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Circadian characteristics of mice depleted with GPR7

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ABSTRACT

GPR7, now known as a receptor of neuropeptide B and neuropeptide W, is expressed in neurons of the suprachiasmatic nucleus (SCN), the mammalian circadian center. By the quantitative *in situ* hybridization, we demonstrated that GPR7 mRNA showed a significant circadian rhythm in the SCN showing a peak at early subjective night in both light-dark and constant dark. We characterized the circadian feature of GPR7-knockout mice, but the period length and the phase-dependent phase shift to light exposure were not disordered in GPR7-knockout mice. Moreover, the food-anticipatory behavior in restricted feeding schedule was observed in this gene-deleted mouse similar to wild-type. These results indicate that the role of GPR7 may be subtle or limited in relation to the circadian clock despite its robust expression in the SCN.

Most organisms display a physiological and behavioral rhythm with periods of nearly 24 hours. In mammals, the central circadian oscillator is located in the hypothalamic suprachiasmatic nucleus (SCN) (12). Here the circadian rhythm is generated in the oscillator cells by a transcription/translation based autoregulatory negative feedback loop in which the protein products of a set of clock genes inhibit their own transcription (23, 26). In SCN, this cellular rhythm is synchronized through the interaction of oscillating cells (32), and entrained to the environmental light-dark cycle by the photic and non-photic signals (9, 12). Many receptors and their intracellular signal transductions may be enrolled in this intercellular communication, and a G-protein-coupled receptor GPR7 might be this candidate, since GPR7 is highly expressed in the SCN (13). GPR7 is originally described by O'Dowd *et al.* (1995) (21) as a receptor having close structural resemblance to both the somatostatin-3 (SST3) receptor, and the μ-, δ-, and κ-opioid receptors. Recently, its two endogenous ligands, neuropeptide B (NPB) and neuropeptide W (NPW), were identified (3, 6, 28, 30). Although roles of this receptor on feeding, prolactin-release and pain were reported (29), the roles of GPR7 on biological rhythms have not been clarified. In the present study, we demonstrated the circadian change of GPR7 expression in the SCN, and further examined the circadian characteristics of mice deleted with GPR7 gene (10).

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MATERIALS AND METHODS

Animals and behavioral rhythm monitoring. For examining the expression of GPR7, we purchased male C57B16 mice at 6 weeks age (JAPS, Osaka, Japan). Mice were housed in 12 h light/dark (LD) cycles (fluorescent light, 300 lux) at least for 2 weeks at 22 ± 2°C with freely provided diet and water. Six mice were examined for assessing the brain expression of GPR7 at ZT 16 (ZT stands for Zeitgeber time in a LD cycle; ZT0 is light-on and ZT12 is lights-off). For quantitative analysis of GPR7 in the

SCN, mice were examined in the LD and in constant darkness (DD) at every 4 h (n = 4–5 at each time point for both experiments; total n = 58), starting at ZT0 or CT0 (CT stands for circadian time; CT0 is subjective dawn and CT12 is subjective dusk).

We examined the behavioral characteristics of *GPR7*$^{-/-}$ mice in relation to the wild-type mice (10). Locomotor activity was detected by passive (pyroelectric) infrared sensors (FA-05 F5B; Omron, Kyoto, Japan) (27). Period length was monitored at least 4 weeks in DD. Moreover, phase-dependent phase-shift by the brief light exposure, and the effect of restriction feeding (RF) were also examined. Mice were entrained to a 12 h: 12 h LD cycles for at least 1 week before any experimental manipulation. All data were monitored and analyzed by Chronobiology kit (Stanford Software Systems, Stanford, CA). The all experimental procedures were approved by the Committee for Animal Research of Kobe University and the Animal experimentation Committee of Kyoto University.

In situ hybridization. Mouse GPR7 cDNA fragment (positions 330–768 (438 bp) of Gene Bank accession no. XM_136404; primers (5'-CGTGTTCAT CCTCAACCTGGCTATC-3' and 5'-GATGGTGGT CACCGGGATGGCAAG-3') was subcloned into the pBluescript KS (−) vector (Stratagene, La Jolla, CA) and sequenced to verify their identity and orientation. These cDNA fragment containing vectors were linearized with restriction enzymes and used as templates for antisense cRNA probes. Radiolabeled probes for GPR7 mRNA were made using ^{33}P-UTP (New England Nuclear, Boston, MA) with a standard protocol for cRNA synthesis.

Mice were deeply anesthetized with ether, and intracardially perfused with 10 mL of ice cold 0.1 M phosphate buffer (PB) (pH 7.4), followed by 20 mL of a fixative containing 4% paraformaldehyde in 0.1 M PB. For the mice housed in darkness, anesthesia was performed under safe dark red light. The brains were removed, post-fixed in the same fixative for 24 h at 4°C and placed in 0.1 M PB containing 20% sucrose for 48 h. These brains were frozen using dry ice and stored at −80°C until use. Mouse brain sections were made 40 μm in thickness by a cryostat (Leica, Solms, Germany). To minimize technical variations throughout the hybridization procedure, sections from different experimental conditions were gathered into one group and processed simultaneously. *In situ* hybridization histochemistry using the free-floating sections were performed according to the method detailed previously (27). Briefly, tissue sections were processed with 1 μg/mL proteinase K (0.1 M Tris-HCl, pH 8.0, 50 mM EDTA, 10 min) at 37°C and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then incubated in the hybridization buffer (60% formamide, 10% dextran sulphate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.6 M NaCl, 0.2% N-laurylsarcosine, 500 mg/mL transfer RNA, 1 × Denhardt's solution and 0.25% sodium dodecyl sulphate) containing the ^{33}P-UTP-labeled antisense cRNA probes for 16 h at 60°C. Sections for free floating *in situ* hybridization were mounted onto gelatin-coated microscope slides, air-dried, and dehydrated. These sections together with ^{14}C-acrylic standards (Amersham, Buckinghamshire, U.K.) were exposed to Biomax film (Kodak, Rochester, NY) for five days and subjected to the image analysis procedure.

Quantification of hybridization. The radioactivity of each SCN on film was analyzed using a microcomputer interface to an image analysis system (MCID, Imaging Research, St Catherines, Ontario, Canada) after conversion into the relative optical densities produced by the ^{14}C-acrylic standards. Data were normalized with respect to the difference between signal intensities in equal area of the SCN and the corpus callosum. The intensities of the optical density of the sections from the rostral to the caudal most part of the SCN (8 sections per mouse brain) were then summed; the sum was considered to be a measure of the amount of GPR7 mRNA in this region. The intensity values are presented as mean ± SEM (n = 4–5). We indicate relative mRNA abundance, which means that the peak intensity value in the LD or DD condition was adjusted to 100. Data were analyzed by one-way ANOVA followed by Scheffe's multiple comparisons.

Phase-response curve to the light pulse. For the phase response curve, on the last LD cycle, mice received a 30 min light pulse of 300 lux at designated CT times (CT2, CT6, CT10, CT14, CT18, and CT22). The mice remained in DD for at least 2 week after the pulse. The response to light from ZT12–24 or 20–24 were tested by Student's *t*-test. The phase response curve was tested by two-way ANOVA. Bonferroni *post hoc* tests were used for the comparison between the values of wild-type and *GPR7*$^{-/-}$ groups at the same time points. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA).

Entrainment to the food restriction. Age matched mice were used. Mice were entrained in 12 h: 12 h LD cycle for more than 7 days, and food was given *ad libitum* before entering RF schedule. Lighting conditions remained the same and water was freely available throughout all experiments. On the first day of RF, food was withdrawn for complete fasting. Then, mice were allowed access to food for 4 h from ZT5 to ZT9 for 7 consecutive days. On the day 9, food was again withheld for the entire day.

RESULTS

Distribution of GPR7 mRNA in the mouse brain

Strong expression of GPR7 mRNA was found in sporadic regions of mouse brain (Fig. 1A). In hypothalamus, GPR7 mRNA signals were found at high level in the SCN, paraventricular nucleus (PVN), supraoptic nucleus (SON), and hypothalamic dorsomedial nucleus (DMH) (Fig. 1A, a–d). High level signals were also found in central nucleus of the amygdala (AmyCe), insular cortex (InsCx) and zona incerta (ZI) in the forebrain (Fig. 1A, c and d). In the other area, GPR7 mRNA signals were found especially in superior colliculus (SC) and nucleus of the solitary tract (NTS) in the midbrain and dorsal horn of the spinal cord (DH) (Fig. 1A, e–g).

Circadian expression of GPR7 mRNA in the SCN

A robust circadian expression of GPR7 mRNA was

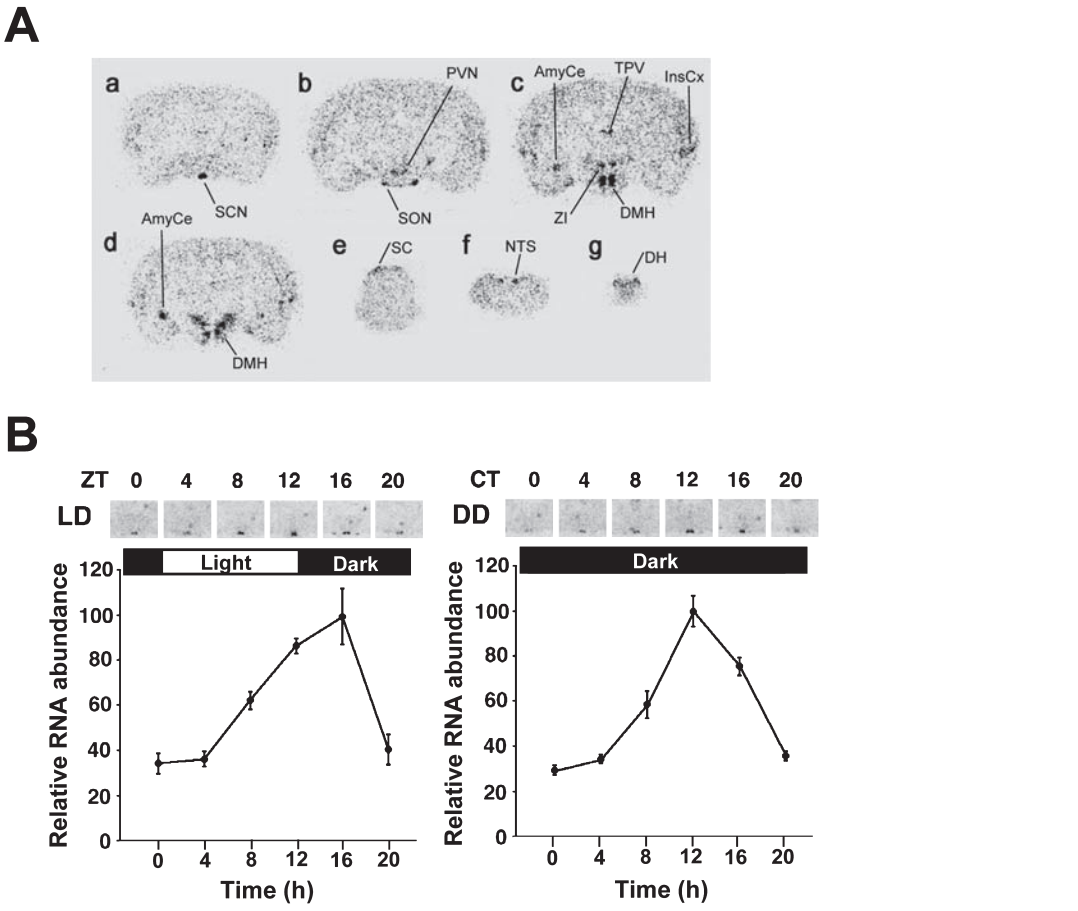

Fig. 1 GPR7 mRNA in the mouse brain. **(A)** Distribution of GPR7 mRNA in the mouse brain. Strong signals are distributed in specific regions of the brain. Weak signals diffusely detected in the brain are non-specific. AmyCe, central nucleus of the amygdale; DMH, dorsomedial nucleus of the hypothalamus; DH, dorsal horn of the spinal cord; InsCx, insular cortex; NTS, nucleus of the solitary tract; PVN, paraventricular hypothalamic nucleus; SON, supraoptic nucleus; SCN, suprachiasmatic nucleus; SC, superior colliculus; TPV, thalamic paraventricular nucleus; ZI, zona incerta. **(B)** Circadian expression of GPR7 mRNA in the mouse SCN. Quantitative analysis of GPR7 mRNA in LD (left) and DD (right) conditions (mean ± SEM; n = 4–5). Values were determined by quantitative *in situ* hybridization using isotope-labeled probes with the mean peak value being adjusted to 100. Representative *in situ* hybridization autoradiograms are shown in upper side. Numbers on each autoradiogram indicate the sampling time (h).

observed in both LD and DD conditions. By the quantitative *in situ* hybridization, in LD condition, signals showed a peak at ZT16 and a trough at ZT0–ZT4 (ANOVA; $F[5, 22] = 17.671$, $P < 0.0001$) (Fig. 1B, left). In the DD condition, the signals of GPR7 mRNA also showed a peak at CT12 and a trough at CT0 (ANOVA; $F[5, 23] = 65.354$, $P < 0.0001$) (Fig. 1B, right). The maximum GPR7 mRNA level is about three fold-higher than the minimum under both LD and DD conditions.

Circadian behavior and its response to light in GPR7$^{-/-}$ mice

To examine the effect of GPR7-defect on behavioral rhythms, wild-type and *GPR7*$^{-/-}$ mice (10) were entrained in LD condition for at least 1 week, followed by DD condition in which the circadian period was measured. There was no significant difference between wild-type and *GPR7*$^{-/-}$ mice in circadian period (23.74 ± 0.04 h in wild-type and 23.76 ± 0.03 h in GPR7-knockout, $P > 0.05$, Student's *t*-test).

Next, we examined the effect of light (300-lux) on the behavioral phase-shift of *GPR7*$^{-/-}$ mice. First, we tried the one day 12 h-extension schedule. Mice were entrained in LD condition at least one week, and then light period was extended to a 12 h after the end of the scheduled lighting period for one day, followed by exposing DD condition (Fig. 2A, upper). The average phase delays of wild-type and GPR7-knockout were 4.68 ± 0.27 h and 4.33 ± 0.25 h respectively ($P > 0.05$, Student's *t*-test). Next, we tried the 4 h-exposure of light on the time expected to have phase-advance. Concretely, in the last light-day cycle, mice were exposed to 4 h light pulse at ZT20–24. Wild-type and *GPR7*$^{-/-}$ mice were phase advanced with 0.21 ± 0.13 h and 0.23 ± 0.12 h, respectively, and there was no statistical significant difference ($P > 0.05$, Student's *t*-test).

To examine the phase-dependent phase-shift by the exposure of light more precisely, we constructed phase response curve (PRC) of wild-type and *GPR7*$^{-/-}$ mice by light pulses (30 min, 300 lux) at every 4 h circadian time. As shown in Fig. 2B, the light pulse at the CT14 and CT18 produced phase delays and at CT22 produced phase advance in both wild-type and *GPR7*$^{-/-}$ mice. PRC of *GPR7*$^{-/-}$ mice showed no difference to that of wild-type ($P > 0.05$, two-way ANOVA).

Anticipation of feeding in restricted feeding schedule in GPR7$^{-/-}$ mice

Not only to light, circadian rhythms are entrained to non-photic stimuli including melatonin and feeding (2, 4). Here, we examined the effect of RF on the circadian rhythm in *GPR7*$^{-/-}$ mice. Behavioral activity under *ad libitum* feeding or 4 h RF in the middle of the light phase (ZT5–9) was measured. A robust increased food seeking activity was observed about 2 h before food preparation in wild-type mice within a few days after start of RF schedule (Fig. 3, left). This anticipatory behavior was also shown in the *GPR7*$^{-/-}$ mice (Fig. 3, right).

DISCUSSION

The present quantitative investigation has demonstrated that GPR7 mRNA is rhythmically expressed in the SCN in a circadian fashion. The mRNA expression level at a peak in subjective night was about three fold higher than that at a trough in subjective day. Although the generation mechanism of this clear night-peak rhythm has not been clarified, it is speculated that the core oscillation of clock genes induces the circadian transcription of clock controlled genes via the E-box, D-box, and RRE (RORE Responsive Element) in their promoters (23, 26, 31). Since BMAL1, a representative night-peak rhythmic gene (22), is known to show this characteristic expression profile via its RRE site on its promoter (25), similar mechanism is possible for GPR7 gene. By the search of genomic sequence of possible promoter region, we found one RRE site at 5,316-base upstream of the transcription initiation site of GPR7 gene in mice, but this site is not evolutionally conserved in the human GPR7 gene. Thus, the mechanism of night-peak pattern of GPR7 in the SCN is not known at present, and the indirect regulation of clock genes or regulation of signal transduction molecules such as cAMP (24) should be considered to understand the rhythmic nature of GPR7 transcripts.

Since the ablation of receptors which are expressed abundantly in the SCN such as VPAC2 shows the abnormality of rhythm (8), we examined GPR7-knockout mice. However, the period length of the knockout mice in constant dark conditions was virtually the same as that of wild type. We next characterized the effect of light-induced phase-shift. First we characterized gross change of phase-advance and phase-delay effects, by adopting the schedules of 4 h light at ZT20/24 and 12 h extension light, respectively (5, 15). However, there were no difference between *GPR7*$^{-/-}$ and wild type. For the more precise evaluation of light effect on circadian rhythm, we made a phase response curve to

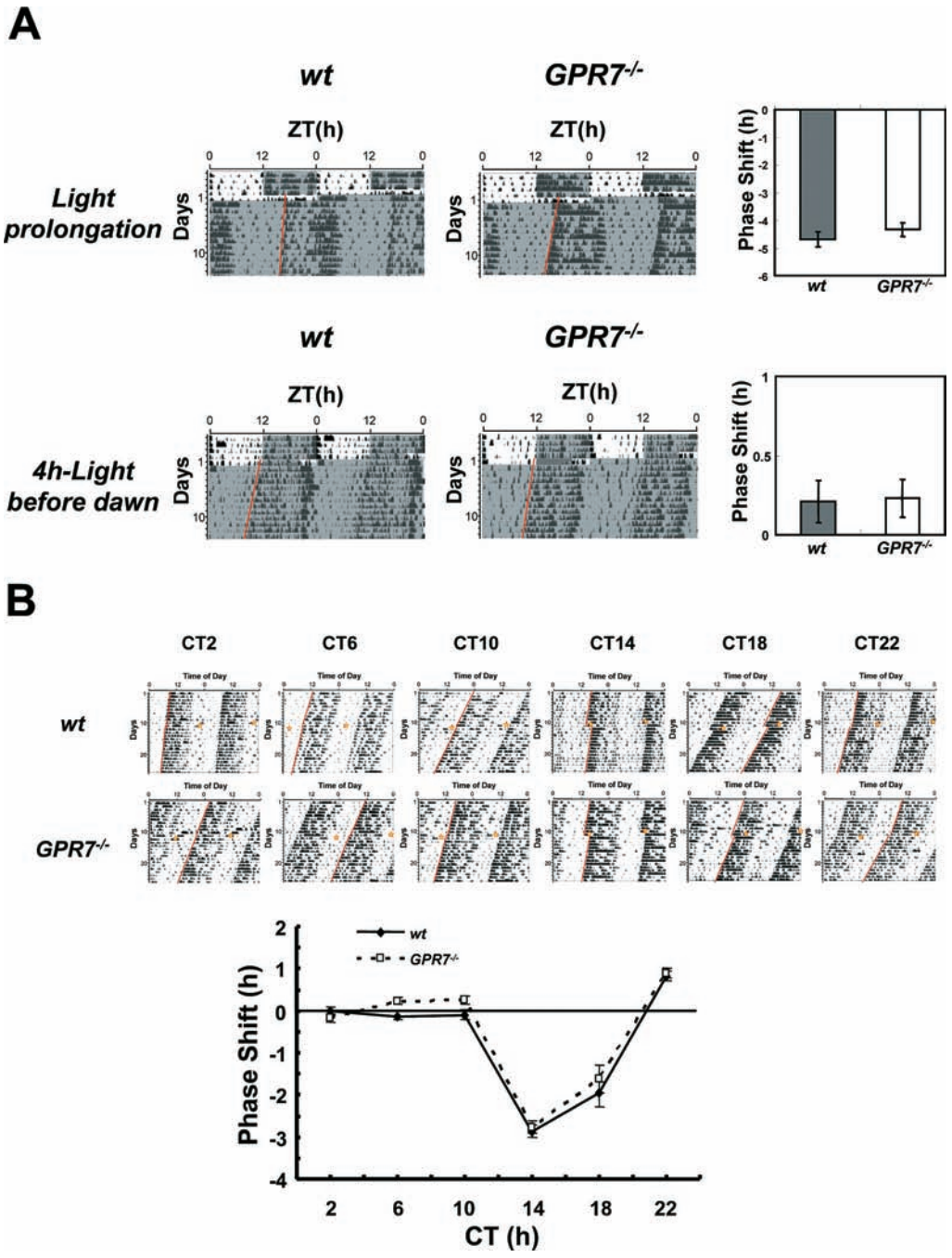

Fig. 2 *GPR7*$^{-/-}$ mice behavioral response to light. **(A)** Response to light from ZT12–24 or 20–24. Double-plotted locomotor activity rhythms of representative wild-type mice and *GPR7*$^{-/-}$ mice. Mice were initially entrained to a fixed 12 h LD cycle of 300 lux. Mice were then moved to DD after 12 h of light prolongation (ZT12–24) or 4 h light pulse before dawn (ZT20–24) at the last day of LD cycle. Locomotor activities were expressed in the histogram. Periods of darkness are indicated by grey backgrounds. The red lines delineate the phase of activity onset after the light manipulation. The bar graphs at right show mean ± SEM of the phase shifts for each genotype. Positive values denote phase advances, whereas negative values denote phase delays. No significant difference $P > 0.05$, Student's *t*-test). **(B)** Phase response curve (lower) and representative actograms (upper) of wild-type and *GPR7*$^{-/-}$ mice. Wild-type (filled diamond) and *GPR7*$^{-/-}$ (unfilled square) mice were entrained to a fixed 12 h LD cycle of 300 lux. On their final LD cycle, the mice received a 30 min light pulse of 300 lux at designated CT times (CT2, 6, 10, 14, 18, 22) and subsequently maintained in DD for at least 14 days. The red lines delineate the phase of activity onset before or after the light pulse. Values are presented as mean ± SEM phase shift (in hours). Positive values denote phase advance, whereas negative values denote phase delays. n = 3–8 per group. No significant difference at all time points tested ($P > 0.05$, 2-way ANOVA).

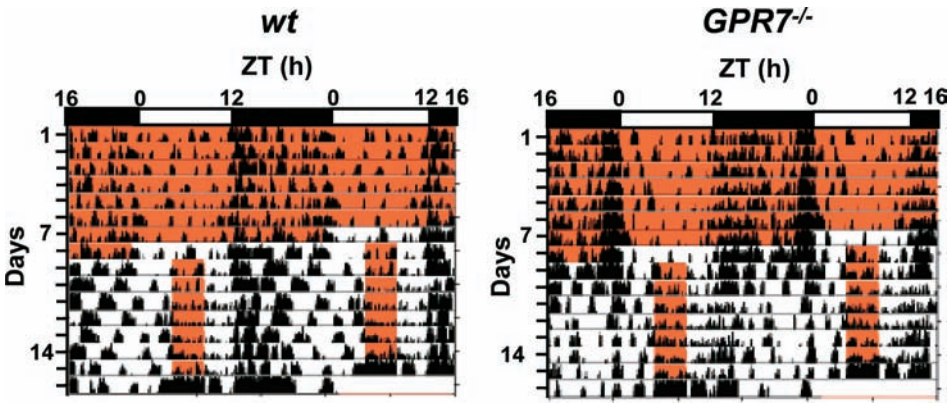

Fig. 3 Food-anticipatory activity under restriction feeding in *GPR7*$^{-/-}$ mice. Representative double-plot actograms show locomotor activity of wild-type **(left)** and *GPR7*$^{-/-}$ **(right)** mice, respectively. Wild-type and *GPR7*$^{-/-}$ mice were fed *ad libitum* for 7 days then followed by fasting at day 8 and restriction feeding starting at day 9. Areas shaded with orange color in the actogram indicate the time when food is available. The light phase (ZT0–12) and the dark phase (ZT12–24) are indicated as top black and white bar respectively.

30 min light pulse (300 lux), but we found no difference of *GPR7*$^{-/-}$ and wild-type mice.

Above findings demonstrate that there are no abnormalities for the generation and entrainment of the circadian behavioral rhythms in *GPR7*$^{-/-}$ mice in spite of the high expression of GPR7 in the SCN. Since *GPR7*$^{-/-}$ mice are reported to show mild adult-onset obesity (10), this gene deletion can surely show some physiologically relevant phenotype for energy metabolism. Thus, our present results indicate that GPR7-deficiency is compensated by other factors, or GPR7 receptor is functionally inactive.

As the candidates of compensatory substance, the GPR8 is one candidate, because of its 60% amino acid identity with showing the similar binding affinity to NPB/NBW (13). However, GPR8 gene was absent in rodent (13). Somatostatin receptors and opioid receptors, which are abundantly expressed in the SCN, might be other candidates since 40% of sequence similarity was noted. However, since no biochemical and pharmacological data are available at present, this hypothesis remains to be proved in future studies.

Since an autoradiographic study has shown that high levels of ^{125}I-labeled NPB or NPW bindings are detected in the SCN (11, 29), functional receptors may be expressed in the SCN. However, there are many evidences that they are functionally inactive. First, the intracerebroventricular injection of NPW induces c-fos in many brain structures expressing GPR7, but never induces c-fos in the SCN neurons (14). This suggests that SCN neurons are unresponsive to NPW or that SCN neurons specifically use another signal cascade to transduce GPR7-mediated signals. Second, it is possible of no existence of endogenous ligands in the SCN and nearby structure, since both NPB and NPW have not been found in the SCN and nearby nuclei (11, 30). Third, it is also possible that GPR7 in the SCN may be involved in unknown non-rhythmic functions in the SCN.

Since we found no abnormality for circadian rhythms and its entrainments to light, we broaden the examination to the non-photic entrainment of circadian rhythms (9). Here we examined prefeeding activity during RF: if feeding is restricted to a single meal scheduled at a fixed time for several days, mice develop intense locomotor activity before this mealtime (2), and this prefeeding activity persists even when food is withheld for several days (18). Several brain structures are involved in these phenomena including dorsomedial hypothalamic nucleus (DMH) (7), limbic system (1), and SCN (16). Although the primary brain locus for this phenomenon is still in argue (19, 20), recent evidence suggests the special role of DMH (7, 17, 20). Since GPR7 is also strongly expressed in DMH as in Fig.1, we examined the preceding activity to feeding. When 4 h RF schedule was applied to *GPR7*$^{-/-}$ mice, anticipatory behavior was also observed in the *GPR7*$^{-/-}$ mice (Fig. 3. right). This strongly indicates that GPR7 do not play a role in feeding induced entrainment.

In the SCN, GPR7 mRNA reveals significant circadian rhythmicity with night-peak pattern in constant dark condition. However, there is no significant difference about circadian rhythm phenotype between wild-type and GPR7-knockout mice. These

results indicate that defect of GPR7 in the SCN will be completely compensated by other factors, functional ligands do not exist in nearby regions, or NPB/NPW-GPR7 system will be involved in other functions.

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