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

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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 phosphorothioate 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 respond 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 junB, 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 (Shigeoyoshi et al., 1997). The induction of mPer1 (<20 min) by light is more rapid than the accumulation of c-fos protein (Shigeoyoshi 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 phosphorothioate 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.
PHOTOPHOSPHOTIOTIOATE 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 ODNs (18-mer) were 5'-TAT GGG ACC ACT CAT GTC T-3' and 5'-A GAC AGT 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 GTC AGC TGA C-3' and 5'-CAT CCT GCC 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 h 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 stereotactic 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 nl/μl/min 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, when 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 other two ODNs, sense and random ODNs, lacked specific sequences of mPer1. The antisense ODN on the other hand, was found to be specific to mPer1 antisense ODN, and we examined the effects of intracerebroventricular administration of four different ODNs and anisomycin on behavioral rhythms (Fig. 1A,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. 1C). 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 (Shigyoshi et al., 1997). Therefore, we examined the effect of mPer1 antisense ODN on light-induced phase shifts (Fig. 2A,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 h. 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 with 20% sucrose for 24 hr. The distributions of ODN in the serial cryostat sections (30 μm) containing SCN were determined using a Vectastain 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 cut into the buffer containing glutamate (10 mM). Total RNA from the SCN (n = 4) was extracted in each product 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 TTG TTT-3' and 5'-CGA AGT TTC ACC AGC GTG CTG G-3' (mPer1); 5'-ACA CCA CCC CTT ACA AGC TTC TCT-3' and 5'-CGC TGG ATG ATG TCT GGC TTC-3' (mPer2); and 5'-CTC AAC TAGT GTC TAC A-3' and 5'-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. 1B). Significant phase delays were observed when mPer1 antisense ODN was administered at CT1. There were no significant differences between mPer1 antisense ODN administered 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. 1A,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. 1C). 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.
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. 2B). 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. 2B). 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
these experiments, the mean peak of electrical activity on the subsequent day occurred at ZT6–7 (ZT6.0 ± 0.5; n = 4) (Fig. 3A,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. 4A). 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. 4B,C). Administration of 6 nmol of mPer1 antisense significantly reduced the expression of...
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
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, junB, 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 (Kashiwara 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.
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 affected by ODN injection. We also demonstrated that ODNs injected intracerebroventicularly 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 molecular bases of these genes in detail.

REFERENCES


