximal phase delays ($P < 0.01$) and *mPer1* expression ($P < 0.01$) in the SCN (Fig. 3B, right panel). Interestingly, *mPer2* expression was also induced in the SCN in a light intensity-dependent manner (70 and 300 lux for 5 min, $P < 0.01$) (Fig. 3B, right panel). Comparatively speaking, an association was found between light-induced phase delays and *mPer* expression.

Involvement of NMDA receptors in PACAP-induced mPer1 expression and phase delays

PACAP-induced *mPer1* mRNA expression in the SCN was blocked by i.p. administration of (+)MK-801

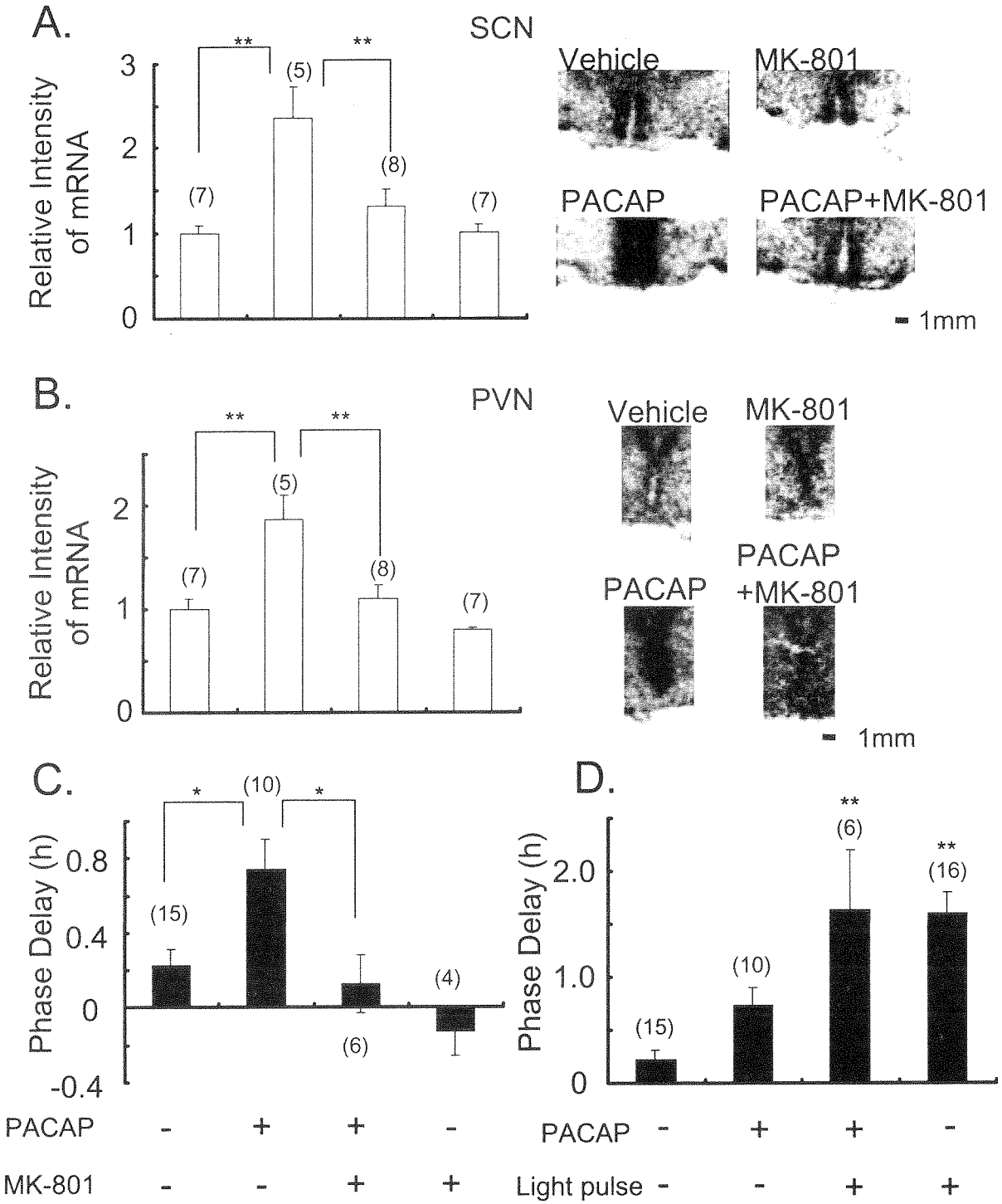

Fig. 4. Effect of (+)MK-801 and light exposure on PACAP-induced *mPer1* mRNA and phase delays. (A) Effect of (+)MK-801 on PACAP-induced *mPer1* expression in the SCN. (+)MK-801 (0.5 mg/kg) was administered i.p. 30 min before PACAP (200 pmol) injection. Value for vehicle injection at ZT16 was set to 1. Representative autoradiograms show blocking effect of (+)MK-801 on PACAP-induced *mPer1* mRNA expression in the SCN. (B) Effect of (+)MK-801 on PACAP-induced *mPer1* expression in the PVN. Value for vehicle injection at ZT16 was set to 1. Representative autoradiograms show similar blocking effect of (+)MK-801 on *mPer1* mRNA in the PVN. (C) Effect of (+)MK-801 on PACAP-induced phase delays in circadian activity rhythm. (D) Effect of light on PACAP-induced phase delays in circadian activity rhythm. PACAP (200 pmol) was injected at CT16 and light pulse was given 15 min after the injection. $*P < 0.05$, $**P < 0.01$ vs. value of PACAP injection (Tukey–Kramer test).

(0.5 mg/kg) 30 min before PACAP (200 pmol) injection ($P<0.01$, PACAP injection vs. (+)MK-801 and PACAP administration, Fig. 4A). In addition, PACAP-induced *mPer1* mRNA in the PVN ($P<0.01$, PACAP injection vs. (+)MK-801 and PACAP administration, Fig. 4D) and Pe (Fig. 4D and data not shown) was significantly attenuated by (+)MK-801.

Behavioral phase delays by PACAP were also significantly blocked when co-administrated with (+)MK-801 (0.5 mg/kg) (0.74 h for PACAP alone vs. 0.12 h for (+)MK-801 and PACAP, $P<0.05$) (Fig. 4C). (+)MK-801 administration alone had no effect on *mPer1* mRNA expression or the phase of circadian activity. Previous papers demonstrated that PACAP and 8-bromo-cAMP potentiated glutamate-induced phase delays of circadian SCN neuronal rhythm *in vitro* (Chen et al., 1999; Tischkau et al., 2000). To confirm the results of these two papers *in vivo*, we examined the effect of PACAP on light-induced phase delays in mouse circadian activity rhythm and found that PACAP failed to augment the delays (Fig. 4D). A non-saturating light pulse combined with PACAP was utilized to determine if the two stimuli had an additive effect, but such an effect was not observed even when light intensity was set at 10 lux [light only, 0.82 ± 0.06 h ($n=4$); PACAP+light, 0.77 ± 0.12 h ($n=4$), $P>0.05$].

DISCUSSION

In the present experiments, we elucidated that i.c.v. administration of PACAP in the mouse brain causes moderate phase delays in behavioral rhythm that are accompanied by the induction of *mPer1* and *mPer2* in the SCN when applied during early subjective night under DD conditions. Our finding that PACAP phase-delays mouse circadian rhythms in the early subjective night (CT16) coincides with previous results showing that PACAP causes phase delays at this phase in hamsters both *in vivo* and in SCN sections *in vitro* (Harrington et al., 1999). Nielsen et al. reported that PACAP induces *Per1* and *Per2* expression in the rat SCN late night *in vitro* (Nielsen et al., 2001). Thus, PACAP can induce *Per1* and *Per2* in the SCN both *in vivo* and *in vitro* late and early subjective night. We found that PACAP has a dose-dependent (75 pmol and 200 pmol) effect on both behavior and *mPer1* expression, while Piggins et al. noted no obvious concentration dependency of the PACAP-induced phase delay in hamster wheel-running rhythm (Piggins et al., 2001). Harrington et al. found that only a low concentration of PACAP (0.001 μM *in vitro* and *in vivo*) delays SCN firing rate and activity rhythms in hamsters during early subjective night (Harrington et al., 1999). Higher concentrations (0.1–1 μM) of PACAP are reportedly ineffective at phase shifting the hamster SCN *in vitro* during the subjective night. The apparent discrepancies among these studies may be attributable to the different experimental conditions used, thereby making it difficult to formulate direct comparisons. For example, our experiments were conducted in mice using a high concentration of PACAP while Piggins et al. and Harrington et al. utilized hamsters and low concentrations. On the other hand, our finding that PACAP may be released from RHT terminals during the early subjective night and contribute to the phase-delaying effect of light at this phase is consistent with the results of both studies.

In the present experiment, PACAP at any concentration did not affect the phase of mouse free-running rhythm, *mPer1*, or *mPer2* expression in the SCN when administered during the day (CT8 or ZT8). This result agrees with recent behavioral results (Piggins et al., 2001). *In vitro* studies of the SCN, however, showed that application of 1 μM PACAP during this phase evokes large phase advances in neuronal firing rate rhythms of ~3.5 h in rats (Hannibal et al., 1997) and ~3.2 h in hamsters (Harrington and Hoque, 1997). These conflicting results may reflect differences between the *in vivo* and *in vitro* test situations. Anyway we need to attempt to estimate actual tissue concentrations of injected PACAP. Alternatively, the removal of afferent fibers in SCN sections may up-regulate PACAP receptor expression or cause other changes that alter the sensitivity of the SCN to the effects of PACAP.

The present results demonstrate that PACAP induces moderate expression of *mPer1* and slight expression of *mPer2* at ZT16 in the SCN *in vivo*, whereas light application causes strong phase delays corresponding with strongly expressed *mPer1* and *mPer2* in the SCN. A recent report by Albrecht et al. denotes the importance of light-induced *mPer2* expression in causing phase delays during the early night in *mPer1* and *mPer2* mutant mice (Albrecht et al., 2001). Based on this report, we suppose that the slight expression of *mPer2* ($P=0.068$ vs. vehicle, Student's *t*-test) in the SCN reflects weak phase delays produced by PACAP injection. A recent paper by Hannibal et al. (2001) suggests dissociation between the light-induced phase shift of the circadian rhythm and *mPer1* and *mPer2* gene expression in mice lacking the PACAP1 receptor. Because of the small phase delay produced by PACAP in the present results, we decided to further investigate the effect of weak light intensity (10 lux) on phase delays and *mPer* gene expression in the SCN. We found that weak light intensity causes a small phase delay similar to PACAP injection with a significant increase of *mPer1* and *mPer2* gene expression (data not shown). All together, these findings indicate PACAP-induced *mPer* gene expression in the SCN and phase shift are not always interdependent phenomena. In this experiment, gene expression was measured after 2 days in DD, while phase shifts were measured after 2 weeks in DD. As Mistlberger and Holmes (2000) reported a change in circadian responsiveness to light over time under DD conditions, the directness of the comparison of phase shifts with gene expression is weakened. We previously reported that after 1 week in DD, light-induced *mPer* gene expression in the SCN slightly increased (Aida et al., 2002). Therefore, further experiment is required to examine both phenomena using the same experimental protocol.

The mechanisms underlying these phase-shifting effects of PACAP on the mouse SCN circadian pacemaker are

not known. We would like to propose a tentative mechanism regarding *mPer1* expression in the SCN, PVN, and Pe. Yagita and Okamura (2000) showed that forskolin-induced *Per1* expression in Rat-1 cells accompanies cAMP response element (CRE) binding protein (CREB) phosphorylation. Recently we reported that addition of PACAP to the medium also induces transient *Per1* gene expression in cerebellar culture cells (Akiyama et al., 2001). This induction is also mimicked by dibutyryl-cAMP and suppressed by a protein kinase A (PKA) inhibitor but not by MEK inhibitors (Akiyama et al., 2001). Both PACAP and forskolin activate adenylate cyclase, enhance the synthesis of cAMP, and result in the activation of PKA. In addition to PKA activation, PACAP enhances Ca^{2+} entry into the supraoptic nucleus neurons via voltage-gated Ca^{2+} channels (Shibuya et al., 1998). Increased Ca^{2+} then activates Ca^{2+}-dependent protein kinases such as Ca^{2+}/calmodulin-dependent protein kinase II/IV (CaMKII/IV) and mitogen-activated protein kinase (MAPK). The transcription factor CREB specifically binds to CREs and activates the transcription of target genes when phosphorylated by PKA (Haus-Seuffert and Meisterernst, 2000). Interestingly, it is reported that the promoter of the *mPer1* gene contains four CREs (Yamaguchi et al., 2000). Thus, PACAP-induced activation of *mPer1* in the SCN may be involved in PKA and/or CaMKII/IV activation followed by CREB phosphorylation, thereby playing a role in signal transduction.

Although previously unanticipated, i.c.v. injection of PACAP dose-dependently caused an elevation of *mPer1* gene expression in the Pe and PVN of the mouse. It is well known that the distribution of PACAP immunoreactivity is detected not only in the SCN but also in the Pe area and PVN of the hypothalamus (Piggins et al., 1996). PACAP injection produces an increase in the hybridization signal of arginine vasopressin mRNA in the PVN (Nomura et al., 1999). Legradi et al. (1998) identified innervation of PVN corticotropin-releasing hormone (CRH) neurons by PACAP nerve fibers. In addition, Grinevich et al. (1997) found that PACAP i.c.v. injection results in the up-regulation of CRH gene expression in the PVN. Although the physiological roles of *mPer1* gene expression in this area are still unknown, we recently demonstrated that physical and inflammatory stressors to mice caused an increase of *mPer1* gene expression in the CRH-positive neurons in the PVN (Takahashi et al., 2001). Further experiment may elucidate whether the activation of PACAPergic neurons is involved in stress-induced *Per* gene expression in the PVN. We recently reported that exposure to light at night but not during the day caused the elevation of *Per1* gene expression in the hamster PVN (Yamamoto et al., 2001). Similarly, the present data demonstrates that injection of PACAP at night but not during the day increases *mPer1* gene expression in the Pe and PVN, suggesting that *Per1* gene expression in these brain areas is also under the control of the circadian clock system.

In this experiment, (+)MK-801 attenuates both PACAP-induced phase delays and *mPer1* expression in the SCN, PVN, and Pe. Controversial results indicated that PACAP can potentiate NMDA-induced inward current in the SCN neurons *in vitro* (Harrington et al., 1999), but there is no effect on NMDA-induced Ca^{2+} increase in the SCN cells (Kopp et al., 2001). We suggest that activation of an NMDA receptor may be involved in producing PACAP-induced phase delays and *mPer1* expression in the SCN. Since (+)MK-801 was given systemically, and the fact that glutamate is the major excitatory neurotransmitter in the brain, the blockade is likely much broader than that of glutamatergic transmission from RHT. This should be considered especially in the case of the Pe and PVN *mPer1* activation, which could be hardly attributable to the RHT, but more to the local effects of i.c.v. injected PACAP, as this structure lies close to the ventricular surface. Therefore, the fact that (+)MK-801 prevents the overall effect of PACAP would nevertheless indicate the global importance of glutamatergic neurotransmission for the full effect of PACAP.

Previous papers demonstrated that PACAP and 8-bromo-cAMP potentiate glutamate-induced phase delays of circadian SCN neuronal rhythm *in vitro* (Chen et al., 1999; Tischkau et al., 2000) and intracellular Ca^{2+} increase in the SCN cells (Kopp et al., 2001). Our present results showed no augmentative or additive effect of PACAP on light-induced phase delays. The discrepancy between our data and that of the above papers may be attributed to differences in experimental conditions (*in vitro* vs. *in vivo*) as well as the use of different species (rats and mice).

In summary, the present results demonstrate that PACAP induces phase delays in mouse circadian rhythm in association with an increase of *mPer* expression in the SCN via activation of NMDA receptors.

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also induce a phase advance of *Pai-1* mRNA expression. We further investigated whether normal *Clock* gene function is required for RF-induced resetting of the peripheral clock by using *Clock/Clock* mice.

2. Materials and methods

2.1. Animals and housing

Clock mutant mice were purchased from Jackson Laboratory (Stock No. 002923) (Bar Harbor, ME, USA) and interbred in our laboratory. Genotypes were determined by PCR [20]. Animals were maintained on a light–dark (LD) cycle (12 h light, 12 h dark, with lights on at 8:30 a.m.) at a room temperature of 23°C and given food and water *ad libitum* except for RF experiments. All animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government. Some mice were transferred to DD conditions at ZT12 (Zeitgeber time; ZT0 is defined as lights-on time and ZT12 as lights-off time), and sampling was initiated 24 h after transfer.

2.2. RNA Isolation and RT-PCR

Mice ($n=3$–6 for each time point) were deeply anesthetized with ether and intracardially perfused with ice-cold saline. After perfusion, the heart was rapidly isolated, frozen in liquid nitrogen, and stored at −80°C until RNA isolation. Total RNA was extracted using ISOGEN Reagent (Nippon Gene, Tokyo, Japan). 100 ng of total RNA was reverse transcribed and amplified using the Superscript One-Step RT-PCR System (Invitrogen, CA, USA) in a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). Specific primer pairs were designed from published data of the *mPer1*, *mPer2*, *mBmal1*, *mNpas2*, *Pai-1*, and *β-actin* genes in GenBank as follows: *mPer1* [289 bp]: 5′-CAAGTGGCAATGAGTCCAACG-3′ (forward) and 5′-CGAAGTTTGAGCTCCCGAAGT-3′ (reverse); *mPer2* [381 bp]: 5′-CAGACTCATGATGACAGAGG-3′ (forward) and 5′-GAGATGTACAGGATCTTCCC-3′ (reverse); *mBmal1* [344 bp]: 5′-CACTGACTACCAAGAAAGTATG-3′ (forward) and 5′-ATCCATCTGCTGCCCTGAGA-3′ (reverse); *mNpas2* [243 bp]: 5′-CTCAGTGGTCAGTTACGCAG-3′ (forward) and 5′-TGGAGGTGGGTTCTGACATG-3′ (reverse); *Pai-1* [539 bp]: 5′-TCAGAGCAACAAGTTCAACTACACTGAG-3′ (forward) and 5′-CCCACTGTCAAGGCTCCATCACTTGCCCCA-3′ (reverse); and *β-actin* [452 bp]: 5′-GAGGGAAATCGTGCGTGACAT-3′ (forward) and 5′-ACATCTGCTGGAAGGTGGACA-3′ (reverse). We used a semi-quantitative RT-PCR method for measuring the expression level of mRNA. All PCR products were placed under linear amplification from cycles 26 through 30; however, from the 32nd cycle products levels plateaued (data not shown). Therefore, we obtained PCR products in the 28th cycle for quantification. PCR was performed under the following conditions: cDNA synthesis at 50°C for 30 min followed by 94°C for 2 min, PCR amplification for 28 cycles with denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min. The target clock gene cDNA was co-amplified with *β-actin* cDNA in a single PCR tube. The PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and analyzed by an EDAS-290 system (Kodak, NY, USA). The intensity of PCR product of the target gene was normalized to the intensity of *β-actin*. The amplitude (ratio of peak and trough) and phase determined by this method were reproduced in a different experiment and found comparable as determined by Northern blot [5], which suggests that the present experimental conditions can detect a circadian change of *mPer1* and *mPer2* gene expression in the mouse liver.

2.3. RF experiment

The RF experiment was performed as previously described [22]. In brief, after 1 day of fasting (termed as day 0), mice were allowed access to food for 4 h from ZT5 to ZT9 for 6 consecutive days (day 1 to day 6). On day 7, food was again withdrawn for the entire day. Animals were sacrificed at ZT5, 11, 17, and 23 on day 6, and ZT5 on day 7.

2.4. Statistical analysis

The values are expressed as means ± S.E.M. For statistical analysis, one-way ANOVA was applied followed by Dunnett's two-tailed test or the Student's *t*-test.

3. Results

3.1. Daily expression of mPer1, mPer2, mBmal1, mNpas2, and Pai-1 genes in the heart

Using the semi-quantitative RT-PCR method, all clock genes examined in the mouse heart under LD conditions showed a clear daily mRNA rhythm (Fig. 1 and Table 1). *mBmal1* (Fig. 1B) and *mNpas2* (Fig. 1C) mRNA levels peaked at night, while *mPer1* (Fig. 1D) and *mPer2* (Fig. 1E) mRNA levels peaked during the day. *Pai-1* mRNA, the basal expression of which is thought to be under the control of a circadian clock, also showed a clear daily change in the heart, with a peak at late evening (Fig. 1F). On the other hand, *mBmal2* (*Clif*) expression did not show a daily oscillation as previously reported (data not shown) [3].

The daily rhythm of clock genes under LD conditions was attenuated or even absent in the heart of *Clock/Clock* mice (Fig. 1 and Table 1). *mBmal1* mRNA did not show significant daily variation in *Clock/Clock* mice; as previously shown, there was a constant high expression [21]. The *mPer1* mRNA rhythm was also dampened. *mPer2* mRNA was still expressed in a circadian fashion under our experimental conditions, but there was a severely dampened amplitude and delayed peak compared to wild-type mice (Fig. 1E) *mNpas2* mRNA also showed a weak but significant daily rhythm (Fig. 1C), whereas *Pai-1* mRNA expression lacked significant daily variation in *Clock/Clock* mice (Fig. 1F).

To rule out the effect of light, the circadian patterns of clock genes and *Pai-1* gene expression were also studied under DD conditions. Similar to LD conditions, circadian expression of these genes was observed under DD conditions in wild-type mice (Table 1). In *Clock/Clock* mice, *mPer1*, *mPer2*, and *Pai-1* gene expression exhibited no significant rhythmicity when assessed by one-way ANOVA (Table 1).

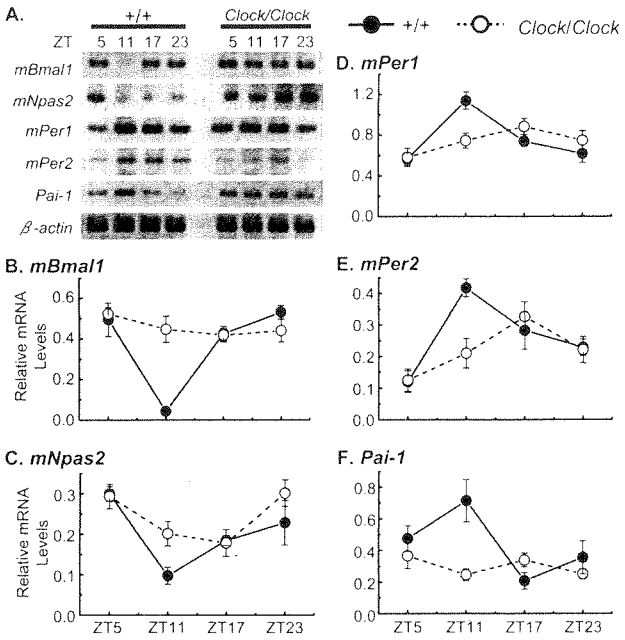

Fig. 1. Daily fluctuation of *mPer1*, *mPer2*, *mBmal1*, *mNpas2*, and *Pai-1* mRNA in the heart of wild-type and *Clock/Clock* mice. A: Representative electrophoresis photographs of PCR products from each genotype (wild-type and *Clock/Clock*) at ZT 5, 11, 17, and 23. B: Daily mRNA abundance of each clock gene was plotted as a relative mRNA level that was normalized to *β-actin* mRNA ($n=4$–6).

Restricted feeding induces daily expression of clock genes and *Pai-1* mRNA in the heart of *Clock* mutant mice

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Abstract **Plasminogen activator inhibitor-1 (PAI-1) is a key factor of fibrinolytic activity. The activity and mRNA abundance show a daily rhythm. To elucidate the mechanism of daily *Pai-1* gene expression, the expression of *Pai-1* and several clock genes was examined in the heart of homozygous *Clock* mutant (*Clock/Clock*) mice. Damping of the daily oscillation of *Pai-1* gene expression in *Clock/Clock* mice was accompanied with damped or attenuated oscillations of *mPer1*, *mPer2*, *mBmal1*, and *mNpas2* mRNA. Daily restricted feeding induced a daily mRNA rhythm of all clock genes and *Pai-1* mRNA in *Clock/Clock* mice as well as wild-type mice. The peaks of clock genes and *Pai-1* mRNA were phase-advanced in the heart of both genotypes after 6 days of restricted feeding. The present results demonstrate that daily *Pai-1* gene expression depends on clock gene expression in the heart and that a functional *Clock* gene is not required for restricted feeding-induced resetting of the peripheral clock.**

Key words: Circadian; *Clock* mutation; Heart; Plasminogen activator inhibitor-1; Restricted feeding

1. Introduction

An endogenous circadian rhythm controls various physiological and behavioral phenomena. The molecular circadian clock system is thought to be based on transcriptional/translational feedback loops consisting of 'clock' genes and their products [1]. In mammals, CLOCK, a member of the bHLH-PAS transcription factors, heterodimerizes with BMAL1 and binds to E-box DNA motifs in the promoter of *Per1/2* and *Cry1/2*, thereby activating their transcription. On the other hand, CRY1/2 proteins negatively regulate the E-box binding ability of CLOCK:BMAL1. Concurrently, PER2 enhances *Bmal1* transcription [1]. Moreover, when BMAL2 (also called CLIF), which is highly homologous to BMAL1 [2], is expressed with CLOCK, E-box mediated transcription is accelerated [3]. NPAS2, a transcription factor that is highly homologous to CLOCK, heterodimerizes with BMAL1 and binds to the E-box motif, thereby activating *Per1/2* transcription [4]. Both NPAS2:BMAL1 and CLOCK:BMAL2 might also function as positive regulators in place of CLOCK:BMAL1. These clock genes are expressed not only in the suprachiasmatic nucleus (SCN) of the hypothalamus where the master clock exists, but also in other brain regions and various peripheral tissues. For example, in the rat heart, these clock genes are abundantly expressed and show clear circadian rhythms [5,6].

In previous studies, an abnormal circadian rhythmicity was demonstrated in *Clock* mutant mice. Heterozygous *Clock* mutant mice (*Clock/+*) demonstrated a lengthened and less stable circadian period, whereas homozygous mutant mice (*Clock/Clock*) showed a gradual loss of circadian rhythmicity in constant darkness (DD) [7]. Positional cloning and subsequent analysis showed that mutant CLOCK lacks 51 amino acids [8]. Furthermore, mutant CLOCK failed to activate E-box mediated transcription [9].

The onset of myocardial infarction frequently occurs from 6 a.m. to 12 p.m. in humans [10]. This phenomenon results in part because of a down-regulation of fibrinolytic activity. Plasminogen activator inhibitor type 1 (PAI-1) is the primary regulator of the fibrinolytic cascade [11,12], and its activity changes in a circadian fashion such that it is high in the morning and low in the evening [13]. *Pai-1* mRNA expression appears in the adiposities, the liver, the kidneys, and abundantly in the heart [14]. The *Pai-1* gene has two functional E-box motifs in the promoter [15]. Maemura et al. [3] demonstrated that *Pai-1* mRNA shows a daily rhythm in vivo, and *Pai-1* transcription is up-regulated by CLOCK:BMAL2 through E-box sites and down-regulated by mPER2 and mCRY1 in cultured cells. To confirm the importance of the *Clock* gene in circadian expression of the *Pai-1* gene, we investigated *Pai-1* gene expression in the heart of *Clock/Clock* mice.

Under restricted feeding (RF) conditions, animals are allowed daily access to food for only a limited time. RF-induced anticipatory locomotor activity rhythm is known to be SCN-independent because SCN-lesioned animals still maintain this activity rhythm [16]. Recent reports demonstrate that circadian clocks expressed in peripheral tissues such as the liver are reset by daily RF during the day in both normal and SCN-lesioned animals [17–19]. We examined whether an RF-induced phase advance in the expression of clock genes can

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Abbreviations: DD, constant dark; LD, light–dark; PAI-1, plasminogen activator inhibitor-1; RF, restricted feeding; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time

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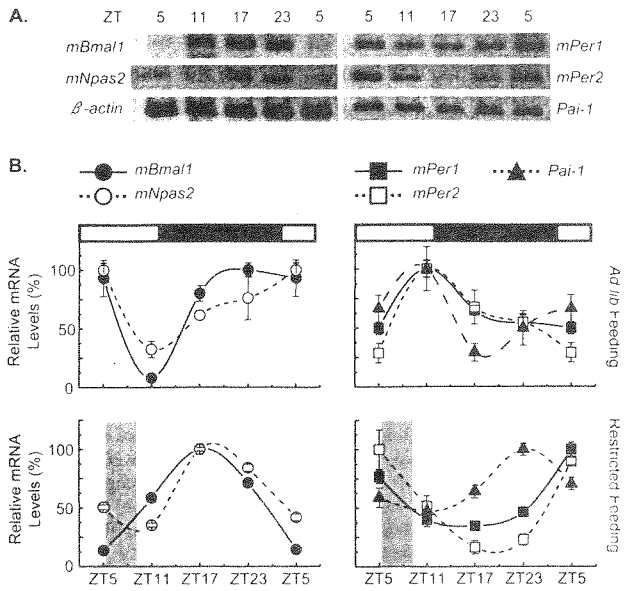

Fig. 2. Effect of RF on the daily clock gene expression in the heart of wild-type mice. A: Representative electrophoresis photographs of PCR products from wild-type mice at ZT5, 11, 17, and 23 on day 6 (final day of RF) and at ZT 5 on day 7. B: Expression of clock genes of wild-type was plotted as a relative mRNA level that was normalized to *β-actin* expression. The peak value was set to 100% ($n = 3$–6). Points from the *ad libitum* feeding schedule at ZT5 on day 7 were re-plotted as data at ZT5 on day 6.

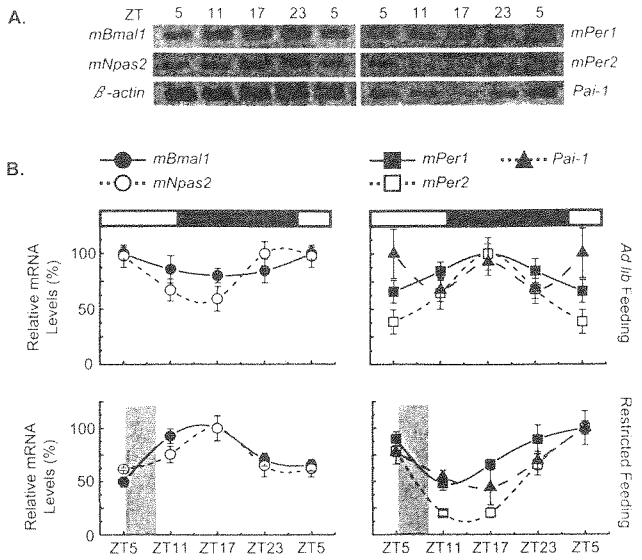

Fig. 3. Effect of RF on daily clock gene expression in the heart of *Clock/Clock* mice. A: Representative electrophoresis photographs of PCR products from *Clock/Clock* at ZT5, 11, 17, and 23 on day 6 (final day of RF) and at ZT5 on day 7. B: Expression of clock genes of *Clock/Clock* was plotted as a relative mRNA level that was normalized to *β-actin* expression, and the peak value was set to 100% ($n = 3$–6). Points from the *ad libitum* feeding schedule at ZT5 on day 7 were re-plotted as data at ZT5 on day 6.

3.2. *RF-induced entrainment of clock genes and Pai-1 gene expression in Clock/Clock mice*

Wild-type and *Clock/Clock* mice were placed on a RF schedule under LD conditions. As shown in Fig. 2, wild-type mice placed under RF for 6 days showed a daily variation in all clock genes as well as *Pai-1* gene expression in the heart (lower panels). Compared to mice with free access to food (upper panels), the phase of the peaks and troughs of clock genes was advanced by 6–12 h with RF. The *Pai-1* mRNA expression pattern also showed a 12-h change under an RF schedule.

When *Clock/Clock* mice followed a RF schedule, both *mPer2* and *mNpas2*, that showed a weak daily rhythmicity under *ad libitum* conditions, exhibited a robust phase-advanced rhythmicity in the heart. Interestingly, *mPer1*, *mBmal1*, and *Pai-1* mRNAs exhibiting no significant rhythmicity under *ad libitum* conditions, showed a clear daily rhythmicity under RF conditions in *Clock/Clock* mice. The peak expression of these mRNAs also showed a phase-advanced rhythmicity that was similar to wild-type mice under RF conditions (Fig. 3 and Table 1).

4. Discussion

In the present experiment, we found that the amplitude of daily oscillations of *mBmal1* and *mPer1* gene expression was blunted in the heart of *Clock/Clock* mice. In addition, *Pai-1* mRNA oscillation completely disappeared in the heart of mutant mice, suggesting that expression of the *Pai-1* gene is closely clock-controlled in the heart. The present in vivo results support those of a recent paper in which CLOCK: BMAL2 up-regulated the *Pai-1* gene and PER2 and CRY1 down-regulated *Pai-1* in cultured cells [3]. As PAI-1 activity regulates the fibrinolytic cascade [11], possibly a circadian change of *Pai-1* expression underlies the circadian rhythmicity of myocardial infarction occurrence.

In the present experiment, we demonstrated a daily expression of *mNpas2* in the wild-type mouse heart. However, the oscillation of *mNpas2* expression in *Clock/Clock* mice is not as severely dampened as that of *mBmal1* expression, which is lost under LD conditions. NPAS2 possesses high homology to CLOCK, and co-expression of NPAS2 and BMAL1 activates transcription of *Per1*, *Per2*, and *Cry1* genes, suggesting that

Table 1
Daily variation and peak time of clock genes and *Pai-1* mRNA expression

	Feeding conditions	Lighting conditions	Positive regulator		Negative regulator		Output
			mBmal1	*mNpas2*	*mPer1*	*mPer2*	*Pai-1*
+/+	*Ad lib*	DD	pZT23***	pZT23***	pZT11***	pZT11***	pZT17**
	Ad lib	LD	ZT23***	ZT5***	ZT11***	ZT11***	ZT11*
	RF	LD	ZT17***	ZT17***	ZT 5***	ZT 5***	ZT23*
Clock/Clock	*Ad lib*	DD	pZT 5***	pZT 5*	–	–	–
	Ad lib	LD	–	ZT23*	–	ZT17*	–
	RF	LD	ZT11-17***	ZT17*	ZT5***	ZT5***	ZT23*

Ad lib: free feeding schedule (four time points); pZT: projected ZT; RF: RF schedule (five time points). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. –: not significant.

NPAS2 is capable of replacing CLOCK as a BMAL1 partner in areas other than the SCN [4]. Therefore, *mPer1* and other clock genes in which the expression is regulated through the E-box are not only controlled by CLOCK:BMAL1 but also by NPAS2:BMAL1 (or CLOCK:BMAL2) in the heart.

Since RF does not affect the daily or circadian expression of clock genes in the SCN [17,22], RF-induced oscillation of the peripheral clock does not require oscillation of the master SCN clock. Furthermore, lesion of the SCN does not affect the RF-induced oscillation of *mPer* gene expression in the liver [19]; however, the necessity of *Clock* gene function in the RF-induced circadian rhythm had yet to be determined. We demonstrated that the rhythmicity of *mPer1*, *mBmal1*, and *Pai-1* mRNA, of which the daily oscillation dampened under *ad libitum* conditions, reappeared under RF conditions in *Clock/Clock* mice. Recently, it was shown that methamphetamine (MAP) restores locomotor activity rhythm in behaviorally arrhythmic *Clock/Clock* mice [23]. These results suggest that intact CLOCK is not necessary for inducing either wheel-running activity rhythm with chronic MAP treatment or food entrainment of the peripheral oscillator through RF. Interestingly, the MAP-induced locomotor activity rhythm is also independent of the circadian oscillator in the SCN [24,25]. Functional CLOCK might not be necessary for these SCN-independent rhythms. Perhaps, NPAS2 compensates for the functional deficit observed in *Clock/Clock* mice.

Pai-1 mRNA expression showed a RF-induced phase advance in the mouse heart. This result indicates the possibility that the timing of fibrinolytic activity may be regulated through *Pai-1* gene expression by changing the feeding time schedule. Some Ca^{2+} channel blockers [26] and β-adrenergic receptor antagonists [27] have reportedly caused a time of day-dependent effect on myocardial disease. Therefore, a controlled feeding time may help with the success of this type of chronopharmacological treatment. Further studies are needed to confirm the interaction of peripheral clock gene function and fibrinolytic activity through *Pai-1* gene expression.

In summary, we demonstrated that *Pai-1* mRNA expression had a daily rhythmicity in the mouse heart, the expression of which is under the control of clock genes. Furthermore, feeding schedule can affect the rhythmic phase of not only clock gene expression but also *Pai-1* gene expression in both wild-type and *Clock/Clock* mice. Thus, molecular clock resetting in the mouse heart under RF conditions may not require the functional *Clock* gene.

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Extended Action of MKC-242, A Selective 5-HT_{1A} Receptor Agonist, on Light-Induced *Per* Gene Expression in the Suprachiasmatic Nucleus in Mice

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We reported previously that (*S*)-5-[3-[(1,4-benzodioxan-2-ylmethyl)amino]propoxy]-1,3-benzodioxole hydrochloride (MKC-242) (3 mg kg^{-1}, i.p.), a selective 5-HT_{1A} receptor agonist, accelerated the re-entrainment of hamster wheel-running rhythms to a new 8 hr delayed or advanced light-dark cycle, and also potentiated the phase advance of the wheel-running rhythm produced by light pulses. The molecular mechanism underlying MKC-242-induced potentiation of this phase shift, however, has not yet been elucidated. We examined the effects of MKC-242 on light-induced *mPer1* and *mPer2* mRNA expression in the suprachiasmatic nucleus (SCN) of mice. MKC-242 (5 mg kg^{-1}, i.p.) potentiated light-induced *mPer1* and *mPer2* expression in the SCN of mice housed in constant darkness for 2 days, when mRNA levels were observed 3 hr after light-exposure. More potentiating action of MKC-242 on *mPer2* expression in the SCN was observed in mice housed in constant darkness for 9–10 days. This facilitatory action of MKC-242 on *mPer1* expression was antagonized by WAY100635, a selective 5-HT_{1A} receptor blocker, indicating that MKC-242 activated 5-HT_{1A} receptors. Other drugs such as 8-hydroxy-dipropylaminotetralin (10 mg kg^{-1}, i.p.), paroxetine (10 mg kg^{-1}, i.p.), buspirone (10 mg kg^{-1}, i.p.), and diazepam (10 mg kg^{-1}, i.p.) did not display a potentiating action on light-induced *mPer1* and *mPer2* expression in the SCN. In the behavioral experiments, we found that MKC-242 (5 mg kg^{-1}, i.p.) potentiated light-induced phase delays of free-running rhythm in mice. The present results suggest that prolonged increase of *mPer1* or *mPer2* expression in the SCN by MKC-242 may be involved in the potentiation of photic entrainment by MKC-242 in mice.

Key words: circadian rhythm; light-entrainment; *Per* gene; suprachiasmatic nucleus; 5-HT_{1A} receptor; MKC-242

The biological clocks of mammals, located in the suprachiasmatic nuclei (SCN) of the hypothalamus, control various physiological daily rhythms, and are reset by environmental light exposure (Inouye and Shibata, 1994). The SCN are densely innervated by 5-HT neurons in the midbrain raphe nuclei in rodents (Cagampang et al., 1993; Cagampang and Inouye, 1994; Meyer-Bernstein and Morin, 1996). Systemic or local injections of 8-hydroxy-dipropylaminotetralin (8-OH-DPAT), an agonist for 5-HT_{1A} and 5-HT_7 receptors, suppress the light-induced phase shifts of hamster activity rhythms (Rea et al., 1995; Mintz et al., 1997; Weber et al., 1998; Belenky and Pickard 2001; Smith et al., 2001), light-induced *c-fos* expression in the hamster SCN (Selim et al., 1993; Glass et al., 1994, 1995; Picard et al., 1997), and the firing rates of light responsive cells in the hamster SCN (Ying and Rusak, 1994, 1997). In in vitro studies, Liou et al. (1986) and Rea et al. (1994) reported that 5-HT suppressed the optic nerve stimulation-evoked field potentials in the rat SCN and hamster SCN, respectively. This evidence suggests that 5-HT neurons from the midbrain raphe nuclei possess an inhibitory regulation on the photic entrainment of the biological clock in mammals via 5-HT_{1A} or 5-HT_7 receptor activation.

Morin and Blanchard (1991) reported that depletion of hamster brain serotonin increased the circadian activity rhythm response to light. MKC-242, (*S*)-5-[3-[(1,4-Benzodioxan-2-ylmethyl)amino]propoxy]-1,3-

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benzodioxole hydrochloride, a selective 5-HT_{1A} receptor agonist, potentiated light-induced phase shifts in hamster circadian rhythms by decreasing the turnover of 5-HT in the SCN (Moriya et al., 1998). Thus, a decrease in 5-HT activity may result in a facilitator action on light-induced phase shift.

Recent studies on the molecular aspects of clock genes have produced a functional model for circadian rhythms (Dunlap, 1999; Young, 2000 for review). For example, the expression of *mPer1*, *mPer2*, and *mPer3* mRNAs in the SCN occurs in a circadian fashion (Shearman et al., 1997; Takumi et al., 1998; Zylka et al., 1998). We found that brief exposure to light during the subjective night resulted in a large and rapid induction of *mPer1* expression (Shigeyoshi et al., 1997) and *mPer2* mRNA expression in the SCN (Shearman et al., 1997; Takumi et al., 1998). Thus, *mPer1*, *mPer2*, and *mPer3* are rhythmically expressed in the SCN, and *mPer1* and *mPer2* exhibit circadian photoresponses. In addition, we demonstrated recently that a light-induced phase delay was inhibited significantly by *mPer1* and *mPer2* antisense oligonucleotides before light exposure (Akiyama et al., 1999; Wakamatsu et al., 2001). Therefore, we suggest that expression of the *mPer1* or *mPer2* genes may be an important step in causing photic entrainment.

It remains to be clarified how MKC-242 potentiates light-induced phase shifts on the molecular bases of circadian clock regulation. In this experiment, we investigated whether the potentiation action of MKC-242 on light-induced phase delays accompanied with an increase in *mPer1* or *mPer2* expression in the mouse SCN. To elucidate the specificity of MKC-242 as a 5-HT_{1A} receptor agonist (Matsuda et al., 1995a,b; Suzuki et al., 1995; Abe et al., 1996; Asano et al., 1997), we further examined the effects of 8-OH DPAT, paroxetine, buspirone, and diazepam on light-induced *mPer* gene expression in the SCN. Although in previous study we used hamsters for behavioral assessment of MKC-242-induced potentiation (Moriya et al., 1998), we used mice in this experiment. The reasons why we selected other species include generalization of MKC-242 effect on circadian clock and easily comparison of effective doses with other behavioral pharmacological data (Matsuda et al., 1995a; Abe et al., 1996).

MATERIALS AND METHODS

Animals

Male ddY mice weighing 20–35 g were maintained under controlled environmental conditions (23 ± 2°C room temperature; 12:12 hr light:dark cycle, lights on at 8:30) for at least 2 weeks before use in the experiments. The light intensity was almost 200 lux at the level of the animal cage. Food and water were provided ad lib. Animals were treated in accordance with the Law (No. 105) and Notification (No. 6) of the Japanese Government. Under the light:dark cycle, zeitgeber time (ZT) referred to the animal colony light:dark cycle. ZT0 was designated as lights-on and ZT12 as lights-off. In free-running conditions under constant darkness (DD), circadian time (CT) was defined instead of ZT, and CT12 referred to the onset of activity.

Recording of Locomotor Activity Rhythm

Mice were housed individually in transparent plastic cages (35 × 20 × 20 cm), and their locomotor activity rhythms under DD were measured by area sensors (FA-05 F5B Omron, Japan) with a detector system of a thermal ultra red radiation from mouse (Oshima and Ebihara, 1988). Locomotor activity was continuously recorded in 6 min epochs by a PC-9801 computer. After free-running for at least 2 weeks in DD, mice were randomly assigned to injections of MKC-242 (5 mg kg^{-1}) or vehicle at CT15.5, 30 min before light exposure (70 or 300 lux, 5 min) at CT 16. The phase of the rhythm was assessed visually by applying a straight edge to the onset of activity on successive days before and after drug injection and, determining the difference in phases on the day of drug injection (Akiyama et al., 1999). Activity data was analyzed "blind" to the experimental treatments.

Sample Preparation

In situ hybridization was utilized to quantify or determine the histochemical distribution of the expression of *mPer1* and *mPer2* mRNA expression in coronal sections of the hypothalamus. Mice were entrained to the light:dark cycle for at least 2 weeks then kept in DD conditions. On the second day of DD at CT15.5, mice were i.p. injected with MKC-242 (5 mg kg^{-1}), 8-OH DPAT (10 mg kg^{-1}), paroxetine (10 mg kg^{-1}), buspirone (10 mg kg^{-1}), diazepam (10 mg kg^{-1}) or vehicle, and then light was delivered to some of these mice (70 lux, 5 min) or not at CT16. Some mice were entered into DD condition with monitoring the phase of free-running of locomotion. On Day 9–10 of DD at CT15.5, these mice were i.p. injected MKC-242 or vehicle and then light was delivered to all mice (70 lux, 5 min) at CT16. Next, mice were deeply anesthetized with ether 1 or 3 hr after light exposure and intracardially perfused with 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde. In some cases, WAY100635 was injected 30 min before MKC-242 administration. Brains were excised, post-fixed in 0.1 M PB containing 4% paraformaldehyde for 24 hr at 4°C, and transferred into 20% sucrose in phosphate buffer saline for 24 hr at 4°C.

In Situ Hybridization Protocol

Frontal sections (30 μm thick) were collected and transferred into PBS for 30 min after treatment with 6× SSC for 30 min. Sections were incubated in hybridization buffer [50% formamide, 6× SSC, 0.1 mg/ml denatured salmon sperm DNA, 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA) and 10% dextran sulfate] containing labeled cRNA probes overnight at 60°C. Radio isotope [RI: α[^{33}P]UTP (New England Nuclear/Dupont, Boston, MA)]-labeled antisense cRNA were made according to a standard protocol for cRNA synthesis.

The sequences of cRNA probes for *mPer1*, *mPer2*, and *mPer3* were as described in our previous studies (Nikaido et al., 2001, Wakamatsu et al., 2001). After hybridization, these sections were rinsed in 2× SSC/50% formamide for 45 min and again for 15 min at 60°C, treated with RNase A for 30 min at

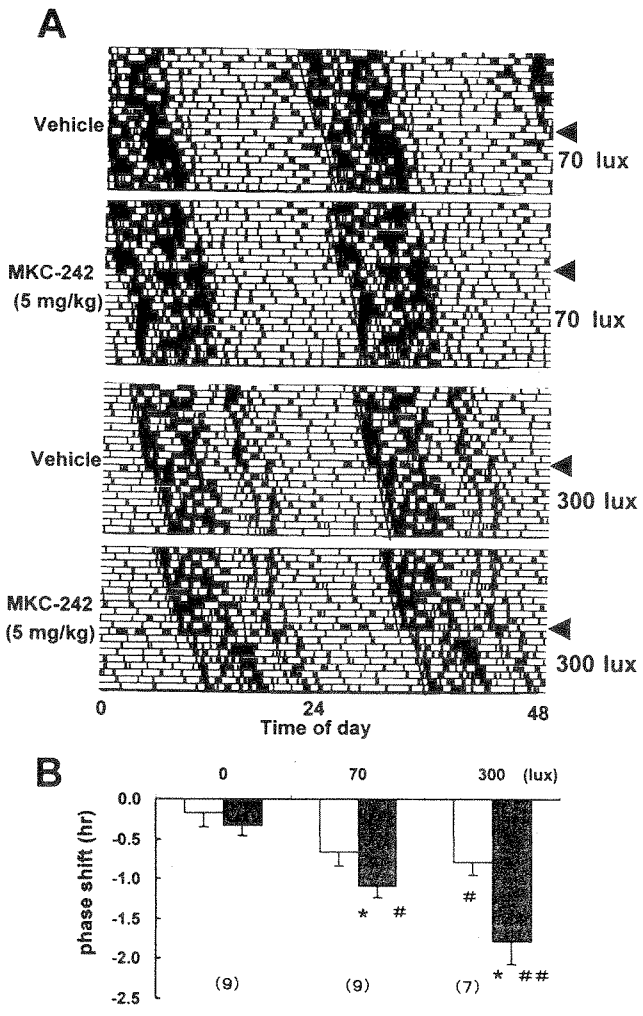

Fig. 1. Effect of MKC-242 on light-induced phase delays in mouse circadian activity rhythm. **A:** Double-plotted actograms. If the mouse exhibits locomotion in each 6 recording min, vertical thin bar is plotted in the actogram, and high active period is expressed by dense bars. Each line in the figure shows the onset of activity. Arrows exhibit the day of drug injection and light exposure. MKC-242 or vehicle was injected into each mouse at CT15.5, then 30 min later light (70 or 300 lux) was applied for 5 min at CT16. **B:** Summary of phase delays. Individual animals that received vehicle (open column) or MKC-242 (5 mg kg^{-1} i.p) (hatched column). Zero lux group, no light group. Numbers in parentheses represents the number of animals. $*P < 0.05$ vs. vehicle (Student's paired *t*-test). $\#P < 0.05$, $\#\#P < 0.01$ vs. non-light (Dunnett's test).

37°C, treated two times with 2× SSC/50% formamide for 15 min at 60°C, and then treated with 0.4× SSC for 30 min at 60°C. For RI in situ hybridization, tissue sections were collected into 2× SSC and then treated with proteinase K [1.0 μg ml^{-1}, 10 mM Tris buffer (pH 7.5), 10 mM EDTA] for 10 min at 37°C, 4% PFA in 0.1 M PB for 5 min, 2× SSC for 5 min followed by 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, and then treated two times with 2× SSC for 5 min. The radioactivity of each SCN on BioMax MR film (Eastman

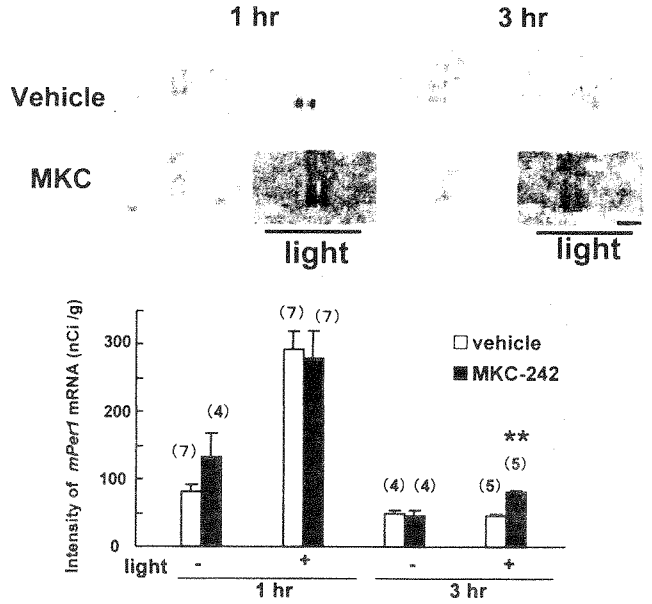

Fig. 2. Effects of MKC-242 on light-induced *mPer1* expression in the SCN of mouse kept in DD for 2 days. MKC-242 (5 mg kg^{-1} i.p) or vehicle was injected at CT15.5, then 30 min later light (70 lux for 5 min) was applied at CT16. One or 3 hr after light exposure, mice were sacrificed. **Upper panel:** in situ hybridization signals of *mPer1* in the SCN. Calibration, 0.5 mm. **Lower panel:** summary of *mPer1* mRNA expression. Numbers in parentheses represents the number of animals. $**P < 0.01$ vs. vehicle (Student's *t*-test).

Kodak, Rochester, NY) was analyzed using a microcomputer interface to an image analysis system (MCID, Imaging Research Inc., St. Catherine's, ON) after conversion into optical density by ^{14}C-autoradiographic microscales (Amersham, Buckinghamshire, UK). For data analysis, we subtracted the intensities of the optical density in the corpus callosum from those in the SCN of each section and regarded this value as the net intensity in the SCN as reported previously (Moriya et al., 2000). The intensity values of the sections from the rostral-most to the caudal-most part of the SCN (five sections per mouse brain) were then summed; the sum was considered to be a measure of the amount of *mPer1*, *mPer2* and *mPer3* mRNA in the SCN.

Drugs and Reagents

MKC-242, WAY100635 (N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride), paroxetine, buspirone and diazepam were generously donated by Mitsubishi Pharma Corporation (Yokohama, Japan). (±)8-OH-DPAT was purchased from Research Biochemicals Inc. (Natick, MA). The rest of the chemicals were of the highest grade.

All drugs were freshly prepared. MKC-242 and diazepam were suspended in 0.5% carboxymethylcellulose (CMC) and other drugs were dissolved in saline, before injection at the dose indicated (1 ml kg^{-1}).

Data and Statistical Analysis

Values are expressed as the mean ± SEM. Statistical analysis was conducted using the Student's *t*-test or one-way

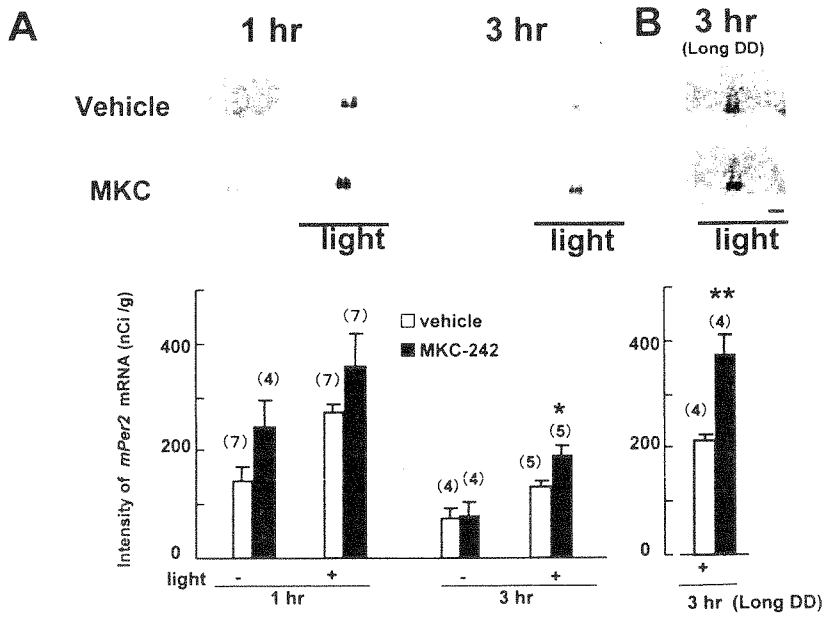

Fig. 3. Effects of MKC-242 on light-induced *mPer2* expression in the SCN of mouse kept in DD for 2 days (**A**) or 9–10 days (**B**). MKC-242 (5 mg kg^{-1} i.p) or vehicle was injected at CT15.5, then 30 min later light (70 lux for 5 min) was applied at CT16. One or 3 hr after light exposure, expression of the *mPer2* signal was observed. **Upper panel:** in situ hybridization signals of *mPer2* in the SCN; scale bar = 0.5 mm. **Lower panel:** summary of *mPer2* mRNA expression.. Numbers in parentheses represents the number of animals. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. vehicle (Student's *t*-test).

ANOVA followed by Dunnett's test. *P*-values of 5% or less were considered statistically significant.

RESULTS

Effect of MKC-242 on Light-Induced Phase Delays of Mouse Activity Rhythm and *mPer1* Expression in the SCN

Light exposure at CT16 produced phase delays of the mouse circadian rhythm in a light intensity-dependent manner ($F[2,22] = 3.8$, $P < 0.05$ for one-way ANOVA) (Fig. 1). Post-hoc analysis revealed difference between 300 ($P < 0.05$) lux group and non-treatment. Consistent with our previous results in the hamster (Moriya et al., 1998), administration of MKC-242 (5 mg kg^{-1}, i.p.) 30 min before light stimulus significantly potentiated light (70 lux or 300 lux, 5 min)-induced phase delays ($F[2,22] = 15.6$, $P < 0.01$ for one-way ANOVA). One hour after light exposure (70 lux or 300 lux, 5 min) at CT16, *mPer1* and *mPer2* gene expression were observed in the SCN. Hybridized signals of *mPer1* and *mPer2* also increased with light intensity (*mPer1*: 300% and 500% of control non-light group for 70 lux and 300 lux; *mPer2*: 170% and 370% of control non-light group for 70 lux and 300 lux). Because we expected MKC-242 would augment *mPer* expression, we examined the effect of MKC-242 (5 mg kg^{-1}, i.p.) on low intensity light (70 lux, 5 min)-induced *mPer* gene expression. In the vehicle administration group, *mPer1* expression was strongly induced by light 1 hr after exposure, but this elevation returned to control basal level 3 hr after light exposure (Fig. 2). This time course of *mPer1* expression after light exposure was similar to the data of previous studies (Takumi et al., 1998; Zylka et al., 1998). MKC-242 did not potentiate light-induced *mPer1* expression when observed 1 hr after light exposure, whereas the level of *mPer1* remained significantly high 3 hr after light in comparison with the vehicle administration group (Fig. 2). Interestingly, injection of MKC-242 (5 mg kg^{-1}, i.p.) alone without light exposure did not affect *mPer1* expression in the SCN at either 1 or 3 hr after drug administration (Fig. 2).

Effect of MKC-242 on Light-Induced *mPer2* Expression in the SCN

Hybridized signals were obtained from the same animals described in Figure 2. In the vehicle administration group, light exposure increased *mPer2* expression to 200% and 250% compared to mice of the control group at 1 hr and 3 hr, respectively (Fig. 3). This time course of *mPer2* expression after light exposure was similar to the data of previous studies (Takumi et al., 1998; Zylka et al., 1998). Although MKC-242 increased light-induced *mPer2* expression 1 hr after light exposure, this increment did not reach significant level ($P = 0.08$). Three hours after light exposure, however, a significant increase of *mPer2* expression was observed in MKC-242-treated mice ($P < 0.05$). The expression of *mPer2* was also unaffected by MKC-242 administration alone.

Effect of MKC-242 on Light-Induced *mPer2* Expression in the SCN of Mice Housed in Constant Darkness for 9–10 Days

Behavioral experiment was conducted under DD conditions for at least 10 days. In a recent study, we reported that light caused 12% increase of *mPer2* gene expression without affecting *mPer1* in mice that had kept in DD condition for 10 days in comparison with those for 2 days (Aida et al., 2002). In addition, MKC-242 potentiated *mPer2* ($P = 0.08$) but not *mPer1* expression 1 hr after light exposure (Figs. 2, 3). Therefore, we examined the effect of MKC-242 on light-induced *mPer2* gene expres-

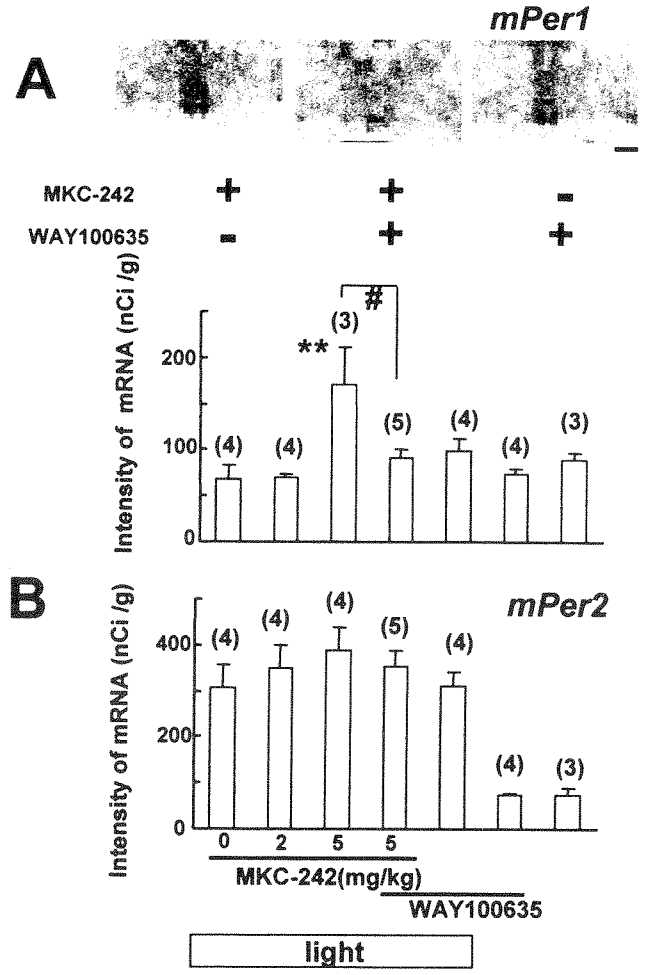

Fig. 4. Antagonistic effect of WAY100635 on MKC-242-induced potentiation of *mPer1* (**A**) or *mPer2* (**B**) mRNA expression with light exposure. **Upper left panel:** in situ hybridization signals of *mPer1* in the SCN. All autoradiographs were obtained from groups exposed to light. Scale bar = 0.5 mm. Vehicle or WAY100635 (10 mg kg^{-1}) was injected at CT15, followed by MKC-242 (5 mg kg^{-1}) or vehicle injection at CT 15.5. Some animals were exposed to light. Three hr after light exposure, mice were sacrificed. A summary of *mPer* gene expression is shown individually in each column figure. Numbers in parentheses represents the number of animals. $^{**}P < 0.05$ vs. light (Dunnett's test). $\#\#P < 0.01$ vs. MKC-242 + light (Student's *t*-test).

sion in the SCN of mice kept in DD for 9–10 days. MKC-242 increased light exposure (70 lux, 5 min at CT16)-induced *mPer2* (1.73 times higher than light only, $P < 0.01$ Student's *t*-test) gene expression in the SCN 3 hr after light exposure (Fig. 3).

Antagonistic Effect of WAY100635 on Facilitatory Action of MKC-242 in *mPer1* and *mPer2* Expression

A lower dose of MKC-242 (2 mg kg^{-1}, i.p.) did not elevate light-induced *mPer1* or *mPer2* expression 3 hr after light exposure (Fig. 4A). One of our previous studies demonstrated that 3 mg/kg but not 1 or 0.1 mg /kg of MKC-242 potentiated a light-induced phase advance in hamster circadian rhythm (Moriya et al., 1998). As shown in Figure 4, pre-injection of WAY100635 (10 mg kg^{-1}), a selective 5-HT_{1A} receptor blocker, antagonized the potentiating action of MKC-242 on *mPer1* expression. In this experiment, light strongly increased the *mPer2* gene expression 3 hr after light exposure and therefore, potentiation of MKC-242 (5 mg kg^{-1}) on light-induced *mPer2* expression did not reach a significant level (Fig. 4B). WAY100635 slightly antagonized the MKC-induced potentiating action on *mPer2* gene expression. WAY100635 (10 mg kg^{-1}) itself did not affect either the light pulse-induced or basal level *mPer1* or *mPer2* expression in the SCN. Light exposure did not induce *mPer3* expression in the mouse SCN 3 hr after light. Both doses of MKC-242 (2 or 5 mg kg^{-1}, i.p.) did not affect *mPer3* expression with or without light exposure (data not shown).

Effect of 8-OH-DPAT, Paroxetine, Buspirone, and Diazepam on Light Pulse-Induced *mPer1* and *mPer2* Expression

8-OH-DPAT (10 mg kg^{-1}), a 5-HT_{1A}/5-HT_7 receptor agonist, paroxetine (10 mg kg^{-1}), a serotonin selective re-uptake inhibitor, buspirone (10 mg kg^{-1}), a 5-HT_{1A} receptor agonist and diazepam (10 mg kg^{-1}), a benzodiazepine receptor agonist, failed to affect light pulse (70 lux for 5 min at CT16)-induced *mPer1* and *mPer2* expression 3 hr after light exposure (Fig. 5).

DISCUSSION

The present experiments demonstrate that MKC-242, a selective 5-HT_{1A} receptor agonist, prolongs the photic induction of *mPer1* and *mPer2* in the mouse SCN in a manner corresponding to the potentiation of phase delays. In recent studies, we demonstrated that injection of antisense oligonucleotides for *mPer1* and *mPer2* into the lateral ventricle attenuated light-induced phase delays of mouse circadian rhythm (Akiyama et al., 1999; Wakamatsu et al., 2001). Therefore, the rapid induction of *mPer1* or *mPer2* mRNAs in the SCN after light exposure during subjective night should be an important step for clock resetting. Non-photic stimuli such as benzodiazepine, 8-OH-DPAT, or novel wheel-running during the subjective day reset the circadian rhythm (Hastings et al., 1997). It has been demonstrated recently that administration of triazolam/brotizolam, 8-OH-DPAT, or novel wheel-running during subjective day reduces *Per1* and *Per2* mRNA in the hamster SCN (Maywood et al., 1999; Horikawa et al., 2000; Yokota et al., 2000). It is strongly suggested that non-photic stimuli presented during the subjective day cause a phase advance by reducing *Per1* and *Per2* mRNA in the hamster SCN. Taken together, these observations suggest that rapid induction or reduction of *Per1* or *Per2* gene expression in the SCN is required to reset the circadian clock.

In the present experiment, MKC-242 potentiated the light-induced SCN *mPer1* and *mPer2* expression under 2 days of DD and *mPer2* expression under 9–10 days of

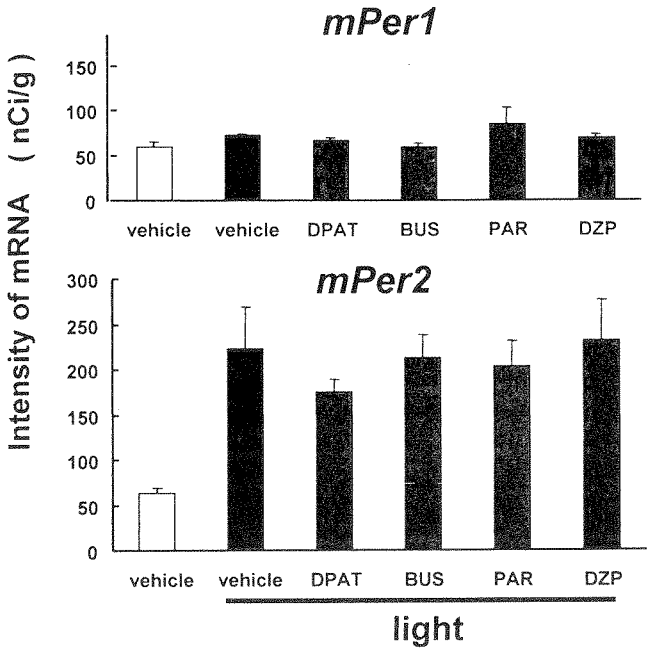

Fig. 5. Effects of various other drugs on light-induced *mPer1* and *mPer2* expression in the SCN. Each drug was injected at CT15.5, then light (70 lux for 5 min) was applied. Three hours after light exposure, mice were sacrificed. The dose of all drugs was prepared as 10 mg kg^{-1}. DPAT, 8-OH DPAT; BUS, buspirone; PAR, paroxetine; DZP, diazepam. Values are given as mean from four animals. $*P < 0.05$ vs. vehicle + light (Dunnett's test).

DD. As behavioral experiment of light-induced phase delays was done in more than 10 days in DD, MKC-242-induced potentiation of *mPer2* gene expression may be associated with behavioral results. We reported that light exposure augmented *mPer2* (12% increase) but not *mPer1* (6% decrease) gene expression in the SCN after 10 days of DD housing (Aida et al., 2002). Recent studies have demonstrated that *mPer2* but not *mPer1* gene is essential for circadian core oscillation in the SCN (Cermakian et al., 2001; Zheng et al., 2001) using mice with a targeted mutation in the *mPer1* and *mPer2*. In addition, *mPer1* is not involved in light-induced phase shift of behavioral rhythm (Cermakian et al., 2001). Albrecht et al. (2001) suggests the importance of *mPer2* expression in the phase delays using *mPer1* and *mPer2* gene knock-out mice. Taken together, it is suggested that prolonged expression of *mPer2* in the SCN may be associated with augmentation of MKC-242 on light-induced phase delays.

We have shown that there is a regional difference in the regulation of *mPer1* and *mPer2* in the mouse SCN (Aida et al., 2002; Shigeyoshi et al., 1997). *mPer1* and *mPer2* in the ventral SCN is inducible by light whereas the *mPer1* and *mPer2* in the medial SCN is not induced by light but rather demonstrates a strong circadian rhythm in expression. Although it is possible that MKC-242 differentially acts on these two different populations of *mPer1* and *mPer2* expressing neurons, the present in situ hybridization data could not clear this argument. Further experiment using emulsion autoradiography may help us to dissolve this question.

In the present experiment, MKC-242 blocked the recovery process of *mPer1* and *mPer2* expression 3 hr after light exposure, but it did not affect the level of *mPer1* and *mPer2* expression 1 hr after light exposure. We can rule out the possibility that a ceiling effect by MKC-242 failed to potentiate *mPer1* and *mPer2* expression 1 hr after light, because high intensity light (300 lux, 5 min) increased *mPer1* and *mPer2* expression in the SCN. We suppose that MKC-242 increases the stability of *mPer1* or *mPer2* mRNA or decreases the destruction of *mPer1* or *mPer2* mRNA. Therefore, it is important to investigate whether *mPer1* or *mPer2* mRNA changes after MKC-242 administration are translated into differences in the levels of mPER proteins. If *mPer* expression observed after light exposure to 70 and 300 lux light pulses (*mPer1*, 300% and 500% of control non-light levels; *mPer2*, 170% and 370%) was compared to behavioral phase delays (400% and 450%), *mPer1* or *mPer2* gene expression are roughly associated with behavioral phase delays.

MKC-242 strongly augmented the light-induced phase shifts in hamsters (Moriya et al., 1998) in comparison with present mice data. We do not know the reason for such species differences. Although difference in 5-HT inputs or 5-HT receptors between the two animal species has not well been elucidated, 8-OH DPAT caused robust phase advances in hamsters (Horikawa et al., 2000) but not in mice (our unpublished observation). Light-induced phase delays in ddY mice were smaller than those in ICR (Akiyama et al., 1999) or Balb/c mice (Shigeyoshi et al., 1997). Small phase delays of ddY mouse may be explained by its longer free-running period (24 hr<) than other strains (24 hr>). In this experiment we used ddY mice, because MKC-242 was expected to potentiate light-induced phase shift. Thus, such strain and species differences may refer to function of other clock genes or clock controlled genes and their products.

The site of action or the intra/intercellular mechanism underlying the potentiating action of MKC-242 on photic entrainment are still unclear. MKC-242 may selectively activate 5-HT_{1A} receptors in the midbrain raphe nuclei, which are widely known to possess autoreceptors suppressing the activity of 5-HT neurons. In the previous experiments, we showed that peripheral injection of MKC-242 suppressed 5-HT turnover in the SCN (Moriya et al., 1998). Systemic injection of a 5-HT_{1A} receptor antagonist, NAN-190, or 5-HT-terminal destruction restricted to the SCN resulted in a potentiation of photic entrainment of the biological clock in rodents (Rea et al., 1995; Bradbury et al., 1997). Furthermore, the light pulse-induced phase shift of activity rhythms was dramatically increased after monoamine (including serotonin) depletion by reserpine (Penev et al., 1993). This evidence suggests that serotonergic innervation to the

SCN exerts tonic suppressing actions on photic entrainment of the biological clock. Therefore, we propose that MKC-242 activates 5-HT_{1A} autoreceptors in the midbrain raphe and suppresses the activity of 5-HT neurons projecting to the SCN. This reduction of 5-HT activity in the SCN then leads to the potentiation of photic entrainment. The physiological roles in the 5-HT_{1A} receptors in the SCN in relation to its function on clock gene expressions are not fully understood. When this receptor in the SCN is selectively stimulated, photic entrainment and induction of *mPer1* and *mPer2* expression must be inhibited, because some reports have suggested a suppressing action of 5-HT_{1A} receptor activation on photic entrainment (Gannon et al., 2001).

In contrast to MKC-242, 8-OH-DPAT and buspirone, well known 5-HT_{1A} receptor agonists, failed to potentiate light-induced *mPer1* expression. This difference in the action of MKC-242 and 8-OH-DPAT, however, may reflect the specificity of these chemicals for 5-HT receptor subtypes. MKC-242 reportedly has a high affinity for 5-HT_{1A} receptors (K_i = 0.35 nM; Matsuda et al., 1995a) and a relatively low affinity for 5-HT_7 receptors (K_i >30 nM; unpublished observation). In addition, the potentiating action of MKC-242 on light-induced *mPer1* expression (present result) and phase shifts (Moriya et al., 1998) was reversed by co-administration of WAY100635, a 5-HT_{1A} receptor blocker. 8-OH-DPAT has a high affinity for both 5-HT_{1A} and 5-HT_7 receptors (Lovenberg et al., 1993). The importance of 5-HT_7 receptors in regulating photic and non-photic entrainment of the biological clock is becoming abundantly clear. Ying and Rusak (1997) reported that 8-OH-DPAT suppressed firing rates of light-responsible SCN neurons via the activation of 5-HT_7 receptors in the SCN. Furthermore, the phase advancing action of 8-OH-DPAT on firing rhythms in an SCN slice were abolished by the 5-HT_2/5-HT_7 blocker, ritanserin, but not the 5-HT_{1A} antagonist, pindolol, suggesting a functional role of 5-HT_7 receptors in the SCN. Possibly 8-OH-DPAT suppresses rather than augments photic entrainment via 5-HT_7 receptor activation in the SCN. In the present experiment, we used paroxetine as a serotonin selective re-uptake inhibitor, because of its potential to increase synaptic 5-HT in the SCN when administered peripherally, thereby activating 5-HT_{1A}/5-HT_7 receptors and inhibiting *mPer* expression in the SCN. Thus, MKC-242 can discernible from both 8-OH DPAT and paroxetine.

Buspirone and MKC-242 behave as full and partial agonists at pre- and postsynaptic 5-HT_{1A} receptors, respectively (De Vivo and Maayani, 1986; Sharp et al., 1989; Hoyer and Boddeke, 1993; Matsuda et al., 1995b). There is a rapid metabolism of buspirone to the alpha2-adrenergic receptor antagonist, 1-(2-pyrimidinyl-piperazine) (1-PP) (Caccia et al., 1986; Engberg, 1989; Gobert et al., 1997) . Thus, progressive generation of 1-PP by buspirone metabolism leads to a compensation of buspirone's inhibitory influence upon 5-HT levels via 5-HT_{1A} autoreceptors through the facilitatory influence mediated by 1-PP at α2-aderenoceptors. Therefore, buspirone should increase *mPer1* and *mPer2* expression in the SCN, but this *mPer1* and *mPer2* increase may be canceled by its metabolites, 1-PP.

The geniculohypothalamic pathway has been reported to inhibit the regulation of the photic entrainment of the biological clock via the release of neuropeptide Y (NPY) and γ-aminobutyric acid (GABA) in the SCN (Ying et al., 1993; Biello, 1995). In the present experiment, diazepam did not potentiate light-induced *mPer1* expression in the SCN.

MKC-242 reportedly possesses anti-depressant effects (Matsuda et al., 1995a). Therefore, the circadian rhythm disorders observed in depressed subjects may improve after treatment with MKC-242 via a potentiation of photic entrainment. In addition, MKC-242 exhibits a different drug profile, if compared to 8-OH-DPAT, paroxetine, buspirone, and diazepam in terms of circadian regulation.

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特集 生体時計と概日リズム

概日リズムの薬理*

柴田重信**

時差ぼけ軽減のためメラトニンや睡眠導入薬のベンゾジアゼピンが臨床的に使われてきたが，その作用機序は不明のままであった。また，うつ病のリズム異常仮説から，抗うつ薬が体内時計機構に何らかの作用を及ぼすことは十分考えられてきたが，やはりその作用機構についてはわからなかった。ところで，分子生物学的手法の成功により体内時計機構の分子レベルの解明は急速に進んでいる。したがって，種々の薬物やあるいは神経伝達物質の体内時計作用機構をモレキュラーレベルで説明できる可能性が出てきた。ここでは，体内時計の発振，同調，出力機構を，伝達物質—細胞内情報伝達—時計遺伝子といった一連の動きから説明し，さらに，先に述べた薬物の作用機構を時計遺伝子の発現変化で説明している。とくに，セロトニンと抗うつ薬，GABAとベンゾジアゼピンという組み合わせで，体内時計の薬理学的視点について解説している。

キーワード：セロトニン，時計階層性，ベンゾジアゼピン，時計遺伝子

はじめに

体内時計機構は便宜上，約24時間周期で発振する発振機構，これを24時間にあわせる同調機構，さらにこの時計情報を外部に伝える出力機構から成り立っている。この同調，発振，出力の各機構についてその分子機構が明らかになりつつある。したがって，この分子機構に影響を及ぼす薬物も見出されており，このような薬物は体内時計機構に影響を及ぼす可能性がある。あるいは臨床的にリズム障害や，時差ぼけに有用な薬物や逆に副作用としてリズム障害を引き起こす薬物が時計遺伝子に作用する可能性も考えられる。したがって，ここでは，薬物がどのような機構で体内時計に影響を及ぼすかにつき，薬理学的観点から，すなわちこれらの時計機構にどのような受容体，細胞内情報伝達物質，遺伝子がかかわっているかを薬理学的な立場から解説する。さらに薬理学的観点からの，体内時計機構に作用する薬物の創薬的視点についても言及する。

Ⅰ．概日リズムの発振機構

哺乳動物体内時計は約24時間の発振周期を有するが，その基本的な周期形成には，*Period* (*Per1*, *Per2*, *Per3*) 遺伝子産物による *Per* 遺伝子へのネガティブフィードバック機構によるモデルが提唱されている (Dunlap, 1999；Reppert & Weaver, 2001)。実際，カゼインカイネースイプシロンⅠの変位により時計の発振周期が短くなるタウミュータントハムスターの存在が知られている (Lowrey et al, 2000)。また，*Cry1* や *Cry2* のノックアウトマウスではフェノタイプとして輪回し行動のフリーラン周期がそれぞれ短いあるいは長いことが知られている。このように哺乳動物でも発振周期を調節する時計遺伝子の存在が明らかになってきつつある。一方，以前より，重水やリチウム，エストロゲ

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* Pharmacological study on circadian rhythms.

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ンが発振周期を延長させることが知られているが，これらの薬物の発振機構に対する作用については不明である。リチウムは双極性のうつ病や，躁病の治療薬として臨床応用されているが，リチウムの周期延長作用がこの臨床効果を説明できるか否か分かっていない。面白いことに，リチウムは行動上のフリーラン周期を延長するのみならず，視交叉上核（SCN）分散培養下の神経活動のリズムも周期延長を引き起こす（Abe et al, 2000）。重水はSCNのカルシウムオシレーションの発振周期も延長することが知られている。したがって，これら周期を延長する薬物はSCNレベルで作用しているものと考えられるが，時計遺伝子発現に対する作用については不明のままである。

II．概日リズムの光同調

1．グルタメート

光同調を担う物質，すなわち視神経からSCNへの神経経路の主要な神経伝達物質はグルタミン酸であることが知られている（表1）。そこでこの伝達における*Per*遺伝子の役割について述べる。いずれの*Per*遺伝

表1　体内時計同調を担う伝達物質，化合物，処置

視交叉上核依存性リズム	光同調	非光同調
	光パルス	暗パルス
	視神経刺激	メラトニン
	グルタメート	5-$HT_{1A/7}$
	PACAP	GABA/BDZ
	SP	運動
	カルバコール	NPY
	D1受容体	
	ニコチン受容体	
視交叉上核非依存性リズム		
	メタンフェタミン	
	一定時刻給餌	

PACAP：pituitary adenylate cyclase activating polypeptide, SP：substance P, D1：dopamine D1 receptor, 5-HT：serotonin, BDZ：benzodiazepine, NRY：neuropeptide Y.

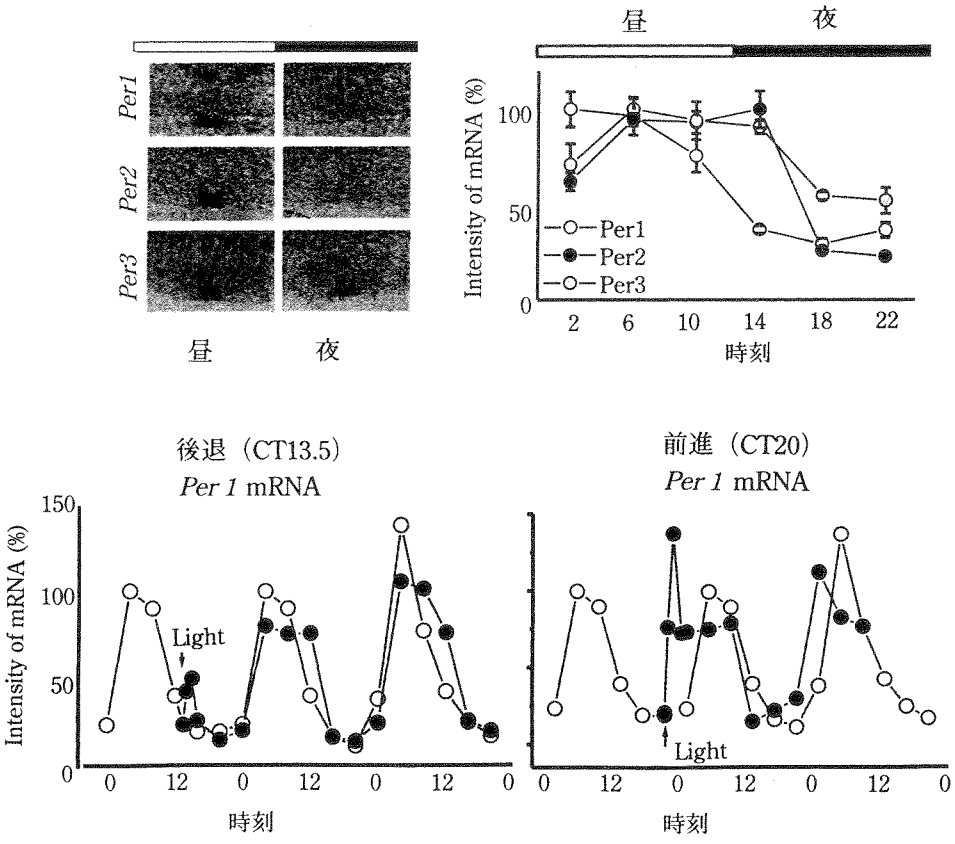

図1　ハムスターの視交叉上核の*Per1*，*Per2*，*Per3*遺伝子発現の日内変動（上側パネル）と，光照射による遺伝子発現増大とその後の位相変化（下側パネル）。昼間の動物の*Per*遺伝子発現は強く，夜間は弱い。夜の初めに光照射すると，その後の*Per1*発現リズムの位相が後退し（左下），夜の終わりに光照射する（右下）と前進する。

子発現もSCNに強く，また主観的昼間に高く，主観的夜間に低いというリズミカルな発現パターンを示す(図1)。SCNにおける*Per*遺伝子発現に対する光照射の効果を調べた結果，主観的夜間の短時間の光照射が，*Per1*のmRNA量を一過性に上昇させ，その後，発現リズムの位相を変えることが観察された(図1)。また*Per1*遺伝子発現上昇作用における光刺激の時刻依存性や照度依存性は，同系統マウスにおける行動リズム位相変化のそれとほぼ一致しており，この発現上昇が光によるリセットと相関していることが明らかになった(Shigeyoshi et al, 1997)。また，*Per2*遺伝子発現も*Per1*と同様，光によって上昇することが明らかとなった(Zylka et al, 1998)。一方で*Per3*遺伝子発現は光刺激によってまったく影響されないことから，光の同調における1次的な反応には関与していないと考えられる(Takumi et al, 1998)。

われわれは光による*mPer1*遺伝子の発現が光の同調における必要条件であることを証明するために翻訳開始点を含む18塩基からなるアンチセンス-S-オリゴヌクレオチドを作成し，その光同調に及ぼす作用を検討した(Akiyama et al, 1999)。まずCT16(CT12を活動開始時刻とする)の光照射による行動リズム位相後退作用に対する効果を検討したところ，アンチセンスは有意に位相変化を抑制し，溶媒投与やランダムオリゴヌクレオチド投与はまったく影響しなかった。さらに視交差上核を含む急性スライスを用い，グルタミン酸適応による神経活動リズムの位相変化に対してもアンチセンスは有意に抑制作用を示した。また最近*mPer2*のアンチセンス-S-オリゴヌクレオチドを作成し，光照射前に投与すると，行動の光による位相後退を阻害した(Wakamatsu et al, 2001a)。これらの実験より光による同調には少なくとも*mPer1*や*mPer2*遺伝子の発現上昇反応が必要であることを証明できた。

光同調はグルタミン酸受容体のうちNMDA受容体の活性化に基づくことを生理学的実験により明らかにしていたので，このNMDA受容体に注目し，NMDA受容体拮抗薬の光による*Per*遺伝子誘導に対する影響を検討した。その結果，NMDA受容体の非競合的拮抗薬MK-801の末梢投与や競合的拮抗薬D-APVの脳室内投与が光による発現上昇を抑制することを観察した。また，NMDAの視交叉上核への局所投与だけで*Per*遺伝子の誘導が惹起されることを明らかにした(Moriya et al, 2000)。

次にNMDA受容体活性化に伴い細胞内へのカルシウムの流入が惹起されるとカルモジュリンキナーゼなどタンパク質リン酸化酵素が活性化される。このリン酸化シグナルが光の*Per*遺伝子発現に関与しているかどうか明らかにするために，カルモジュリンキナーゼ阻害薬のKN-93の効果を調べてみると，キナーゼ活性の抑制と共に光による*Per*遺伝子発現上昇も抑制されることがわかった(図2)。さらに光刺激によってSCNのカルモジュリンキナーゼ活性も上昇すること

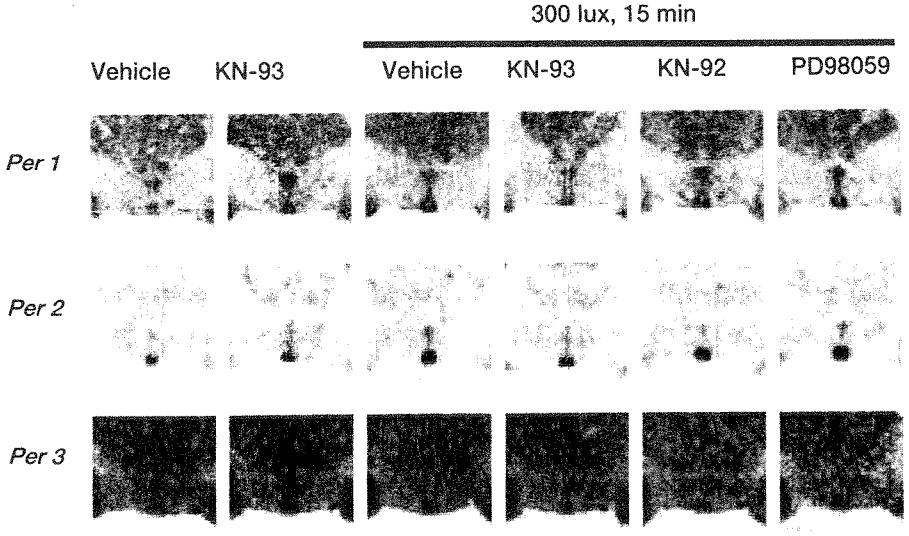

図2　光刺激によるハムスターの視交叉上核の*Per1*, *Per2*, *Per3*遺伝子発現に対するカルシウムカルモジュリンキナーゼ阻害薬の作用。Calcium/calmodulin-dependent protein kinase阻害薬のKN-93は光照射による*Per1*, *Per2*の遺伝子発現を抑制したが，その不活性体であるKN-92では抑制されなかった。またmitogen-activated protein kinaseのカイネース阻害薬のPD98059は光照射による*Per*の発現を拮抗しなかった。

が，免疫組織化学的手法やイムノブロット法によっても確認された。一方，rat-1細胞で指摘されているMAPKの関与についても調べた。その結果，MEKの阻害薬であるPD98056は光による行動上の位相変化もまた*Per1*や*Per2*の一過性の上昇もいずれも抑制しなかった。したがって，光照射RHTの活性化，グルタミン酸の放出，NMDA受容体の活性化，カルモジュリンとカルモジュリン依存性プロテインキナーゼ(CaMK)の活性化といった一連のシグナル系が，光による*Per*遺伝子発現上昇を伝達していることを明らかにすることができた(図2)(Yokota et al, 2001)。

2. PACAP

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)が視神経の伝達物質であり，RHTのグルタミン酸の伝達を調節することが知られている。位相後退が起こる主観的暗期のはじめの光照射による位相後退をPACAPが促進し，一方，主観的暗期の後半の光刺激による位相前進をPACAPは抑制するという(Harrington et al, 1999)。PACAPはPKAの活性化を引き起こすことから，PKAの活性化とそれに引き続くCREBのリン酸化が光り同調を促進する可能性が考えられる。実際*mPer1*の光による発現増大はPKAの同時活性化で促進されることをわれわれは明らかにした。表1には光同調に関わる薬物を列挙した。

Ⅲ. 概日リズムの非光同調

光刺激が夜間に特異的に体内時計の位相を動かすのに対して，多くの非光刺激は昼間に作用して体内時計をリセットする。このような非光同調因子としては，強制的運動，あるいは薬物による覚醒レベルの上昇や恒常明飼育下の暗パルス刺激などが知られている(表1)。神経伝達物質の関連から述べると，外側膝状体からSCNへの伝達物質であるGABAやneuropeptide Yの投与や$GABA_A$受容体の刺激，ベンゾジアゼピン化合物の投与によりこのような同調が引き起こされるが，メラトニンやセロトニン神経もこのような同調にかかわっている。

1. セロトニン神経系

SCNへの神経入力系としては前述したRHTや外側膝状体のほかに，中脳縫線核群の腹内側からSCNへ直接的な神経支配が，また背側から外側膝状体への豊富なセロトニン神経投射があり，外側膝状体を経由してSCNへ情報を供給している。SCNに存在するセロトニンの受容体は，$5\text{-}HT_{1A}$，$5\text{-}HT_{1B}$，$5\text{-}HT_{2A}$，$5\text{-}HT_{2C}$，$5\text{-}HT_{5A}$，$5\text{-}HT_7$が知られている(表2)。体内時計の機構に影響を及ぼすことが分かっているセロトニン受容体の役割について述べる。

表2 視交叉上核に発現するセロトニン受容体と機能

受容体サブタイプ	局在	機能
1A	＋	非光同調
1B	＋＋	光同調抑制
2A	＋＋＋	なし
2C	＋	光同調
5A	＋＋	不明
7	＋＋	非光同調

＋：検出可，＋＋：かなり豊富，＋＋＋：非常に豊富。

1) $5\text{-}HT_{1A}/5\text{-}HT_7$

$5\text{-}HT_7$受容体はその遺伝子発現も蛋白質発現もいずれもSCNに見られることが確認されている。われわれはセロトニン受容体($5\text{-}HT_{1A}/5\text{-}HT_7$)のアゴニストである8-OH-DPATの末梢投与が行動リズムの位相変化を時刻依存的に変化させ，その位相反応曲線は光同調刺激によるものと180°位相を異にしていることを明らかにした(Tominaga et al, 1992)。最近のわれわれの研究では$5\text{-}HT_7$受容体に対してより親和性が高い(＋)8-OH-DPATの方が(－)8-OH-DPATより強力に位相変異を起こすこと，この作用が$5\text{-}HT_7$受容体特異的拮抗薬DR4004で拮抗されることを明らかにした。さらに，SCNの神経活動リズムも行動リズムと同様に非光同調型の位相変化を惹起した。次にわれわれは$5\text{-}HT_{1A}/5\text{-}HT_7$受容体アゴニストの8-OH-DPATが*Per*遺伝子の形成するネガティブフィードバックループに対してどのような影響を与えるのかについてmRNAレベルで検討した(Horikawa et al, 2000)。まずハムスターの輪回し行動リズムを指標にして，8-OH-DPATの位相変異作用を調べると，主観的昼間のCT6において有意な位相前進作用を惹起することを確認した。同条件で*Per*遺伝子発現量を調べると，CT6における8-OH-DPAT投与が*Per1*と*Per2* mRNA量を減少させることが観察された。一方，位相前進を引き起こさないCT1やCT20の8-OH-DPAT投与はmRNA量に影響しなかった。いずれの時間帯に8-OH-DPATを投与しても，光刺激の時と同様*Per3*遺伝子の発現は影響されなかった。非光型の同調時には*Per1*と*Per2*遺伝子発現の低下が重要な過程であることが示唆された。さらにHastingsらのグループも新規輪回しの提示による運動量増加が*Per1*遺伝子の発現低下を惹起することを報告しており，*Per*遺伝子の低下が生理的状態でも惹起されることが判明した(Maywood et al, 1999)。

光同調と非光同調はその位相応答曲線が180度ず

れていることから，両者は相互作用する可能性が考えられる。実際，8-OH-DPATの末梢投与あるいはSCNへの直接投与は光刺激による行動の位相変化やSCNでのFOS発現を抑制する。SCNのセロトニン放出を増大させると，そのセロトニンが5-HT_7受容体を刺激し光同調を抑制するならば，セロトニン放出を低下させると，光同調を促進しそうである。実際MKC-242(5-HT_{1A}受容体アゴニスト)はSCNのセロトニン放出を低下させその結果，光同調を促進させた(Moriya et al, 1998)。

2) 5-HT_{1B}

5-HT_{1B}受容体は視神経の終末に強く発現し，視神経からSCNへの光同調入力を調節している。実際5-HT_{1B}受容体刺激薬のTFMPPを投与すると，光による行動の位相変化を抑制するし，視神経刺激で誘発されるEPSCも抑制する(Pickard et al, 1999)。眼球摘出動物ではこの受容体の発現が消失することからも，5-HT_{1B}受容体の視神経終末の発現が示唆される。面白いことに，この受容体のノックアウトマウスでは，TFMPPによる抑制効果が見られないことから，5-HT_{1B}受容体が光同調に抑制性に制御していることが明らかとなった。

3) 5-HT_{2A}/5-HT_{2C}

前述してきたように，セロトニン神経の活性化あるいは受容体刺激は光同調と正反対の振る舞いを示してきた。ところが，ラットでは5-HT_{2A}/5-HT_{2C}の刺激薬のDOIがSCNのFOS発現を引き起こしたり，松果体のメラトニン分泌を抑制したりし，まるで光刺激と類似した作用を示すことが報告された(Kennaway & Moyer, 1998)。さらに，ラットの背側縫線核を破壊すると，光刺激によるFOSの誘導が減弱し，セロトニンが光刺激に対して促進的に作用する可能性を指摘している。このような差異は動物種差に起因する可能性が考えられる。われわれも，8-OH-DPATによる非光同調がICR系のマウスでは見られないが，C57black系マウスでは見られることを見出している。

4) 5-HT_{5A}

本受容体のサーカディアンリズムにおける関与はごく最近述べられてきている(Duncan et al, 2000)。したがって，この受容体のリズムにおける関与の具体的事例については知られていない。この受容体はSCNに豊富に発現しているし，外側膝状体，さらに縫線核にも強く発現していることから，今後その機能が明らかになっていくものと考えられる。

2. 抗うつ薬

選択的セロトニン再取り込み阻害薬の感情障害(うつ病)治療を考えてみても，セロトニンとうつ病は非常に関係が深い。また，うつ病は不眠や，早朝覚醒など睡眠あるいは睡眠—覚醒リズムの障害の症状を呈する場合が多い。また，高緯度地方では冬季に気分が優れないなど季節性感情障害が起こることもよく知られており，うつ病とサーカディアンリズム異常には何らかのかかわりが存在する可能性が指摘されている。しかしながら実際抗うつ薬のサーカディアンリズム機構に対する明確な作用は知られていない。抗うつ薬がサーカディアンリズム周期を短くするという報告があるがこれに反対する論文もある。抗うつ薬，抗不安薬のゲピロンは5-HT_{1A}/5-HT_7受容体刺激薬のようにそれ自身が非光同調を引き起こし，光同調を抑制するという報告がある。また，フルオキセチンの慢性投与は5-HT_7受容体の不活性化を引き起こすことも報告され，さらに，パロキセチンやデシプラミンの慢性投与がSCNの5-HT_{1B}受容体の不活性化を引き起こすことが報告され，抗うつ薬がセロトニン神経を介してSCNに直接作用する可能性を指摘している。動物のサーカディアンリズム異常に対して抗うつ薬フルオキセチンの慢性投与がこれを改善するという報告もある。これらの結果が，うつ病のサーカディアンリズム異常仮説にどのように関わってくるのかは不明なままである。

3. GABA神経系

SCNにはGABA神経細胞が豊富に含まれる。実際60〜70%がGABA陽性あるいはGAD陽性細胞である。したがって，SCNのGABAは重要な役割を演じている可能性がある。まず，GABAが昼間に興奮性の作用を，夜間に抑制性の作用をすることで，SCNの神経活動を昼高く夜低くしている可能性が示唆された(Wagner et al, 1997)。しかしこれに反論し，GABAは常に抑制性の伝達をするという論文もある。また，GABAはSCNの個々の神経発火を同期化する働きがあることが示された(Liu & Reppert, 2000)。また，SCNの神経核としての働きはギャップジャンクションの働きによるという報告もある。ギャップジャンクションをダイカップリングで調べた研究によると，昼間はダイカップリングが強く夜は弱いという。また，ギャップジャンクションはハロタンや$GABA_A$受容体刺激薬のムシモールでブロックされるという。したがって，GABAは活動依存的のみならず，ギャップジャンクションの働きを介して神経の同期を制御している可能性が考えられる。一方，外側膝状体の伝達物質であるGABAは非光同調を担う物質でもある。実際GABAやムシモールの投与は非光同調と類似した時計のリセットを引き起こした(Tominaga et al, 1994)。先に述べた

ように8-OH-DPATが光同調を抑制したように，ムシモールのSCNへの直接投与や末梢投与は光刺激による行動の位相変化やSCNでのFOSの発現を抑制する。

4. ベンゾジアゼピン

ベンゾジアゼピンはGABAの作用を増強することはよく知られた事実であるが，実際フルニトラゼパムがSCNのGABAによるIPSCを増強することが知られている。したがってベンゾジアゼピンの作用部位はSCNに存在する可能性が考えられる。トリアゾラム(Van Reeth & Turek, 1989)を主観的昼間に投与すると，顕著な位相前進が起こり，この作用はムシモールと類似している。われわれはブロチゾラムでも同様の位相前進作用を認めており(図3)，このような薬物が本当に*Per*遺伝子の発現を変えて時計機構に働いているのか興味が持たれる点である。実際トリアゾラムやブロチゾラムといったベンゾジアゼピン系薬物は主観的昼間に投与するとハムスターの*Per1*や*Per2*遺伝子発現量を低下させることが判明した(Horikawa et al, 2000；Yokota et al, 2000)。したがって，ブロチゾラムはSCNに直接作用した可能性が考えられる。ところで，トリアゾラムの位相前進作用はセロトニン神経の破壊で消失することから，この作用にセロトニン神経系が関わっている可能性が指摘されている。実際，ブロチゾラムによる*Per*遺伝子発現低下作用は8-OH-DPATとよく類似していた。したがってブロチゾラムはセロトニン神経の活性化を通して，SCNの*Per1*や*Per2*発現を低下させた可能性も考えられる。このことを実証するために，8-OH-DPATとブロチゾラムを同時投与したところ，これらの薬物の単独投与と併用投与はほとんど同程度の位相前進，*Per1*や*Per2*発現低下を引き起こした。このことから，8-OH-DPATとブ

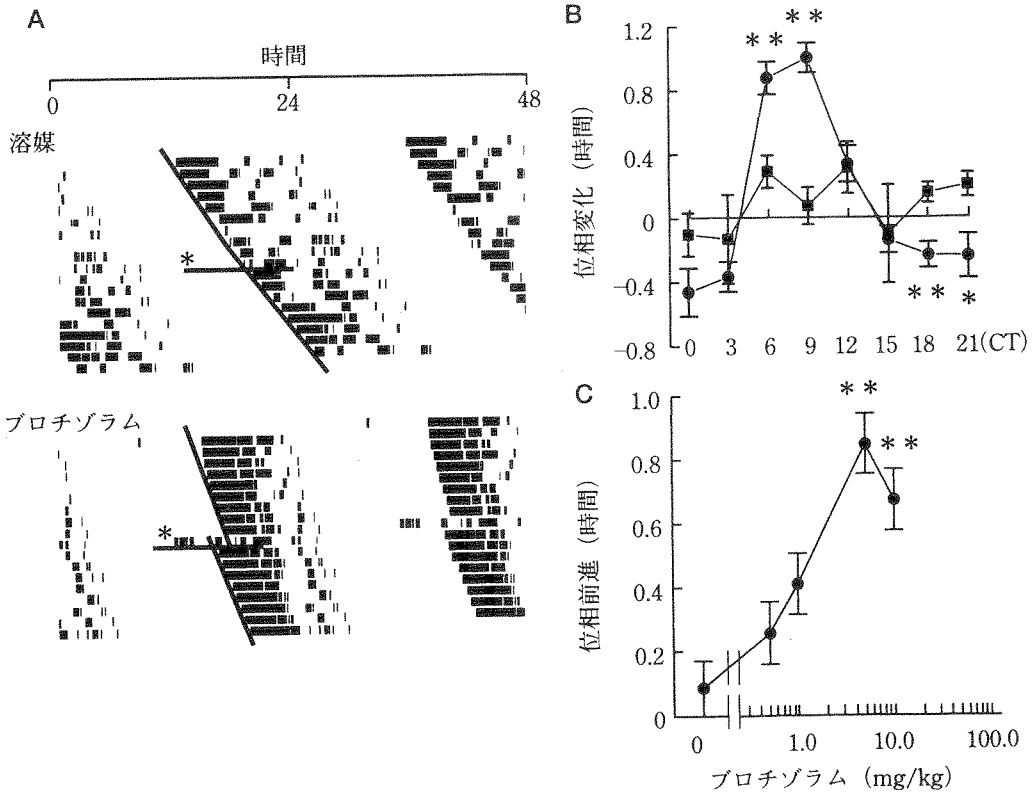

図3 ハムスターのサーカディアン輪回し行動リズムに対するブロチゾラムの作用。Aは行動リズムのダブルプロットで，代表例を示す。溶媒投与では活動開始の位相はまったく影響を受けなかった(上図)，一方，ブロチゾラムの投与は活動の位相開始を早めた(下図)。*印は薬物投与時刻を示す。Bはブロチゾラムの位相前進作用の時刻依存性を示す。黒丸がブロチゾラム(5 mg/kg)投与群で黒四角が溶媒投与群である。CT6，CT9のブロチゾラム投与により有意な位相前進が，CT18，CT21投与で有意な位相後退が見られる。縦軸のプラスは位相前進を，マイナスは位相後退を表す。それぞれの点は5〜10匹のハムスターの平均値である。**$P<0.01$，*$P<0.05$ (Student's t-test)。Cはブロチゾラムの CT6投与の用量依存性を表すグラフである。それぞれの点は4〜10匹のハムスターの平均値である。**$P<0.01$ (Dunnett's test)，ブロチゾラム(0 mg/kg)との差。

ロチゾラムが有する位相前進作用や *Per1* や *Per2* の発現低下は共通した細胞内情報伝達機構を介しているものと考えられた。

トリアゾラムやブロチゾラムは不眠症治療に広く使用されている薬物である。このように，これらベンゾジアゼピン系薬物は不眠症治療に広く用いられるだけでなく，体内時計の位相前進を期待できる薬物でもある。したがって，位相前進を強いる時差間の移動時に出現する不眠症に対してベンゾジアゼピンを使用することは，不眠症軽減，時差ぼけ軽減ならびに位相前進促進作用のいずれも期待できる。このことを図4で模式的に説明する。昼から夕方さらに夜半にかけてブロチゾラムを服用すると，*Per1*，*Per2* の一過性の低下が起こり次の周期の位相が前進する。つまり，*Per1*，*Per2* 遺伝子発現が減少する時間帯に薬物によりより早く低下させると，次の発現が早く起こることになる（図4A）。したがって，時差ぼけ以外にも，睡眠相位相後退症の患者を対象に，夜の前半にブロチゾラムを服用させると，位相が前にずれ，睡眠相位相後退症が軽減される可能性が強く示唆される。

ところで，光による行動の位相前進やSCNにおける *Per1*，*Per2* 遺伝子の発現増大がブロチゾラムの投与により消失することがわかった（図5）。したがってここでも再び光同調と非光同調の相互作用が *Per* mRNA の発現量の変化で説明し得る可能性が示唆された。われわれの体内時計は24時間より長いため，通常は早朝の光暴露で体内時計を30～60分前進させ，外界の24時間周期に合わせているわけであるが，ブロチゾラムの服用があまりに遅く，朝の光で体内時計をリセットする時にもこの薬物が体内に十分に存在すると，そのリセットが悪くなることが示唆された。このことは図4Bで模式的に説明している。したがって，朝の光による体内時計のリセット時にこれらのベンゾジアゼピンが少なくともSCNには存在しないことが望まれる。

脳に発現するニューロステロイドはGABAの作用に類似していることが知られているが，dehydroepiandrosterone sulphate（DHEAS）は主観的昼間投与で位相前進を，また光による位相変位作用を抑制することが報告された。つまり，ニューロステロイドはGABAやベンゾジアゼピンの働きに類似していた。

Ⅳ．視交叉上核非依存性リズムの同調

1．時刻記憶の形成方法と特徴

ラットやマウスに1日のある決まった時刻に餌を提示すると動物は徐々にその時刻を覚え，餌提示の2～3時間前より活動が活発になってくる，いわゆる予知行動が出現してくるようになる（Hara et al, 2001）。

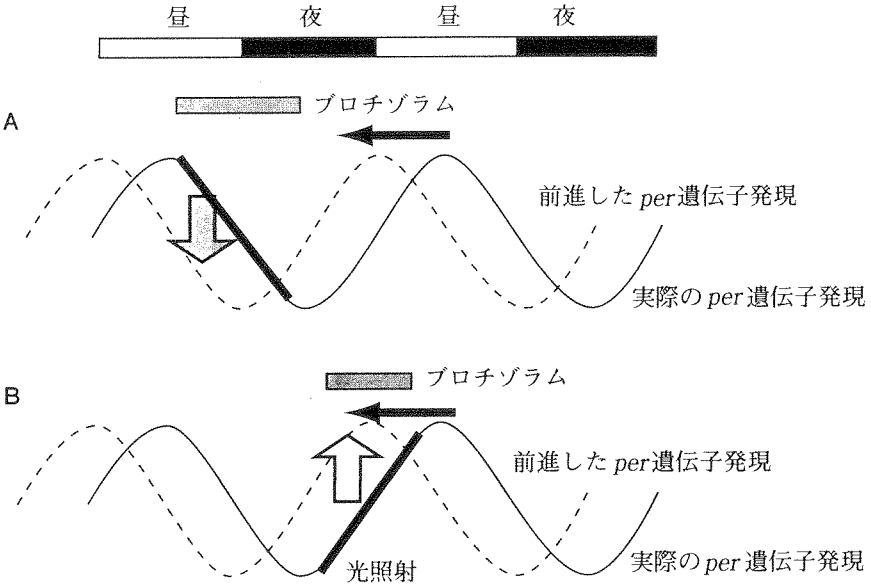

図4　ブロチゾラムによる体内時計前進作用を説明する模式図。ブロチゾラムは *Per* 遺伝子発現が減少する時間帯に作用すると位相前進を引き起こす可能性が考えられる（**A**）。また，光による *Per* 遺伝子の発現増大を起こす時期にブロチゾラムが存在すると，それを抑制する可能性がある（**B**）。実線は実際の *Per* の発現を示し，破線はブロチゾラムや光照射で起こる位相前進を表す。

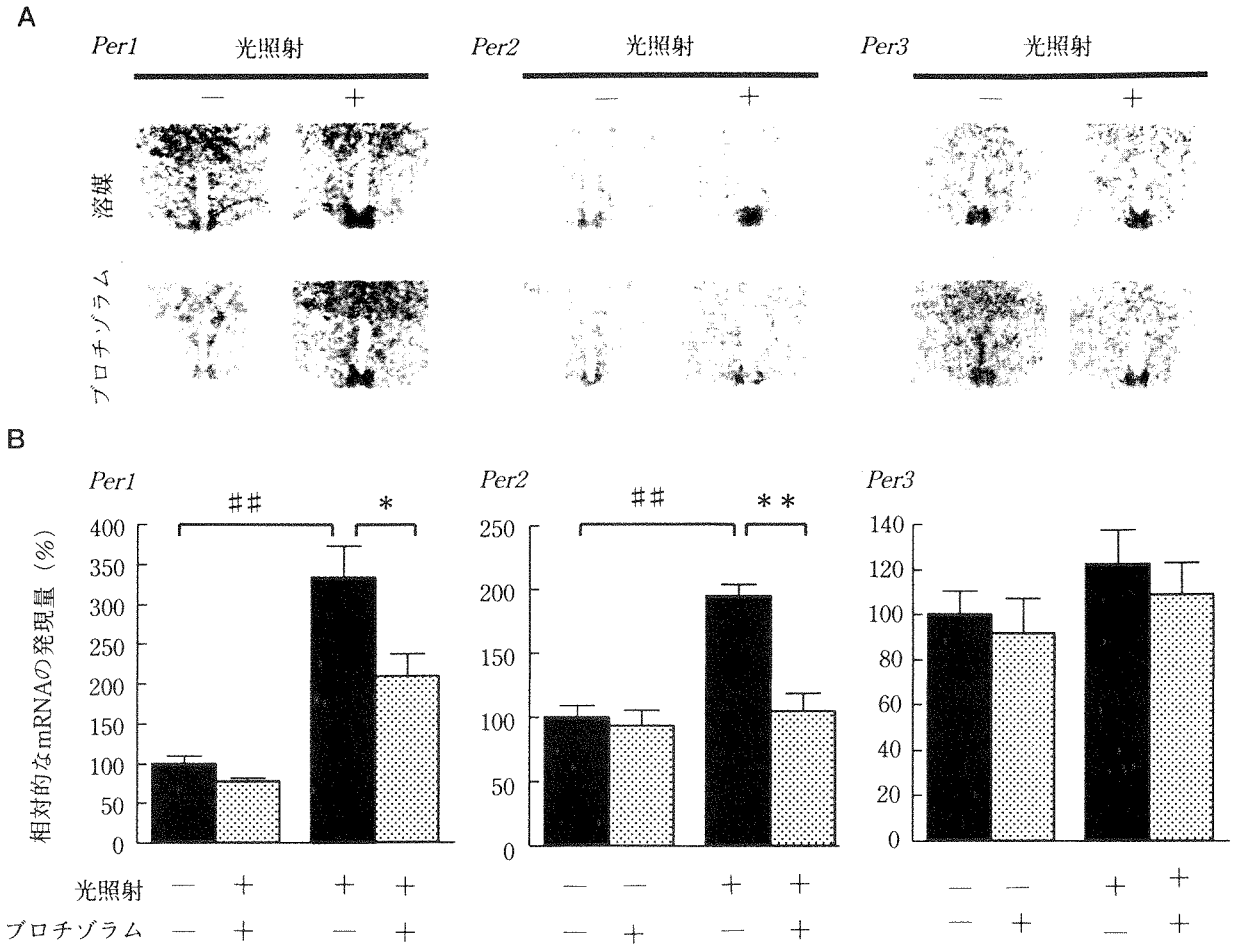

図 5 光照射による視交叉上核 *Per1*, *Per2* および *Per3* の発現に対するブロチゾラムの作用。溶媒もしくはブロチゾラム（5 mg/kg）を CT19.5 に投与し，CT20 に光（5 lux）を 15 分照射した。CT21.5 に脳を還流固定し，視交叉上核の *Per* 遺伝子発現について調べた。**A** は実際のシグナルを示す。**B** は 4 匹の動物の平均値を示す。##P＜0.01，**P＜0.01，*＜P0.05（Tukey's test）。

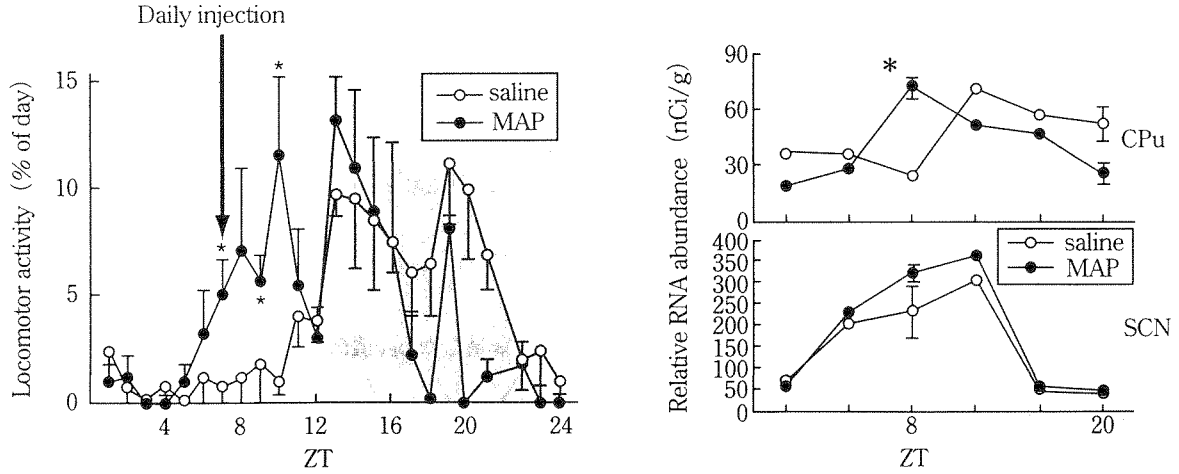

図 6 毎日一定時刻のメタンフェタミン（MAP）投与の予知行動（左側）ならびに *Per1* 遺伝子発現に及ぼす影響（右側）

メタンフェタミン 5 mg/kg を昼間の ZT7 に 6 日間毎日投与し，7 日目に活動量を測定する。MAP 群は以前投与していた時間帯に活動量の増大が見られる。行動観察とまったく同一のスケジュールで，線条体（CPu）と視交叉上核（SCN）の *Per1* 遺伝子発現を調べた。線条体の *Per1* 遺伝子発現は MAP 投与によりピーク位相が前進した。

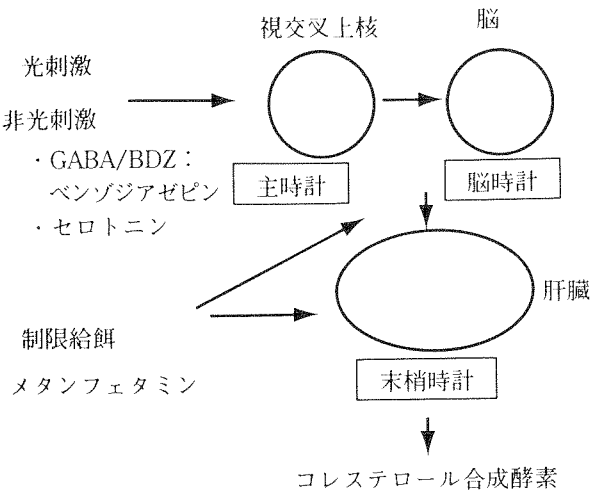

図 7　体内時計階層構造の模式図。視交叉上核に主時計があり大脳皮質や小脳などに脳時計があり，肝臓，心臓などに末梢時計がある。これらの脳と末梢時計はローカル時計としてその組織の時間情報を発現し，主時計の視交叉上核がローカル時計の位相のタイミングを調整している。

この行動はSCNを破壊した動物でも見られることから，SCN非依存性リズムと称される。齧歯類は夜行性動物であるため，夜間に活動が盛んになり，かつ摂食行動も盛んになるので，一般的には昼間の2〜4時間を制限摂食時間とする場合が多い。また，覚醒剤であるメタンフェタミンを毎日一定時刻に投与しても，制限給餌と同様に時刻の記憶が形成できる（図6左側）。制限給餌やメタンフェタミンの投与開始から3日目くらいから時刻の記憶ができ始め，1週間でほぼ完成

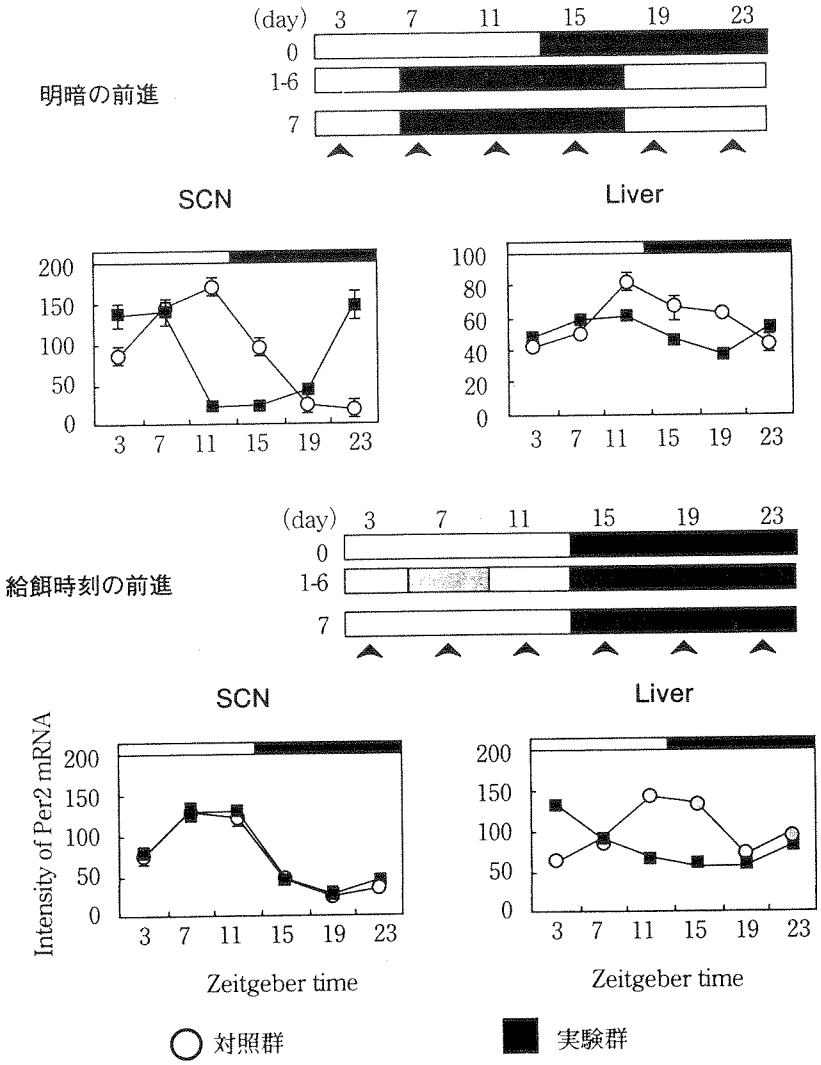

図 8　明暗環境を7時間前進（上側）させたときと給餌時間帯を7時間前進（下側）させたときの視交叉上核ならびに肝臓の *Per2* 遺伝子発現リズム。肝臓の *Per2* 発現リズムは光環境より給餌環境の変化により敏感に反応する。

する。このように完成した動物を翌日絶食させたり，メタンフェタミンを投与しなくても，以前に処置していた時間帯に動物の活動量は増大してくる。しかしながら，このような動物を自由摂食に戻したり，メタンフェタミンを投与しなかったら，時刻記憶に基づく，活動の増大は消失してくる。したがって，餌を提示したりメタンフェタミンを投与することを条件刺激と考えると，この時刻認知の学習には習得曲線と消去曲線が得られることになる。この給餌性リズムはSCN性のリズムによく類似している。たとえば給餌の周期が22～31時間の範囲内でないと同調できない，給餌性リズムも絶食させるとフリーランし，餌を提示する時刻を変更すると移行期がみられる。唯一SCN性リズムと異なる点は，能動的な発振系でない点である。

2. 体内時計遺伝子と時刻記憶

*Per*遺伝子はSCN(主時計)に明瞭に発現するのみならず，大脳皮質，海馬，小脳などの脳時計にも強く発現することが知られている。さらに脳のみならず，心臓，肝臓，骨格筋など末梢時計にも幅広く発現し，かつ明瞭なリズムを刻むことがよく知られている(図7)。先に述べたように給餌性リズムはSCN性のリズムとよく似ていることから，この給餌性リズムにも*Per*遺伝子発現が関わっている可能性が示唆される。

マウスに制限給餌を6日間行い，次の日に絶食下に動物の脳と肝臓を取り出し，*Per1*と*Per2*のmRNA発現を調べたところ，大脳皮質や室傍核では夜の始まりをピークとする*Per1*，*Per2*のmRNA発現リズムが昼間に制限摂食させることにより，そのピークを昼間に移すことがわかった(Wakamatsu et al, 2001b)。しかしながらSCNの*Per*遺伝子発現は制限給餌により何ら影響を受けることはなかった。SCNを破壊すると大脳皮質の*Per*の発現リズムは消失するものの，制限給餌を行うと，大脳皮質の*Per*の発現リズムは再びリズム性を取り戻し，その発現パターンは当然昼間にピークとするものであった。さらに，肝臓の*Per*遺伝子の発現リズムが明暗環境の位相前進と給餌時間の位相前進といずれの方により反応するかを調べてみた(図8)。SCNは光環境のシフトに反応するが，給餌のシフトに反応しなかった。一方，肝臓では逆に，給餌のシフトにより強く反応した。したがって，恐らく，脳時計や末梢時計に発現している*Per*遺伝子は予知行動を引き起こす給餌性リズム形成にも重要な役割を果たしているものと考えられる(Hara et al, 2001；Damiola et al, 2000)(図8)。

メタンフェタミンも毎日1回投与すると，約1週間の投与で予知行動が現れるようになる(図6)。予知行動が完成したこの時期に*Per*遺伝子の発現を調べて見ると，*Per1*も*Per2*の両方の遺伝子ともに線条体では昼間にピークを有するようなリズムを形成するようになる(図6右側)。しかしながらこの場合でもSCNの*Per*遺伝子発現リズムにはまったく影響を及ぼさなかった。

V. 体内時計出力

SCNからの出力機構は不明な点が多いが，神経系としてはバソプレッシンやVIPが考えられている。SCNは室傍核にGABA，グルタミン酸神経を介して出力信号を出し，それがシナプスを介した松果体のメラトニン分泌に影響を及ぼす。最近の研究では室傍核のGABA神経が実際メラトニン分泌に関わっていることを報告している。また，SCNからの出力は視索前野に興奮性と抑制性の出力を出し，睡眠リズムに関わる可能性が指摘されている。さらに神経の逆行性のトレースでSCN出力が室傍核を介して心臓や肝臓，副腎などへ出ているとの研究があり，これらのローカル時計の位相調節に関わっている可能性が示唆される。このことはSCNが中心時計として働き，他の部位に発現している時計遺伝子はローカル時計として働き，SCNからの情報が体内時計システムとして働いているものと考えられる。

おわりに

Per1, *Per2*, *Per3*は中枢神経のみならず心臓，骨格筋，肝臓，副腎などに強く発現していることが知られている。時計遺伝子はそれぞれ発現している器官での「ローカル時計」の働きに寄与し，SCNの主時計は「時計管理」を行っているものと考えている。すなわち生体はこの時計の階層構造をうまく利用し，生体のホメオスタシス機構を維持しているものと考えられる。そこで，生体リズム分子機構の急速な解明が脳疾患治療薬の開発に大いに役立つことを期待したい。

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Abstract

Pharmacological study on circadian rhythms

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Precise, rhythmic, daily change of the internal milieu is a conspicuous feature of all living organisms. It affects temporal patterns of all kinds of behaviors during a day and deeply influences both social structure and daily life of individual human beings. These daily variations arise from the internal circadian mechanisms. Three functions of the endogenous clock are discriminated as a rhythm generation, entrainment to light-dark cycle and output from the clock. Endogenous clock is localized in the suprachiasmatic nucleus (SCN) in mammals. Recent papers demonstrated strong expression of clock genes such as *Per1, Per2* and *Per3* in the SCN. As serotonin/antidepressant and GABA/benzodiazepine drugs affect the light and non-light-induced entrainment, these drugs can regulate the circadian oscillation of clock genes and environmental stimuli-induced change of *Per* gene expression in the SCN.

There are two main stimuli that entrain circadian rhythm ; light-dark cycle (LD) and restricted feeding. Light resets circadian clock with induction of *Per1* and *Per2* gene in the SCN, the locus of a main oscillator. We examined which stimuli, restricted feeding (RF) or lighting, reset clocks in the mouse brain clock such as cerebral cortex and/or peripheral clock such as liver because of the widespread expression (hippocampus, cerebellum and also peripheral tissue) of *Per* genes. Mice were allowed access to food for 4 h during daytime (7 h advance of feeding time) under LD or constant darkness. The peaks of *Per1* and *Per2* mRNA in the cerebral cortex and liver were advanced 6-12 h after 6 days of RF, whereas those in SCN were unaffected. The increase of *Per* expression by RF treatment was observed in SCN-lesioned mice. The present results suggest that RF strongly entrained the expression of *Per* and clock-controlled gene in the cerebral cortex and liver without affecting light-dependent SCN clock function. Moreover, I mentioned the possibility that some drugs are useful to keep physiological homeostasis through their action on biological systems (main, brain and peripheral clocks).

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見が急速に蓄積している．さらに時計遺伝子の変異によるヒトにおけるリズム異常が報告され，これまでアプローチが困難であったヒトの時差症候群（ジェットラグ），老人の睡眠障害，睡眠相遅延症候群，うつ病，心臓疾患，高血圧などのリズム異常と関連の深い疾患の原因解明および診断，治療への応用の道も見出されつつある．哺乳類研究におけるマウスなど実験動物の有用性は明らかであるが，いまや，臨床検査項目としての個人における体内時間の決定法や，上にあげた臨床疾患に対しての新しい時間治療法の導入も射程距離内に入ったといえる．

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特 集
概日リズムの分子精神医学

概日リズムの薬理学

柴田重信*

リズム障害や，時差ぼけ軽減のためメラトニンや睡眠導入薬のベンゾジアゼピンが臨床的に使われてきたが，その作用機構についてはわからなかった．ところで，分子生物学的手法の成功により体内時計機構の分子レベルの解明は急速に進んでいる．ここでは，体内時計の発振，同調，出力機構を，伝達物質—細胞内情報伝達—時計遺伝子といった一連の流れから説明し，さらに，先に述べた薬物の作用機構を時計遺伝子の発現変化で説明している．とくに，セロトニンと抗うつ薬，GABAとベンゾジアゼピンという組み合わせで，体内時計の薬理学的視点について解説している．

KEY WORDS
視交叉上核
セロトニン
時計階層性
ベンゾジアゼピン
時計遺伝子

1．視交叉上核依存性リズム

1）光同調系に作用する薬物

光同調を担う物質，すなわち視神経からSCNへの神経経路の主要な神経伝達物質はグルタミン酸であることが知られている（表1）．いずれの*Per*遺伝子発現もSCNに強く，また主観的昼間に高く，主観的夜間に低いというリズミカルな発現パターンを示す．SCNにおける*Per*遺伝子発現に対する光照射の効果を調べた結果，主観的夜間の短時間の光照射が，*Per 1*のmRNA量を一過性に上昇させ，その後発現リズムの位相を変えることが観察された（図1A）．*mPer 1*や*mPer 2*のアンチセンス-S-オリゴヌクレオチドを光照射前に投与すると，光による行動の位相後退を阻害した[1)2)]．これらの実験より光による同調には少なくとも*mPer 1*や*mPer 2*遺伝子の発現上昇反応が必要であることを証明できた．最近の*Per*遺伝子ノックアウトの研究によれば，光による位相後退にはより*Per 2*が，位相前進には*Per 1*遺伝子の活性化が重要であるという．

NMDA受容体拮抗薬の光による*Per*遺伝子誘導と行動に対する影響を検討した．その結果，NMDA受容体の非競合的拮抗薬MK-801の末梢投与や競合的拮抗薬D-APVの脳室内投与が光による*Per 1*，*Per 2*いずれの発現上昇もまた行動の位相変化も抑制することを観察した．また，NMDAのSCNへの局所投与でも*Per*遺伝子の誘導が惹起されることを明らかにした[3)]．NMDA受容体活性化以降のCaMK II/IVやMAPKの情報伝達

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けることはなかった．SCNを破壊した動物でも類似の結果が得られた．また，肝臓でも，遺伝子発現は給餌のシフトにより強く反応した．したがって，脳時計や末梢時計に発現している*Per*遺伝子は予知行動を引き起こす給餌性リズム形成にも重要な役割を果たしているものと考えられる（図2）[8)10)]．中枢時計から末梢時計への連絡を薬理学的観点から調べてみると，①交感神経系，②ACTH—副腎皮質ホルモン系がその候補にあげられている．

3．明暗環境シフトによる睡眠障害に対する食事時間の変更

以上の研究から時差ぼけが起こるメカニズムとして，視交叉上核の主時計が光環境にすばやく同調するのに対して，脳時計や末梢時計は時間がかかり，この再同調の時間的なずれが原因であるとする考えがある．そこで，ハワイ旅行のため位相を前進させる必要がある海外旅行者に対して，3日前より食事時間を前倒しにしたところ，とくに高齢者（60歳以上）の時差ぼけに関連した睡眠障害が軽減することがわかった[11)]．このことは睡眠相後退症候群の患者に対して，同じような食餌療法が有効である可能性を示唆するものであった．

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型の同調時には *Per 1* と *Per 2* 遺伝子発現の低下が重要な過程であることが示唆された．さらに Hastings らのグループも新規輪回しの提示による運動量増加が *Per 1* 遺伝子の発現低下を惹起することを報告しており，*Per* 遺伝子の低下が生理的状態でも惹起されることが判明した．

ii）5-HT_{1B}

5-HT_{1B}受容体は視神経の終末に強く発現し，視神経から SCN への光同調入力を調節している．実際 5-HT_{1B}受容体刺激薬の TFMPP を投与すると，光による行動の位相変化を抑制するし，視神経刺激で誘発される EPSC も抑制する．眼球摘出動物ではこの受容体の発現が消失することからも，5-HT_{1B}受容体の視神経終末の発現が示唆される．面白いことに，この受容体のノックアウトマウスでは，TFMPP による抑制効果がみられないことから，5-HT_{1B}受容体が光同調に抑制性に制御していることが明らかとなった．

iii）5-HT_{2A}/5-HT_{2C}

前述してきたように，セロトニン神経の活性化あるいは受容体刺激は光同調と正反対の振る舞いを示してきた．ところが，ラットでは 5-HT_{2A}/5-HT_{2C}の刺激薬の DOI が SCN の FOS 発現を引き起こしたり，松果体のメラトニン分泌を抑制したりし，まるで光刺激と類似した作用を示すことが報告された．さらに，ラットの背側縫線核を破壊すると，光刺激による FOS の誘導が減弱し，セロトニンが光刺激に対して促進的に作用する可能性を指摘している．このような差異は動物種差に起因する可能性が考えられる．

iv）5-HT_{5A}

本受容体のサーカディアンリズムにおける関与はごく最近述べられてきている．したがって，この受容体のリズムにおける関与の具体的事例については知られていない．この受容体は SCN に豊富に発現しているし，外側膝状体，さらに縫線核にも強く発現していることから，今後その機能が明らかになっていくものと考えられる．

3．抗うつ薬

選択的セロトニン再取り込み阻害薬の感情障害（うつ病）治療を考えてみても，セロトニンとうつ病は非常に関係が深い．また，うつ病は不眠や，早朝覚醒など睡眠あるいは睡眠—覚醒リズムの障害の症状を呈する場合が多く，うつ病とサーカディアンリズム異常には何らかのかかわりが存在する可能性が指摘されている．しかし抗うつ薬のサーカディアンリズム機構に対する明確な作用は知られていない．抗うつ薬がサーカディアンリズム周期を短くするという報告があるがこれに反対する論文もある．抗うつ薬，抗不安薬のゲピロンは 5-HT_{1A}/5-HT_7受容体刺激薬のようにそれ自身が非光同調を引き起こし，光同調を抑制するという報告がある．また，フルオキセチンの慢性投与は 5-HT_7受容体の不活性化を引き起こすことも報告され，さらに，パロキセチンやデシプラミンの慢性投与が SCN の 5-HT_{1B}受容体の不活性化を引き起こすことが報告され，抗うつ薬がセロトニン神経を介して SCN に直接作用する可能性を指摘している．動物のサーカディアンリズム異常に対して抗うつ薬フルオキセチンの慢性投与がこれを改善するという報告もある．これらの結果が，うつ病のサーカディアンリズム異常仮説にどのようにかかわってくるのかは不明なままである．

4．GABA 神経系

SCN には GABA 神経が豊富に含まれ，実際 60～70%が GABA 陽性あるいは GAD 陽性細胞である．したがって，SCN の GABA は重要な役割を演じている可能性がある．まず，GABA が昼間に興奮性の作用を，夜間に抑制性の作用をすることで，SCN の神経活動を昼高く夜低くしている可能性が示唆された．しかしこれに反論した，GABA はつねに抑制性の伝達をするという論文もある．また，GABA は SCN の個々の神経発火を同期化するはたらきがあることが示された[6]．外側膝状体の伝達物質である GABA は非光同調を担う物質でもある．実際 GABA やムシモールの投与は非光同調と類似した時計のリセットを引き起こした．先に述べたように 8-OH-DPAT が光同調を抑制したように，ムシモールの SCN への直接投与や末梢投与は光刺激による

行動の位相変化やSCNでのFOSの発現を抑制する．

5．ベンゾジアゼピン

ベンゾジアゼピンがGABAの作用を増強することはよく知られた事実であるが，実際フルニトラゼパムがSCNのGABAによるIPSCを増強することが知られている．したがってベンゾジアゼピンの作用部位はSCNに存在する可能性が考えられる．トリアゾラムを主観的昼間に投与すると，顕著な位相前進が起こり，この作用はムシモールと類似している．トリアゾラムやブロチゾラムといったベンゾジアゼピン系薬物は主観的昼間に投与するとハムスターの*Per 1*や*Per 2*遺伝子発現量を低下させることが判明した[5)7)]．したがって，ベンゾジアゼピン系薬物はSCNの*Per 1*や*Per 2*遺伝子発現量を一過性に低下し，位相を変えるものと考えられた．位相前進を強いる時差間の移動時に出現する不睡症に対してベンゾジアゼピンを使用することは，不眠症軽減，時差ぼけ軽減ならびに位相前進促進作用のいずれも期待できる．このことを**図1**で模式的に説明する．昼から夕方さらに夜半にかけてベンゾジアゼピンを服用すると，*Per 1*，*Per 2*の一過性の低下が起こり，つぎの周期の位相が前進する．つまり，*Per 1*，*Per 2*遺伝子発現が減少する時間帯に薬物によりより早く低下させると，つぎの発現が早く起こることになる（**図1B**）．

脳に発現するニューロステロイドはGABAの作用に類似していることが知られているが，dehydroepiandrosterone sulphate（DHEAS）は主観的昼間投与で位相前進を，また光による位相変位作用を抑制することが報告された．つまり，ニューロステロイドはGABAやベンゾジアゼピンのはたらきに類似していた．

2．視交叉上核非依存性リズムと同調

時計遺伝子は主時計であるSCNに発現するのみならず，大脳皮質，小脳，さらに心臓，肝臓，骨格筋といった末梢臓器にも強い発現があることが知られている．つまり「主時計」，「脳時計」，「末梢時計」といった時計の階層構造の仕組みが想定される（**図2**）．主時計のSCNを壊すとすべての時計が止まることから，主時計から末梢時計に向かって，何らかの時間の情報が伝わっ

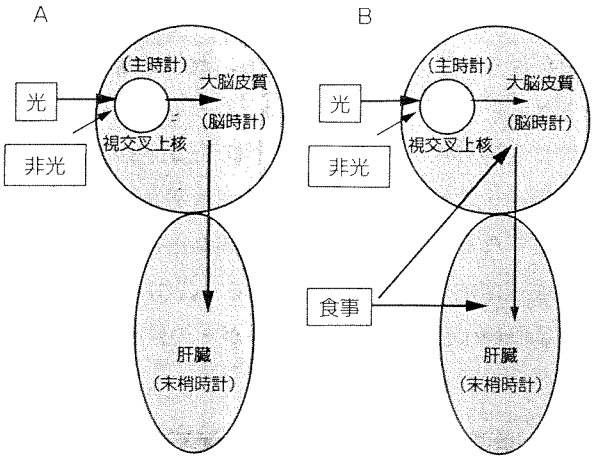

図2．体内時計階層構造の模式図ならびに光（非光）同調と給餌性同調の作用様式
（A）視交叉上核に主時計があり大脳皮質や小脳などに脳時計があり，肝臓，心臓などに末梢時計がある．これらの脳と末梢時計はローカル時計としてその組織の時間情報を発現し，主時計の視交叉上核がローカル時計の位相のタイミングを調整している．
（B）一定時刻の給餌刺激は，主時計と無関係に脳時計や末梢時計の位相変化を引き起こす．

ているものと考えられる．この情報システムの詳細は不明であるが現在考えられていることについて述べる．ラットやマウスに1日のある決まった時刻に餌を提示したり，メタンフェタミンを投与すると動物は徐々にその時刻を覚え，餌や薬物の提示の2〜3時間前より活動が活発になってくる，いわゆる予知行動が出現してくるようになる[8)]．この行動はSCNを破壊した動物でもみられることから，SCN非依存性リズムと称される．この給餌性リズムはSCN性のリズムによく類似している．たとえば給餌の周期が22〜31時間の範囲内でないと同調できない，給餌性リズムも絶食させるとフリーランし，餌を提示する時刻を変更すると移行期がみられる．唯一SCN性リズムと異なる点は，能動的な発振系でない点である．

マウスに制限給餌を6日間おこない，つぎの日に絶食下に動物の脳と肝臓を取りだし，*Per 1*と*Per 2*のmRNA発現を調べたところ，大脳皮質や室傍核では夜のはじまりをピークとする*Per 1*，*Per 2*のmRNA発現リズムが昼間に制限摂食させることにより，そのピークを昼間に移すことがわかった（**図2**）[9)]．しかしながらSCNの*Per*遺伝子発現は制限給餌により何ら影響を受

表 1. 体内時計同調機構にかかわる薬物

視交叉上核依存性リズム		視交叉上核非依存性リズム
光同調	非光同調	同調
光	強制運動	制限給餌
グルタメート	GABA	メタンフェタミン
(NMDA)	ベンゾジアゼピン	グルココルチコイド
PACAP	(ニューロステロイド)	NADPH/NPAS2
Substance P	メラトニン	
5-$HT_{2A/2C}$	Neuropeptide Y	
	5-$HT_{1A/7}$	
	5-HT_{1B}	

それぞれの同調を引き起こす薬物，化合物，受容体を示す．

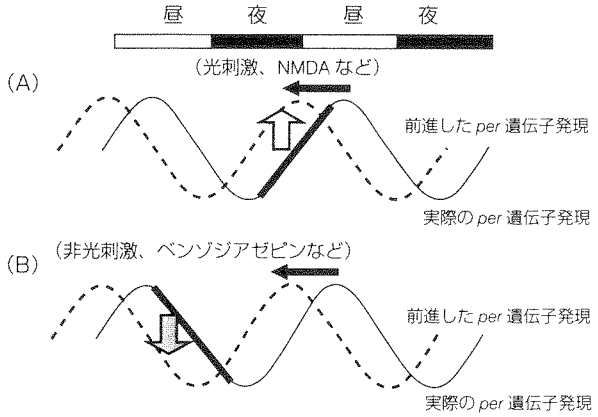

図 1. Per 遺伝子発現を指標とした光同調ならびに非光同調の模式図
光照射により視交叉上核 *Per 1* および *Per 2* 遺伝子発現は一過性に増大し，その後の *Per* 遺伝子発現リズムの位相を変える（A）．逆に，ベンゾジアゼピンの投与により，*Per 1* や *Per 2* 遺伝子発現が一過性に抑制され，その後の *Per* 遺伝子発現の位相が変化する（B）．

における関与を調べた．MEK 阻害薬と異なり，CaMK II/IVの阻害薬は光による行動上の位相変化もまた *Per 1* や *Per 2* の一過性の上昇もいずれも抑制した．したがって，光照射，視神経からのグルタミン酸の放出，NMDA 受容体の活性化，カルモデュリンと CaMK II/IVの活性化といった一連のシグナル系が，光による *Per* 遺伝子発現上昇を伝達していることを明らかにすることができた[4]．

2）非光同調系に作用する薬物

光刺激が夜間に特異的に体内時計の位相を動かすのに対して，多くの非光刺激は昼間に作用して体内時計をリセットする．このような非光同調因子としては，強制的運動，あるいは薬物による覚醒レベルの上昇や恒常明飼育下の暗パルス刺激などが知られている（**表 1**）．神経伝達物質の関連から述べると，外側膝状体から SCN への伝達物質である GABA や neuropeptide Y の投与や $GABA_A$受容体の刺激，ベンゾジアゼピン化合物の投与によりこのような同調が引き起こされるが，メラトニンやセロトニン神経もこのような同調にかかわっている．

a．セロトニン神経系

SCN への神経入力系としては前述した視神経以外に，中脳縫線核群の腹内側から SCN へ直接的な神経支配が，また背側から外側膝状体への豊富なセロトニン神経投射があり，外側膝状体を経由して SCN へ情報を供給している．SCN に存在するセロトニンの受容体は，5-HT_{1A}，5-HT_{1B}，5-HT_{2A}，5-HT_{2C}，5-HT_{5A}，5-HT_7 が知られている（**表 1**）．体内時計の機構に影響を及ぼすことがわかっているセロトニン受容体の役割について述べる．

i）5-HT_{1A}/5-HT_7

5-HT_7受容体はその遺伝子発現も蛋白質発現もいずれも SCN にみられることが確認されている．セロトニン受容体（5-HT_{1A}/5-HT_7）のアゴニストである 8-OH-DPAT の末梢投与が行動リズムの位相変化を時刻依存的に変化させ，その位相反応曲線は光同調刺激によるものと 180°位相を異にしていることが明らかとなった．つぎにわれわれは 5-HT_{1A}/5-HT_7受容体アゴニストの 8-OH-DPAT が *Per* 遺伝子に対してどのような影響を与えるかについて mRNA レベルで検討した[5]．位相前進作用を惹起する明期の後半における 8-OH-DPAT 投与が *Per 1* と *Per 2* mRNA 量を減少させることが観察された．一方，位相前進を引き起こさない時間帯の 8-OH-DPAT 投与は mRNA 量に影響しなかった．いずれの時間帯に 8-OH-DPAT を投与しても，光刺激のときと同様 *Per 3* 遺伝子の発現は影響されなかった．非光

一 鼎　談 一

体内時計と疾患

島　田　和　幸
柴　田　重　信
（司会）前　村　浩　二

鼎談

体内時計と疾患

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鼎談

前村(司会)：ヒトの生理機能やさまざまな疾患は日内変動を呈することはよく知られています．そしてこの日内変動は，脳に内在する体内時計によってコントロールされていると考えられています．最近，ほ乳類で *clock* など体内時計に関する遺伝子が次々にクローニングされ，体内時計の分子メカニズムが随分明らかにされてきました．そして，現在この体内時計の分野は非常にホットな領域となっております．

本日は体内時計について基礎的研究をされていらっしゃる柴田先生，臨床の仕事をされていらっしゃいます島田先生にお越しいただき，疾患の日内変動を体内時計の観点から見直してみたいと思います．先生方，きょうはお忙しいところをありがとうございます．

まず，柴田先生に体内時計研究の歴史について，体内時計の遺伝子がクローニングされる以前の研究も含めてお教えいただければと思います．

体内時計研究の歴史と分子メカニズム

柴田：ショウジョウバエの時計遺伝子として *period* (*per*) 遺伝子が1984年にクローニングされましたが，そのショウジョウバエの遺伝子がクローニングされてからマウスの時計遺伝子がクローニングされるまでに13年もかかってしまいました．結局，その間は何が行われたかというと，マウスの世界では生理学的な研究が精力的にやられていて，ショウジョウバエの方も *period* 遺伝子はクローニングしたのはいいんだけれども，さてそれがどういう機構になっているか，というのがなかなか見当がつかないということだったんです．

体内時計遺伝子発現の経過を**表1**に示しておきました．

表 1. 体内時計遺伝子発現の経過と視交叉上核の体内時計としての確率

年	ショウジョウバエ (時計遺伝子)	マウス (時計遺伝子)	げっ歯動物 (視交叉上核)
1972			破壊
1979			アイランド
1982			スライス培養
1984	*per*		
1990			移植
1994	*tim*		
1996			液性出力
1997		*per*, *clock*	
1998	*clock*, *cycle* *double time*, *cry*	*bmal1*, *tim* *casein kinase Iε*	
1999		*cry*	

1990年に，時計遺伝子の遺伝子産物が，自分の遺伝子を制御するという，いわゆるフィードバック機構が提唱されました．もしその系が24時間で閉じることができれば，24時間の時計のモデルができ上がるということです．その後，1994年にショウジョウバエでは *timeless* 遺伝子，今は *tim* ともいうのですが，*period* と *tim* という二つの遺伝子がみつかって随分研究が進展しました．両者の遺伝子産物が蛋白結合するわけですが，その結合によりフィードバックの核内移行が決まるということが分かるようになってきたんです．

その後大きな変化というのは1997年にマウスの *clock* 遺伝子と *period* 遺伝子がクローニングされたことです．東京大学(医科学研究所)の程(肇)先生を中心としたこのマウスの *period* 遺伝子のクローニングはかなりセンセーショナルだったということもあって，当時，新聞などで大きく取り上げられました．それ以降は研究も一気に進みまして，1997，98，99年と立て続けにそれに関連する遺伝子が次から次にみつかってきたわけです．先ほどでてきました *period*，*tim*，*clock* に加え *bmal1* や *cry* などです．それから蛋白のリン酸化というのが時計のフィードバック機構の時間設定に重要ですが，それがカゼインキナーゼ1εという酵素の機能であることが分かってきて，約24時間のサーカディアンリズム(概日リズム)のフィードバックモデルが確立された，という状況かと思います．

前村：どうもありがとうございます．そういう遺伝子がいろいろ発見されてきたわけですが，それらがどのように相互作用あるいはフィード

島田先生　　前村先生　　柴田先生

バックをして体内時計が分子的に調節されていると今，考えられているのでしょうか．

柴田：そのことについてはいろんなモデルが提唱されています．時計としての約24時間周期を，ずっと永久に刻み続けないといけないので，自己完結型でないといけない，ということが一つです．それからもう一つは，とくに *period* 遺伝子がみつかった時に大きな問題になったのですが，この遺伝子産物は転写制御できる場所(DNA結合配列)がなかったのが問題になったんです．それでいろんなことを調べていくと実はそれがCLOCK，BMAL1の結合に対して制御するということが分かったんです．

そうやって考えてみますと，フィードバックモデルで自己完結的に24時間周期を作る時に，たとえばどうしても振動が減衰しそうになる．その時に促進性の制御としてBMAL1，CLOCKというのが *period* の発現を持ち上げるような形にする．そうすると時計としては減衰しなくてずっと永久に回り続けやすいということになります．電池を入れて時計が止まらないようにするという考え方をすれば考えやすくなると思います．

島田：それでもかなり難しいです(笑い)．*period* 遺伝子とか，*clock* 遺伝子が体内時計を司っている遺伝子だ，という最初のきっかけはどういうことで分かったんですか．

柴田：それはmutation(突然変異)の実験です．

島田：なるほど．行動の変化が先で，その後のいろんな転写の部分は後で分かったということですね．

柴田：そうです．だから一番重要な発見としては，ショウジョウバエにmutationをかけた時に，時計がいかにもないようなショウジョウバエがとれてきちゃったんです．それをずっと調べてみたら，それがある遺伝子のmutationが起こっている場所が分かった．そこが *period* と名づけられました．

島田：その遺伝子が作った産物が上流に働いて，ネガティブフィードバックをかけて自分自身の発現をブロックするということですね．

柴田：転写が起こって *period* 遺伝子の産物ができます．それがまたある時間経って核の中に入ってきて，結局自分自身を抑えるんです．抑えているとまた *period* 遺伝子産物の量が減ってくるので抑制が利かなくなって，また作るようになってくる．要するにそういう意味では何か物が増えてきたら抑える，いわゆる生理学的にいろんなホルモンでいわれているフィードバック機構，たとえばCRF-ACTH-コーチコステロン系などと同じですね．

島田：要するに *period* 遺伝子が，自分の作った産物で自分を制御して，それで回転する系を作っているということですね．

柴田：そうです．これはメインのところですから，岡村(均)先生がこの特集号でも記述されていると思うんですが…．

疾患と日内変動

前村：体内時計研究の歴史と体内時計の分子メカニズムについて込み入った話を大変分かりやすくお話をしていただきありがとうございました．続いて，臨床的にも日内変動を呈するような生理機能やさまざまな疾患があると思います．島田先生はとくに循環器をご専門になさっていらっしゃいますが，臨床的にはどういうことが分かっているのでしょうか．

島田：睡眠，覚醒あるいは体温が変動するという生理的な現象がよく知られているわけです．心血管系疾患に限っていうと，たとえば心筋梗塞というのは最初，身体の限界を尽くした時にその心臓が，その状態が耐えきれずに起こすんだ，というふうに考えられていたんです．どういう時期に心筋梗塞が起こるのかという登録をしてみると，非常に見事に一つのピークがでてきたんです．それは朝方，とくに起床後数時間以内にピークがでてきていて，どうも心筋梗塞の発症というのは決して1日のうちでランダムに起こるのではなく，ある時間帯，覚醒して行動を開始するという時期に集中して起こっているといえます．狭心症，突然死，重症不整脈，それから脳卒中など，そういったものも同じような時期に集中しているということです．では，なぜそうなっているのか，ということが問題なわけです．それが分かれば予防に非常に役立つわけです．

今まで分かっているのは，血管に血栓を形成するといったようないくつかの生理的あるいは病態生理的なメカニズム，たとえば血圧が高いとか，心拍数が増えるとか，血栓傾向が高まるとかなどのいろんな血管障害を構成する因子がどうもすべてこの時期にピークを作っているのではないかといわれています．

前村：いろいろな生理機能のピークが明け方にあって，それが心筋梗塞などの発症に関係しているということですね．

島田　和幸 先生

島田：まさかそれが遺伝子だとか(笑い)が原因だったら，これは大変なことで，新たな概念をまた作らないといけないので，ぜひ本日はその辺をお聞きしたいと思います．

体内時計の中枢の調節

前村：従来，それらの調節は視床下部の視交叉上核に体内時計のセンターがあって，そこからの調節によって体全体のホルモンや，自律神経系で調節されていると考えられていたかと思うのですが，いかがでしょうか．

島田：臨床的には，一番代表的な部分は交感神経と副腎皮質のホルモンですね．

柴田：我々が体内時計といった時は視交叉上核をまずは指していたわけです．というのはその視交叉上核というのは視神経が交差している場所ですが，1972年の報告では，そこを破壊してしまうと行動リズムとか，ホルモン分泌のリズムが消えてしまうということで，そこがセンターだといわれたわけです．その後1979年にアイランド実験というもので，視交叉上核を視床下部から切り離して，その神経活動性を観察すると昼はきちんと活動して，夜は活動しないという活動がみえるということで，視交叉上核に体内時計の本体があるのだろう，ということが分かったんです．実際，視交叉上核を取りだして，スライス培養系に持っていく実験が1980年代に行われたのですが，ちゃんと時計として生き続けるんです．もし，そこが時計の本体でなければそういう条件であれば消失してなくなっちゃうわけです．それでより確定的になったのは，1990年代に，視交叉上核を破壊し，まったくリズムがない動物に新しい視交叉上核を移植する実験がありました．その時，mutantのハムスターの視交叉上核を移植する実験も行われたんです．そうするとその移植したハムスターのリズムに従った新しいリズムができたんです．ですから壊したところが回復してリズムが再びでてきたんじゃないか，ということが否定されたということで，視交叉上核がメインの時計だというのが確立されたのです．ですから，遺伝子絡みの分子生物学的研究がちょうど始まる前までにかなり生理学的な意味での視交叉上核の位置づけがはっきりしていたということです．表1の右側を参考にして下さい．

島田：そうすると，先生がおっしゃったいくつかの遺伝子のメカニズム

は視交叉上核のmRNAが増えている，そういうふうに理解していいわけですね．

柴田：ええ，基本的にはそうです．ほ乳動物では視交叉上核ですが，ショウジョウバエの場合はlateral neuronがメインだといわれています．

島田：たとえば日が昇る，沈むとか，光が入ってくるとか，というのは，この時計とはあまり関係ないわけですか．

柴田　重信 先生

柴田：本質的に時計が発振する，つまりほぼ24時間の時を刻むのに地球自転に伴う外界の明暗変化は関係ないんです．話題がずれて申しわけないですが，毛利(衛)さんが宇宙飛行にアカパンカビを持って行って実験をしたんです．それはなぜかというと，その当時は先ほど島田先生がおっしゃったように，日が昇って沈むという，要するに受け身的にただ反応しているだけだ，という可能性が捨て切れていなかったんです．

アカパンカビ：
アカパンカビ（*Neurospora*）は下等真核生物の体内時計の研究材料として用いられており，分生子形成リズムの関わる時計遺伝子として*frq*がクローニングされている．これを用いた時計の温度補償性の研究が盛んである．

島田：非常に考えやすいですね．

柴田：それを証明するために宇宙に持って行くと，人工衛星は丁度90分位で周回しますから，そういう条件だと時計も90分になっちゃうと，そうするとまさにそっちの方が正しいことになるわけです．

ところが実際にやってみると，やはりほぼ24時間だったんです．だからやはり遺伝的に組み込まれた性質であるということが分かったんですね．

島田：けれども24時間ということはおそらく，できた時の原因は元々そこにあるんじゃないでしょうか．そうでないと24時間じゃないですよね．

柴田：そうですね．24時間に近いわけだから，やはり地球が育ててくれたのだろう，というのはよく分かるわけですね．

前村：なるほど．そうしますと，自律的に24時間の時を刻んでいて，むしろ光が時を刻んでいるというよりは時計を同期させる(約24時間周期のリズムを24時間きっかりに合わせる)というふうに考えればよろしいんでしょうか．

柴田：ええ，だから光は何のためにあるかというと，やはり同期させるためにあるといえるでしょう．

島田：今，約24時間のリズムがあります．その時に光がぽんぽんと6時間ごとに点滅したとしても，24時間のリズムはちょっと狂うかも

しれないけれども，基本的には残っている，そういう意味でしょうか．

柴田：そうです．しかし6時間刻みというのは，体内時計を同調させるのはちょっと難しいんです．やはり大体24時間に近い刻みで光をやらないとだめです．

末梢時計の果たす役割

島田：体内時計に関連した遺伝子の発現は視交叉上核以外の場所ではないわけですか．

柴田：実際，調べてみるとほとんどの臓器に時計遺伝子のmRNAが発現していて，やはりリズムを刻んでいます．当然，mRNAの量的な変化などをみると，中心時計の視交叉上核はやはり一番大きく昼と夜の差が変化しますが，たとえば肝臓，心臓，血管でもそうですが，その発現ピークはずれていても実はリズムを刻んでいるんです．

前村：我々臨床家にとっては，たとえば心臓に個別に時計があって，血管にあって，それがいろんな生理現象を動かしているというのは大変魅力的な仮説です．私どもは，末梢にも体内時計があるんじゃないか，という最近の考え方を踏まえまして，島田先生が先ほどおっしゃられた心筋梗塞が朝多い原因について検討をしてみました．PAI-1，これは血栓の線溶系を抑制する物質ですが，この活性が朝高い，つまり朝に血栓ができやすいことが，心筋梗塞が朝に多い一因であると知られています．実際マウスで実験してみますとPAI-1の遺伝子発現は明らかな日内変動を呈しています．この*PAI-1*遺伝子の日内変動は，もちろんホルモンや他の液性因子の影響もあるのでしょうけれども，少なくとも一部は末梢の体内時計によって直接調節されているのではないかと実験的に提唱させていただきました．

PAI-1：
plasminogen activator inhibitor-1.

末梢時計の存在とその意義については，現在非常に注目されている領域だと思うのですが，柴田先生，現時点でどのように考えられておられるのでしょうか．

柴田：直接的な証明はなかなかないんです．現象論的なものはたくさんありまして，去年から今年にかけていくつか報告された中で，おもしろい実験がありました．末梢の時計といえば肝臓などが代表だと思うのですが，そういった時計は食事というのが光と同じように同調させる要因になる，というのが分かってきたんです．1日の中で一定の時間だけ食事をさせると，その時刻に合わせた位相で時計が動くようになっている．たとえばネズミは普通夜行性だから夜摂食するんですが，食事の時間をずらして毎日きちんと昼間に食事をやるんです．そうすると視交叉上核の時計遺伝子発現はまったくそういうこととは何の関係もないのですが，肝臓などの時計は給餌時刻の方に引っ張られ


前村　浩二 先生

てきて，そちらに位相を合わせた時を刻むんです．ところがおもしろいことに，その時に下流の遺伝子がいくつか調べられていて，たとえばコレステロールを合成あるいは分解する酵素のそれぞれのmRNA量をみてみると，実はそのリズムも給餌時刻に引っ張られていることが分かったのです．

島田：末梢の血管の中にある遺伝子とか，心臓にあって時を司る遺伝子というのは視交叉上核の遺伝子と同一ですか．

柴田：それは基本的には同じものです．

島田：同じmRNAがでているということが分かったんですね．

柴田：そうです，そのとおりです．

活動と日内リズム

島田：ところで心血管系の場合は同期させるファクターというのは食事ではないですね．行動はどうですか．たとえば立ち上がるとか…．

柴田：行動と一言でいってもそれは難しいです．たとえば運動についての研究があります．光が視交叉上核の時計を同調しますが，もう一つ光ではないものでも視交叉上核を同調するものがあり，有名なのがメラトニンです．メラトニンは時差ぼけにいいとかいいますが，その理由は，光とちょっと仕組みは違うんですが，時計を同調させるからです．

島田：そのメラトニンはホルモンですか．

柴田：松果体からでるホルモン，つまり液性因子です．それは時計を同調させますので，それと同じような仕組みで，実は運動があるんです．それは覚醒レベルを上げるような自発的な運動をさせると，やはり時計を同調できるのです．ただ，おもしろいことに光が圧倒的に強く同調させるんです．ところがメラトニンは実際は弱いんです．

島田：運動がリセットする強さはどうですか．

柴田：メラトニンと同じようにそんなに強くないです．けれども可能性はあるということです．我々の研究室ではトレッドミルとか，水泳をさせると末梢時計が同調するんじゃないか，なんて思ったりもして研究しています．

島田：臨床で分かっているのは，たとえば，朝方血小板機能が亢進する

といわれますが，疾患が発症するためには決して目覚めただけじゃだめで，必ず起きないといけない，行動しないといけない，その二つが揃った時に初めてそれが起きます．狭心症とか，心筋梗塞の発症の仕方も目覚めてかつ起き上がって，しかもその間隔がシャープというか，急にピュッと起き上がるような時に非常に起こりやすいといわれているわけです．ですからそれを今まで僕らが説明するのは，交感神経の刺激がその時に急激に起こったとか，あるいはさまざまな行動に伴う生理的なホルモンなり，液性因子が関連したりと考えていたわけですが，実際に，外から同期させるようなものが絶えず調節していて，たまたまそういう時期に病気を持った血管があった時にポッといろんなものがでてくるというか，そういうことがあるのかも知れないですね，よく分かりませんけれども…．

前村：今，先生がおっしゃられた実際の体内時計＋行動というか，環境も大事だということで，たとえばシフトワークしている看護婦さんや夜間労働者でそういうスタディをされているんでしょうか．

島田：ええ，だから僕は今まではそういった体内時計とは別ものだと思っていたわけです．あくまでもそれは行動が支配していることであって，環境因子と内因性のものが絡み合って，一つの生理的な血圧などの現象を生じるものだと思っていたわけですが，今のお話を聞いて実は行動自身も，単に一時的に血圧に影響するだけじゃなしに，末梢の時計なり，何らかの時計なりを動かすようなものがあって，それが長い間にリズムを作る．だからいわば一元化的に説明するということを考えてもいいのかしら，とちょっと思ったのですが…．

柴田：実際，ヒトの場合，朝いつも散歩する人は，それが時計の同期機構に本当にいいのか，というようなところはまだ実はあまり研究されていないんです．

島田：すごくおもしろいと思います．食事をするということが肝臓の酵素のリズムを同期する．大体環境因子というのは食事と運動です．

柴田：食事と運動というのはやはり重要だと思うんです．普通，規則正しい生活は基本だからといった時は，睡眠をきちんととり，きちんと起きて食事をして，適度な運動をするということになるわけですね，結局は．

島田：そうです．だから今お話を聞いて，循環器疾患というのは一次予防にしろ，二次予防にしろ，まさに食事と運動をいつも生活習慣にしているということがありますので，コレステロールなり，あるいは交感神経なり，あるいは血栓因子，PAI-1とか，さまざまなもの自身が実はregulationしているものが別にあるんですよ，という話になったら，また新たな見方をしなきゃいけないというふうにちょっと思いましたね．

臨床へのフィードバック

前村：そうしますと，生理機能，疾患にリズムがあるという考え方で見直した場合，臨床的に何か疾患の予防，治療を行う際に変えるべきことはありますでしょうか．

島田：この問題は臨床的に，我々がいかに疾患の発症を防ぐか，ということを考えてみた場合に，リスクがある状況においてそのリスクが高まらないようにしてやらないといけない，そういう話になるわけでしょう．ですから今，morning surge といって血圧が上がったり，あるいは心拍数が上がったり，あるいは血栓傾向が高まったりした場合に降圧薬，β遮断薬，あるいは抗血小板薬とかで治療をしています．現象論的に上がってくるものを抑える，そのこと自身が確かに効いているという事実はあるんですね．疾患の発症を抑えられているわけです．さらにそういったリスクが高まっている時期に，焦点を合わせた治療をする価値があるというか，効果があるということが分かっています．確かに行動ももちろんですが，どういうふうに行動すればリスクが高まるような生体の環境にならないのか．あるいはそういったリスクが高まらないようにする modification ができるのかどうか．それが分かればまた新たなものがでてきますね．

前村：そうしますと，いろんな疾患あるいは生理機能が日内変動を呈するメカニズムを踏まえた上で予防あるいはタイミングに合った治療をするともっと有効性が高まるという可能性がありますね．

島田：そうですね．疾患に関しては，日本人にとくに多い異型狭心症，vasospastic angina がなぜあんなふうに起床する直前に起こるのか非常に不思議ですけれども，ほかにも心房細動とかの不整脈でも非常に activity が高まった時に起こるものと，逆に低い時に起こるものがあります．心臓以外では気管支喘息などが非常に代表的な疾患ですが，そういうことが統一的に理解できるようなことがくればいいのですが，まだもう一つ分からないというのが現状でしょうか．今，疾患ではっきりとこれは遺伝子というか，時計のリズムが狂った結果の病気だというのはありますか．

体内時計遺伝子変異と疾患

柴田：一番はっきりしていて，この論文がでたから時計の研究をやっていてよかった，といわせたのが 2001 年の Science 誌に報告された睡眠位相が非常に早くなる睡眠-覚醒リズム障害を呈する一群の家系の研究です．*period 2* という時計遺伝子があるのですが，その産物がカゼインキナーゼ 1ε によりリン酸化を受けて，その蛋白質の安定性が変わり，核への移行速度が変わると考えられています．そこのサイ

トにmutationが起こっていたのです．それが非常におもしろいのは，遺伝的に非常に朝型のハムスターがいるんです．遺伝的には変だということは分かっていたんだけれど，ハムスターであるため分子機構解析がやれなかったんです．ところがヒトの報告が発表されるその前の年に実はそのハムスターが*period 2*という遺伝子側じゃなくて，カゼインキナーゼに変異が起こっている，つまりキナーゼとしての働きを持ち得ないということが分かったんです．でも出現する症状は非常によく似ていたんです．フェノタイプというか，現象論的にはネズミも見事に早起きで，ヒトもえらい早起きだったということです．

島田：この時計遺伝子に関するSNP(1塩基多型)はどの程度分かっているんですか．

前村：今のところはそういう特殊な家系でmutationがみつかったという段階ですが，確かに周りを見回しても，朝型の人とか，夜型の人とかいます．そういう軽い睡眠，覚醒の傾向はSNPで多少影響されている可能性はあると思うのですが，むしろ環境の要因の方が大きいのかも知れません．いかがでしょうか．

柴田：そうですね．実際日本でも，埼玉医大の海老澤(尚)先生が，いつまで経っても宵っ張りで寝られないというタイプの人(睡眠相後退症候群)と，ヒト*period 3*遺伝子mutationに相関があることを報告されています．

島田：これは非常に夢みたいな話だけれども，近い将来，遺伝子を調べて，あなたは何時間のタイプですよとか(笑い)，あなたの起床時間はこれですよ，といえる時代がくる可能性がありますね．それでそれにもかかわらず，違う行動をするという，いわば生体にストレスがかかるようなことをすると心筋梗塞が起こるとか(笑い)．ストレスというのは心血管系のものすごく大きなトリガーです．ストレスでよく眠れない，過労死というのがありますね．あれはまさにリズムを無視した，その人のいわゆる生体の血圧なり，血液なり，血管系なりがこうあるべきだという状況をまったく無視してフリーランしたわけでしょう，ある意味で…．

柴田：そうですね．最近，行動リズムを調べる簡易型の腕時計みたいなものを売っていて，これで行動リズムや睡眠状態をある程度みることができます．そういうのと，先ほど先生がおっしゃられたように，自分の遺伝子の発現パターンとの絡みで，たとえばこのパターンだとこうだから，今日はもう早く寝るようにしようとか…(笑い)．

時間薬理学と時間変更薬

前村：先ほど，島田先生の方から心血管系に限らず疾患の予防あるいは治療に，生体リズムを考えるとより効果があるんじゃないか，という

お話があったのですが，時間薬理学ということで薬をどのタイミングで使ったらいいか，というお話がありますが，現状ではどうなっていますでしょうか．

柴田：いろいろなところで実践されつつあります．実践効果として有名なのは気管支喘息薬のβ刺激薬であるツロブテロール貼付薬やテオフィリンのユニフィル錠などの薬です．丁度喘息発作が起こりやすい時間を狙って，血中濃度のピークをそこに持っていくようにあらかじめ貼付したり，飲んでもらうように剤形設計をわざわざしたものなのです．今回の特集にも組んである癌の治療でも，1回投与する時に朝昼夜のいずれがよいかというような問題も重要になってくる可能性があります．

その時に時間薬理の考え方としては大きく二つあって，一つは先ほどから随分話題になっていますように，主効果，つまり薬理効果を最大限発揮させるという考えと，もう一つは主作用はそんなに高まらないとしても，副作用を減らすという考えです．副作用が減れば随分よくなることがありますよね，たとえば抗うつ薬は，吐き気などの消化器系の副作用がかなりでやすいといわれていますが，そういう副作用が起こりにくい時間，たとえば食事をしない夜に投与すると多分抑えられるだろうと考えるわけです．だからその辺はやはり重要なポイントになってくると思うし，適正な時刻に薬を使うことは患者さんにとっては非常にいいことだと思います．

島田：そうですね．我々が手にする薬剤は最近は，吸収，分布を設計する時にいろいろ工夫がされています．とくに心血管系の薬剤はそういうのが多くなってきています．効かせたい時に効かせるように服用時間を決めたりとか，剤形を工夫しての長時間作用型とか，短時間作用型に長時間作用型を組み合わせるとかですね．インスリンを含め，多くの薬剤をかなりそういう気持ちで我々は処方していると思うんです．副作用についても，たとえば心拍数を適正にするためには，夜中は自然に徐脈になりますから，そういう時には心拍数が夜中でも下がらないような薬を投薬して，心拍数が上がる時には下がるような，α遮断薬とか，β遮断薬とか，交感神経系の薬剤を投与するとか，ということもやっていますので，時間薬理学的な概念はかなり浸透しつつあると思うんです．

柴田：確かに時計絡みでは時間薬理という言葉があるように，「時間と薬」とを考えた時にもう一つの考え方としてさっきおっしゃられたように，薬が時間を変え得るかという問題です．この辺に関しては実は今のところまだ薬がないんです．唯一あるといえばメラトニンですが，これは薬としては認可されておらず，健康食品としてアメリカでは売られていますけれども，日本では売られていないので普通は使え

ないんです.

そういう意味ではまさに今，molecularがこれだけ分かったわけですから，製薬企業も含めてベンチャーとか，そんなところもかなり力を入れて，それこそ1粒飲んだら1日が随分短くなるとか，長くなるとか(笑い).

島田：さっきいいましたように，ゲノムを使ってtailored medicineといいますか，この方の場合は6時に起きればいい．こっちの人はちょっと交感神経が遅いから6時半にしてください，というふうになる可能性もありますね.

柴田：そうですね．それは大いにあると思いますよ．それと，たとえば時計を少し動かしたりすることができるということになれば，先ほどおっしゃられたようにリスクファクターがいっぱい重なりそうな時は，若干後ろめにやるとか，前めに引っ張るとか，そういうこともできる可能性もあるわけなので，薬としては両方の面があるんじゃないかと思います.

体内時計研究の今後の方向性

前村：現在，体内時計に関連する遺伝子は大体もうクローニングされ尽くされて，将来的な流れとしてはそれら相互のネットワークをみるとか，あるいは体内時計と疾患との結びつきをみるとか，各個体の遺伝子と日内変動，体内時計の関連をみるとか，そういう方向になっていくんでしょうか.

そうして，各個人の時間のパターンに合わせた疾患の予防，治療に繋がっていくのだと思います.

先生方，本日はありがとうございました.

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