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Running Title: Casein kinase inhibition delays peripheral clocks

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Key words

Circadian; Transcription factors; Suprachiasmatic nucleus; melatonin; corticosterone;

Abbreviations:

Actb, Beta actin;
Bmal1, Brain and muscle ARNT-like protein 1, *Arntl* and *Mop3*;
Nr1d1, nuclear receptor subfamily 1, group D, member 1, also known as *Rev erb alpha*;
Per1, Period 1;
Per2, Period 2;
Cry1, Cryptochrome 1;
Cry2, Cryptochrome 2;
Dbp, D site of albumin promoter (albumin D-box) binding protein;

Abstract

Circadian rhythms are generated through a transcription-translation feedback loop involving clock genes and the casein kinases CSNK1D and CSNK1E. In this study we investigated the effects of the casein kinase inhibitor PF-670462 (50 mg/kg) on rhythmic expression of clock genes in the liver, pancreas and suprachiasmatic nucleus as well as plasma corticosterone, melatonin and running behaviour in rats and compared them to the responses to a 4 hour extension of the light phase. PF-670462 acutely phase delayed the rhythmic transcription of *Bmal1*, *Per1*, *Per2* and *Nr1d1* in both liver and pancreas by 4.5 ± 1.3 hours and 4.5 ± 1.2 hours respectively 1 day after administration. In the suprachiasmatic nucleus the rhythm of *Nr1d1* and *Dbp* mRNA expression was delayed by 4.2 and 4 hours respectively. Despite these changes the time of peak plasma melatonin secretion was not delayed, although the plasma corticosterone rhythm and onset of wheel running activity were delayed by 2.1 hours and 1.1 hours respectively. These changes are in contrast to the effects of the 4 hour light extension, which resulted in delays in peak expression of the clock genes of less than 1 hour and no change in the melatonin or corticosterone rhythms. The ability of the casein kinase inhibitor to bring about large phase shifts in the rhythms of major metabolic target tissues may lead to new drugs being developed to rapidly phase adjust circadian rhythms to alleviate the metabolic impact of shift work.

1. Introduction

The circadian timing system of animals ensures that a wide range of physiological processes are appropriately timed across the day and night. In mammals, the suprachiasmatic nucleus (SCN) is the site of the master biological clock with its endogenous rhythmicity generated through clock gene transcription factors [1]. Through a transcription/translation feedback loop (TTFL) system the cycle of gene expression has a period close to 24 hours. The retinae are connected to the SCN via the optic nerve such that the TTFL system in the SCN is entrained to the solar day/night cycle. The SCN then alters a range of physiological systems via neuronal and hormonal output signals to the rest of the body [2] allowing the organism to respond to changes in season, trans-meridian travel or shift work. One such important neural pathway is a multisynaptic connection to the pineal gland which influences its production of the hormone melatonin [3]. Acute exposure to light during the night suppresses the production of melatonin in an intensity and wavelength dependent manner. The same light exposure also causes the induction of genes that are part of the TTFL system in the SCN resulting in a shift in the phase of the endogenous rhythm on subsequent cycles. The impact of the light is temporally gated such that exposure during the early dark period will delay the phase of the SCN rhythm, whereas light during the late dark period will result in an advance in the rhythm. An interesting feature of the circadian timing system and its response to light is that even under the most favourable experimental conditions, the largest acute phase shifts achieved with bright light are up to 4 hours [4-10] and vary with the timing and duration of the light pulse [11]. As a consequence, to shift rhythms by 12 hours as required with transmeridian travel, can take many days to achieve. Furthermore, there is growing evidence that during light induced phase shifting, the SCN and its peripheral targets respond at different rates, with liver and muscle taking up to a week to fully adapt to even moderate changes in lighting conditions [12]. A likely cause for this disconnection is the presence of an entrainable clock TTFL system in virtually all other cells and organs.

1 The basis of cellular rhythmicity in the SCN and peripheral organs has been extensively

2 researched, and while new modulators are still being discovered, the core mechanism operating

3 in mammals is quite clear [13]. In brief, the transcription factors CLOCK/BMAL1 drive the

4 expression of the period genes (*Per1* and *Per2*) and the cryptochrome genes (*Cry1* and *Cry2*).

5 The period and cryptochrome proteins in turn repress the CLOCK/BMAL1 induction of their

6 own transcription, thereby creating a negative feedback loop. An accessory loop involving

7 NR1D1 (REV ERB alpha) and NR1F1 (ROR alpha) repress or induce respectively *Bmal1* gene

8 expression to help the process to be self-sustaining. Stability of the proteins involved in the

9 transcription-translation feedback loop is a key factor in the maintenance of the near 24 hour

10 cyclicity found in all organisms. In particular phosphorylation of the period proteins, which

11 primes them as targets for degradation, is a key event.

12

13 The casein kinases CSNK1D and CSNK1E have a pivotal role in ensuring that clock gene

14 expression is rhythmic by phosphorylating the Period proteins and thereby marking them for

15 subsequent degradation [14, 15]. Inhibition of the enzymes results in retention of nuclear PER

16 protein [16], leading to prolonged repression of *Per* and *Cry* gene expression, effectively

17 delaying the cycle until the inhibition of the enzyme wanes and the PER/CRY complex finally

18 degrades. The potent casein kinase 1δ/ε inhibitor PF-670462 has been shown to delay rhythms in

19 an alga [17], *Neurospora* [18], a marine crustacean [19], zebra fish [20], rats [21, 22], mice [16,

20 23, 24] and cymologous monkeys [25]. The widespread cross species effects of casein kinase

21 inhibition is clear evidence of its conservation as an integral component of the circadian timing

22 system. While PF-670462 is considered primarily a casein kinase inhibitor, there is the potential

23 for inhibition of other kinases including PKACα (protein kinase A catalytic subunit), p38

24 cascade (p38 cascade, p38 coupled to MAPKAPK2), HGK (HPK/GCK-like kinase), LCK,

25 (lymphocyte-specific protein tyrosine kinase) and EGFR (epidermal growth factor receptor

26 tyrosine kinase [23]. The effects of PF-670462 inhibition of casein kinase have been studied at

1 the cellular level of mammals using PER2:: Luc reporters [16, 23] and clock gene expression in

2 the whole hypothalamus [24], but there have been no studies addressing the effects of *in vivo*

3 CSNK1D and CSNK1E inhibition on clock gene expression in key organs or hormone secretion.

4

5 In this study we investigated the effect of a single dose of PF-670462 administered at the time of

6 lights off on rhythmic clock gene expression in the liver, pancreas and SCN over the subsequent

7 36 hours. We chose the liver and pancreas because of the critical role rhythms in these organs

8 have in maintaining metabolic homeostasis [26-28]. In addition we assessed the impact of casein

9 kinase inhibition on melatonin and corticosterone secretion and wheel running rhythmicity. The

10 impacts on gene expression and hormone secretion were compared with those occurring

11 following acute exposure to 4 hours of light at the expected time of lights off, which was

12 predicted to result in approximately a 3 hour delay in the melatonin rhythm [29].

13

14 **2. Material and methods**

15 *2.1. Animals and experimental design*

16 Male albino Wistar rats (4 weeks of age on arrival) were obtained from the University of

17 Adelaide Laboratory Animal Services Facility where they had been maintained on a 12L:12D

18 photoperiod (lights off at 2000h). The rats were then group housed (n = 5) in light controlled

19 environment chambers for one week with *ad libitum* access to food and water. The studies were

20 approved by the University of Adelaide Animal Ethics Committee.

21

22 To investigate the effects of acute inhibition of casein kinase 1δ/ε on the expression of clock and

23 other genes in the suprachiasmatic nucleus, liver and pancreas, groups of rats were injected with

24 PF-670462 (Lundbeck Research USA; 50 mg/kg; s.c.) or vehicle (20% 2-Hydroxypropyl-β-

25 cyclodextrin; Sigma, St Louis, MI) just prior to the time of lights off (2000h). The dose and

26 timing of administration was chosen on the basis of previous dose response studies conducted in

rats [21]. Following the injections, the lights remained off for the remainder of the experiment.

Groups of rats were killed by rapid decapitation; 5 vehicle treated rats at 2400h, 0400h, 0800h,

1200h, 1600h and 2000h (4, 8, 12, 16, 20 and 24 hours post treatment) and 3 rats at 2400h,

0400h and 0800h (28, 32 and 36 hours post treatment); 10 PF-670462 treated rats were killed at

2400h and 2000h (4 and 24 hours post treatment) and 5 rats at the other times. Brain, liver and

pancreas tissue was immediately dissected and placed in RNAlater® (Ambion, Texas, USA) for

24 hours at 4ºC and then stored at -20ºC until processed. Blood was collected into heparinised

tubes, centrifuged and plasma stored at -20ºC.

To investigate the effects of an acute 4 hour extension of the light phase on the expression of

clock and other genes in the liver and pancreas, the lights in the environment chambers remained

on until 2400h and the rats thereafter remained in darkness until killed. For the control rats,

lights were turned off as usual at 2000h. Groups of 5 control rats were killed at 2000h, 2400h,

0400h, 0800h, 1200h, 1600h and 2000h (0, 4, 8, 12, 16, 20 and 24 hours after lights off) and

groups of 3 rats were killed at 2400h, 0400h and 0800h (28, 32 and 36 hours after lights off).

Light exposed rats (n = 5 per time point) were killed at 4 hourly intervals from 2400h. Liver,

pancreas and blood were collected and stored as above.

2.2. RNA Isolation and Real Time RT-PCR

Rat liver and pancreas tissue was homogenised in TriReagent (Sigma) using the

PowerLyzer24™ bench-top homogeniser (Mo Bio Laboratories Inc, Carlsbad, CA) at 6500rpm

(30 s x 2, 30 s) and total RNA was isolated according to the manufacturer's instructions. For the

brains, a 1-mm coronal section including the SCN was prepared using a Vibroslice (Campden

Instruments, London, UK), placed on a slide and frozen on dry ice for approximately 2 min.

SCN from both hemispheres were subsequently punched out using a modified 22-gauge needle

and expelled into 100 μl RNAqueous lysis buffer (Ambion) [30] and stored at −20°C. The

remaining brain sections were subsequently examined under a dissecting microscope to confirm

that both SCN had been collected. Ambion RNAqueous® micro kits (Ambion) were used to

extract RNA from the SCN samples, which were further processed as previously described [30].

Potential residual DNA was digested using a DNA-free kit (Ambion) according to the

manufacturer's instructions. Punching of the SCN was not always successful and this together

with occasional reverse transcription failures resulted in some time points having reduced data

points.

Because of the small amount of tissue obtained from the punches, all of the extracted SCN RNA

and 2μg of the liver and pancreas RNA were reverse-transcribed using Super Script III

(Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions, with a total

reaction volume of 39μl, made up to 100μl following reverse transcription. After the addition of

primers and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), genes of

interest were amplified using a GeneAmp 7500 Sequence Detection System (Applied

Biosystems) in duplicate, using primers designed and optimised in our laboratory (Table 1). The

expression of genes within each sample was normalised against *Actb*, and expressed relative to a

calibrator group (2400h vehicle treated rats or 2400h control rats) using the formula $2^{-(\Delta\Delta Ct)}$.

2.3. Wheel running behaviour.

To assess the effects of PF-670462 on voluntary physical activity, rats were housed individually

in cages fitted with running wheels (25 cm diameter) within light controlled chambers. A data

acquisition system (LabPro, Data Sciences, St. Paul, MN) was used to record the number of

wheel rotations in 10 minute bins. After 10 days acclimatisation to the running wheels, 5 rats

were administered PF-670462 (50 mg/kg; s.c.) and 5 rats were vehicle treated at the time of

lights off (2000h). The rats then remained in continuous darkness for 9 days to determine the

effects of the drug on the phase of their running rhythms. Periodograms were prepared using

1 Actiview software (MiniMitter, Bend, OR) using data for the 1st to 9th days after treatment. The

2 onset of activity (defined as the time when there were more than 10 revolutions/10 minutes) was

3 then calculated for each of the first 8 days before and the 9 days after treatment for each rat and

4 their times adjusted to account for the free running periods. The differences between the

5 averaged individual pre-treatment and post-treatment onsets were calculated and then the

6 average shifts for the control and PF-670462 treated groups were determined.

7

8 *2.4. Hormone assays*

9 Melatonin was assayed in 250 μl plasma and assayed by double antibody RIA [31] (Buhlmann

10 Laboratories, Allschwil, Switzerland) according to the manufacturer's instructions.

11 Corticosterone was assayed in 10 μl plasma by double antibody RIA from MP Biomedicals

12 Australia (Seven Hills, Australia) according to the manufacturer's instructions.

13

14 *2.5. Statistics*

15 The gene expression and hormone data was fitted to sine curves using CircWave;

16 (http://hutlab.nl/ [32]). For the determination of phase for both the PF-670462 and extended light

17 experiments only data from the last 24 hours was used to eliminate any acute drug effects on

18 gene expression. The time of peak expression refers to the acrophase when there was a

19 significant ($P < 0.05$) fit of the data to a sine curve with a period of 24 hours and phase shifts are

20 the differences between the acrophases. Differences in expression at each time point were

21 analysed by 2-way ANOVA with *post hoc* Bonferroni tests.

22

23 **3. Results**

24 *3.1 Effects of PF-670462 on the liver*

25 During the 12 hours following PF-670462 administration, the liver *Bmal1* mRNA expression

26 increased slower than in the control rats as dawn approached such that the peak was delayed by 7

1   hours (Figure 1). In addition peak *Bmal1* mRNA expression was almost 2 fold higher than the

2   controls. Expression of *Nr1d1* mRNA was also affected by the casein kinase inhibitor, with peak

3   expression occurring 3.9 hours later than the controls and the level of expression more than 50%

4   lower at the time of the peak. Following PF-670462 administration, the pattern of decreasing

5   *Per1* mRNA expression across the night and into the subjective light period followed that of the

6   controls, but the subsequent increase towards the time of subjective lights off was slowed such

7   that the time of peak expression was delayed by 1.2 hours and the level of expression was

8   approximately 50% lower than the control rats (Figure 1; $P < 0.001$). A similar pattern of

9   expression occurred for *Per2* and *Cry2* mRNA but with a larger delay in the timing of peak

10   expression (Table 2). Rhythmic expression of *Cry1* mRNA was lost following PF-670462

11   treatment (Figure 1; $P > 0.05$) with the level of expression lower than in the controls throughout

12   the subjective night ($P < 0.05$).

13

14   *3.2 Effects of PF-670462 on the pancreas*

15   In the pancreas, PF-670462 administration delayed the expected morning peak in *Bmal1* mRNA

16   expression by 7.2 hours compared to the controls, but there was no difference in the level of

17   expression at the time of the peak (Figure 2). The timing of the afternoon/evening peaks, but not

18   the levels of expression of *Nr1d1, Per1* and *Per2* mRNA expression were delayed by up to 5.7

19   hours (Table 2). While *Cry1* and *Cry2* mRNA expression was rhythmic in vehicle treated rats,

20   the rhythm was lost following PF-670462 administration and *Cry1* mRNA expression remained

21   low throughout the subjective dark period (Figure 2; $P < 0.05$).

22

23   *3.3 Effects of PF-670462 on hormone secretion*

24   Plasma melatonin was highest during the night and had decreased to its lowest level by 4 hours

25   after subjective lights on in the controls and increased again during the subjective night. PF-

26   670462 administration had no impact on either the timing or the amplitude of the melatonin

1   rhythm (Figure 3a). The pattern of plasma corticosterone in control and PF-670462 treated rats

2   was similar until the subjective night when the time of peak secretion was delayed by 2.1 hours

3   (Figure 3c).

4

5   *3.4 Effects of PF-670462 on the suprachiasmatic nucleus*

6   Rhythmic expression of *Bmal1*, *Nr1d1* and *Dbp* mRNA was apparent in the SCN punches from

7   the control rats (Figure 4). For *Per1* and *Per2* mRNA expression, the data did not fit a sine

8   curve, although highest expression occurred at the time of subjective lights off (2000h) and 4

9   hours before (1600h) respectively. PF-670462 administration had no effect on either the timing

10   or levels of *Bmal1* mRNA (Figure 4). By contrast *Nr1d1* and *Dbp* mRNA expression

11   progressively decreased following PF-670462 administration to be lowest at the time of

12   subjective lights on. Expression then increased such that the time of peak expression occurred

13   4.2 and 4 hours later than the controls respectively (Figure 4). *Per2* mRNA expression

14   progressively decreased following PF-670462 administration, to be lowest at 1000h, and

15   thereafter expression increased to reach a maximum at 2400h. The timing and levels of *Per1*

16   mRNA expression were not altered by PF-670462.

17

18   *3.5 Effects of PF-670462 on wheel running behaviour*

19   To investigate the effects of PF-670462 inhibition of casein kinase on circadian behavioural

20   rhythmicity, rats were monitored for 9 days after administration of the drug. In the 8 days prior

21   to treatment, all rats established consistent patterns of wheel running characterised by an onset of

22   running activity within 20 minutes of the lights going off and a predominance of running during

23   darkness. In the first 12 hours following administration of PF-670462 or vehicle, the number of

24   wheel revolutions was similar in both groups (873 ± 141 revolutions for the controls vs. $1004 \pm$

25   119 revolutions for PF-670462 treated rats; $P > 0.05$). The subsequent onset of running activity

26   for the drug treated rats was delayed, compared to the pre-treatment period (25 ± 9 minutes for

1   the controls vs. 93 ± 14 minutes for PF-670462 treated rats; Figure 5; P = 0.004). The period of

2   the free running rhythm following the treatment was not altered (24 hours 12 minutes for the

3   controls vs. 24 hours 7 minutes for PF-670462 treated rats; P > 0.05).

4

5   *3.5 Effects of a 4 hour extension of the light period on rhythms*

6   Prolonging the light phase by 4 hours resulted in no or small delays in the rhythm of expression

7   of liver *Bmal1*, *Per1*, *Per2* and *Nr1d1* mRNA (Figure 6). The mean delay of peak expression of

8   these 4 genes was 0.8 ± 0.5 hours compared to 4.5 ± 1.3 hours for the same genes following PF-

9   670462 administration. The peak in liver *Cry1* mRNA expression was delayed by 0.28 hours,

10   whereas *Cry2* mRNA expression was not rhythmic in either control or light exposed rats. In the

11   pancreas, the expression of *Bmal1*, *Per1*, *Per2* and *Nr1d1* mRNA was delayed by 0.9 ± 0.5 hours

12   (Figure 7), compared to a delay of 4.5 ± 1.2 hours for the same genes following PF-670462

13   administration. *Cry1* and *Cry2* mRNA expression was delayed by 0.52 and 2.72 hours

14   respectively following the light exposure.

15

16   Extension of the light period resulted in the suppression of the melatonin rise until 0400h (Figure

17   3b) but had no effect on the timing of peak secretion during the following subjective night

18   (0430h versus 0456h). Following the light exposure, unlike the control rats, there was no clear

19   plasma corticosterone rhythm (Figure 3d), but the highest levels of corticosterone in the 2 groups

20   occurred at the 2000h sampling time, suggesting that there was little effect of light.

21

22   **4. Discussion**

23   In the current study we showed that administration of the casein kinase 1δ/ε inhibitor PF-670462

24   prior to lights off resulted in large delays (3.2 – 7.2 hours) in rhythmic clock gene expression in

25   peripheral tissues of rats (liver and pancreas). Smaller shifts in the expression of some clock

26   genes were detected in SCN punches. Interestingly there was no acute effect of PF-670462 on

1   the secretion of melatonin or the timing of the rhythm the following night, whereas the

2   corticosterone rhythm was delayed by 2.1 hours. As expected, administration of PF-670462

3   delayed the onset of wheel running activity by approximately 1.1 hours which is consistent with

4   previous studies in rats at the dose used (50 mg/kg) [21], although less than that reported in mice

5   [24].

6

7   In contrast to the liver and pancreas, the acute effect of PF-670462 treatment on the SCN was a

8   steady suppression of *Per1*, *Per2*, *Nr1d1* and *Dbp* mRNA expression over the initial 12 hours.

9   This is consistent with prolonged transcriptional repression of *Bmal1* mRNA due to reduced

10   phosphorylation of the Period and Cryptochrome proteins. Subsequent degradation of the

11   proteins and loss of inhibition of the enzymes and translation of new enzyme protein would

12   facilitate the resumption of transcription of *Per1*, *Per2*, *Nr1d1* and *Dbp*. Interestingly there was

13   no effect of PF-670462 on *Bmal1* mRNA expression levels or timing and changes in the timing

14   of peak *Per1* or *Per2* mRNA expression in the SCN on the second subjective night were not

15   clear. The rhythms of *Nr1d1* and *Dbp* mRNA expression were, however, delayed. A recent study

16   in mice reported similar shifts in *Nr1d1*, *Dbp* and *Per2* mRNA expression in hypothalamic

17   blocks following administration of PF-670462 (30 - 100 mg/kg) [24]. Small or no shifts were

18   reported for *Bmal1*, *Per1*, *Dec1*, *Nr1f2* and *Prok2* mRNA. It is unlikely that the small changes

19   are due to a lack of a central effect of the drug since PF-670462 is reported to have good brain

20   penetrance and a short half-life (approximately 30 minutes) [21]. Furthermore at the

21   concentration used in this experiment, it may be predicted from pharmacokinetic and *in vitro*

22   studies in rats and mice that most of the casein kinase 1δ/ε activity would be inhibited for several

23   hours [21, 23]. The inhibitor used in the current study has been reported to affect the 2 casein

24   kinase enzymes casein kinase 1δ and casein kinase 1ε differently [23]. While the relative levels

25   of the enzymes in various tissues are not known, it is possible that the different phase delays

26   apparent in the SCN and the peripheral tissues studied is a reflection of variations in the degree

1   of inhibition of phosphorylation of the clock proteins. Limitations of the current rat and the

2   previous mouse studies are that the tissues included both core and shell regions of the SCN

3   which have quite different functions [33]. Furthermore the amount of RNA obtained from the

4   SCN punches is quite low and can result in greater variability in RT PCR results and loss of

5   statistical power to detect rhythm changes. The failure of PF-670462 to change the timing of the

6   plasma melatonin rhythm, in contrast to the 1.1 hour delay in wheel running activity is

7   interesting and consistent with the relatively small change in SCN function. Another

8   consideration is that the 4 hour sampling interval and curve fitting approach may not be optimal

9   for determining small shifts of melatonin secretion.

10

11   A secondary aim of the current study was to compare the effects of the inhibition of casein

12   kinases with the effects of an extension of the light period by 4 hours. We have previously

13   shown that a 6 hour light extension resulted in a 3.7 hour delay in the nocturnal rise of urinary 6-

14   sulphatoxymelatonin on the following subjective night [10]. In mice exposed to a 6 hour light

15   extension there was induction of SCN *Per2* and *Cry1* mRNA expression, (but not *Per1* mRNA),

16   followed by a 6 hour delay in the expression rhythm [34]. We reasoned that prolonged light

17   would suppress melatonin secretion, phase delay the melatonin rhythm the next night and delay

18   clock gene expression in the liver and pancreas. As predicted, prolonging light exposure

19   suppressed the normal nocturnal rise in melatonin secretion, with high levels only appearing at

20   0400h, but no subsequent delay in peak secretion was detected, perhaps in part due to the 4 hour

21   sampling that was used. Nevertheless the changes in liver and pancreas gene expression rhythms

22   following light exposure were smaller than those following PF-670462 administration. Although

23   we did not analyse clock gene expression in the SCN of the rats following light exposure, the

24   minimal changes in timing of the pineal melatonin rhythm and the liver and pancreas gene

25   rhythms suggests that acute inhibition of casein kinase has a more powerful impact on the SCN

26   than 4 hours of light and that the response is greater in peripheral tissues.

2 There are some limitations in the current study. We did not investigate the effects of PF-670462

3 on casein kinase enzyme activity following administration and so must rely on previous studies

4 that have shown that this compound does indeed prevent phosphorylation of key clock proteins.

5 The study was conducted at a single dose (50 mg/kg) which was shown previously to not

6 produce maximum phase shifts in rats. Higher doses of PF-670462, for example 100 mg/kg,

7 resulted in delays in the onset of wheel running of up to 4 hours in rats [21]. However, in

8 preliminary gene expression studies at this higher dose, we observed that 3 of 15 rats died within

9 12 hours of administration (Kennaway, unpublished results). This toxicity has not been observed

10 previously (Dr Jeffrey Sprouse, personal communication), but we were compelled to use the

11 lower dose. The study did not address the impact of the changes in liver and pancreas clock gene

12 rhythmicity on the function of these organs, for example the impact on rhythms of glucose

13 tolerance and insulin sensitivity, but this is an area that will be pursued in the future. Finally it

14 would be very interesting to know how long the clock gene changes in the liver and pancreas

15 persist and whether chronic administration would bring about larger shifts in rhythmicity as has

16 been observed in behavioural studies in mice [24] and rats [22], although limited access to and

17 the high price of the drug may limit these types of study in rats.

19 The development of drugs that alter the intrinsic timing system of cells is opening up an exciting

20 new area of pharmacology with important potential applications. One such possibility is that the

21 drugs could be used to facilitate the adaptation of shift workers to their artificial lifestyle of

22 nocturnal wakefulness and meals and diurnal sleep opportunities. This may help to lower the risk

23 of developing the shift work-related metabolic and cardiovascular disorders that are increasingly

24 being reported [35, 36]. In the current study acute administration of PF-670462 caused phase

25 shifts in gene expression simultaneously across central and peripheral tissues as well as the

26 previously documented change in behavioural rhythmicity. Future studies should investigate the

1 long term impact of the alterations in liver and pancreas function following acute and chronic

2 administration of these drugs to determine if there are adverse effects of rapidly phase shifting

3 rhythms in this manner.

4

5 In conclusion, acute administration of the casein kinase inhibitor PF-670462 to rats at the

6 beginning of the dark period resulted in small delays in clock gene rhythms in the SCN and

7 corticosterone secretion and had no effect on melatonin secretion. The small changes in gene

8 expression observed in the SCN may, however, be due to the incorporation of both core and shell

9 regions of the SCN in the punches. By contrast there were large phase delays in clock gene

10 rhythms in the liver and pancreas. The effects of acute casein kinase inhibition on liver and

11 pancreas rhythmicity were considerably greater than the effects of a 4 hour light extension.

12

13 **Acknowledgements**

18

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Figure legends

Figure 1

The expression of *Bmal1*, *Per1*, *Per2*, *Nr1d1*, *Cry1* and *Cry2* mRNA in the liver of rats treated with PF-670462 or vehicle. Rats were injected at 2000h and tissue collected at 4 hour intervals for 36 hours. The relative expression (mean ± SEM) is plotted with the expression data of vehicle treated rats at the initial 2400h mark set at 1. Vehicle treated animals are shown as filled symbols and continuous lines, while PF-670462 data is represented by open symbols and broken lines. Where no error bar is evident, it is obscured by the symbol. The shaded area from 0800h to 2000h represents the subjective light period. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2

The expression of *Bmal1*, *Nr1d1*, *Per1*, *Per2*, *Cry1* and *Cry2* mRNA in the pancreas of rats treated with PF-670462 or vehicle. Data is displayed as for Figure 1. ** $P < 0.01$; *** $P < 0.001$

Figure 3

Plasma melatonin (a, b) and corticosterone (c, d) levels in rats treated with PF-670462 or exposed to 4 hours extended light at the beginning of the dark period. (a, c) Rats were injected with PF-670462 or vehicle at 2000h and blood collected at 4 hour intervals for 36 hours in darkness. The data are the mean ± SEM. Vehicle treated animals are shown as filled symbols and continuous lines while PF-670462 data is represented by open symbols and broken lines. Where no error bar is evident, it is obscured by the symbol. The shaded area from 0800h to 2000h represents the subjective light period. (b, d) The lights remained on from 2000h until 2400h (light shaded area) and then remained off throughout the experiment. The data for the control rats are shown as filled symbols and continuous lines while data from light expose rats is represented by open symbols and broken lines. The shaded area from 0800h to 2000h represents the subjective light period. *** $P < 0.001$.

Figure 4

The expression of *Bmal1*, *Nr1d1*, *Per1*, *Per2* and *Dbp* mRNA in the suprachiasmatic nuclei (SCN) of rats treated with PF-670462 or vehicle. Rats were injected at 2000h and tissue collected at 4 hour intervals for 36 hours. The relative expression (mean ± SEM) is plotted with

1   the expression data of vehicle treated rats at the initial 2400h mark set at 1. The number of SCN

2   punches analysed from vehicle treated and PF-670462 treated rats respectively was 4 & 9 at

3   2400h, 4 & 3 at 0400h, 5 & 4 at 0800h, 5 & 4 at 1200h, 5 & 5 at 1600h, 4 & 9 at 2000h, 2 & 5 at

4   2400h, 3 & 2 at 0400h and 3 & 4 at 0800h. Vehicle treated animals are shown as filled symbols

5   and continuous lines, while PF-670462 data is represented by open symbols and broken lines.

6   Where no error bar is evident, it is obscured by the symbol. The shaded area from 0800h to

7   2000h represents the subjective light period. Data is displayed as for Figure 1.* $P < 0.05$.

8

9   Figure 5

10   Wheel running records for rats injected with vehicle (a – e) or PF-670462 (f – j). Each line

11   represents the record for a rat for 24 hours for 10 days before and 10 days after treatment (time

12   of injection indicated by the asterisk). The shading indicates the period of darkness with the

13   lights remaining off continuously immediately after vehicle or drug administration.

14

15   Figure 6

16   The expression of *Bmal1*, *Per1*, *Per2*, *Nr1d1*, *Cry1* and *Cry2* mRNA in the liver of rats exposed

17   to 4 hours extended light at the beginning of the dark period (light shaded area), followed by

18   continuous darkness to the remainder of the experiment. The data for the control rats are shown

19   as filled symbols and continuous lines while data from light exposed rats is represented by open

20   symbols and broken lines. Data is displayed as for Figure 1. * $P < 0.05$; ** $P < 0.01$; *** $P <$

21   0.001.

22

23   Figure 7

24   The expression of *Bmal1*, *Nr1d1, Per1*, *Per2*, *Cry1* and *Cry2* mRNA in the pancreas of rats

25   exposed to 4 hours extended light at the beginning of the dark period. The data for the control

26   rats are shown as filled symbols and continuous lines while data from light exposed rats is

27   represented by open symbols and broken lines. Data is displayed as for Figure 1. * $P < 0.05$; ** P

28   < 0.01; *** $P < 0.001$.

29

30

31

Figure 1
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Figure 1
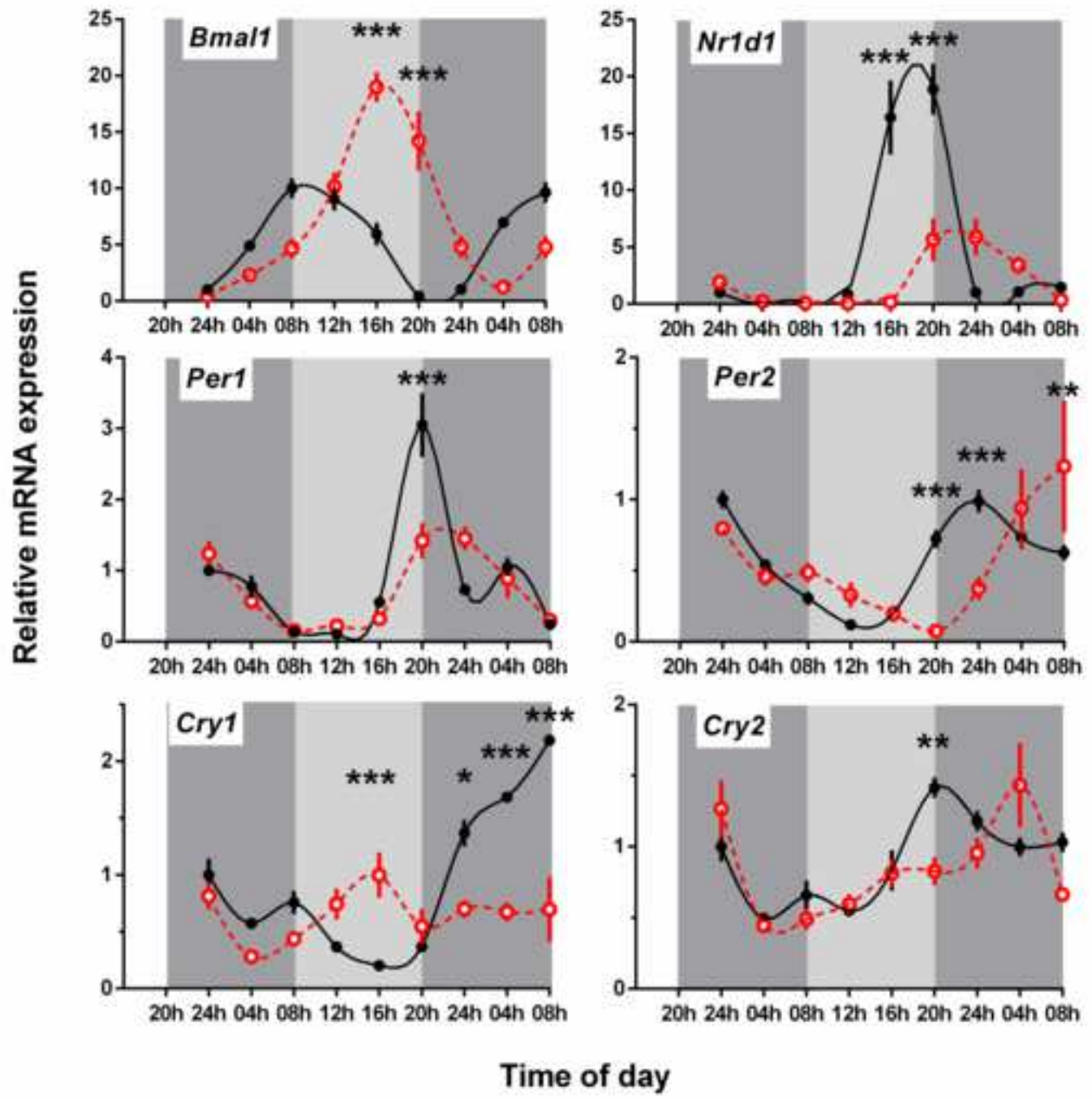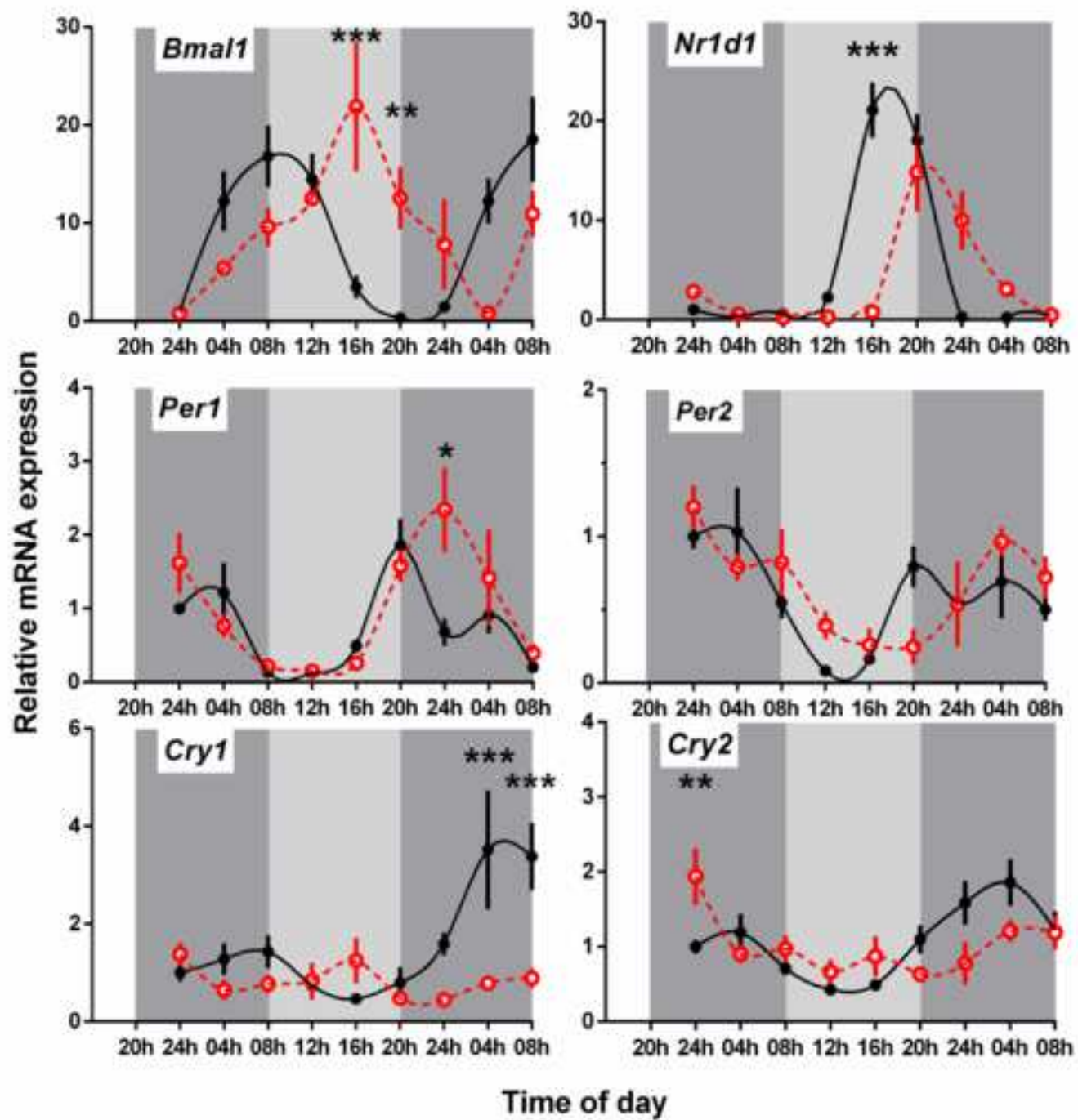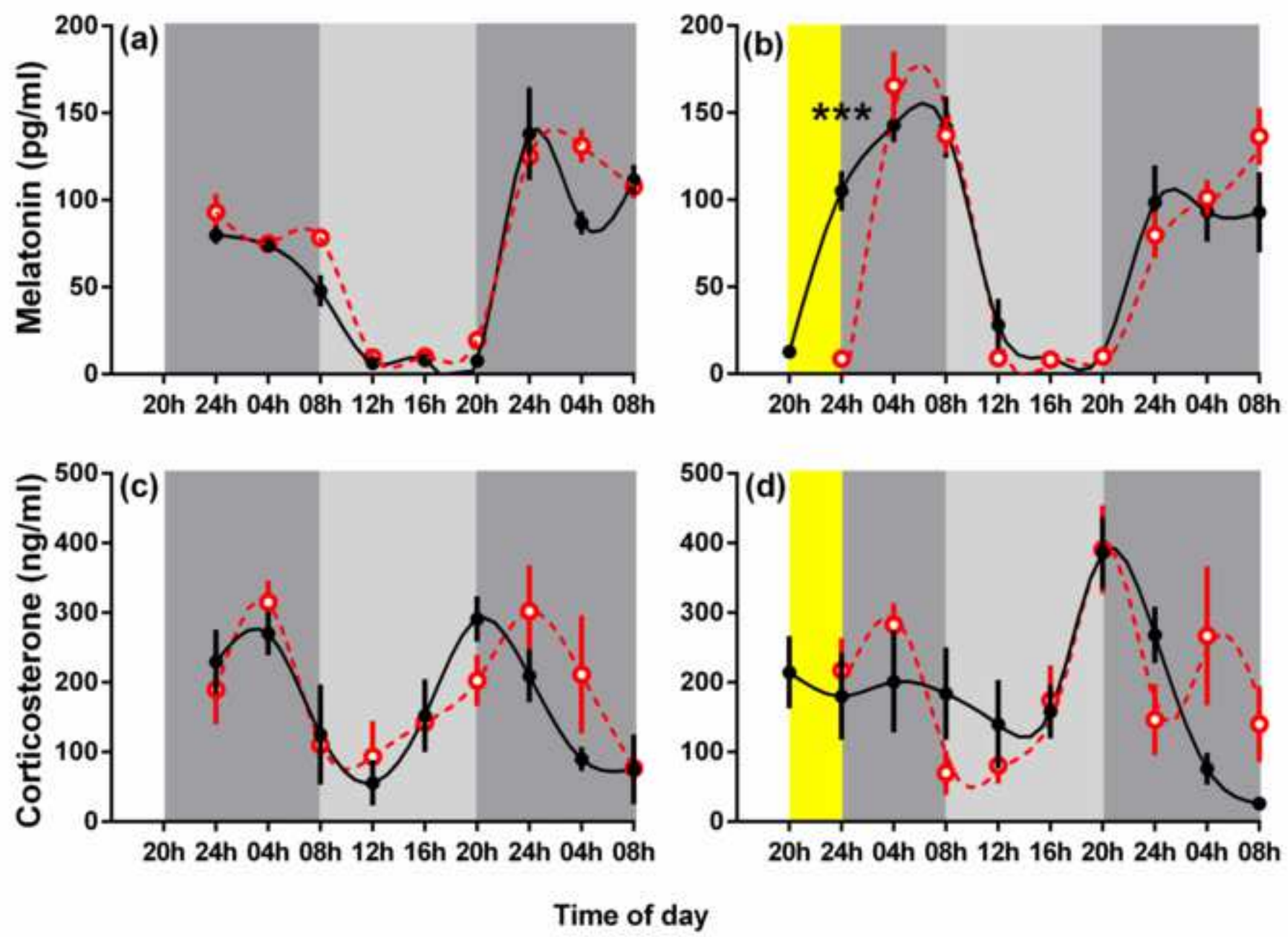
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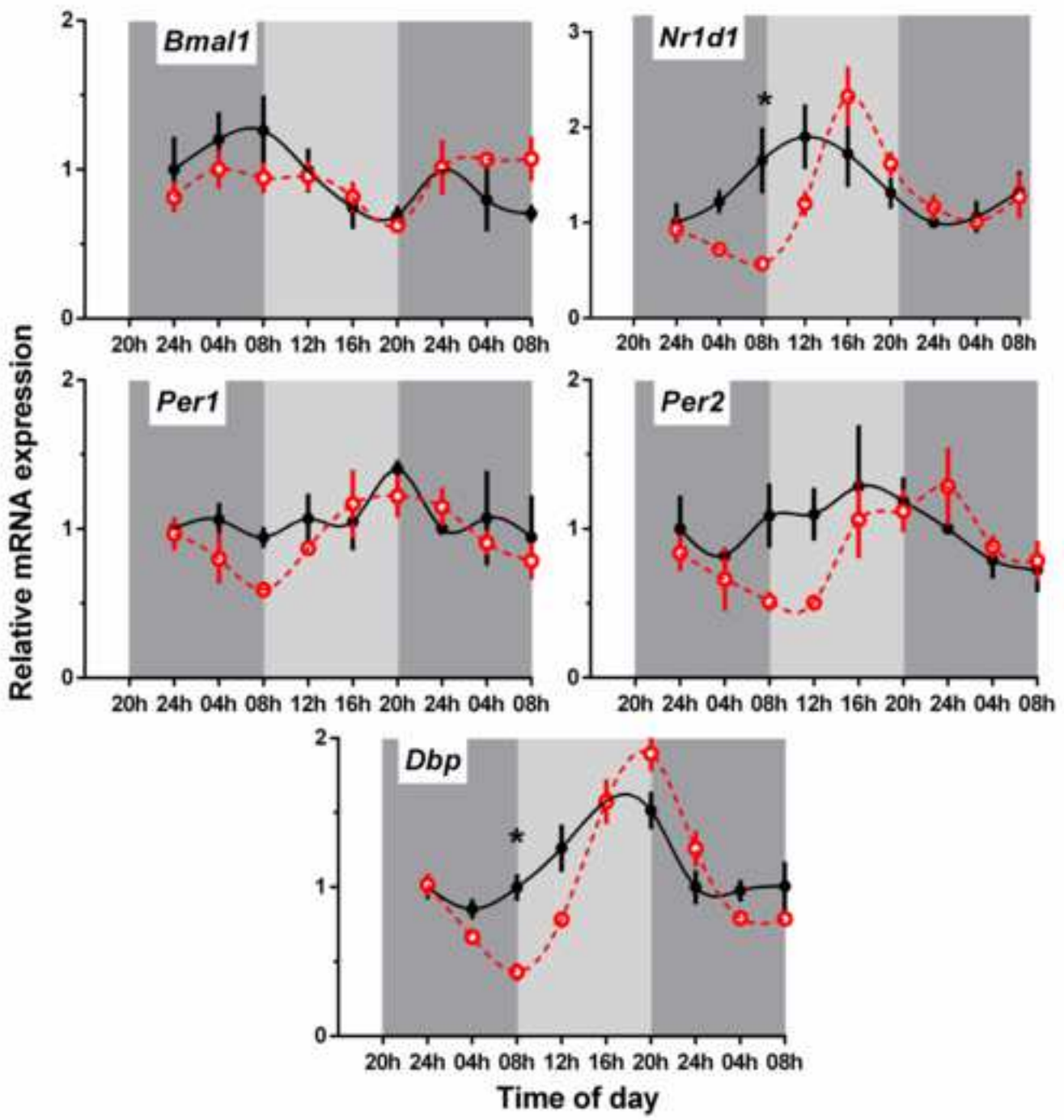
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Figure 5

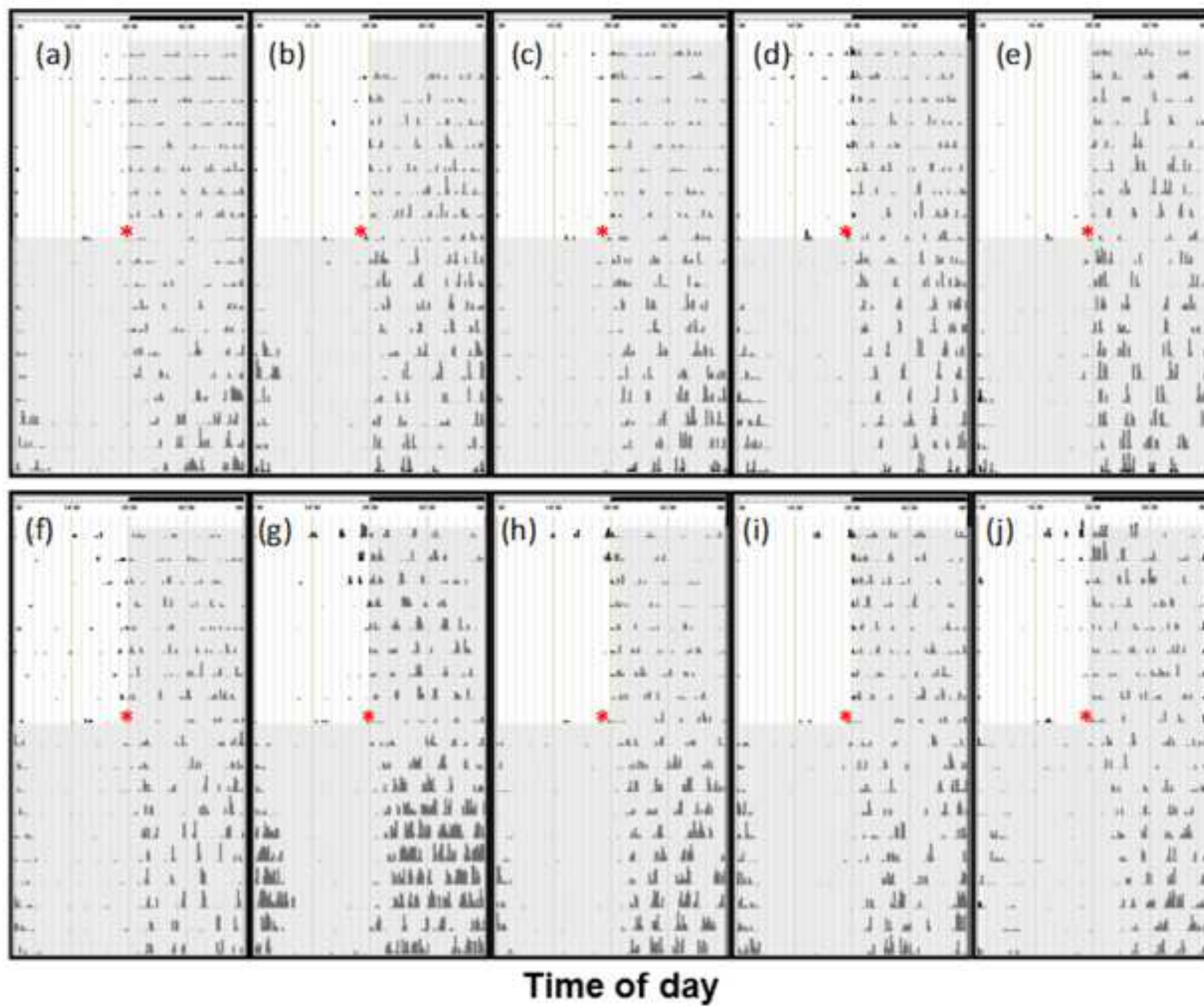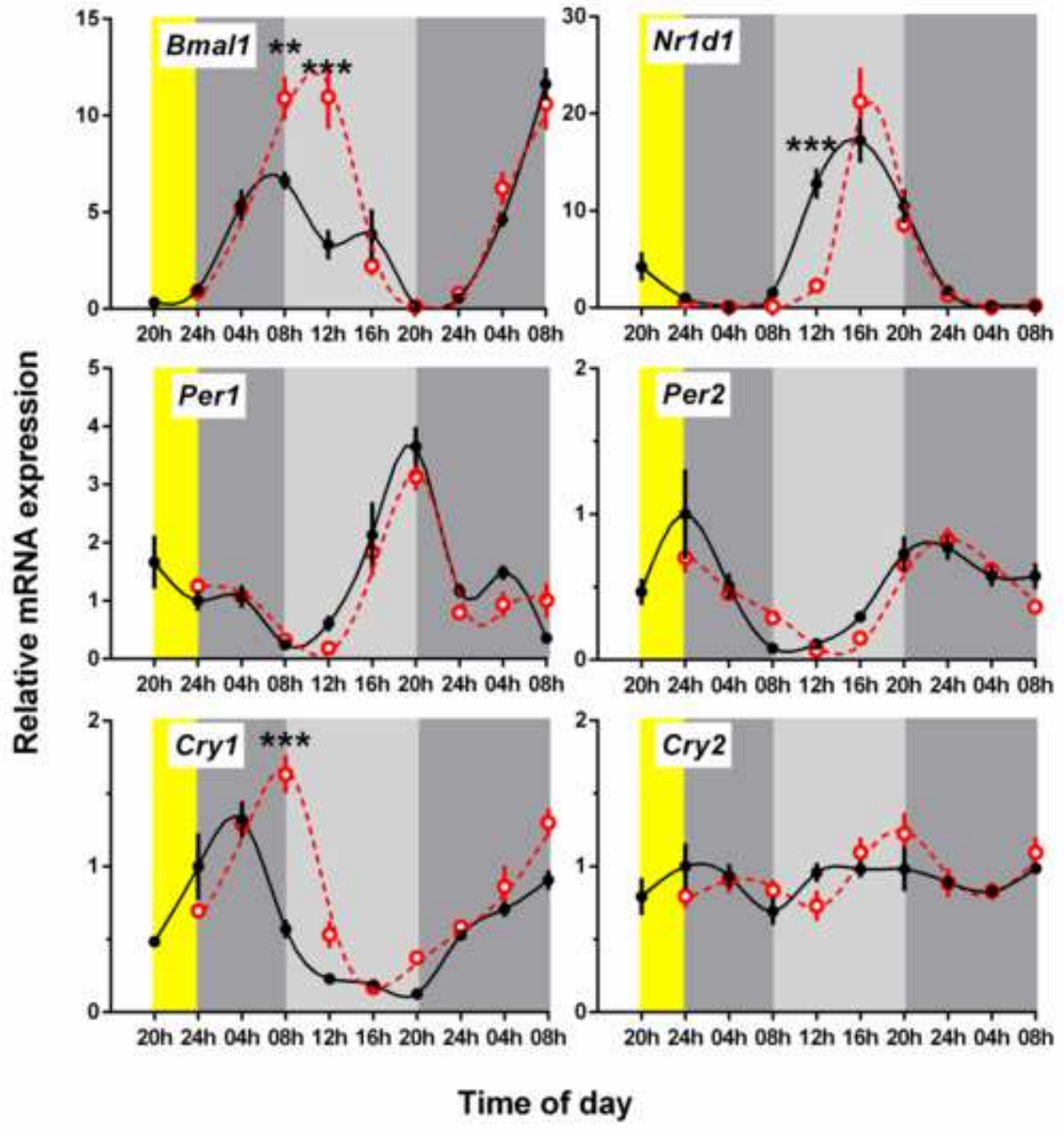**Time of day**

Figure 6
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Figure 7
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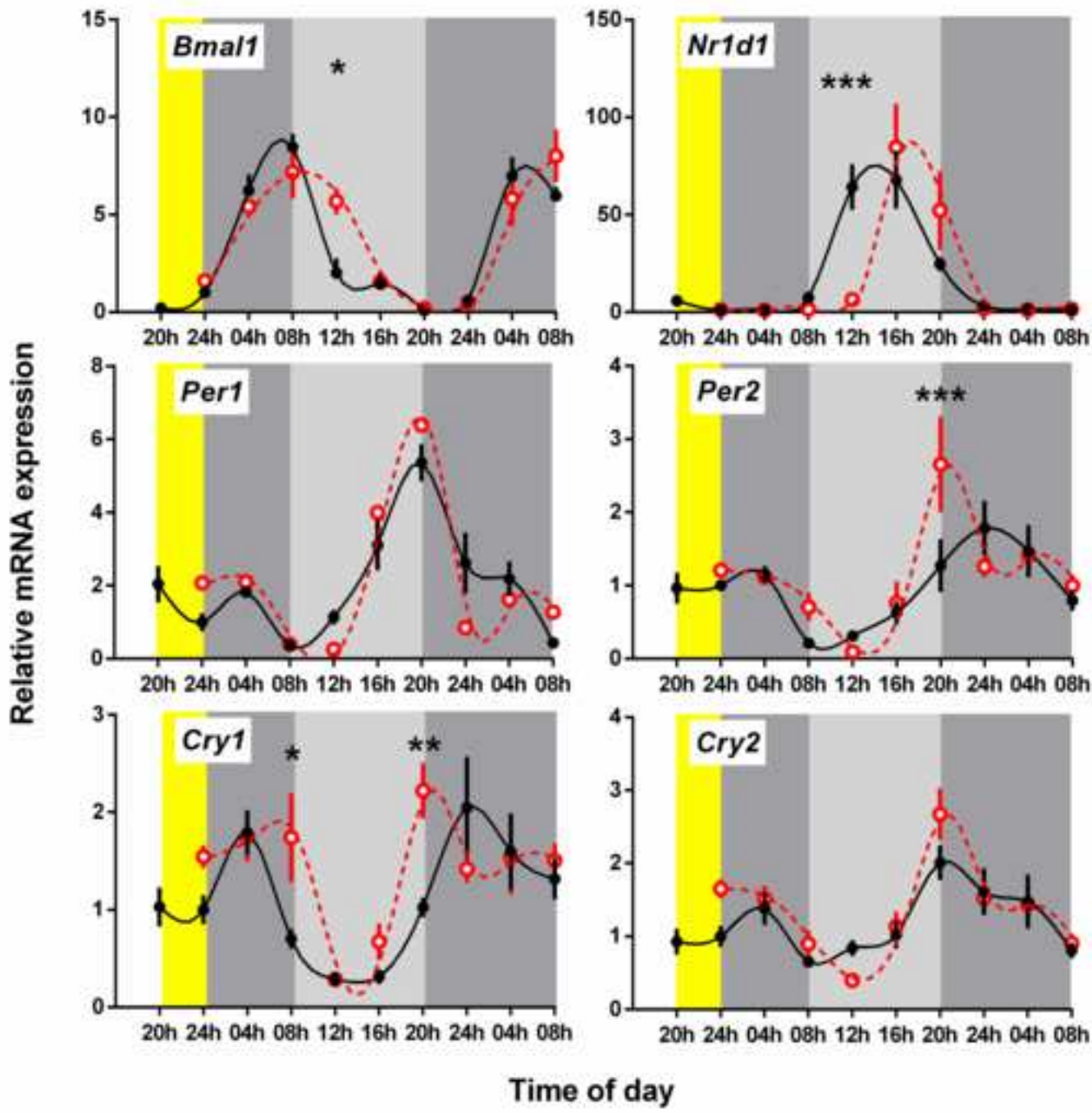Relative mRNA expression

Time of day

Table 1

The genes, their accession numbers, forward and reverse primer sequences, amplicon lengths and primer gene sequence locations used for the Real Time RT-PCR analysis of gene expression in the liver, pancreas and SCN of rats.

Gene	Accession #		Sequence	Amplicon length (bp)	Primer location
Actb	NM_031144	Fwd	CCTCTGAACCCTAAGGCCAA	89	325 - 344
		Rev	AGCCTGGATGGCTACGTACA		414 - 395
Bmal1	NM_024362.2	Fwd	TCCACAGCACAGGCTACTTGAA	104	1433 - 1454
		Rev	TTGCAACGAGGCAGCTCAGAT		1537 - 1517
Nr1d1	NM_145775	Fwd	ACAGCTGACACCACCCAGATC	100	996 - 1016
		Rev	CATGGGCATAGGTGAAGATTTCT		1096 - 1074
Per1	NM_001034125	Fwd	GCGTTGCAAACGGGATGT	100	783 - 800
		Rev	GCAGGCGAGATGGTGTAGTAGA		883 - 862
Per2	NM_031678.1	Fwd	AGCAGTCCCCTACAGCTTAACCT	128	3042 - 3064
		Rev	CCGAGATGCGCCAGATGT		3170 - 3153
Cry1	NM_198750.2	Fwd	GGGAAGCGCCCAAGTCA	90	2232 - 2248
		Rev	CCTCCCGCATGCTTTCGTAT		2322 - 2303
Cry2	NM_133405.1	Fwd	TTCAGAAGGCCGCTAATTG	101	1427 - 1445
		Rev	AGATCTGCTTCATCCGCTCAA		1528 - 1508
Dbp	NM_012543.2	Fwd	CCCGAGGAACAGAAGGATGA	100	1115 - 1134
		Rev	ATCTGGTTCTCCTTGAGTCTTCTT		1215 - 1192

Table 2
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Table 2

The time of peak expression of liver and pancreas genes and the changes in the peaks of vehicle treated, PF-670462 treated, control and light exposed rats.

Liver	Vehicle	PF-670462	Δ (h)	Control	Light	Δ (h)
Bmal1	1102h ± 2.00h	1802h ± 1.56h	-7.00	0917h ± 1.81h	0907h ± 1.55h	+0.17
Per1	2026h ± 1.45h	2140h ± 1.51h	-1.23	1923h ± 1.88h	1940h ± 2.41h	-0.28
Per2	2333h ± 2.61h	0538h ± 2.28h	-6.08	2234h ± 2.70h	0031h ± 2.18h	-1.95
Nr1d1	1807h ± 0.91h	2159h ± 1.07h	-3.87	1548h ± 1.35h	1651h ± 0.80h	-1.05
Cry1	0502h ± 2.36h	No peak	N/A	0553h ± 2.53h	0610h ± 2.41h	-0.28
Cry2	1944h ± 3.01h	2143h ± 2.65h	-2.00	No peak	No peak	N/A
Pancreas						
Bmal1	0926h ± 1.61h	1641h ± 2.40h	-7.25	0722h ± 1.93h	0753h ± 1.50h	-0.52
Per1	2047h ± 1.77h	2233h ± 1.66h	-1.77	1917h ± 1.92h	1916h ± 2.25h	+0.02
Per2	2256h ± 2.59h	0436h ± 2.92h	-5.67	2240h ± 2.69h	2326h ± 2.64h	-0.77
Nr1d1	1748h ± 0.78h	2100h ± 0.78h	-3.20	1453h ± 1.08h	1719h ± 0.80h	-2.43
Cry1	0623h ± 2.02h	No peak	N/A	0058h ± 2.64h	0129h ± 2.87h	-0.52
Cry2	0256h ± 3.14h	No peak	N/A	1959h ± 2.80h	2242h ± 2.78h	-2.72