Period1 gene expression in the olfactory bulb and liver of freely moving streptozotocin-treated diabetic mouse

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ABSTRACT

Clock genes express circadian rhythms in most organs. These rhythms are organized throughout the whole body, regulated by the suprachiasmatic nucleus (SCN) in the brain. Disturbance of these clock gene expression rhythms is a risk factor for diseases such as obesity. In the present study, to explore the role of clock genes in developing diabetes, we examined the effect of streptozotocin (STZ)-induced high glucose on *Period1* (*Per1*) gene expression rhythm in the liver and the olfactory bub (OB) in the brain. We found a drastic increase of *Per1* expression in both tissues after STZ injection while blood glucose content was low. After a rapid expression peak, *Per1* expression showed no rhythm. Associated with an increase of glucose content, behavior became arrhythmic. Finally, we succeeded in detecting an increase of *Per1* expression in mice hair follicles on day 1 after STZ administration, before the onset of symptoms. These results show that elevated *Per1* expression by STZ plays an important role in the aggravation of diabetes.

1. Introduction

Diabetes has become one of the most important health problems of modern society, affecting millions of people worldwide. Circadian disturbances have recently been identified as contributors to metabolic diseases such as diabetes. Alternations in circadian rhythm derived from modern lifestyles have been shown to be related to obesity and metabolic syndrome [1-2]. Disturbance of clock gene expression rhythm is a risk factor for diseases like obesity [3-4]. Disruption of the clock components CLOCK and BMAL1 leads to hyperinsulinemia and diabetes [4]. Interestingly, recent study has shown that all clock and clock-controlled genes examined possess circadian advanced and dampened rhythms of gene expression in streptozotocin (STZ)-induced type 1 diabetic rat heart [5] and mouse liver [6-7]. STZ disrupts pancreatic beta cells and induces type 1 diabetes [8]. The blood glucose level markedly increases for several days and remains at a high level until death.

In the present study, we explore the temporal change of *Per1* expression until and during the onset of diabetes after STZ injection to clarify when it affects the expression of clock genes. To analyze the circadian system of *Per1* expression, we need long recording times of each organ throughout the whole body. It is especially important to measure the rhythm of peripheral organs for diseases which arise from circadian disruption [3-4]. In previous studies we reported on a multiple recording system for the central nervous system and peripheral tissues [9-12]. In this report, we describe a double recording system of clock gene expression in the OB and liver while monitoring

locomotor activity. We examined temporal *Per1* expression change in the OB and liver of freely moving mice in developing sever diabetes after STZ treatment. In addition, we confirmed elevated *Per1* expression after STZ treatment using our newly developed recording system using mouse hairs.

2. Materials and methods

2.1 Animals

Mice were born and reared in our animal quarters where environmental conditions were controlled: 12 hr light /12 hr dark (LD) cycle with lights on 8:00 – 20:00, temperature (23 ± 1 °C) and humidity (50 ± 5%). Animals were given food and water ad libitum. C57BL/6J mice carrying a *Per1*-promoter driven firefly-luciferase reporter gene (*Per1-luc*) were used [9]. The *Per1-luc* reporter was constructed as follows: a 6.7 kb region upstream of the translation-initiation codon of *mPer1* was fused to the firefly luciferase (*Luc*) coding region [13]. We checked the LUC expression in the mouse by luciferase and luciferin reaction. Before *in vivo* recoding, the mouse ear was punched out (Nadox KN-292-2, diameter 1.5mm) and LUC assay was done according to Kit protocol (Pica Gene, Toyo B-net co., LTD).

All animal work was performed in accordance with Guidelines for the Care and Use of Laboratory Animals in International University of Health and Welfare with the permission #18014 from the Committee for Animal Experimentation.

2.2. STZ injection

Streptozotocin (STZ) was purchased from Wako Chemical (Fujifilm Wako Chemical, Osaka, Japan). STZ was dissolved in saline solution and immediately intraperitoneally injected (200 mg/kg) into adult *Per1-luc* transgenic (Tg) mice at Zeitgeber time (ZT) 9-10.

2.3 Recording of Per1 gene expression in the OB and liver of freely moving mice

A portable optical detection (POD) device [10] was set in a custom-made recording box (W 400 x D 500 x H 500 mm) equipped with a ventilation and monitor system which measured temperature, humidity and lux. The environmental conditions were controlled at temperature 23±2℃ and humidity 50±5%.

For recording the OB, animals were anesthetized with isoflurane (Zoetis Japan) and a

small hole was drilled in the skull. A stainless steel guide cannula (φ 0.90 mm) was stereotaxically implanted into the OB using the following coordinates: 4.0 mm anterior, 1.0 mm lateral to the bregma at a depth of 3.0 mm below the skull. After surgery, the mouse was housed in LD and allowed to establish stable daily activity rhythms. After at least 5 days of stable activity rhythm, a plastic optical fiber (diameter 0.50 mm, surface cladding 0.5 mm thick), was inserted into the guide cannula and fixed by dental cement (Hy-Bond Temporary Cement, Shofu Inc.)[9-11]. For recording the liver, a tissue contact optical sensor (TCS) [11] was attached with the median and left lobe using a suture to hook the xiphoid process of the sternum and internal abdominal wall. The optical fiber was passed under the skin and out the neck area. We reconfirmed the position of the TCS by performing a laparotomy after finishing the experiment.

At least 7 days after surgery, the optical fibers were then connected to the POD, and bioluminescence recording began in constant darkness. D-luciferin (20 mg/ml) (Fujifilm Wako, Japan) was intraperitoneally injected into *Per1-luc* Tg mice with a controlled flow rate with modifications from our previous reports [9] to get enough photon counts. A controller (RE-C100, Aquatech Co., Ltd., Osaka, Japan) regulated the micro pump (RP-TX, Aquatech Co., Ltd., Osaka, Japan) and was connected to a modified free moving animal system (Eicom, shimotoba, Kyoto, Japan) which consisted of a liquid swivel (TCSI-20, 11μl single channel swivel, 0.65 mm connections), balance arm, crisscross mouse harness and a tube that was guided into the intraperitoneal cavity through a subcutaneous tunnel from an incision in the dorsal neck to the ventral abdomen. The micro pump speed was 10~15 ul/hr. The detection ability of bioluminescent was the same as our previous system [9] (Supplementary Fig. 1).

Photons emitted by the target areas of freely moving mice were integrated over 10 sec intervals and averaged for 30 min. These data were plotted and an approximate curve was made using Excel. The primary peak time of *Per1-luc* expression was determined as the time of highest intensity of *Per1-luc* expression around a continuous high-intensity period during each circadian cycle.

Per1-luc Tg mice were housed in transparent plastic cages (W175 x D245 x H 125 mm) and their locomotor activity rhythm was monitored by an infrared sensor located 30 cm above the surface of the cage using an on-line PC (Chronobiology Kit, Stanford Software Systems) [9]. Food and water were provided ad libitum. Daily activity onset was visually estimated from standard double-plot actograms of locomotor activity behavior. The locomotor activity onset time was designated as CT12.

2.4 Detection of STZ-induced Per1 expression in hairs

Food and water were provided ad libitum. Mouse back skin hairs were collected before STZ injection (day 0), day 1 and day 7 after STZ injections. Glucose content and drinking activity measurements were also performed just before sampling time. For measuring glucose content, blood samples from the mouse tail were analyzed by LAB Gluco (RIJ, Japan). The range for the LAB Gluco was 10-600 mg/dL, and any value greater than 600 mg/dL was registered as 'HI'. After STZ administration, mice showed 'HI' on day 9, 11 and 14 in Fig. 1B. Mouse back skin hairs were isolated by forceps at ZT10 and incubated in PicaGene cell culture reagent Luc (Toyo B-net Co., Ltd) over several hours. After centrifugal spinning, supernatant fluid was mixed with PicaGene (Toyo B-net Co., Ltd). Bioluminescence of mixed solution was measured every 10 sec by an optical fiber connected PMT system using 96 well plates as previously described [10]. The mixed solution was also measured with a micro PMT system set in the dark box. Bioluminescence on the photoelectric surface of the micro PMT was measured every 10 sec. After the measurement, the number of back skin hairs was counted. The value of bioluminescence was then calculated per hair.

3. Results

3.1 Effects of STZ on blood glucose content, body weight and water intake

Fig. 1A shows images of *Per1-luc* Tg mice after STZ injection. After STZ injection, the condition of the mice gradually changes; they assume a curled up posture and hair coat quality decreased (Fig. 1A day 14). The glucose content in the blood at ZT10~11 under ad libitum feeding was over 400 mg/dl 3~4 days after STZ injections. At this point the mice were considered to have developed diabetes [14]. The body weight significantly decreased after STZ injections on day 3. The body weight at day 7 decreased 19.3% compared to that of day 0. The amount of water intake gradually increased after STZ administration. At 7 days, the amount of water intake was 6 times than that of day 0. (**$p<0.01$, ***$p<0.001$ vs. saline, two-way ANOVA followed by Bonferroni's multiple comparisons test).

3.2 Recording of *Per1* gene expression in the OB and liver of freely moving mice

To record photon counts for long duration, we developed the luciferin application system modified from our previous reports [9-13]. In this study, a micro pump connected with a controller was used to supply D-luciferin intraperitoneally into freely moving *Per1-luc* Tg mice. The optical fiber was inserted into the OB and the TCS was attached with the liver. Bioluminescence rhythm of *Per1-luc* in the OB and liver are shown in Supplementary Figs. 1A and 1B. Clear circadian rhythms are detected in both

organs in freely moving mice. The present luciferin application system shows no locomotor activity or behavioral activity rhythm (Supplementary Fig. 1C).

Next, to explore the relationship between *Per1* expression rhythm and diabetes, we examined temporal *Per1* expression change after STZ injection in both the OB and liver until sever diabetes developed. Supplementary Fig. 2A shows *Per1* expression rhythm in the OB before, immediate after and several days after STZ injections in freely moving mice. Supplementary Figs 2B, C and D show magnified areas ①, ② and ③ in supplementary Fig. 2A, respectively. Bioluminescence rhythm peak of *Per1-luc* in the OB was at CT11.2 ± 1.2 h. STZ administration induced elevated *Per1* expression and maintained high expression in the OB. The increased *Per1* expression rate after STZ administration was 1.5 (150% increase). After the first peak of elevated *Per1* expression, rhythmic *Per1* expression disappeared.

Similarly, STZ had an effect on *Per1* expression in the liver (Fig. 2) of the same mouse in supplementary Fig. 2 measured by double recording. Bioluminescence rhythm peak of *Per1-luc* in the liver had a peak at CT13.6 ± 1.1 h. STZ increased *Per1* expression 4.1-fold and induced no rhythmic *Per1* expression. The *Per1* expression in the liver is more sensitive to STZ compared with that of OB. Table 1 shows the summarized data of *Per1* expression rhythm in the OB and liver of *Per1-luc* Tg mice.

3.3 Recording of activity rhythm

Behavior activity rhythm of the same mouse measured *Per1* expression rhythm in supplementary Fig. 2 and Fig. 2 is shown in Fig. 3. Before STZ administration (① in Fig. 3A), *Per1-luc* Tg mouse show stable rhythmic *Per1* expression with the period of 23.8 hr. However, on day 3 after STZ administration, locomotor activity rhythm became arrhythmic.

Analysis of behavior activity rhythm is shown in Fig. 3B.

Circadian rhythmicity of locomotor activity is shown by the ratio of subjective night (SN) or subjective day (SD) relative to total activity (Fig. 3C). Before STZ administration, there is a clear circadian rhythm between SN and SD ($P<0.0001$ Student's t-test). But there is no difference of total activity/ day between pre and post STZ administration.

3.4 STZ induce *Per1* expression in hairs

Finally, we confirmed elevated *Per1* expression after STZ treatment using our newly developed recording system with mouse hairs. Mouse back skin hairs before STZ injection (day 0), day 1 and day 7 after STZ injections were examined. On day 1, elevated *Per1* expression was detected although glucose content in the blood was normal (Fig. 4A, B, C). On day 7, *Per1* expression, glucose content and amount of water consumption were significantly higher than that of day 0 (*$p<0.05$, one-way ANOVA followed by Dunnet's test).

4. Discussion

Circadian disturbances have been identified as contributors to metabolic diseases such as diabetes and obesity [3-4]. We have shown in this study how *Per1* rhythmicity and locomotor activity rhythm change in response to changes in blood glucose content *in vivo* in real time using STZ-induced diabetic mice.

STZ is taken up by pancreatic β cells via glucose transporter GLUT2 [15], and causes DNA alkylation and eventual β cell death. The mice were induced to type 1 diabetes [8]. We designated a non-fasting blood glucose level of 400 mg/dL as our criteria for severe diabetes [14], where the mice drink a lot of water and loose body weight.

Like the metabolic system, the circadian system is a complex feedback network that involves interactions between the central nervous system and peripheral tissues. In the present study, to reveal how *Per1* expression rhythm in the brain and peripheral tissues under diabetic conditions are affected, we examined the temporal change of *Per1* expression in the OB and liver. The OB of rodents has been suggested to possess a self-sustaining circadian oscillator which functions independently from the master circadian clock in the SCN of the hypothalamus [16]. *Per1* mRNA is highly expressed in the mitral cell layer of the main OB in rats [17] and mice [18]. The liver also has high *Per1* expression and circadian controlled rate-limiting metabolic enzymes [19]. In freely moving mice using our recording system, the OB and liver have shown rhythmic *Per1* expression [9,10]. On day 1 after STZ administration, it was elevated, but *Per1* expression rhythm was dampened thereafter. These phenomena also were detected in the cortex and skin (cortex, 1.4 ± 0.3; skin, 1.4 ± 0.1). The ratio of peak value was highest in the liver and occurred earliest. Interestingly, blood glucose content on day1 had no high expression (day 0, 204mg/dL; day 1, 147 mg/dL) and the mice did not yet indicate diabetes. After day 3, *Per1* expression remained high and had no rhythmic expression in these tissues. Locomotor activity rhythm disappeared on day 3 after STZ administration, although total activity showed no difference on day 3 comparing with that of pre STZ administration. Previous reports have shown that STZ has no effect on

clock gene expression in the brain cortex [20] or locomotor activity rhythm [6,7]. The reason is still unclear. One possibility is that different mouse strains (ddY [7] and ICR [6]) were used for those experiments. STZ seems to have some strain-dependent sensitivity [21,22]. In addition, age, weight and the time of day that STZ is administered alters diabetes induction [23,24].

The present results show *Per1* response to subtle blood glucose levels on day 0 ~ day 1. These responses are different among tissues. Elevated *Per1* expression in back skin hairs on day 1 after STZ administration was detectable by our system. We established a method to measure abnormal clock gene expression (risk factor) before the onset of severe diabetes using whole hair root tissues. Finally, our experimental procedure is extremely simple and makes it easy to detect risk factors for severe diabetes.

Acknowledgments

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Figure legends

Figure 1. The effects of STZ on blood glucose content, body weight and water intake.

A. Photos of a *Per1-luc* Tg mouse after STZ injection. **B.** Blood glucose content after STZ injection. Each plot indicates the mean ± S.E. (n= 5). **C, D.** Body weight and water intake changes after STZ injection. Each plot indicates the mean ± S.E. (n=3). Statistical significance as detected by two-way ANOVA followed by Bonferroni's multiple comparisons test (**p<0.01, ***p<0.001 vs. saline). Open circles indicate saline treated group; closed circles indicate STZ treated group.

Figure 2. Effects of STZ on *Period1* gene expression rhythm of liver in a freely moving mouse (double recording).

A. *Per1* gene expression in the liver before and after STZ (200 mg/kg, i.p.) injection of *Per1-luc* Tg mouse in a freely moving mouse. Triangles indicate STZ injection time. The mouse is the same animal in supplementary Fig. 2. The *Per1* gene expression in the OB and liver were measured by double recording. **B, C, D.** *Per1* gene expression in the liver, magnified areas ①, ② and ③ in the Fig. 2A, respectively. Day 0 indicates STZ injected day.

Figure 3. Effects of STZ on locomotor activity rhythm

A. Double-plotted actograms showing a *Per1-luc* Tg mouse in constant darkness. Activity records are expressed in black histograms of activity counts in 5 min bins and double-plotted so that 48 h are shown on the x-axis and consecutive days on the y-axis. The red (strait) line shows the regression line fitted to the onset of activity time (CT12). Triangle indicates STZ injection time. The duration of recording *Per1* gene expression in supplementary Fig. 2 and Fig. 2 are indicated by vertical lines ①, ② and ③.

B. Analysis of $\chi 2$ periodograms for behavior activity rhythm. Horizontal axis shows the period (hours), the vertical axis shows the amplitude. The oblique line in the periodogram indicates a significance level of p=0.01. **C (left)**. Locomotor activity analysis of *Per1-luc* Tg mice. Each plot indicates the mean ± S.E. (n=6). Statistical significance as detected by Student's test (***P < 0.001 vs. D). N and D indicated subjective night and day respectively. **C (Right).** Period of locomotor activity. Each plot indicates the mean ± S.D. (n=6).

Figure 4. STZ induced *Per1* expression in back skin hairs.

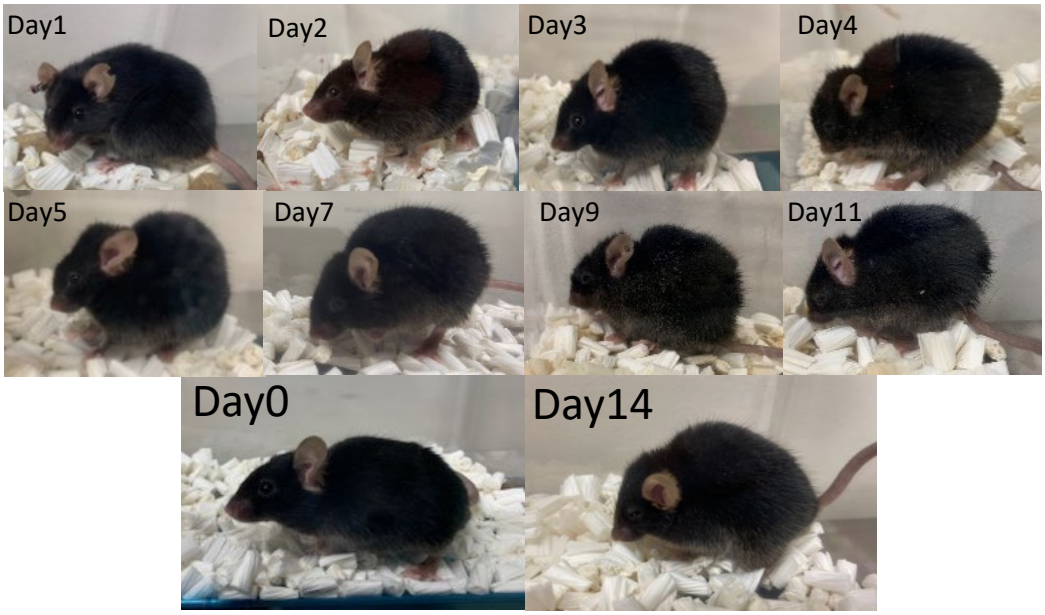
A. STZ-induced *Per1* expression in hairs. Day 0 is the STZ injection day. Bioluminescence of back skin hairs was measured every 10 sec and expressed as a value per hair (n=5). **B**. Glucose content in the blood after STZ injection (n=5). **C.** Drinking amount after STZ injection (n=5). Each value represents the mean ± S.D. *$p<0.05$, one-way ANOVA followed by Dunnet's test.

Table 1.

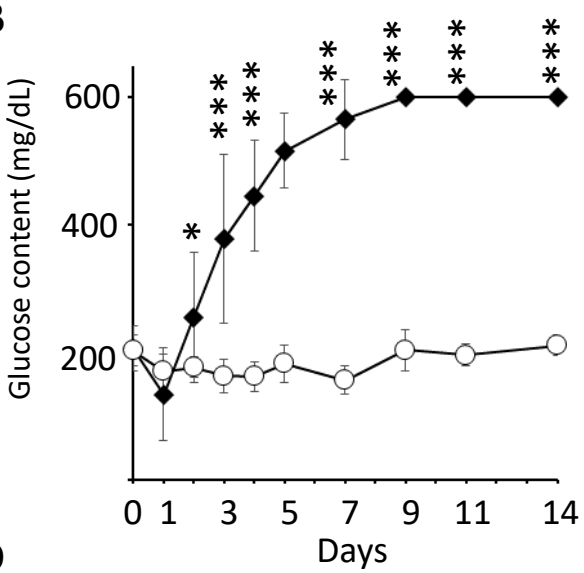
Effects of STZ on circadian *Per1* expression and locomotor activity.

OB: Olfactory bulb, Pre: before STZ administration, Post: after STZ administration, - : no rhythmic expression.

A



B



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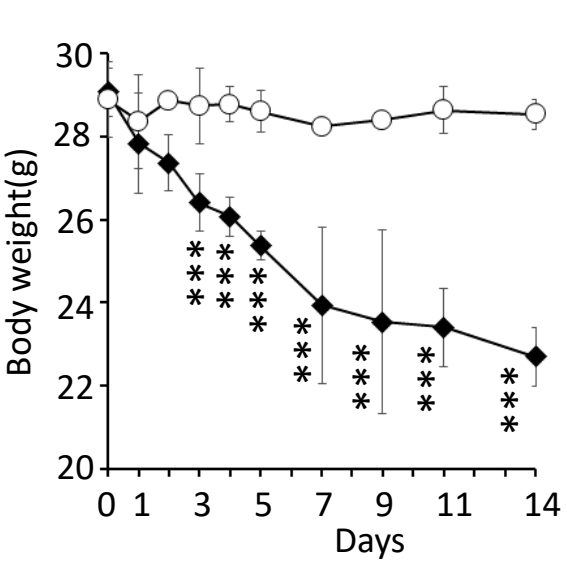


D

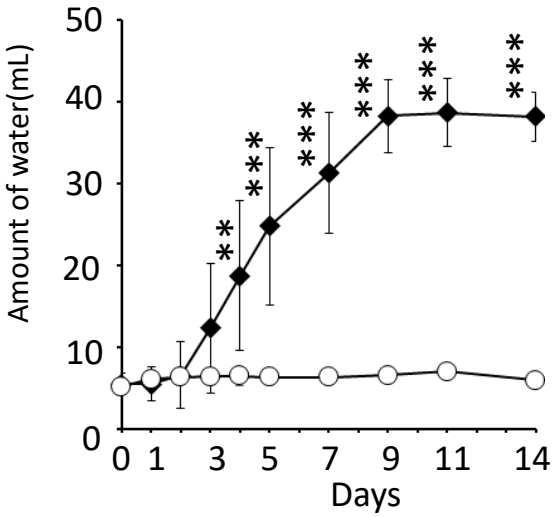
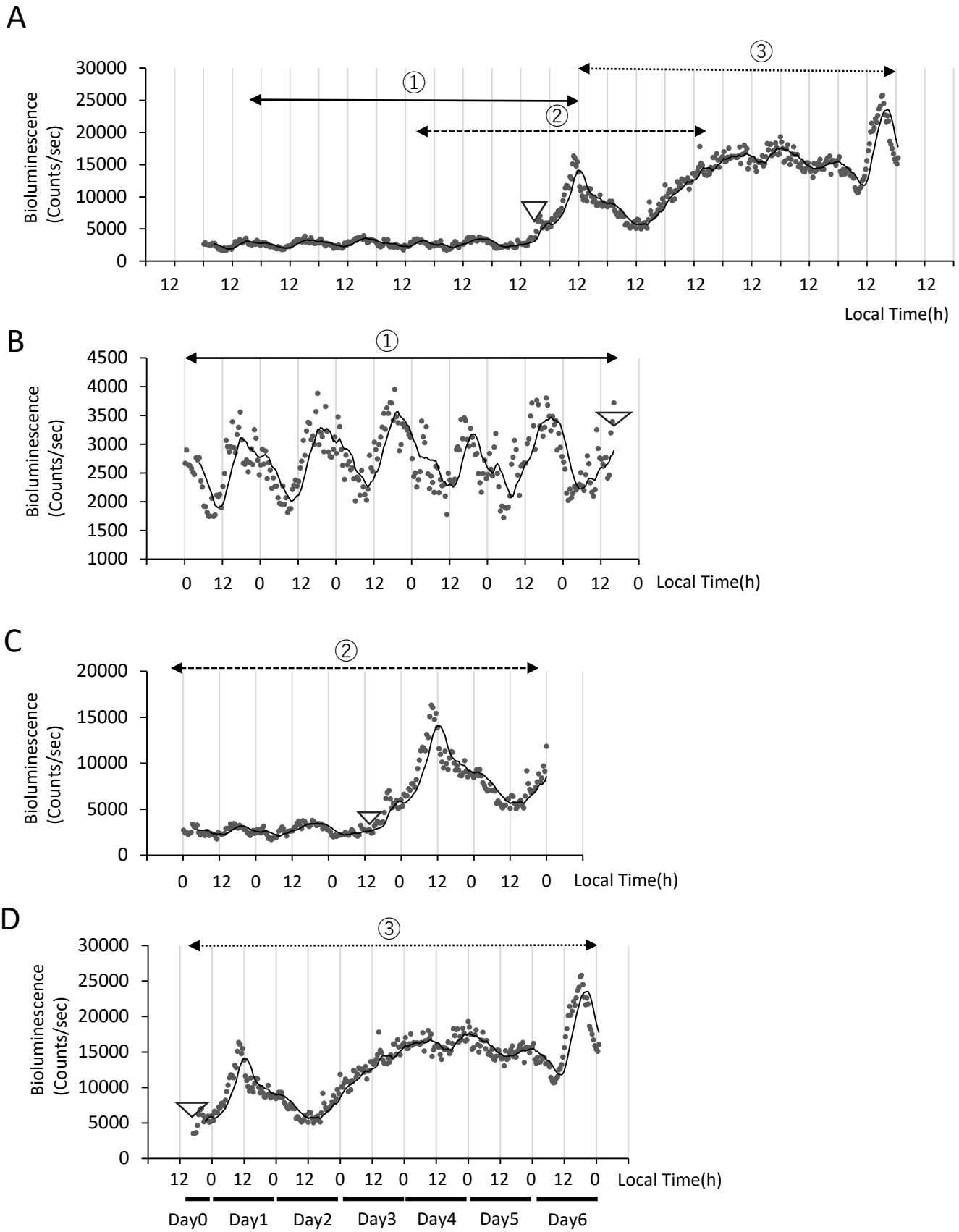


Figure 1


A
Bioluminescence (Counts/sec)
30000
25000
20000
15000
10000
5000
0
①
②
③
12 12 12 12 12 12 12 12 12 12 12 12 12 12
Local Time(h)
B
Bioluminescence (Counts/sec)
4500
4000
3500
3000
2500
2000
1500
1000
①
0 12 0 12 0 12 0 12 0 12 0 12 0
Local Time(h)
C
Bioluminescence (Counts/sec)
20000
15000
10000
5000
0
②
0 12 0 12 0 12 0 12 0 12 0
Local Time(h)
D
Bioluminescence (Counts/sec)
30000
25000
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15000
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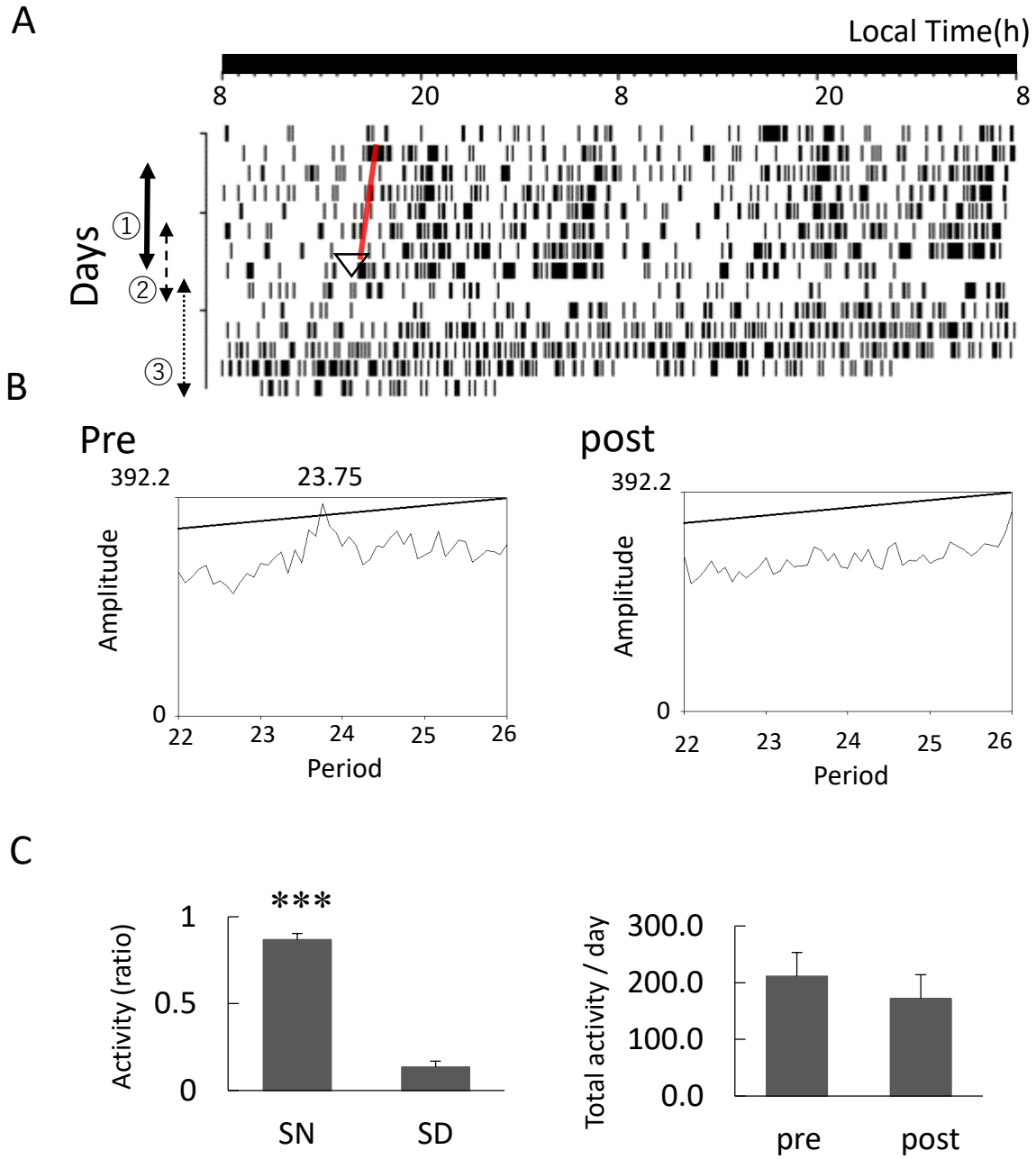
Day0 Day1 Day2 Day3 Day4 Day5 Day6

Figure 2


A
Local Time(h)
8
20
8
20
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Days
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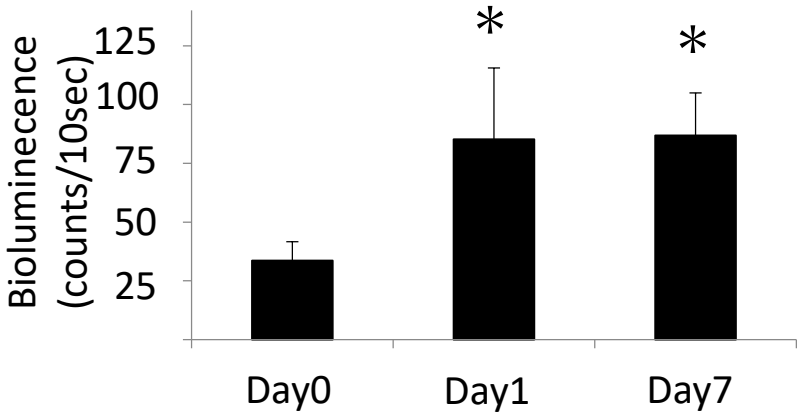
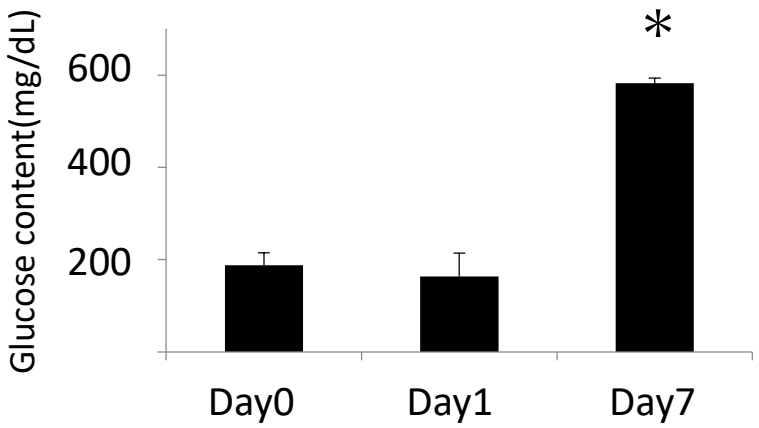
B
Pre
post
392.2
23.75
Amplitude
0
22
23
24
25
26
Period
392.2
Amplitude
0
22
23
24
25
26
Period
C
***
Activity (ratio)
1
0.5
0
SN
SD
Total activity / day
300.0
200.0
100.0
0.0
pre
post

Figure 3

A *Per1* expression



B Glucose content



C Drinking activity

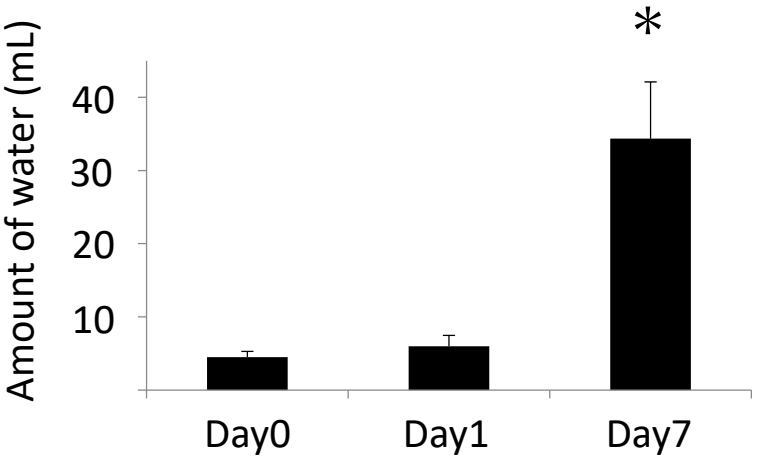


Figure 4

Pre	OB (n=8)	Liver (n=8)	Behavior (n=8)
	Mean ± S.D.	Mean ± S.D	Mean ± S.D.
Peak time(CT)	10. 8 ± 0. 5 h	13. 0 ± 1. 1 h	
Period	23. 8 ± 1. 2 h	24. 4 ± 0. 8 h	23.6 ± 0. 2 h

Post	OB (n=8)	Liver (n=8)	Behavior (n=8)
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Peak time(CT)	–	–	
Period	–	–	–

Photon counts	OB (n=4)	Liver (n=4)
(ratio : Post/Pre)	Mean ± S.D.	Mean ± S.D.
Pre	1703. 5 ± 811. 8	3427. 6 ± 1806. 7
Post	3220. 9 ± 2411. 7	11988.7 ± 6035. 1
Ratio	1. 7 ± 0.5	3. 5 ± 0.5