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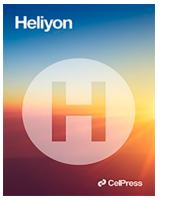

Research article

Cholecystokinin receptor type A are involved in the circadian rhythm of the mouse retina

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A B S T R A C T

The retina is the only organ projecting external light to the suprachiasmatic nucleus. Cholecystokinin receptor type A (Cckar/*Cckar*) is one of the essential factors for light reception in retinal cells. As there was a lack of literature on the matter, we aimed to elucidate the cause of the time-dependent phase change in clock gene expression. We found that *Cckar* mRNA expression in retinal cells exhibited diurnal variations. The rhythm of expression of the clock gene *Per1/Per2* in retinal cells was altered in *Cckar*$^{-/-}$ mice. The light sensitivity of retinal cells was evaluated in wild-type mice, which showed c-Fos was activated in the ganglion cell layer more than in the inner granular layer. This increase in the number of c-Fos-positive cells was suppressed by lorglumide, a Cckar antagonist. Treatment of rat retina primary cells with lorglumide suppressed *Per2* transcription, which was altered in a time-dependent manner relative to the *Per2* expression. Light irradiation studies in *Cckar*$^{-/-}$ mice did not exhibit an increase in *Period* expression in the suprachiasmatic nucleus. These results indicate that Cckar is among the factors that regulate the cycle of clock genes on the retina. *Cckar* knockout attenuates the light responsiveness of suprachiasmatic nucleus and reduces the expression amplitude of *Period* genes in the retina. Thus, Cckar may contribute to entrainment of the light environment and maintenance of the expression cycle of *Period* gene, which is one of the core clock genes.

1. Introduction

Circadian rhythms with a cycle of approximately 24 h are recognized in physiological phenomena such as the sleep-wake cycle, body temperature, and hormone secretion, and play a fundamental role in maintaining homeostasis in living organisms. In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus of the midbrain is the central clock and regulates the clocks of peripheral organs throughout the body via hormones and neurotransmission [1,2]. The circadian clock synchronizes the phase of biological rhythms to the external environment by external factors such as light, diet, and drugs [3]. Light entrainment is the most powerful entrainment signal in the external environment. Light activates photoreceptors in retinal cells that project information to the SCN via the retinohypothalamic tract [4,5]. However, the detailed molecular mechanisms have not been established.

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Abbreviations

ANOVA	Analysis of variance
CCK	Cholecystokinin
CCK-8s	Cholecystokinin Octapeptide
Cckar	Cholecystokinin receptor type A
CT	Circadian time
DEX	Dexamethasone
GCL	Ganglion cell layer
INL	Inner granular layer
ipRGC	Intrinsically photosensitive retinal ganglion cells
LD	light-dark cycle
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
ZT	Zeitgeber time

Cholecystokinin (CCK), a neurotransmitter peptide stimulating the satiety center, is present in the peripheral organs and the brain. CCK and its receptors are widely distributed in the brain, including the cerebral cortex, hypothalamus, and brainstem [6,7]. Furthermore, it was reported that CCK is distributed in the SCN of rodents [8–10]. CCK receptors are present in the retinas of monkeys and amacrine cells in rats [11,12]. CCK receptors expressed on amacrine cells in the retina reportedly enhance the sensitivity to light signals [11,13].

Cck receptor type A (Cckar) was previously reported to be expressed in retinal amacrine cells, while functional Cckar expression was not observed in SCN [13]. Amacrine cells are neurons with more than 60 morphological differences that suppress retinal neurotransmission [14]. Obese model animal rats with spontaneous deletion of Cckar showed an attenuated response to light stimulation [15,16]. In particular, a decrease in resynchronization in response to changes in the light environment has been reported [17,18]. Light stimulation-induced pupillary reflexes were attenuated in *Cckar* knockout mice (*Cckar*$^{-/-}$ mice). Furthermore, light-induced phase changes in behavioral rhythms and enhancement of clock gene expression are attenuated in *Cckar*$^{-/-}$ mice [13,19].

Thus, previous reports indicate that Cckar are involved in the light signaling. However, it is unclear how the expression of clock genes in the retina is altered by the presence or absence of CCK signaling. In this study, we used *Cckar*$^{-/-}$ mice to analyze changes in clock gene expression in the retina. We also evaluated the effects on clock gene expression by treatment of primary cultured rat retinal cells with an agonist or antagonist of Cckar.

2. Materials and methods

2.1. Animals

To further clarify the relationship between the biological clock system and Cholecystokinin, we conducted our experiments using male mice, which are less affected by the sex cycle. Male *Cckar*$^{-/-}$ in an C57BL/6J background mice and wild-type C57BL/6J mice were generated as previously reported [13]. For each experiment, *Cckar*$^{-/-}$ male mice and age-matched wild-type male mice were used at 2–6 months of age. The mice were housed in a constant-temperature room at 23 ± 2 °C, fed ad libitum, and maintained under 12 h:12 h light/dark (LD) cycle conditions. The light-on time (07:00) was assigned as zeitgeber time 0 (ZT 0). These mice were bred alone for two weeks to complete synchronization with the light environment. All the animal experiments were approved by the Animal Welfare Committee of Kyushu University (A26-016-2).

2.2. Daily expression rhythm

At six time points (ZT 2, 6, 10, 14, 18, and 22), the mice were sacrificed by cervical dislocation under isoflurane hyperesthesia, and the retina and brain were rapidly removed. Coronal brain sections (1 mm) were prepared using a brain matrix (MBS-S1C; BrainSienceIdea) and SCN sections were harvested by scalpel excision. The excised organs were used for immunostaining and gene expression analyses.

2.3. Light-induced expression

Light-induced expression experiments were conducted as previously described [20]. Mice were kept under LD (light, 12 h: dark, 12 h) conditions for 2 weeks to acclimate to the light/dark environment and then switched to a constant-dark environment with a 24 h dark period. Two days after the change to the constant-dark condition, the mice were exposed to 20 or 80 lx light for 15 min at Circadian Time 16 (CT16: sleep on-set time was CT0). One hour after irradiation, the mice were sacrificed for retinal and SCN sampling.

2.4. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Retinal samples were homogenized, and total RNA content was extracted as per the protocol of the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA purity was determined by an absorbance ratio of 1.8–2.0 at 260/280 nm, and quality was confirmed by the rRNA band ratio by an Experion™ automated electrophoresis station (Bio-Rad Life Science Research, Hercules, CA, USA). A total of 200 ng of total RNA was reverse-transcribed according to the protocol of the SuperScript® VILO cDNA Synthesis Kit (Invitrogen; Thermo Fisher, Waltham, MA). Quantification of *Cckar*, *Per1*, and *Per2* mRNA was performed by real-time RT-PCR using an Applied Biosystems® 7500 real-time PCR (Applied Biosystems, Foster City, CA). Relative quantification was performed using the ΔΔCt method with β-actin as an internal standard. Primer/probe sets were purchased from Applied Biosystems (Table 1 and Table 2).

2.5. Intravitreal administration

Mice housed for two weeks under 12 h:12 h light/dark conditions were switched to constant dark conditions, and a mixed injectable anesthetic of medetomidine hydrochloride (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg) was administered intraperitoneally at CT16 on the following day. Subsequently, 10 μL of 3 μM lorglumide (Sigma-Aldrich, St Louis, MO, USA) or saline was administered intraocularly. Following 30 min of intraocular administration, the eyeball and SCN were irradiated with 80 lx light for 15 min. Transperfusion fixation was performed with 4 % PFA; the samples were removed, placed 8 h in 4 % PFA, and then soaked in 20 % sucrose. Sections (10 μm thick) were prepared using a cryostat (Thermo Fisher Scientific) and washed with 0.1 % Triton-PBS (PBS containing 0.1 % Triton X-100 (Sigma-Aldrich)); endogenous peroxidase was removed using 0.5 % H_2O_2. After blocking with 0.3 % Triton-PBS with 1 % goat serum (Vector Labs, Burlingame, CA, USA), the cells were exposed to 1:2500 anti-*c*-Fos rabbit IgG (Calbiochem, Nottingham, UK) as the primary antibody and incubated at 4 °C for 48 h. Sections were incubated with 1:200 biotin-conjugated anti-rabbit IgG (Vector Labs) as a secondary antibody at room temperature for 1 h, and with avidin and biotin-conjugated peroxidase (Vector Labs) at room temperature for 1 h. Staining was performed using metal-enhanced DAB (Thermo Fisher Scientific), and the sections were attached to glass slides. The sections were dehydrated with ethanol and xylene and mounted using Entellan® New (Merck Millipore, Billerica, MA, USA). For each section, a 200 μm wide region was randomly selected, and the number of cFos-positive cells expressed in the ganglion cell layer (GCL) and the inner granular layer (INL) of that region was counted. The average number of positive cells was calculated in 3 fields per mouse and the quantitative evaluation was performed from 6 mice per group (18 fields per group).

2.6. Cell culture and in vitro experiments

Primary rat retinal cells were purchased from Lonza Japan Ltd. (R-Ret-Neo: Tokyo, Japan). Cells were maintained in PNGM™ Primary Neuron Growth Medium BulletKit™ (Lonza) supplemented with 5 % FBS and 0.5 % penicillin-streptomycin at 37 °C in a humidified 5 % CO_2 atmosphere.

Cells were suspended in PNBM™ Primary Neuron Basal Medium and seeded into a 48-well plate (Falcon, NY, USA) at a density of 50,000 cells per well. Cells were treated with 100 nM dexamethasone for 2 h to synchronize the intracellular clock mechanism. 24 h later, Cckar analogs Cholecystokinin Octapeptide sulfated (CCK-8s; 300 nM; Abcam, Cambridge, UK) and Lorglumide (300 nM), a Cckar blocker, was administered. Cells were collected 4 h after addition and total RNA was extracted with the aforementioned kit. After reverse transcription, the expression level of *Per2* mRNA was measured by qRT-PCR.

Cells were seeded under the same conditions as above. Cells were treated with 100 nM dexamethasone for 2 h to synchronize the

Table 1
Primer sets for qPCR analysis of gene expression for mouse sample.

Gene	Reporter sequence	Assay ID	Amplicon length(bp)
Cck1r	CACCTACTTCATGGGCACTTCCGTG	Mm00438060_m1	63
Per1	AGCCCCTGGCTGCCACCATGGCCCC	Mm00501813_m1	106
Per2	CCTCCAACATGCAACGAGCCCTCAG	Mm00478113_m1	73
Actin	ACTGAGCTGCGTTTTACACCCTTTC	Mm00607939_s1	115

Table 1**Probe sets for qPCR analysis of gene expression.** List of probes for qRT-PCR to measure each mRNA expression level in mice.

Table 2
Primer sets for qPCR analysis of gene expression for rat sample.

Gene name	Accession number	F/R	Primer sequence	Product length(bp)
Per2	XM_039084159.1	F	ACACCAAACTGCCTGTTCCA	140
		R	TTCTTGTGGATGGCGAGCAT	
Actin	NM_031005.4	F	GCTGCACCCTTCAACAACTG	93
		R	GGTCAGTCCCTGGATCTCCT	

Table 2 **Primer sets for qPCR analysis of gene expression.** Primer list for qRT-PCR to measure the expression level of each gene in rat retinal cells.

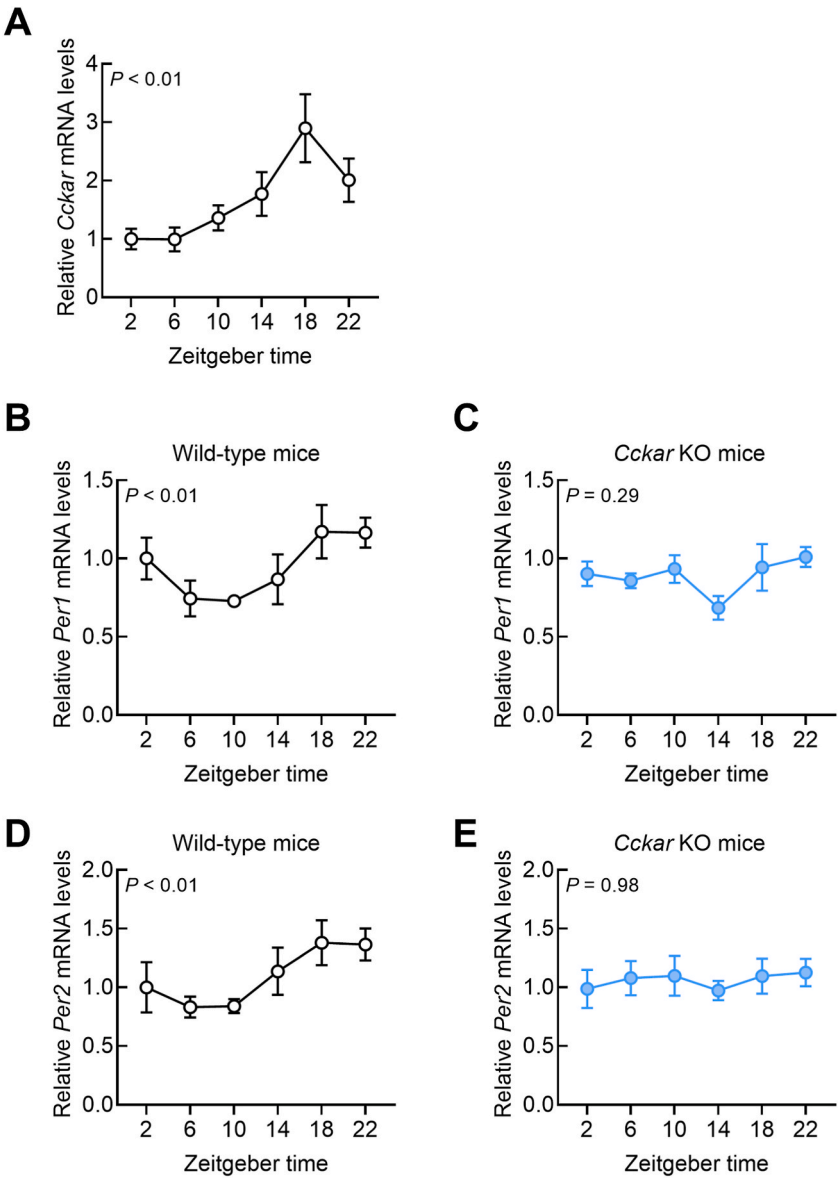

Fig. 1. Influence of *Cckar*$^{-/-}$ mice on the amplitude of clock gene expression in the retina. (A) Temporal-expression profiles of *Cckar* mRNA in the retina of wild-type mice. Each value represents the mean with S.E.M. of results for six mice. There were significant time-dependent variations in *Cckar* mRNA levels in wild-type mice ($P < 0.01$; cosinor method). (B, C) Temporal-expression profiles of *Per1* mRNA in the retina of wild-type (B) and *Cckar*$^{-/-}$ mice (C). (D, E) Temporal-expression profiles of *Per2* mRNA in the retina of wild-type (D) and *Cckar*$^{-/-}$ mice (E). Each value represents the mean with S.E.M. of results for six mice. The probe list used for quantification is described in Table 1. *P*-values in the graph were the result of the cosinor method.

intracellular clock mechanism. The culture medium was then changed to new medium with 1 % FBS. DMSO (Vehicle), CCK-8s (300 nM), and Lorglumide (300 nM) were added 4 h after 100 nM Dexamethasone (DEX) synchronization. Each reagent (DMSO, CCK-8s, Lorglumide) was added at a final concentration of 0.5 % per well. Cells were collected at 20, 24, 28, 32, 36, 40, 44 and 48 h after the DEX synchronization and total RNA was extracted with the aforementioned kit. The expression level of *Per2* mRNA was measured by qRT-PCR.

2.7. Statistical analysis

Results are expressed as the mean ± SEM. Each value expresses the value of ZT2 of the wild-type mouse as a standard (1.0). Statistical analyses were performed using GraphPad Prism software (ver. 8; GraphPad Software, San Diego, CA, USA). Differences among groups were analyzed using two-way ANOVA followed by Tukey's post-hoc tests; $P < 0.05$ was considered statistically

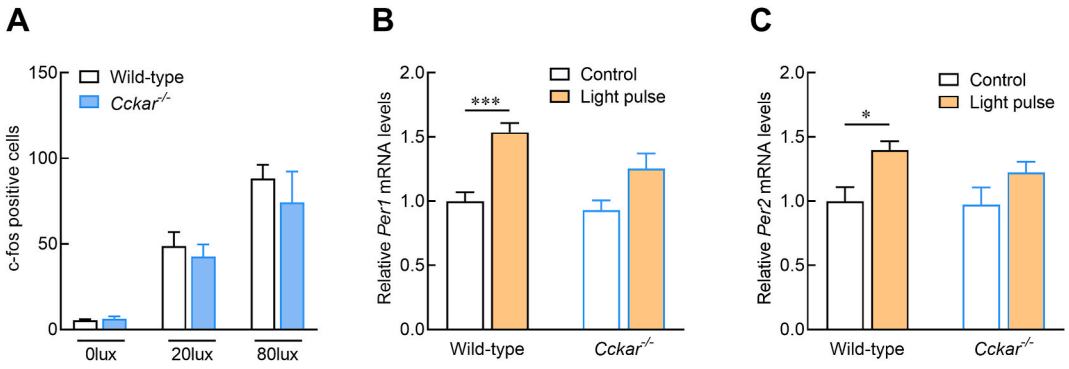

Fig. 2. Light-induced alterations in clock gene expression in the SCN of wild-type and *Cckar*$^{-/-}$ mice. (A) Influence of light intensity on the number of c-fos positive cells in the suprachiasmatic nucleus (SCN) of wild-type and *Cckar*$^{-/-}$ mice. The number of c-fos positive cells did not significantly change with light intensity. Each value represents the mean with S.E.M. of results for six to eight mice (wild-type: six, *Cckar*$^{-/-}$: eight). (B, C) Light-induced changes in *Per1* mRNA (B) and *Per2* mRNA (C) expression in the SCN of wild-type and *Cckar*$^{-/-}$ mice. Each value represents the mean with S.E.M. of results for eight mice. The probe list used for quantification is described in Table 1. *$P < 0.05$, ***$P < 0.001$; significant difference between the two groups (two-way ANOVA with Tukey's post-hoc test).

significant. For the analysis of diurnal variation, the cosinor method analysis was performed using the cosinor program (R. Refinetti, http://www.circadian.org/softwar.html), and there was a significant diurnal variation at $P < 0.05$.

3. Results

3.1. Disappearance of diurnal variation of clock genes Per1 and Per2 due to Cckar deficiency

To examine the variation in Cckar expression in the retina, we quantified *Cckar* mRNA expression every 4 h under 12:12 h light/dark conditions (Fig. 1A). There was a significant diurnal variation in Cckar expression in the retina of wild-type mice (cosinor method, $P < 0.01$; mesor, 1.67; acrophase, 17.84; amplitude, 0.87). *Cckar* mRNA expression peaked at ZT18 and was up to three-fold higher than that at the trough.

It is unclear how the rhythm of Cckar expression affects the expression of clock genes. To investigate the temporal biological effects of Cckar deficiency, we compared the diurnal variation in the expression of clock genes *Per1* and *Per2* in the retinas of wild-type and *Cckar*$^{-/-}$ mice. In wild-type mice, significant diurnal variation and increased nocturnal expression of both *Per1* and *Per2* were observed (Fig. 1B and D, cosinor method; *Per1* in wild-type mice; mesor = 0.95, amplitude = 0.25, acrophase = 20.70, $P < 0.01$, *Per2* in wild-type mice; mesor = 1.09, amplitude = 0.31, acrophase = 19.40, $P < 0.01$). In contrast, in *Cckar*$^{-/-}$ mice, there was no statistically significant diurnal variation in *Per1* or *Per2* in the retina (Fig. 1C and E, *Per1* in *Cckar*$^{-/-}$ mice; $P = 0.290$, *Per2* in *Cckar*$^{-/-}$ mice; $P = 0.981$). These results indicate that Cckar expression is closely related to the amplitude of retinal clock gene expression.

3.2. Alteration of per gene expression in SCN-induced light irradiation in Cckar$^{-/-}$ mice

Light stimulation induces neurotransmission originating from the influx of Ca^{2+} in retinal cells [21]. Ca^{2+} influx signaling causes induction of CREB, ERK, and cfos expression [22]. The expression rhythm of the Cckar correlated with *Per* expression in the mouse retina. Since the Cckar is involved in neural activation via Ca^{2+} influx in the retina [13], we hypothesized that the presence or absence of the receptor alters the expression level of clock genes in the SCN due to light sensitivity. To clarify the relationship between the Cckar and light stimulation, we evaluated how clock genes in the SCN are altered upon light irradiation in wild-type and *Cckar*$^{-/-}$ mice.

Mice reared under constant dark conditions were exposed to 20 or 80 lux light for 15 min. The number of cFos-positive cells in the SCN was unchanged between the wild-type and *Cckar*$^{-/-}$ mice (Fig. 2A). However, the expression levels of *Per1* and *Per2* in the SCN of wild-type mice were significantly elevated (Fig. 2B and C). In contrast, the induction of *Per* expression was weak in the SCN of *Cckar*$^{-/-}$ mice (*Per1*, $P = 0.059$; *Per2*, $P = 0.312$). These results indicate that the conventional pathway is well-functioning for photosensitive nerve firing in the SCN of *Cckar*$^{-/-}$ mice. However, *Period* expression in the SCN in *Cckar*$^{-/-}$ mice was not upregulated, indicating that Cckar contribute significantly to the induction of *Per* expression.

3.3. Effect of Cckar antagonists on photostimulation response in the retina

We examined how the response to light stimulus changed under the administration of a Cckar antagonist. The number of cFos-positive cells in the retina was examined in the INL and GCL, respectively (Fig. 3A–C).

In the GCL, a significant increase in the number of cFos positive cells by light irradiation was observed in both the saline group and the lorglumide (Cckar blocker) administration group (Fig. 3B, Saline; 2.15 ± 0.20 vs 6.53 ± 0.05, $P < 0.001$; Lorglumide; 2.31 ± 0.20

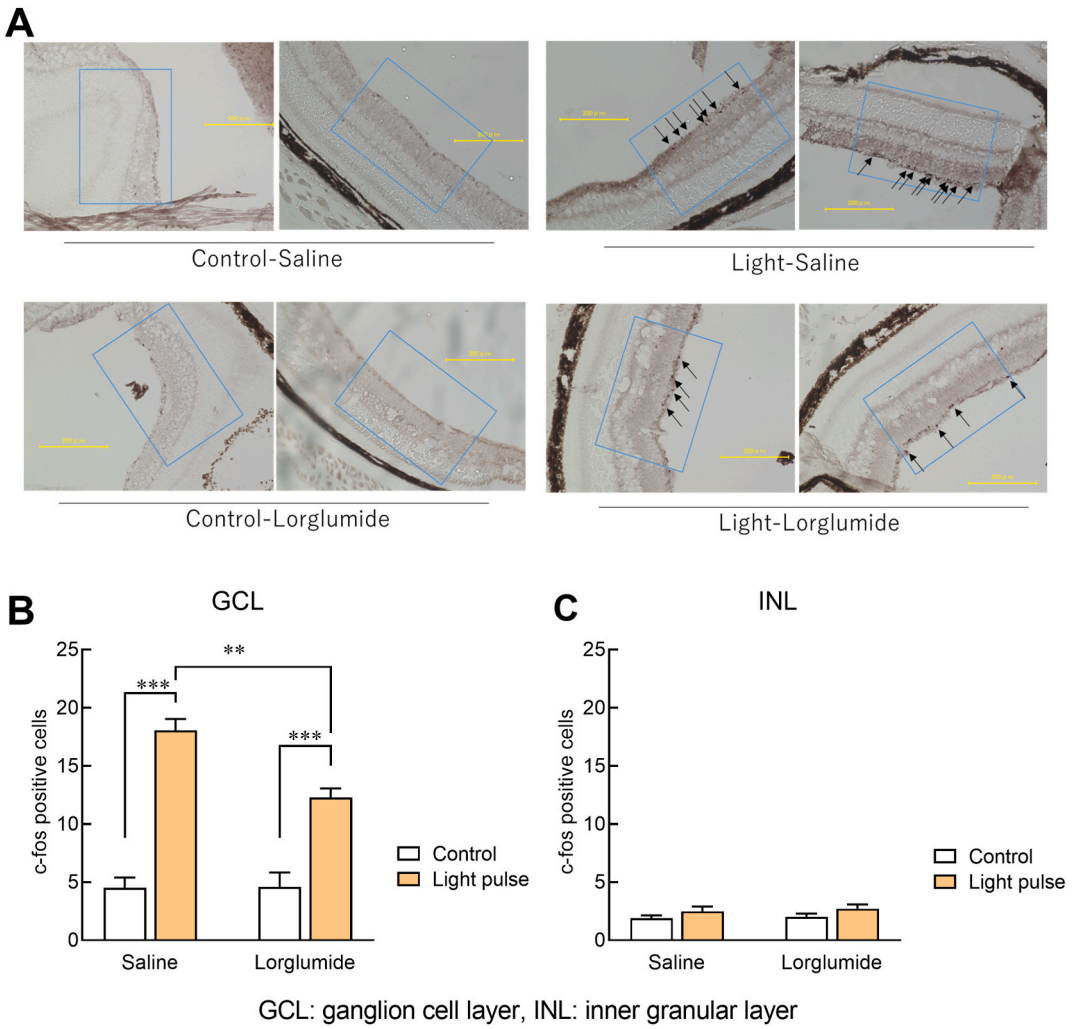

Fig. 3. Light-induced c-fos positive cell expression in the retina of mice with lorglumide. (A) Microscopic images of c-fos-positive cells in the mouse retina; arrowheads in the GCL indicate c-fos-positive cells. A 200 μm wide region was randomly selected, and the number of cFos-positive cells expressed in GCL and INL of that region was counted. (B, C) Effect of lorglumide on light-induced c-fos-positive cells in GCL (B) and INL (C). Each value represents the mean with S.E.M. of results for six mice. $^{**}P < 0.01$, $^{***}P < 0.001$; significant difference between the two groups (two-way ANOVA with Tukey's post-hoc test). GCL: ganglion cell layer, INL: inner granular layer.

vs 4.85 ± 0.13, $P = 0.002$). However, the increase in the number of positive cells in the lorglumide-treated group was significantly suppressed ($P = 0.014$) compared to that in the saline group. In contrast, in the INL, there was a tendency to increase the number of positive cells by light irradiation in both groups, but the difference was not significant (Fig. 3C).

3.4. Changes in Per2 mRNA expression in rat retinal cells in response to Cckar stimulation and blockade

Although Cckar expression is related to the amplitude of clock gene expression in the retina, it remains unclear whether CCK signaling is related to *Per2* expression. To determine the extent to which CCK signaling affects Per2 expression, we evaluated the effects of CCK-8s, a Cckar analog, and lorglumide on primary cultured rat retinal cells. In the CCK-8s-treated group, a temporal expression of *Per2* mRNA was increased 4 h after CCK-8s treatment (Fig. 4A). In contrast, a decrease in *Per2* mRNA expression was detected in the lorglumide-treated group, and a significant decrease in *Per2* mRNA expression was observed after 4 h compared to that in the DMSO group ($P < 0.01$).

Moreover, we measured the effects of CCK-8s and Lorglumide on *Per2* mRNA expression levels synchronized by 100 nM dexamethasone. Rat retinal cells were synchronized by addition of DEX, then CCK-8s or lorglumide was added 4 h after medium change. The mRNA was then collected every 4 h starting 20 h after synchronization. The expression of *Per2* mRNA exhibited a significant circadian variation in rat retinal cells from 20 to 48 h after the treatment with DEX for 2 h (Fig. 4B and C; cosinor method, $P < 0.001$). In the CCK-8s-added group, the amplitude was larger and the expression of Per2 was significantly higher than in the DMSO group

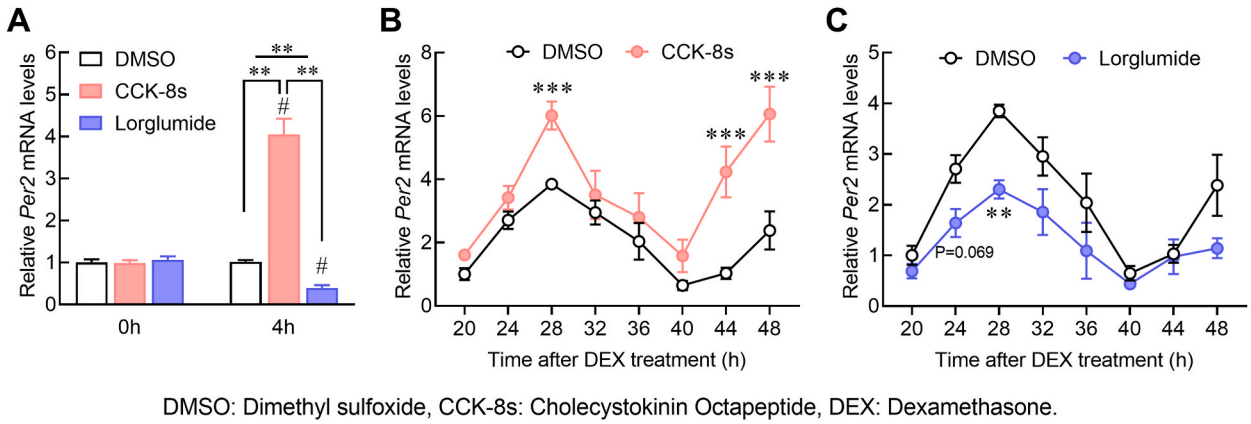

Fig. 4. Influence of CCK receptor ligands on Per2 expression activation. (A) Effects of CCK-8s and lorglumide on *Per2* mRNA expression. Each value represents the mean with SEM of results for four samples. $^{**}P < 0.01$; significant difference between the two groups; $\#P < 0.05$; significant difference from 0 h at corresponding groups (two-way ANOVA with Tukey's post-hoc test). (B) Temporal expression profiles of the *Per2* mRNA in rat retinal cells after DEX treatment with DMSO or CCK-8s. Each value represents the mean with S.E.M. of results for three samples. The mean of 20 h Per2 expression levels in DMSO group were set to 1. (C) Temporal expression profiles of the *Per2* mRNA in rat retinal cells after DEX treatment with DMSO or lorglumide. Each value represents the mean with S.E.M. of results for three samples. The mean of 20 h Per2 expression levels in DMSO group were set to 1. The results for the DMSO-treated groups in Fig. 4B and C are represented identically. The primer list used for quantification is described in Table 2. $^{**}P < 0.01$, $^{***}P < 0.001$; significant difference between the two groups at the corresponding time (two-way ANOVA with Tukey's post-hoc test). The results of the analysis by the Cosinor method are listed in Table 3. DMSO: Dimethyl sulfoxide, CCK-8s: Cholecystokinin Octapeptide, DEX: Dexamethasone.

Table 3
Analysis details in Fig. 4.

1) Two-way ANOVA						
	Time		Treatment		Interaction	
	F value	P value	F value	P value	F value	P value
Fig. 4A	F(1,3) = 35.31	**P = 0.009**	F(1.13,3.39) = 62.30	**P = 0.003**	F(1.01,3.03) = 112.4	**P = 0.002**
Fig. 4B	F(7,28) = 8.349	**P < 0.0001**	F(7,28) = 35.85	**P < 0.0001**	F(1,4) = 435.0	**P < 0.0001**
Fig. 4C	F(7,28) = 3.945	**P = 0.004**	F(2.23,8.93) = 39.49	**P < 0.0001**	F(1,4) = 67.32	**P = 0.001**

2)Cosinor analysis					
Fig. 4B and C	Amplitude	Mesor	Acrophase (h)	Period (h)	P value
DMSO	1.674	2.175	29.53	16.8	**P < 0.0001**
CCK-8s	2.275	3.575	30.2	15.5	**P < 0.0001**
Lorglumide	0.710	1.361	25.53	17.2	**P = 0.003**

Table 3 **Statistics for the data listed in Fig. 4.** 1) Two-way ANOVA results for the data listed in Fig. 4A–C. 2) Statistical analysis of the time variation of *Per* 2 mRNA expression levels using the Cosinor method, as shown in Fig. 4B and C.

(Fig. 4B, cosinor method; DMSO group; mesor = 2.175, amplitude = 1.674, $P < 0.001$, CCK-8s group; mesor = 3.575, amplitude = 2.275, $P < 0.001$). In contrast, *Per2* mRNA in the lorglumide group showed a smaller amplitude and significantly lower expression than in the DMSO group. (Fig. 4C, cosinor method; lorglumide group; mesor = 1.361, amplitude = 0.710, $P < 0.01$). Furthermore, compared to the *Per2* mRNA period in the DMSO group, the period was shorter in the CCK-8s group and longer in the lorglumide group (DMSO, 16.8 h; CCK-8s, 15.5 h; Lorglumide, 17.2 h). This suggests that CCK signaling affects changes in Per2 expression in retinal cells.

4. Discussion

Although Cckar influence light sensitivity and retinal nerve activation [13,23], their effect on retinal clock gene expression is unclear. In this study, diurnal variation was observed in the expression of Cckar in the retina of mice. Deletion of the Cckar caused loss of rhythm and low expression of *Per* in the retina (Figs. 1 and 4). The Cckar inhibitor varied the expression rhythm of the *Per2* mRNA in retinal cells (Fig. 4C). These results indicate that the rhythm of Cckar expression may be involved in the temporal biological regulation of the retina.

Fig. 1A shows that Cckar expression was upregulated in mouse retinal cells at night (ZT18). The Cckar is localized to glycine receptor-positive amacrine cells in the retina [13]. The main function of amacrine cells is to receive glutamatergic input from bipolar cells and inhibit neurotransmission in ganglion cells [24]. Amacrine cells are characterized by individual neurotransmitter

receptor-positive cells that differ from one another [25]. Therefore, the inhibitory system in these cells is controlled by transmitters such as GABA, glycine, and dopamine [24]. Intrinsically photosensitive retinal ganglion cells (ipRGCs), which are blocked by signals from amacrine cells, exhibit neuronal firing even without light stimulation [26]. Therefore, amacrine cells play a fundamental role in stabilizing information transmission in the retina. Previous studies have shown that retinal amacrine cells in mice undergo neuronal firing induced by CCK-8s [13], and Cckar signal rhythm may exist in vivo due to fluctuations in receptor expression. Although the temporal biological role of this fluctuation in signal intensity requires further study, it is likely that the increased expression of the Cckar during the dark period enhances the transmission of the inhibitory system and inhibits spontaneous nerve firing of ipRGCs.

In *Cckar*$^{-/-}$ mice, there was no diurnal variation in the mRNA expression of retinal *Per1/Per2* (Fig. 1B–E). Time-dependent photosensitivity is reduced in mice with mutations in clock genes [27]. The behavioral cycle of *Cckar*$^{-/-}$ mice under constant dark conditions did not differ from that of wild-type mice [13]. Therefore, it is assumed that the expression of clock genes in the SCN of *Cckar*$^{-/-}$ mice is nearly unchanged. The loss of the expression rhythm of clock genes in the retina is considered to be a retina-specific phenomenon due to the loss of the expression rhythm of the Cckar, and not of the SCN-derived biological clock. As Cckar expression is associated with photosensitivity in the retina, the receptor may be involved in the generation of time-dependent photosensitivity.

The expression of c-Fos in the inner granular layer did not significantly change in either the VEH or lorglumide groups, and no significant differences were observed with or without light irradiation. We have not performed any validation on the number of cfos-positive cells after photoreception using *Cckar*$^{-/-}$ mice, however, we consider that cfos-positive cells are reduced in *Cckar*$^{-/-}$ mice as well as in the lorglumide-added group. We originally reported that light sensitivity is reduced in *Cckar*$^{-/-}$ mice [13]. These reports suggest that CCK-Cckar signaling contributes to the increase in cfos-positive cells in photosensitivity.

In light irradiation experiments in the ganglion cell layer, the expression of clock genes in dopaminergic amacrine cells increased [28,29]. Amacrine cells regulate the neuroinhibitory system not only in bipolar and ganglion cells but also in amacrine cells. Dopaminergic neurons have receptors for glutamate, GABA, and glycine [30]. The stimulation of AMPA/Glutamate pathway through glycinergic amacrine cells is mainly a signal from on-type dipolar cells from rods by weak light [31,32]. One reason Cckar deficiency was not associated with the effect of light stimulation in this study may be that there is a non-glycinergic amacrine-cell-mediated pathway for signals from the cones. In contrast, a loss of diurnal variation in Cckar-derived Cck signaling may disrupt the rhythm of clock gene expression in dopaminergic neurons in *Cckar*$^{-/-}$ mice. Further investigation of the network between amacrine cells is required to elucidate the mechanism of circadian rhythm maintenance in the retina.

In vitro studies on the association between Cckar signaling and clock genes showed that Per2 expression was promoted by CCK-8s, a Cckar analog. CCK is secreted by neurons, especially from astrocytes and the cornea, as well as the gastrointestinal tract [33,34]. Since secretion on the retina has not been reported, CCK function as a neurotransmitter from other organs to the retina. In view of the results in Fig. 4, CCK-Cckar signaling on the retina might be a hormone that affects the expression level of Per2 in Cckar-positive cells.

Primary cultured rat retinal cells, composed of many cells derived from the retina, were used in this study. Therefore, a limitation of this study is that it is not possible to define which cells in the retina can become a phenomenon. Amacrine cells expressing Cckar are present in primary cultured rat retinal cells [35,36]. The percentage of glycinergic amacrine cells in the cells used in this study is unknown; however, they may be present. In this study, only the Period gene was evaluated as the clock gene to be evaluated. This is because it is representative of clock genes that respond to cFos. The expression sites of the period gene and the cfos-positive sites in the retina were in close proximity [37]. We have not yet evaluated whether cFos-positive cells are linked to cells with increased expression of the period gene. Future studies of the relationship between cfos positivity and changes in *Period* gene expression in Cckar expression-positive cells may clarify the physiological significance of Cckar.

The amplitude of clock gene expression in retinal cells is affected by stimuli from the external light environment and the SCN [38]. The retina is also a peripheral organ; however, it is known to have its own clock mechanism, separate from entrainment signals from the SCN [39]. Although multiple pathways are known to be involved in the process of neurotransmission from the SCN to peripheral organs [40,41], the clock mechanism in retinal cells remains unclear. The results of this study present a regulatory process for the retinal cell clock mechanism through photoreception. This study indicated that CCK signaling is one of the regulatory mechanisms of clock genes in retinal cells. Blockade of Cckar-CCK signaling attenuated the light-responsive clock-firing mechanism in retinal cells and SCN. Taken together, the disruption of Cckar-CCK signaling may lead to abnormalities in circadian clock machinery associated with altered light responsiveness. Because abnormalities in the machinery can cause the disruption of physiological homeostasis, further detailed analysis is expected to lead to the development of therapies targeting Cckar-CCK signaling for diseases with biological rhythm abnormalities.

Ethics statement

The animals used in this study were treated according to the guidelines stipulated by the Animal Care and Use Committee of Kyushu University. All experiments were conducted according to the protocol approved by the Internal Committee for Animal Experiments at Kyushu University (protocol ID A26-016-2).

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Data availability statement

The original manuscript presented in this study is included in the article. Further inquiries can be directed to the corresponding author.

CRediT authorship contribution statement

Yusuke Yamakawa: Writing – original draft, Project administration, Data curation, Conceptualization. **Yuya Tsurudome:** Writing – review & editing, Visualization, Software, Funding acquisition, Data curation. **Masaki Tamada:** Project administration, Methodology, Investigation. **Yuki Tsuchimochi:** Software, Resources, Methodology, Investigation. **Yuya Umeda:** Software, Investigation, Data curation. **Yuya Yoshida:** Visualization, Validation, Data curation. **Daisuke Kobayashi:** Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis. **Takehiro Kawashiri:** Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Toshio Kubota:** Visualization, Validation, Resources, Methodology. **Naoya Matsunaga:** Writing – review & editing, Software, Funding acquisition, Data curation. **Takao Shimazoe:** Writing – review & editing, Visualization, Validation, Resources, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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