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Changes in light-induced phase shift of circadian rhythm in mice lacking PACAP

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is one of the neurotransmitters that transfers light signals from the retina to the hypothalamic suprachiasmatic nucleus (SCN) where the master clock of mammalian circadian rhythm locates, and is suggested to be implicated in the mechanism of light-induced phase shift of the circadian clock. Here, we examined changes in the phase shift of circadian rhythm in behavioral activity in mice lacking PACAP ($PACAP^{-/-}$). The phase advance in $PACAP^{-/-}$ mice by a light stimulation at late subjective night was significantly attenuated, but the phase delay due to the illumination at the early subjective night slightly diminished. In contrast, the induction of c-Fos in the SCN by the illumination at the early subjective night but not that at the late subjective night was significantly blunted in $PACAP^{-/-}$ mice. These data provide new aspects about the roles of PACAP in light-induced phase shift of the circadian clock.

Keywords: Pituitary adenylate cyclase-activating polypeptide; Suprachiasmatic nucleus; Circadian rhythm; Entrainment; c-Fos

Mammalian circadian rhythms of physiological phenomena and behaviors are driven by a master clock located within the suprachiasmatic nucleus (SCN) in the hypothalamus [1,2]. The retinohypothalamic tract (RHT), a direct axonal projection of a subset of retinal ganglion cells (RGCs) to the SCN, transfers the light signal to the SCN and synchronizes the circadian rhythm to exactly 24 h solar cycle [3,4]. Recent report revealed that both nonvisual photoreception by melanopsin (Opn4) and classical visual photoreceptor systems are important in the entrainment of the circadian clock [5].

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon family of peptides, and it is co-expressed with glutamate, an essential modulator of light entrainment, in a subset of RGCs included in the RHT [6]. Interestingly, the PACAP-containing RGCs are identical to the subset of cells expressing melanopsin [7].

In respect to PACAP receptors, there are two types of receptors reported to be expressed in the SCN. One is PACAP-specific (PAC_1) receptor and the other is $VPAC_2$ receptor which shares with that of VIP [8,9]. In vitro and in vivo studies show that PACAP has different effects on the photoentrainment of circadian rhythms in a dose-dependent manner. Namely, PACAP in nanomolar concentrations causes phase shifts and induces expression of *per* genes in the SCN which is similar to those after the light stimulation, but PACAP in micromolar concentrations enhances phase delay and suppresses phase advance [10–14]. Interestingly, PAC_1 receptor knockout ($PAC1^{-/-}$) mice showed abnormalities in both light-induced phase shift and light-induced expressions of *c-fos* and *per* genes, whereas these abnormalities were dissociated [15]. In respect to $VPAC_2$ receptor, one of the authors previously found evidence suggesting that VIP-receptive neurons in the SCN are responsible for the generation of circadian rhythm in rats [16]. Consistent with this, recently it was found that mice lacking $VPAC_2$ receptor ($Vipr2^{-/-}$) lost behavioral circadian rhythm

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under constant conditions and the photic induction of *per* gene was also abolished [17]. However, it is not easy to understand mechanisms of the generation and entrainment of circadian rhythm from these data, further analysis was necessary to clarify the role of PACAP in the photoentrainment of circadian clock.

In this study, we examined the behavioral circadian rhythm and photic phase shift of mice lacking PACAP gene (PACAP$^{-/-}$). PACAP$^{-/-}$ mice were reported to exhibit hyperactive psychomotor behaviors, high early-mortality rate, and abnormalities in glucose and lipid homeostasis [18–20]. We obtained evidence suggesting that PACAP signaling is involved in the mechanism of the light-induced phase shift of the circadian clock in a time-dependent manner and that induction of c-Fos is less important in the light-induced phase shift of the clock. We describe the details.

Materials and methods

Animals. PACAP$^{-/-}$ mice on the background of Institute of Cancer Research (ICR) strain were used [18,21]. Wild-type mice and mice homozygous for the mutant PACAP gene were obtained from the intercross of heterozygous animals and experiments were conducted with the adult male mice. Animals were maintained individually in cages under controlled illumination with food and water ad libitum. All animal cares and handling procedures were approved by the Institutional Animal Care and Use Committee of Osaka University.

Behavioral analysis. Animals were entrained to a 12 h light:12 h dark cycle for about 1–3 weeks. Subsequently, animals were transferred to a constant dark (DD) condition. The activity of individual mouse was monitored by the far-infrared monitoring apparatus (Bio-Medica, Japan). Free-running period (τ) was assessed from the behavioral data later than 4 days after starting DD using the χ^2 periodogram [22]. For experiments of light-induced phase shift, mice were exposed to a 30 min light pulse (20 lx) at three different circadian times (CTs) 6, 15, and 21 at which CT12 was designated as activity onset time. The phase shift was assessed by the method of Daan and Pittendrigh [23].

Immunohistochemistry. The induction of c-Fos protein in the SCN by the illumination was investigated in the second DD cycle. Mice were exposed to a 30 min light pulse or no pulse at CT15 or CT21. One hour after the start of illumination, mice were deeply anesthetized under a dim red light and perfused intracardially with ice-cold saline and then with 4% paraformaldehyde (PFA, Sigma, St. Louis, MO) in phosphate-buffered saline (PBS). Brains were removed, postfixed in 4% PFA in PBS at 4 °C overnight, and cryoprotected in 20% sucrose for two overnights. Then, brains were sliced using a cryomicrotome (CM1900, Leica, Germany) in 20-μm-thick sections. Immunohistochemical analyses of c-Fos, VIP, and arginine–vasopressin (AVP) in the SCN were conducted using specific polyclonal rabbit antibodies against c-Fos (in 1:1000 dilution; Santa Cruz, South San Francisco, CA), VIP (1:2000 dilution), and AVP (1:1000 dilution) as primary antibodies. Both anti-VIP and anti-AVP antibodies were kindly provided by Dr. R.M. Buijs (Netherlands Institute for Brain Research) [24]. The immunoreactivities were visualized with Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine (Sigma) as chromogen. To determine the area of the SCN, adjacent sections in experimental animals were stained by cresyl violet (Sigma) and the SCN was delineated. Images of the slices were examined under a microscope (BX51, Olympus, Japan) and the number of c-Fos immunoreactive nuclei in the SCN was counted using Photoshop imaging program (Adobe Software).

Results

Immunohistochemical analysis of neuronal structures in the SCN of PACAP$^{-/-}$ mice

The architecture of the SCN in PACAP$^{-/-}$ mice was examined by the immunohistochemistry before analyzing behavioral circadian rhythms. VIP-immunoreactive neurons were located exclusively in the ventrolateral region and in a small dorsomedial part of the SCN. Intense staining of VIP-immunoreactive fibers was observed in all the regions of the SCN up to a subparaventricular zone in PACAP$^{-/-}$ mice as well as in the wild-type mice (Fig. 1A, upper panels). AVP-immunoreactive neurons

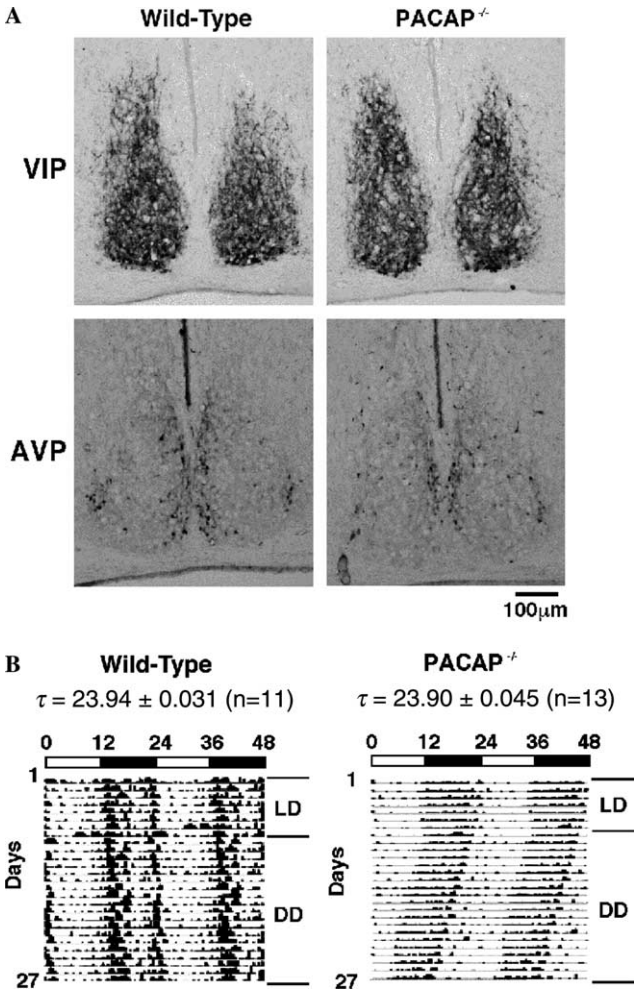

Fig. 1. Analysis of the histological architecture of the SCN and behavioral free-running period in PACAP$^{-/-}$ mice. (A) Immunostaining of VIP-IR (upper panels) and AVP-IR (lower panels) in the SCN of the wild-type (left panels) and PACAP$^{-/-}$ mice (right panels) are shown. Scale bar = 100 μm. (B) Representative activity records of individual wild-type (left) and PACAP$^{-/-}$ mouse (right) are presented in double-plotted format. Mice maintained in a 12 h:12 h LD cycle (indicate by the *top black–white bar*) were transferred to constant darkness (DD) conditions on the day indicated by the *black bar* to the *right* in each figure. Values above the actograms denote means ± SEM of free-running period in each genotype.

were detected mainly in the dorsomedial region in PACAP$^{-/-}$ mice similar to the wild-type (Fig. 1A, lower panels). These results indicated that the basic structure of the SCN was not impaired in PACAP$^{-/-}$ mice.

Behavioral circadian rhythmicity in PACAP$^{-/-}$ mice

Then behavioral circadian rhythmicity in wild-type and PACAP$^{-/-}$ mice was examined. The circadian

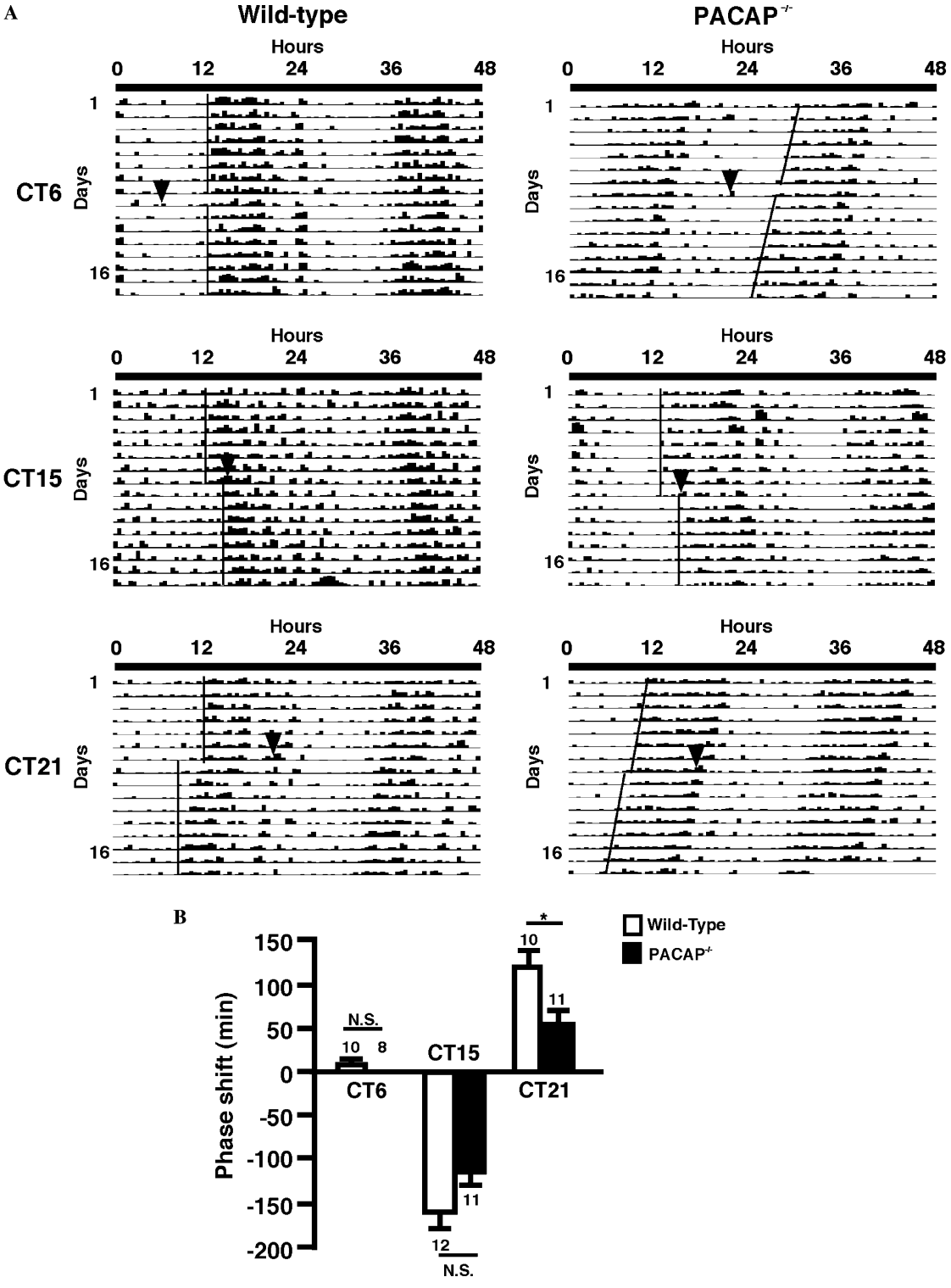

Fig. 2. Light-induced phase shift of the circadian rhythms in PACAP$^{-/-}$ mice compared with the wild-type. (A) Representative actograms of individual wild-type (left) and PACAP$^{-/-}$ mice (right) are shown. Arrowheads indicated light stimulations (30 min, 20 lx) at indicated circadian time. The lines show the onset of activity before and after the light pulses. (B) Quantification of light-induced phase shifts of circadian behavior. The values are expressed as means ± SEM. *$p < 0.05$ (Mann–Whitney U test). Numbers above or beyond the bars show those of animals used.

rhythm of PACAP$^{-/-}$ mice could be entrained to a 12 h:12 h LD cycle that was comparable to that of wild-type mice (Fig. 1B). PACAP$^{-/-}$ mice displayed similar circadian period to that of the wild-type mice under the DD condition ($p = 0.521$, unpaired Student's t test) (Fig. 1B). These results indicate that loss of PACAP signaling does not affect the intrinsic circadian rhythmicity and entrainment to the external light:dark cycle.

Light induced phase shifts and expression of c-Fos in PACAP$^{-/-}$ mice

In order to investigate the role of PACAP in the clock response to light, we examined the effect of 30 min photic stimulation at CT6 (middle of subjective day), CT15 (early subjective night), and CT21 (late subjective night), respectively (Fig. 2A). When illuminated at CT15 and 21, both of PACAP$^{-/-}$ and wild-type mice exhibited a phase delay and advance of the circadian rhythm, respectively. The phase delay of PACAP$^{-/-}$ mice due to the photic stimulation at CT15 showed a tendency to be attenuated compared with that of wild-type mice [PACAP$^{-/-}$ mice vs. wild-type: 109.8 ± 16.5 min vs. 156.5 ± 19.5 min; not significant ($p = 0.110$, Mann–Whitney U test)]. In contrast, the light pulse at CT21 produced a significantly smaller phase advance in the rhythm of PACAP$^{-/-}$ mice than that in the wild-type (52.3 ± 16.5 vs. 116.5 ± 19.9 min; $p < 0.05$, Mann–Whitney U test). The pulse at CT6 did not evoke a phase shift in the behavioral rhythm in both wild-type and PACAP$^{-/-}$ mice. These results suggest that PACAP signaling is implicated in the mechanism of the phase shift of the circadian rhythm by light stimulation at the subjective night, especially in regard to the phase advance (see Fig. 2B).

Next, we examined the light-induced c-Fos expression in the SCN. In the wild-type mice, the illumination at

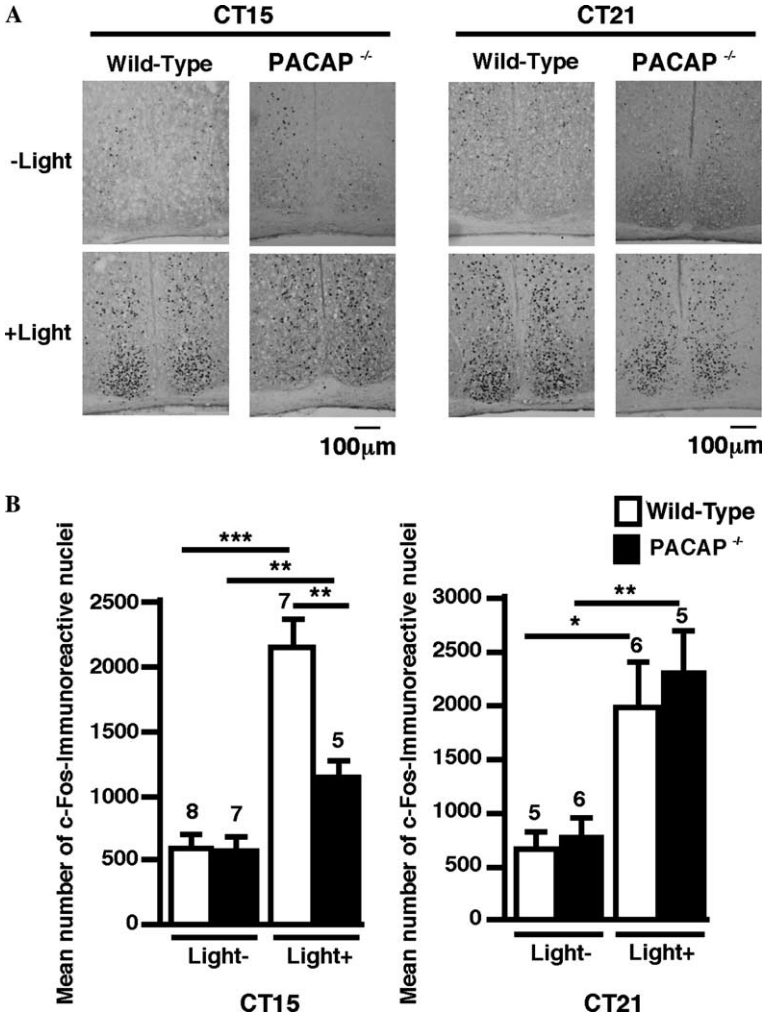

Fig. 3. c-Fos induction by photic stimulation in PACAP$^{-/-}$ and wild-type mice (A) Photomicrographs represented c-Fos-IR in the SCN 1 h after light stimulation (30 min, 20 lx) at CT15 (left panels) or CT21 (right panels). Scale bars = 100 μm. (B) Quantification of c-Fos expression induced by light pulse at CT15 (left) of CT21 (right). Numbers of c-Fos immunoreactive nuclei in the SCN were counted. Numbers of experimental animals used are shown above bars. The values are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (unpaired Student's t test).

both CT15 and CT21 increased the number of c-Fos-immunoreactive cells in the retinorecipient SCN (Fig. 3A). In contrast, the induction of c-Fos-immunoreactivities (IR) by photic stimulation at CT15 was significantly attenuated in PACAP$^{-/-}$ mice, especially in the ventrolateral (retinorecipient) part of the SCN (Fig. 3A). On the other hand, the increase in c-Fos-IR due to the illumination at CT21 was similar in both groups of mice. These results suggest that PACAP signaling is involved in the photic induction of c-Fos at the early subjective night but not at the late subjective night.

Discussion

Since roles of PACAP in the generation and photoentrainment of circadian rhythm are still poorly understood, we examined circadian rhythmicity, light-induced phase shift, and an immediate early gene expression in the SCN of PACAP$^{-/-}$ mice. At first, we checked the histological construction of the SCN in PACAP$^{-/-}$ mice, because PACAP is suggested to be involved in the neural development [25]. Results of immunohistochemical analyses of VIP-IR and AVP-IR in the SCN suggest that there is no drastic change in the architecture of the SCN in PACAP$^{-/-}$ mice (Fig. 1A).

Transgenic mice related to PACAP signaling, *PAC1*$^{-/-}$ mice, and transgenic mice overexpressing human $VPAC_2$ receptor were reported to exhibit significantly but slightly shorter free-running period than that of wild-type mice [17,26]. In contrast, PACAP$^{-/-}$ mice did not show significant change in free-running period compared with that of wild-type mice (Fig. 1B). Thus the present findings suggest that PACAP may not be involved in the generation of the intrinsic circadian rhythm.

PACAP$^{-/-}$ mice exhibited the entrainment of the circadian behavioral rhythm to the LD cycle (Fig. 1B). Moreover, when the environmental LD cycle was delayed or advanced 6 or 10 h, the resynchronization of the rhythms in PACAP$^{-/-}$ mice to the new environment occurred within similar time period to that of wild-type mice (data not shown). Although transgenic mice overexpressing human $VPAC_2$ receptor exhibit quick resynchronization compared with wild-type [26], our data suggest that VIP but not PACAP might be important in the resynchronization of the behavioral circadian rhythm via $VPAC_2$ receptor.

In the present experiment, we demonstrate that PACAP$^{-/-}$ mice show a significant reduction in the phase advance of the circadian rhythm responding to the light pulse at the late subjective night (CT21) and a moderate but insignificant decrease in its phase delay at the early subjective night (CT15) when compared with those of wild-type mice. We performed similar experiment using a running-wheel apparatus to monitor locomotive activity and found that PACAP$^{-/-}$ mice exhibited similar phase delay by photic stimulation at CT15 compared with that in wild-type mice (98.0 ± 23.9 min in PACAP$^{-/-}$ vs. 111.4 ± 13.8 min in the wild-type). However, the phase advance by the light pulse at CT21 in PACAP$^{-/-}$ mice was impaired (50.0 ± 23.6 and 110.8 ± 12.3 min in PACAP$^{-/-}$ and wild-type mice, respectively). Taken together, the present findings indicate that the phase advance but not phase delay of the circadian behavioral rhythm due to illumination is severely attenuated in PACAP$^{-/-}$ mice. In contrast to the phase shift, the induction of c-Fos protein in the SCN in PACAP$^{-/-}$ mice due to the illumination at the early subjective night (CT15) but not the late subjective night (CT21) was significantly blunted (Fig. 3). Inductions of clock genes, *per1* and *per2*, and an immediate early gene, *c-fos*, in the SCN are thought to be important in phase shift of the circadian clock [27,28]. However, the dissociation of the changes in the phase shift and c-Fos induction in PACAP$^{-/-}$ mice suggests that the induction of c-Fos might not be essential for the phase shift. Our results are in good agreement with the results of *PAC1*$^{-/-}$ mice, in which extents of inductions of *per1*, *per2*, and *c-fos* are dissociated with the extent of the phase response curve due to the light stimulation [15].

The administration of PACAP into the SCN in nanomolar concentrations causes phase shifts similar to those of light or glutamate stimulations [10], but PACAP at micromolar concentration can reinforce the glutamate- or light-induced phase delay and diminish phase advance [11]. *PAC1*$^{-/-}$ mice exhibited enhanced phase delay by a light pulse at the early subjective night and showed phase delay, but not phase advance, in response to the photic stimulation at the late subjective night [15]. It is difficult to explain these previous results in a straight way. One of the problems found in the previous pharmacological studies is that they cannot discriminate two pathways of PACAP signaling through PAC_1 receptor and $VPAC_2$ receptor. Administration of PACAP into the SCN in vitro or in vivo may also stimulate the VIP signaling pathway that can cause phase shifts similar to light stimulations [29]. Comparing phenotypes of PACAP$^{-/-}$ mice and *PAC1*$^{-/-}$ mice, it is possible that these phenotypes are contingent on PAC_1 and $VPAC_2$ signaling in the phase shifting mechanism. Since both PACAP$^{-/-}$ and *PAC1*$^{-/-}$ mice exhibited severe impairment in phase advance by the light pulse at the late subjective night, it seems that PACAP–PAC_1 receptor signaling is important for the phase advance mechanism. In contrast, PACAP–PAC_1 receptor signaling might be implicated in the induction of c-Fos at the early subjective night but not the late subjective night, because in both of PACAP$^{-/-}$ (Fig. 3) and *PAC1*$^{-/-}$ [15] mice the induction of c-Fos in the SCN at the early subjective night was impaired, while that at the late subjective night was unimpaired. Among the clock

genes, inductions of *per1* and *per2*, and the degradation of BMAL1 protein in the SCN were shown to be linked to the light-induced phase shifts [30–33]. To clarify roles of these clock proteins in the phase shift mechanism, further analysis about these clock proteins in $PACAP^{-/-}$ mice should be required.

In conclusion, the present findings suggest that PACAP signaling is important in the light-induced phase shift of circadian rhythm, especially in the one of the phase advance. The dissociations between phase response curve and induction of c-Fos might become a clue for the understanding of the molecular mechanism of phase shift.

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