神経—グリア複合体としての視交叉上核の時計機構

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研究代表者　　柴田重信

（早稲田大学人間科学部教授）

はしがき

研究組織

研究代表者：　柴田重信（早稲田大学人間科学部教授）

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鈴鹿

研究成果」

サーカディアンリズム（概日リズム）は約２４時間周期のリズム現象であり、原核生物のシアノバクテリアからヒトに至るまで生物界に普遍的に見られる基本的な生命現象である。この約２４時間変動は非常に安定しており周期間の誤差は約１％と少ない。哺乳動物のサーカディアンリズムを支配する体内時計が視交差上核に存在することは、破壊、培養、移植等の研究から明らかにされている。生物は自分自身の体内時計で生み出されるサーカディアンリズム変動を地球の自転周期である 24 時間周期に合わせること（同調、リセット）により、１日の生活リズムを営むことができる。哺乳動物のでは光刺激が網膜ー視床下部路を通して視交差上核の体内時計をリセットしていることになる。このような光同調は、時差ボケ、うつ病や老人性痴呆患者に応用されつつある高照度光療法の作用機序の説明にもなっている。現在、視交差上核の時計機構は「同調性入力ー時計発振ー時計出力」で表されるような一見単純な神経回路でできあがっていると思われている。しかしながら、視交差上核の体内時計を作り上げている物質基盤、分子機構ならびに核内神経回路網などは実際のところ殆ど分かっていない。この研究の困難さは１）視交差上核が視床下部の中でも約 0.5mm の球状の小さな神経核であり、２）約１万個の神経細胞から成り立つが、GFAP

染色が際だって高いことから分かるようにグリア細胞も豊富に存在し、3）この神経核は神経細胞とグリア細胞が高密度の局所的な細胞間コミュニケーション社会を形成しているからであろうと考えられる。

これまで神経細胞とグリア細胞の研究は独自に進められてきた。特にグリア細胞は発生、分化といった分野や、成長後の神経細胞死や神経修復機構など病理学的分野で関心があり研究が進められてきた。近年、グリア細胞に種々の受容体や、イオンチャンネル、細胞内情報伝達系、ギャップジャンクションなどの存在やその役割が続々と明らかにされるに従ってグリア細胞の生理学的側面にも注意が払われ出してきた。視交差上核は小さな神経核にもかかわらずグリア細胞が発達し、いわゆる「神経ーグリア複合体」を形成しているので、視交差上核の体内時計機構を明らかにするには視交差上核を複合体と考える研究アプローチが是非必要である。視交差上核では発達したグリア細胞が神経細胞に積極的に影響を及ぼすことにより、発振周期を約２４時間にしかも安定的に保ったり、光による時計リセット時に極端な位相変化をもたらさないような工夫をしているものと考えられる。したがって本研究では視交差上核における体内時計の時計機能の中でも発振機構と同調機構を取り上げ「神経ーグリア複合体」としての視交差上核という観点からの研究を進めた。

これらの研究成果を発表論文に照らし合わせて記述していく事にする。

(A) 本研究の成果は大部分論文（１）に記載されている。すなわちグリア細胞の骨格タンパクである Glial Fibrillary Acidic Protein (GFAP)ノックアウト動物のサーカディアンリズムを調べた成果である。GFAP ノックアウト動物は正常明暗下では正常な日内リズムをまた恒暗下でも正常なフリーランリズム示すが、恒明下ではサーカディアンリズムの周期の延長が著しくまた、リズムの消失が早く起こる事がわかった。このようなノックアウト動物の中脳縫線核や視交叉上核のセロトニン代謝が低下していることが明らかとなった。実際セロトニン神経を破壊すると GFAP ノックアウト動物のような現象が見られる事が知られており、一致する。また、実際正常動物を恒明下に飼育すると、視交叉上核の GFAP の発現量が低下する事もわかった。この事は GFAP ノックアウト動物がすでにある意味で恒明下に飼育されているのと類似した状態になっていることが示唆された。

(B) 我々は時計遺伝子 m*Per1* の光同調機構への関与を調べるため，光照射の m*Per1* mRNA の発現への影響を調べた (19)。その結果，行動リズムに位相変化を引き起こすサーカディアンタイム (CT) において m*Per1* mRNA は SCN において急速に誘導されることが分かった。位相変化の CT 依存性を gating と呼ぶが、m*Per1* の発現にも gating が見られた。この誘導は一過性であり、照射 30 分後にはピークに達し、180 分後にはもとのレベルに戻る．注目されるのはその結果引

き起こされる *mPer1* 転写産物の概日性発現リズムの位相変化が行動リズムの位相変化に対応していることである。ただし、発現リズムの位相変化が 1 サイクルで完了しているのに対、行動リズムの位相変化には数日かかる。さらに光による *mPer1* 誘導と位相変化の大きさは相関しており、その閾値もほぼ同一であった。*mPer1* 遺伝子の概日性の発現は SCN 全体に見られるのに対して、光による *mPer1* 誘導は光情報を SCN へ伝達している RHT が投射する腹外側部に限局していた。我々は *mPer1* アンチセンスをを用いて SCN 内の光による *mPer1* 誘導を特異的に抑制した時 (6)、に行動の位相変化も抑制されることを見出している。これらのことから *mPer1* 遺伝子の光誘導が、光による行動の位相変化に必要とされることが示唆される。この *mPer1* 遺伝子の光誘導は *Drosophila* では見られず、位相変化は光による TIM の急速な分解によって引き起こされると考えられている。このことは, *Drosophila* と哺乳類でリズムに関与する遺伝子(産物)は共通であっても、その調節機構が必ずしもすべて保存されているわけではないことを示している。

(C) 視交叉上核への神経入力系としては中脳縫線核群の腹内側から視交叉上核へ、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知られており、また視交叉上核には数種のセロトニン受容体が存在する。我々はセロトニン受容体 ($5HT_{1A}$ /$5HT_7$)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。最近の我々の研究では 5-HT7 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-HT7 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、視交叉上核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。一方、セロトニン神経の活動性を低下させる薬物や破壊は体内時計の光同調を促進し、セロトニン神経の活性化は光同調を逆に抑制することが知られている。実際 MKC-242 ($5HT_{1A}$ 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。また、DR4004 ($5HT_7$ 受容体アンタゴニスト)も光同調を促進させることが確かめている。

以下に本研究成果の発表論文の主なものの別刷りを掲載した。

Moriya T, Yoshinobu Y., Kouzu Y., Katoh A., Gomi H., Ikeda M., Yoshioka T., Itohara S. and Shibata S., The essential role of Glial Fibrillary Acidic Protein (GFAP)-expressing astrocytes in the mouse circadian oscillation under constant lighting condition. Eur.J.Neurosci.,in press

Abstract

In order to clarify the role of GFAP-expressing astrocytes in the circadian clock, we compared the activity rhythms of GFAP mutant mice to those of wild-type mice in various lighting conditions. GFAP mutant mice exhibited stable circadian rhythms both in light-dark cycles and constant darkness and showed normal entrainment to environmental light stimuli. However, under constant lighting conditions, the period of the activity rhythm in GFAP mutant mice was longer and more disrupted than in wild-type mice. HPLC analysis revealed that serotonergic activity in the suprachiasmatic nucleus, which is the center of the circadian clock, and the raphe nuclei was reduced in GFAP mutant mice. Furthermore, housing for 80 days under constant light decreased GFAP expression in the suprachiasmatic nucleus of C57BL/6J mice while increasing GFAP expression in the intergeniculate leaflet. These results demonstrate that the activities of GFAP expressed in astrocytes were changed in the suprachiasmatic nucleus and the intergeniculate nucleus under constant lighting conditions. Astrocytes in the suprachiasmatic nucleus may play important roles in the maintenance of circadian rhythms under constant lighting conditions via regulation of serotonergic activity.

Introduction

Daily physiological rhythms, such as locomotor activity and body temperature, persist under conditions without environmental time cues, suggesting the existence of endogenous time-keeping systems in animals (Inouye & Shibata, 1994). Various studies revealed that mammalian circadian clocks were located in the suprachiasmatic nucleus (SCN) of hypothalamus (Ralph et al., 1990). Recent studies are beginning to describe the molecular and cellular mechanisms that generate the circadian rhythm in the SCN (Welsh et al., 1995).

SCN neurons play important roles in generating circadian rhythms. The frequency of SCN neuronal firing shows circadian rhythm, with high during the daytime, low during the nighttime under both *in vivo* (Inouye & Kawamura, 1979) and *in vitro* (Green & Gillette, 1982; Groos & Hendriks, 1982; Shibata et al., 1982). The blockade of firing in the SCN by tetrodotoxin abolished the circadian rhythm *in vivo* (Schwartz et al., 1987) and *in vitro* (Shibata & Moore, 1993) and the firing rhythm reappeared exactly on the phase predicted by the rhythm before the administration of tetrodotoxin. Recently, it was reported that dissociated SCN neurons displayed a circadian firing rhythm and their rhythms did not synchronize in spite of functional synapses (Welsh et al., 1995). This evidence demonstrates that the circadian rhythm-generating system is located in individual SCN neurons.

Glial cells in the SCN may also play roles in the circadian rhythms (Van den Pol et al., 1992; Prosser

et al., 1994; Jiang et al., 1997). Glial fibrillary acidic protein (GFAP), which is an astrocyte-specific intermediate filament protein, is abundantly existed in the SCN (Lavialle & Serviere, 1995). Suppression of glial metabolism or the inhibition of GAP junctions disrupted the SCN firing rhythm (Prosser et al., 1994), and the rhythms of vasoactive intestinal polypeptide and vasopression release from cultured SCN slices was not synchronized when antimitotic drugs were treated (Shinohara et al., 1995). These reports suggest that astrocytes synchronize circadian rhythms individually generated in SCN neurons. However, the role of astrocytes remain unclear because of the lack of specific agents to manipulate astrocytes activity.

Recently, mice lacking GFAP gene were generated (Gomi et al., 1995). GFAP mutant mice do not show any abnormalities in their development, reproducibility, brain structure nor the morphogenesis of astrocytes. However, long-term depression in the cerebellum and the eyeblink conditioning were clearly deficient in GFAP mutant mice (Shibuki et al., 1996), indicating that GFAP has neuro-modulating functions in addition to its functions as a part of the cytoskeletal structure.

In order to clarify the role of GFAP expressing astrocytes in the circadian clock, we examined the circadian activity rhythm of GFAP mutant mice in various lighting conditions. We also examined the concentration of serotonin (5-hydroxytryptamine; 5-HT), an important neurotransmitter in regulating the circadian clock (Cagampang & Inouye, 1994; Cutrera et al., 1994; Rea et al.,1994; Moriya et al., 1996; 1998) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in GFAP mutant mice. We also examined effects of lighting conditions on GFAP expressions in normal C57BL/6J mice to confirm the role of GFAP in circadian systems.

MATERIALS AND METHODS

Animals

Wild-type and mutant mice (3 months old at the beginning of the experiments) were produced as described previously (Shibuki et al., 1996). Mice used in this experiment were the littermates intercrossed between male and female heterozygotes that have been backcrossed to C57BL/6J mice for at least five generations. Targeting of GFAP was verified by southern blot analysis using tail DNA. We also used male C57BL/6J mice for immunohistochemical and western blotting studies. These mice were maintained under controlled environmental conditions (23 ± 2℃ room temperature; 12-12 hr light-dark cycle (LD), lights on at 8:30 A.M.) for at least 2 weeks before being used for the experiments. Light intensity was set to 50 lux over the all experiments. Food and water were provided *ad libitum*. Animals were treated in accordance with the Law (No.105) and Notification (No.6) of the Japanese Government.

Recording of wheel-running rhythm

Mice were housed individually in transparent plastic cages (31 x 20 x 13 cm), each equipped with a

running wheel (10 cm diameter), that triggers a microswitch on each revolution. Wheel-running activity was continuously recorded in 6 min epochs by a PC-9801 computer. For measurements of free-running rhythms, mice were maintained under LD conditions for at least 2 weeks and followed by constant darkness (DD) or constant light (LL) condition for 3 and 4 weeks, respectively. The free-running period was determined by χ^2 periodgram. In order to examine differences in the photic entrainment, we employed two experiments paradigms. To obtain the light pulse-induced phase shift in DD, mice were exposed to a light pulse (50 lux, 15 min) at CT16 approximately 1 week after being released from LD into a DD condition and their wheel-running rhythm were continuously recorded. Phase shifts were calculated from distance between two regression lines drawn through the daily onsets of wheel-running activity for at least 1 week before and after light pulse. For measuring re-entrainment to a 6 hr advanced LD cycle, mice were maintained in a 12:12 hr LD cycle for at least 10 days, and the LD cycle was advanced 6 hr. The phase shifts 5 days after a LD shift were calculated. Both regression lines for light pulse-induced phase shifts and the curved line for re-entrainment were drawn by a blinded observer.

Measurement of 5-HT and 5-HIAA concentration

Measurements of 5-HT and 5-HIAA contents in the SCN and the raphe nuclei were done by HPLC as previously reported (Ono et al., 1996). The SCN and the raphe nuclei of wild-type and mutant mice were dissected as described above. Monoamines were extracted with 250 μl of 0.5 M $HClO_4$ by sonication on ice. After centrifugation at 15,000 rpm for 10 min at 4℃, supernatants were collected for measurement of monoamine contents. Eicompak MA-5ODS (4.6 x 150 mm) (Eicom, Kyoto, Japan) and an electrochemical detector (ECD-300, Eicom, Kyoto, Japan) were used for 5-HT and 5-HIAA assays. The mobile phases were as follows: 50 mM sodium-acetate-citrate buffer (pH 3.9) containing 150 mg/l sodium 1-octanesulfonate, 5 mg/l EDTA and 10 % methanol. The data were analyzed with a Powerchrom 2.0.6 system.

GFAP immunohistochemistry

Mice were deeply anesthetized with Nembutal (80 mg/kg i.p.) and perfused intracardially with 50 ml of saline (37℃) containing 16 units/l heparin, followed by 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.2; 4℃). Brains were removed from the skull and fixed with 20 ml of 4% paraformaldehyde in 0.1 M PBS and transferred to 20% and 30% sucrose solutions in 0.1 M PBS each for 24 hr. Brains were cut into 30 μm slices from rostral to caudal SCN, intergeniculate leaflet (IGL) and median raphe with a freezing microtome. Alternate sections were incubated with anti-GFAP antibody (GF12.24, Progen Biotechnik, Heidelberg, Germany) for 24 hr at 4℃. All sections were then washed 3 times with 0.1 M PBS (10 min each) and incubated for 2 hr with biotinylated anti-mouse goat antibody (diluted to 1:200 with PBS including 1% normal goat serum and 0.3% Triton X-100; Vectastain). The sections were washed 3 times with 0.1 M PBS and incubated for 2 hr in an avidin-biotin complex

solution (Vectastain ABC kit). After 3 washes with 0.1 M PBS, sections were visualized with diaminobenzidine as a chromogen and mounted on gelatin-coated glass slides. All procedures were performed at room temperature except for the incubation with the primary antibody.

GFAP western blot analysis

Wild and GFAP mutant mice were anesthetized with ether and killed by decapitation. The brain was rapidly removed from the skull and the SCN and the IGL were dissected free and quickly frozen by liquid N_2. Then, samples were homogenized with 100 □l of following buffer: 50 mM MOPS, 100 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 0.32 M sucrose, 1 mM vanadate, 1mM DTT, 0.2 mM ABSF, 5 □g/ml pepstatin A, 50 □g/ml leupeptin, 100 nM calyculin A. After centrifugation at 15,000 rpm for 10 min at 4℃, supernatants were collected and added to 5X reducing Laemmli buffer (Laemmli, 1970) (2.4% SDS, 6% 2-mercaptoehanol) followed by boiling for 5 min. Samples were electrophoresed on 7.5 % polyacrylamide gel and transferred to Immobilon-P membrane (Millipore, Bedford, MA), and incubated with anti-GFAP antibody (GF12.24, Progen Biotechnik, Heidelberg, Germany) for 3 hr at room temperature. GFAP on the membrane was visualized by an Immun-Star Chemiluminescent Protein Detection Systems (Bio-Rad, Hercules, CA) and analyzed by an image analyzing system (GS-250 Molecular Imager, Bio-Rad, Hercules, CA).

Statistics

The data are presented as mean ± SEM. Statistical analysis was conducted by Student's *t*-test or Fisher's exact probability test. P values of 0.05 or less were considered as statistically significance.

Results

Wheel-running rhythm

We compared the circadian activity rhythms of wild-type and GFAP mutant mice under LD and DD conditions. Both wild-type and GFAP mutant mice showed LD-entrained wheel-running rhythm and their wheel-running activities were restricted to the dark period (Fig. 1). The periods of wheel-running rhythm were exactly 24 hr in both wild-type and GFAP mutant mice (Table 1). Under the DD condition, both wild-type mice and GFAP mutant mice exhibited a stable free-running rhythm and we did not observe a significant difference in the period of wheel-running rhythm (Fig. 1, Table 1).

We next investigated the photic entrainment of the circadian clock. Light pulses (15 min) at circadian time 16 (CT; CT12 was defined to activity onset time) induced a phase delay of wheel-running rhythm in both wild-type and GFAP mutant mice. There was no significant difference in degree of phase delay between wild-type and GFAP mutant mice (Wild-type mice: 84.73±6.58 min (n = 11), GFAP mutant mice: 81.14± 12.19 min (n = 14); $p > 0.05$ (Student's *t*-test)). Furthermore, no

differences in the re-entrainment of wheel-running rhythm following a 6 hr advanced LD cycle were observed between wild-type and GFAP mutant mice (phase shifts on 5 days after a LD shift were 3.37± 0.29 hr (Wild-type mice; n = 9) and 3.27±0.41 hr (GFAP mutant mice; n = 7); $p > 0.05$ (Student's *t*-test)).

Under LL conditions (Fig. 2), the period of wheel-running rhythm rapidly increased in both wild-type and GFAP mutant mice. One month of housing in LL conditions resulted in the disruption of circadian rhythmicity in some animals. $\square^2$ periodgram and Fisher's exact probability test revealed that GFAP mutant mice tended to be significantly arrhythmic under LL conditions (Fig. 2 and Table 2). Furthermore, GFAP mutant mice had a significantly longer circadian period compared to that of wild-type mice when we averaged the data of only those mice that still showed circadian rhythmicity in LL.

5-HT and 5-HIAA concentration

We measured 5-HT and its metabolite, 5-HIAA contents in the SCN and the raphe nuclei, that is known to be important for circadian rhythmicity under LL condition. The 5-HIAA contents in both the SCN and the IGL of GFAP mutant mice were significantly lower than of wild-type mice, while there was no difference in the 5-HT content in either the SCN or the raphe nuclei (Table 3).

GFAP expression

We used male C57BL/6J mice for GFAP immunohistochemistry and western blot analysis because both wild-type and GFAP mutant mice had the similar genetic background of C57BL/6J mice.

The SCN contained the intensive GFAP immunoreactivity within the hypothalamic nuclei. The intensity and the expression pattern of GFAP did not show any changes through various zeitgeber times (Fig. 3). We next examined the effect of LL on GFAP immunoreactivity in the SCN, IGL and raphe nuclei. During LL housing for 80 days, longer free-running and more arrhythmic circadian locomotor activity rhythms were observed if compared with LD condition (data not shown). GFAP immunoreactivity was decreased in the SCN but increased in the IGL by LL housing for 80 days (Fig. 4). On the other hand, there is no change in the GFAP immunoreactivity in the median raphe nuclei during LL housing.

In order to analyze the GFAP immunoreactivity quantitatively, we employed the western blot analysis of the GFAP content in the SCN and IGL under LL or LD conditions. GFAP mutant mice gave no detectable band of GFAP (50 kDa)(Fig. 5). The LL housing for 80 days significantly decreased GFAP content in the SCN and significantly increased it in the IGL of wild mice (Fig. 5).

Discussion

We demonstrated that, under constant lighting conditions (LL), the circadian rhythm of GFAP mutant mice tended to be disrupted and the free-run period was found to be longer than that of wild-type mice.

It is known that the circadian period becomes longer under LL than DD and long-term LL housing decreased the amplitude of circadian rhythms in rats and mice and elicited the splitting into two components in hamsters (Pickard et al., 1993; Pickard, 1994; Depres-Brummer et al., 1995). Since GFAP were abundantly expressed in several brain regions including the SCN, IGL and raphe nucleus, all which are important for regulating circadian rhythm (Morin et al., 1989; Botchkina & Morin, 1995), we could not conclude exactly which brain regions, were crucial for the abnormality of circadian rhythm in GFAP mutant mice under only the LL conditions. However, as is well known that GFAP is very specific protein for astrocytes, the abnormality in the neuronal behavior can be described to loss of glial activity and consequently loss of interaction between glia and neuron.

As SCN is a main oscillator in the circadian systems, we would like to know how GFAP regulates neuronal activity in the SCN. It is reported by several authors that neuronal firing rate or glucose utilization in the SCN slice also displays disrupted rhythms when animals are maintained under LL condition, indicating that the disruption of circadian rhythms under LL occurred in the SCN (Schwartz et al., 1977; Shibata et al., 1984; Zlomanczuk et al., 1991; Yu et al., 1993). Thus, the SCN is involved in the arrhythmicity under LL condition. We demonstrated that serotonergic activity in the SCN and the raphe nuclei were markedly decreased in GFAP mutant mice. Depletion of brain serotonin by 5,7-DHT increased the period length produced abnormalities of activity rhythm under LL conditions but not under DD conditions (Morin & Blanchard, 1991). This report is consistent with our observations. GFAP expressing astrocytes play crucial roles in the development of serotonergic neuron via S-100□ protein (Ueda et al., 1994a; 1994b), indicating the important interaction between astrocytes and serotonergic neurons. Therefore, we conclude that GFAP in the SCN may be involved in the neuronal regulation of astrocytes and lack of GFAP resulted in the decrease of serotonergic activity in the SCN followed by disruption of circadian rhythms under LL condition.

As shown in our experiments, LL condition strongly decreased the GFAP protein expression in the SCN. Considering GFAP mutant mice lack the activity of GFAP in the SCN, the reduction of GFAP in the SCN may induce the arrythmicity of locomotor activity under LL condition, by unknown molecular mechanism.

Effects of GFAP on the neuronal function was examined in another brain region, as follows. In the cerebellum, GFAP mutant mice are deficient in the ability to produce long-term depression (Shibuki et al., 1996), and long-term potentiation in the hippocampus was enhanced in GFAP mutant mice (McCall et al., 1996). These reports suggested that GFAP played a role in regulating neuronal synaptic plasticity. Recently, we reported that optic nerve-SCN synaptic transmission was potentiated after the tetanic stimulation (Nishikawa et al., 1995; 1996). Therefore, one possible interpretation of our results is that the lack of GFAP might affect synaptic plasticity in the SCN via modulation of neuronal function, such as serotonergic neurons, followed by disruption of circadian rhythms in LL conditions.

In addition to those, we would like to stress that the IGL also might be involved in the appearance of

abnormal rhythmicity in GFAP mutant mice under LL condition. Retina ganglion neurons, that received the photic signal for circadian clock, have been reported to innervate to the SCN and also to the IGL of the thalamus. Then, IGL sends its light information to the SCN using transmitters of NPY and/or GABA. We, in the present experiment and other groups (Morin et al., 1989) observed that GFAP was highly expressed in the IGL. The ablation of the IGL prevents the elongation or disruption of the circadian activity rhythm under LL conditions in hamsters (Harrington M.E. & Rusak, 1988; Pickard et al., 1987) but not in mice (Pickard, 1994), suggesting that the IGL may be involved in limiting the light signal to the SCN under LL conditions.

We showed that long-term LL housing led to increase of GFAP expression in the IGL. Increase of GFAP in the IGL under LL condition might be results of compensation to long-term light exposure. Therefore, GFAP mutant mice exhibited the arrhythmicity under LL, because of lack of compensatory role of GFAP on disruption of circadian locomotor rhythm under LL.

In our study, GFAP mutant mice did not exhibit any abnormality of wheel-running rhythm under LD and DD conditions and there was no difference in the light pulse-induced phase shift nor re-entrainment to 6 hr advanced LD cycle. These results indicate that GFAP does not have a crucial role in the generation nor photic entrainment of the circadian clock. Although it is not so easy to show clearly the significance of astorocytes in circadian changes, there are some reports about involvement of astrocytes in synchronization of circadian rhythms (Prosser et al., 1994; Shinohara et al., 1995) by using inhibitors of astrocytes metabolism and GAP junction or antimitotic drugs. These reports would appear to disagree with our findings. It is, however, important to note that the circadian normality observed in GFAP mutant mice do not imply the non-contribution of astrocytes to the functioning of the circadian clock, because mice devoid of GFAP did not show apparent abnormalities in the morphogenesis of astrocytes in brains (Gomi et al., 1995; Pekny et al., 1995; Wang et al., 1997). We can also exclude the possibility that the compensatory expressions of other intermediate filaments rescue the effect of lacking GFAP, since neither vimentin nor nestin was up-regulated in GFAP mutant mice (Gomi et al., 1995; McCall et al., 1996; Wang et al., 1997; Pekny et al., 1998).

We, in the present experiment, and other groups (personal communication with Dr. Okamura in Kobe University) observed no daily variation of GFAP expression in the mouse SCN, while other researchers observed daily variation in hamsters (Lavialle & Serviere, 1993). The difference of animal species, such as mice, rats and hamsters, may account for this discrepancy. Anyway in mice, GFAP possesses a meaning role in the generation of circadian rhythmicity only under LL conditions. The physiological significance of the changes of GFAP expression in the SCN and the IGL could be achieved by studies using microinjection of GFAP antisence into specific brain areas.

In conclusion, it became clear that GFAP-expressing astrocytes regulate the photic signal of LL to the biological clock in the SCN or the IGL. We demonstrated that serotonergic activity in the SCN was regulated by GFAP-expressing astrocytes and this may be lead to the abnormality of the circadian rhythm

under LL condition in GFAP mutant mice. Furthermore, GFAP expressions in the SCN and the IGL are regulated by consecutive light exposure, suggesting its important roles in the circadian clock under LL condition. Further studies will be required to clear the cellular mechanisms how GFAP-expressing-astrocytes regulate the circadian rhythm in the SCN.

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Tables

Table 1. Free-running period of wheel-running rhythm of wild-type and GFAP mutant mice in LD and DD

	Wild-type		Mutant	
Lighting condition	Period (hr)	n	Period (hr)	n
LD	24.01±0.01	12	24.00±0.01	12
DD	23.68±0.06	12	23.78±0.04	12

Wheel-running rhythms of wild-type and GFAP mutant mice were recorded as described in the legend for Fig. 1. Free-running periods during last 10 days (LD) or 15 days (DD) were calculated by periodgram. Data are mean±SEM. Student's *t*-test revealed no significant difference between wild-type and mutant mice.

Table 2. Difference in tendency toward the disruption of circadian rhythmicity in LL and free-running period of wheel-running rhythm of wild-type and GFAP mutant mice in LL

	Wild-type		Mutant	
	rhythmic	arrythmic	rhythmic	arrythmic
Number of mice #	11	4	4	8
Free-running period in LL (hr)	25.64±0.15		*26.25±0.06	

Wheel-running rhythms of wild-type and GFAP mutant mice were recorded as described in the legend for Fig. 2. Mice were divided into rhythmic and arrhythmic groups using periodgrams recorded during the last 15 days in LL. Free-running periods during the last 15 days in LL were calculated by periodgram. Data are mean±SEM. #: significant difference ($p < 0.05$) in ratio of number of rhythmic and arrhythmic mice between wild-type and GFAP mutant mice (Fisher's exact probability test). *: significant difference ($p < 0.05$) from wild-type (student's *t*-test).

Table 3. 5-HT and 5-HIAA contents and ratio (5-HIAA/5-HT) in the SCN and the raphe nuclei of wild-type and GFAP mutant mice

		Wild-type		Mutant	
		contents (ng/mg) or ratio	n	contents (ng/mg) or ratio	n
SCN	5-HIAA	58.20±5.53	6	*37.39±4.37	6
	5-HT	20.85±2.85	6	21.50±4.26	6
	Ratio	2.97±0.33	6	2.22±0.67	6
Raphe	5-HIAA	858.87±68.86	6	*639.03±33.75	6
	5-HT	1098.93±120.39	6	983.08±33.34	6
	Ratio	0.83±0.10	6	0.65±0.03	6

Mice were maintained under LD for at least 2 weeks. During the light period, the SCN and the raphe nuclei were prepared and the monoamine content was measured by HPLC as described in Materials and Methods. Data are expressed as mean±SEM. *: significant difference ($p < 0.05$) from wild-type (student's *t*-test).

Figure legends

Figure 1. Representative double-plot actograms of wild-type (A) and GFAP mutant (B) mice in LD followed by DD conditions. Time of day is indicated horizontally and consecutive days vertically. The open and solid bars on the top of actogram represent the light and dark period, respectively. Mice were maintained under LD for 2 weeks followed by DD for 3 weeks and their wheel-running activity was continuously recorded as described in Materials and Methods.

Figure 2. Representative double-plot actograms of wild-type (A) and GFAP mutant (B) mice in LD followed by LL conditions. Details were described in the legend for figure 1. Mice were maintained under LD for 2 weeks followed by LL for 1 months.

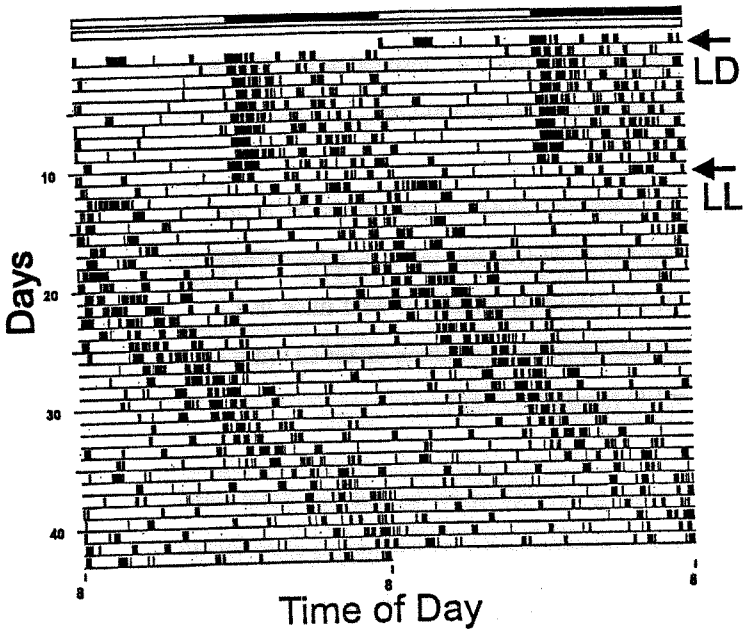
Figure 3. GFAP immunoreactivity in the SCN of C57BL/6J mice at various time of day. Mice maintained under LD were perfused transcardially with saline followed by 4% paraformaldehyde at various ZTs (A) ZT2 (B) ZT6 (C) ZT10 (D) ZT14 (E) ZT18 (F) ZT22. Coronal sections (30 □m) through the SCN were processed for immunohistochemistry. There was no variation in GFAP immunoreactivity among various ZTs. These experiments were conducted repeatedly 4 times. Scale bar = 500 □m.

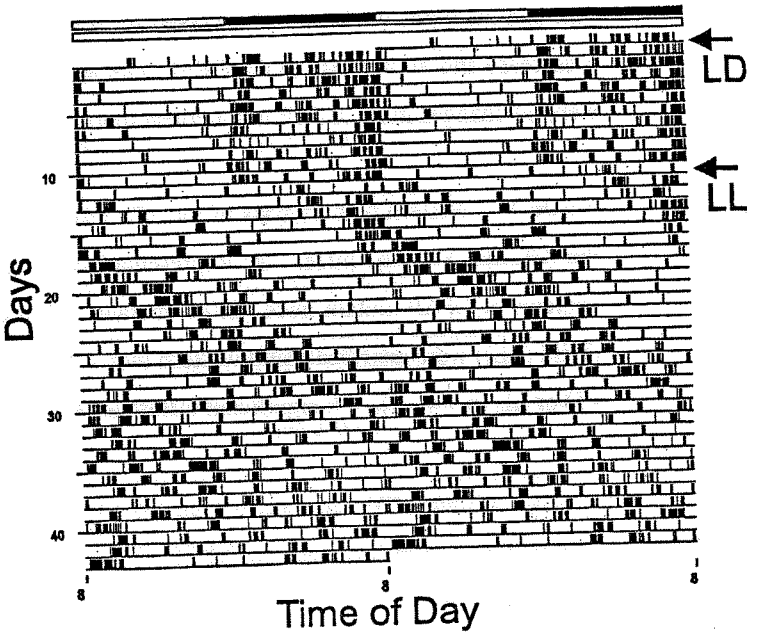
Figure 4. Effect of LL housing on GFAP immunoreactivity in the SCN (A, B), the IGL (C, D) and the raphe nuclei (E, F) of C57BL/6J mice. Mice were maintained under LD (n = 6)(A, C, E) or LL (n = 5)(B, D, F) for 80 days and perfused transcardially and coronal sections (30 □m) through the SCN, the IGL and the raphe nuclei were processed for immunohistochemistry. Long-term LL housing resulted in a decrease and an increase in the GFAP immunoreactivity in the SCN and the IGL, respectively. There were no changes in the GFAP immunoreactivity in the raphe nuclei. Scale bar = 500 □m.

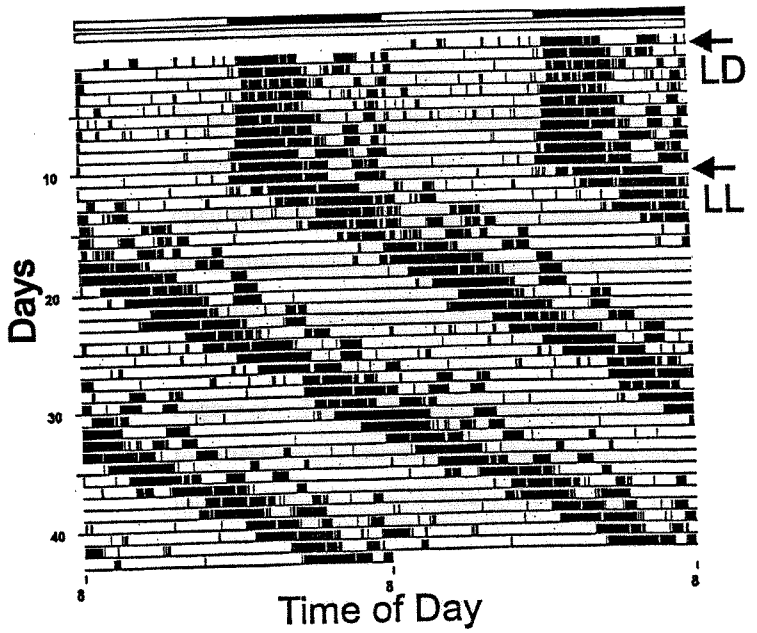
Figure 5. Western blot analysis of the effect of LL housing (80 days) on GFAP content in the SCN and the IGL of C57BL/6J mice. A: Representative western blot for the SCN (A) and the IGL (B). GFAP contents of GFAP mutant mice (GFAP mutant), of wild mice in LD (Wild (LD)) and of wild mice in LL (Wild (LL)) were analysed using western blot. C: Summarized data of mice maintained under LD (n = 4 - 5) or LL (n = 4 - 5). *: significant difference ($p < 0.05$) from LD (student's *t*-test).

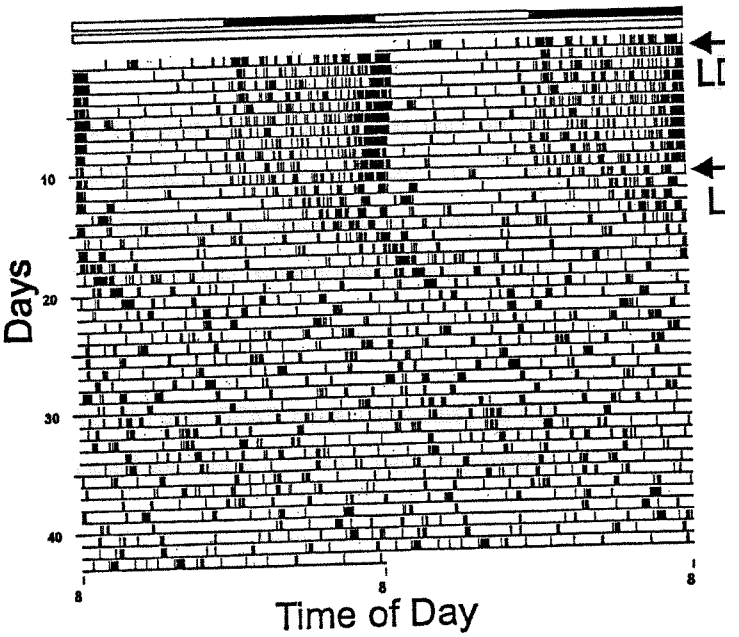
A. Wild

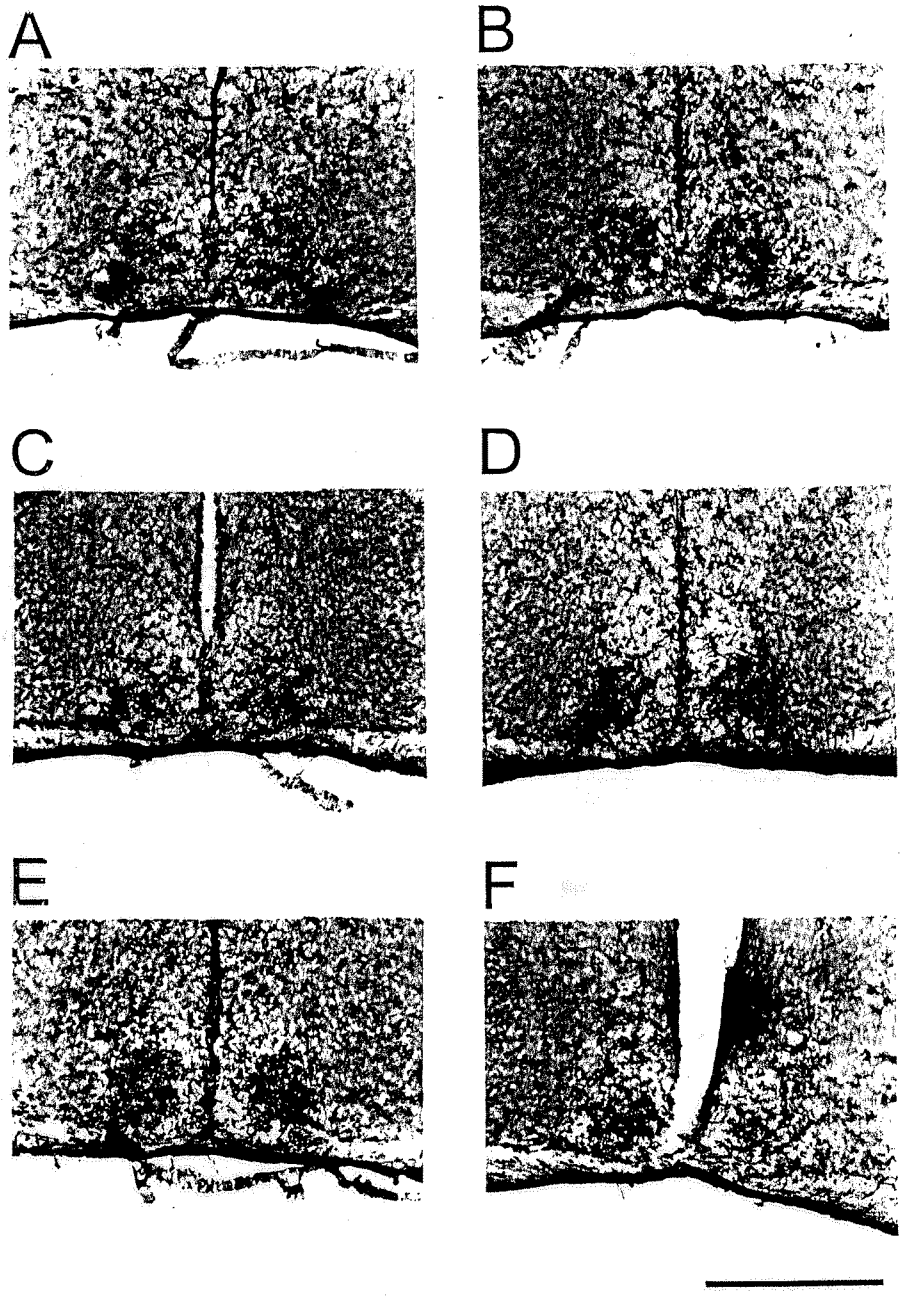
B. GFAP mutant










A
B
C
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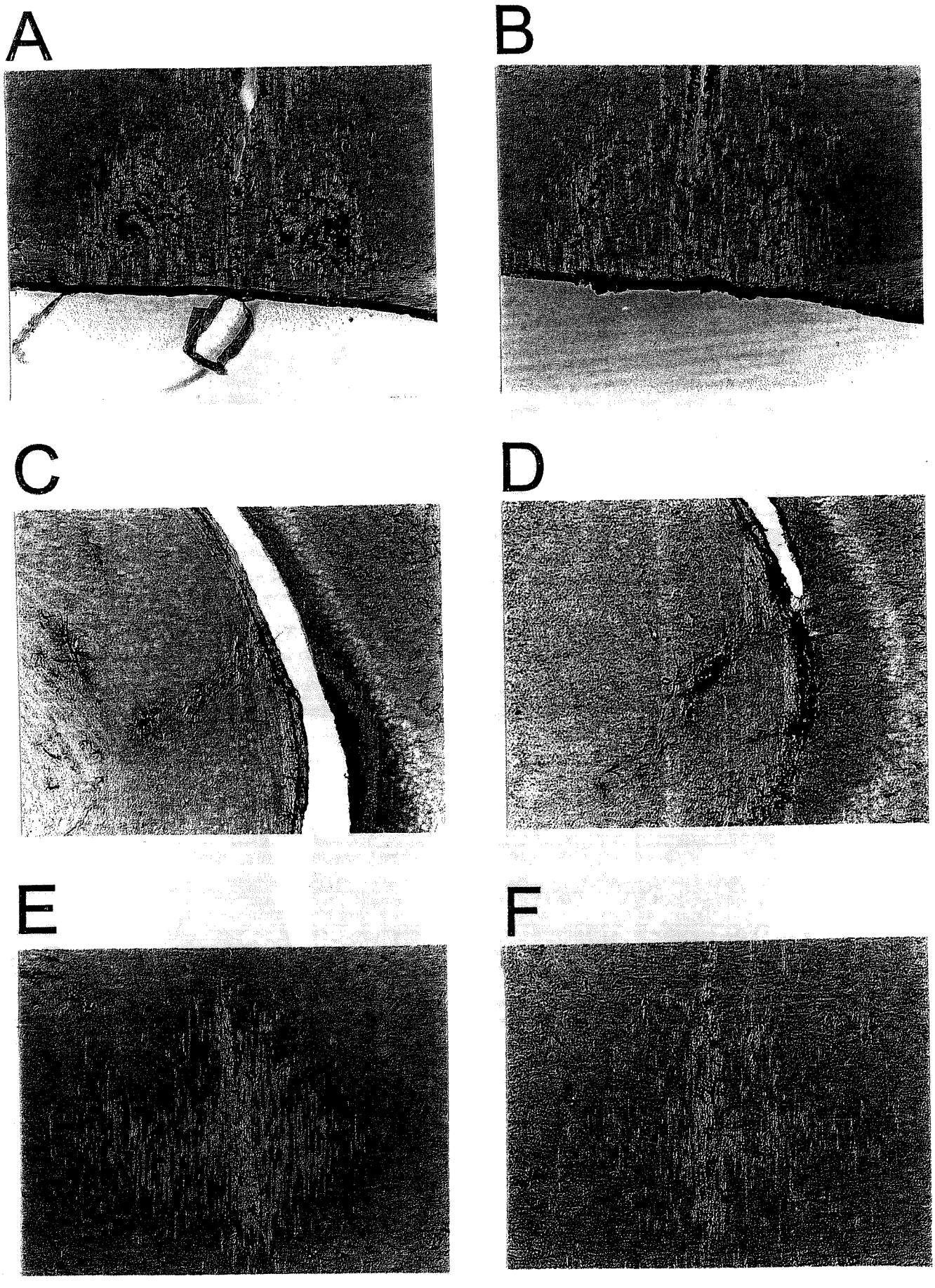
A
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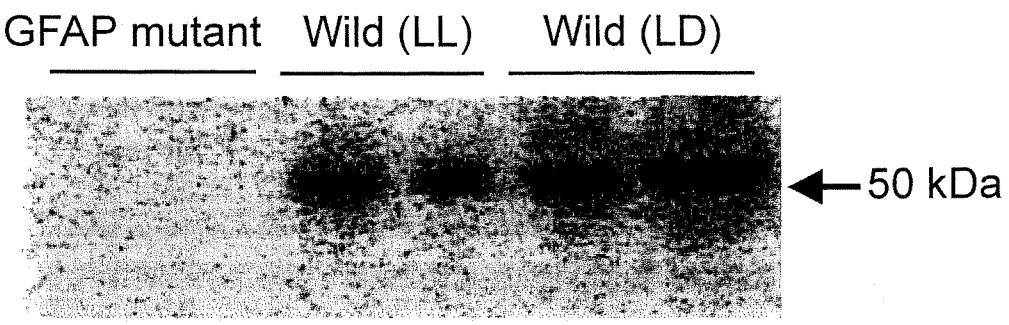
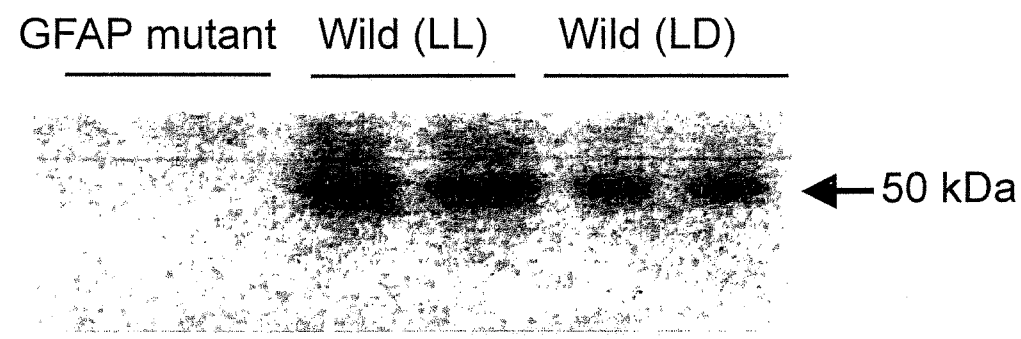
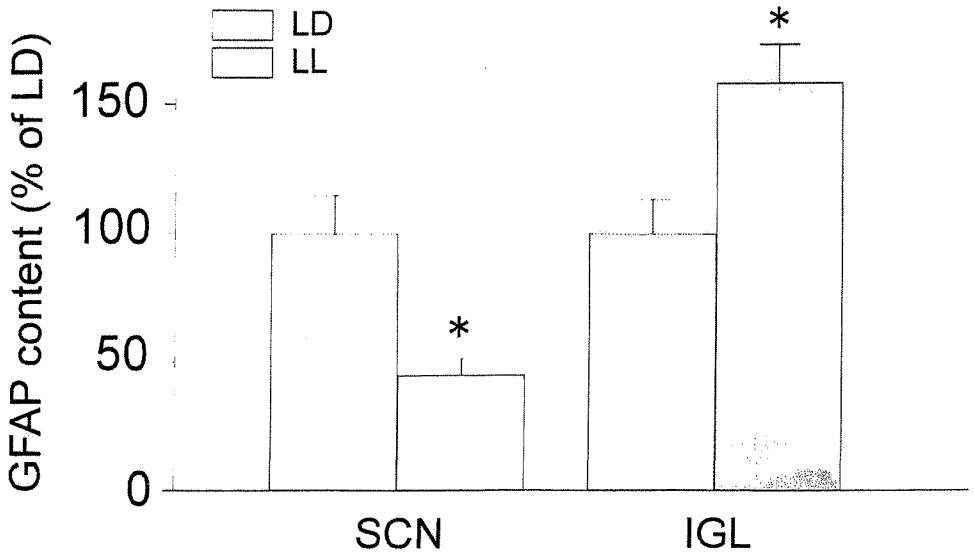
A


GFAP mutant
Wild (LL)
Wild (LD)
50 kDa

B


GFAP mutant
Wild (LL)
Wild (LD)
50 kDa

C


LD
LL
GFAP content (% of LD)
150
100
50
0
*
*
SCN
IGL

定性がある)．また，変温動物や植物にも体内時計が備わっていることからも想像できるように，体内時計は外界の温度変化に対して補償性が高い(Q 10 が 1 に近い)．行動の発振周期が 24 時間より極端に長い，短い，無周期などのミュータントのショウジョウバエを用いた研究から時計遺伝子として per と tim 遺伝子が分離同定され，これらの遺伝子産物の会合，リン酸化，核内移行の時間経過から 24 時間の周期変動機構が解き明かされようとしている．時計発振には遺伝子-蛋白質-プロモーターへのフィードバック制御-遺伝子というループ機構の存在が提唱されているが，現在の役者だけでは 24 時間という長周期の制御機構を説明しきれていない．哺乳動物の時計遺伝子としては clock が見いだされた．これは人為的にミューテーションをかけた突然変異マウスからその候補遺伝子をクローニングして得られたものである.[1,2] 一方，per 遺伝子をもとにケトゲノム遺伝子のクローニングから時計遺伝子 m per，h per が昨年秋に同定された.[3] これらの遺伝子産物の mRNA は視交差上核に豊富に存在し，外界の明暗情報がなくてもサーカディアンリズム変動を示すことが分かった．

我々は普通地球上の明暗が変化するところに住んでいるので，24 時間より長い部分つまり 30～60 分間毎日針を進め，自転の 24 時間周期に合わせている．このような情報入力を同調といい，同調を引き起こすいくつかの方法の詳細は後述する．このように明暗変化がないときの約 24 時間変動のリズムを概日リズム「サーカディアンリズム(サーカ：約，ディアン：1 日)」といい，明暗条件下にある昼夜変動を日内リズムという．哺乳動物の体内時計が視交差上核(図 2)にあることは破壊，培養，移植等の研究から 1990 年代の初頭には確立された事実となった.[4]

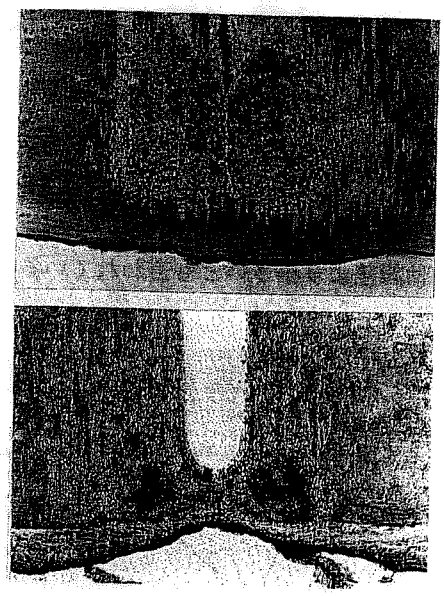


図 2 視交差上核の免疫組織化学(上側，ハムスター；下側，マウス)
上側：体内時計の位相変化を起こす時刻に光照射を行ったときの FOS 蛋白質の発現を示す．網膜からの神経性の投射が豊富な部位(腹外側部)に FOS の発現も多い．
下側：GFAP(grial fibrillary acidic protein)陽性アストロサイトが視交差上核に豊富に存在することが分かる．アストロサイトが体内時計の光同調に関わっていることが GFAP ノックアウト動物を用いた研究から分かった．

体内時計からの周期変動情報は脳全体に伝わり，体温，睡眠覚醒，ホルモン分泌，自律神経反応リズムなどを形成しているが，その神経性，ホルモン性制御機構の詳細は不明である．視交差上核は松果体へも多シナップス性に出力情報を出し，体内時計支配でメラトニンの分泌を制御している．ところがそのメラトニンがフィードバック機構で逆に視交差上核の時計機構を制御しており，その詳細も後述する．

光とメラトニン

Light and Melatonin in the Circadian Rhythms

話題

早稲田大学人間科学部　教授
柴田　重信
SHIGENOBU SHIBATA

① はじめに

早起きは三文の得という諺がありますが，早起きし朝日に当たることは頭の中にある体内時計をリセットし，睡眠覚醒リズムなどを正常な状態に保つのに重要であることが分かってきた．外界の光情報は色彩，明暗，形体視などの視覚情報に大切であるのみならず，明暗情報は視床下部の視交差上核にある体内時計にも伝えられ地球自転の24時間周期よりずれない工夫に役立っている．松果体ホルモンのメラトニンは夜間に分泌され，かつ睡眠誘発作用があることから，光が明暗情報の明を，メラトニンが暗の情報を体内時計に伝達制御していると考えることもできる．ここでは，(1)体内時計の仕組み，(2)時計のリセット(同調)メカニズム，(3)リズム障害と，それに対する高照度光療法ならびにメラトニンなどの薬理療法の可能性について以下に述べる．

② 体内時計の仕組み

図1に体内時計の仕組みを模式的に記述した．中央には約24時間の周期変動を起こす発振時計がある．この時計の周期はヒトの場合24.5～25時間と24時間より長いが，マウスはほとんどの系統が24時間より短く，ラット，ハムスターはヒトに似ている．しかし周期の長短の種による違いの生理学的意味はよく分かっていない．またいずれの動物種においてもこの発振周期は老化ともに24時間に近づくことが知られている．時計の発振周期の変動誤差は非常に小さく，心臓の拍動誤差より小さいとも言われている(発振周期は非常に安

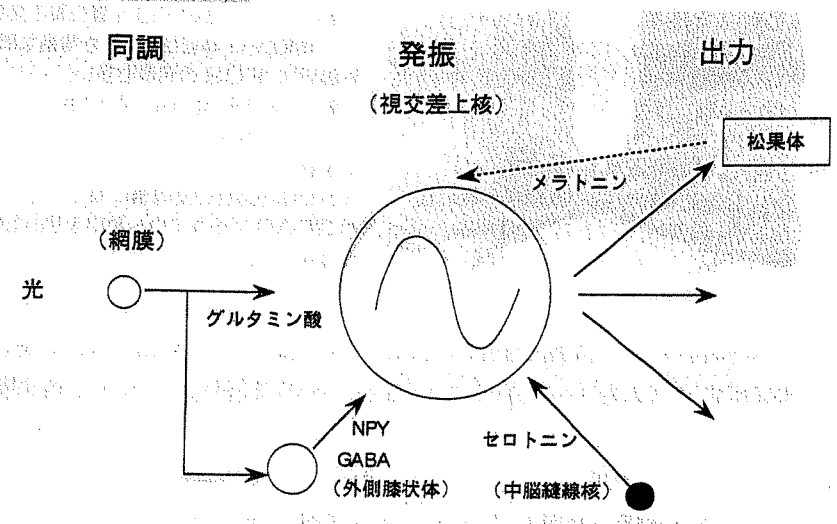


図1　哺乳動物体内時計の模式図
NPY，ニューロペプチドY；GABA，ガンマアミノ酪酸．

3) 光とメラトニンによる同調

(1) 光同調と非光同調　体内時計の振動周期を24時間に同調させる刺激を同調因子という．光が最も強力な同調因子であるが，光以外にも，強制的覚醒，メラトニン，種々の薬物あるいは胎児や新生児期の場合の母体などが非光の同調因子として働くことが知られている(図3)．横軸に光や視神経刺激のサーカディアン時刻を，縦軸にその時の位相の変化を前進と後退で表すと，位相反応曲線が得られる．したがってヒトのように周期が長い場合には位相反応曲線の前進部分を使い，マウスの場合は位相後退部分を使って体内時計を毎日リセットしていることになる．実際，ヒトやハムスターの場合は前進の部分が大きくマウスの場合は後退の部分が大きい．

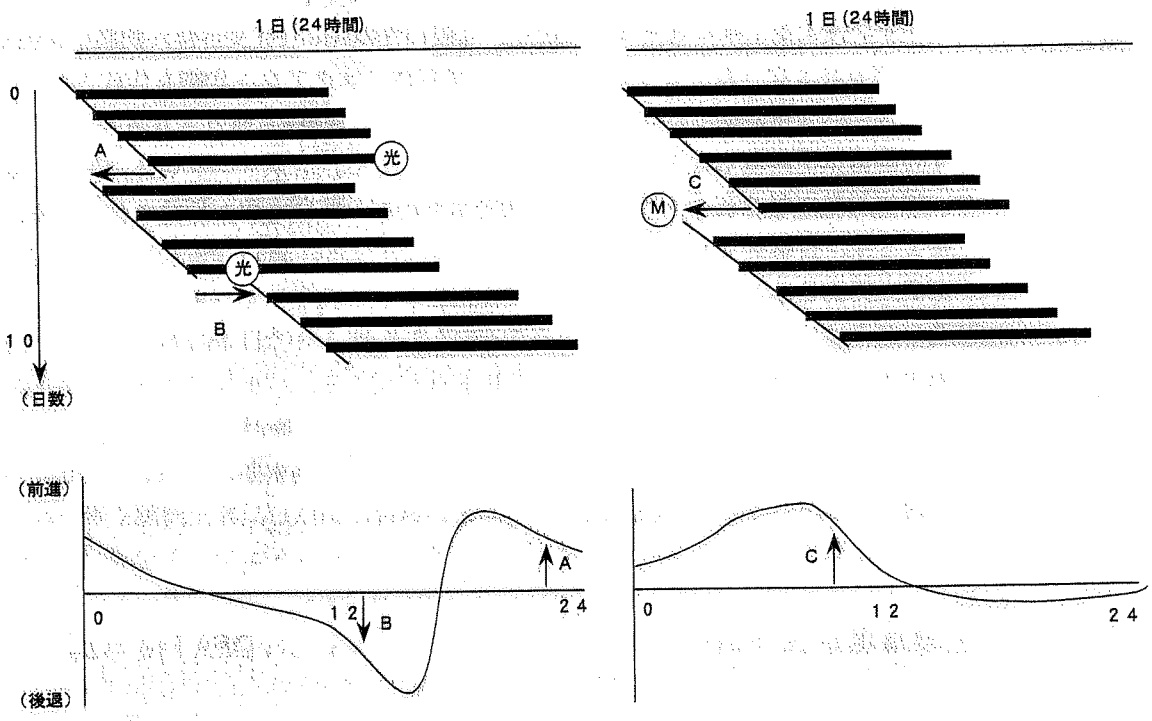


図3　サーカディアンリズム同調(リセット)の模式図
左側，光による同調；右側，メラトニンなど光によらない同調．
ネズミの活動期を黒いバーで示している(ヒトの場合睡眠時間を表していると考えても良い)．恒常暗期で飼育しているため活動期が日数の経過とともに右側へずれていく(体内時計の遅れによる)．活動期の終わりに光照射すると次の日の活動開始が早くなり(位相前進)，活動開始まもなく光照射を行うと遅くなる(位相後退)．一方，メラトニンは活動期の始まる前に投与すると位相前進が起こる．このような結果を，横軸にサーカディアンリズム時刻(個体によって周期が24～25時間と異なるため，例えば24.5時間を24時間に縮めて表す時刻をサーカディアン時間と定義する．一般的には動物の活動開始時刻をサーカディアン時刻12とする)を取り，縦軸に位相の変化量を分もしくは時間で表す．サーカディアン時刻13～15の光照射で位相後退が，19～24では位相前進が見られる．つまり，我々は毎日明け方(動物の活動期の終わり)の光照射により，地球自転の24時間周期からずれないようにしていることが分かる．また，メラトニンによる位相反応曲線は光照射の場合に比較して180度位相がずれている．

(2) 光同調の細胞内情報伝達系　外界の光情報は網膜のガングリオン細胞から視交差上核へ至る直接経路(網膜視床下部路)と，外側膝状体を経由して入力する経路(外側膝状体視床下部路)の両者によって体内時計へ伝えられている．前者の伝達物質はグルタミン酸で，後

者はニューロペプチドYやGABAだと考えられている．光同調に働いているのはグルタミン酸神経系が主であり，その受容体としてはAMPA/KAとNMDA受容体が重要である．その後CREBのリン酸化，カルモデュリンカイネース，NOの活性化とc-fos遺伝子の発現が見られるが，その後どのようなカスケードがclockやm per，h per遺伝子の制御を行い，光同調が完成するのかは分からない．[5]

(3) メラトニンを中心に非光同調について　松果体除去ラットや，メラトニン合成能力の低い系統のマウスの実験から，サーカディアンリズムに対するメラトニンの働きについては長い間否定的な見解であった．最近，恒常暗のフリーラン条件下にメラトニンを毎日投与すると，フリーランリズムが24時間周期に固定され，この作用が視交差上核破壊動物では出現しないことから，メラトニンが視交差上核に作用し時計を同調させる可能性が指摘された．その後，メラトニン受容体がクローニングされ，メラトニンに高親和性のMel 1 a受容体が視交差上核に高密度に発現し，低親和性のMel 1 b受容体が網膜に発現していることが明らかとなった．ごく最近Mel 1 A受容体ノックアウト動物も作成され，メラトニンの生理学的役割が明らかになりつつある．[6] さらに，睡眠覚醒リズム障害とヒトメラトニン受容体遺伝子異常との関連性の研究も始まっている．メラトニンが体内時計の前進作用や，睡眠誘発作用を有することから，時差ボケ治療の臨床応用が期待されている．図4に示すように，日本からハワイやアメリカ西海岸に行くと，5〜8時間位相を前進させる必要がある．アメリカでは日本時間の夜明けに相当するところに光があたることによって位相前進のドライビングフォースがかかることになる．ところで日本時間の午後にメラトニンを服用すると，睡気が生ずるとともに位相前進が起こる．つまりメラトニン服用により光による位相前進と協力的に働いて，早く現地の時間に体内時計を合わせることができるようになる．ニューロペプチドYやセロトニン1A受容体刺激薬，強制的な覚醒も体内時計を同調させるが，その作用様式はメラトニンの場合に類似し，非光同調や暗パルス型同調やarousal型同調などとも呼ばれている．[7] 図4にはセロトニン1A受容体刺激薬8-OH

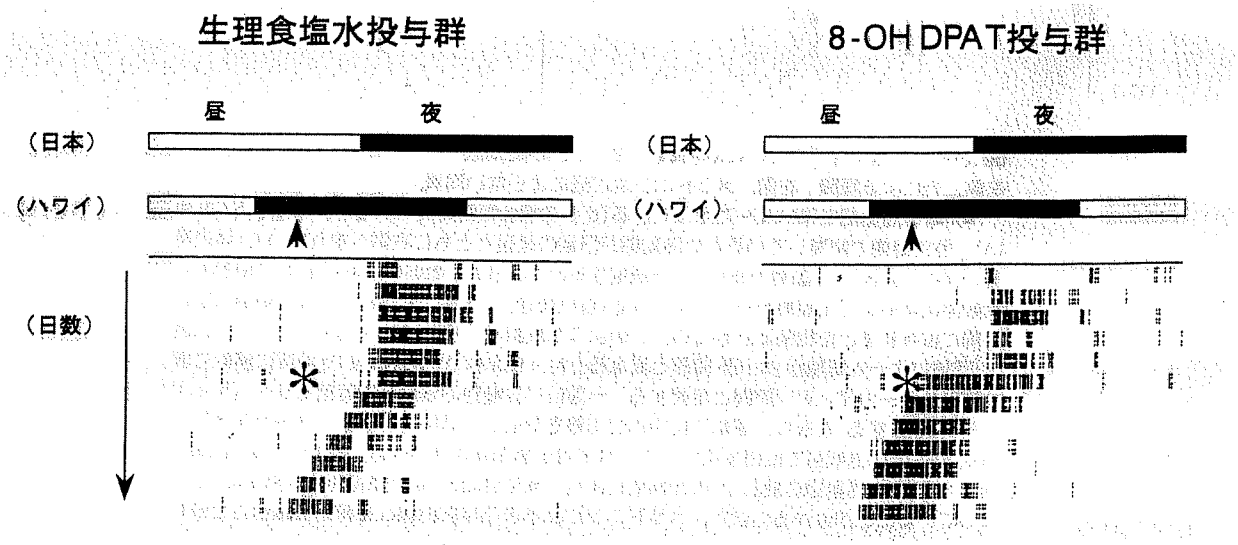


図 4　セロトニン関連薬物の位相前進促進作用
図は日本時間の明暗環境下に5日間飼育し，6日目に明暗環境を6時間位相前進させたときに動物が新しい明暗環境に慣れる時間経過を示す．8-OH DPATには再同調促進作用が見られる．短い黒バーはハムスターの輪回し行動を示し，密なほど活動が盛んなことを表す．

DPATのハムスター輪回し行動に対する作用を示した．

4 光，メラトニン同調と治療

冬季うつ病(seasonal affective disorder：SAD)は典型的なリズム病で，冬季に日照不足を起こす高緯度地域に見られる．秋から冬にかけて炭水化物の摂取量が増し，うつ症状が出現し，春になると寛解してくる．[8] 体内時計のリセットには光が最も重要であることから，これらの患者に高照度光を照射したところ，うつ症状が良くなったという報告がある．現在，夜間徘徊を伴う痴呆老人や睡眠相後退症候群の患者に対するメラトニンの投薬でも有効であったという報告があいついでいる．また，ビタミンB12の誘導体であるメチルコバラミンやセロトニン関連化合物が光同調を著明に促進させることも動物やヒトを対象にした研究で明らかになりつつあり，光同調を調節する化合物が今後薬物として開発される可能性がある．メラトニンは時差ボケや老人の睡眠障害の治療，不登校児の治療に有効かもしれないという研究もあるが，特許の問題があるので，現在はメラトニンそのものではなく，メラトニン類似化合物の検索が進められている．一方，メラトニンは日長を計測するホルモンだとも言われており，繁殖などの概年リズムの調節作用，また母児間同調作用にも関わっているのではないかと考えられているので，産婦人科領域の研究者にも興味が持たれ始めている．サーカディアンリズムの研究は歴史が浅いが，リズム障害やリズム病と言った概念や疾病の診断が最近確立されつつあり，今後この分野の研究を応用した薬物が登場するものと考えている．[8]

5 おわりに

ヒトの体はホメオスタシスにより維持されていることは周知の事実であるが，これはあくまで体内時計の発振によるリズム変動を基盤とした変化であることを知っておく必要がある．光とメラトニンは体に陽と陰を照らしているが，最近の研究によりその体内時計への関わりがずいぶん分かるようになってきた．生体リズム機構が明らかになれば，時間治療学(生理現象，疾病症状の時刻依存性)，時間薬理学(薬効の時刻依存性)などの学問が盛んになり，更にこのような研究が副作用軽減に役立つ治療法の確立にも寄与すると考えられる．最後に，読者のサーカディアンリズムに対する関心が芽生えれば幸いだと思う．

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視交叉上核における NMDA受容体と長期増強現象

守屋　孝洋　もりや　たかひろ／九州大学薬学部薬理学教室 博士課程／〒812　福岡市東区馬出3-1-1
柴田　重信　しばた　しげのぶ／早稲田大学人間科学部薬理学教室 教授／〒359　埼玉県所沢市三ヶ島2-579-15

はじめに■

体内時計の存在する視交叉上核（SCN）へは外界の明暗情報が視神経－視床下部路を通して入力しており，この入力神経の伝達物質はグルタミン酸であることが明らかになってきた。この入力神経は体内時計の重要な性質の1つである時刻依存性のリセット機構に働いていることがわかってきた。そこで本稿ではこのグルタミン酸神経の体内時計リセット機構の役割を，NMDA受容体を中心に解説した。また，グルタミン酸神経の高頻度刺激はシナプス電位の長期増強現象を引き起こすことが，海馬などで広く認められている。そこで，SCNでも長期増強現象の発現が起こることを，著者らが得られた結果を中心に述べる。

1　体内時計の光同調■

生物の様々な生理機能には，1日を1サイクルとする変動が観察される。たとえば，夜行性動物であるラットは昼間は睡眠をとり，夜間に覚醒し行動する。また，体温や心臓の活動あるいは血中ホルモン濃度といったほとんど全ての生理機能も，ある決まった位相関係を保ちながら変化する。明暗条件下の1日の中でのこのような変化を日内変動という。一方，外界の1日の手掛かりのない状態，たとえば恒暗条件下では，体内時計は独自の周期に従って発振するが，このような状態のリズムをフリーランリズム（自由継続リズム）という。この状態の約1日のリズムをサーカディアンリズム（サーカ；約，ディアン；1日）といい，脳内に存在する体内時計がこのリズムを発振することが明らかになっている。

ヒトの体内時計のサーカディアンリズム周期は24.5時間であるために，隔離状態にある人間では，起床時間や就眠時間が毎日0.5時間ずつ遅れていくことになる。ところが日常ではこのようなことは起こらない。それは1日の明暗の変化すなわち光の変化が体内時計を同調しているからである。光は最も強力な同調因子であり，これが体内時計を同調するときには位相の変化を引き起こすことができる。

サーカディアンリズムの研究では，動物を恒常条件下に飼育した後も，以前に昼だった時間，つまり動物が昼と感じている時間帯を主観的昼，逆に夜と感じている時間帯を主観的夜という。光による同調すなわち位相変化作用は，主観的夜においてのみ起こる。すなわち，光は主観的夜の始まりでは体内時計の位相後退（時計の早送り）を引き起こし，逆に主観的夜の終りでは体内時計の位相前進（時計の巻き戻し）を惹起する。先程述べたヒトの場合では，フリーラン周期が24.5時間であるために，主観的夜の終り（まさに目を覚まそうとする時間帯）に太陽光が0.5時間分の位相前進を起こし，体内時計を1日に同調するしくみになっている。

その他にも，気温，食事，母親，運動などの非光同調因子も体内時計を同調する働きがあるが，その度合は光

同調に比較すると弱いことが知られている。

II　光同調因子としてのグルタミン酸■

哺乳類の体内時計は，視床下部に位置する視交叉上核（Suprachiasmatic Nucleus；SCN）という左右一対の神経核に存在する。SCNを外科的に他の脳部位との神経連絡を切断しても，またSCNを含む脳スライスを作製しても，その神経の電気的活動は約24時間を1周期とするサーカディアンリズムを示すことから，SCNに存在する体内時計は自律性があるということができる[11, 31]。

光同調のための神経連絡は主に2種類が知られている。1つは光受容器である網膜からSCNに至るモノシナプス経路で，Retinohypothalamic Tract（RHT）という。もう1つは網膜から外側膝状体を経由しSCNに至る経路で，Geniclohypothalamic Tract（GHT）という。この両経路の存在は解剖学的，電気生理学的研究によって証明されている[20]。外側膝状体を電気的に破壊しても体内時計の光同調はたいした影響を受けないことが報告されており，光同調に重要な経路はRHTであり，GHTは光同調を補助もしくは調節する役割をしているものと推測されている。

SCNが体内時計であることが明らかになって以来，光同調を担う物質，すなわちRHTの神経伝達物質を同定しようとする研究が続けられてきたが，以下のような報告から興奮性アミノ酸であるグルタミン酸がRHTにおける神経伝達物質であることが一般に受け入れられている。①順行性トレーサーを網膜に注入し，RHT終末を可視化した状態で，グルタミン酸の局在を免疫電子顕微鏡レベルで解析すると，RHT終末にはグルタミン酸陽性反応が豊富に検出された[3, 33]。②SCNを含む脳スライスを用いた実験で，視神経の電気刺激によりSCNからトリチウムラベルされたグルタミン酸が放出された[15]。③同じくSCNを含む脳スライスを用いた実験で，グルタミン酸の還流応用が，SCN神経活動リズムの位相変化を惹起し，その位相反応曲線は光刺激による行動リズムの位相反応曲線と極めて類似していた[32]。④CNQXやMK-801などの様々なグルタミン酸受容体拮抗薬が光刺激による位相変化やSCN内でのFOS蛋白質誘導のいずれも抑制する。

また，グルタミン酸の他にも，アセチルコリンやヒスタミンあるいはニューロペプチドであるサブスタンスPも光同調に関与する物質の候補としてあげられている。ところが，これらの物質による光同調作用はグルタミン酸受容体拮抗薬によって抑制されることから[4]，光同調に関与する1次的な伝達物質としてはグルタミン酸が有力視されている。

III　光同調におけるNMDA受容体の役割■

グルタミン酸受容体はその薬物の親和性の違いからN-methy-D-aspartate（NMDA）に高親和性を示すNMDA受容体，Kainic acid（KA）や（R, S）-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid（AMPA）に高親和性を示すAMPA／KA受容体，さらに代謝型グルタミン酸受容体に大きく3つに分類される。代謝型グルタミン酸受容体がいわゆるG－蛋白質とカップルしてセカンドメッセンジャーの生成を惹起するのに対して，NMDA受容体やAMPA／KA受容体はそれ自身がカチオン透過型のイオンチャネルを形成するイオンチャネル内蔵型受容体であることが特徴的である。

その中でもNMDA受容体は，虚血時の遅発性神経細胞死[34]や脳発生におけるシナプス形成[17]，さらには海馬における長期増強現象（Long-term potentiation；LTP）など多種の神経可塑性に関与しており，注目されている[16]。通常の静止膜電位では，生理的濃度のMg^{2+}によってNMDA受容体のチャネル活性は抑制されているが，リガンドの結合と膜の脱分極の両方に依存してチャネルは開き，主にNa^{+}とCa^{2+}の両方を細胞内に流入させ，その結果，NMDAは持続時間の長い興奮性シナプス後電位を発生させる。流入したCa^{2+}は多くのCa^{2+}結合蛋白質や様々なリン酸化酵素を活性化し，細胞内情報伝達経路のスイ

ッチをONにする。また，NMDA受容体のリン酸化酵素によるリン酸化によりチャネル活性が調節されることも報告されている。

体内時計の光同調はグルタミン酸が神経伝達物質として関与していることは前に述べたが，3つのグルタミン酸受容体の中でもNMDA受容体が重要であることが知られている。①NMDA受容体拮抗薬のMK-801は光刺激によるげっ歯類の行動リズムの位相変化を抑制する[5,6]。②光刺激は，SCNにおいて，位相変化を引き起こす時刻特異的に早期発現遺伝子の1つであるc-FOS蛋白質の発現を惹起するが（また，光照射前のc-fos mRNAアンチセンスのSCN内投与は，光刺激による位相変化を完全に抑制することが最近報告されている。），MK-801，DGGやAP5などのNMDA受容体拮抗薬はこれらの光刺激によるc-fos mRNAやc-FOS蛋白質の発現を抑制する[1,2]。③グルタミン酸還流応用によるSCN神経自発放電リズムの位相変位作用もMK-801によって抑制される。光同調の同様の抑制は，AMPA／KA受容体拮抗薬であるCNQXによっても生じることから，少なくともNMDA受容体とAMPA／KA受容体の両者が体内時計の光同調に関与していると考えられている。

このように光刺激による位相変位作用は，NMDA受容体拮抗薬で抑制される。したがって，当然NMDA受容体刺激薬は光刺激と似たような位相変位作用を引き起こすはずである。そこで著者らは脳スライスを用い，この可能性を確かめる研究を行った。すなわち，SCN神経活動やそのリズムの位相を指標とし，NMDA受容体刺激薬の効果を検討した[28,29]。まず，SCN神経細胞に対する作用を2-deoxyglucose（2-DG）の取り込みを指標とした急性実験を行い，NMDAが2-deoxyglucoseの取り込みを増加させることを見出した。すなわちNMDAはSCN神経細胞の代謝活動を上昇させることを明らかにした。また，NMDAの還流適用によってSCN内でc-FOS蛋白質の発現が引き起こされることも観察した。同様にNMDAの還流適用はSCN神経リズムの位相変化を引き起こし，その位相反応

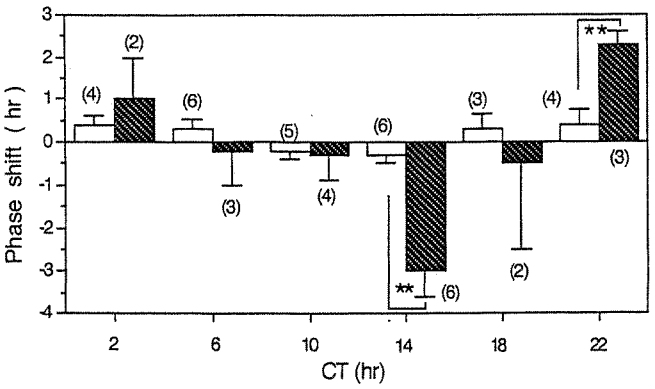

図1　SCNニューロン活動サーカディアンリズムのNMDA投与による位相反応
白ぬきカラムは溶媒投与群，黒ぬりカラムはNMDA（10 μM）投与群。NMDA（10 μM）を特定の時刻（CT）に還流応用後洗浄し，翌日SCNから自発放電活動を記録する。活動のピーク時刻が無処置群に比較して早くくれば位相前進（縦軸（＋）），遅くくれば位相後退（縦軸（－））で表す。NMDAはCT14投与で位相後退を，CT22投与で前進を引き起こす。このような位相変化は*in vivo*の行動サーカディアンリズムの位相変化と一致する。

曲線は視神経電気刺激によるSCN神経リズム位相変位や，*in vivo*での光刺激による行動リズムの位相変化と非常に類似していた（図1）。これらの受容体刺激薬を用いた実験や先程の受容体拮抗薬を用いた実験により，少なくともNMDA受容体が体内時計の光同調に深く関わっているということができる。

それではNMDA受容体活性化後，どのような細胞内情報経路が働き，体内時計を同調させるのであろうか。カルモジュリン阻害薬のcalmidazoliumやカルモジュリン依存性プロテインキナーゼ阻害薬のKN-62は光刺激による行動リズムの位相変化やc-FOS蛋白質発現，転写因子であるCREBのリン酸化，さらにグルタミン酸負荷によるSCN神経活動リズムの位相変化のすべてを抑制することを著者らは明らかにした。また，NMDAによるSCN神経活動リズムの位相変化作用は蛋白質合成阻害薬であるシクロヘキシミドによって抑制されることも見出した[30]。カルモジュリン依存性プロテインキナーゼがCREBをリン酸化し得ることを考えあわせると，光同調のメカニズ

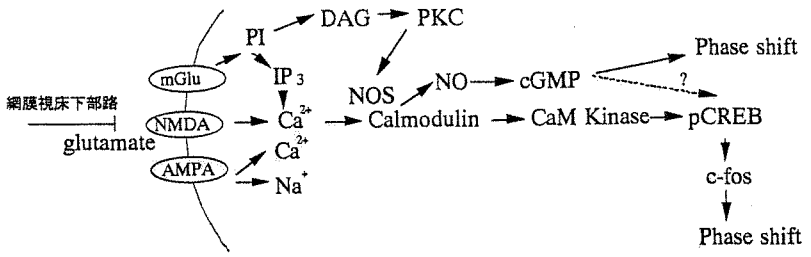

図2　SCNサーカディアンリズムの光同調におけるグルタミン酸受容体の役割
mGlu：代謝型グルタミン酸受容体，NMDA：NMDA受容体，AMP：AMPA／KA受容体，PI：ホスファチジルイノシトール，DAG：ジアシルグリセロール，PKC：プロテインキナーゼC，IP3：イノシトールトリスリン酸，NOS：NO合成酵素，NO：一酸化窒素

ムは以下のように考えられる（図2）。まず，光によって視神経終末より遊離されたグルタミン酸がNMDA受容体などのグルタミン酸受容体の活性化を引き起こし，その結果細胞内に流入したCa^{2+}が，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を活性化し，CREB蛋白質がリン酸化される。リン酸化され活性化されたCREB蛋白質は遺伝子上のCRE領域に結合し，c-FOSなどの早期発現遺伝子の発現を惹起し，新たな蛋白質の発現あるいは未知のリズム発振遺伝子の発現を調節する結果，体内時計の同調が引き起こされる。最近，ガス状伝達物質である一酸化窒素（NO）の合成酵素阻害薬によって光刺激による行動リズムの位相変化やNMDAによるSCN神経活動リズムの位相変化が抑制されることが報告された[7, 35]。したがって，NOの産生とそれに引き続くcGMPの増加が，NMDA受容体刺激後によるc-FOS蛋白質発現の下流において重要な働きをしていることが示唆された。

ところで，第3の眼ともいわれる松果体で生成され分泌されるメラトニンは，SCNに存在する体内時計によってその産生量が調節される一方，外界の光によって急性的にその産生が抑制されることが広く知られている。この光による急性的なメラトニン産生抑制の神経経路は破壊実験などから，SCNがその中継点として機能することが明らかになっている。すなわち，光受容器である網膜で入力した光情報は，順にSCN，Paraventricular Nucleus（PVN），Intermediolateral Cell Column（IML），Superior Cervical Ganglion（SCG）を経由して松果体に達する。SCGからは最終的にノルエピネフィリンが神経伝達物質として使われ，これがβ-受容体を介してメラトニン合成の律速酵素であるN-Acethyltransferase（NAT）の不活性化を引き起こす。高橋らのグループは，NMDA受容体の拮抗薬であるD-AP5やN-[1-(2-thienyl)-cyclohexyl]-piperidineが，光刺激による血中メラトニン濃度の減少やNAT活性の低下を抑制することを明らかにした[25]。したがって，NMDA受容体は，同調因子としての光情報だけでなく，メラトニン産生抑制などの他の光情報にも広く関わっていることが示唆された。

IV　NMDA受容体のサブタイプとSCN内分布，機能的役割

現在までのところ，NMDA受容体は5つのサブタイプがラット，マウスよりクローニングされている[21]。各サブタイプはNMDAR1（ζ1），R2A（ε1），R2B（ε2），R2C（ε3），R2D（ε4）と命名されており，それぞれは4つの疎水性領域をもち細胞膜貫通型受容体を形成することが推定されている。アフリカツメガエル卵母細胞にNMDAR1サブユニットを発現させると，それ単独ではチャネル活

性をほとんど示さないが，いずれかの1つのNMDAR2サブユニットをNMDAR1と共に発現してやると非常に活性の高いチャネル活性が現れるようになる[18]。また，NMDAR1サブユニットに対する特異的な抗体によって，NMDAR2AサブユニットやNMDAR2Bサブユニットなど他のNMDAR2サブユニットが免疫沈降されてくるというような報告を考えると[27]，NMDA受容体は数種類のサブユニットが会合したヘテロオリゴマーを形成していることが推測される。また，NMDAR2サブユニットのカルボキシ末端にはリン酸化などの様々な修飾部位が存在することから，NMDAR1が受容体として機能する上での構成サブユニットを形成し，NMDAR2は調節サブユニットとして働くことも考えられている。

脳内の発現パターンをみてみると，NMDA受容体各サブユニットが機能的多様性を有していることを連想させる[36]。まず，NMDAR1 mRNAは胎生期より脳全体に発現する。一方，NMDAR2BやNMDAR2D mRNAは胎生期のみに発現が認められ，生後急速に消失する。したがってこれらのNMDAR2サブユニットは神経細胞の分化や神経回路の形成に深く関与しているであろうと考えられる。それに対してNMDAR2AやNMDAR2C mRNAは生後に発現が認められ，その脳内分布はそれぞれ多様的である。

SCNにおけるNMDA受容体サブユニットmRNAの発現パターンも*in situ*ハイブリダイゼーション法を用いて詳細に検討されている（表1）。NMDAR1 mRNAはSCN全体に発現しているが，SCN以外の視床下部領域にも同様に発現しており，その発現はSCNに特異的ではない。NMDAR2サブユニットmRNAの発現パターンは使用する動物種や報告によってまちまちである。マウスでは，①ノザンブロットによる解析によってNMDAR2BとNMDAR2A mRNAがSCNに強く発現しているが，NMDAR2CとNMDAR2D mRNAは検出できないという報告や[24]，②*in situ*ハイブリダイゼーション法によって，NMDAR2BとNMDAR2D mRNAは検出されないが，NMDAR2C mRNAがSCNの腹外側部と背内側部に限局して存在するという報告がされている[36]。またラットでは，③いずれのNMDAR2サブユニットmRNAの発現も認められないという観察や[9]，④NMDAR2C mRNAがSCNの背内側部に存在するという報告がある[19]。一般的に，体内時計を維持する上で，SCN内の各部位が機能的な役割分担をしており，腹外側部では主に光同調の入力を，また背内側部では体内時計の発振そのものや他の脳部位への出力をになっていると考えられている。したがって，NMDAR2サブユニットのSCN内での局在は興味がもたれるところである。またラットにおいて，⑤NMDAR1とNMDAR2C mRNA量が日内変動し，光刺激が位相変化を引き起こす時刻特異的にこれらの発現量を増加することも報告されており[12]，体内時計の発振，同調にこれらの

表1　SCNにおけるNMDA受容体mRNAの発現と分布

文献	動物，方法	NMDAR1	NMDAR2A	NMDAR2B	NMDAR2C	NMDAR2D
19	Rat, *In situ* *	SCN全体に豊富に存在	検出されず	検出されず	背内側部に限局して存在	N.T.***
12	Rat, *In situ*	SCN全体に豊富に存在，光によって誘導	N.T.	N.T.	SCNに豊富に存在，光によって誘導	N.T.
9	Rat, *In situ*	SCN全体に豊富に存在	検出されず	検出されず	検出されず	N.T.
36	Mouse, *In situ*	SCN全体に豊富に存在	N.T.	検出されず	背内側部と腹外側部に限局して存在	検出されず
24	Mouse, Northern**	豊富に存在	比較的多い	豊富に存在	少ない	少ない

*：*In situ* Hybridization，**：Northern Blotting，***：Not Tested.

NMDA受容体が深く関与していることが示唆されている。

最近，相同的組換法により，これら各NMDA受容体サブユニットを欠損したノックアウトマウスが相次いで作製されている。NMDA受容体は，光同調，その情報の残りのSCN神経細胞への伝達，また発振機構そのもの，あるいはメラトニン合成抑制などの光情報の伝達など，いくつかの体内時計における機能を担っていると考えられている。したがって，これらのノックアウト動物の行動リズムの詳細な検討により，NMDA受容体の体内時計における機能的分担が，各NMDA受容体サブユニットの発現によって説明されることが期待される。

V　SCN内長期増強現象■

グルタミン酸などの興奮性神経伝達物質を有する神経を高頻度，短時間刺激すると，興奮性シナプス後電位（EPSP）の大きさが高頻度刺激前より増大し，これが数時間以上持続するという長期増強現象（LTP）が生じることはよく知られている。

このようなLTP現象は海馬において詳細に検討され，シナプスの可塑性現象の1つであると考えられており，LTPが海馬の重要な機能である学習，記憶の基礎過程を表していると解釈されている。このようなLTP現象は興奮性シナプスを有する脳部位で広く認められている。前述したように網膜からSCNへはグルタミン酸を有する興奮性シナプス入力があり，これが体内時計の同調に関わっていると考えられている。したがってこの網膜－SCN路においてもLTP現象が発現するのか，またここで得られるLTPは体内時計のリセット機構とどのように関連しているのか興味があるところである。そこでSCNで得られるLTP現象についての著者らの実験結果について以下に述べる。

1．SCN内LTP現象の時刻依存性

視神経とSCNを含むラットのスライス標本を水平面切断により作製する。視神経刺激でSCNからEPSPの複合電位としての電場電位を記録する。電場電位が安定的に記録された後，100Hz，1秒間の高頻度刺激を行い，その後この電位を120分間観察する。その結果，**図3，4**に示すように，昼間の高頻度刺激により主観的昼の前半をピークとした著明なLTP現象が発現した。一方，主観的夜間に刺激した場合，LTP現象はわずかに発現したのみであった。また，同一個体から得られた海馬切片を用いてSchaffer側枝刺激によるCA1領域からの電場電位を記録した。視神経の場合と同一条件で高頻度刺激すると刺激直後から約150％に増大するLTP現象が出現したが，この場合主観的昼間でも夜間でも得られる電場電位の大きさはほとんど同じであった。SCNのLTPと海馬で得られるLTPを比較すると，SCNのLTPは発現までの時間経過が非常にゆっくりしているという特徴を有した。実際，視覚に関わる脳部位として，外側膝状体や大脳皮質視覚領域でもLTPが発生することが知られているが，この場合のLTPもその発現過程がゆっくりとしておりSCNの場合と類似しているが，その理由は不明である。また前述したように，視神経刺激やNMDAによるSCN神経活動リズムの位相変化はこれらの処置を主観的夜に行ったときに起こる。したがって，SCNのLTP発生は体内時計のリセット機構とは直接的な関係はないものと考えられるが，その生理学的意味はわからない。外界の明暗サイクルの変化や，恒常条件の変化などには，以前の明暗環境を記憶していたような現象すなわち履歴現象がみられることがよく知られている。したがって，ここで得られたLTPはこのような明暗環境の学習効果となんらかの関係があるものと思われる。

2．SCNのLTPと老化

老齢動物では学習，記憶の機能が低下することはよく知られている。また，明暗サイクルへの再同調も低下する。このように老齢動物ではSCNシナプス伝達が低下しているものと考えられるので，老齢ラットのSCNのLTP

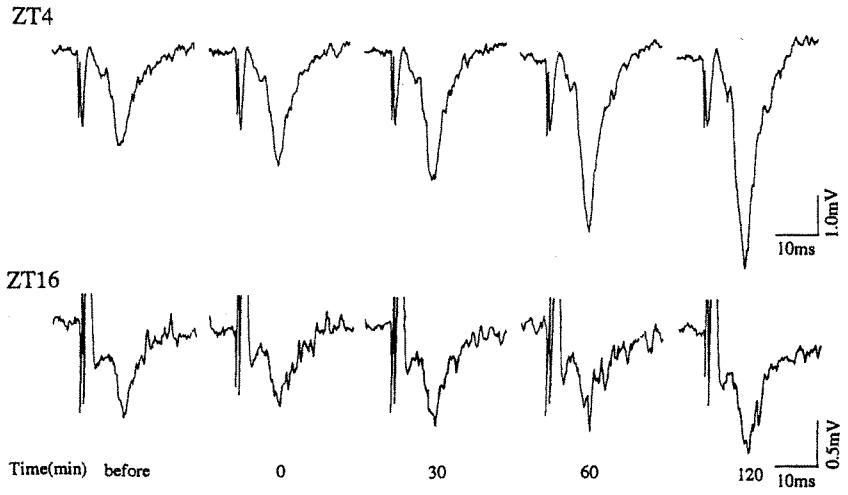

図3　SCN電場電位の長期増強現象
図は視神経を100Hz1秒間高頻度刺激をした後の視神経－SCN電場電位の変化を示す。
主観的昼間のZT4にテタヌス刺激を行うと，その後の電場電位の振幅は徐々に増大する。
一方，主観的夜間のZT16に同様の処置を行っても，振幅の変化は小さい。

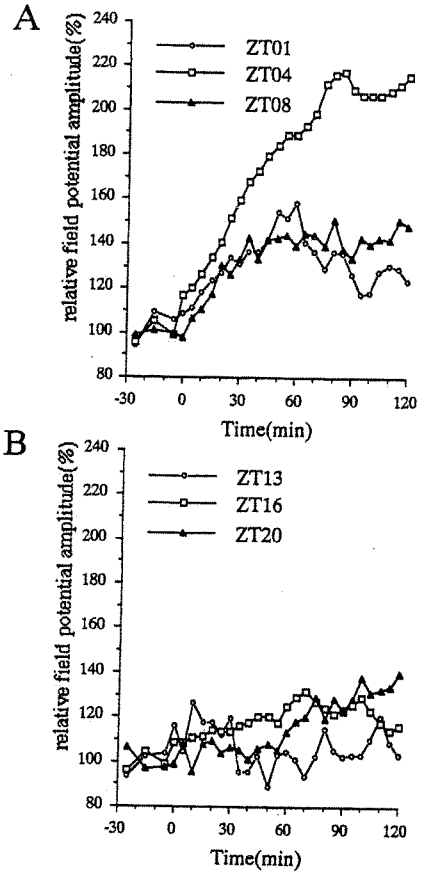

図4　SCN電場電位の長期増強現象の時刻依存性
Aは主観的昼間に，Bは主観的夜間に高頻度刺激し，
その後の電場電位の大きさを時間経過で示している。
主観的昼間の高頻度刺激により，電場電位の振幅が
ゆっくりと増大する。

発生について調べた。老齢ラット（1.5－2.0年齢）ではSCNから安定したシナプス電位を記録できない場合が多かった。また，記録できた標本の視神経を高頻度刺激したが，若齢ラットのようなLTPは観察されず，刺激後一過性の増大が認められるのみであった。これは老齢動物の海馬で得られるLTP現象と似ていた。

3．SCNのLTPと薬物

SCNで発生するLTPの神経機構を明らかにする目的で，LTPに対するNMDA受容体拮抗薬のAPVの作用を調べた。APVの投与により，視神経刺激によるLTP発生は完全に拮抗された。また，視神経刺激のかわりに，グルタミン酸（10mM）を還流液中に応用すると，この場合にもLTP発生がみられた。したがって視神経刺激により，グルタミン酸放出とそれに続くNMDA受容体の活性化がSCNのLTP発生には重要であることがわかった。

ビタミンB_{12}のアナログであるメチルコバラミンが動物行動のサーカディアンリズムの光同調を促進させるという報告がある。つまりメチルコバラミンが視神経－SCNのシナプス伝達を増強させうる可能性がある。そこでメチルコバラミンの視神経－SCNの電場電位に対する作用を調べた結果，この電位は増大しその効果はメチルコバラミンの洗浄後も1時間以上持続し，このLTPもNMDA受容体拮抗薬のAPVで拮抗された。つまりメチルコバラミン投与は視神経の高頻度刺激と類似した効果をもたらすことが判明した。したがって，メチルコバラミンの動物行動の光同調促進効果はSCNでの視神経－SCNのシナプス伝達増大に基づく効果であろうと解釈された。

前述したようにSCNにはNMDA受容体のサブタイプが多数発現していることがわかっているので，このようなLTP発現がどのNMDA受容体のサブタイプに関わっているかを明らかにする必要がある。また，このLTPがSCNの時計機構の何を反映しているのかを明らかにする必要がある。

おわりに■

体内時計の光同調や，LTP発生におけるNMDA受容体の役割の重要性について述べてきた。NMDA受容体は脳に広く分布し，様々な機能に関わっていることが明らかにされており，体内時計におけるNMDA受容体の機能は他の脳機能とどこが類似し，またどこが相違しているのかを明らかにし，時計機構におけるNMDA受容体を特徴づける必要がある。また，SCNにはmGlu受容体も発現しており，これらの受容体とNMDA受容体との関連性についても研究することにより体内時計におけるグルタミン酸神経の役割の全容が明らかになるものと考えられる。このような研究は，最近リズム性疾患の治療に用いられている高照度光療法などの作用機序解明にも寄与するものと考えている。

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BRAIN RESEARCH

Research report

Methylcobalamin amplifies melatonin-induced circadian phase shifts by facilitation of melatonin synthesis in the rat pineal gland

Masayuki Ikeda [a,b,*], Makoto Asai [c], Takahiro Moriya [a], Masami Sagara [b,c], Shojiro Inoué [b], Shigenobu Shibata [c]

[a] *Advanced Research Center for Human Sciences, Waseda University, Saitama, Japan*
[b] *Institute of Medical and Dental Engineering, Tokyo Medical and Dental University, Tokyo, Japan*
[c] *School of Human Sciences, Waseda University, Saitama, Japan*

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Abstract

Effects of methylcobalamin (methyl-B_{12}), a putative drug for treating human circadian rhythm disorders, on the melatonin-induce circadian phase shifts were examined in the rat. An intraperitoneal injection of 1–100 μg/kg melatonin 2-h before the activity onset tim (CT 10) induced phase advances of free-running activity rhythms in a dose-dependent manner ($ED_{50} = 1.3$ μg/kg). Injection c methyl-B_{12} (500 μg/kg) prior to melatonin (1 μg/kg) injection induced larger phase advances than saline preinjected controls, while th injection of methyl-B_{12} in combination with saline did not induce a phase advance. These results indicate amplification of melatonin-ir duced phase advances by methyl-B_{12}. Pinealectomy abolished the phase alternating effect of methyl-B_{12}, suggesting a site of actio within the pineal gland. In fact, methyl-B_{12} significantly increased the content of melatonin in the pineal collected 2-h after activity onse (CT 14). In contrast, no difference in melatonin content was found at CT 10, indicating that the effect of methyl-B_{12} may be gated afte the activity onset time when endogenous melatonin synthesis is known to increase. These results suggest that methyl-B_{12} amplifie melatonin-induced phase advances via an increase in melatonin synthesis during the early subjective night at a point downstream from th clock regulation.

Keywords: Circadian phase shift; High performance liquid chromatography; Melatonin; Methylcobalamin; Monoamine assay; Pineal gland; Rat; Vitami B_{12}

1. Introduction

Following the accidental discovery of an improvement in the non-24 h sleep–wake syndrome by the vitamin B_{12} analog, methylcobalamin (methyl-B_{12}) [18], systematic approaches to understanding the mechanisms have been examined. Methyl-B_{12} is also clinically effective for treating the delayed sleep phase syndrome, which is thought to be caused by the inability of the endogenous clock to entrain to environmental time cues (zeitgebers) [26]. Light-induced circadian phase shifts are amplified by the administration of methyl-B_{12} in rats and humans [10,12,14]. Field potentials recorded in the hypothalamic suprachiasmatic nucleus (SCN) evoked by the optic nerve stimulation are potentiated by the application of methyl-B_{12} in vitro [25]. These results indicate a positive effect of methyl-B_{12} on th photic entrainment pathway. In contrast, Ebihara et al. [5 reported that the circadian entrainment by daily intraper toneal (i.p.) injections of saline are attenuated by methy B_{12} administration. Since i.p. injections of saline induce a acute elevation of motor activity, the result indicates a inhibitory effect of methyl-B_{12} on the activity-depende circadian entrainment which is thought to be independe of the photic pathway [30]. Therefore, it seems likely tha methyl-B_{12} modulates circadian entrainment depending o the type of zeitgeber.

The pineal hormone, melatonin, is an endogenous zei geber. Single or daily melatonin administration induce circadian phase shifts of locomotor rhythms in mammal [2,29,34]. Since melatonin is normally synthesized in th pineal only during the dark phase [6] and the diurn administration of melatonin induces wakefulness (thus ele vates motor activity) in nocturnal rodents [11], circadia entrainment by melatonin is thought to be related to bot

* Corresponding author. Advanced Research Center for Human Sciences, Waseda University, 2-579-15, Mikajima, Tokorozawa, Saitama 359, Japan. Fax: +81-424-67-4377; E-mail: msikeda@mn.waseda.ac.jp

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photic and activity-dependent entrainment pathways. However, circadian entrainment by melatonin is essentially independent of these pathways since the effective time window to induce circadian phase shifts by melatonin is different from the windows for photic and activity-dependent entrainment pathways. Melatonin induces phase advances late in the resting phase (subjective day) [1], while photic stimulus induces phase shifts in the active phase (subjective night) [28] and evoked motor activity induces phase advances maximally in the middle of the subjective day [30]. In addition, melatonin induces circadian phase shifts of firing activity rhythm of the SCN [8,22,23] and melatonin receptors are abundantly found in the SCN [4,7,32], indicating a direct regulation of the circadian clock by melatonin.

Although melatonin and methyl-B_{12} are used therapeutically to treat circadian rhythm sleep disorders, the interrelation between these clock regulators has not been studied in animal models. In the present study, we analyzed the effect of methyl-B_{12} on melatonin-induced circadian phase shifts as well as on melatonin biosynthesis in the rat.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (310–450 g, 60–70 days old) reared under LD 12:12 in a constant temperature and humidity (25°C and 60 ± 6%) were used. Food and water were available ad libitum.

2.2. Recording of melatonin-induced circadian phase shifts

In order to record free-running activity rhythms, the animals were maintained in constant dim illumination (dim LL; 0.5–1.0 lux). The free-running period and the activity onset time (CT 12) were estimated by an eye-fitting method as reported previously [16]. Melatonin (Sigma) was initially dissolved in methanol and diluted with physiological saline to a final concentration of 1–100 μg/ml (less than 0.2% methanol). The melatonin solution was injected i.p. 2-h before the activity onset time (CT 10) which is the most effective for inducing phase shifts [1]. The magnitude of the phase shift was estimated as the difference in the activity onset time (CT 12) of pre- and postinjection days by three experienced investigators. In order to determine an accurate activity onset time following melatonin injection, activity recording was continued for at least 10 days after drug injection.

The effect of an i.p. injection of methyl-B_{12} on melatonin-induced phase shifts was analyzed using a crossover experimental design. Intact rats ($n = 8$) and pinealectomized (PINX) rats ($n = 6$) were maintained in dim LL for 10–14 days before the experiments. The rats were then randomly assigned to two treatment groups ($n = 4$ for intact rats and $n = 3$ for PINX rats). Each group was used during two trials separated by at least 3 weeks. In the first trial, rats received either saline or methyl-B_{12} (500 μg/kg) injection 30-min prior to a melatonin (1 μg/kg) injection at CT 10. In the second trial, the injection of saline or methyl-B_{12} was reversed between groups. In another set of experiments ($n = 6$), a methyl-B_{12} (500 μg/kg) injection was given 30 min prior to a saline injection and the rats monitored for 10–14 days under dim LL conditions to determine the effects of methyl-B_{12} on circadian activity. The volume of each injection was adjusted to 1 ml/kg body weight.

2.3. Pinealectomy

At least 2 weeks prior to the recording of drinking rhythms, rats were anesthetized with an i.p. injection of pentobarbital sodium (50 mg/kg) and placed into a stereotaxic instrument. A round cranial incision (7 mm ϕ, center at the ramda) was made and the dura mater was cut to expose the pineal gland. The pineal body was removed with fine forceps according to methods reported elsewhere [34]. After surgery, the incision was treated with 5000 U penicillin streptomycin. The dissected cranial cap was wiped with ethanol and then fixed on the cranium with dental resin. At the end of recording of drinking rhythms, the rats were sacrificed with a lethal dose of pentobarbital sodium, and the accuracy of the pinealectomy was visually confirmed.

2.4. Analysis of melatonin synthetic activity in the pineal gland

Rats were kept in individual cages and locomotor activities were recorded under constant darkness (DD). The activity was detected by infrared sensor (F5B-GA18M, Omron) and the free-running period was eye-estimated as described above. After a week under DD, 1 mg/kg methyl-B_{12} dissolved in saline or saline (control group) was i.p. injected at CT 9.5 and then the animals were sacrificed at CT 10 or CT 14 with lethal dosage of ether under dim red light (< 0.5 lux). Following decapitation and eyeball removal, the pineal body was exposed by a cranial incision, removed with fine forceps and immediately immersed in 30 μl of ice cold 0.3 M PCA in 50% methanol. The pineal gland was homogenized by a pellet mixer for 5 min and additionally by an ultrasonic mixer for 10 min at 4°C. The sample was centrifuged at 13,000 g for 15 min at 4°C and the supernatant (20 μl) was used to assay indoleamines by high performance liquid chromatography with electrochemical detection (HPLC-ECD). For melatonin assay, the sample (14 μl) was directly injected into the mobile phase with 0.1 M sodium acetate, 0.1 M citric acid monohydrate, octanesulfonic acid (80 mg/l) and EDTA (5 mg/l) adjusted to pH 3.9 in 30% methanol. The mobile phase was circulated at a flow rate of 1

ml/min. For evaluation of serotonin (5-HT), 5-hydoxyindoleacetic acid (5-HIAA) and *N*-acethylserotonin (NAS), the resting sample (6 μl) was diluted 20-times with the mobile phase and then injected into the chromatography column. Each target substance was filtered by a pre-column, divided in the 4.6 mm$\phi \times$ 150 mm chromatogram column (MA-50DS, Eicom) at 24°C and detected by the electrode (ECD-300, Eicom). The detected electrical signal was fed into a computer via an analog–digital converter (Power Chrome, AD Instruments) for peak area and retention time analysis.

3. Results

3.1. Dose responsiveness of melatonin-induced phase-advances in dim LL free-running rats

Rats kept under dim LL had stable free-running rhythms of drinking behavior (Fig. 2A). A single injection of 1–100 μg/kg melatonin at CT 10 induced a circadian phase advance in a dose-dependent manner ($F(3,22) = 3.50$, $p < 0.05$ by one-way ANOVA, $ED_{50} = 1.3$ μg/kg) (Fig. 1). The mean phase shift induced by 1 μg/kg melatonin (15.0 $\pm$ 10.2 min) was not significantly different from the saline injected control (-2.5 ± 13.1 min) while the injection of 10 μg/kg (40.0 $\pm$ 10.2 min) or 100 μg/kg (41.4 $\pm$ 11.2 min) melatonin induced significantly larger responses ($p < 0.05$ for each group by Tukey's post-hoc test following one-way ANOVA) (Fig. 1).

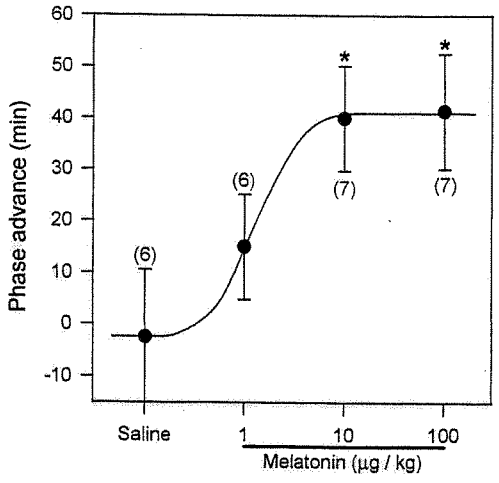

Fig. 1. Dose response curve for melatonin-induced phase advances. Melatonin or saline was injected intraperitoneally at CT 10 in free-running rats maintained in continuous dim illumination. An approximate sigmoidal curve was fitted by nonlinear regression using Sigma Plot™ 3.0. Note that 1 μg/kg melatonin did not induce a significantly larger phase shift than saline. The phase advances caused by the 10 or 100 μg/kg melatonin injections are significantly larger than the saline injected control ($* p < 0.05$, by one-way ANOVA followed by Tukey's post-hoc analysis).

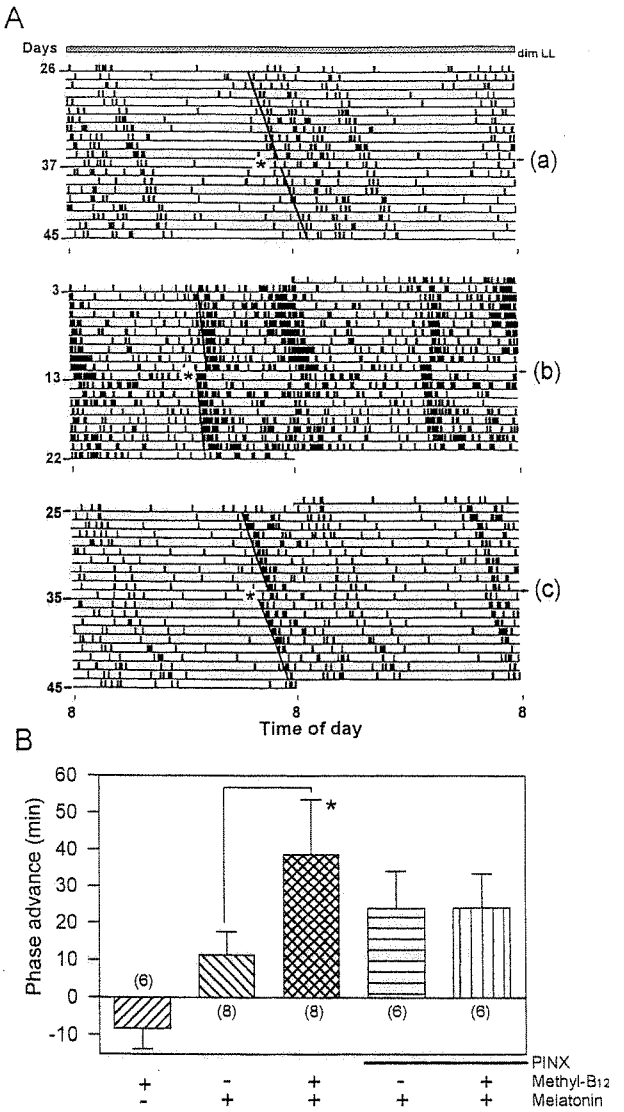

Fig. 2. (A) A double-plotted actigram of drinking behavior in a rat free-running under continuous dim illumination (dim LL). There was no circadian phase shift induced by a melatonin (1 μg/kg) injection in combination with a saline injection (a). Actigram showing a 70-min phase advance by a melatonin (1 μg/kg) injection in combination with a methyl-B_{12} (500 μg/kg) injection (b). This magnitude of phase advance was comparable to that induced by 100 μg/kg melatonin (c). Asterisks indicate the time of melatonin injection (CT 10). Bold lines on each actigram indicate the eye-estimated activity onset time (CT 12). (B) Means $\pm$ SEM of the circadian phase advance caused by combinatory injections of methyl-B_{12} (500 μg/kg) and melatonin (1 μg/kg): + and − on the bottom indicate drug and saline injection, respectively. Underline indicates the groups of pinealectomized (PINX) rats. Significant differences were observed between methyl-B_{12} + melatonin-injected group and saline + melatonin-injected group in intact rats ($* p < 0.05$ by Student's *t*-test), but an equivalent effect of methyl-B_{12} was voided in the PINX rats.

3.2. Effects of methylcobalamin on the melatonin induced phase-shift in intact and pinealectomized (PINX) rats

An i.p. injection of methyl-B_{12} (500 μg/kg), 30-min prior to the melatonin (1 μg/kg) injection significantly

amplified the melatonin-induced phase advance in intact rats ($p < 0.05$ by Student's t-test) (Fig. 2A, B). The phase advance amplified by the methyl-B_{12} was 3.4 times that of saline-pretreated rats and comparable to the shift induced by 10 or 100 μg/kg melatonin (Figs. 1 and 2A). On the other hand, injection of saline following a 500 μg/kg methyl-B_{12} injection did not induce a significant phase shift (Fig. 2B). In PINX rats, injection of the methyl-B_{12} (500 μg/kg) did not amplify the melatonin (1 μg/kg)-induced phase advance (Fig. 2B). The saline-preinjected controls showed slightly larger (+12.8 min) shifts in PINX rats than in intact animals (n.s. by Student's t-test).

3.3. Circadian variation in melatonin synthesis

The content of melatonin in the pineal gland had a manifest circadian rhythm with a gradual increment during the subjective night (CT 14–22) and a trough during the subjective day (CT 10) ($F(3,16) = 5.53$, $p < 0.01$ by one-way ANOVA) (Fig. 3A–D, Table 1). Similar to the melatonin rhythm, the intermediate substrate, N-acetylserotonin

Table 1
Circadian time (CT) variations in the contents of indoleamines in the rat pineal gland

	n	Serotonin	5-HIAA	NAS	Melatonin
CT 10	6	31.36±8.26	4.59±1.52	1.02±0.39	0.24±0.14**
CT 14	6	9.59±3.22	1.02±0.42	1.76±0.82	0.53±0.22*
CT 18	4	14.87±7.67	1.69±0.58	1.13±0.15	1.76±0.93
CT 22	4	11.37±2.81	1.62±0.28	1.45±0.17	2.65±0.63

Mean content (ng/gland)±SEM of serotonin, 5-HIAA (5 hydroxy indole acetic acid), NAS (N-acetyl serotonin), and melatonin. Animals sacrificed at CT 10 or CT 14 underwent a saline injection at CT 9.5 as a control experiment of methyl-B_{12} injection. * $p < 0.05$, ** $p < 0.01$ in comparison with melatonin content at CT22 by Tukey's post-hoc test following one-way ANOVA.

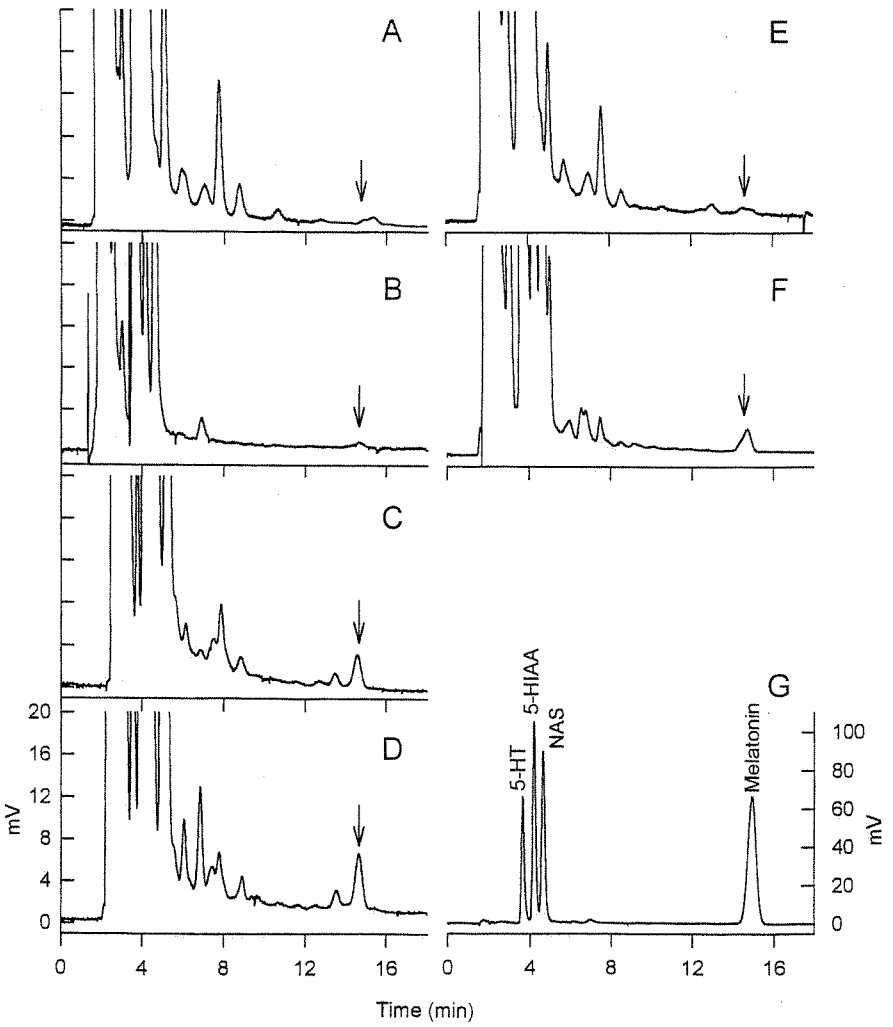

Fig. 3. Representative chromatographs of a rat pineal extract. Arrows indicate the retention time (RT) of melatonin defined by a standard solution (G) which contains 2.5 μg/ml serotonin, 2.5 μg/ml 5-HIAA, 2.5 μg/ml NAS and 25 μg/ml melatonin. The melatonin peak was small in the subjective day (A: CT 10) or early in the subjective night (B: CT 14) and was elevated from middle (C: CT 18) to late (D: CT 22) subjective night. An i.p. injection of methyl-B_{12} (1 mg/kg) elevated the melatonin content early in the subjective night (F: CT 14) but not in the subjective day (E: CT 10).

(NAS) levels were high during the subjective night and low in the subjective day, although the day–night difference was not significant due to the large inter-individual variations ($F(3,16) = 0.40$, $p = 0.74$, n.s. by one-way ANOVA). In contrast, the concentration of the substrate 5-HT indicated an inverse circadian pattern being 3.3 times higher at CT 10 than that at CT 14 (Table 1), although the time course difference was not significant ($F(3,16) = 2.86$, $p = 0.069$, n.s. by one-way ANOVA) (Table 1). The concentration of 5HT oxide, 5HIAA, indicated a similar circadian pattern to the 5HT rhythm ($F(3,16) = 2.95$, $p = 0.064$, n.s. by one-way ANOVA).

3.4. Effect of methylcobalamin on the melatonin synthetic activity in the pineal gland

A methyl-B_{12} (1 mg/kg) injection 30-min prior to the pineal sampling at CT 10 did not affect any of the evaluated monoamines (Fig. 3A, E, Fig. 4). However, methyl-B_{12} significantly enhanced (+0.56 ng/gland) the melatonin content of the pineal gland at CT 14 ($p < 0.05$ by Student's t-test) (Fig. 3B, F, Fig. 4). Similarly, NAS (+0.81 ng/gland) and 5-HIAA (+1.26 ng/gland) levels were increased while 5-HT levels were slightly decreased (−0.60 ng/gland) at CT 14 although these differences were not statistically significant (n.s. by Student's t-test) (Fig. 4).

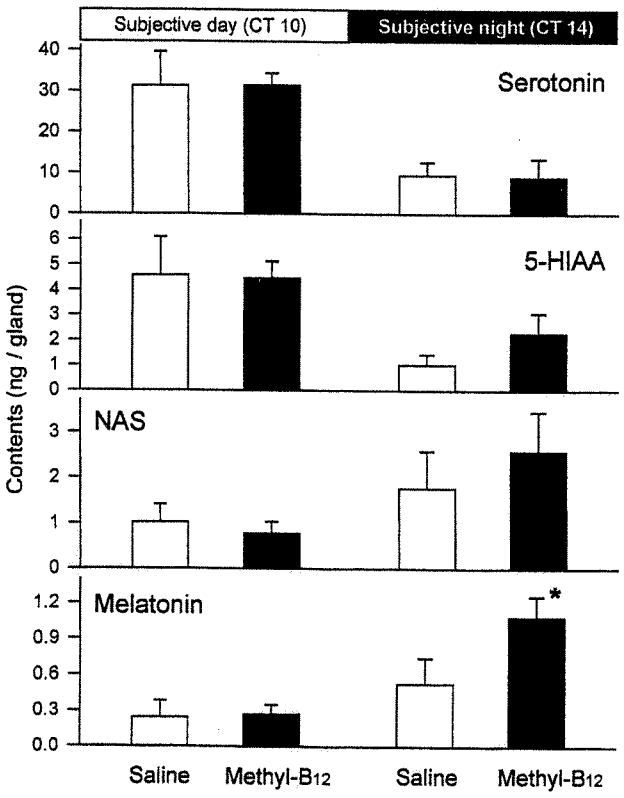

Fig. 4. Effects of methyl-B_{12} on the melatonin synthetic activity in the rat pineal gland. White bars indicate the mean content of monoamines in the pineal gland sampled after saline injection at CT 9.5 and dark bars indicate the mean contents after methyl-B_{12} (1 mg/kg) injection at CT 9.5. Significant differences between the saline and methyl-B_{12} groups were observed in the melatonin content in the subjective night (CT14) but not in the subjective day (CT 10). Mean + SEM, $n = 6$ for each group, * $p < 0.05$ by Student's t-test.

4. Discussion

The present study addressed the effect of methyl-B_{12} on the melatonin-induced circadian phase-shifts in the rat. A 1–100 μg/kg melatonin injection late in the subjective day (CT 10) induced a dose-dependent phase-advance in free-running rats maintained in dim LL. A methyl-B_{12} injection 30-min prior to the injection of melatonin (1 μg/kg) amplified the phase-advance. Pinealectomy eliminated the effect of methyl-B_{12} on the melatonin-induced phase shifts although the basal sensitivity for melatonin was slightly increased in the PINX rats.

The dose-responsiveness of melatonin for behavioral phase shifts and the sensitization for melatonin in the PINX rats observed in the present study were similar to those reported for free-running rats under constant darkness [34]. The magnitude of the phase advance caused by 1 μg/kg melatonin following preinjection of methyl-B_{12} was comparable to that caused by 10 or 100 μg/kg of melatonin alone. Methyl-B_{12} injection in combination with a saline injection did not induce apparent phase shifts, suggesting amplification of melatonin-induced phase shifts by methyl-B_{12}. Two possible interpretations may explain the effects of methyl-B_{12}. First, the sensitivity of the circadian clock to melatonin is amplified (e.g., melatonin receptors in the SCN are sensitized) by methyl-B_{12}. Second, methyl-B_{12} promotes endogenous melatonin synthesis augmenting the subthreshold dosage (1 μg/kg) of melatonin. The latter possibility is more plausible since the effect of methyl-B_{12} on the melatonin-induced phase shift was eliminated by pinealectomy which removes the primary locus for melatonin biosynthesis.

According to the above hypothesis, subsequent experiments addressed whether methyl-B_{12} injected at CT 9.5 affects melatonin synthesis in the pineal gland. To observe the melatonin synthetic activity, we evaluated the pineal gland for levels of melatonin, 5-HT, NAS, and 5-HT oxide, 5-HIAA by HPLC-ECD. The contents of these monoamines were detectable in the extracts from a single pineal gland by our HPLC-ECD method. The 5-HT and 5-HIAA contents at CT 10 were larger than those at CT 14, while the NAS and melatonin levels indicated inverse rhythms, being consistent with previous reports which used reversed-phase HPLC-ECD in combination with the radio immunoassay [24].

Interestingly, there were no significant differences in the content of melatonin and its substrates in the pineal gland which was collected 30-min after the methyl-B_{12} injection (CT 10). CT 10 corresponds to the time of the

melatonin injection in the first experiment. These results indicate that the amplification of melatonin-induced phase advances observed in the first experiment were not caused by an acute elevation of the endogenous melatonin content. On the other hand, early in the subjective night (CT14), the content of melatonin was increased while the 5-HT content was decreased by a methyl-B_{12} injection. These data indicate that methyl-B_{12} promotes melatonin synthesis. During melatonin biosynthesis, the intermediate substrate, NAS is synthesized from 5-HT by the rate-limiting enzyme, *N*-acethyltransferase (NAT). NAT activity was increased early in the night (or subjective night) by β-adrenergic innervation from the superior cervical ganglion which undergoes efferent regulation by the SCN (Fig. 5) [19–21]. Therefore, melatonin synthesis undergoes upstream regulation by NAT and methyl-B_{12} may accelerate the melatonin synthesis only when NAT is activated by the SCN clock.

Methyl-B_{12} is known to be a major methyl-base donor for the *S*-adenosyle methionine (SAM) which is involved in methylation reactions [27]. During melatonin synthesis, 5-hydroxy-indole-*O*-methyl-transferase (HIOMT) requires SAM as a methyl donor to convert NAS to melatonin [31]. Therefore, transmethylation by methyl-B_{12} may promote melatonin biosynthesis (Fig. 5). This hypothesis is supported by our preliminary observation in which cyanocobalamin, an analog of vitamin B_{12} which has a cyan-base in place of a methyl-base of methyl-B_{12}, was ineffective in modulating the melatonin content of the rat pineal gland (data not shown).

Although the phase response curve for single i.p. injections of melatonin which is based on the locomotor activity rhythms has a narrow window for the phase advance late in the subjective day [1], phase shifts of SCN firing rhythm recorded in vitro indicates a broad window for phase advances stretching from late in the subjective day to early in the subjective night [8,22,23] and an additional window late in the subjective night to early in the subjective day [23]. Therefore, it is conceivable that amplification of the melatonin-induced phase advances by methyl-B_{12} were caused by a secondary impact of melatonin rise during the early subjective night. However, since methyl-B_{12} in combination with saline did not produce a phase advance, the acceleration of melatonin synthesis in the early subjective night alone seems to be insufficient to induce a phase advance in vivo.

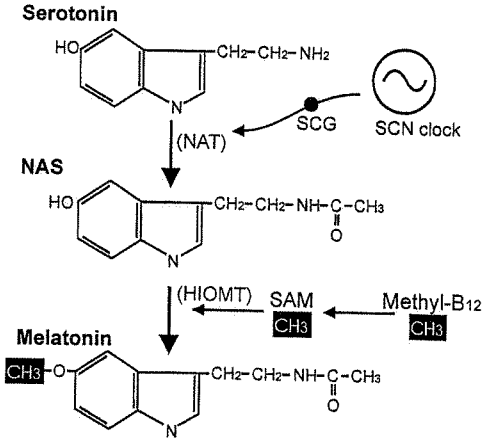

Fig. 5. A model of methyl-B_{12} action on melatonin synthesis in the pineal gland. In melatonin synthesis, the intermediate substrate, *N*-acethylserotonin (NAS) is synthesized from serotonin by the rate-limiting enzyme, *N*-acethyltransferase (NAT). NAT is activated early in the subjective night by β-adrenergic innervation from the superior cervical ganglion (SCG) which is regulated by the SCN clock. The methyl-B_{12} may serve as a methyl-base donor for the *S*-adenosyle methionine (SAM) which supplies a methyl-base for 5-hydroxy-indole-*O*-methyl-transferase (HIOMT).

The present results demonstrated an increase in melatonin synthesis by methyl-B_{12} in the pineal gland. However, with respect to our previous reports which indicated the somnogenic action of methyl-B_{12} in rats [13,17], further careful discussion will be required since endogenous melatonin is known to act as the 'darkness hormone' and thus induce wakefulness in the nocturnal animals. In our previous experiments, temporal sleep promotion and reduction of brain temperature was observed during or immediately after the nocturnal intracerebroventricular (i.c.v.) infusion of methyl-B_{12} [13,17]. In such cases, infusion of methyl-B_{12} into the third ventricle may directly affect the surrounding hypothalamic area [33], such as the medial preoptic area which is known as a sleep- and thermo-regulatory center. Interestingly, the day after a nocturnal 10-h i.c.v. infusion of methyl-B_{12}, diurnal sleep promotion and nocturnal sleep reduction were observed in the previous report [17] or similar reversed phase modulation was observed by the intravenous infusion of methyl-B_{12} [3]. In these cases, methyl-B_{12} may spread widely in the central nervous system and may affect pineal metabolism. Therefore, the reported function of methyl-B_{12} for sleep promotion may not be contradictory to the melatonin increase observed in the present study, although the most effective site of action of methyl-B_{12} still has to be determined.

We recently demonstrated that the total content of vitamin B_{12} analogs in the rat brain are decreased at night (or subjective night) and increased during the day (or subjective day) [15]. Interestingly, the circadian variations seen in the vitamin B_{12} content were similar to those of SAM activity and inverse to the melatonin content in the pineal gland [9,31]. Since SAM acts as a methyl-base donor for HIOMT in the process of melatonin synthesis (Fig. 5), these reports together with the present results suggest that endogenous methyl-B_{12} may be partially utilized for endogenous melatonin synthesis.

In addition to the reported functions for the photic and the activity-dependent entrainment pathways, the present study indicates that the melatonin-induced circadian entrainment is also modulated by the methyl-B_{12}. This effect may be caused by an acceleration of melatonin synthesis in the early subjective night when endogenous melatonin

synthesis is initiated by the circadian clock, although it is also possible that methyl-B_{12} increased the sensitivity of the clock for melatonin. The present results shed light on the understanding of the consequence as well as the mechanisms of methyl-B_{12} therapy for circadian rhythm sleep disorders.

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総 説

生体時計の生理学的，薬理学的，分子生物学的解析

秋山 正志[1]，守屋 孝洋[2]，柴田 重信[1]

はじめに

生物は外界の1日を単位とする環境サイクルに対応するために生体の内部状態を1日周期でリズミックに変化させている．こうした日周リズムのうちのいくつかは定常条件下でも24時間に近い周期で長期間持続することから生物自身に自律的にリズムを生起する機構が存在していることがわかる．このリズムを概日リズムと呼び，その機構を体内時計と呼ぶ．体内時計によって生み出されるリズムは，外界の様々な環境因子（同調因子）に同調し，正確な24時間周期のリズムとなる．哺乳類ではおよそ25年前に移植・破壊実験等から，視床下部の視交叉上核（suprachiasmatic nucleus: SCN）に概日性リズムを駆動する振動体が存在することが明らかにされた．SCNは名前の通りげっ歯類では視交叉の真上に位置する一対の直径が0.5 mmにも満たない卵形の小さな神経核である．SCNには様々な脳部位からの神経が投射している．特に，網膜から直接SCNにいたる網膜視床下部路（Retinohypothalamic tract: RHT）は，外界の明暗変化の情報を体内時計へ伝達し，内因性に生み出されるリズムを正確に外界の明暗サイクルに同調させるという重要な働きを有する．また，網膜から中脳の外側膝状体中隔葉（IGL）を中継してSCNにいたる外側膝状体視床下部路（Geniculohypothalamic tract: GHT）は，RHTと同様に外界の明暗情報を伝えたり，覚醒レベルの上昇に伴う体内時計の位相変化に重要な役割を果たしているものと考えられている．前者のように明暗（光）の変化による体内時計のリセットを光同調といい，後者のように光以外の因子によるリセットを非光同調という．光同調と非光同調の伝達経路は前述したように中継する神経経路も異なり，またSCN神経細胞内のシグナル伝達についても対照的であることが知られている．ここでは，[1]概日リズムの生理機構として光同調と非光同調に関わる情報伝達系を概観し，最近進展著しい[2]哺乳類における生物時計の分子遺伝学について説明し，最後に[3]リズム異常と創薬に関わる話題を取り上げる．

キーワード：概日リズム，視交叉上核，光同調，*per*遺伝子，薬剤開発

[1]早稲田大学人間科学部薬理学教室
[2]早稲田大学人間科学部人間総合研究センター
（〒359-1164 埼玉県所沢市三ヶ島2-579-15）

1. 概日リズムの生理学

(1) 光同調

光同調を担う物質，すなわちRHTの神経伝達物質はグルタミン酸であることが以下の知見から支持されている．すなわち，1）免疫組織化学的に解析すると，RHT終末にはグルタミン酸が豊富に含まれている．2）脳スライスを用いた実験で，視神経の電気刺激によりSCNから放射ラベルされたグルタミン酸が放出される．3）グルタミン酸のスライスへの還流適用による神経活動リズムの位相反応曲線は光刺激による行動リズムの位相反応曲線と極めて類似している．4）様々なグルタミン酸受容体拮抗薬が光刺激による行動リズムの位相変化やSCN内でのc-FOSタンパク質発現を抑制する(1)．

グルタミン酸受容体は薬物に対する親和性の違いからNMDA受容体，AMPA/KA受容体さらに代謝型グルタミン酸受容体に分類される．我々は脳スライスを用いてNMDA受容体刺激薬の効果を検討した(2)．2-Deoxyglucose（2-DG）の取り込みを指標とした急性実験では，NMDAが2-DGの取り込みを増加させること，NMDAの還流適用はSCN神経活動リズムの位相変化を引き起こし，その位相反応曲線は視神経電気刺激によるSCN神経リズム位相変位や，in vivoでの光刺激による行動リズムの位相変化と非常に類似していること，またc-FOSタンパク質の発現が引き起こされることを観察した．このNMDAの位相変化作用はin vivoの系でもMintzらによって確認された．

NMDA受容体はいわゆるイオンチャネル連結型受容体ファミリーに属し，活性化されると細胞外から細胞内へとCa^{2+}を流入させることが知られている．海馬などではテ

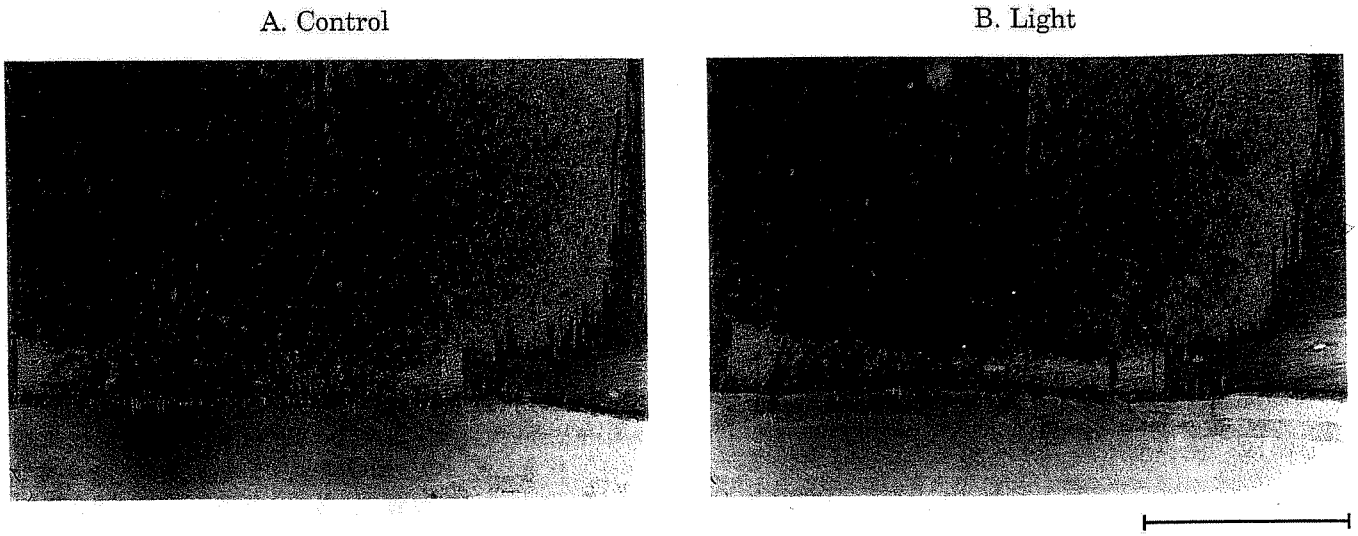

Fig. 1 Light pulse-induced phosphorylation of calcium/calmodulin dependent protein kinase II (CaMKII) in the hamster suprachiasmatic nucleus (SCN). Hamsters were exposed to a light pulse (300 lux) for 5 min at ZT 13.5 and perfused transcardially with saline and paraformaldehyde. Brain slices including the SCN were processed for immunohistochemistry with antibody to phospho-CaMKII. Scale bar = 500 μm.

タヌス刺激によりNMDA受容体が活性化され，Ca^{2+}流入とそれに引き続く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）の活性化がシナプス長期増強現象に重要な役割を果たしていることが知られている．我々はカルモジュリン阻害薬のカルミダゾリウム，トリフルオペラジンやCaMK阻害薬のKN-62が光刺激による行動リズムの位相変化やc-FOSタンパク質発現，転写因子であるcAMP Responsible Element Binding Protein (CREB)のリン酸化，さらにグルタミン酸負荷によるSCN神経活動リズムの位相変化のすべてを抑制することを明らかにした(3)．また動物行動の位相変化が起こる時刻の光刺激によってCaMKIIが活性化されることを見出した（図1)．最近，*c-fos*や同じ転写因子でありc-FOSタンパク質とAP-1複合体を形成する*junB*のアンチセンスオリゴヌクレオチドの脳室内投与が，光による行動リズムの位相変化を抑制することが報告された(4)．したがって，グルタミン酸受容体の活性化は，Ca^{2+}の流入とそれに続くカルモジュリンおよびCaMKの活性化，CREBのリン酸化を惹起し，c-FOS，JunBタンパク質などの早期発現遺伝子産物を発現させ，体内時計の同調が引き起こされると考えられる．

一酸化窒素合成酵素（NOS）が，CaMKによるリン酸化によって活性化されることが報告されている．NOSはRHTが投射しているSCNの腹外側部に多量に存在している．NOS阻害薬のL-NAMEがNMDA還流適用によるSCN神経活動リズムの位相変化を抑制すること(5)，L-NAMEは光照射によるハムスター輪回し行動リズムの位相変化を抑制し，この効果はNOの基質であるL-arginineによって消失することが明らかになった(6)．NOは細胞質中に存在する可溶性のグアニル酸シクラーゼを活性化し，細胞内のcGMPの濃度を増加させる．Weberらはc GMP依存性プロテインキナーゼの選択的阻害薬であるKT-5823のSCNへの局所投与が光刺激による行動リズムの位相前進を有意に抑制するが，行動リズムの位相後退に対しては全く作用しないことを報告した．これらの結果は，光による体内時計のリセットにはカルモジュリン－NOS－NO－可溶性グアニル酸シクラーゼ－cGMP－cGMP依存性プロテインキナーゼという細胞内シグナルが重要な役割を果たしていることを示唆している．

(2) 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，制限給餌や強制的運動，あるいは薬物による覚醒レベルの上昇や母親との接触などが知られている．SCNへの神経入力系としては前述したRHTやGHTのほかに，中脳縫線核群からの豊富なセロトニン神経投射が知られており，SCNには数種のセロトニン受容体が存在する．我々はセロトニン受容

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非光同調刺激によるものと極めて類似していることを明らかにした(7)．最近の我々の研究では5-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-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(8)．一方，IGLからはneuropeptide Y（NPY）神経が投射しているが，SCNへのNPYの投与は非光同調型の体内時計位相変化を引き起こし，さらにこの効果はテトロドトキシンで拮抗されないことから，NPYの位相変異作用はSCN内の神経細胞への直接的な作用であると思われる．Gilletteらは，cAMPアナログがSCNの神経活動リズムの位相を時刻依存的に変化させ，その作用は非光同調型であることを明らかにした(9)．さらにアデニル酸シクラーゼを促進させるフォルスコリンやホスホジエステラーゼ阻害薬であるIBMXも同様な位相変異作用を示すことから，内因性のcAMPが非光同調型の体内時計位相変異作用を有することがはっきりした．

2. 概日リズムの分子生物学

定常条件下でそれぞれの生物種が固有の周期のリズムを持っていることは，概日リズムが遺伝的な制御を受けていることを示している．概日リズムの分子機構の研究は最近までショウジョウバエ（*Drosophila*）やアカパンカビなどの下等生物を用いて行われてきた．なぜならこれらの生物ではリズムの周期の異なる突然変異体，いわゆる時計突然変異体の分離が容易であったこと，また分子生物学的手法が容易に適用できたからである．一方哺乳動物では最近まで生理学的・行動学的側面からの研究が主であった．ところが1997年に入り相次いで哺乳類のリズム発振自体に深くかかわっていると考えられる遺伝子がクローニングされ事態は一変しつつある．以下ではリズム発振および入力系に関わる遺伝子について説明する．

高等生物でも単一の遺伝子変異によって周期が変化することが示されたのは，ハムスターの*tau*変異体が最初であった(10)．この変異は常染色体半優性変異であり，正常なハムスターはおよそ24時間の活動リズム周期を示すのに対し，この変異遺伝子をヘテロで持つハムスターは22時間，ホモで持つハムスターは20時間の活動周期を示す．外界の温度に関わらず概日リズムの周期はほぼ一定に保たれている（温度補償性）が*tau*変異ハムスターではその機能が一部損なわれていることが報告された（哺乳動物のような恒温動物でも組織培養実験で温度補償性が確認されている）(11)．ハムスターではポジショナルクローニングのための遺伝的多型マーカーが整備されていないため，そのクローニングは今のところ成功していない．

遺伝マーカーを多数利用できるマウスを用いて，概日リズム異常をおこす変異マウスを分離し，その原因遺伝子を同定しようとする，いわゆるforward geneticなアプローチがJ. Takahashiらのグループによって進められた．1994年，Vitaternaらはおよそ300匹のオスのマウスを変異原であるアルキル化剤*N*-ethyl-*N*-nitrosoureaで処理し，正常のメスと交配して遺伝子に変異を持つ多数のマウスを作成し行動リズムを計測した結果，およそ300匹中1匹だけ1時間周期の長くなるマウスを発見した(12)．解析の結果この長周期の表現系は第5染色体上の半優性変異によって引き起こされることが明らかとなり，その原因遺伝子は*Clock*と名づけられた．Homozygousの*Clock*変異マウスはDD下で24時間の長周期の活動リズムを示すとともに2，3週間のうちにリズムが消失する．一方リズムが消失したマウスに光パルスを与えると再び長周期のリズムが発現してきた．1997年KingらとAntochらはBACのcontigによるポジショナルクローニングとトランスジェニックマウスで発現させたBACクローンの*Clock*変異のin vivo complementation実験によって*Clock*遺伝子をクローニングした(13,14)．*Clock*遺伝子は24のエクソンからなるおよそ100 kbにおよぶ大きな遺伝子で新規のbHLH-PASファミリーの転写遺伝子をコードしていた．また，C末には転写活性化に重要と考えられるグルタミン酸リッチ(Q-rich)な領域を含んでいた．さらに，*Clock*変異はspliceドナー部位の点突然変異によりexon19の転写がスキップされ，CLOCKタンパク質（CLOCK）のC末のQ-rich領域から51残基のアミノ酸が欠失して引き起こされていることが明らかとなった．このことは変異タンパク質では転写活性化が起こりにくくなる可能性を示唆している．このようにforward genetic的なアプローチは実際に活動周期の異なる変異体マウスを得られること，まったく新規の遺伝子を分離できるという反面，その形質が劣性であった場合には実験の性質上分離することは不可能である．

これらと同時に下等生物で得られた知見をもとにまず遺伝子を分離しその機能を調べる，reverse genetic的なアプローチとして，哺乳動物における*Drosophila*時計遺伝子ホモログの探索が試みられてきた．*Drosophila*ではこれまでに時計の振動体を構成する遺伝子として*period*（*per*）および*timeless*（*tim*）遺伝子が同定されている（ここでは*Drosophila*の*per*をd*Per*と呼ぶ）．d*Per*遺伝子のミスセンス変異によるPERタンパク質（PER）の単一のアミノ酸置換によってハエの活動リズムの周期は長くな

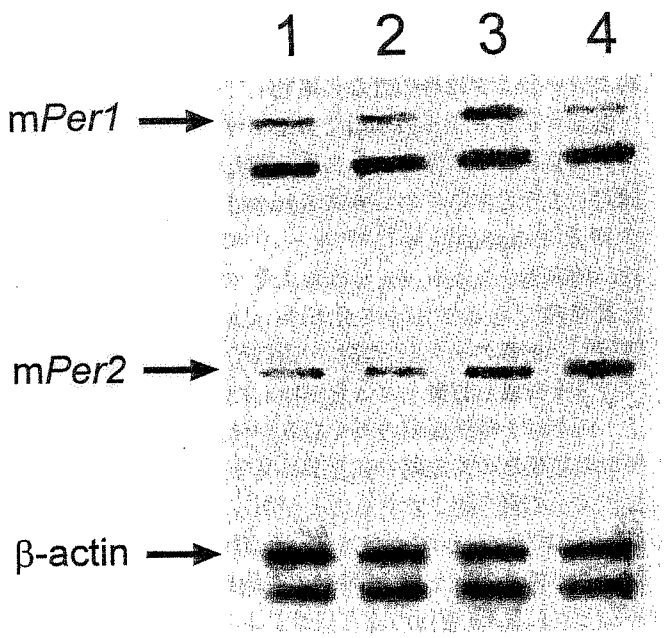

Fig. 2 Light induction of m*Per1* transcript in the suprachiasmatic nucleus (SCN) was inhibited by in vivo m*Per1* antisense oligonucleotide (ODN) treatment. The m*Per1* and random antisense ODNs were administrated i.c.v. at ZT15. Mice were placed in constant darkness (DD) for 1 hr and half of the mice were exposed to a light pulse (20 lux) for 15 min at ZT16 and transferred to DD for additional 1 hr. Brains were removed, and the SCN were punched out from the frozen slices (0.5-mm-thick). Total RNAs were isolated and m*Per1*, m*Per2* and β-actin cDNA were quantified by a competitive PCR method. Lane 1, treated with random ODN; lane 2, treated with antisense ODN; lane 3, treated with random ODN following light pulse; lane 4, treated with antisense ODN following light pulse. Lower lanes in m*Per1* and β-actin panels represent PCR products of respective competitors.

Table 1 Clock genes isolated from *Drosophila* and mammals were conserved through evolution. +indicates the existence of the PAS domain in the gene products.

Drosophila	mammals	PAS
	Per1	+
per (*period*)	*Per2*	+
	Per3	+
	(*Per4*?)	?
tim (*timeless*)	?	−
doubletime	?	−
d*Clock*	*Clock*	+
cycle	BMAL1 (MOP4)	+

ったり短くなったりする．また，ナンセンス変異によるPERの一部欠失では無周期となる．d*Per* および *tim* 遺伝子のmRNAとタンパク質はどちらも概日性の変動がみられ，その変動リズムはmRNAがタンパク質に較べ6時間近くも先行している．その後の研究から，PERとTIMタンパク質（TIM）はお互いに会合して二量体を形成し核へ移行し，d*Per*（および，おそらく *tim*）遺伝子自身の転写を（間接的に）抑制するものと考えられるようになった．このネガティブフィードバックグループが概日リズム振動体の実体であるとする考え方は他の生物でも適用できると現在のところ考えられている．

哺乳動物においてはこれまでd*Per*ホモログの分離が何度も試みられてきたが成功していなかった．しかし，程・岡村らのグループは特殊なPCR法を用いてヒトゲノムから相同遺伝子（h*Per1*）を分離することに成功した(15)．ほぼ同時にSunらによっても同一遺伝子が同定された(16)．さらに97年末から98年はじめにかけて3つのグループからほぼ同時に第二の *Per* ホモログ（*Per2*）のクローニングを報告した(17〜19)．現在までにさらにもうひとつのホモログ（*Per3*）の存在が確認されている（マウスおよびヒトのd*Per*相同遺伝子をm*Per*およびh*Per*と呼ぶ）．m*Per1* およびm*Per2* 遺伝子の脳内での発現を調べたところSCNにおいてもっとも強い発現が見られた．また，m*Per1*，m*Per2* の発現はd*Per*同様概日性の発現を示していたが，そのピークはm*Per1* では主観的明期の前半，m*Per2* では主観的明期の後半に高く，いずれも主観的暗期にピークがくるd*Per*とは位相が逆であった．m*Per1* とm*Per2* のSCN内での発現を細胞レベルで調べた実験では，SCNにおいてこれら遺伝子の両方が単一細胞中に発現していた．どちらも主観的明期において発現していることから，これら遺伝子産物が相互作用する可能性が示唆される．興味深いことに *Drosophila* でも哺乳類でも *Per* 遺伝子はさまざまな器官・組織に発現している．*Drosophila* では各種器官で自律的なリズム発現が見られることが明らかにされている(20)が，哺乳動物での役割は今のところ不明である．

Shigeyoshiらはm*Per1* の光同調機構への関与を調べるため，光照射のm*Per1* mRNAの発現への影響を調べた(21)．その結果，行動リズムに位相変化を引き起こすCT

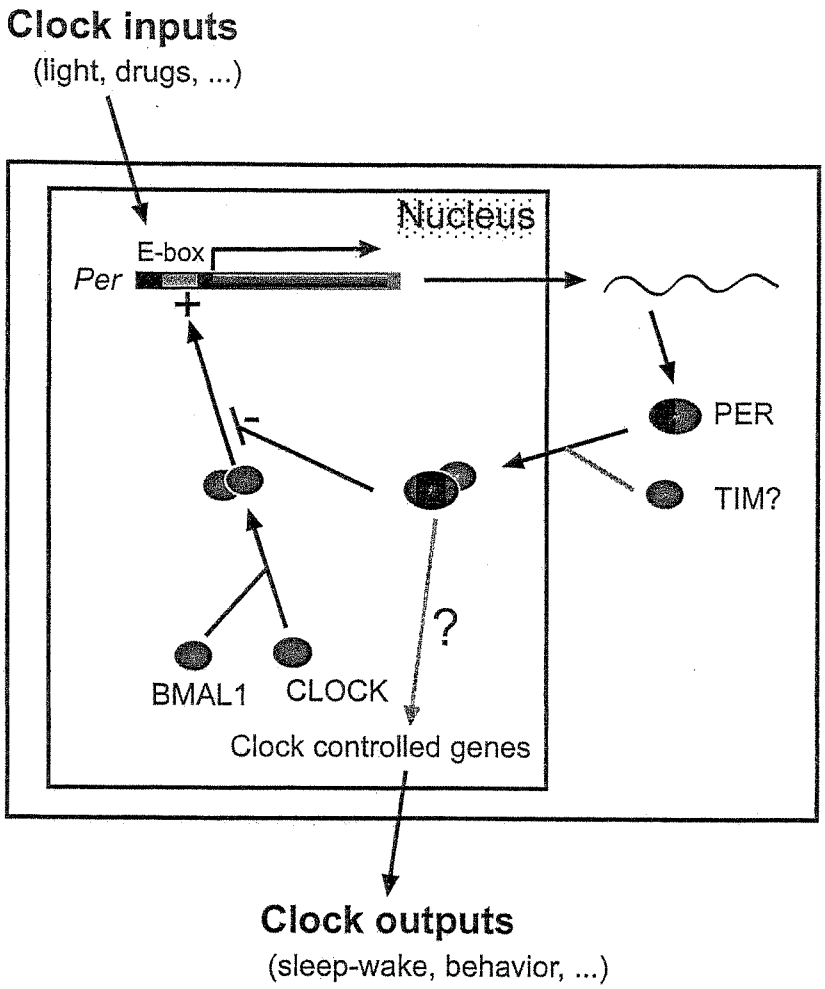

Fig. 3 Schematic illustration of the mammalian circadian timing system in an oscillator cell. The feedback loop comprising the oscillator was shown with arrows.

において m*Per 1* mRNA は SCN において急速に誘導されることが分かった．位相変化の CT 依存性を gating と呼ぶが，m*Per 1* の発現にも gating が見られた．この誘導は一過性であり，照射 30 分後にはピークに達し，180 分後にはもとのレベルに戻る．注目されるのはその結果引き起こされる m*Per 1* 転写産物の概日性発現リズムの位相変化が行動リズムの位相変化に対応していることである．ただし，発現リズムの位相変化が 1 サイクルで完了しているのに対し，行動リズムの位相変化には数日かかる．さらに光による m*Per 1* 誘導と位相変化の大きさは相関しており，その閾値もほぼ同一であった．m*Per 1* 遺伝子の概日性の発現は SCN 全体に見られるのに対して，光による m*Per 1* 誘導は光情報を SCN へ伝達している RHT が投射する腹外側部に限局していた．我々は m*Per 1* アンチセンスを用いて SCN 内の光による m*Per 1* 誘導を特異的に抑制した時（図 2）に行動の位相変化も抑制されることを見出している．これらのことから m*Per 1* 遺伝子の光誘導が，光による行動の位相変化に必要とされることが示唆される．この m*Per 1* 遺伝子の光誘導は *Drosophila* では見られず，位相変化は光による TIM の急速な分解によって引き起こされると考えられている．このことは，*Drosophila* と哺乳類でリズムに関与する遺伝子（産物）は共通であっても，その調節機構が必ずしもすべて保存されているわけではないことを示している（表 1）．

ところでこれまで分離された d*Per* および哺乳動物の *Per 1*，*Per 2* および *Clock* 遺伝子産物は PAS ドメインと呼ばれる共通の領域を持つことが分かっている．PAS ドメインは d*Per*，ヒトの *Arnt*，ショウジョウバエの *Sim*

の各遺伝子産物に共通する領域として名づけられたもので，PER以外のタンパク質ではタンパク質同士の会合に関わっていると考えられる．ただし，PERとTIMは二量体を形成するがTIMはPASドメインを持っていないことから，これらタンパク質での明確な役割は明らかではなかった．また，PERもTIMも転写制御に必要とされるドメインが確認されていないことから，そのネガティブフィードバック機構には別の転写調節因子が介在していると考えられてきた．つい最近*Drosophila*においても*Clock*ホモログ（d*Clock*）が存在することが明らかにされる(22,23)とともに，マウスと*Drosophila*の両方でCLOCKが二量体を形成するときのパートナーが明らかとなった(24,25)．これらはハエではcycle，哺乳類ではBMAL1（MOP3）と呼ばれる遺伝子でこれらは互いにホモログであり，どちらの遺伝子産物（CYCLE, BMAL1）もCLOCK同様PASドメインを有していた．d*Clock*, *cycle*遺伝子のどちらか一方の変異をホモで持つ*Drosophila*では概日リズムが消失していた．PERには転写活性化ドメインが存在しないことは述べたが，CLOCK(dCLOCK)，BMAL1(CYCLE)ともbHLHドメインを持っている．dPERのプロモーター領域にはd*Per* mRNAの正常なサイクリングに必要とされるE-boxと呼ばれるヌクレオチド配列が存在しているが，この配列は実験によって決定されたCLOCK-MOP3の結合配列とほとんど同一である．さらに，この配列がm*Per1*遺伝子上流に存在しCLOCK-BMAL1ヘテロ二量体がこのE-box配列に結合し転写を活性化することが明らかにされた．同じことがdCLOCKで確認されるとともに，dCLOCKによる転写誘導の結果できたPER，TIMがdCLOCKのE-boxを介した転写活性化つまり*per*, *tim*自身の転写を抑制することが明らかにされた(26)．*Drosophila*で得られた結果もあてはめると哺乳類の時計振動体の機構は以下のようになる（図3）．まず*Per*遺伝子の転写がCLOCK-BMAL1によって活性化され徐々にPERが蓄積していく．PERはパートナータンパク質(哺乳類TIMホモログか？）と結合して核内へ移行しCLOCK-BMAL1の転写活性化機能を阻害し，結果として*per*遺伝子の発現は抑制されPERが減少する．その結果，再びCLOCK-BMAL1による*Per*遺伝子の転写の活性化がはじまり，次の概日サイクルが始まる．

ところでm*Per1*遺伝子は光照射後，SCNで急速に一過性の誘導を受けるが，これまでにも一連の早期発現遺伝子が同様に光によって誘導されることが示されている(27)．これら遺伝子の誘導にもm*Per*同様誘導にgatingが見られることが知られている．現在までにマウスでSCNにおいて光によって誘導されることが知られている遺伝子はm*Per1*，m*Per2*に加え*c-fos*，*fos-B*，*jun-B*，*zif268*（NGFI-A），*nur77*（NGFI-B），*egr-3*の8種である．このうち，*egr*-3はm*Per*同様光による発現誘導が光同調に重要であると考えられている腹側部にのみ見られる点が注目される．

ところで，これまで既知の光受容体をすべて欠いた*rd*変異体でも光による位相変化が起こることから概日リズムの光受容体は謎であった(28)．つい最近，哺乳動物に2種の青色光受容体（クリプトクローム），CRY1，CRY2が存在し，CRY2はマウス網膜のganglion細胞とinner nuclear層に特異的に発現し，CRY1はSCN内で高レベルで発現しかつ概日性の変動を示すことが明らかとなった(29)．このことは生物が光情報を視覚情報はビタミンA由来色素（オプシン），概日時計の同調にはビタミンB2由来色素（クリプトクローム）を介して受容している可能性を示唆している．最近，ある種の培養細胞で高血清処理により時計遺伝子が誘導され，その後発現が22.5時間の周期で3周期ほど振動することが明らかとなった(30)．この処理によって*Per*とともに*c-fos*も急性的に誘導され，その誘導パターンがSCNでの光による遺伝子誘導と似ていることが分かった．したがってこの系は概日リズムの機構の解明に重要な役割を果たす可能性がある．

3. 概日リズムの薬理学

体内時計の変調は時差ぼけなどで経験するところであるが，高度な社会構造による夜間労働の増大（米国では実に500万人の従事者がいるという）とこれに伴うヒューマンエラーの増大，さらに睡眠・覚醒リズム障害，うつ病や痴呆の夜間徘徊に代表されるリズム障害を伴う精神疾患の増大が指摘されている．体内時計の同調を促進する薬物としては光の作用を増強するタイプとその薬物自身が同調を起こすタイプの2種類が考えられる．前者の例としてはMKC-242($5HT_{1A}$受容体アゴニスト)(31)やDR4004($5HT_7$受容体アンタゴニスト）があげられるし，後者の例ではメラトニンをあげることができる．また，m*Per1*やm*Per2*はSCNのみならず，海馬の歯状回や小脳顆粒細胞層，梨状葉皮質などにも発現している．これらの脳部位に発現している時計遺伝子の機能についてはまだ不明であるが，おそらく海馬の機能（情動・覚醒・記憶・てんかん）や小脳の機能（運動調節）に何らかの働きをしているものと考えられる．さらにm*Per1*やm*Per2*は中枢神経のみならず心臓，骨格筋，生殖腺，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．したがって

ホオスタシス維持のための生体の時計機構に視点を置いた新しいタイプの薬物を開発できる可能性があると考えている．

おわりに

ここ数年来脳研究が花盛りになってきているがその高次機能の解明への道のりはまだ程遠いのが現状である．一方，動物の概日リズム行動を直接支配するであろう時計遺伝子の発見が相次ぎ，生体リズムの仕組みが分子の言葉で解き明かされそうな勢いを感じることができる．我々はこのように急速に進み出した概日リズム研究が脳研究の先導的役割を担っているものと自負している．最後に生体リズム研究の急速な進歩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 – Physiological, pharmacological and molecular aspects of mammalian biological clocks. Masashi AKIYAMA[1], Takahiro MORIYA[2] and Shigenobu SHIBATA[1] ([1]Department of Pharmacology, School of Human Sciences, Waseda University, and [2]ARCHS, Waseda University, Tokorozawa, Saitama 359-1164, Japan). *Folia Pharmacol. Jpn.* (Nippon Yakurigaku Zasshi) **112**, 243-250 (1998)

Circadian rhythm is an endogenous rhythm that persists in constant conditions with a period of nearly but not identical to 24 hr. Under natural conditions, the circadian clock is precisely entrained to the daily (24 hr) cycle, because environmental stimulus (especially light) induces a phase shift of the clock. In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus has been shown to be the primary pacemaker that drives daily rhythms of behavioral and physiological activity. Photic information is conveyed from the retina to the SCN directly by the retinohypothalamic tract (RHT) and indirectly by the geniculohypothalamic tract (GHT). The transmitter of the RHT is glutamate, while the GHT is GABA and neuropeptide Y. Serotonergic innervation from the median raphe and melatonin from the pineal body are likely to provide non-photic information to the SCN. Single gene mutations that dramatically alter circadian phenotype were found in the hamster (*tau*) and mouse (*clock*). Moreover, the homologous genes of the *Drosophila* clock gene, *per,* were found in mammals and the homologue of the mammalian *clock* was found in *Drosophila*. These data suggest that the some constitutes of the biological clock may be conserved between *Drosophila* and mammals, and a transcription-translation feedback loop involving some clock gene products may be a oscillator itself.

Keywords: circadian rhythm; suprachiasmatic nucleus; photic entrainment; *per* gene; drug development

特集■生命にとって時間とは何か

記憶と生物時計

柴田重信

蜜蜂が蜜を採取に行くとき花の場所だけでなくその時刻もよく覚えて，より効率的に蜜を採取することが知られている．これは蜂は空間学習と時間学習ができることを意味する．また，犬の散歩を毎日ある時刻におこなっていると，その時刻に近づいてくると犬はそわそわして飼い主に散歩をねだるようになる(時刻認知学習)．このようなことから，脳には時間の事象を生物時計との関わりをもって学習・記憶するメカニズムが存在すると考えられるが，その詳細については不明である．

時間知覚や時刻知覚に関連した学習・記憶と生物時計

時間学習や時刻学習を遂行するには，それぞれ時間認知および時刻認知が必要性である．ここではミリ秒から秒の単位の時間を感覚する機構として“時間認知”を，またいわゆるいま何時という時刻の感覚を司る機構を“時刻認知”と便宜上区別する．このような区別ができうるのか，また，この両者に密接な関連があるのかといったことに関しては不明な点が多い．図1には記憶と生物時計の関係を模式的に示した．

時間を意識するためには内的時間が必要であり，この時間意識には以下の二つの事象が考えられる．“時間知覚(time perception)”と“時間評価(time estimation)”である．時間知覚は直接的に把握される時間，つまり継時的に呈示されたいくつかの事象が一つのまとまりとして，知覚される時間の範囲内の知覚である．

一方，時間評価はより長い時間範囲での時間の意識を意味している．過去に起こった二つの事項の間の経過時間を評価したり，さらに過去の記憶の体系を利用することで未来を見通したりするということである．ここでいう時間認知はおもに時間知覚のことであり，時刻認知には時間評価の機構と関連性がありそうであることが推察される．時間知覚と時間評価は7～8秒を境に区別できるとされている．以下，研究が進んでいる時間知覚について述べる．

時間知覚に関連した学習・記憶

時間知覚を反応方法の違いによって分類すると以下のようになる．

(1)言語評価法: 呈示された時程を1分とか2秒とかいうように被検者が時計時間で評価する．

(2)産出法: 実験者が示した時計時間に(1分とか2秒とか)見合った時程を，計時装置に接続されたキーを押すなどして被検者が作り出す．

(3)再生法: 呈示された時程を，何らかの行動によって，被験者が作り出す．

(4)比較法: 呈示された二つの時程を，被検者が比較して，どちらの時程が長いか，短いかなどの判断をする．

また，反応方法を2大別すると，“時程の尺度化(duration scaling)”と“時程の弁別(duration discrimination)”になる．

時間知覚をもたらす内的過程として何らかの計時システムの存在が考えられる．短期の記憶障害と時間認知の関係が知られている．例えばコルサコフ症候群では3～96秒のインターバルの時間評価のタスクが完成できない．これはおそらく前頭葉皮質の障害と関係があるのではないかと考えられている．また時間評価は種々の薬物によって影響を受けることが知られている．とくにアルコールは時間評価が長くなりやすいことが知られている．

動物実験では低頻度差別強化による時間認知学習系(differential reinforcement of low response rate; DRL)や2種類の時間を時間評価する時間評価学習系が提唱されている．DRLは20秒や30秒おきにレバーを叩けば一番効率的に報酬が得られるので，20秒や30秒の時間認知学習が成

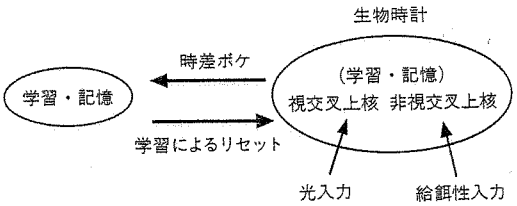

図1　記憶と生物時計の関係を示した模式図．哺乳動物の能動振動の体内時計は視交叉上核にある．外界の光刺激は，この時計をリセットする情報としてだけでなく，光の履歴効果の情報として視交叉上核に伝わる．一方，一定時刻の給餌性刺激やメタンフェタミンの一定時刻投与刺激は非視交叉上核性の生物時計を駆動するが，この時計の中に時刻認知記憶系が存在する可能性がある．また，生物時計と外部の学習記憶系は“時差ボケ”や“学習による時計リセット”で表わせるように相互関係がある．

立する系である．このDRLは種々の中枢神経作用薬によって影響を受けることが知られている．DRLはグルタミン酸受容体の一つのNMDA受容体の拮抗薬であるMK-801によって顕著に障害されるという．空間認知に基づく学習行動や，シナプスレベルの可塑的変化を反映している海馬の長期増強現象(LTP)がMK-801で障害されることを考えると，時間認知にも脳内グルタミン酸NMDA受容体の活性化が必要であることが推察される．さらに後述するように，時刻記憶にもNMDA受容体が重要であることは非常に興味深い．

時刻認知に関連した学習・記憶

動物は，ある一定時刻に餌や水を呈示してやると，この時刻を記憶するようになる．このような一連の行動を時刻認知行動という[1]．この時刻認知行動は体内時計の視交叉上核を破壊した動物にも起こることから，視交叉上核の体内時計とは異なった脳内神経機構で発現するものと考えられている．この時刻認知行動の特徴を述べてみると，(1)制限給餌同調のTサイクル(時間周期)(例えば23時間おきに餌を呈示したり28時間おきに呈示する)は，視交叉上核依存性のリズムをもった光のTサイクルの同調範囲に類似している．(2)制限給餌(Tサイクル)と給餌性運動リズムには正の位相関係が存在することが報告されている．これも視交叉上核依存性のリズムをもった光のTサイクル実験での位相関係に類似している．したがって，給餌性の時刻認知に基づくリズム形成の神経機構は視交叉上核性のリズム形成機構に類似しているものと考えられる．カテコールアミンの破壊薬である6-OHDAの処置によって，給餌性のコーチコステロンのリズムが障害されるという報告や，カテコールアミンの再取り込みや，遊離を促進させるメタンフェタミンの一定時刻投与が時刻認知を引き起こす[2]ことを考えると，時刻認知には脳内カテコールアミン神経系が重要な働きをしているものと考えられる．しかしながら，ドーパミン神経が豊富な線状体，側坐核の破壊では時刻認知は障害されないという報告がある．また，時間感覚を障害した前頭葉皮質の破壊や，空間認知に重要である海馬の破壊でも時刻認知は障害されず，給餌行動に密接に関係のある室傍核や腹内側核の視床下部を破壊しても障害されない．一方，時間感覚を障害したNMDA受容体拮抗薬のMK-801は給餌性，メタンフェタミン性の時刻認知を障害した[3]．さらに，われわれはNMDA2AあるいはNMDA2A/2Cの受容体のノックアウトマウスで，給餌性の時刻認知障害が出現することをみいだした(図2)[4]．このように脳内NMDA受容体の活性化が時間認知だけでなく時刻認知にも重要であると考えられている．

時間認知と時刻認知の関係

人における時刻認知の研究は数少ない．ASCHOFFの研究によれば，約1時間の時間評価は睡眠覚醒リズムの長さに対応しているという．つまり睡眠覚醒リズムの周期が長くなると，1時間の時間評価も長くなるという．また，1分の時間評価の長短は1日の中で変わるし，また季節によっても変わるという．したがって，体内時計が時間感覚や時間評価に影響を与えている可能性があるものの，動物実験などを使用した直接的な証明はいまのところない．これらの研究を通して，特定の感覚器を有しない時間，空間の感覚の神経機構が明らかになるものと考えられる．痴呆等で時間や時刻の見当識障害が報告されているが，時間/時刻認知の研究はこのような障害治療のための基

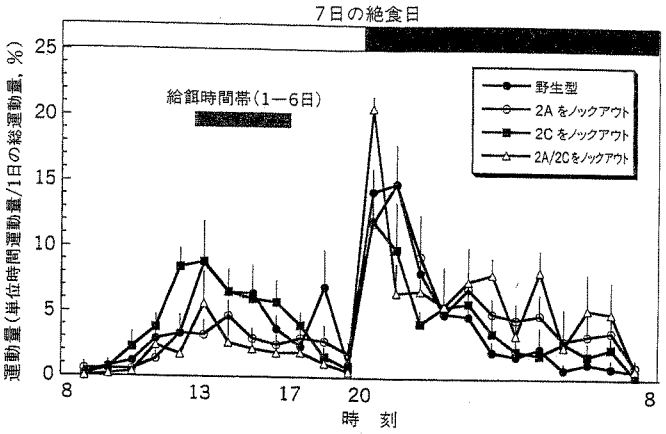

図2 NMDA受容体サブタイプのノックアウト動物における給餌性の予知行動.
NMDA受容体サブタイプのノックアウト動物を2日間絶食させ，その後，13時から17時の4時間だけ餌が得られるようにする．この操作を6日間おこない，7日目は再び絶食させる．夜行性動物であるマウスはこの時間帯の活動量は低いが，この時間に餌を呈示されることによって時刻認知学習が成立する．すなわち7日目の絶食日でも11時から17時にかけてマウスの運動量は亢進したままである．NMDA2AとNMDA2A/2Cのノックアウト動物はこの時間帯の上昇が低く，時刻認知学習が成立しにくい．

礎的研究に役立つものと考えている．

生物時計をリセットする光の履歴記憶

体内時計への光入力が時計を強力にリセットすることはよく知られている[5]．一方，視交叉上核が光照射時間を記憶し，光環境の季節性変動をこの部位の可塑的変化で捉えている可能性がある．また，明暗条件下に飼育していた動物を，恒暗条件下においても，しばらくの間は以前明期であったはずの時刻の活動量が少ないなどのいわゆる光の履歴効果の存在が知られている．網膜から視交叉上核へはグルタミン酸を有する興奮性シナプス入力があるので，この網膜-視交叉上核路において長期増強現象(LTP)が発現するか否かについて調べた[6][7]．グルタミン酸などの興奮性神経伝達物質を有する神経を高頻度，短時間刺激すると，興奮性シナプス後電位(EPSP)の大きさが高頻度刺激前より増大し，これが数時間以上持続するというLTPが生じることはよく知られている．このようなLTP現象は海馬において詳細に検討され，シナプスの可塑性現象の一つであると考えられており，LTPが海馬の重要な機能である学習・記憶の基礎過程を表わしていると解釈されている．視神経刺激で視交叉上核からEPSPの複合電位としての電場電位を記録する．その結果，図3に示すように，高頻度刺激によって昼の前半をピークとした著明なLTP現象が発現した．一方，夜間に刺激した場合，LTP現象はわずかに発現しただけであった．また同一個体から得られた海馬切片を用いてシェーファー側枝刺激によるCA1領域からの電場電位を記録した．視神経の場合と同一条件で高頻度刺激すると，刺激直後から昼でも夜でもほぼ同じ大きさのLTP現象が出現した．視交叉上核と海馬のLTPを比較すると，視交叉上核のLTPは時間経過が非常にゆっくりしていた．

視交叉上核で発生するLTPの神経機構を明らかにする目的で，LTPに対するNMDA受容体拮抗薬のAPVの作用を調べた．APVの投与によって，視神経刺激によるLTP発生は完全に拮抗された．また，視神経刺激のかわりに，グルタミン酸(濃度10 mM)を還流液中に応用すると，この場合にもLTP発生がみられた．したがって視神経刺激によって，グルタミン酸放出とそれに続くNMDA受容体の活性化が視交叉上核のLTP発生には重要であることがわかった．外界の明暗サイクルの変化や，恒常条件の変化などに以前の明暗環境を記憶していたような現象すなわち履歴現象がみられることから，ここで得られたLTPはこのような明暗環境の履歴学習効果となんらかの関係があるものと思われる．

時差ボケ

時差があるところを急激に移動すると，現地の明暗環境の位相と自分自身の体内時計の位相がずれる状態が起こる．このようなとき睡眠覚醒リズムがうまくいかず，注意力低下に伴う認知障害等が現われ，いわゆる時差ボケ症状が起こる(本特

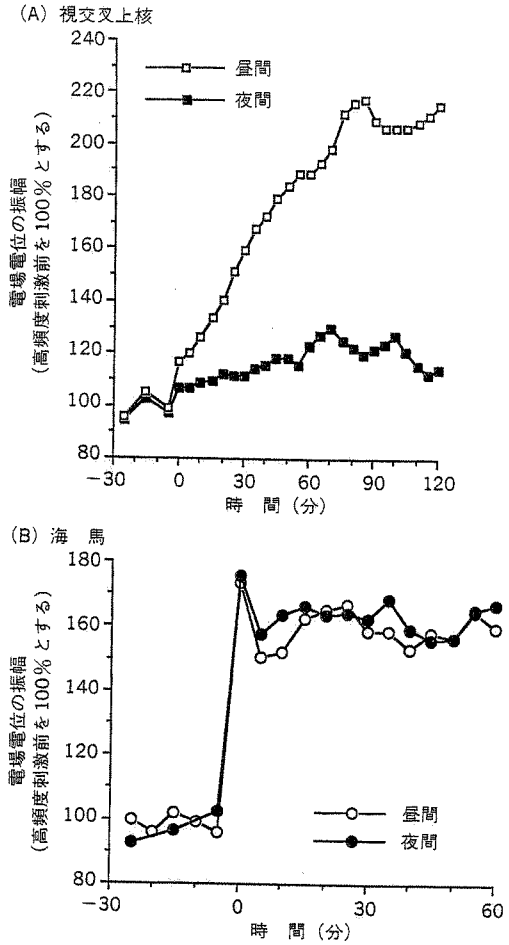

図3　視神経の高頻度刺激による視交叉上核電場電位の長期増強現象. 視神経を含む視交叉上核と海馬の薄切切片を作成後，視神経や，シェファー側枝の電気刺激で，それぞれ視交叉上核と海馬CA1領域から電場電位を記録する．一定の大きさの振幅の電位が記録できたら，これらの部位の高頻度刺激をおこなう(100 Hz, 1 sec)．その後，再び一定の間隔で刺激をおこなうと，電場電位の振幅が大きくなり，これが数時間続くいわゆる長期増強現象(LTP)が観察される．視交叉上核ではLTPは昼間に顕著に現われゆっくりとした時間経過で起こるが，海馬のLTPは昼夜関係なく急速に増大する．

集本間研一氏の解説参照)．実際動物の飼育環境の明暗の位相を変えたら，受動的回避学習に障害が出たという報告がある．しかしながらこの場合，時差そのものが学習の成立に影響を及ぼしたのか，時差による注意力の低下が学習障害を起こしたのかはよくわからない．臨床上，時差ボケ防止には学習・記憶障害を治療する方法ではなく，高照度光療法やメラトニン服用による体内時計の現地時刻への速やかな適応が考案されている[8]．いずれにしても，生物時計が睡眠覚醒リズム等の機能を介して学習・記憶の成立に影響を及ぼすことは十分に考えられる．

学習・記憶による体内時計リセット

時差ボケにみられるように，生物時計は学習・記憶に何らかの影響を及ぼすことを述べたが，学習・記憶機構が逆に生物時計に影響を及ぼす可能性について述べる．前述したように外界の光刺激は体内時計を強力にリセットする．そこで，この光刺激を無条件刺激とし，動物に風を当てることを条件刺激として，パブロフ型の連合学習を成立させた[9]．つまり毎日光照射に先行して動物に風を当て，この学習を成立させた．このような動物はその1週間後に風を当てられただけでも体内時計をリセットさせてしまう．当然，風と光とを同時に与えない，あるいは風だけを当てた動物の体内時計はリセットされない．このような変化は行動学的に現われるだけでなく，初期遺伝子発現の視交叉上核における変化としても捉えられている．このことから，体内時計のリセットは光だけでなく後天的に学習された条件刺激でも起こすことができることが明らかとなったが，条件刺激と無条件刺激の統合が視交叉上核で起こるのか，あるいは他の脳部位で学習が成立した結果が視交叉上核に伝達されるのか，その点については不明である．

(Shigenobu SHIBATA
早稲田大学人間科学部薬理学)

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NMDA induced glutamate release from the suprachiasmatic nucleus: an in vitro study in the rat

Toshiyuki Hamada[a], Rie Sonoda[a], Akihito Watanabe[a], Michiko Ono[a], Shigenobu Shibata[b,*], Shigenori Watanabe[a]

[a]*Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyushu University 62, Fukuoka 812, Japan*
[b]*Department of Pharmacology and Brain Sciences, School of Human Sciences, Waseda University, Tokorozawa, Saitama 359-1164, Japan*

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Abstract

The suprachiasmatic nucleus (SCN) has been identified as a pacemaker for mammalian circadian rhythms. Excitatory amino acid receptors, especially *N*-methyl-D-aspartate (NMDA) receptors, have been considered to play an important role in the transmission of light information from the retina to the circadian clocks in the SCN. In the present study, we showed that application of NMDA at circadian time (CT) 12–15 induced significant glutamate release from the SCN region in vitro. The NMDA-induced glutamate release was blocked by co-application of the NMDA receptor antagonist MK-801, but not by that of tetrodotoxin. These results suggested that glutamate stimulated its own release by activating NMDA receptors. This NMDA-induced glutamate release through NMDA receptor-mediated mechanisms might be involved in NMDA-induced potent phase shifts.

Keywords: Circadian rhythm; Suprachiasmatic nucleus; *N*-Methyl-D-aspartate; Glutamates; Rat

The suprachiasmatic nucleus (SCN) of the hypothalamus has been identified as a primary pacemaker for circadian rhythms in mammals. Entrainment of circadian rhythms to the environmental light-dark cycle is mediated by the direct retinohypothalamic tract (RHT) [6]. Excitatory amino acids (EAAs) are involved in the transmission of photic information from the retina to the SCN [6,8,12,13,18]. Electrical stimulation of the optic nerve has been shown to induce calcium-dependent release of [^{3}H]glutamate in in vitro hypothalamic slice preparations containing the SCN [13]. Application of glutamate produces changes in the firing rhythm of SCN neurons in subjective night, but glutamate shows no effect during subjective day [5,8]. This effect is similar to the light-induced phase resetting effect.

Light-induced phase shifts of activity in free-running hamsters and c-fos expression in the SCN are blocked by a systemic injection of MK-801 [1,3], a non-competitive antagonist of the *N*-methyl-D-aspartate (NMDA) receptors, while focal administration of NMDA induces Fos protein in the SCN [7] and phase shifts in vivo [14]. Thus, NMDA-type receptors have been suggested to be involved in mediating photic inputs to the SCN. Recently, we reported that application of glutamate, NMDA and non-NMDA receptor agonist kainate caused an increase in 2-deoxyglucose uptake in the SCN, and that NMDA produced changes in the phase of firing rhythms of SCN neurons in vitro with a phase-response curve similar to the one demonstrated after treatment by optic chiasm stimulation [16–18]. These lines of evidence suggest that glutamate is a neurotransmitter in the RHT, and that NMDA receptor subtypes play important roles in the transmission of light information.

It has been demonstrated that NMDA induces the release of endogenous EAAs from striatum in vivo and in vitro, and that this effect is blocked by non-competitive NMDA receptor antagonist, MK-801 [11]. It has also been reported that NMDA receptors are present on glutamatergic terminals

* Corresponding author. Tel.: +81 429 476732; fax: +81 429 476806; e-mail: shibata@human.waseda.ac.jp

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[19]. These findings indicate that NMDA causes the release of glutamate by a mechanism involving NMDA receptor action in the presynaptic nerve. Thus, in the SCN, activation of NMDA receptors may induce an increase in glutamate release, which may stimulate NMDA receptors. In the present study, we examined whether NMDA might induce glutamate release from SCN slices during early subjective night, because NMDA produces remarkable phase delays in early subjective night [18].

Wistar rats weighing 200–300 g were housed in a normal (lights on 0800 h) 12:12 h light-dark cycle for at lease 2 weeks prior to the study. To eliminate direct effects of light on neuron activity within the slice, all rats were placed in constant darkness for 2 days prior to killing by decapitation in dim red light. The rats were decapitated under ether anesthesia and the brains quickly removed. Coronal hypothalamic slices (450 μM thickness) through the SCN and anterior hypothalamic area were prepared with a tissue chopper as reported previously [9,18]. Then the SCN region was dissected out from each slice.

Glutamate releases from SCN were measured by the batch method [15]. After 1–2 h of preincubation in Krebs-Ringer solution equilibrated with 95% O_2/5% CO_2, each slice was placed in a dish with 1 ml control Krebs-Ringer or Mg^{2+}-free Krebs-Ringer containing drug and incubated for 15 min. The composition of the control Krebs-Ringer solution, equilibrated with 95% O_2/5% CO_2, was (in mM): 129 NaCl, 1.3 $MgSO_4$, 22.4 $NaHCO_3$, 1.2 KH_2PO_4, 4.2 KCl, 10.0 glucose, 1.5 $CaCl_2$. Mg^{2+}-free Krebs-Ringer contained Na_2SO_4 instead of $MgSO_4$. These buffers were maintained at pH 7.3–7.4. The slices were obtained 2 h before application of agents at a specified circadian time (CT). CT0 refers to lights-on and CT 12 to lights-off in the colony. Extracellularly released glutamates in the solution were assayed using HPLC with a precolumn derivatization technique by o-phtal-aldehyde/2-mercaptoethanol. The derivatized amino acids were quantified by fluorescence detection (excitation 334 nm, emission 425 nm). In enucleation experiments, the rats were enucleated and 5 days later slices were prepared as described earlier. All data are expressed as mean ± SEM and significant differences between groups were determined using Student's *t*-test.

We examined whether NMDA might induce glutamate release from the SCN region at early subjective night. First, we examined the spontaneous release of glutamate in normal Krebs-Ringer and in Mg^{2+}-free Krebs-Ringer, because NMDA receptors were blocked by Mg^{2+} in a voltage-dependent fashion. The average release of glutamate in Mg^{2+}-free-Krebs-Ringer did not different from that in normal Krebs-Ringer (Fig. 1A) (106 ± 12.1%, with 100% equaling release of glutamate in normal Krebs-Ringer; $n = 3$). The average glutamate release per 15 min was 197.7 ± 22.8 pmol/SCN in the Mg^{2+}-free-Krebs-Ringer. Mg^{2+}-free Krebs Ringer was used for the remaining NMDA receptor experiments.

Application of NMDA induced glutamate release from the SCN region in a dose-dependent manner. Significant release of glutamate was observed after application of 1 μM (124 ± 5.6%; $n = 7$) or 10 μM (186 ± 8.5%; $n = 8$) NMDA. High K^+ (50 mM) treatment for 15 min also induced a significant release of glutamates from the SCN region (Fig. 1B) (277 ± 53.8%; $n = 3$). In the next experiment, we examined the effect of the NMDA receptor antagonist MK-801 on NMDA-induced glutamate release from the SCN region. The application of MK-801 (1 μM) for 15 min around CT13 produced a slight release of glutamate from the SCN region (129 ± 4.0%; $n = 3$), whereas NMDA (10 μM)-induced glutamate release was significantly blocked by MK-801 (1 μM) (Fig. 2A) (109 ± 7.4%; $n = 3$). However, NMDA (10 μM)-induced glutamate release (178.1 ± 6.26, $n = 4$) was still seen in TTX (1 μM) supplemented Ringer solution (165.3 ± 8.35, $n = 4$). The application of TTX (1 μM) for 15 min at CT13 did not produce any glutamate release from the SCN region (105.5 ± 17.22, $n = 4$) (Fig. 2B).

In order to elucidate whether NMDA-induced glutamates originated from axon terminals of the optic nerve, we examined the influence of enucleation on NMDA-induced glutamate release from the SCN. Enucleation was conducted under ether anesthesia. Five days after enucleation, no significant changes were observed in spontaneous release of

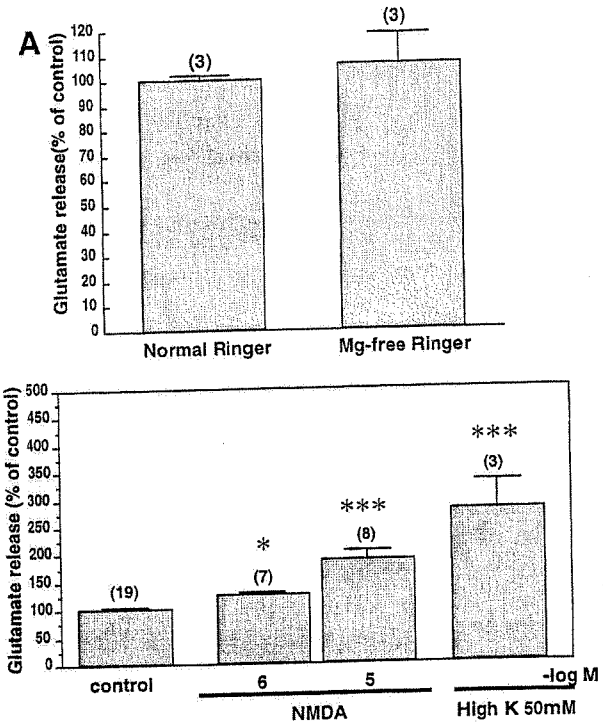

Fig. 1. NMDA-induced glutamate release from the SCN region in vitro. (A) The spontaneous release of glutamate from the SCN in normal Ringer and in Mg^{2+}-free Ringer. Values are expressed with 100% equaling glutamate release in normal Ringer. (B) NMDA (1 and 10 μM) or high K^+ (50 mM) treatment in a bath for 15 min at CT 13. Values are expressed with 100% equaling control release. Numbers in parentheses indicate the number of animals. $^*P < 0.05$, $^{***}P < 0.005$ vs. control group (Student's *t*-test).

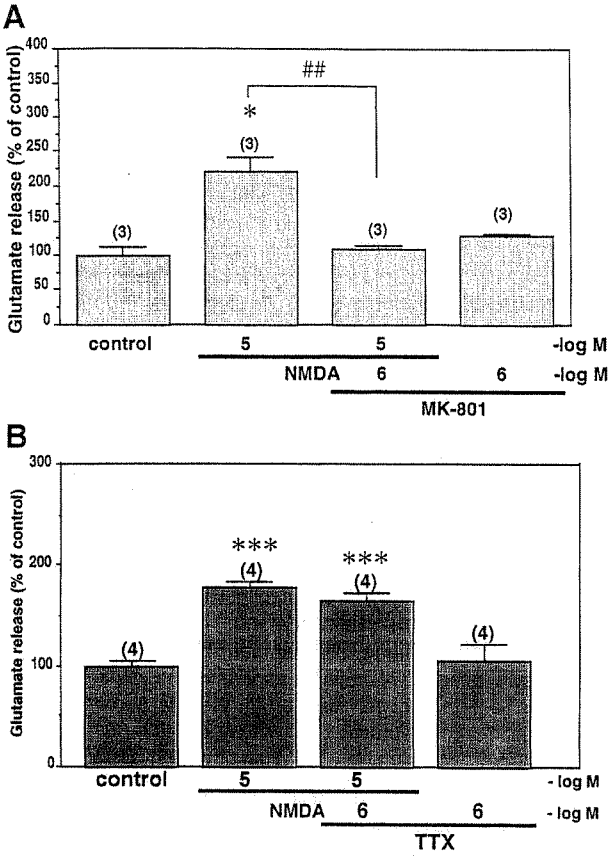

Fig. 2. Effect of MK-801 or TTX on NMDA-induced glutamate release from the SCN region in vitro. MK-801 (100 μM) or TTX (1 μM) treatment in a bath 5 min prior to NMDA application and for 15 min of incubation time at CT 13. Numbers in parentheses indicate the number of animals. (A) NMDA-induced glutamate release was significantly blocked by MK-801 (100 μM). $^{*}P < 0.05$ vs. control group, $^{\#}P < 0.01$ vs. NMDA + MK-801 (Student's *t*-test). (B) NMDA-induced glutamate release was not significantly blocked by TTX (1 μM). $^{**}P < 0.01$ vs. control group (Student's *t*-test).

glutamates in the enucleated rats (94.9 ± 8.54% of release of glutamates of the normal rats; $n = 3$)(Fig. 3A). NMDA-induced glutamate release in enucleated rats was not signifcantly changed (86.32 ± 5.72% of NMDA-induced glutamate release of the normal rats; $n = 3$) (Fig. 3B).

The present results indicate that NMDA induces glutamate release from the SCN region in a dose-dependent manner, and that NMDA-induced glutamate release is blocked by MK-801. It has also been reported that 2-amino-5-phosphopentanoic acid (a competitive NMDA receptor antagonist) and MK-801 (a non-competitive NMDA antagonist) reduce the high K^+-evoked release of endogenous EAAs from brain slices in a tetrodotoxin insensitive manner [4]. These data strongly suggest that NMDA enhances EAA release and then the released glutamates stimulate NMDA receptors. Thus, NMDA-induced glutamate release through NMDA-receptor-mediated mechanisms might be implicated in NMDA-induced phase shifts in vivo [14] and in vitro [18].

It has been reported that NMDA receptors are present on glutamatergic terminals [2]. These results indicate that NMDA-induced glutamate releases are mediated by presynaptic receptors, and that NMDA enhances EAA release, which in turn stimulates NMDA receptors. Support for this hypothesis has come from the study [11] showing that NMDA induces the release of EAAs from striatal slices, and that this effect is blocked by MK-801.

In the present study, no significant changes were observed in spontaneous and NMDA-induced release of glutamates in enucleated rats. This suggested that glutamates released from the SCN without RHT terminals were mainly measured. [^{3}H]MK-801 binding sites have been reported to be distributed homogeneously in the SCN [10]. In addition, it was recently reported that NMDA-receptor 1 subunit mRNA-expressing cells are distributed evenly throughout the SCN [6]. Glutamate-like immunoreactivity has been demonstrated in both retinal and non-retinal terminals in the SCN [2].

We found that [^{3}H]glutamates were abundantly released from the SCN after local stimulation of the SCN in comparable amount with that observed after optic nerve stimulation [13]. The present results also suggested that non-RHT glutamatergic terminals might play an important role in NMDA-induced release of glutamates from the SCN region. Thus, in summary, it is indicated that NMDA induces glutamate release from the SCN region, and that released glutamates may play a role in NMDA-induced phase shifts.

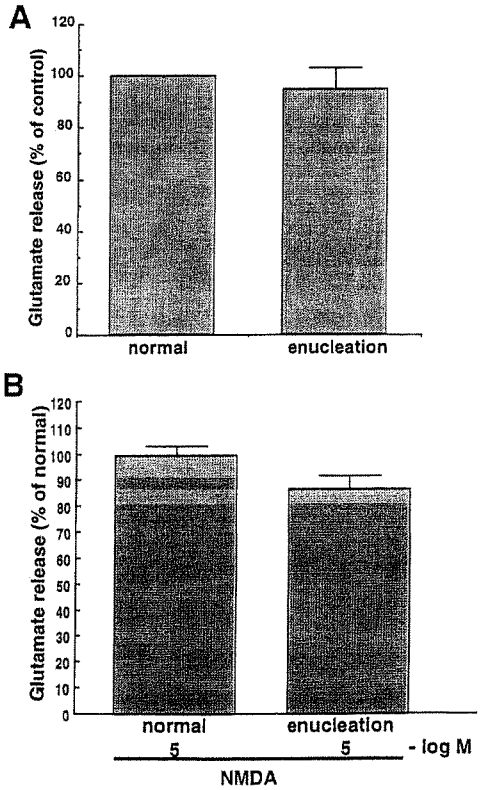

Fig. 3. Effect of NMDA on glutamate release from enucleated rat SCN. (A) The difference in spontaneous release of glutamate between the normal and the enucleated rats was not significant. (B) NMDA-induced glutamate release from the normal or the enucleated rats.

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Circadian rhythms and serotonergic neurons

池田真行[1)]　Masayuki Ikeda　専門：時間生物学，睡眠生理学
趣味：パソコン，魚釣り
柴田重信[2)]　Shigenobu Shibata　専門：神経薬理学，時間生物学
趣味：テニス，魚釣り
1）早稲田大学人間総合研究センター，2）同人間科学部薬理学研究室（所沢市三ケ島 2-579-15 〒359）


Ikeda M

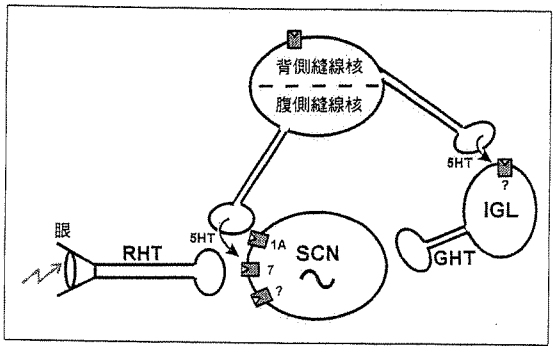

図1　視交叉上核（SCN）の光情報入力経路におけるセロトニン支配
SCN の光情報入力経路には，主に網膜視床路（RHT）および外側膝状体（IGL）を介した外側膝状体視床路（GHT）の２つの経路があるが，そのいずれにも中脳縫線核からのセロトニン（5HT）支配が及んでいる．前者では腹側縫線核（median raphe）からの，後者では背側縫線核（dorsal raphe）からの投射が関与していると考えられている．1A，７などはセロトニンレセプターを示す．

膝状体（intergeniculate leaflet；IGL）に向かう背側縫線核（dorsal raphe）の突起がその役割を担っていると考えられている（図 1）[1,2)]

SCN への縫線核セロトニン神経の投射は，SCN のとくに腹外背側部位に対してであり，この部位は眼からの光情報が RHT を介して伝達される場所でもある．この部位にはバソアクティブ・インテスティナル・ポリペプチド（VIP）が豊富に存在し，RHT のグルタミン酸神経の終末と縫線核からのセロトニン神経の終末はともにこの VIP 神経にシナプスを形成している．また，SCN のセロトニン含量には明期の中間に高く夜間に低い概日リズムがみられることが知られている[3)]．したがって，セロトニンは時計への光入力を制御している可能性が考えられるが，セロトニン神経活動は逆に体内時計の支配下にあるともいえる．

ところで，概日システムにおけるセロトニン神経の役割を解明すべく 1970 年から 1980 年代にかけて，縫線核の電気破壊実験や 5，7 ジヒドロキシトリプタミン（5-7DHT）による化学破壊実験が数多く行われた．しかしながら，これらのセロトニン神経の機能低下の試みは概日リズムの振幅や位相をわずかに変えるにすぎなかった．ところが，1990 年代に入ると，多様なセロトニンレセプターとそれに基づくレセプター刺激薬が発見され，これを契機に概日リズムに対するこれらの薬物の作用評価を行う研究が積極的に行われるようになった．

II　光同調に対するセロトニン神経の役割

光同調に対するセロトニン神経の役割は以下の代表的な研究により明らかにされている．まずはじめに，光刺激や視神経刺激によって引き起こされる SCN の複合興奮性シナプス後電位（複合 EPSP）がセロトニン 1A レセプターのアゴニストである（±）-2-dipropylamino-8-hydroxy-1, 2, 3, 4-tetrahydronaphthalene（8-OH-DPAT）やカルボキサミドトリプタミン（5-CT）などによって抑制され

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特集 時間薬理学—新しい投薬の指針

REVIEW

概日リズムとセロトニン神経系

Key words：circadian rhythm, serotonin, suprachiasmatic nucleus, entrainment, pharmacology

はじめに

植物から哺乳動物に至るほとんどすべての生物は，体内に時刻を知るための時計（体内時計）をもっており，たとえ環境から時刻の情報が得られなくても，1日のほぼ決まった時刻に活動と休止をすることが可能である．この自律的な活動休止リズムは，約24時間周期をもつことから概日リズム（サーカディアンリズム*）という．哺乳類では体内時計は視床下部視交叉上核*（SCN）に存在することが知られており，SCNを破壊された動物は，環境からの時刻情報なしでは概日リズムを維持することが困難である．通常の場合，SCNの振動は主に昼夜の明暗周期に同調しており，ほぼ正確に24時間の時を刻んでいる．また，時計を同調させるための光の情報は，必ずしも12時間交代といった明暗サイクルである必要はなく，毎日十数分間の光パルスを与えることでも可能である．一方，環境光を恒常暗や恒常明状態においた動物の体内時計はフリーラン状態を示す．たとえば，恒暗条件下におけるハムスターの輪回し行動リズムのフリーラン周期*は約24.5時間であり，活動時間は毎日30分程度うしろにずれていく．このような状態の動物にさまざまな時刻で光照射をすると，時刻依存的に活動の位相を変えることができる．すなわち，恒常条件下にする以前夜間だった時間帯（主観的夜）の前半に光照射を行うと位相後退を，後半から朝方に行うと位相前進を起こすことができる．こうした位相反応の解析は，体内時計の同調能力を計測する有用な手法として確立している．さらに，位相反応を引き起こす時刻での光刺激はSCN内にc-*fos*遺伝子の発現を引き起こすことが知られているが，これは実際にSCNに不可逆的な変化（つまりは時計の針が動いたこと）を表す1つの指標と考えられている．よって，光刺激とともに任意の薬物を投与しSCNに発現するFosタンパクの免疫応答を解析すれば，その薬物が光同調に対してどのように作用するのかを推定することが可能である．本稿では，こうしたアプローチによってこれまで明らかにされたセロトニンによる概日リズムの制御について説明する．

I SCNにおけるセロトニン神経支配

光情報が体内時計の調節にとって重要であることは先に述べたとおりである．光による体内時計の同調は，網膜で受容した明暗の情報を，網膜視床路（retinohypothalamic tract；RHT）あるいは外側膝状体視床路（geniculohypothalamic tract；GHT）の神経連絡によってSCNに伝達することにより行われると考えられている．RHTの神経伝達は伝達物質として主にグルタミン酸が働いており，一方，GHTではγ-アミノ酪酸（GABA）やニューロペプチドY（NPY）が働いていると考えられている．しかしながら，いずれの神経連絡も中脳縫線核からのセロトニン支配を受けていると考えられている．さらに最近の報告では，RHT側の支配はSCNに向かう腹側縫線核（median raphe）のセロトニン神経の突起が，またGHT側の支配は外側

サーカディアンリズム
日本語で概日リズム（約1日のリズムという意味）である．このリズムは外界の明暗周期がない状態のリズム（体内時計の発振でのみ刻まれるリズム）であるために，われわれが普通に生活している，あるいは動物を普通に飼育している場合には日内リズムというのが正しい．したがって高血圧患者の血圧のサーカディアンリズムという言い方は誤りで，正確には高血圧患者の血圧の日内リズムというような言い方が正しい．

視交叉上核
哺乳動物のサーカディアンリズムをコントロールする体内時計が存在すると考えられている脳内部位．視床下部で視神経がまさに交差する直上にある片側約1万個の神経細胞から成る神経核である．

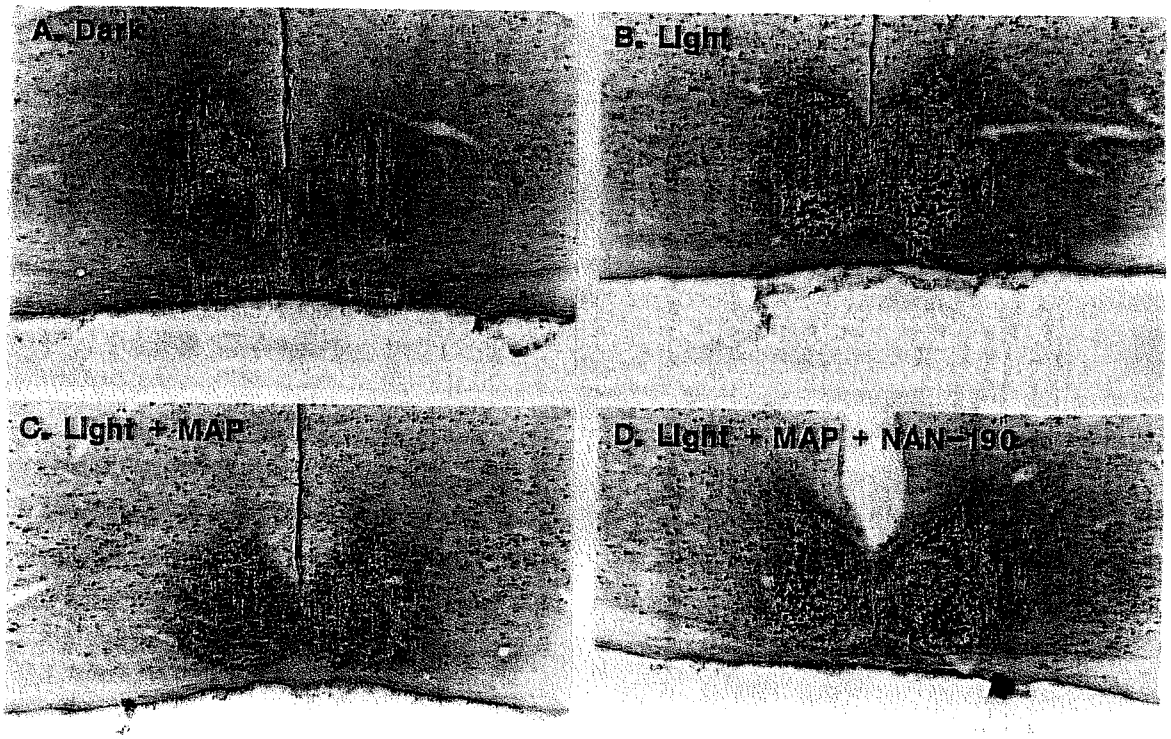

図2 光刺激によるSCNのFosタンパク発現に対するメタンフェタミンの作用
図はそれぞれ（A）恒暗条件下，（B）15分間の光パルスを与えたもの，（C）光パルスの30分前にメタンフェタミン（MA, 10 mg/kg）を腹腔内投与したもの，（D）光パルスの30分前にセロトニン1Aレセプターアンタゴニストである NAN-190（10 mg/kg）とメタンフェタミン（10 mg/kg）を腹腔内投与したものにおけるFosタンパク発現を示している．メタンフェタミンは光刺激によるSCNのFos発現を抑制するが，1Aレセプターアンタゴニストはメタンフェタミンによる抑制効果を拮抗した．
(Moriya T et al, 1996[9]より引用)

ることが報告されている[4,5]．また，セロトニンの1Aレセプターの特異的アゴニスト〔8-OH-DPATまたはブスピロン（buspirone）〕がFosタンパクの免疫応答を抑制することや，セロトニン1B，2，3レセプターのアゴニストでは抑制作用がみられないことが報告されている[6]．これらのことはセロトニン1Aレセプターが光による体内時計の調整に深く関与することを示している．

こうした研究をふまえて，最近筆者らは神経終末のセロトニン放出促進作用ならびにセロトニン再取

1972年にこの脳部位の破壊によりサーカディアンリズムが消失することが発見され，また培養，移植などの実験から，哺乳動物の場合視交叉上核だけが唯一無二の体内時計だと考えられている．

フリーラン周期 ──────
恒明（暗）条件下に体内時計の支配下でのみ動いているリズムである．この周期は動物の種や個体によって異なり，普通23～25時間ぐらいのなかに入る．マウスは一般に24時間より短く，ハムスター，ラット，ヒトでは長い．恒暗条件より恒明条件で周期は延長する．老化に伴いいずれの動物でも周期が24時間に近づく．ハムスターの輪回し行動の周期（開始時刻）は非常に安定しているため，ハムスターはリズムの研究に繁用されている．

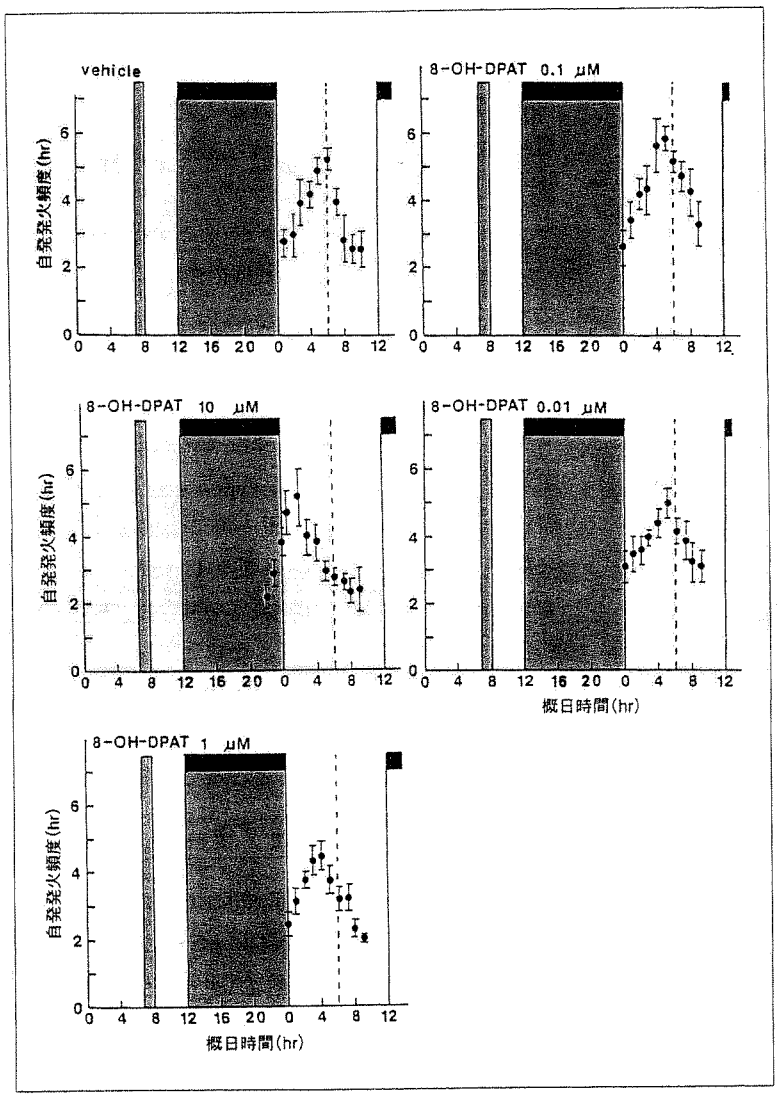

図3　SCN神経活動の自発発火頻度にみられる概日リズムに対するセロトニン1Aレセプターアゴニストの作用
SCNを含む脳スライスに対して主観的昼（CT 7～8時）に8-OH-DPAT（0.01～10 μM）を投与すると，用量依存的にSCNの発火リズムの位相が前進した．なお，破線は溶媒のみを投与した場合のSCN発火リズムの頂点位相を示している．
（Shibata S et al, 1992[12]より引用）

込み阻害作用の強いメタンフェタミンの光同調における役割について調べ，以下のような興味深い結果を得た．つまり，メタンフェタミンは視神経刺激によるSCNの複合EPSPを抑制すると同時に視神経刺激によるVIPの放出も抑制した．また，行動レベルの作用として，恒暗条件下のハムスターの輪回しリズムの光による位相変化も抑制した[7]．さらに，メタンフェタミンは光刺激によるSCNのFosタンパクの発現を抑制したが，この効果はセロトニン1AレセプターのアンタゴニストであるNAN-190により拮抗された（図2）[8]．これらの結果から，メタンフェタミンはセロトニン遊離を促進することにより光同調を抑制する可能性があることが明らかにされた．

セロトニンレセプターアゴニストが光同調を抑制することは疑いようのないものとなった．このことは言い換えると，光による時計の同調がセロトニンレセプターアンタゴニスト薬を投与することによって促進される可能性があることを意味している．実際，1Aレセプターアンタゴニストが光同調を促進させることが報告されている．こうしたことは，概日リズム

メラトニン
メラトニンは松果体でセロトニンからNアセチルトランスフェラーゼにより合成される内分泌ホルモンの1つであり，非常に古くから知られていたホルモンである．最近，抗ラジカル作用や寿命延長作用があるとの報告からメラトニンが奇跡のホルモンとして騒がれているが，裏付けのデータが曖昧である．一方，メラトニンレセプターがクローニングされ，これが視交叉上核に高密度に発現していることがわかって以来メラトニンのリズム調整作用は非常に注目されている．

の同調障害に対する高照度光療法の作用秩序解明への有用な薬理学的アプローチになるといえるであろう．

III セロトニンレセプター刺激による同調と非光同調系との相関

ハムスターに輪回しを強制するとその施行時間に依存した位相変化が起こることが知られている．この場合，位相応答の時刻依存性は，前述したような光同調の場合と明らかに異なっている．すなわち主観的昼間に施行すると顕著な前進が起こるが，主観的夜間にはほとんど作用しないのである．このことは，体内時計の調節機構には光同調系とは独立した（非光同調）システムが存在することを意味している．また，ラットにおいて運動量とSCNのセロトニン含量に正の相関がみられることが報告されており[9]，非光同調系とセロトニンの関係は興味のもたれるところであった．

Tominagaらはセロトニン1Aレセプターのアゴニストである8-OH-DPATがハムスターの輪回し活動リズムの位相変化を引き起こすことを報告したが[10]，この位相反応の時刻依存性は，強制輪回しのそれと相似していた．このことは1Aレセプターが体内時計の非光同調系の神経伝達にも関与することを示唆している．また，脳スライスSCNの自発発火頻度リズムを指標とした解析では，非特異的なセロトニンレセプターのアゴニストであるquipazineや8-OH-DPATの投与がSCNリズムの位相前進を引き起こしたが，この場合も強制輪回しの場合と相似した時刻で位相前進を引き起こした（図3）[11,12]．したがって，これらセロトニンレセプターアゴニストはSCNそのものに対する作用により位相変化を引き起こしたと考えられる．

セロトニンレセプター刺激薬以外にも，IGLからSCNへの伝達物質であると考えられているGABAやNPY，あるいはGABAレセプターを修飾するムシモール（muscimol）やベンゾジアゼピン（benzodiazepine）のトリアゾラム（triazolam）なども非光同調系型の位相変化を引き起こすことが報告されている．このことは，GHTが非光同調系の神経伝達に重要である可能性を示唆している．モノアミン神経毒素であるPCA（*p*-クロロアンフェタミン）は視床下部モノアミンのとくにセロトニンとその代謝物である5-ヒドロキシインドール酢酸（5HIAA）やドーパミンの含量を有意に減少させるが，このPCA処理はトリアゾラムによって引き起こされる位相反応を減少させることが報告されている[13]．よって，セロトニンは，トリアゾラムの作用する同調系にも関与するものと思われる．セロトニン神経とGHT神経活動の相互作用については興味がもたれるところである．

IV セロトニンレセプター

上記のような一連の生理学的なアプローチは，セロトニンのとくに1Aレセプターが概日リズムの制御に関与していることを示している．一方，RocaらはSCNにおいてセロトニンレセプターのさまざまなサブタイプの遺伝子発現を調べたところ，次の興味深い結果を得た[14]．つまり，SCNには1Cレセプターが主に発現しており，1Aレセプターの発現は認められたものの，希薄であったのである（表1）．SCNの自発発火リズムの位相変化に対してはセロトニンの2，1Cあるいは3，4レセプターのアゴニストあるいはアンタゴニストの作用は否定され

高照度光療法

サーカディアンリズムは光によりリセットされるが，季節性感情障害や痴呆患者の徘徊や睡眠—覚醒リズム障害の患者では光同調があまりうまくいかない可能性が指摘されている．これらの疾患に高照度光の早期照射が改善効果をもたらすことが報告されて，最近ビタミンB_{12}のリズム疾患を改善させる効果が光同調の促進効果である可能性も指摘されている．

表1　セロトニンレセプターサブタイプと概日システムとのかかわり

サブタイプ	Gタンパク	エフェクター経路	SCNにおけるレセプターの局在	レセプター刺激による効果	
				SCN	IGL
1A	G_i, G_o	アデニル酸シクラーゼの抑制 K^+チャネルの開放 Ca^{2+}チャネルの閉鎖	+	*c-fos* 発現 神経活動の位相変化 グルタミン酸濃度の減少	神経活動の抑制
1B	G_i	アデニル酸シクラーゼの抑制	+	*c-fos* 発現	?
1C	G_q	ホスファチジルイノシトールの加水分解	+++	×	?
1D, 1E	G_i	アデニル酸シクラーゼの抑制	−	?	?
2A, 2B, 2C	G_q	ホスファチジルイノシトールの加水分解	+	×	?
3	なし	イオンチャネルのリガンド-ゲート	−	×	?
4	G_s	アデニル酸シクラーゼの活性化	?	?	?
5A, 5B	?	?	?	?	?
6	G_s	アデニル酸シクラーゼの活性化	?	?	?
7	G_s	アデニル酸シクラーゼの活性化	?	GABA内向き電流の抑制 グルタミン酸濃度の減少	?

+：検出，+++：多量に検出，−：未検出，?：不明，×：効果なし

ており[15]，この解釈については現在でも議論の余地がある．しかしながら最近では，新しくみつかったセロトニン7レセプターが1Aレセプターアゴニストである8-OH-DPATにも高い親和性をもつことから，これまで1Aレセプターに対する作用と考えられていた一部はおそらく7レセプターを介したものであることが示唆されている[16~18]．

V　メラトニン前駆体としてのセロトニン

松果体のホルモンであるメラトニン*は夜間に多く昼間に少ない明瞭な概日リズムを示し，さまざまな生理現象のリズムを調節すると同時に，SCNや網膜にもフィードバックして作用し概日時計の位相や光感受性を調節することが知られている．このメラトニンは松果体内でセロトニンを前駆体として*N*-アセチルトランスフェラーゼにより合成される．松果体のセロトニンは，その他の脳部位のセロトニン機構とは独立して存在しており，その組織重量あたりの含量は他の約50倍以上にも及ぶ[19]．したがって，セロトニン代謝にかかわる薬物を末梢より作用させる場合などについては，メラトニンを介する概日リズムの調節機構にもその作用が及ぶことを十分に考慮しなくてはならないであろう．

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Inhibition of Light- or Glutamate-Induced *mPer1* Expression Represses the Phase Shifts into the Mouse Circadian Locomotor and Suprachiasmatic Firing Rhythms

Masashi Akiyama,[1] Yasuko Kouzu,[1] Satomi Takahashi,[1] Hisanori Wakamatsu,[1] Takahiro Moriya,[2] Miyuki Maetani,[3] Shigenori Watanabe,[3] Hajime Tei,[4] Yoshiyuki Sakaki,[4] and Shigenobu Shibata[1,2]

[1]Department of Pharmacology and Brain Science and [2]ARCHS, School of Human Sciences, Waseda University, Tokorozawa, Saitama 359–1192, Japan, [3]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812–82, Japan, and [4]Human Genome Center, Institute of Medical Sciences, University of Tokyo, Tokyo, Japan

mPer1, a mouse gene, is a homolog of the *Drosophila* clock gene *period* and has been shown to be closely associated with the light-induced resetting of a mammalian circadian clock. To investigate whether the rapid induction of *mPer1* after light exposure is necessary for light-induced phase shifting, we injected an antisense phosphotioate oligonucleotide (ODN) to *mPer1* mRNA into the cerebral ventricle. Light-induced phase delay of locomotor activity at CT16 was significantly inhibited when the mice were pretreated with *mPer1* antisense ODN 1 hr before light exposure. *mPer1* sense ODN or random ODN treatment had little effect on phase delay induced by light pulses. In addition, glutamate-induced phase delay of suprachiasmatic nucleus (SCN) firing rhythm was attenuated by pretreatment with *mPer1* antisense ODN, but not by random ODN. The present results demonstrate that induction of *mPer1* mRNA is required for light- or glutamate-induced phase shifting, suggesting that the acute induction of *mPer1* mRNA in the SCN after light exposure is involved in light-induced phase shifting of the overt rhythm.

Key words: antisense oligonucleotide; circadian rhythm; firing rhythm; mPer1; phase shift; suprachiasmatic nucleus

Circadian rhythms, which persist in the absence of environmental cues, are observed in a wide variety of organisms (Edmunds, 1988). To maintain synchrony with the daily environmental cycle, organisms responded to environmental cues, especially light, to reset or entrain their circadian rhythms. In mammals, the suprachiasmatic nucleus (SCN) of hypothalamus has been shown to be a primary circadian pacemaker of locomotor activity and various physiological phenomena (Hastings, 1997). The genetic and molecular mechanisms that control circadian rhythms were initiated by studies of *Drosophila* rhythms (Konopka and Benzer, 1971). The circadian rhythms evident in the locomotor activity of adult flies and in gating of eclosion were altered by mutations in two genes, *period* (*dPer*) and *timeless* (*tim*) (Hall, 1998; Young, 1998). Protein levels and mRNA levels of these genes undergo robust circadian oscillation, and both proteins co-regulate their own regulation by negative feedback loops. In mammals, previous studies have demonstrated that mRNAs of immediately early genes (IEGs), including c-fos and *jun*B, are markedly induced by light in the SCN (Rusak et al., 1990; Morris et al., 1998). However, the molecular component of the circadian clock and the mechanism by which the light entrains the circadian clock have only been recently elucidated. The recent isolation of *dPer* homologous genes, *Per1* (Sun et al., 1997; Tei et al., 1997), *Per2* (Albrecht et al., 1997; Shearman et al., 1997; Takumi et al., 1998b), and *Per3* (Zylka et al., 1998; Takumi et al., 1998a) from human and mouse have significantly clarified the molecular mechanisms of the circadian clock in mammals. These genes are rhythmically expressed in the SCN. We showed that brief exposure to light during subjective night results in a large and rapid induction of *mPer1* expression (Shigeyoshi et al., 1997). The induction of *mPer1* (<20 min) by light is more rapid than the accumulation of c-fos protein (Shigeyoshi et al., 1997). This suggests that c-fos protein is not directly involved in the rapid induction of the *mPer1* gene.

To investigate whether induction of *mPer1* transcripts by light exposure is necessary for light-induced phase shifts, we injected an antisense phosphotioate oligonucleotide (ODN) to *mPer1* mRNA intracerebroventricularly 1 hr before light exposure. We found that inhibition of light-induced *mPer1* expression by antisense oligonucleotide *in vivo* significantly represses light-induced phase shifts of the mouse circadian locomotor rhythm. We have reported that treatments with glutamate, NMDA, or substance P, or stimulation of the optic chiasm produce changes in the phase of the firing rhythm of SCN neurons *in vitro* with a phase-response curve similar to that induced by light exposure *in vivo* (Shibata et al., 1992, 1994; Shibata and Moore, 1993). Direct application of *mPer1* antisense ODN to the SCN in hypothalamic slices *in vitro* produced an attenuation of the glutamate-induced phase shift in a manner similar to the reduction of the light-induced phase shifts observed *in vivo*. These results suggest that acute induction of *mPer1* mRNA in the SCN after light exposure is involved in light-induced phase shifts of overt rhythms.

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Correspondence should be addressed to Shigenobu Shibata, Department of Pharmacology and Brain Science, School of Human Sciences, Waseda University, Tokorozawa, Saitama 359–1192, Japan. E-mail: shibata@human.waseda.ac.jp

MATERIALS AND METHODS

Phosphotioate ODNs. The published sequence of *mPer1* was used to design an antisense ODN targeted to the region of the mRNA containing the initiation ATG. The sequences of the *mPer1* antisense and sense ODNs (18-mer) were 5′-TAG GGG ACC ACT CAT GTC T-3′ and 5′-A GAC ATG AGT GGT CCC CTA-3′, respectively. The sequences of random ODN (18-mer) and vasopressin precursor gene (AVP) antisense ODN (18-mer) were 5′-CCG TTA GTA CTG AGC TGA C-3′ and 5′-CAT CCT GGC GAG CAT AGG T-3′, respectively. The random ODN contained an equivalent GC content as the antisense and sense ODNs of *mPer1*. All ODNs were purified by HPLC to reduce the possible toxicity of phosphotioate ODNs.

Animals and surgery. Male *Balb/c* mice (Takasugi Saitama, Japan) purchased 6 weeks postpartum were maintained on a 12 hr light/dark cycle with light on at 8:30 A.M. Animals were given food and water *ad libitum*. Stainless steel guide cannulas (6.0 mm, 23 gauge) were implanted bilaterally intracerebroventricularly (4.5 mm anterior and 1.1 mm lateral to lambda and depth of 2.1 mm below the skull) using a stereotaxic frame (Narishige, Tokyo, Japan). After 2 d recovery, animals were moved to continuous darkness for at least 2 weeks before ODN administration. For assessment of the locomotor activity, mice were housed individually, and their locomotor activity rhythm was measured by area sensors (model FA-05 F5B; Omron, Tokyo, Japan) with a thermal radiation detector system, and data were stored on a personal computer.

After free-running for 14–20 d in constant darkness, mice were randomly assigned to *mPer1* antisense ODN, *mPer1* sense ODN, *mPer1* random ODN, AVP antisense ODN, or vehicle (sterilized saline). A 5 μl aliquot of each ODN (2–6 nmol) was unilaterally injected into the lateral ventricle via an injection cannula (external diameter, 0.35 mm) extending 0.5 mm below the tip of the guide cannula at a rate of 1 $\mu l\ min^{-1}$ using a 10 μl Hamilton syringe. Injection was performed at circadian time 1 (CT1; CT12, onset time of locomotor activity), CT4, CT8, CT15, or CT21, then animals were returned to their individual cages. For light exposure experiments, implanted mice were again randomly assigned an ODN, and 60 min after the injection, each animal was exposed to a light pulse lasting 15 min at CT16. Light (20 lux) was administered while the mice were in a Plexiglas cylinder. After treatment, animals were returned to constant darkness. Some mice were first exposed to the light, and then ODNs were administered 0 or 120 min after light exposure. Each group received a repeated intracerebroventricular injection (3 or 4 times for each animal) after at least 14 d. Injections were randomly administered into the right or left ventricle. To verify that ODNs were administered into the cerebral ventricle, mice were injected with 5 μl of saturated fast green, and their brains were examined macroscopically after sectioning. Anisomycin (50 μg in 5 μl of saline) and MK-801 (10 μg in 5 μl of saline) were also intracerebroventricularly injected in the same manner. After treatment, animals were returned to constant darkness. The phase of the rhythm was assessed visually by applying a straight edge to the onset of activity on successive days before the light pulse and again beginning ~3 d after a light treatment and determining the difference in phases on the day of the light exposure (Daan and Pittendrigh, 1976). At least four independent experiments using different mice were done at each group.

Slice culture and measurement of neural activity rhythm. On the first day, coronal hypothalamic slices (400 μm thickness) including SCN were obtained between zeitgeber time 9–11 (ZT9–11) from male *Balb/c* mice (10–14 weeks). Then, slices were preincubated and treated with vehicle or *mPer1* antisense, sense, or random ODNs (each 20 μM) in Krebs' Ringer's solution (in mM: NaCl 129, KCl 4.2, $MgSO_4$ 1.3, KH_2PO_4 1.2, $CaCl_2$ 1.5, glucose 25, $NaHCO_3$ 22.4, and HEPES 25, with gentamycin 0.5 mg/ml, pH 7.3–7.4) for 4 hr (ZT12–16). At ZT16, the slices were removed into the buffer containing glutamate (10 μM) for 15 min. After drug treatment, perfusion with normal medium was reinstated. The spontaneous action potentials of single SCN cells were recorded extracellularly through glass electrodes filled with 3 M NaCl during the second day *in vitro*. Stable single unit activity was recorded over 5 min intervals. The activities of all cells recorded during a single experiment were averaged into 2 hr intervals using 1 hr lags. Previous studies have shown that this procedure yields a pattern of electrical activity for the population of SCN neurons that varies little between animals, and that the time of peak electrical activity is a reliable marker of the phase of the SCN pacemaker (Shibata and Moore, 1993; Shibata et al., 1994).

Biochemical analysis. To detect injected biotinylated ODN, anesthetized mice were perfused intracardially with ice-cold saline and 4% paraformamide in 0.1 M phosphate buffer (PB), pH 7.4, and then their brains were removed, post-fixed for 24 hr at 4°C and placed in 0.1 M PB with 20% sucrose for 24 hr. The distributions of ODN in the serial cryostat sections (30 μm) containing SCN were determined using a Vecstatain ABC Elite kit (Vector Laboratories, Burlingame, CA).

RT-PCR analysis. The effect of *mPer1* antisense ODN on *mPer1* expression in the SCN was examined by RT-PCR. Mice were entrained to light/dark cycle for 2 weeks. Mice were transferred to constant darkness for one extra daily cycle, and at ZT15, mice were administered antisense ODN (2, 4, and 6 nmol) and vehicle. Half of both groups received light treatment (20 lux, 15 min) at ZT16. At ZT17.5, brains ($n = 4$ for each group) were removed and placed in ice-cold saline. Slices (0.5-mm-thick) of mice brain that contained SCN were frozen on dry ice, and the SCNs were punched out with a 26 gauge needle. Total RNA from the SCN ($n = 4$) was extracted in each group by Trizol solution (BRL, Bethesda, MD). A one-step RT-PCR system (BRL) was used for reverse transcription of ~100 ng of RNA, and *mPer1*, *mPer2*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were amplified by PCR. RT-PCR reactions were performed for 21 cycles with *mPer1*, *mPer2*, and GAPDH primers in a single tube. The primer pairs used for the amplification of each product are as follows: 5′-CCA GGC CCG GAG AAC CTT TTT-3′ and 5′-CGA AGT TTG AGC TCC CGA AGT G-3′ (*mPer1*); 5′-ACA CCA CCC CTT ACA AGC TTC-3′ and CGC TGG ATG ATG TCT GGC TC-3′ (*mPer2*); and 5′-GAC CTC AAC TAC ATG GTC TAC A-3′ and TGG CCG TGA TGG CAT GGA CT-3′ (GAPDH). The sizes of the PCR products of *mPer1*, *mPer2*, and GAPDH were 402, 779, and 436 bp, respectively. PCR products were run on 3% agarose gels, and DNA in the appropriate bands were detected with an EDAS-120 system (Eastman Kodak, Rochester, NY).

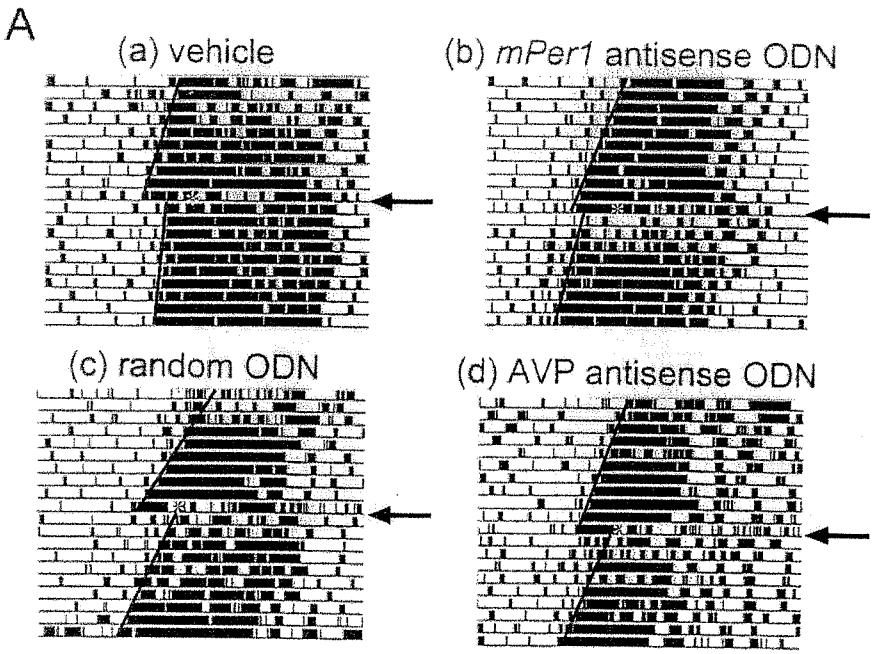
RESULTS

Phase shift effects of *mPer1* antisense ODN on various CTs

Administration of 6 nmol of *mPer1* antisense ODN at various CTs (CT1, 8, 15, 21) were compared with vehicle administration (Fig. 1*B*). Significant phase delays were observed when *mPer1* antisense ODN was administrated at CT1. There were no significant differences between *mPer1* antisense ODN administrated at other CTs. To examine whether this ODN shifting effect is specific to *mPer1* antisense ODN, we examined the effects of intracerebroventricular administration of four different ODNs and anisomycin on behavioral rhythms (Fig. 1*A*,*C*). Administration of anisomycin, which inhibits protein synthesis, has been shown to induce phase shifts when it was injected into the SCN region (Inouye et al., 1988). Two ODNs, *mPer1* antisense ODN and AVP antisense ODN, had specific mRNA targets, whereas the other two ODNs, sense and random ODNs, lacked specific mRNA targets. We found that phase delays were observed when anisomycin (50 μg) or *mPer1* antisense (6 nmol) ODN was administered ($p < 0.01$; Student's t test). No significant phase shifts were observed after injection of the other ODNs or vehicle (Fig. 1*C*). The magnitude of the phase shifts by *mPer1* antisense ODN were dose-related, with injection of 4 nmol of the *mPer1* antisense ODN producing a phase shift approximately half the size of the one at a 6 nmol dose. No phase delays were observed at 2 nmol doses.

Effect of ODN on light-induced phase shifts

We previously demonstrated that *mPer1* induction by light is strongly correlated with phase shifts in behavioral rhythms (Shigeyoshi et al., 1997). Thus, we examined the effect of *mPer1* antisense ODN on light-induced phase shifts (Fig. 2*A*,*B*). Mice injected with vehicle at CT15 followed by exposure to a light pulse for 15 min at CT16 had a marked phase delay in the circadian rhythm of locomotor activity of ~2 hr. MK-801, an NMDA receptor antagonist, injected intracerebroventricularly at CT15 markedly depressed the light-induced phase delay at CT16, as previously reported ($p < 0.01$; Student's t test) (Colwell et al., 1990; Shibata et al., 1994). Injection of *mPer1* antisense ODN at



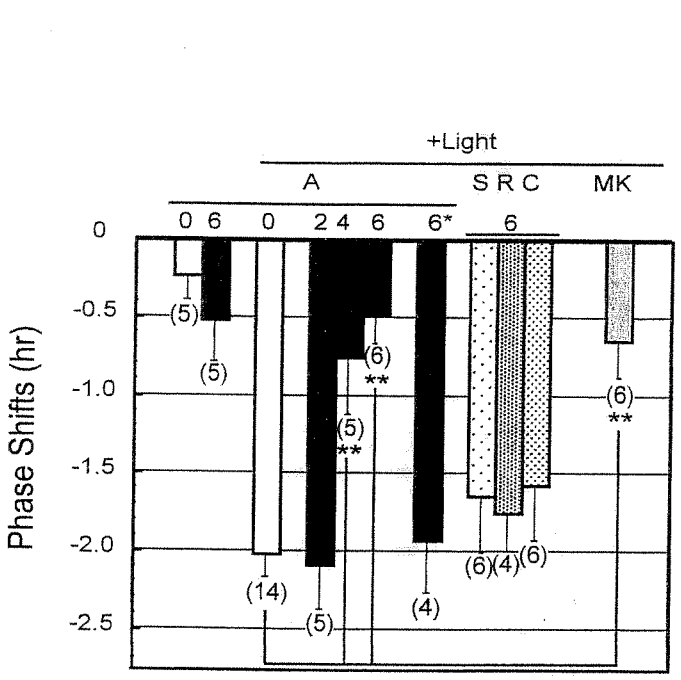


Figure 2. Effect of ODN injection on light-induced phase delay of locomotor activity rhythm. Mice were injected with ODNs at CT15 under the safety light, 1 hr after injection, mice were exposed to light (20 lux) for 15 min and returned to constant darkness. *A*, Locomotor activity records of vehicle (*a*), *mPer1* antisense (*b*), random ODN (*c*), and AVP antisense ODN (*d*)-injected mouse. *B*, Light-induced phase shifts in *mPer1* antisense ODN (*A*), sense ODN (*S*), random ODN (*R*), AVP antisense ODN (*C*), and MKC-801 (MK)-injected mouse. * indicates that antisense ODN was administered 2 hr after the light pulse. The *number* in the figure indicates the amount (nanomoles) of injected ODN. *0* indicates the vehicle administration. *Numbers* in parentheses indicate the number of experiments. Preinjection of *mPer1* antisense ODN (4 and 6 nmol) and MK-801 significantly reduced light-induced phase shift (**$p < 0.01$; Student's t test). Injection of *mPer1* antisense ODN 2 hr after light exposure did not have any effects.

these experiments, the mean peak of electrical activity on the subsequent day occurred at ZT6–7 (ZT6.0 ± 0.5; $n = 4$) (Fig. 3*A*,*B*). For slices treated with glutamate *in vitro* at ZT16 on day 1, the peak was around ZT9 on day 2 (Fig. 3). Glutamate-induced phase delay at ZT16 was significantly blocked by 4 hr pretreatment with *mPer1* antisense ODN (ZT12–16) but not by pretreatment with random ODN. However, *mPer1* antisense ODN did not produce phase changes when applied alone for 4 hr (ZT12–16).

Distribution of ODN in the brain and effect of antisense ODN on mRNA

Distribution of antisense ODN was examined by injection and staining of biotinylated ODN in the fixed slice section of the brain. The ODN were most extensively distributed around the third ventricle, including the SCN (Fig. 4*A*). Inhibition of *mPer1* induction by *mPer1* antisense ODN in the SCN 1.5 hr after light exposure at CT16 was examined by RT-PCR. Before amplification, RNA were preliminarily tested for possible genomic DNA contamination. Gel analysis showed bands of expected lengths. Light exposure at CT16 induced expression of *mPer1* mRNA (180 ± 24% of nonlight group; $n = 4$) and *mPer2* mRNA (160 ± 25%; $n = 4$) 90 min after light pulse. Light induction of *mPer1* mRNA was considerably inhibited when 4 or 6 nmol of *mPer1* antisense ODN was administrated (Fig. 4*B*,*C*). Administration of 6 nmol of *mPer1* antisense significantly reduced the expression of

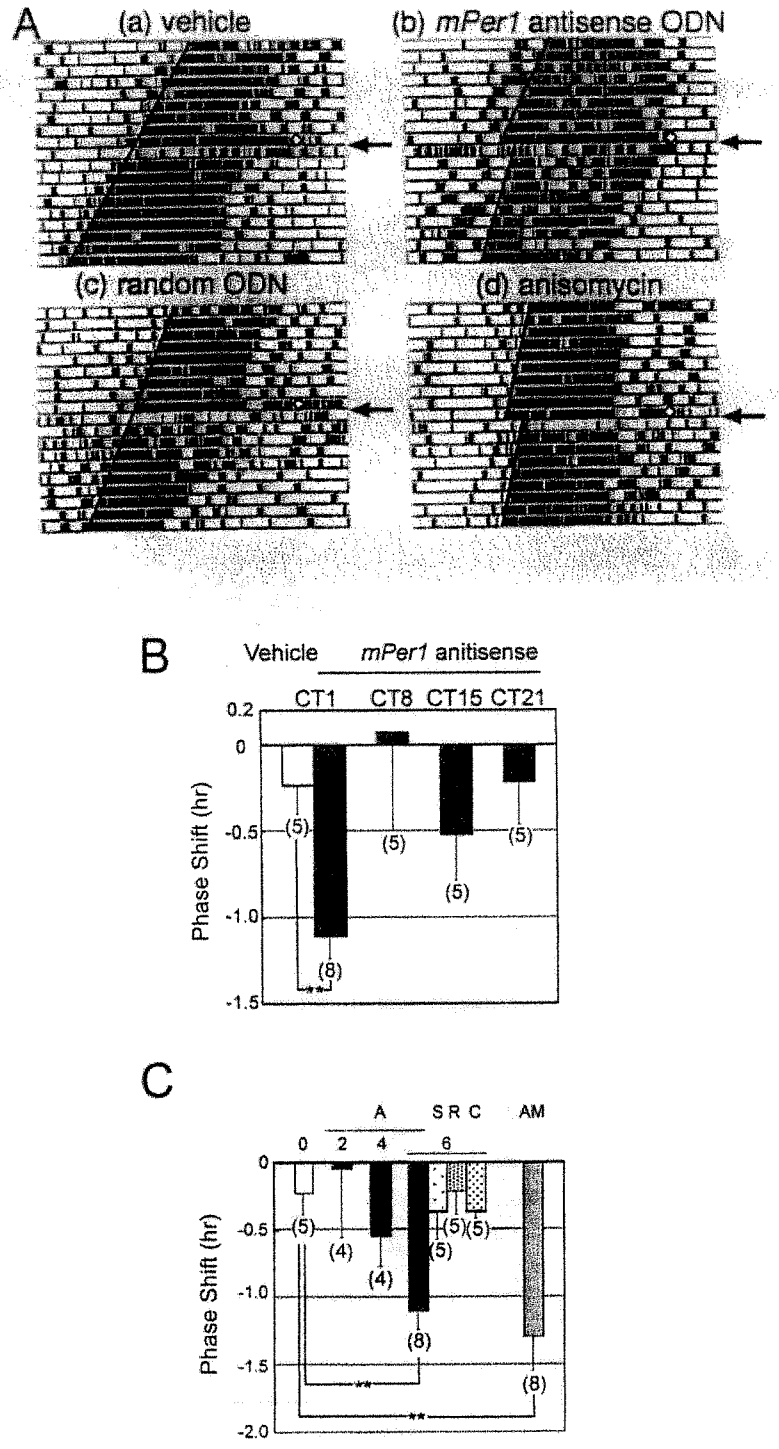


Figure 1. Effect of ODN administration at CT1 on the mouse circadian locomotor rhythm. *A*, Locomotor activity records of vehicle (*a*), *mPer1* antisense ODN (*b*), random ODN (*c*), and anisomycin (*d*)-injected mice. Each animal was injected at CT1 (◇ in the figure) intracerebroventricularly (5 μl; 1 μl min^{-1}) and returned to constant darkness. *B*, Phase–response curve for *mPer1* antisense ODN administration at CT1, CT8, CT15, and CT21. *Numbers* in parentheses indicate the number of experiments. Injection of *mPer1* antisense ODN at CT1 induced a significant phase delay (**$p < 0.01$; Student's t test). *C*, Phase shifts of mouse locomotor rhythm by various ODNs or anisomycin injection at CT1. *0*, Vehicle; *A*, *mPer1* antisense ODN; *S*, sense ODN; *R*, random ODN; *C*, AVP antisense ODN. The *number* in the figure indicates the amount (in nanomoles) of injected ODN. *Numbers* in parentheses indicate the number of experiments. Injection of *mPer1* antisense ODN and anisomycin significantly phase delayed locomotor rhythm (**$p < 0.01$; Student's t test).

CT15 attenuated the light-induced phase delay at CT16 in a dose-dependent manner [phase shift, −0.480 ± 0.194 hr (6 nmol injection of antisense ODN) vs −2.204 ± 0.141 hr (vehicle injection); $p = 0.0001$]. However, injection of *mPer1* antisense alone at CT15 did not alter locomotor activity (Fig. 2*B*). *mPer1* antisense administration immediately after light exposure (CT16.3) also inhibited the light-induced phase shift, but less efficiently, and administration of it 2 hr after light exposure (CT18) did not inhibit the phase delay (Fig. 2*B*). The other ODNs injected at CT15 did not affect the light-induced phase delay at CT16.

Effect of antisense ODN on *in vitro* SCN neural activity rhythm

We and other researchers have reported that treatments with glutamate produce changes in the phase of the firing rhythm of SCN neurons *in vitro* with a phase–response curve similar to that induced by light exposure *in vivo* (Shibata et al., 1994; Shirakawa and Moore, 1994). Thus, we examined the effects of *mPer1* antisense ODN *in vitro*. In control experiments, coronal hypothalamic slices containing whole SCN were treated *in vitro* for 4 hr on day 1 between ZT12 and ZT16 with drug-free perfusion medium. In

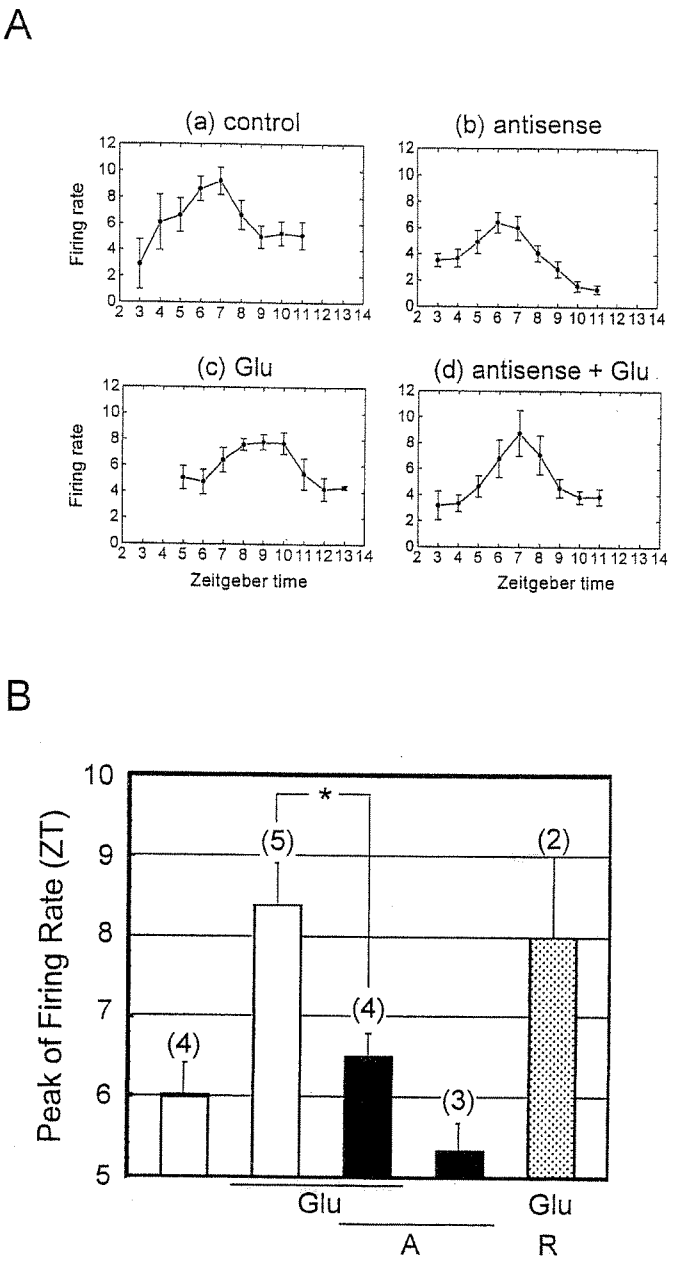


Figure 3. Effect of *mPer1* antisense ODN on glutamate-induced phase delay of SCN firing rhythm *in vitro*. *A*, The average neuronal activity rhythms in the SCN recorded from mice slice on day 2. Each *point* indicates the 2 hr means ± SEM of firing rate of single SCN cells from ZT2–14. *B*, Average phase shifts induced by glutamate and glutamate plus *mPer1* antisense ODN. Each *bar* indicates the peak of firing rate (mean ± SEM). *Numbers* in parentheses indicate the number of slices. Preincubation of *mPer1* antisense ODN significantly reduced glutamate-induced phase shift (*$p < 0.05$ vs glutamate alone; Student's *t* test). *Glu*, Glutamate; *A*, *mPer1* antisense ODN; *R*, *mPer1* random ODN.

mPer1 mRNA (68 ± 8.7% of random ODN treatment; $n = 4$; $p < 0.05$; Student's *t* test) but not that of *mPer2* mRNA (99 ± 11% of random ODN treatment; $n = 4$; $p > 0.05$; Student's *t* test). This result suggests that phenotypic effects of *mPer1* antisense ODN treatment on light-induced phase delay are mediated by the specific inhibition of *mPer1* expression in the SCN.

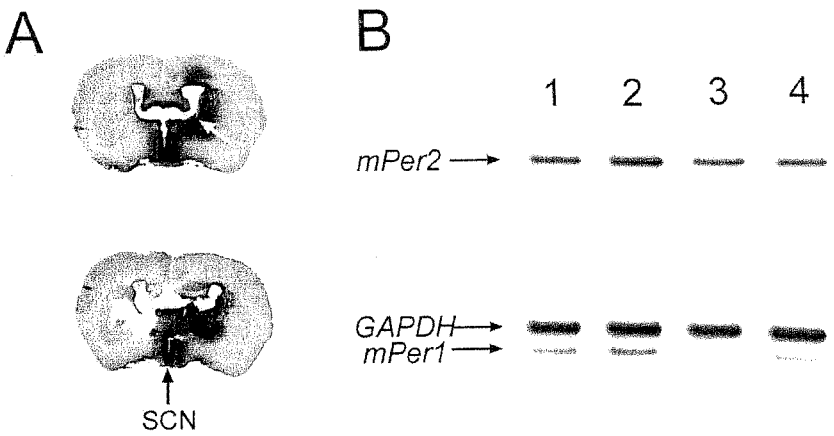
DISCUSSION

Administration of *mPer1* antisense ODN at CT1, but not at other CTs, significantly delayed the locomotor rhythm of mice. Injection of vehicle, *mPer1* sense ODN, or *mPer1* random ODN at CT1 had little effect. It is currently believed that circadian oscillators, including those in mammals, are comprised of transcription/translation-based negative feedback loops controlled by clock genes (Hall, 1998; Reppert, 1998; Young, 1998). Peripheral or intra-SCN injections of translation inhibitors such as anisomycin or cycloheximide at early subjective days (CT1–4) have been reported to produce a phase delay in wheel-running rhythms (Takahashi and Turek, 1987; Inouye et al., 1988). Transcript of *mPer1* is endogenously rhythmic with a consistent peak of expression in the subjective day at CT4 (Tei et al., 1997). We observed that the injection of *mPer1* antisense ODN 4 hr before the light pulse did not block the light-induced phase delay of locomotor rhythm (data not shown). Therefore, the largest reduction of *mPer1* expression by antisense ODN might occur when antisense ODN is injected 2–3 hr before the *mPer1* peak and may be the reason why antisense ODN delayed the circadian rhythm only at CT1.

In this study, we demonstrated that blockade of acute *mPer1* induction after light exposure by antisense ODN prevents the light-induced phase shifts of the circadian activity rhythm. This block in light-induced phase shift was caused by selective inhibition of *mPer1* induction, because *mPer1* antisense ODN alone did not interfere with the free-running rhythm at CT16. Moreover, it is interesting that both *mPer1* mRNA expression and phase delay of locomotor activity induced by light at CT16 were reduced by 4 nmol of *mPer1* antisense ODN but not by 2 nmol. Thus, we observed the parallel reduction of *mPer1* expression and phase delay. In the present experiment, 4 and 6 nmol of *mPer1* antisense ODN reduced to 60–70% of *mPer1* RNA expression induced by light exposure. Although we do not detect the protein production of mPer1 after light exposure, we can estimate 30–40% reduction of *mPer1* mRNA may affect the light-induced phase changes in mouse behavior. Present results suggest that the reduction of light-induced phase delay by antisense ODN *in vivo* is a result of the inhibition of light-induced acute induction of *mPer1* gene in the SCN. Further experiments are needed to locate the specific region of antisense ODN action (for example, direct antisense ODN injection into the SCN or immunostaining of mPer1 antibody there).

Transcript of *mPer1* is rapidly induced by light in a time-of-day-dependent manner (Shigeyoshi et al., 1997). The responsiveness of *mPer1* mRNA to light is gated so that little or no increase was seen during the subjective day, whereas robust induction was seen during the subjective night. Gating is also present in light-induced phase shifts of behavioral rhythm. Their dose and threshold is closely correlated with *mPer1* inducibility in the SCN. These results with our present results suggest that *mPer1* plays a central role in the circadian clock. *mPer2* gene was also shown to be induced by light but in a delayed manner compared with *mPer1* (Takumi et al., 1998b), possibly reflecting a different regulatory mechanism. Recently, *mPer3* has been isolated and shown not to be light inducible (Takumi et al., 1998a; Zylka et al., 1998), suggesting that *mPer* genes have different roles in the light-induced phase shift. Therefore, injection of *mPer2* or *mPer3* antisense ODN or cocktails containing antisense ODNs of *mPer* genes may be a good strategy for determining the roles of these genes.

To exclude the possibility that other regions of the brain might have added to the effects of *mPer1* antisense ODN treatment, we examined the neural rhythm of SCN using slice culture. Administration of *mPer1* antisense ODN blocked the glutamate-induced



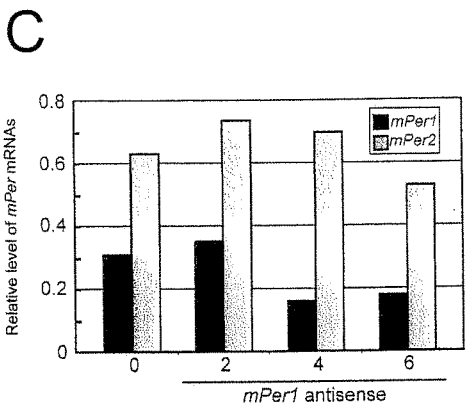


Figure 4. Effects of *mPer1* antisense ODN on the *mPer1* expression in the SCN. *A*, Distribution of biotinylated ODN 2 hr after injection into the brain. *mPer1* antisense ODN (5′-biotnylated; 6 nmol in 5 ul) was microinjected intracerebroventricularly. Mice were killed 2 hr later, followed by detection of biotinylated ODN. An *arrow* on the *top* slice shows antisense ODN injection site. An *arrow* on the *bottom* slice shows the position of SCN. The ODNs were most extensively distributed around the third ventricle including the SCN. *B*, Inhibition of light induction of *mPer1* transcript in the SCN by *in vivo mPer1* antisense ODN treatment. Total RNA was isolated 1.5 hr after light exposure from *mPer1* antisense ODN-pretreated mice, and *mPer1*, *mPer2*, and GAPDH RNA were amplified by an RT-PCR method. *Lane 1*, Treated with vehicle; *lane 2*, treated with 2 nmol of antisense ODN; *lane 3*, treated with 4 nmol of ODN; *lane 4*, treated with 6 nmol of antisense ODN. The PCR products of *mPer1*, *mPer2*, and GAPDH gene are indicated by *arrows*. *C*, Semiquantitative analysis of RT-PCR products shown in *B*. The band intensity of RT-PCR products of *mPer1* and *mPer2* mRNA was measured by one-dimensional analysis software (Eastman Kodak), and their amounts were normalized against GAPDH.

phase delay of the SCN circadian firing rhythm. Thus, glutamate-induced phase shifts may be involved in the expression of *mPer1* mRNA in the SCN. SCN is entrained to the environmental light/dark cycle via a retinal projection, the retinohypothalamic tract (RHT). Glutamate is a transmitter of the RHT (de Vries et al., 1993). Glutamate and NMDA application to rat SCN *in vitro* have been reported to cause phase delays in SCN firing rhythms when applied at early subjective night (Shibata et al., 1994; Shirakawa and Moore, 1994). Furthermore, glutamate receptor antagonists and inhibitors of nitric oxide synthase, calmodulin, or calcium calmodulin kinase II antagonize phase shifts in the SCN firing rhythm induced by glutamate or NMDA *in vitro* (Shibata et al., 1994; Watanabe et al., 1994; Fukushima et al., 1997). Therefore, we cannot rule out the possibility that *mPer1* antisense ODN interferes with these biochemical steps. However, the sequence specificity of the ODNs on light- or glutamate-induced phase delay strongly suggest this is not the case.

Light-induced phase shifts of circadian rhythms induce immediately early genes (IEGs) such as *c-fos*, *jun*B, and NGFI-A mRNAs specifically in the SCN (Rusak et al., 1990; Morris et al., 1998). Blockade of expression of c-fos or Jun B expression in the SCN has been shown to inhibit light-induced phase shifts in mammalian circadian clocks (Wollnik et al., 1995). These proteins are believed to dimerize and bind to AP-1, which are CRE/CaRE consensus sequences that are present in the promoters of many genes (Takeuchi et al., 1993). The light-induced induction of IEGs is also gated as *mPer1* and *mPer2*. The time courses of c-fos and *mPer1* mRNA induction are similar, but it is unknown whether c-fos protein is involved in transcription of *mPer1* (or *mPer2*) or the induction of the c-fos and *mPer* are simultaneous.

In this study, we used antisense ODN as pharmacological tools to inhibit *mPer1* expression *in vivo* and *in vitro*. The mechanism of inhibition of physiological systems by antisense ODNs is believed to be the result of specific hybridization of the antisense ODN to its complementary mRNA, causing disruption of the translation of the mRNA into protein (Talamo, 1998). We have not determined the amounts of mPer1 protein expression, because we have not obtained anti-mPer1 antibody. Antisense ODN is also believed to bind to the genomic DNA region of the corresponding gene and inhibit binding of transcription factors and to bind to mRNA and accelerate degradation of targeted mRNA by RNaseH (Kashihara et al., 1998). Both of these mechanisms should lower the level of mRNA. These effects may be sequence-specific; arising from inhibition of imperfectly matched target genes, or sequence-independent effects on gene expression. Antisense ODNs may also effect nontargeted genes or even be toxic to physiological systems (Talamo, 1998). In the present study, we showed that *mPer1* mRNA in the SCN was reduced by treatment with *mPer1* antisense ODN, but treatment did not affect *mPer2* and GAPDH mRNA levels, demonstrating that the antisense ODN used in this study specifically effects only *mPer1* gene expression. We used phosphotioate-substituted ODNs, which have longer biological half-lives than unsubstituted ODNs but

may be toxic (Agrawal et al., 1991). In this study, some animals exhibited altered locomotor activity for the first several hours after injection. However, this effect was observed in both *mPer1* antisense ODN-injected animals and control ODNs-injected animals, suggesting this change is caused by a toxic effect of the administration of ODNs. In all cases, locomotor activity was restored to normal under constant darkness. In our previous experiments (Ono et al., 1996, Watanabe et al., 1996), methamphetamine and adenosine antagonists inhibited the light-induced phase shift, although these chemicals increase or decrease motor activity, respectively. Thus, the circadian oscillator may be unaffected by ODN injection. We also demonstrated that ODNs injected intracerebroventricularly were distributed in specific regions of the brain after 2 hr, especially around the third ventricle including the SCN. However, other regions of the brain might have added to the effects of *mPer1* antisense ODN treatment.

In summary, the present results indicate that acute induction of *mPer1* mRNA after light exposure is necessary for light-induced phase shifting of the mouse locomotor rhythm. Further genetic dissection of *mPer* genes, possibly with knock-out mice is useful to identify the role of these genes in detail.

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Potentiating action of MKC-242, a selective 5-HT_{1A} receptor agonist, on the photic entrainment of the circadian activity rhythm in hamsters

[1,3]T. Moriya, [2]Y. Yoshinobu, [1]M. Ikeda, [2]S. Yokota, [2]M. Akiyama & [2]S. Shibata

[1]Advanced Research Center for Human Sciences; [2]Department of Pharmacology and Brain Science, School of Human Sciences, Waseda University, Tokorozawa, Saitama 359-1192, Japan

1 Serotonergic projections from the midbrain raphe nuclei to the suprachiasmatic nuclei (SCN) are known to regulate the photic entrainment of circadian clocks. However, it is not known which 5-hydroxytryptamine (5-HT) receptor subtypes are involved in the circadian regulation. In order to verify the role of 5-HT_{1A} receptors, we examined the effects of 5-{3-[((2S)-1,4-benzodioxan-2-ylmethyl)amino]-propoxy}-1,3-benzodioxole HCl (MKC-242), a selective 5-HT_{1A} receptor agonist, on photic entrainment of wheel-running circadian rhythms of hamsters.

2 MKC-242 (3 mg kg^{-1}, i.p.) significantly accelerated the re-entrainment of wheel-running rhythms to a new 8 h delayed or advanced light-dark cycle.

3 MKC-242 (3 mg kg^{-1}, i.p.) also potentiated the phase advance of the wheel-running rhythm produced by low (5 lux) or high (60 lux) intensity light pulses. In contrast, 8-hydroxy-dipropylaminotetralin (8-OH-DPAT)(5 mg kg^{-1}, i.p.), a well known 5-HT_{1A}/5-HT_7 receptor agonist, only suppressed low intensity (5 lux) light-induced phase advances.

4 The potentiating actions of MKC-242 on light pulse-induced phase advances were observed even when injected 20 or 60 min after the light exposure.

5 The potentiating action of MKC-242 was antagonized by WAY100635, a selective 5-HT_{1A} receptor blocker, but not by ritanserin, a 5-HT_2/5-HT_7 receptor blocker, indicating that MKC-242 is activating 5-HT_{1A} receptors.

6 Light pulse-induced c-*fos* expression in the SCN and the intergeniculate leaflet (IGL) were unaffected by MKC-242 (3 mg kg^{-1}, i.p.).

7 HPLC analysis demonstrated that MKC-242 (3 mg kg^{-1}, i.p.) decreased the 5-HIAA content in the SCN.

8 The present results suggest that presynaptic 5-HT_{1A} receptor activation may be involved in the potentiation of photic entrainment by MKC-242 in hamsters.

Keywords: Circadian rhythm; light-entrainment; 5-Hydroxytryptamine (5-HT); 5-HT_{1A} receptor

Introduction

The biological clocks of mammals, which are located in the suprachiasmatic nuclei (SCN) of the hypothalamus, control various physiological daily rhythms such as feeding, drinking, locomotor activity, sleep-wakefulness, plasma adrenal corticosterone levels and the body temperature cycle (Inouye & Shibata, 1994). It is well known that daily light-dark cycles strongly entrain the circadian rhythms generated by the biological clock. Light signals for photic entrainments reach the SCN *via* a direct projection from the retina (retinohypothalamic tract (RHT)) and *via* an indirect projection from the retina through the intergeniculate leaflet (IGL) (geniculohypothalamic tract (GHT)) (Inouye & Shibata, 1994).

Both the SCN and the IGL are innervated by 5-hydroxytryptamine (serotonin, 5-HT) neurons in the midbrain raphe nuclei in rats (Cagampang *et al.*, 1993; Cagampang & Inouye, 1994), and in hamsters (Meyer-Bernstein & Morin, 1996). Systemic or local injections of agonists for 5-HT receptor subtypes suppress the light-induced phase shifts of hamster activity rhythms (Rea *et al.*, 1994; Pickard *et al.*, 1996; Mintz *et al.*, 1997), light-induced c-*fos* expression in the hamster SCN (Selim *et al.*, 1993; Glass *et al.*, 1994, 1995; Pickard *et al.*, 1996), the extracellular glutamate concentration (Selim *et al.*, 1993; Srkalovi *et al.*, 1994) and the firing rates of light responsible cells in the hamster SCN (Ying & Rusak, 1994). 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. Furthermore, Morin & Blanchard (1991) reported that depletion of hamster brain serotonin increased the circadian activity rhythm response to light. This evidence suggests that 5-HT neurons from midbrain raphe nuclei regulate the photic entrainment of the biological clock in mammals in an inhibitory manner.

However, it remains to be clarified how various 5-HT receptor subtypes are involved in circadian regulation. Recently, Pickard *et al.* (1996, 1997) reported that selective 5-HT_{1B} receptor agonists inhibit the light-induced phase shift of hamster wheel-running activity rhythm and light-induced c-*fos* expression in the SCN. Bilateral enucleation reduces the density of 5-HT_{1B} receptors in the SCN. Based on these observations, they suggested that activation of 5-HT_{1B} receptors, which are localized presynaptically on retinal terminals in the SCN, suppress the photic entrainment of the biological clock.

On the other hand, the roles of 5-HT_{1A} or 5-HT_7 receptor are still vague because of a lack of subtype-specific ligands. For example, 8-hydroxy-dipropylaminotetralin (8-OH-DPAT),

[3] Author for correspondence.

which is an agonist for both 5-HT_{1A} and 5-HT_7 receptors suppressed photic responses of the circadian clock, and these actions are antagonized by both 5-HT_{1A} and 5-HT_7 receptors antagonists (Weber *et al.*, 1996). In contrast, Ying & Rusak (1997) reported that the suppression of the firing rates of light-responsive SCN neurons induced by 8-OH-DPAT were mediated *via* activation of 5-HT_7 receptors but not 5-HT_{1A} receptors. Furthermore, 5-HT_{1A} receptor mRNA is sparsely distributed in the rat SCN (Roca *et al.*, 1993).

In order to establish the role of 5-HT_{1A} receptors in regulating the photic entrainment of the biological clock, we examined the effects of 5-{3-[((2S)-1,4-benzodioxan-2-ylmethyl)amino]propoxy}-1,3,-benzodioxole HCl (MKC-242), a selective 5-HT_{1A} receptor agonist (Matsuda *et al.*, 1995a,b; Suzuki *et al.*, 1995; Abe *et al.*, 1996; Asano *et al.*, 1997) on the photic entrainment of the hamster wheel-running rhythm. The K_i values of MKC-242 for 5-HT_{1A} and 5-HT_7 receptors are 0.35 nM (Matsuda *et al.*, 1995a) and >100 nM (personal communication from Mitsubishi Chemical Co.), respectively. We also investigated the effects of MKC-242 on light-induced c-*fos* expression in the SCN and the IGL, and on 5-HT turnover in the SCN to confirm neuronal and cellular mechanisms of serotonergic regulation of photic entrainment.

Methods

Animals

Male Syrian hamsters (*Mesocricetus auratus*) weighing 120–200 g were maintained under controlled environmental conditions (23±2°C room temperature; 12-12 h light-dark cycle, lights on at 08:30 h) for at least 2 weeks before being used for the experiments. The light intensity was almost 200 lux at the level of the animal cage. Food and water were provided *ad libitum*. 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 animal colony light-dark cycle. ZT0 was designated as light-on and ZT12 as light-off. In free-running conditions under constant darkness, circadian time (CT) was defined instead of ZT, and CT12 referred to the onset of wheel-running.

Recording of wheel-running rhythm

Hamsters were housed individually in transparent plastic cages (35×20×20 cm), each equipped with a running wheel of 15 cm diameter, which closes a microswitch on each revolution. Wheel-running activity was continuously recorded in 6 min epochs by a PC-9801 computer.

Fos immunohistochemistry

After 2 days of constant darkness, hamsters were injected intraperitoneally with drugs 30 min prior to a light pulse (0, 5 or 60 lux for 15 min) at projected ZT 20 and returned to darkness. Sixty minutes after the light pulse onset, the animals were deeply anaesthetized with Nembutal and perfused intracardially with 100 ml of saline (37°C), followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.2; 4°C). Brains were removed from the skull and fixed with 50 ml of 4% paraformaldehyde in 0.1 M PBS and transferred to 20 and 30% sucrose solutions in 0.1 M PBS for 24 and 48 h, respectively. Brains were cut into 40 μm slices from rostral to caudal SCN or IGL with a freezing microtome. Alternate sections were incubated for 48 h with anti-Fos antibody (OA-11-824, Cambridge Research Biochemical. U.S.A.) diluted to 1:1000 with 0.1 M PBS containing 1% normal rabbit serum and 0.3% Triton X-100 (PBSRT). All sections were then washed three times with 0.1 M PBS (10 min each) and incubated for 2 h with biotinylated anti-sheep rabbit antibody (diluted to 1:200 with PBSRT; Vectastain). The sections were washed three times with 0.1 M PBS and incubated for 2 h in an avidin-biotin complex solution (Vectastain ABC kit). After three washes with 0.1 M PBS, sections were visualized with diaminobenzidine as a chromogen and mounted on gelatin-coated glass slides. All procedures were performed at room temperature. The number of cells which expressed Fos immunoreactivity was counted by an unnotified observer. Average cell numbers in the bilateral SCN or the IGL per one slice were calculated.

Measurement of 5-HT and 5-HIAA content

Measurements of 5-HT and 5-HIAA contents in the SCN were done by HPLC as previously reported (Ono *et al.*, 1996). Hamsters were anaesthetized with ether and killed by decapitation. The brain was rapidly removed from the skull and the SCN was dissected free. Monoamines in the SCN was extracted with 200 μl of 0.5 M $HClO_4$ by sonication on ice. After centrifugation at 15,000 r.p.m. for 10 min at 4°C, supernatants were collected for measurement of monoamine contents. Eicompak MA-50DS (4.6×150 mm) (Eicom, Kyoto) and an electrochemical detector (ECD-300, Eicom) were used for 5-HT and 5-HIAA assays. The mobile phases were as follows: 50 mM sodium-acetate-citrate buffer (pH 3.9) containing 80 mg l^{-1} sodium 1-octanesulphonate, 5 mg l^{-1} EDTA and 10 (v/v)% methanol. The data were analysed with a Powerchrom 2.0.6 system.

Drugs and reagents

MKC-242 and WAY100635 (N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride) were a kind gift from Mitsubishi Chemical Co. (Yokohama, Japan). (±)8-OH-DPAT and ritanserin were purchased from Research Biochemicals (Natick, MA, U.S.A.). All other chemicals were of the highest grade.

All drugs were freshly prepared. MKC-242 was suspended in 0.5% carboxymethylcellulose (CMC) and injected i.p. at the dose indicated (1–3 ml kg^{-1}). All other drugs were dissolved in saline and injected i.p. at the indicated doses (1 ml kg^{-1}).

Data and Statistical analysis

The data are presented as means±s.e.mean. Statistical analysis was conducted by one- or two-way ANOVA followed by Dunnett's test or Student's *t*-test. *P* values of 5% or less were considered as statistically significant.

Results

Effect of MKC-242 on re-entrainment of wheel-running activity rhythms to a new light-dark cycle

Figure 1 shows a representative actogram of the re-entrainment of wheel-running activity to an 8 h advance of light-dark cycle in hamsters. When hamsters were injected with vehicle, it took approximately 2 weeks for complete re-entrainment to a new light-dark cycle. On the other hand, it took only 2–3 days for re-entrainment, when animals were

injected with MKC-242 (3 mg kg^{-1}) at ZT20 on the day of light exposure. Advance and delay experiments were carried out using different animals. MKC-242 (3 mg kg^{-1}) significantly accelerated the re-entrainment of wheel-running activity to both an 8-h-delayed (-6.3 ± 0.3 h for MKC-242 and -4.8 ± 0.3 h for vehicle on 4th day, $P<0.05$, Student's t-test) or -advanced (6.7 ± 0.79 h for MKC-242 and 4.3 ± 0.47 h for vehicle on the 4th day, $P<0.05$, Student's t-test) light-dark cycle.

Effect of MKC-242 on light pulse-induced phase shifts of wheel-running activity in constant darkness

To confirm that MKC-242 accelerated the re-entrainment to new light-dark cycle by potentiating the effects of environmental light, we next examined the effect of MKC-242 on the light pulse-induced phase shift of wheel-running activity in hamsters maintained in constant darkness. As shown in Figure 2a and Table 1, exposure to a light pulse for 15 min (light intensity: 60 lux) at circadian time 20 (CT; CT 12: onset time of wheel-running) caused a phase advance (average values: 1.98 ± 0.13 h) of wheel-running activity in hamsters. One-way ANOVA revealed the significant potentiation of MKC-242 in light-induced phase advance ($F_{4,35}=6.4$, $P<0.01$). Injection of MKC-242 (3 mg kg^{-1}), but not 0.1 or 1.0 mg kg^{-1} 30 min prior to light exposure dramatically potentiated the phase advance of wheel-running activity induced by a light pulse at CT 20 (60 lux) (Dunnett's test, $P<0.05$) (Figure 2b and Table 1). Two to three days are required to get stable phase shifts, when light phase advances the activity rhythm of mammals (Figure 2a). MKC-242 did not affect the time required to complete a phase shift induced by a light pulse at CT20 (data not shown). Whereas, MKC-242 application without light exposure did not affect the phase of wheel-running activity rhythm (Figure 2c and Table 1).

The potentiating action of MKC-242 was dependent on the intensity of the light exposure (Table 2). In the case of vehicle, the phase advance of wheel-running activity increased with an increase of light intensity between 5 and 60 lux. A high

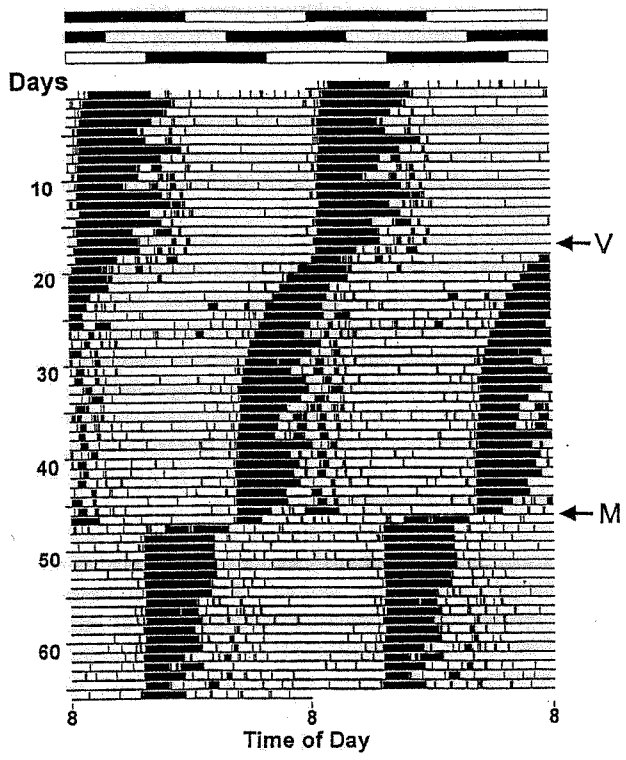

Figure 1 Representative double plot-actogram demonstrating the potentiating effect of MKC-242 on re-entrainment of the wheel-running activity to an 8 h advanced light-dark cycle. Time of day is indicated horizontally and consecutive days vertically. Upper, middle and lower bars on the top of actogram show the light (open bar)-dark (solid bar) cycle during day 1–18, day 19–47 and day 48–66, respectively. Hamsters were maintained in a 12:12 h light-dark cycle at least for 15 days, the light-dark cycle was advanced 8 h (indicated by arrows), then MKC-242 (M) (3 mg kg^{-1}, i.p.) or vehicle (V) were administered at ZT20 of former light-dark cycle for 2 continuous days.

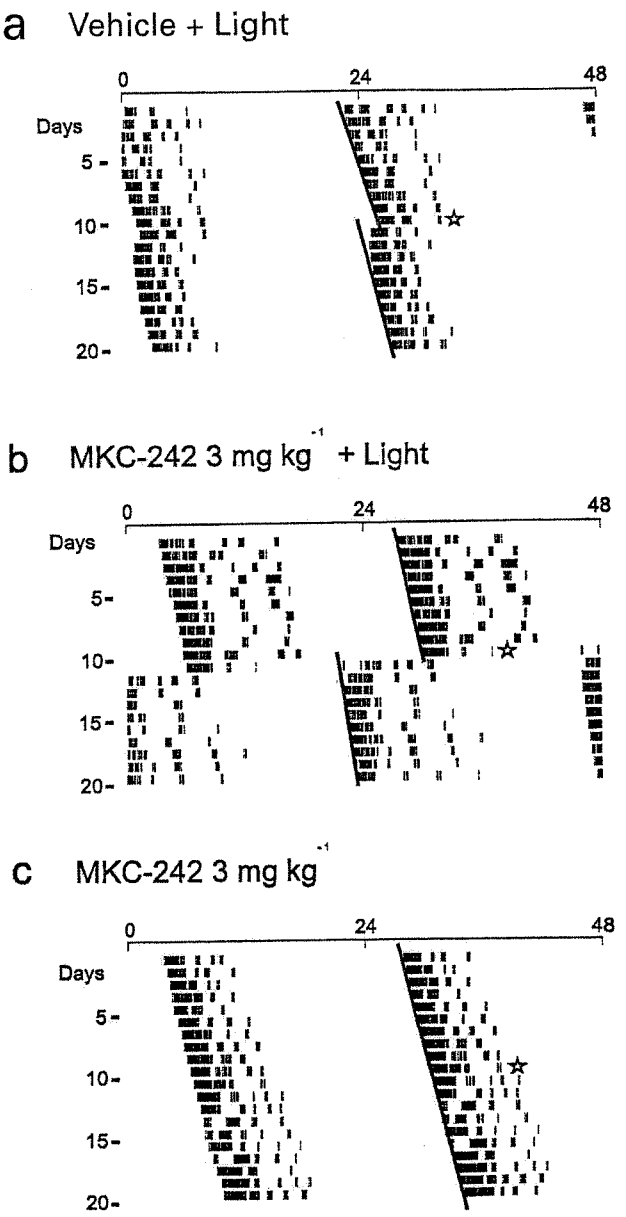

Figure 2 Representative double plot-actograms demonstrating the potentiating effects of MKC-242 on the light pulse-induced phase advance of wheel-running activity of hamsters. The time of day is indicated horizontally and consecutive days vertically. Hamsters were maintained in constant darkness until a stable free-running rhythm was observed for at least 10 days. Hamsters then received an intraperitoneal injection of vehicle (a) or MKC-242 (3 mg kg^{-1}) (b,c) 30 min prior to light exposure (60 lux, 15 min) at CT20 (a,b) or were handled the same without receiving a light pulse (c). Eye-fitted lines to activity onset were also shown in each actogram, and the differences between these two lines were designated as phase changes (h). Approximate treatment times are indicated by stars.

Table 1 Dose-dependent effects of MKC-242 on the light pulse-induced phase advances of wheel-running activity of hamsters

MKC-242 (mg kg^{-1})	*Light pulse (CT20 for 15 min)*	*Phase advance induced by light pulse (h)*
0	+	1.98±0.13 (7)
0.1	+	2.78±0.51 (6)
1	+	3.17±0.63 (8)
3	+	4.25±0.65 (13)*
3	−	0.23±0.21 (6)

Detail of the methods were described in the legend for Figure 2. Data are expressed as means±s.e.mean. The number of animals are shown in parentheses. *, Significant difference ($P<0.05$) compared to vehicle (one-way ANOVA followed by Dunnett's test).

Table 2 Light pulse intensity-dependence for the potentiating actions of MKC-242 on light pulse-induced phase advances of wheel-running activity of hamsters

Light intensity (lux)	*Phase advance induced by light pulse (CT20 for 15 min) (h)* *Vehicle*	*MKC-242* (3 mg kg^{-1})
5	0.83±0.34 (7)	3.12±0.84 (10)*
60	1.98±0.13 (7)	4.25±0.65 (13)*
200	2.30±0.10 (5)	Not tested

Hamsters in constant darkness received either vehicle or MKC-242 (3 mg kg^{-1}) 30 min before a light pulse (5, 60, 200 lux for 15 min at CT20). High intensity (200 lux) light-induced phase shifts were tested for the vehicle group only. Data are expressed as means±s.e.mean. The number of animals are shown in parentheses. *, Significant difference ($P<0.05$) compared to vehicle (unpaired Student's *t*-test).

Table 3 Effect of injection timing on the potentiation action of MKC-242 on light pulse-induced phase advances of wheel-running activity

Time of injection after light pulse onset (min)	*Phase advamce induced by light pulse (CT20 for 15 min) (h)* *Vehicle*	*MKC-242* (3 mg kg^{-1})
−30	1.98±0.13 (7)	4.25±0.65 (13)*
20	2.78±0.51 (5)	5.65±0.85 (6)*
60	2.72±0.33 (6)	5.62±0.096 (6)*

Hamsters maintained in constant darkness received either vehicle or MKC-242 (3 mg kg^{-1}) 30 min before or 20, 60 min after light exposure (60 lux for 15 min at CT20). Data are expressed as means±s.e.mean. The number of animals are shown in parentheses. *, Significant difference ($P<0.05$) compared to vehicle (unpaired Student's *t*-test).

Table 4 Effect of 8-OH-DPAT on light pulse-induced phase advance of wheel-running activity of hamsters maintained in constant darkness

Light intensity (lux)	*Phase advance induced by light pulse (CT20 for 15 min) (h)* *Vehicle*	*8-OH-DPAT* (5 mg kg^{-1})
5	1.13±0.30 (10)	0.25±0.10 (6)*
20	2.28±0.40 (6)	1.52±0.58 (7)
60	2.61±0.44 (6)	2.25±0.50 (6)

Data are expressed as means±s.e.mean. The number of animals are shown in parentheses. *, Significant difference ($P<0.05$) compared to vehicle (unpaired Student's *t*-test).

intensity light pulse (200 lux) caused a ceiling effect on the phase advance (2 h), because more high intensity of light ($10^{11}-10^{15}$ photons cm^{-1} s^{-1}) has reported to cause almost same degree of advance (2 h) (Boulos, 1995). MKC-242 (3 mg kg^{-1}) potentiated not only low (5 lux), but also high (60 lux) intensity light-induced phase advances. MKC-242 caused a large phase advance beyond the ceiling effect, when a 60 lux light pulse was applied. In the next experiment, we observed the effect of 8-OH DPAT on light-induced phase advance. Two-way ANOVA revealed no significant differences between Drug × Intensity of light ($F_{2,35}=0.23$, $P>0.05$), but there are significant increase of phase advance with intensity-dependent manner ($F_{1,35}=10.5$, one-way ANOVA, $P<0.01$). As shown in Table 4, 8-OH-DPAT suppressed low intensity (5 lux) light-induced phase advances, but did not affect high intensity (20 and 60 lux) light-induced phase advances.

In the next experiments, we examined the importance of the timing of MKC-242 injection on the potentiating action on a light pulse-induced phase advance (Table 3). There are significant differences in drug effect ($F_{1,37}=20.6$, $P<0.01$), but in injection timing ($F_{2,37}=1.7$, $P>0.05$). Injection of MKC-242 (3 mg kg^{-1}) 30 min prior to light pulse onset significantly potentiated the phase advance of wheel-running activity, as shown in Figure 3. Furthermore, an injection of MKC-242 (3 mg kg^{-1}) 20 min or 60 min after the light onset, when the light has been turned off, resulted in a strong potentiating action on the light pulse-induced phase advance of wheel-running activity.

As shown in Figure 3, pre-injection of WAY100635 (3–10 mg kg^{-1}), a selective 5-HT_{1A} receptor blocker, antagonized the potentiating action of MKC-242 in a dose-dependent manner. However, WAY100635 (10 mg kg^{-1}) itself did not affect the light pulse-induced phase advance of wheel-running activity. On the other hand, ritanserin (10 mg kg^{-1}), a 5-HT_2/5-HT_7 blocker failed to affect the potentiating action of MKC-242 on light pulse (60 lux for 15 min at CT20)-induced phase advances (vehicle + vehicle; 2.40±0.08 h ($n=5$), vehicle + MKC-242 (3 mg kg^{-1});

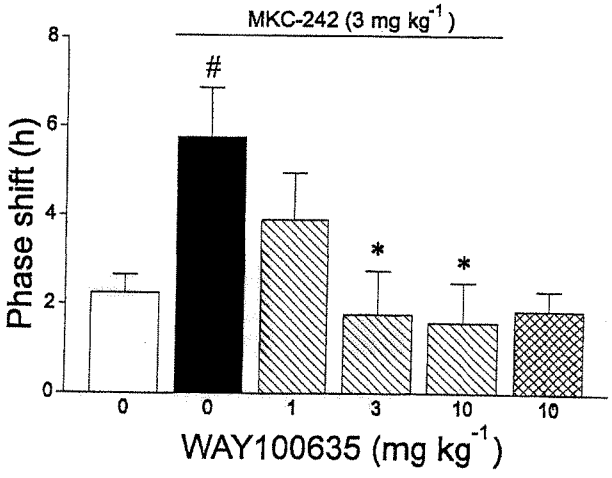

Figure 3 Effect of WAY100635, a selective 5-HT_{1A} receptor blocker, on the potentiating action of MKC-242 on the light pulse-induced phase advance of wheel-running activity. Hamsters received either vehicle or WAY100635 45 min prior to the light pulse, followed by vehicle or MKC-242 (3 mg kg^{-1}) 30 min prior to the light pulse (60 lux for 15 min at CT20). Values are given as means±s.e.mean ($n=6-12$). Cross-hatched column exhibits the injection of WAY100635 without MKC-242. *$P<0.05$, compared with MKC-242 (3 mg kg^{-1}) (closed column) (one-way ANOVA followed by Dunnett's test). #:$P<0.05$, compared with vehicle (open column) (one-way ANOVA followed by Dunnett's test).

5.21±0.66 h (n=13), ritanserin (10 mg kg^{-1})+MKC-242 (3 mg kg^{-1}); 4.81±0.61 h (n=9), ritanserin (10 mg kg^{-1})+vehicle; 2.73±0.58 h (n=5)).

Effect of MKC-242 on light pulse-induced c-fos expression in the SCN and the IGL

Figure 4 shows the effect of MKC-242 on light pulse-induced c-*fos* expression in the SCN and the IGL. Exposure to a light pulse at CT20 for 15 min significantly increased c-*fos* immunoreactive cells both in the SCN ($F_{2,16}$=15.7, P<0.01) and the IGL ($F_{2,7}$=13.1, P<0.01) (Figure 4a,b) in an intensity-dependent manner. However, pre-injection of MKC-242 (3 mg kg^{-1}) did not alter the light pulse (5 lux and 60 lux)-induced c-*fos* expression in the SCN nor in the IGL.

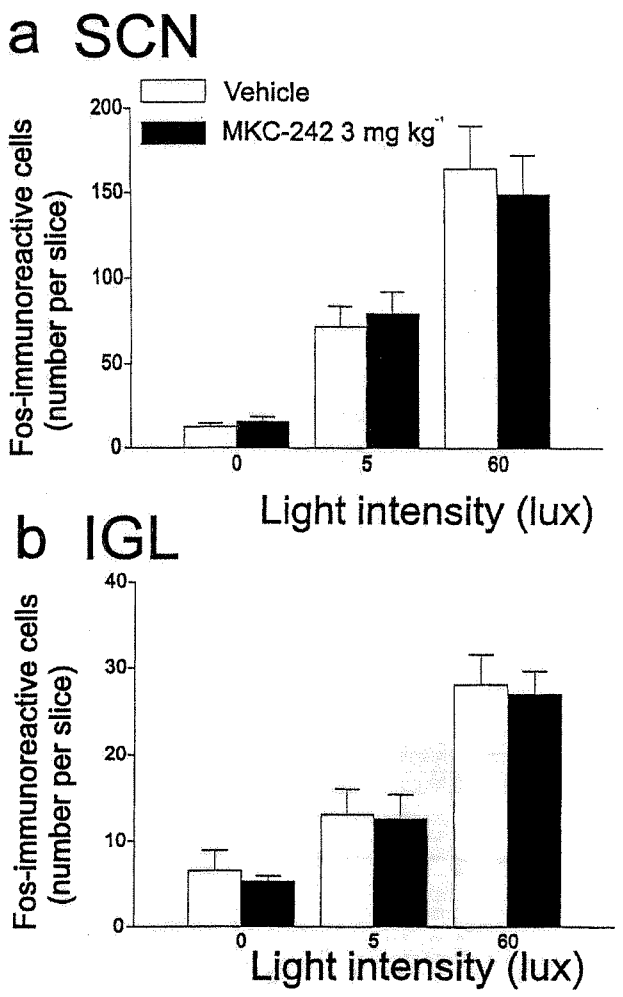

Figure 4 Effect of MKC-242 on light pulse-induced c-*fos* expression in the SCN (a) or the IGL (b) in hamsters. Hamsters maintained on a light-dark cycle were transferred to constant darkness. After 2 days in constant darkness, hamsters were received either vehicle or MKC-242 (3 mg kg^{-1}) 30 min prior to the light pulse (60 lux for 15 min at CT20). Sixty minutes after light pulse onset, the animals were anaesthetized with an overdose of pentobarbital (80 mg kg^{-1}) and perfused transcardially with saline followed by 4% paraformaldehyde. Coronal sections (40 μm) through the SCN or the IGL were processed for immunohistochemistry. Values are given as means± s.e.mean (n=5–9 for the SCN; n=3–4 for the IGL). No significant differences were observed when compared to vehicle group (Student's t-test).

Table 5 Effect of MKC-242 on the 5-HT and 5-HIAA concentrations in the SCN of hamsters

	Vehicle	*MKC-242* (3 mg kg^{-1})
5-HT (ng mg protein^{-1})	60.46±7.61 (7)	59.83±12.17 (8)
5-HIAA (ng mg protein^{-1})	28.50±3.35 (7)	18.89±2.62 (8)*
5-HIAA/5-HT ratio	0.52±0.09 (7)	0.38±0.07 (8)

Hamsters maintained under a light-dark cycle were transferred to a light room at ZT16 (8 h light–dark cycle shift) and received vehicle or MKC-242 (3 mg kg^{-1}) at ZT20 (4 h after transfer to light room). Sixty minutes after injection, brains were prepared and monoamine contents were measured by h.p.l.c. as described in Methods. Data are expressed as means±s.e.mean. The number of animals are shown in parentheses. *, Significant difference (P<0.05) compared to vehicle (unpaired Student's t-test).

Effect of MKC-242 on 5-HT and 5-HIAA concentrations in the hamster SCN

Table 5 shows the effect of MKC-242 on the 5-HT and its metabolite, 5-HIAA concentrations in the hamster SCN. MKC-242 (3 mg kg^{-1}) decreased 5-HIAA content to 73% of control and did not affect the 5-HT content in the SCN.

Discussion

In the present experiments, we demonstrated that MKC-242, a selective 5-HT$_{1A}$ receptor agonist, potentiated the photic entrainment of wheel-running activity in hamsters in a c-*fos* expression-independent manner. Furthermore, MKC-242 decreased the turnover of 5-HT in the SCN.

In contrast to MKC-242, 8-OH-DPAT, a well known 5-HT$_{1A}$ receptor agonist, suppressed the photic entrainment. The differences in actions of MKC-242 and 8-OH-DPAT, however may reflect the specificity of these chemicals for 5-HT receptor subtypes. MKC-242 has been reported to have high affinity for 5-HT$_{1A}$ receptors (K_i=0.35 nM, Matsuda *et al.*, 1995a) and a relative low affinity for 5-HT$_7$ receptor (K_i>100 nM; personal communication from Mitsubishi Chemical Co.). In addition, the potentiating action of MKC-242 on the photic entrainment was not reversed by co-administration of ritanserin, a 5-HT$_2$/5-HT$_7$ receptor blocker. On the other hand, 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 & Rusak (1997) reported that 8-OH-DPAT suppressed firing rates of light-responsible SCN neurons *via* activation of 5-HT$_7$ receptors in the SCN. Furthermore, the phase advancing action of 8-OH-DPAT on firing rhythms in the 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. It may, therefore, be possible that 8-OH-DPAT suppresses photic entrainment *via* 5-HT$_7$ receptor activation.

The site of action or the intra/intercellular mechanism of the potentiating actions of MKC-242 on photic entrainment are still unclear. Although c-*fos* expression in the SCN by light is known to be a biochemical marker of photic entrainment, in the present results, MKC-242 did not affect c-*fos* expression in the SCN. Weber *et al.* (1995) reported that a nitric oxide synthase (NOS) inhibitor blocks light-induced phase shifts of wheel-running activity, but not c-*fos* expression in the hamster SCN. Therefore, the potentiated photic entrainment by MKC-

242 may be the result of an augmented c-*fos*-independent light signal pathway, such as NOS activation. In addition to this possibility, an alternate explanation is that MKC-242 acts downstream of the light-induced c-*fos* expression in the SCN.

We can rule out the following possibility that MKC-242 increases the sensitivity for light in the photo-recipient organ, i.e. retina, because MKC-242 is still able to potentiate photic entrainment, even when it was administered after turning off the light pulse.

5-HT_{1A} receptors were reported to be present in the SCN, IGL and raphe nuclei, all of which are involved in regulating photic entrainment of the biological clocks (Wright *et al.*, 1995). The role of SCN 5-HT_{1A} receptors in photic entrainment is not established at present, although some reports have suggested a suppressing action of 5-HT_{1A} receptor activation on photic entrainment (Rea *et al.*, 1994; Moriya *et al.*, 1996). MKC-242, however, did not suppress but potentiated the photic entrainment by low and high intensity light pulse, suggesting that MKC-242 failed to act on 5-HT_{1A} receptors in the SCN.

The GHT pathway has been reported to inhibitory regulate the photic entrainment of the biological clock *via* the release of neuropeptide Y (NPY) and gamma-aminobutyric acid (GABA) in the SCN (Ying *et al.*, 1993; Biello & Mrosovsky, 1995). The IGL, a relay area of GHT, was reported to be innervated by abundant 5-HT neurons from the raphe nuclei and a 5-HT_{1A} receptor agonist potently suppressed both the spontaneous and light-induced activity of IGL neurons (Ying *et al.*, 1993). Therefore, MKC-242 may act at 5-HT_{1A} receptors in the IGL and block the light signal communicated *via* the GHT. Suppression of GHT activity could potentiate photic entrainment of the biological clock by decreasing the NPY and GABA releases to the SCN. MKC-242, however, failed to affect light-induced c-*fos* expression in the IGL, therefore we believe that MKC-242 is acting in brain region other than the IGL. Alternative explanation is that MKC-242 acts on the IGL-included pathways not involving c-*fos* expression.

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 present experiments, we showed that MKC-242 suppressed 5-HT turnover in the SCN. Systemic injection of 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 has tonic suppressing actions on photic entrainment of the biological clock. Therefore, we propose that MKC-242 activate 5-HT_{1A} autoreceptors in the midbrain raphe and suppress the activity of 5-HT neurons projecting to the SCN, and that the reduction of 5-HT activity in the SCN leads to potentiation of the photic entrainment. To confirm this hypothesis, further experiments, i.e. microinjection study, will be required.

MKC-242 has been reported to possess anti-depressant effects (Matsuda *et al.*, 1995a). Therefore, the circadian rhythm disorders observed in depressed subjects may be improved by treatment with MKC-242.

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