Reduced α-adrenoceptor responsiveness and enhanced baroreflex sensitivity in *Cry*-deficient mice lacking a biological clock

Shizue Masuki[1], Takeshi Todo[2], Yasushi Nakano[3], Hitoshi Okamura[4] and Hiroshi Nose[1]

[1]*Department of Sports Medical Sciences, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, Matsumoto 390-8621, Japan*
[2]*Radiation Biology Center, Kyoto University, Kyoto 606-8501, Japan*
[3]*Kissei Comtec Co., Matsumoto 390-1293, Japan*
[4]*Department of Brain Science, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan*

To reveal the role of clock genes in generating the circadian rhythm of baroreflexes, we continuously measured mean arterial pressure and baroreflex sensitivity in free-moving normal wild-type mice, and in *Cry*-deficient mice which lack a circadian rhythm, in constant darkness for 24 h. In wild-type mice the mean arterial pressure was higher at night than during the day, and was accompanied by a significantly enhanced baroreflex sensitivity of -13.6 ± 0.8 at night compared with -9.7 ± 0.7 beats min^{-1} mmHg^{-1} during the day ($P < 0.001$). On the other hand, diurnal changes in arterial pressure disappeared in *Cry*-deficient mice with remarkably enhanced baroreflex sensitivity compared with wild-type mice ($P < 0.001$): -21.9 ± 1.6 at night and -23.1 ± 2.1 beats min^{-1} mmHg^{-1} during the day. Moreover, the mean arterial pressure response to 10 μg kg^{-1} of phenylephrine, an α_1-adrenoceptor agonist, was severely suppressed in *Cry*-deficient mice regardless of time, while that for the wild-type mice was 10.1 ± 1.9 mmHg in the night, significantly lower than 22.0 ± 3.5 mmHg in the day ($P < 0.01$). These results suggest that CRY genes are involved in generating the circadian rhythm of baroreflex sensitivity, partially by regulating α_1-adrenoceptor-mediated vasoconstriction in peripheral vessels.

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Corresponding author H. Nose: Department of Sports Medical Sciences, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, Matsumoto 390-8621, Japan. Email: nosehir@sch.md.shinshu-u.ac.jp

It has been reported that baroreflexes are involved in generating the circadian rhythm of cardiovascular variability (Makino *et al.* 1997; Di Rienzo *et al.* 2001). On the other hand, in mammals, the oscillation of circadian clock genes has been discovered not only in the suprachiasmatic nucleus of the hypothalamus (Okamura *et al.* 1999) but also in peripheral tissues (Maemura *et al.* 2000; Nonaka *et al.* 2001; Yagita *et al.* 2001; Oishi *et al.* 2003); however, there have been few studies demonstrating how these genes are associated with the circadian rhythm of baroreflex sensitivity.

Arterial pressure is controlled by baroreflexes in the cardiovascular centre of the medulla regulating efferent signals to the heart and the peripheral vessels according to input from the baroreceptors in the systemic circulation. Since the feedback gain of baroreflexes is further controlled by the upper brain regions (Rowell *et al.* 1996), and since clock genes are expressed in these regions (Masubuchi *et al.* 2000; Okamura *et al.* 2002), the central clocks are likely to be involved in the circadian rhythm of baroreflex sensitivity. On the other hand, it has been suggested that α-adrenoceptor responsiveness in the peripheral vessels shows circadian rhythm (Gohar *et al.* 1992; Keskil *et al.* 1996), probably associated with the expression of peripheral clock genes (Nonaka *et al.* 2001). However, there have been no attempts to assess whether central or peripheral clock genes, or both, are involved in the circadian rhythm of baroreflex sensitivity.

Recently, we reported that the baroreflex control of heart rate (HR) was enhanced to stabilize arterial pressure in mice that were genetically deficient in calponin in the vascular smooth muscles, calponin being an intracellular mediator of α-agonist-induced vasoconstriction (Masuki *et al.* 2003*a*), but with normal clock genes. More recently, Masuki *et al.* (2005) reported in human subjects that the baroreflex control of HR was enhanced with attenuated α-adrenoceptor responsiveness at a given time of day. These enhancements compensated for the reduced sensitivity of α-adrenoceptor responsiveness in the peripheral vessels. Therefore, we postulated

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that the circadian rhythm of baroreflex sensitivity would be a secondary adaptation to the primary change in α-adrenoceptor-mediated vasoconstrictor responsiveness, which may be caused by the oscillatory expression of peripheral clock genes.

In this study, we hypothesized that if the circadian rhythm of α-adrenoceptor responsiveness is controlled by clock genes in the peripheral vessels, and if that of baroreflex sensitivity is controlled by other mechanisms unrelated to clock genes, the α-adrenoceptor responsiveness would be severely suppressed in animals genetically deficient in a biological clock while arterial pressure would be well maintained by enhanced baroreflex sensitivity. To examine these hypotheses, we measured the circadian rhythms of baroreflex sensitivity and α-adrenoceptor responsiveness in free-moving *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, genetically deficient in *Cry1* and *Cry2*, since *Cry1* and *Cry2* genes are indispensable for the molecular core oscillator function of circadian clocks in peripheral tissues (Yagita *et al.* 2001) as well as in the suprachiasmatic nuclei (Okamura *et al.* 1999).

Methods

Animals

We used C57BL/6J mice lacking *Cry1* and *Cry2* genes (*Cry*-deficient (*Cry1*$^{-/-}$*Cry2*$^{-/-}$) mice) and mice carrying normal *Cry1* and *Cry2* genes (wild-type mice). *Cry1*$^{-/-}$ mice were generated as previously described (Vitaterna *et al.* 1999). To generate *Cry2*$^{-/-}$ mice, a phage clone carrying a genomic fragment overlapping *Cry2* was isolated by screening a lambda dash mouse genomic library for the 129/Sv strain. The clone carries the C-terminal half of *Cry2* from exons 6 to 11. The nucleotide sequence of the genomic fragment after digestion with a restriction enzyme revealed that a 2.1 kb *Sph* I/*Kpn* I fragment carried exons 7, 8 and 9. We constructed a targeting vector that lacked the 2.1 kb region by subcloning the entire genomic fragment into the *Not* I site of pBluescript and then replacing the 2.1 kb region with a 6 kb *Sph* I/*Kpn* I fragment containing an En2-derived splice acceptor and Ires LacZ-Neo fusion gene (Mountford *et al.* 1994). The resultant targeting vector was used to transform E14tg2a mouse embryonic stem (ES) cells (Hooper *et al.* 1987) as previously described (Vitaterna *et al.* 1999).

Adult male mice were used, aged 9–11 weeks. Body weight was 28.0 ± 0.3 g in wild-type mice ($n = 6$) and 22.3 ± 1.6 g in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice ($n = 5$). The *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice were significantly lighter than the wild-type mice ($P < 0.01$). They were housed at 25°C with food and water *ad libitum* under conditions of light from 7:00 to 19:00 h. The procedures used were approved by the Animal Ethics Committee of Shinshu University School of Medicine.

Catheterization used to measure arterial pressure

As reported previously (Masuki *et al.* 2003*a*), after anaesthetization with pentobarbital sodium (50 mg (kg body wt)$^{-1}$, I.P.), a polyethylene catheter to measure mean arterial pressure (MAP) and HR was inserted into the left femoral artery so that the tip was positioned 5 mm below the left renal artery. The catheter was secured to the surrounding leg muscles, tunnelled subcutaneously and then exteriorized between the scapulae. The exteriorized catheter was connected to a cannula swivel (model TCS2-21; Tsumura, Tokyo, Japan), and the mouse was placed in a cage with a free-moving system (model FM-1121, Tsumura). The arterial catheter was flushed every day with 100 i.u. heparin in 0.2 ml saline.

Measurements

Cry1$^{-/-}$*Cry2*$^{-/-}$ mice lose periodicity in wheel-running behaviour (van der Horst *et al.* 1999) as well as electrophysiological activity in the suprachiasmatic nucleus cells under constant darkness (Bonnefont *et al.* 2003). However, lights have a masking effect on the behaviour: the wheel-running activity is suppressed in the light, and a light–dark cycle results in daily rhythmicity. To exclude any effects of light on the rhythms and to assess mere effects of the circadian clock, we performed the experiments under constant darkness in both *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice and wild-type mice.

Before the experiments, animals were housed in 12 h light : 12 h dark conditions for at least 1 week after implantation with a polyethylene catheter in the femoral artery to measure MAP and HR (Masuki *et al.* 2003*a*). After 36 h they were transferred to constant darkness and MAP and HR were measured for the next 24 h. Here we used the terms 'day' (7:00–19:00 h or circadian time (CT) 0–CT12) and 'night' (19:00–7:00 h or CT12–CT24) as 'subjective day' and 'subjective night', respectively, according to the normal biological clock in wild-type mice. Although *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice completely lack a biological clock, we used '36–48 hours' and '48–60 hours' for day and night after the transition into constant darkness, respectively.

MAP was measured through a catheter connected to a pressure transducer (model TP-400T; Nihon Kohden, Tokyo, Japan). HR was counted from the arterial pressure pulse with a tachometer (model AT-601G, Nihon Kohden). They were recorded with a computer (OptiPlex GX260; Dell, Kawasaki, Japan) every 100 ms through a low-pass filter with an edge frequency of 1.5 Hz to remove pulsatile arterial pressure signals. Activity was monitored with locomotion sensors (model LCM-10M; Melquest, Toyama, Japan) and recorded at 2 min intervals. The criterion for adopting the resting data (Table 1) was activity

Table 1. Mean arterial blood pressure (MAP) and heart rate (HR) in wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice at rest

	Wild-type ($n = 6$)		*Cry1*$^{-/-}$*Cry2*$^{-/-}$ ($n = 5$)	
	Day	Night	Day	Night
MAP				
Mean (mmHg)	79.6 ± 3.6	85.5 ± 3.6**	91.8 ± 4.0*	89.9 ± 3.8*
[a]Variance (mmHg^2)	2.0 ± 0.4	1.7 ± 0.3	1.2 ± 0.2	1.2 ± 0.2
HR				
Mean (beats min^{-1})	435 ± 35	481 ± 27**	529 ± 17*	515 ± 23*
[a]Variance (beats^2 min^{-2})	167 ± 16	299 ± 30***	794 ± 225**	614 ± 155*
Number of data ($\times 10^4$)	18.8 ± 0.8	8.5 ± 1.0***	13.0 ± 1.7**	13.1 ± 1.3**

Values are means ± S.E.M. [a] Variance was calculated from the change in MAP (ΔMAP) or HR (ΔHR) from mean values every 4 s. Number of data, the number meeting the criterion for resting. Total number of data during the day or night was 43.2×10^4. Significant differences from wild-type mice during the day: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Table 2. Spontaneous baroreflex sensitivity (ΔHR/ΔMAP) in wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice

		Day			Night		
	Mouse no.	ΔHR/ΔMAP	R^2	Number of data ($\times 10^4$)	ΔHR/ΔMAP	R^2	Number of data ($\times 10^4$)
Wild-type							
	1	−9.0	0.29	12.1	−14.4	0.28	5.7
	2	−11.7	0.32	14.0	−16.3	0.33	4.4
	3	−10.6	0.33	10.1	−14.5	0.25	3.5
	4	−6.7	0.31	13.3	−10.6	0.30	5.3
	5	−9.8	0.39	14.4	−12.8	0.33	6.1
	6	−10.3	0.41	11.9	−13.1	0.35	9.3
	Mean ± S.E.M.	−9.7 ± 0.7		12.6 ± 0.6	−13.6 ± 0.8***		5.7 ± 0.8***
Cry1$^{-/-}$*Cry2*$^{-/-}$							
	1	−28.6	0.36	4.6	−26.8	0.26	4.2
	2	−25.1	0.32	8.9	−22.4	0.27	8.5
	3	−15.9	0.33	7.1	−16.7	0.30	7.6
	4	−22.3	0.23	8.6	−21.5	0.24	6.8
	5	−23.6	0.24	8.7	−22.2	0.21	8.3
	Mean ± S.E.M.	−23.1 ± 2.1***		7.6 ± 0.8***	−21.9 ± 1.6***		7.1 ± 0.8***

ΔHR/ΔMAP, heart rate response to the spontaneous change in mean arterial pressure (beats min^{-1} mmHg^{-1}). Number of data, the number meeting the criteria for inclusion in spontaneous baroreflex analyses. Total number of data during the day or night was 43.2×10^4. R^2, square of correlation coefficient; ΔHR was highly correlated with ΔMAP in all mice at the level of $P < 0.00001$. ***Significant differences from wild-type mice during the day, $P < 0.001$.

of less than 5 counts (2 min)$^{-1}$ for more than 20 min. The data were also used to calculate spontaneous baroreflex sensitivity (Table 2).

Spontaneous baroreflex sensitivity analyses

Since movement can reduce baroreflex sensitivity in mice (Masuki *et al.* 2003*c*), it was determined only when animals were resting. As in Fig. 1*A*, spontaneous baroreflex sensitivity was determined from HR change in response to the spontaneous change in MAP (ΔHR/ΔMAP) every 4 s, where a cross-correlation function ($R(t)$) between ΔMAP and ΔHR was below the line of $P = 0.05$ (blue), indicating significant negative correlation. Figure 1*B* shows ΔMAP and ΔHR for the parts indicated by the arrows in Fig. 1*A* on an enlarged scale. We found that a rise in MAP caused a fall in HR and inversely a fall in MAP caused a rise in HR after a 0.6 s delay, and the amplitude of the HR response to spontaneous change in MAP was enhanced in the *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse.

HR and MAP during the total resting period of ∼223 min ($n = \sim$134 000) were used to determine spontaneous baroreflex sensitivity in each mouse at rest during the day and night. As spontaneous changes in MAP and consecutive changes in HR were observed at 5–15 cycles min^{-1} (4–12 s cycle^{-1}) as shown in Fig. 1*B*,

we analysed the relation between ΔMAP and ΔHR from the baselines every 4 s of the highest resolution time (τ) using the cross-correlation function given in the following formulas:

$$R(t) = f(\Delta x(t + \Delta t), \Delta y(t))$$

$$\Delta x(t) = x(t) - \overline{x}(t), \quad \Delta y(t) = y(t) - \overline{y}(t)$$

$$\overline{x}(t) = \frac{1}{\tau}\int_{t-\frac{\tau}{2}}^{t+\frac{\tau}{2}} x(t)\,\mathrm{d}t, \quad \overline{y}(t) = \frac{1}{\tau}\int_{t-\frac{\tau}{2}}^{t+\frac{\tau}{2}} y(t)\,\mathrm{d}t$$

where $R(t)$ is the cross-correlation coefficient between x (= MAP) and y (= HR) at the given time of t after correction for the delay time ($\Delta t = 0.6$ s) in response to HR change. The $\overline{x}(t)$ and $\overline{y}(t)$ were averaged values of MAP and HR, respectively, from time $t - \tau/2$ to $t + \tau/2$ ($\tau = 4$ s). Since the cross-correlation function between ΔMAP and ΔHR marked the highest value after correction for the delay time of 0.6 s in all mice, we used this time for the analyses. The detailed numerical analyses were reported previously (Masuki *et al.* 2003*a*). A regression equation was determined from the pooled data during rest in each mouse. The slope of the regression line was used as an index of spontaneous baroreflex sensitivity (Fig. 2).

Variances for ΔMAP and ΔHR were also determined as an index of variability for MAP and HR (Table 1), where ΔMAP and ΔHR were calculated from the same data and equations as in the determination of spontaneous baroreflex sensitivity described above.

Drug-induced baroreflex sensitivity analyses and MAP responses to phenylephrine or sodium nitroprusside

Drug-induced baroreflex sensitivity was determined from the sigmoid response of HR to changes in MAP after an intra-arterial injection of phenylephrine to evoke peripheral α-adrenoceptor-mediated vasoconstriction or sodium nitroprusside for peripheral vasodilatation for each mouse in day and night, respectively. For 3 of 6 wild-type mice and 3 of 5 *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, baroreflex sensitivity in the day was determined at 10:00–12:00 h or CT3–CT5 on the 3rd day after exposure to constant darkness, and then baroreflex sensitivity in the night was determined at 22:00–24:00 h or CT15–CT17 on the same day. For the remaining mice in each group, baroreflex sensitivity was determined at CT15–CT17 in the night on the 3rd day, and then in the day at CT3–CT5 the following day. An alternative order for the determination was adopted to exclude any effects of systemic errors on baroreflex sensitivity due to consecutive drug injections. No significant differences were observed between the sensitivities determined by the two orders.

Before injecting the drugs, we confirmed that the animals were resting according to the range of MAP and HR in Table 1 and no activity was counted by locomotion sensors for at least 5 min. Then, 15 and 30 μg kg^{-1} of sodium nitroprusside, or 5 and 10 μg kg^{-1} of phenylephrine was injected using a 100 μl syringe (Hamilton, Reno, NV, USA) through the bifurcation of a Y-shaped tube (CMA/12; BAS, Tokyo) inserted into the catheter for arterial pressure measurement. Since MAP did not increase with 5 μg kg^{-1} of phenylephrine in the night for wild-type mice or with 10 μg kg^{-1} of phenylephrine in the day and night for *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, 10 μg kg^{-1} was initially injected in the night into wild-type mice, and 50 μg kg^{-1} in the day and night into *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. In addition, 50 μg kg^{-1} were injected in the night into wild-type mice and 200 μg kg^{-1} in the day and night into *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice to increase MAP by ∼20 mmHg, as attained by 10 μg kg^{-1} in the day for wild-type mice. For drug-induced baroreflex analyses, we used the MAP response to phenylephrine seen at 10 μg kg^{-1} in the day and 50 μg kg^{-1} in the night for wild-type mice, and 200 μg kg^{-1} in the day and night for *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. See Fig. 4).

Since the MAP responses to sodium nitroprusside did not change either in the night for wild-type or for *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, we measured the MAP response to 15 and 30 μg kg^{-1} of sodium nitroprusside in all conditions, and the response to 30 μg kg^{-1} was used for the baroreflex analyses. Each dose of sodium nitroprusside or phenylephrine was infused by one trial in each mouse during the day and night in a volume of < 10 μl. The responses were not measured until ∼1 s after the injection because one bifurcation of the Y-shaped tube connected to the pressure transducer was closed during injection into the other bifurcation. MAP and HR responses were recorded thereafter.

Drug-induced baroreflex curves, expressed as the relationship between MAP and HR, were analysed using a logistic sigmoid function according to the following equation (Kent *et al.* 1972; Merrill *et al.* 1996):

$$\mathrm{HR} = \alpha \Big/ \left(1 + \mathrm{e}^{\beta(\mathrm{MAP}-\gamma)}\right) + \delta$$

where α is the range between the upper and lower plateaux, e is an exponential function, β is a coefficient to calculate gain as a function of pressure, γ is MAP at the midpoint of the curve (midpoint), and δ is the lower plateau. Each parameter, the threshold pressure (lowest pressure that produces a significant decline in HR) and the saturation pressure (pressure necessary to achieve maximal inhibition of HR) were determined by fitting the equation to minimize the sum of the y distance between the experimental data (HR) and the y-value (HR) obtained by substituting the x-value (MAP) in the equation.

Statistics

Values are expressed as the means ± S.E.M. The differences in the means and variances of MAP, HR (Table 1),

Table 3. Parameters of drug-induced baroreflex sensitivity analyses

	Wild-type ($n = 6$)		*Cry1*$^{-/-}$*Cry2*$^{-/-}$ ($n = 5$)	
	Day	Night	Day	Night
Gain (beats min^{-1} mmHg^{-1})	-17.4 ± 2.0	$-32.1 \pm 3.9^{**}$	$-41.1 \pm 3.2^{***}$	$-40.6 \pm 1.4^{***}$
Minimum (beats min^{-1})	323 ± 10	323 ± 14	358 ± 25	346 ± 10
Maximum (beats min^{-1})	607 ± 25	$698 \pm 26^{***}$	$752 \pm 28^{**}$	$734 \pm 21^{**}$
Saturation (mmHg)	93.4 ± 2.1	96.2 ± 2.1	101.6 ± 4.2	99.4 ± 2.0
Threshold (mmHg)	68.6 ± 2.5	$77.8 \pm 1.8^{***}$	$86.8 \pm 4.0^{**}$	$85.0 \pm 1.7^{***}$
Midpoint (mmHg)	81.0 ± 2.3	$87.0 \pm 1.8^{***}$	$94.2 \pm 4.0^{*}$	$92.2 \pm 1.8^{**}$

Values are means ± S.E.M. Significant differences from wild-type mice in the day: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Parameters that were used to develop the baroreflex curves in Fig. 3*B* are shown.

activity, spontaneous baroreflex sensitivity (Table 2), the number of data (Tables 1 and 2), MAP responses to phenylephrine or sodium nitroprusside (Fig. 4), and drug-induced baroreflex sensitivity (Table 3) were tested by a 2 (wild-type, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice) × 2 (day, night) ANOVA for repeated measures. Subsequent *post hoc* tests to determine significant differences in the various pair-wise comparisons were performed using Fisher's LSD. The null hypothesis was rejected at $P < 0.05$.

Results

Activity, MAP and HR

Activity in wild-type mice was 6769 ± 629 counts during the night, which was significantly higher than the 4205 ± 435 counts during the day ($P = 0.018$). On the other hand, these diurnal changes were abolished in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice: 4192 ± 885 counts during the day and 3700 ± 510 counts during the night ($P = 0.40$). The total activity for 24 h was $10\,974 \pm 785$ counts in wild-type mice and 7892 ± 1348 counts in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, with no significant differences between them ($P = 0.069$).

In both wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, MAP and HR changed with activity throughout the day and night, increasing during movement and decreasing at rest (Fig. 1*A*). In the wild-type mouse, the baselines of MAP and HR were elevated during the night compared with the day ($P < 0.007$). In the *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse, these diurnal changes disappeared ($P > 0.20$) (Fig. 1*A*, Table 1), demonstrating that the circadian rhythms of MAP and HR were prominent in the wild-type mouse, but absent when CRY genes were absent.

In wild-type mice, HR variability at rest was twofold higher during the night than during the day ($P = 0.0004$), but there was no diurnal difference in MAP variability ($P = 0.31$) (Table 1), indicating that HR fluctuated more in response to spontaneous changes in MAP during the night. However, this diurnal difference in HR fluctuation disappeared in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice ($P = 0.10$), although HR in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice fluctuated more than in wild-type mice ($P < 0.04$) while MAP did not ($P = 0.10$) (Fig. 1*B*). Accordingly, we determined spontaneous baroreflex sensitivity by relating changes in HR (ΔHR) to spontaneous changes in MAP (ΔMAP).

Spontaneous baroreflex sensitivity

In wild-type mice, the number of data meeting the criteria for inclusion in spontaneous baroreflex analyses was significantly smaller during the night than during the day ($P = 0.0009$), but was not different in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice ($P = 0.30$) (Table 2). The total number of data meeting the criteria for inclusion in spontaneous baroreflex analyses during the whole day was not significantly different between wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice ($P = 0.076$). As in Fig. 2 and Table 2, spontaneous baroreflex sensitivity in wild-type mice was enhanced during the night compared with during the day ($P = 0.0002$), while this diurnal difference completely disappeared in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice ($P = 0.15$). Baroreflex sensitivity during the day and night in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice was remarkably enhanced compared with during the day for wild-type mice ($P < 0.0005$).

Drug-induced baroreflex sensitivity

These results were confirmed by the drug-induced baroreflex sensitivity determined from the sigmoid response of HR to changes in MAP after an intra-arterial injection of phenylephrine to evoke peripheral α-adrenoceptor-mediated vasoconstriction, or sodium nitroprusside for peripheral vasodilatation (Fig. 3). In wild-type mice, drug-induced baroreflex sensitivity (gain) in the night was enhanced twofold over that in the day with significantly higher values at the maximum, threshold, and midpoint ($P < 0.0017$) (Table 3). In addition, the HR range in the baroreflex curve (another index of baroreflex gain) for wild-type mice was 375 ± 28 beats min^{-1} in the night, which was significantly higher than the 283 ± 25 beats min^{-1} in the

day ($P = 0.0001$). In contrast, for *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, there were no significant differences in the parameters between the day and night ($P > 0.22$). However, the sensitivity (gain) in the mice was significantly enhanced in the day and night compared with in the day for wild-type mice, with significantly higher values at the maximum, threshold, and midpoint ($P < 0.015$). In addition, the HR range in the baroreflex curve for *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice

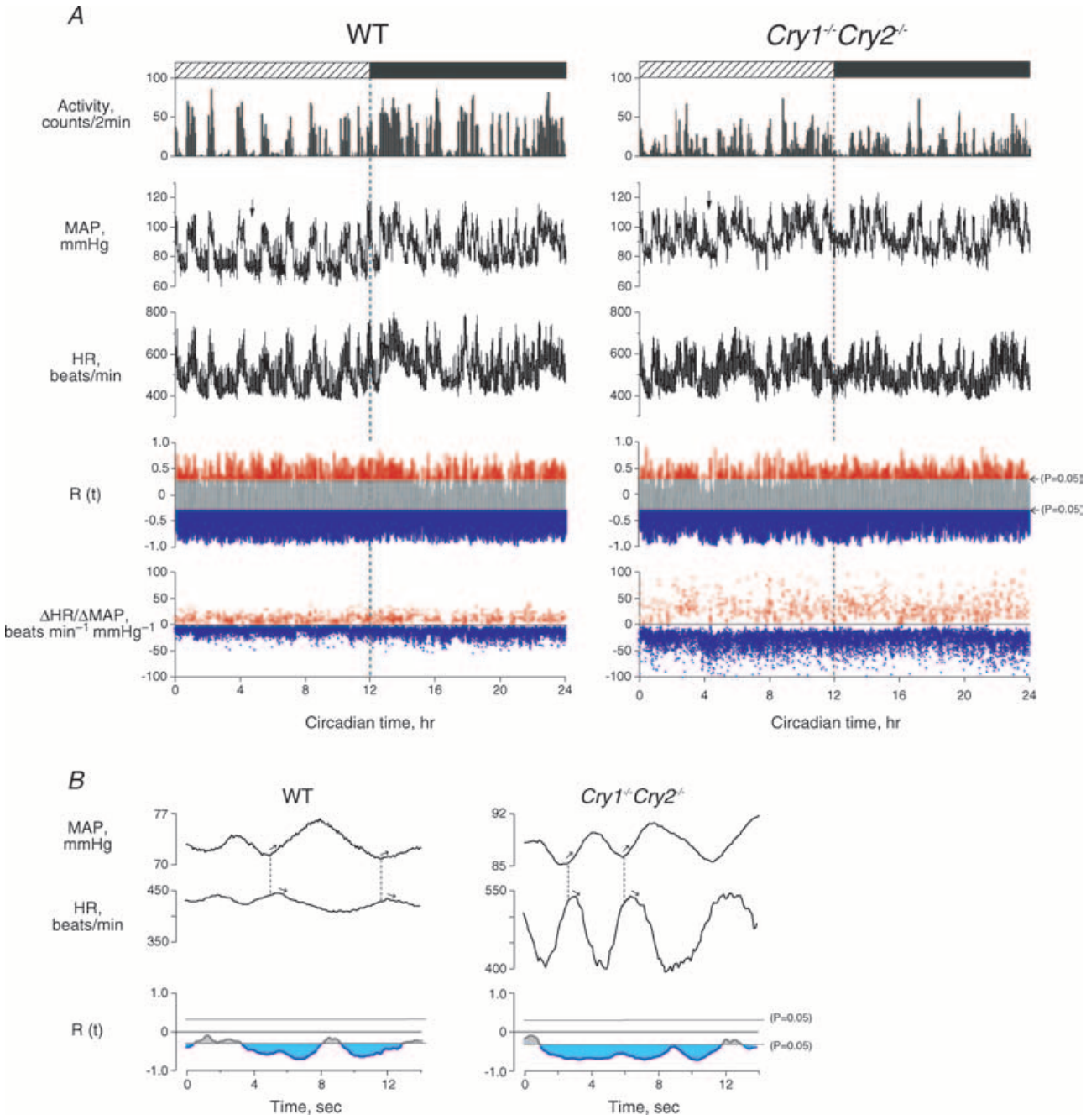

Figure 1. Twenty-four hour profiles of activity, MAP, HR and spontaneous baroreflex sensitivity (ΔHR/ΔMAP) in wild-type (WT) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice

A, typical example of measurements, from top to bottom: activity, MAP, HR, cross-correlation function ($R(t)$) between ΔMAP and ΔHR, ΔHR/ΔMAP in a WT (left) and a *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (right). $R(t)$ above (red) and below (blue) the lines of $P = 0.05$ indicate significantly positive and negative correlations, respectively, which were used to determine positive (red) and negative (blue) ΔHR/ΔMAP. *B*, the areas indicated by arrows in *A* were enlarged to show dynamic change in MAP and HR. The HR response to a given change in MAP was greater in the *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse than in the WT mouse. $R(t)$ in blue indicates a significantly negative correlation at $P < 0.05$, used to determine spontaneous baroreflex sensitivity (ΔHR/ΔMAP).

was 394 ± 10 and 388 ± 12 beats min^{-1} in the day and night, respectively, significantly higher than in the day for wild-type mice ($P = 0.0001$).

MAP responses to phenylephrine or sodium nitroprusside

In wild-type mice, the MAP increase as a result of the injection of 10 μg kg^{-1} of phenylephrine, an α_1-adrenoceptor agonist, was smaller in the night (10 ± 2 mmHg at CT15–CT17) than in the day (22 ± 4 mmHg at CT3–CT5, $P = 0.01$) (Fig. 4). On the other hand, in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, the injection of 10 μg kg^{-1} of phenylephrine did not increase MAP, and even 50 and 200 μg kg^{-1} of phenylephrine increased MAP by only 10–11 and 22–23 mmHg, respectively, in the day and night, with no diurnal differences in the response. In contrast, MAP responses to sodium nitroprusside in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice were almost identical to those in wild-type mice in the day and night ($P > 0.09$). Thus, the pressor responses to phenylephrine in wild-type mice were lower in the night than in the day, and were severely impaired in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice.

Discussion

The major findings in this study are (1) that MAP in wild-type mice was higher during the night than during the day, (2) the α-adrenoceptor-mediated pressor response to phenylephrine in the night for wild-type mice decreased to a half of that in the day, (3) the baroreflex sensitivities, determined by spontaneous or drug-induced methods, were both higher during the night for wild-type mice than those during the day, (4) these diurnal changes disappeared in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, with a severely suppressed α-adrenoceptor-mediated pressor response to phenylephrine, and (5) baroreflex sensitivity was remarkably enhanced in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice.

In mammals, circadian rhythms are created by the hierarchical architecture of the central brain clock at the top and peripheral clocks in various organs at the bottom (Reppert & Weaver, 2002). In either the central or peripheral clocks, circadian clock genes are cyclically expressed by the circadian molecular core oscillator, which is composed of a set of common clock genes with an auto-regulatory transcription–translation-based feedback loop (Balsalobre *et al.* 1998; Yagita *et al.* 2001). Nonaka *et al.* (2001) reported the circadian expression of clock genes in the aorta as well as the cultured vascular smooth muscle

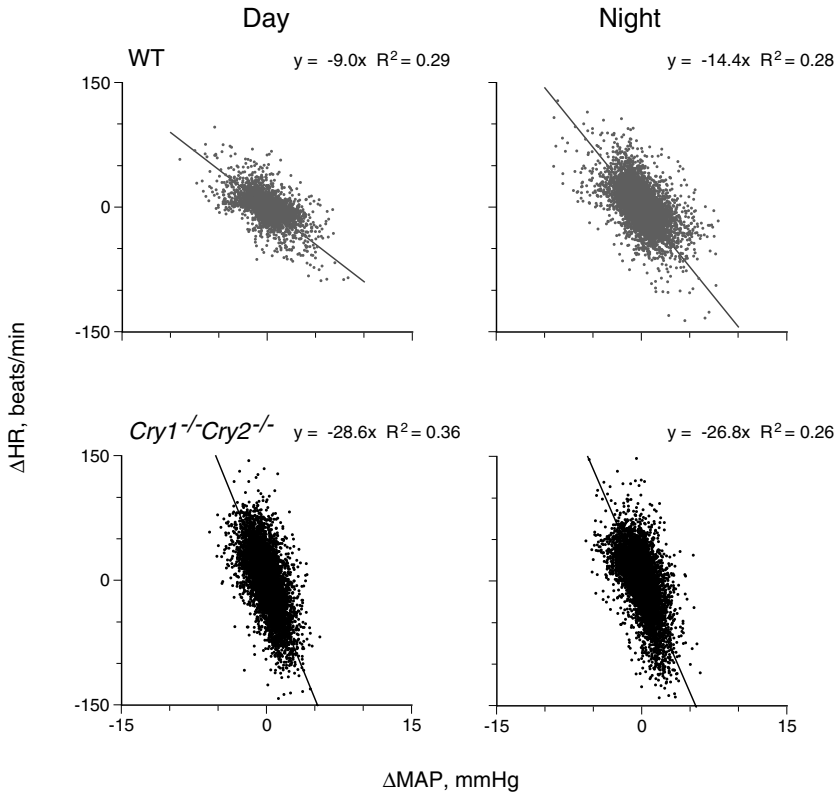

Figure 2. Spontaneous baroreflex sensitivity in wild-type (WT) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice
The slope (ΔHR/ΔMAP), the spontaneous baroreflex sensitivity, was determined from regression analyses (Brace, 1977) on data during periods where ΔMAP was negatively correlated with ΔHR ($P < 0.05$). The number of data was ~83 000 in each graph. Note that ΔHR/ΔMAP is enhanced during the night in a WT mouse while the diurnal difference disappeared in a *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse with enhanced ΔHR/ΔMAP.

cells; however, there have been few studies demonstrating how these clock genes are involved in the circadian rhythm of arterial pressure regulation.

Reduced α-adrenoceptor responsiveness in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice

The extreme suppression of the α-adrenoceptor response in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (Fig. 4) may be caused by an impaired intracellular pathway for α-adrenoceptor-mediated contraction of the vascular smooth muscle cells, including severely reduced expression of α-adrenoceptors. Indeed, clock genes are reportedly involved in the expression of various genes by their transcriptional mechanisms (Panda *et al.* 2002; Oishi *et al.* 2003). In addition, the expression of a set of peripheral clock genes in the liver was reportedly synchronized by electrical stimulation of the hepatic sympathetic nerve (Terazono *et al.* 2003). These results suggest that peripheral CRY genes contribute to circadian changes in arterial pressure regulation by primarily regulating α-adrenoceptor-mediated vasoconstriction in the peripheral vessels although it is unclear whether their expression is controlled by the central clock genes through sympathetic nervous input.

Another possible mechanism for the extreme suppression of the α-adrenoceptor response in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice is the down-regulation of α-adrenoceptors by sustained high sympathetic activity with enhanced baroreflex sensitivity. Experimentally, Grote *et al.* (2000) suggested that sustained higher sympathetic nerve activity and high plasma noradrenaline concentration causes the down-regulation of both α- and β-adrenoceptors. However, as shown in Tables 2 and 3, the HR response to spontaneous or drug-induced changes in MAP was much greater in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice than in wild-type mice during the day and night, indicating that the β-adrenoceptor response in the pacemaker cells of the heart was not reduced in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, but was instead enhanced to compensate for attenuated peripheral α-adrenoceptor-mediated vasoconstriction to maintain MAP. These results suggest that the suppression of the α-adrenoceptor response in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice was caused by mechanisms specific for α-adrenoceptor-mediated vasoconstriction but not by non-specific mechanisms such as the low development of vascular smooth muscle. This idea might be supported by evidence of no significant differences in the vascular responses to sodium nitroprusside between wild-type mice and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (Fig. 4).

These results suggest that a severely suppressed α-adrenoceptor response in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice is not caused by either the down-regulation of α-adrenoceptors due to sustained high sympathetic activity or other non-specific mechanisms in vascular smooth muscles, but is caused by local factors probably associated with the expression of CRY genes in the peripheral vessels.

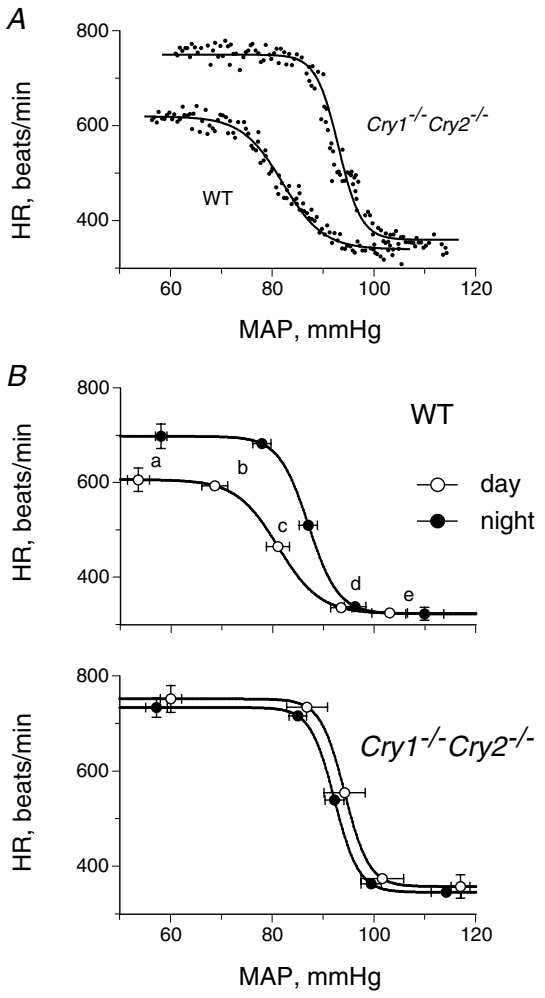

Figure 3. The relationship between MAP and HR after an injection of phenylephrine or sodium nitroprusside
A, typical examples of a wild-type (WT) and a *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mouse in the day. 111 measurements are presented for each mouse. The best-fit sigmoid baroreflex curve generated is also presented as described in the text. *B*, composite baroreflex curves and baroreflex parameters for WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice in the day and night are presented: a, maximum; b, threshold; c, midpoint; d, saturation; e, minimum. Points show means ± S.E.M. for 6 WT and 5 *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice.

Baroreflex sensitivities

This is the first study to determine baroreflex sensitivity from the HR response to spontaneous change in MAP every 4 s for 24 h in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. Using this method, we successfully determined an average sensitivity that was strictly limited to that during the resting periods comprising ∼30% of the total measurement period, since baroreflex sensitivity is reportedly altered

during movement (Burger *et al.* 1998). Experimentally, we previously reported that enhanced baroreflex sensitivity at rest fell by 50% during treadmill exercise in calponin knockout mice (Masuki *et al.* 2003*c*). In the present study, spontaneous baroreflex sensitivity in wild-type mice became less negative during locomotion as shown in Fig. 1*A*. Moreover, since the exogenous administration of phenylephrine or sodium nitroprusside to determine drug-induced baroreflex sensitivity is reported to directly affect HR or baroreflex sensitivity (Peveler *et al.* 1983; Williamson *et al.* 1994; Casadei & Paterson, 2000), we needed to confirm that drug-induced baroreflex sensitivity (Merrill *et al.* 1996) was identical to spontaneous baroreflex sensitivity (Masuki *et al.* 2003*a*). We found that both baroreflex sensitivities in wild-type mice were enhanced during the night compared with during the day, and that they were remarkably enhanced in $Cry1^{-/-}Cry2^{-/-}$ mice regardless of time.

To clarify the mechanisms of these enhanced baroreflex sensitivities, we previously assessed baroreflex sensitivity in mice with targeted destruction of calponin, one of the mediators in the intracellular pathway for α-agonist-induced contraction of the vascular smooth muscle cells (Masuki *et al.* 2003*a*), but with normal clock genes, and suggested that the diurnal rhythm of MAP was flattened with the impairment of peripheral α-adrenoceptor responsiveness and the enhancement of baroreflex sensitivity (Masuki *et al.* 2003*b*; Fig. 5), as observed in $Cry1^{-/-}Cry2^{-/-}$ mice in the present study. Indeed, the enhancement of baroreflex sensitivity during the night in wild-type mice, $Cry1^{-/-}Cry2^{-/-}$ mice, and calponin-deficient mice was highly correlated with the reduction in pressor response to phenylephrine (Fig. 5). Consequently, MAP variability was well controlled in wild-type and $Cry1^{-/-}Cry2^{-/-}$ mice (Table 1). In addition, Masuki *et al.* (2005) recently suggested that the baroreflex control of HR at a given time of day was enhanced in healthy young human subjects with attenuated α-adrenoceptor responsiveness so that arterial pressure was stabilized, consistent with the results in our animal studies. Thus, since baroreflex sensitivity was enhanced with the reduction in α-adrenoceptor responsiveness in both mice and humans with normal clock genes, this enhancement might be a compensatory adaptation to the reduced α-adrenoceptor-mediated vasoconstriction by other mechanisms not related to clock genes.

Baselines of MAP and HR

In this study, the baselines of MAP and HR at rest in wild-type mice were higher during the night than during the day, consistent with the previous results in mice (Li *et al.* 1999; Van Vliet *et al.* 2003) and rats (Zhang & Sannajust, 2000). Since the MAP increase during the night was ∼10% of that during the day, almost identical to the percentage increase in HR, the increase in MAP during the night could be explained by increased HR or cardiac output to supply a higher blood flow to the peripheral organs where the oxygen consumption rate and heat production were enhanced during the night (Nagashima *et al.* 2005).

On the other hand, in $Cry1^{-/-}Cry2^{-/-}$ mice, diurnal changes in the baselines of HR and MAP disappeared and both increased to the same level as during the

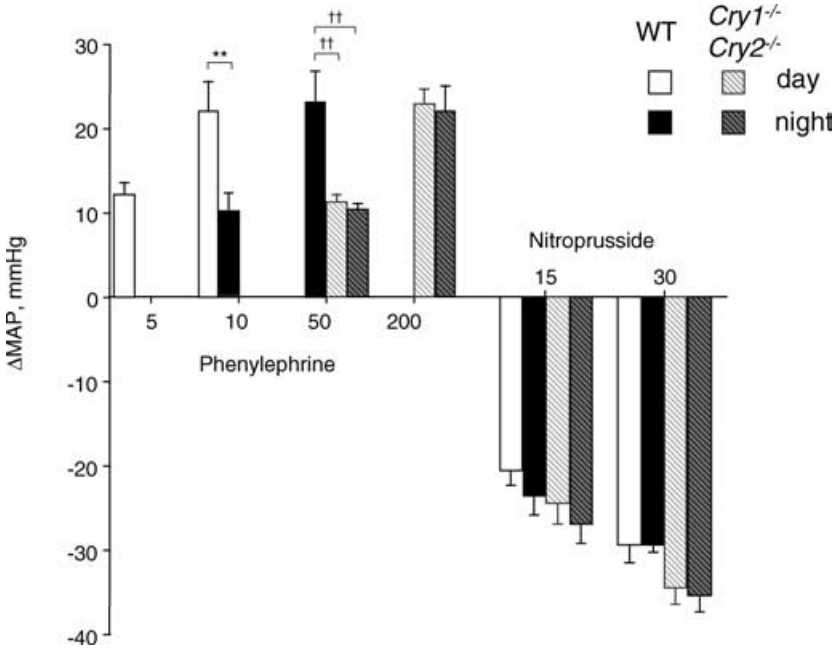

Figure 4. MAP response to 5, 10, 50 and 200 μg kg^{-1} of phenylephrine or 15 and 30 μg kg^{-1} of sodium nitroprusside
Columns show means ± S.E.M. for 6 wild-type (WT) and 5 $Cry1^{-/-}Cry2^{-/-}$ mice. **Significant difference from WT mice in the day at $P < 0.01$. ††Significant differences from WT mice in the night at $P < 0.01$.

night in wild-type mice. We postulated that the oxygen consumption rate and body temperature during the day for *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice would also increase to the same level as during the night in wild-type mice. However, Nagashima *et al.* (2005) measured the circadian rhythms of the core temperature and oxygen consumption rate for 24 h in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice under constant dark conditions and suggested that their circadian rhythms disappeared while the core temperature and oxygen consumption rate averaged for the whole day remained unchanged compared with wild-type mice. Thus, the upward shifts of MAP and HR during the day and the consequent disappearance of their diurnal rhythms in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice are not explained by either the elevated oxygen consumption rate or heat production during the day.

Detailed mechanisms of the well-maintained MAP level throughout the day and night in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice are not clear. Although it is partially explained by the enhanced HR response due to increased baroreflex function (Fig. 3, Table 3), it may not be sufficient for the reduced α-adrenoceptor responsiveness. Similar findings were also reported in recent knockout studies on the subtypes of α-adrenoceptors (α_{1A}, α_{1B}, α_{1D}), suggesting that the level of MAP was relatively well maintained in these mice (Tanoue *et al.* 2002; Rokosh & Simpson, 2002; Hosoda *et al.* 2005). It was also suggested that plasma concentrations of noradrenaline, adrenaline, and angiotensin II did not increase in α_{1B}-, α_{1D}-, or α_{1BD}-adrenoceptor-deficient mice (Hosoda *et al.* 2005) and that the vasoconstrictor response to either angiotensin II or vasopressin was not enhanced in α_{1D}-adrenoceptor-deficient mice (Tanoue *et al.* 2002). Thus, although the maintenance of MAP in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice in this study was partially explained by the enhanced HR response, unknown adaptation mechanisms other than the systemic pressor hormones should be considered.

Limitations

In this study, since the α-adrenoceptor responsiveness was estimated from the increase in MAP after intra-arterial administration of the α-adrenoceptor agonist, reduced α-adrenoceptor responses in the night for wild-type mice and also in the day and night for *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice might be overestimated by the enhanced decrease in HR due to enhanced baroreflex sensitivity (Jones *et al.* 2003). However, these differences in the MAP response were not observed after the administration of sodium nitroprusside, although baroreflex sensitivity was enhanced. These results suggest that HR changes after the administration of these drugs were caused by MAP changes through baroreflexes, and the effects of HR change on MAP were minor. In addition, the 10 μg kg^{-1} injection of phenylephrine was sufficient to increase MAP in wild-type mice but not in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. These results indicate that

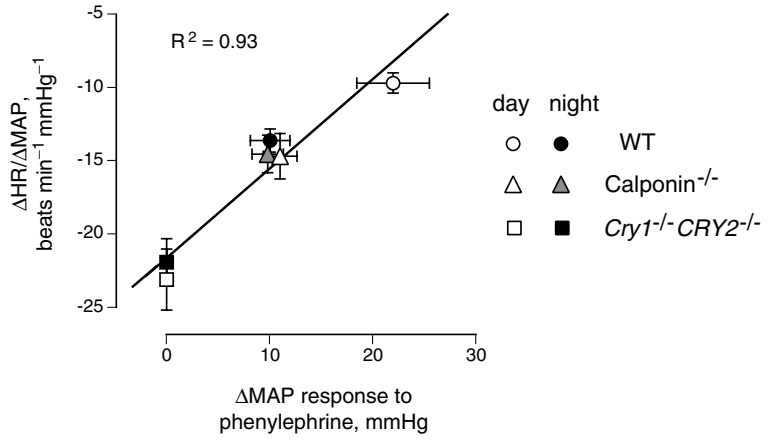

Figure 5. The relationship between the MAP response to 10 μg kg^{-1} of phenylephrine and the spontaneous baroreflex sensitivity in wild-type (WT), *Cry1*$^{-/-}$*Cry2*$^{-/-}$ and calponin-deficient (calponin$^{-/-}$) mice

Means ± S.E.M. bars for 6 WT, 5 *Cry1*$^{-/-}$*Cry2*$^{-/-}$ and 4 calponin-deficient mice. Measurements were performed under dark/dark conditions for WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, and under light/dark conditions for calponin-deficient mice. The average period for which sensitivity was determined was ~223 min (n = 134 000) during the day and night for each mouse. A significantly high correlation was observed between them ($R^2 = 0.93$, $P < 0.01$), suggesting that spontaneous baroreflex sensitivity was enhanced when the α-adrenoceptor-mediated vasoconstrictor response was reduced. Thus, enhanced baroreflex sensitivity may be a compensatory mechanism for reduced α-adrenoceptor-mediated vasoconstriction to maintain arterial pressure, which is not directly related to the oscillation of central clock genes.

α-adrenoceptor responsiveness was severely suppressed in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice.

In summary, the circadian rhythm of baroreflex sensitivity was absent in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice with severely suppressed α-adrenoceptor-mediated vasoconstrictor responses. Thus, CRY genes are involved in generating the circadian rhythm of baroreflex sensitivity, which is partially caused by regulating α-adrenoceptor responsiveness in the peripheral vessels.

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