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

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ABSTRACT

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

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

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

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

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

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

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

Experimental Procedures

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

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

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

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

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

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

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

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

TABLE 1
Period (h) of locomotor activity rhythm in wild-type mice and GRP receptor-deficient mice under LD followed by DD conditions

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

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

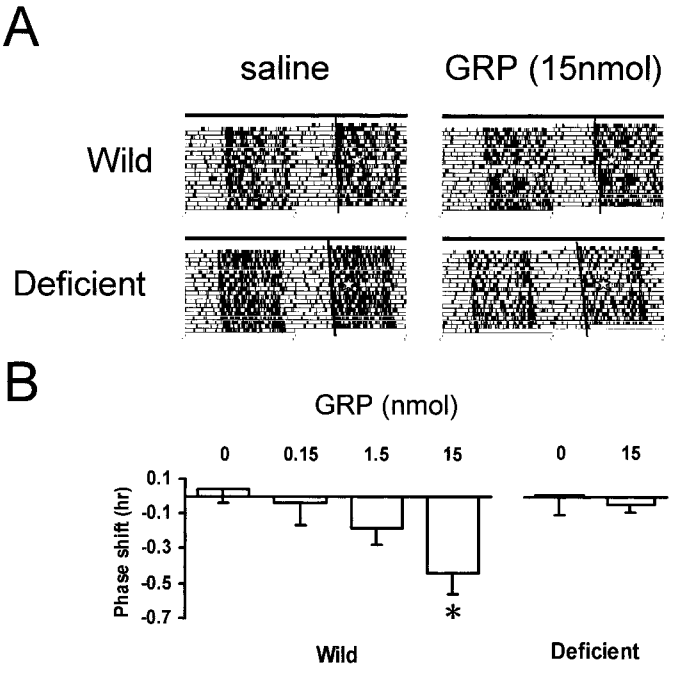

Fig. 1. Phase delays of behavioral activity rhythm by i.c.v. injection of GRP in wild-type (Wild) and GRP receptor-deficient mice (Deficient) under DD conditions. A, representative double-plotted actograms demonstrating that an i.c.v. injection of GRP (15 nmol) phase-delayed the locomotor activity rhythm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

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

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

Results

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

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

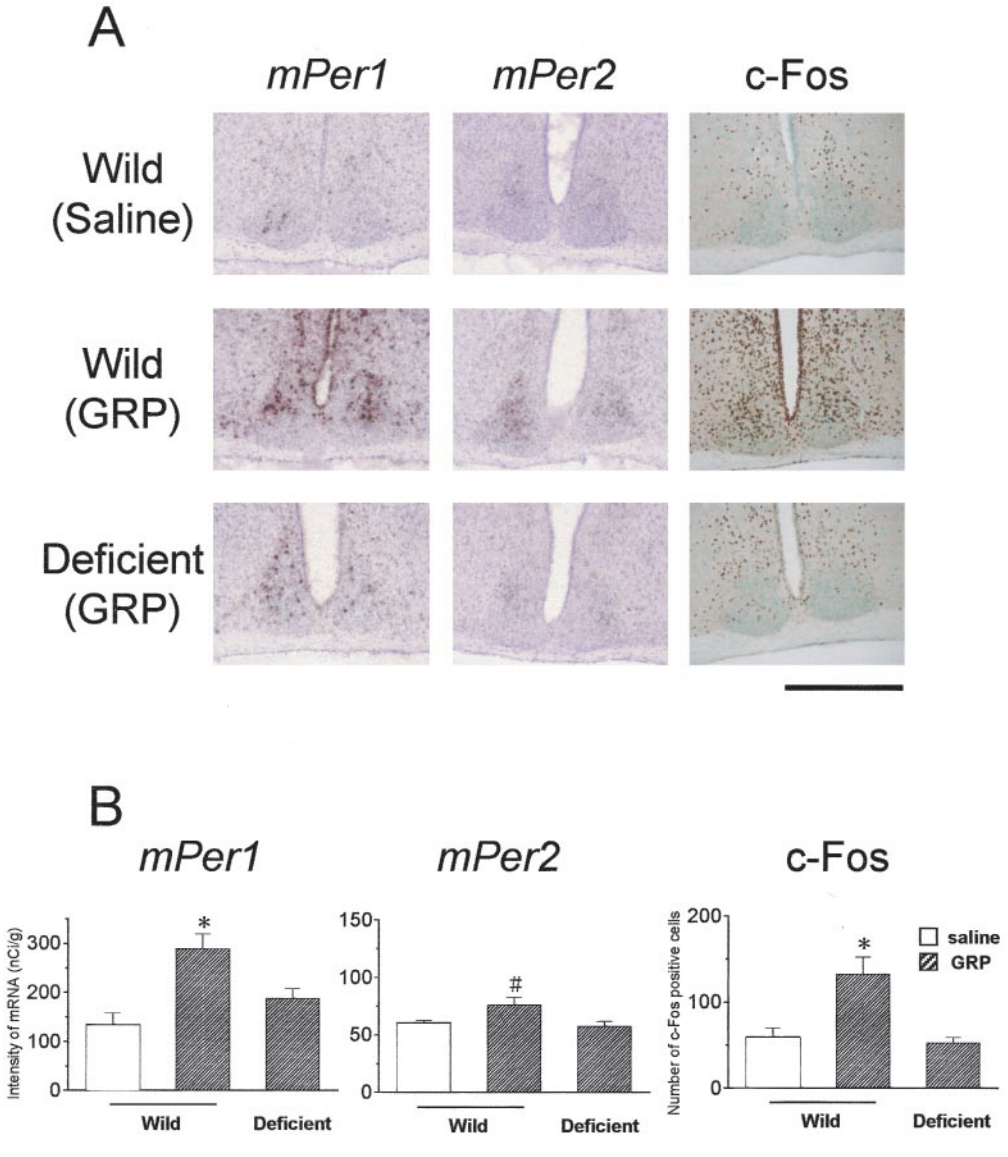

Fig. 2. Induction of *mPer1* and *mPer2* mRNA and c-Fos protein in the SCN by an i.c.v. injection of GRP in wild-type (Wild) and GRP receptor-deficient mice (Deficient). A, representative emulsion autoradiograms or photographs showing that an i.c.v. injection of GRP elicited the induction of *mPer1*,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

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

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

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

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

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

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

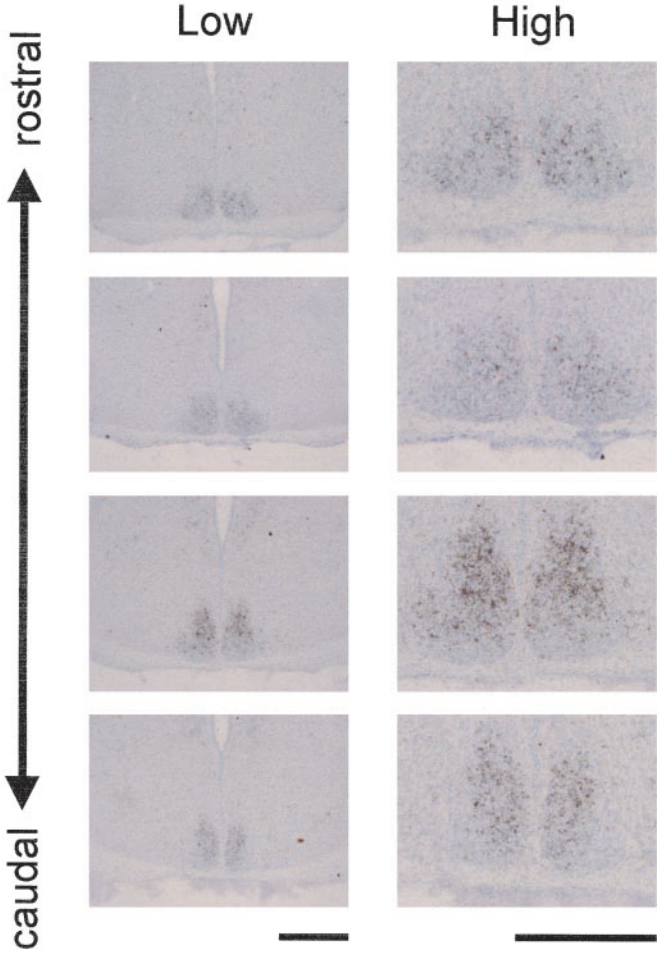

Fig. 3. Topographical analysis for GRP receptor expression in the SCN. Low-power (left) and high-power (right) emulsion autoradiograms showing the rostro-caudal distribution of GRP receptor mRNA in the SCN. C57BL/6J mice were perfused intracardially with 4% PFA at ZT16. Note that the GRP receptor signal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

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

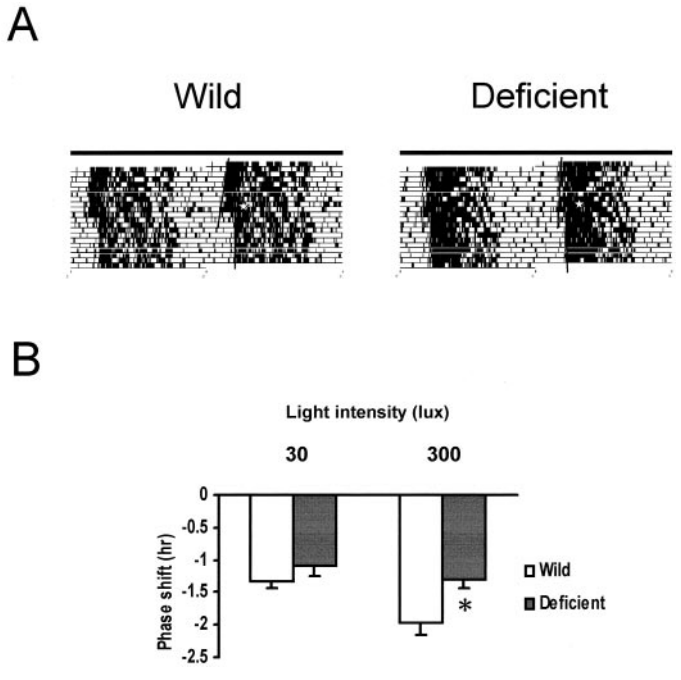

Fig. 4. Attenuated phase shift of locomotor activity rhythm in GRP receptor-deficient mice in response to brief light pulse under DD conditions. A, representative double-plotted actograms demonstrating a phase shift of locomotor activity rhythm in wild-type (Wild) and GRP receptor-deficient mice (Deficient)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

Discussion

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

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

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

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

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

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

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

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

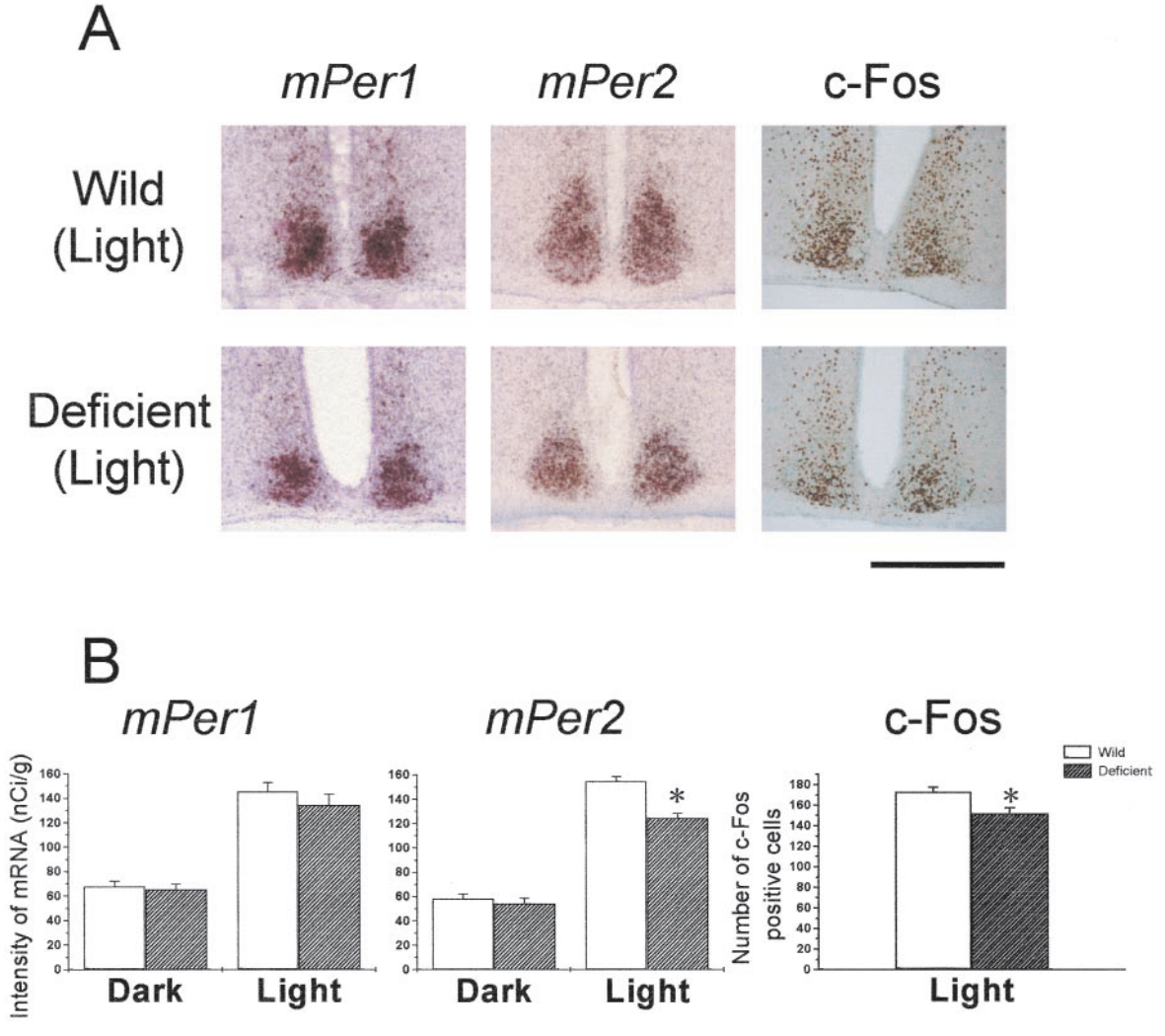

Fig. 5. Light pulse-induced *mPer1*, *mPer2* mRNA, and c-Fos protein expression were partially attenuated in GRP receptor-deficient mice. A, representative emulsion autoradiograms (*mPer1* and *mPer2*) and photographs (c-Fos protein) in wild-type (Wild) and GRP receptor-deficient mice (Deficient). After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

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

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

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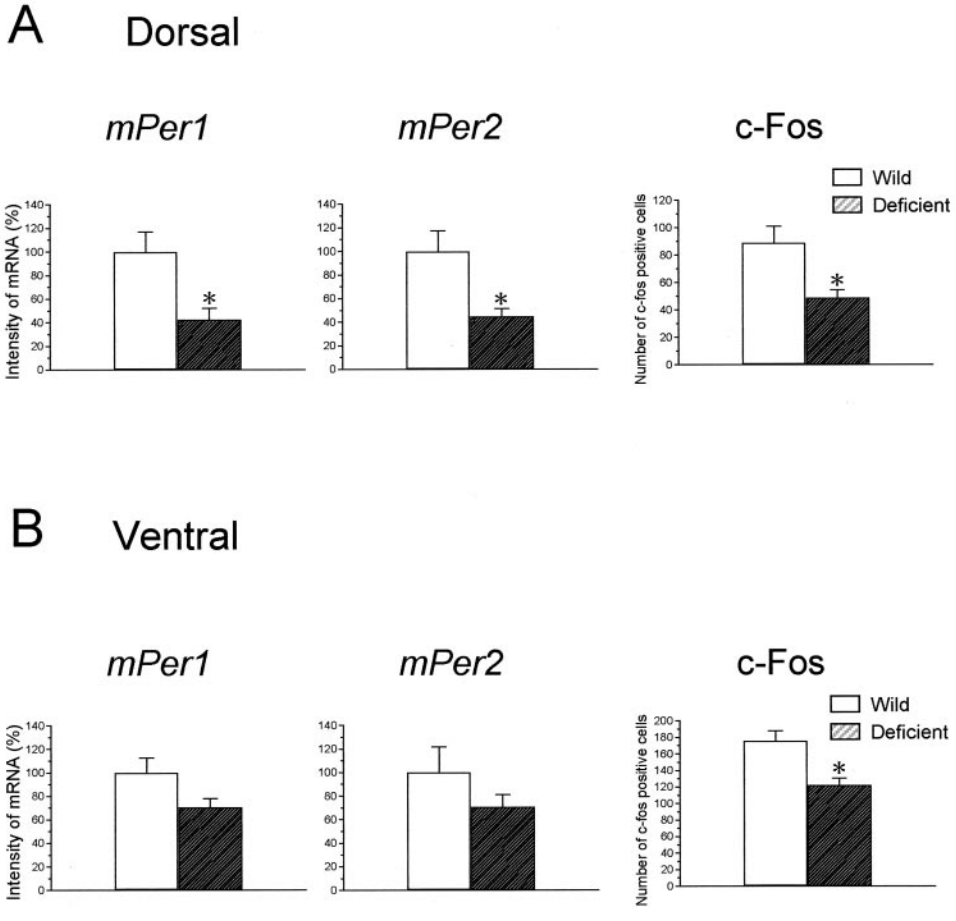

Fig. 6. Light pulse-induced *mPer1*, *mPer2* mRNA, and c-Fos protein expression were attenuated especially in the dorsal SCN of GRP receptor-deficient mice. Quantitative analysis of *mPer* mRNA and c-Fos protein in the dorsal (A) or ventral (B) portion of the caudal SCN. Emulsion autoradiograms (*mPer1*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

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