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Noradrenaline Induces Clock Gene *Per1* mRNA Expression in C6 Glioma Cells Through β_2-Adrenergic Receptor Coupled With Protein Kinase A – cAMP Response Element Binding Protein (PKA–CREB) and Src-Tyrosine Kinase – Glycogen Synthase Kinase-3β (Src–GSK-3β)

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Abstract. Astrocytes in the hypothalamic suprachiasmatic nucleus, site of the master circadian pacemaker, play an essential role in the regulation of systemic circadian rhythms. To evaluate involvement of noradrenergic systems in regulation of circadian variation of clock-genes in astrocytes, we investigated effects of noradrenaline (NA) on expression of several clock genes in C6 glioma cells by using real-time PCR analysis. Treatment with NA (10 μM) induced transient expression of *Per1* mRNA, but not of *Per2*, *Bmal1*, *Clock*, *Cry1*, or *Cry2* mRNA, through activation of β_2 adrenoceptors. Action of NA was partially blocked by H-89 [protein kinase A (PKA) inhibitor] or KG-501 [inhibitor of cAMP response element binding protein (CREB)]. We found that pretreatment with genistein or PP2 (general or Src tyrosine kinase inhibitors, respectively) or LiCl [inhibitor of glycogen synthase kinase-3β (GSK-3β)] significantly inhibited NA-induced *Per1* mRNA expression. In addition, treatment with H-89 and either genistein or LiCl completely blocked NA stimulatory effects. NA markedly induced tyrosine phosphorylation of Src and GSK-3β via activation of β_2 adrenoceptors. Phosphorylation of GSK-3β by NA was completely eliminated by genistein or PP2. These results primarily suggest that two distinct NA-mediating pathways, PKA–CREB and Src–GSK-3β, play crucial roles in regulation of *Per1* expression in astroglial cells.

Keywords: noradrenaline, astrocyte, Per1, tyrosine kinase, glycogen synthase kinase-3β

Introduction

Nearly all mammalian organisms have developed a biological clock system marked by circadian rhythms to synchronize behavioral and physiological functions with 24-h periodicity (1). The pacemaker of such circadian rhythms, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, functions in the pivotal role of biological clock, regulating rhythmic generation of a variety of physiological functions (2). Specific clock genes in this system have been identified and shown to have the crucial responsibility of circadian-rhythm generation. Recent studies of molecular-clock mechanisms have revealed that circadian oscillation of specific clock genes, including *Period* (*Per*), *Cryptochrome* (*Cry*), *Bmal1*, and *Clock*, underlies coordination of circadian rhythmicity in approximately 24-h intervals and consists of a feedback loop in which clock-gene expression is suppressed periodically by the protein products of these genes (3). Furthermore, reports have indicated that such clock genes affect downstream events by regulating rhythmic expression of clock-controlled molecules (4, 5).

Although circadian rhythm generation in SCN is attributable to SCN neurons, evidence suggesting an astrocyte role has been proposed (6). In fact, SCN is comprised of a heterogeneous cell population including neurons and

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glial cells, with astrocytes shown to exist at a rate of about 25% within the SCN (7). One report has shown that inhibition of glial metabolism or glial-gap junctions disturbs circadian rhythms of SCN neurons (8). Such observations have led to speculation that SCN glial cells play pivotal roles in modulating or synchronizing circadian patterns of neuronal activities in SCN. Furthermore, recent reports indicated that astrocytes themselves express circadian oscillation of clock-gene activity and that generation of such rhythms in these cells was sustained when the cells were co-cultured with explants of the adult SCN (9). These observations therefore suggest the possibility that astrocyte circadian activity might serve some function in circadian pacemaking in SCN, with certain diffusible factor(s) produced from the SCN neurons involved in regulation of glial circadian rhythms. Nevertheless, exactly what molecules are associated with modulation of circadian expression in astroglial clock genes is as yet not understood.

SCN molecules such as glutamate, serotonin, and pituitary adenylate cyclase activating peptide have been shown to activate clock-gene transcription (10 – 12). Furthermore, noradrenaline (NA) is a candidate compound for initiation of the cueing of clock-gene expression in astrocytes. Noradrenergic fibers and terminals also are present in the SCN (13). Semba et al. (14) demonstrated that NA levels in rat SCN are high during periods of light and low during periods of darkness, pointing to circadian oscillation of NA levels. Moreover, NA might modulate SCN circadian rhythms by regulating expression of arginine-vasopressin and vasoactive intestinal peptides, substances hypothesized to participate in control of circadian rhythms (15). In addition to the SCN, reports also have demonstrated that NA generates circadian-clock oscillation in peripheral organs and cells (16, 17). Thus, since various roles of NA and several types of adrenergic receptor expression in astrocytes have been reported (18), the possibility also exists that clock-gene expression in astroglial cells might be mediated by NA – adrenergic receptor cascades.

In this study we used C6 glioma cells, which have the potential to express astrocyte-type characteristics (19). Since these cells exhibit circadian oscillation in clock-gene expression after exposure to several reagents (20), we felt it reasonable to try elucidating whether signaling cascades triggered after stimulation with NA also might affect clock-gene expression. As a result of testing this hypothesis, we found that treatment of C6 cells with NA led to transient increase in *Per1* mRNA expression via β_2-adrenergic receptors. Furthermore, this same reaction might be involved in activities of both protein kinase A (PKA) and the Src family of protein tyrosine kinases.

Materials and Methods

Materials

Ham's F12K medium, NA, forskolin, propranolol, CGP20712A, ICI118551, H-89, KG-501, and U73122 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). PP2, KN-93, and Gö6983 were from Calbiochem (La Jolla, CA, USA). Genistein, AG213, U0126, wortmannin, SB202190, and SP600125 were purchased from Tocris Cookson (Bristol, UK). BAPTA-AM was from Dojindo Laboratories (Kumamoto). LiCl was from Nacalai Tesque (Kyoto). NA, propranolol, CGP20712A, ICI118551, H-89, or LiCl were dissolved in distilled H_2O. AG213, U73122, genistein, PP2, KG-501, Gö6983, U0126, SB202190, SP600125, and BAPTA-AM were dissolved in DMSO. The final concentrations of all solvents for treatment of the cells were maintained at 0.5%.

Cell culture

Rat C6 glioma cells were acquired from American Type Culture Collection (Manassas, VA, USA) (CCL-107) and were maintained in Ham's F12K medium supplemented with 15% horse serum, 2.5% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin in an atmosphere of 5% CO_2 / 95% air at 37°C. Twenty-four hours before each experiment, the medium was exchanged with serum-free medium to exclude the undesirable effects of serums. C6 cells between passage numbers 40 and 60 were used for all experiments.

RT-PCR analysis

Total RNAs in C6 cells, rat spinal cord, or adipose tissue were used to synthesize cDNA with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and a random hexamer primer. PCRs were performed with each specific primer indicated in a previous study (21) and AmpliTag Gold™ (Applied Biosystems) at 95°C for 10 min followed by 35 cycles of 95°C for 30 s, each annealing temperature for 30 s, and 72°C for 2 min, with a final extension at 72°C for 5 min. The resulting PCR products were analyzed on a 1.5% agarose gel and had the size expected from the known cDNA sequence.

Real-time PCR analysis

cDNA synthesized using 1 μg of total RNA in each sample were subjected to real-time PCR assays with each specific primer and IQ™ SYBR® Green Supermix (Bio-rad, Tokyo). The sequences of primers are indicated in Table 1. Real-time PCR assays were conducted using a DNA engine Opticon 2 real-time PCR detection system (Bio-Rad). The three-step amplification protocol con-

sisted of a 3 min at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. RNA quantities of target genes were calculated by the C_t method. The C_t values of clock genes amplification were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification.

Western blot analysis

Cells were washed with ice-cold PBS, and solubilized in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The cell lysates were further added to Laemli's buffer and boiled for 5 min. Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. The membranes were blocked in blocking buffer for 1.5 h at room temperature and subsequently incubated with purified polyclonal antibody against Per1 (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA), total cAMP response element binding protein (CREB) (1:1000; Cell Signaling Technology, Beverly, MA, USA), phospho-CREB (1:1000, Cell Signaling Technology), phospho-Src (pY^{418}, 1:1000; Biosource, Camarillo, CA, USA), monoclonal antibody against phospho-glycogen synthase kinase-3β (GSK-3β) (pY^{216}, 1:1000; BD Bioscience, San Jose, CA, USA), or β-actin (1:10000, Sigma) overnight at 4°C. The membranes were washed and then incubated with a horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Then, the membranes were rinsed, and incubated with Luminescence reagent (Bio-rad). Finally, the membranes were exposed to X-ray film to detect the protein. For the quantification of signals, the densities of specific bands were measured with Science Lab Image Gauge (Fuji Film, Tokyo).

Statistical analyses

Data are expressed as the mean ± S.E.M. of at least three independent experiments. Differences between means were determined using a one-way analysis of variance (ANOVA) with a pairwise comparison by the Tukey-Kramer method. Differences were considered to be significant when the *P*-value was less than 0.05.

Results

NA significantly stimulates transient induction of Per1 expression through β_2-adrenergic receptor activation in C6 cells

As shown in Fig. 1a, 1-h incubation of C6 cells with NA (10 μM) induced transient augmentation of expression of only *Per1* mRNA without affecting any other clock genes such as *Per2*, *Bmal1*, *Clock*, *Cry1*, or *Cry2*. Effects of NA (from 0.1 to 10 μM for 1 h) on induction of *Per1* mRNA expression were very likely to occur in a dose-dependent manner, as shown in Fig. 1b. Furthermore, NA also increased the expression level of Per1 protein, and this response peaked at 5 h (Fig. 1c). These data suggest that NA exclusively induces Per1 expression in C6 cells. We therefore decided to focus on association between NA effects and regulation of *Per1* mRNA expression.

We first examined types of adrenergic receptors that might play an indispensable role in NA effects on *Per1* mRNA expression. As indicated in Fig. 2a, expression of α_{1c}-, α_{2d}-, β_1-, or β_2-adrenergic receptors was detected in C6 cells based on RT-PCR analysis. In our study, we used total RNA derived from rat spinal cord and adipose tissue (limited to β_3) as a positive control. We already confirmed that expression of subtypes of adrenoceptor mRNA except for β_3 was detected in rat spinal-cord tissue (21), which thus represents a suitable positive control for examination regarding the presence of α and β adrenoceptors. We then investigated from a pharmacological perspective which of the receptor subtypes might be re-

Table 1. Primer sequences of real-time PCR of clock gene

	Forward primers (5′ → 3′)	Reverse primers (5′ → 3′)
Per1	AGCGCATCCACTCTGGTT	GTCGGTCCTCAGGATGTA
Per2	GACACCCTACCTGGTCAA	CATCCTGGAACAGGCAGT
Clock	CCTGGGAACATCAGGCTA	CTGGCCTTGAGTTCCACT
Bmal1	GCGACTCATCGATGCGAA	AACAGGTGGAGGCGAAGT
Cry1	AATGGAGCCCCTGGAGAT	GAGGACAAGCCATCGGTA
Cry2	GGCAGAAACCACCCCTTA	CTAAGGAAGGCACGCCAT
GAPDH	AGCCCAGAACATCATCCCTG	CACCACCTTCTTGATGTCATC

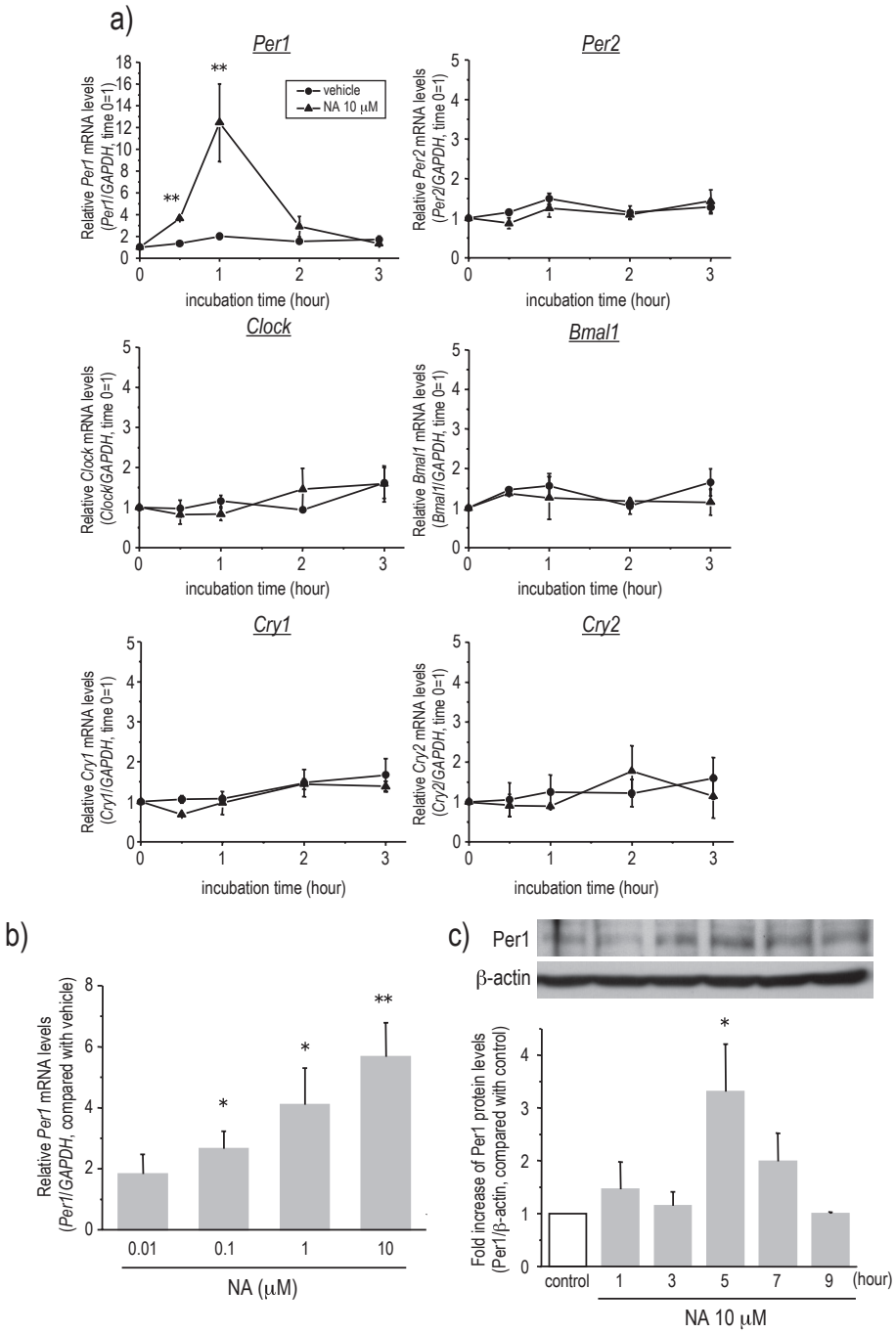

Fig. 1. Treatment of C6 cells with NA led to induction of *Per1* mRNA expression. a) Time-course of NA effects on expression of clock genes in C6 cells. Cells were stimulated with NA (10 μM) for the indicated time periods (h). Data represent the mean ± S.E.M. (bars) for 4 – 5 independent experiments. $^{**}P < 0.01$, compared with the value for vehicle. b) Dose-dependent effect of NA on *Per1* mRNA expression in C6 cells. Cells were incubated with NA (0.01, 0.1, 1, or 10 μM) for 1 h. Data represent the mean ± S.E.M. (bars) for 4 – 7 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the value for vehicle. c) Effect of NA on the expression of Per1 protein. C6 cells were stimulated with NA (10 μM) for the indicated time periods (hour). Data represent the mean ± S.E.M. (bars) for 3 – 7 independent experiments. $^{*}P < 0.05$, compared with the value for the control value.

sponsible for NA-induced *Per1* mRNA expression. As shown in Fig. 2b, any NA effect was completely suppressed by pretreatment with propranolol, a nonselective β-adrenergic receptor antagonist, or ICI118551, a selective β_2 antagonist. On the other hand, pretreatment with CGP20712A, prazocin or yohimbine, antagonists for β_1, α_1, and α_2, respectively, had no effects on NA-induced *Per1* mRNA expression (Fig. 2b). Moreover, treatment with each of these antagonists alone failed to affect *Per1* mRNA expression.

PKA–CREB pathways are partially involved in NA-induced Per1 mRNA expression

As Yagita and Okamura (22) have demonstrated, PKA–CREB is an important signaling cascade in *Per1* transcription regulation. We therefore examined involvement of this pathway in NA-induced *Per1* mRNA expression. Treatment with forskolin, an adenylate cyclase activator, led to induction of *Per1* mRNA expression (Fig. 3: a and b). Moreover, pretreatment with 10 μM of H-89, a potent PKA inhibitor, significantly, but partially,

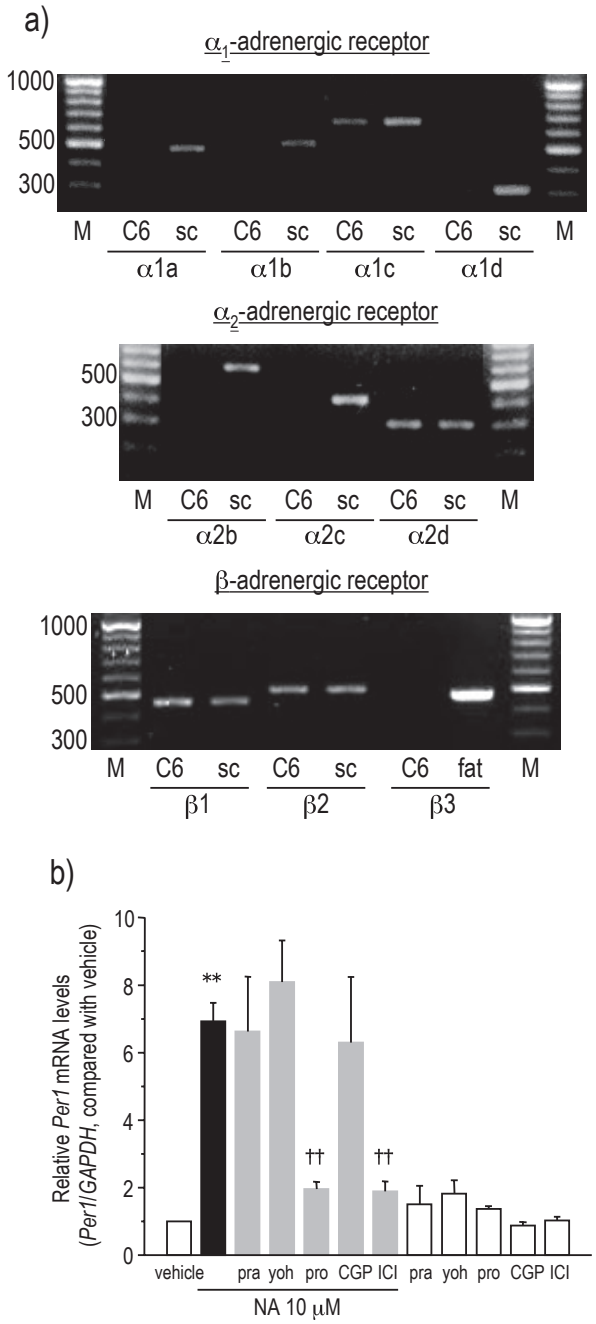

Fig. 2. β_2-Adrenergic receptors are essential for NA-induced *Per1* mRNA expression in C6 cells. a) RT-PCR analysis of adrenergic-receptor mRNA expression in C6 cells. Each lane indicates cDNA fragments of α_1-, α_2-, or β-adrenergic receptors (α1a, 489 bp; α1b, 522 bp; α1c, 661 bp; α1d, 304 bp; α2b, 587 bp; α2c, 425 bp; α2d, 317 bp; β1, 444 bp; β2, 498 bp; β3, 464 bp) amplified from the RNA of C6 cells (C6), rat spinal cord (sc), or rat adipose tissue (fat). Size markers are shown in Lane M. b) Effects of adrenergic-receptor antagonists on NA-induced *Per1* mRNA expression in C6 cells. After incubation with or without prazosin (pra, 10 μM), yohimbine (yoh, 10 μM), propranolol (pro, 10 μM), CGP20712A (CGP, 1 μM) or ICI118551 (ICI, 1 μM) for 30 min, cells were stimulated with NA (10 μM) for 1 h. Data represent the mean ± S.E.M. (bars) for 4 – 6 independent experiments. $^{**}P < 0.01$, compared with the value for vehicle. $^{\dagger\dagger}P < 0.01$, compared with the value for NA alone.

blocked NA effects (Fig. 3a). Despite the fact that a high dose was used, H-89 (20 μM) did not exhibit significantly increased inhibitory effects on NA-induced *Per1* mRNA expression (Fig. 3a). Furthermore, pretreatment with KG-501, which blocks CREB-dependent target-gene transcription through interference with binding between transcription factor and cofactors (23), significantly, but partially, inhibited NA-induced up-regulation of *Per1* mRNA expression (Fig. 3b). We next examined the phosphorylation level of CREB following stimulation by NA. Treatment with NA (10 μM, 5 min) led to significantly increased CREB phosphorylation through stimulation of both β receptors, but not α_1 receptor (Fig. 3c). Moreover, this process was completely suppressed by pretreatment with 10 μM of H-89 (Fig. 3d).

Src tyrosine kinases also contribute to NA-induced Per1 mRNA expression

Since blockade of the PKA–CREB pathway was insufficient for prevention of NA-induced *Per1* mRNA expression, we sought other signal transduction cascades with potential for contribution to NA effects. As shown in Fig. 4a, treatment with genistein, an inhibitor of protein tyrosine kinases, led to significant inhibitory effects on induction of *Per1* mRNA expression by NA or forskolin. Moreover, other tyrosine kinase inhibitors such as AG213 and PP2, a selective inhibitor of Src family tyrosine kinases, also exhibit significant inhibitory activity on NA effects (Table 2 and Fig. 4a). Treatment with each type of tyrosine kinase inhibitor alone had no effect on *Per1* mRNA expression. In addition, pretreatment with U0126, an inhibitor of MAP kinase kinase (MEK), led to partial reversal of NA-induced *Per1* mRNA expression (Table 2). On the other hand, as shown in Table 2, negative results in terms of NA effects were obtained with U73122 (phospholipase C inhibitor), Gö6983 (protein kinase C inhibitor), pertussis toxin ($G_{i/o}$ protein inhibitor), SB202190 (p38 inhibitor), SP600125 (JNK inhibitor), BAPTA-AM (intracellular Ca^{2+} chelator), KN-93 (Ca^{2+}/calmodulin-dependent protein kinase II inhibitor), and wortmannin (phosphatidylinositol 3-kinase inhibitor). Moreover, we found that combined treatment with H-89 and either genistein or PP2 led to additional inhibitory effects on increased *Per1* mRNA expression caused by NA or forskolin (Fig. 4a). In contrast, pretreatment with genistein had no effect on CREB activation by NA (Fig. 4b), suggesting that genistein actions are independent of the PKA–CREB pathway.

Because NA-induced *Per1* mRNA expression was significantly blocked by a Src-specific inhibitor, we next investigated the phosphorylation level of Src tyrosine kinases following stimulation with NA. As a result, treatment with NA (10 μM, 15 min) was found to significantly

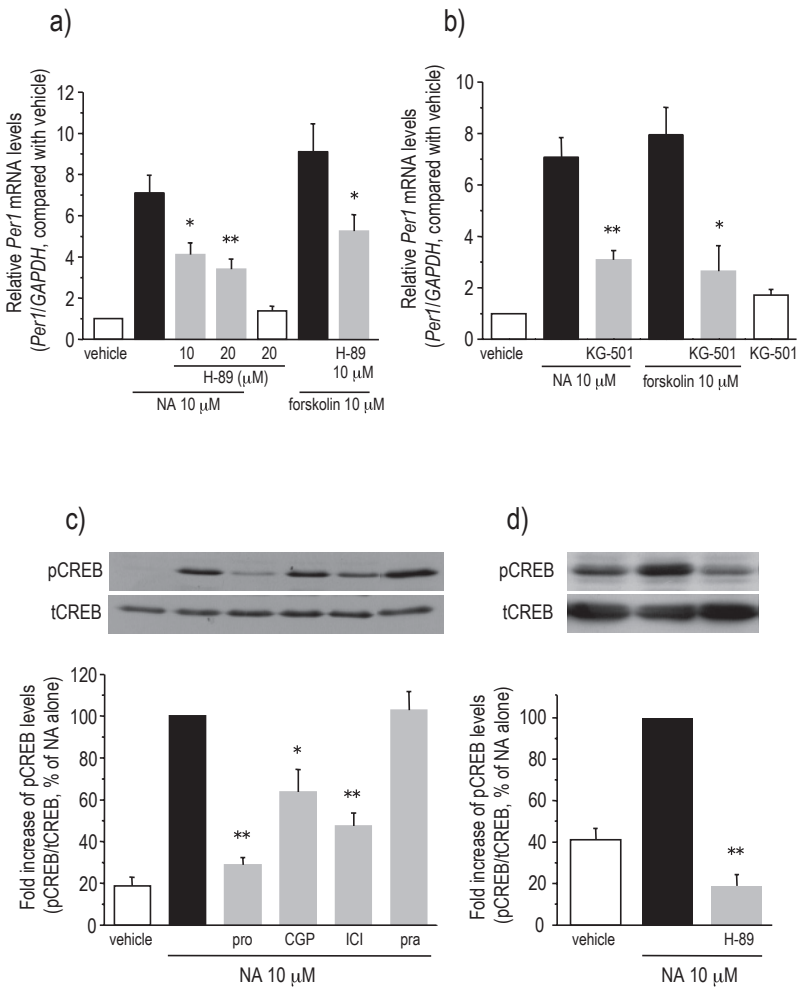

Fig. 3. The PKA–CREB pathway is only partially involved in NA-induced *Per1* mRNA expression. a and b) Effects of H-89 or KG-501 on NA- or forskolin-induced *Per1* mRNA expression. After treatment with or without H-89 (a, 10 or 20 μM) or KG-501 (b, 25 μM) for 1 h, cells were stimulated with NA (10 μM) or forskolin (10 μM) for 1 h. Data represent the mean ± S.E.M. (bars) for 4 – 8 independent experiments. $*P < 0.05$, $**P < 0.01$, compared with the value for NA or forskolin alone. c and d) Phosphorylation of CREB induced by NA is dependent on the β-adrenergic PKA-signaling pathway. After treatment with or without α- or β-adrenoceptor antagonists (c: propranolol, 10 μM; CGP20712A, 1 μM; ICI118551, 1 μM; prazocin, 10 μM, 30 min) or H-89 (d: 10 μM, 1 h), cells were stimulated with NA (10 μM) for 5 min. The upper panel indicates a representative blot. The graph in the lower panel represents quantitative data for each blot in terms of levels of pCREB versus those of tCREB. Data represent the mean ± S.E.M. (bars) for 4 – 9 independent experiments. $*P < 0.05$, $**P < 0.01$, compared with the value for NA alone.

increase phosphorylation of these Src tyrosine kinases (Fig. 5a). The increase was completely blocked by pretreatment with ICI118551 but not with H-89 (Fig. 5: b and c).

Tyrosine phosphorylation of GSK-3β contributes to NA-induced Per1 mRNA expression

Since GSK-3β is known to be a substrate protein for Src tyrosine kinases and to participate in modulation of circadian rhythms (24, 25), we next examined involvement of this enzyme in the circadian process. As represented in Fig. 6a, pretreatment with LiCl, a potent inhibitor of GSK-3β, partially but significantly inhibited NA-induced *Per1* mRNA expression. Furthermore, combined treatment with both LiCl and either H-89 or KG-501 almost completely suppressed stimulatory effects of NA on *Per1* mRNA expression (Fig. 6a). We then further examined NA effects on tyrosine phosphorylation of GSK-3β. Phosphorylation of this enzyme at tyrosine residue (tyr^{216}), located in the kinase domain, was found to increase its activity (26). As shown in Fig. 6b, stimulation with NA (10 μM, 20 min) significantly enhanced tyrosine phosphorylation of GSK-3β, an effect that was blocked by pretreatment with ICI118551, genistein, or PP2, but not H-89 (Fig. 6c). Treatment with these inhibitors alone had no effects on the phosphorylation of GSK-3β (Fig. 6c). Furthermore, we confirmed that LiCl had no effect on NA-induced phosphorylation of CREB (Fig. 6d). These observations thus indicate that Src-tyrosine kinase – GSK-3β is involved in signaling cascades triggered following stimulation of β_2-adrenergic receptors, as well as in regulation of *Per1* mRNA expression in C6 cells.

Discussion

In this study, we have demonstrated that NA stimulated β_2-adrenergic receptors and found that it induces expression of *Per1* mRNA alone among several clock genes tested in C6 cells. This is the first study to report on NA – β_2-adrenergic receptors mediating *Per1* mRNA expression in astroglial cells. We assume that the cas-

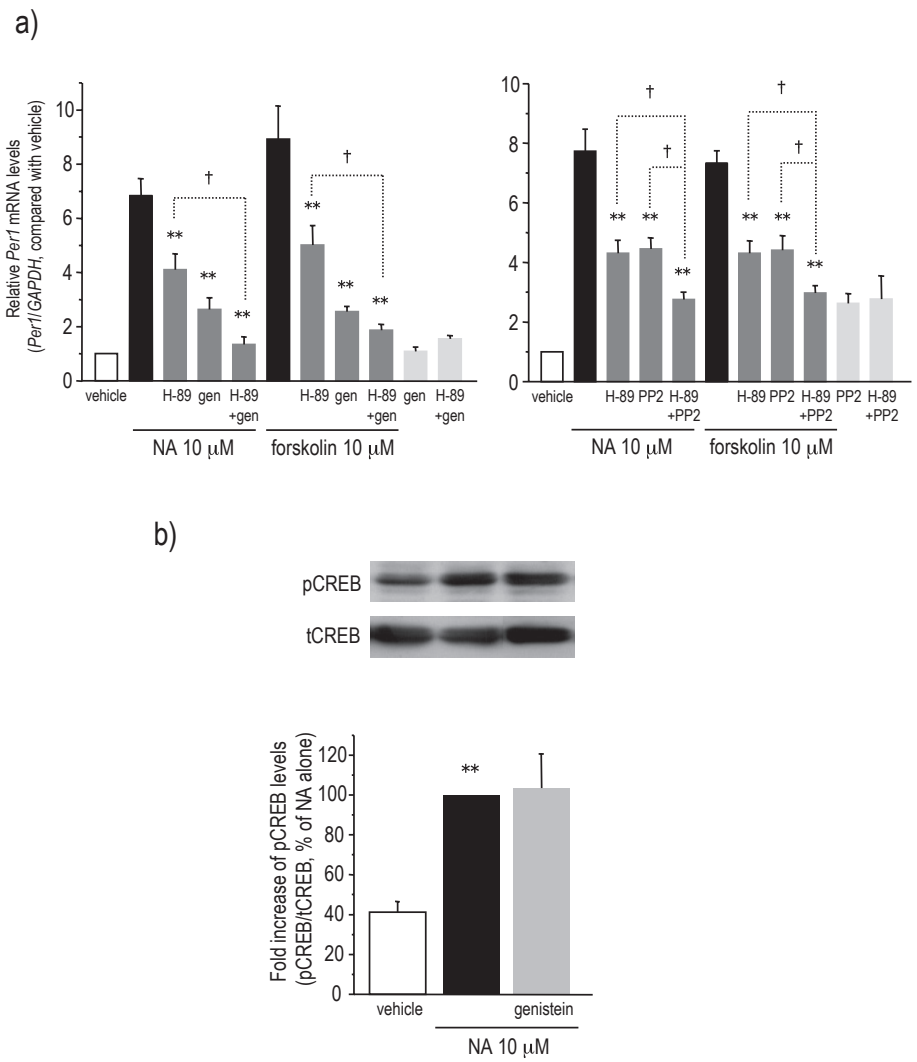

Fig. 4. Protein tyrosine kinases participated in NA-induced *Per1* mRNA expression in C6 cells. a) Effects of genistein (left panel) or PP2 (right panel) on NA-induced *Per1* mRNA expression. After treatment with or without genistein (gen, 50 μM), PP2 (20 μM), H-89 (10 μM) + genistein, or H-89 + PP2 for 1 h, cells were stimulated with NA (10 μM) or forskolin (10 μM) for 1 h. Data represent the mean ± S.E.M. (bars) for 4 – 7 independent experiments. $^{**}P < 0.01$, compared with the value for NA or forskolin alone. $^{\dagger}P < 0.05$, compared between two groups. b) Effects of genistein on NA-induced phosphorylation of CREB in C6 cells. After treatment with or without genistein (50 μM) for 1 h, cells were stimulated with NA (10 μM) for 5 min. Data represent the mean ± S.E.M. (bars) for five independent experiments. $^{**}P < 0.01$, compared with the value for vehicle.

Table 2. Effects of several inhibitors on NA-induced *Per1* mRNA expression in C6 cells

	Per1 mRNA induction (fold alternation of *Per1* mRNA level for vehicle, vehicle = 1)		
	NA (10 μM) alone	NA + inhibitor	Inhibitor alone
Pertussis toxin, 100 ng/ml	11.41 ± 2.09	11.74 ± 1.94	1.63 ± 0.56
U73122, 3 μM	6.14 ± 1.43	8.19 ± 1.69	0.81 ± 0.04
Gö6983, 3 μM	6.59 ± 1.25	5.17 ± 0.89	1.10 ± 0.49
BAPTA-AM, 50 μM	7.68 ± 2.36	6.66 ± 1.73	2.03 ± 0.54
KN-93, 20 μM	5.44 ± 1.02	5.51 ± 0.95	0.82 ± 0.23
AG213, 50 μM	5.92 ± 0.83	3.71 ± 0.52*	0.87 ± 0.23
U0126, 10 μM	5.86 ± 0.78	3.47 ± 0.49*	1.01 ± 0.35
SB202190, 10 μM	6.30 ± 1.32	10.62 ± 2.00	1.09 ± 0.11
SP600125, 10 μM	6.56 ± 0.63	5.83 ± 1.00	1.00 ± 0.20
Wortmannin, 100 nM	7.55 ± 2.68	8.69 ± 1.89	1.37 ± 0.30

Values of increased *Per1* mRNA in C6 cells incubated with or without various inhibitors for 1 h (except for pertussis toxin: 18 h) before treatment with NA (10 μM, 1 h) are expressed as a ratio to vehicle-treated cells. The data represent the mean ± S.E.M. for 3 – 7 independent experiments. $^{*}P < 0.05$, compared with the value for the cells treated with NA alone.

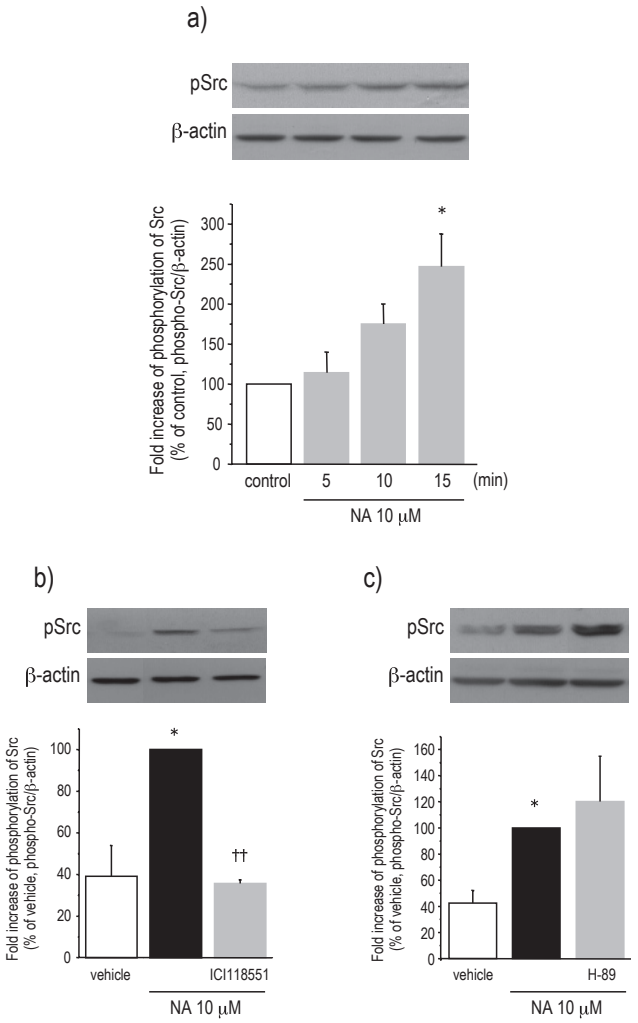

Fig. 5. Stimulation of C6 cells with NA led to phosphorylation of Src tyrosine kinases. a) Time-course of NA effects on phosphorylation of Src tyrosine kinases. Cells were stimulated with NA (10 μM) for the time periods indicated (min). Data represent the mean ± S.E.M. (bars) for 4 – 5 independent experiments. *$P < 0.05$, compared with the control value. b and c) Effects of ICI118551 (b) or H-89 (c) on NA-induced phosphorylation of Src tyrosine kinases. After incubation with ICI118551 (1 μM for 30 min) or H-89 (10 μM for 1 h), cells were stimulated with NA (10 μM) for 15 min. Data represent the mean ± S.E.M. (bars) for 3 – 7 independent experiments. *$P < 0.05$, compared with the value for vehicle. $^{\dagger\dagger}P < 0.01$, compared with the value for NA alone.

cades from NA – β_2-adrenergic receptor activation to *Per1* mRNA induction represent a restricted and specific system, with possible involvement of not only PKA–CREB but also Src tyrosine kinases – GSK-3β–dependent pathways.

In this study, we used C6 cells as a model for astrocytes. A previous study has shown that C6 cells are a good tool for examining the underlying molecular machinery of clock gene expression because several clock genes are expressed and show the circadian oscillation after exposure to dexamethasone in these cells (20). In addition, we and another laboratory have examined various functions of astrocytes by using C6 cells (19, 27). Therefore, although further investigation using by a primary culture of SCN astrocytes is necessary, it is possible that the characteristics of C6 cells might reflect in the SCN astrocytes.

Our results suggest that *Per1* is representative of the immediate inducible gene response to stimuli in glial cells. Induction of *Per1* gene has been demonstrated to represent a crucial component of circadian-clock function in not only SCN but also other tissue and cells (17, 28). In fact, several stimuli initiate robust and immediate increase in *Per1* expression, suggesting that this process might be a key step in initiating circadian rhythms. Thus, demonstration of the signal-transduction pathways involved in regulation of *Per1* mRNA induction in C6 cells might directly resolve questions surrounding roles of astrocytes in generation of such rhythms.

Several previous studies have demonstrated that NA stimulates induction of *Per1* expression through α- or β-adrenergic receptors in various kinds of cells (17, 29). Although expression of several types of adrenergic receptors was detected in C6 cells, our study found involvement of only β_2 adrenoceptors in NA-induced *Per1* mRNA expression. As some reports have suggested a β_2-adrenergic receptor link with multiple signal transduction cascades compared to other types of adrenoceptors (30, 31), we hypothesized that *Per1* gene expression was regulated by a complex process. Furthermore, activation of β_2 receptors with NA might produce superior effects compared to activation with other adrenergic receptors in C6 cells. However, the hypothesis that $\alpha_{1/2}$- or β_1-adrenergic systems might indirectly regulate NA-induced *Per1* mRNA expression in other cells and tissues should not be entirely ruled out. The PKA–CREB signaling cascade coupled with β_2 adrenoceptors has been shown to play an important role in regulation of clock genes including *Per1* in cerebellar granule cells and chondrocytes (32, 33). In our study, this successive cascade was one of the regulatory components in NA-induced *Per1* expression. However, H-89 completely suppressed NA-induced phosphorylation of CREB, with only partial inhibitory action of this reagent on *Per1*-expression induction. Moreover, although stimulation of β_1-adrenergic receptor was also associated with CREB phosphorylation, the NA-induced *Per1* expression was not prevented by the β_1-selective antagonist CGP20712A. Thus, these results, showing that blockage of this pathway was insufficient to prevent NA effects, led us to speculate about the involvement of transduction cascades other than PKA–CREB.

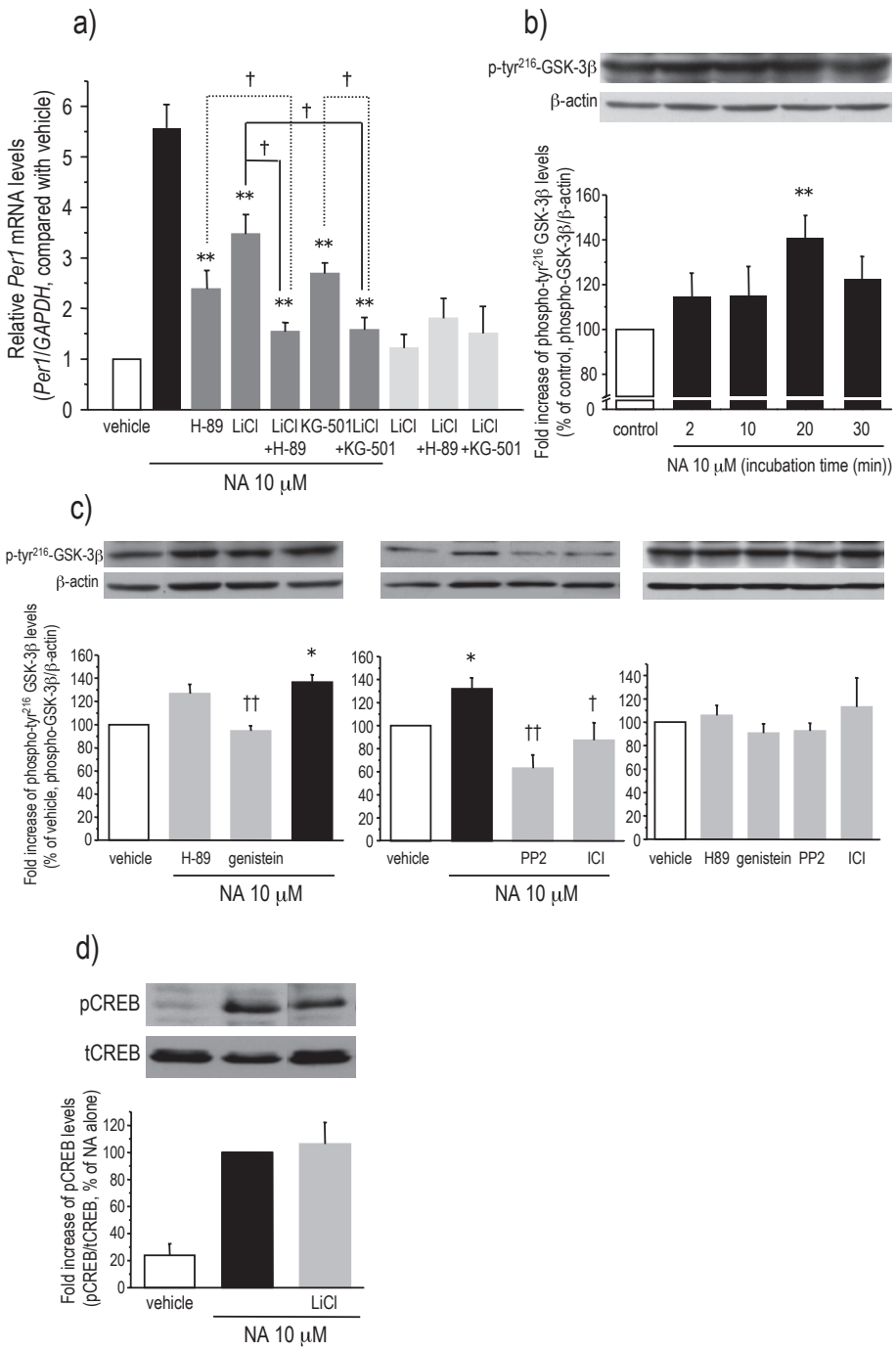

Fig. 6. GSK-3β plays an important role in NA-induced *Per1* mRNA expression in C6 cells. a) Effects of LiCl on NA-induced *Per1* mRNA expression. After preincubation with or without LiCl (10 mM) or LiCl and either H-89 (10 μM) or KG-501 (25 μM) for 1 h, C6 cells were stimulated with NA (10 μM) for 1 h. Data represent the mean ± S.E.M. (bars) for 4 – 11 independent experiments. $^{**}P < 0.01$, compared with the value for NA alone. $^{\dagger}P < 0.05$, compared between two groups. b) Time-course of NA effects on tyr^{216} phosphorylation of GSK-3β. C6 cells were stimulated with NA (10 μM) for the time periods indicated (min). Data represent the mean ± S.E.M. (bars) for four to nine independent experiments. $^{**}P < 0.01$, compared with the control value. c) Effects of tyrosine-kinase inhibitor, H-89 or ICI118551 on NA-induced phosphorylation of GSK-3β. After treatment with or without genistein (50 μM for 1 h), PP2 (20 μM for 1 h), H-89 (10 μM for 1 h), or ICI118551 (1 μM for 30 min), C6 cells were stimulated with NA (10 μM) for 20 min. Data represent the mean ± S.E.M. (bars) for 4 – 8 independent experiments. $^{*}P < 0.05$, compared with the value for vehicle. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, compared with the value for NA alone. d) Effects of LiCl on NA-induced phosphorylation of CREB in C6 cells. After treatment with or without LiCl (10 mM) for 1 h, cells were stimulated with NA (10 μM) for 5 min. Data represent the mean ± S.E.M. (bars) for three independent experiments.

In terms of regulation of *Per1* mRNA expression, the possibility also exists that several β_2 adrenoceptor–triggered signaling cascades other than PKA–CREB contribute to regulation of clock gene mRNA expression (29, 32). In this study, we focused on the Src family of tyrosine kinases as a candidate associated with NA effects, based on our finding that different types of tyrosine kinase inhibitors including PP2, a selective Src inhibitor, exhibited potent inhibitory effects on NA- and forskolin-induced *Per1* expression and that phosphorylation of Src occurred after stimulation of β_2 receptors, independent of PKA activity. These results were similar to results from a previous report by He et al. (34) demonstrating that cAMP-, but not PKA-, dependent mechanisms initiated activation of Src tyrosine kinases. Previous studies also suggested that certain types of Src tyrosine kinases such as c-Src, Fyn, and Lyn are expressed and function in C6 cells (35, 36). These data indicate that Src tyrosine ki-

nases might contribute to regulation of clock-gene expression in conjunction with β-adrenoceptor activation in C6 cells, although it is as yet unclear from our study exactly which types of Src tyrosine kinases are involved. Our observations might therefore provide new insight into involvement of these kinases in glial cell functions through clock regulation.

Our study first showed that NA triggered induction of *Per1* mRNA expression through a Src–GSK-3β–dependent cascade, as indicated in Fig. 7. This hypothesis is supported by the observations that blockage of GSK-3β activity by LiCl significantly inhibited NA-induced *Per1* mRNA expression. In addition, NA-induced phosphorylation of GSK-3β at tyr^{216} was dependent on the activation of β_2 adrenoceptor and inhibited by either genistein or PP2. Furthermore, our results showing that U0126, a MEK inhibitor, had significant inhibitory effect on induction of *Per1* by NA also support involvement of GSK-3β, based on a previous report suggesting that MEK induces tyrosine phosphorylation of GSK-3β in fibroblasts (37). Phosphorylation of GSK-3β at tyr^{216} enhanced the activity of this enzyme and participates in the regulation of cell functions such as neuronal plasticity and apoptosis (24, 38). GSK-3β also is reported to be an important enzyme related to regulation of the mammalian circadian clock (25), with its activation stimulated by β adrenoceptors (38). Phosphorylation of GSK-3β at tyr^{216} is induced by Fyn, which belongs to the Src family of tyrosine kinases and is expressed in C6 cells (24). Interaction between cAMP–PKA–CREB and Src–GSK-3β was not observed in our study, however, because neither genistein nor LiCl generated potent effects on NA-induced CREB phosphorylation, and pretreatment with H-89 failed to affect NA-stimulated phosphorylation of Src or GSK-3β.

Circadian proteins produced by clock genes are involved in regulation of expression of clock-controlled genes (4, 5). Thus, the system also generates physiological rhythms in astrocyte functions. Several studies have suggested the existence of circadian changes in expression levels of glial fibrillary acidic protein (GFAP), a specific astrocyte maker, in SCN astrocytes (39, 40). In addition, Moriya et al. (39) have demonstrated that the expression of GFAP in SCN was dramatically changed under constant lighting conditions, while that was not constant under a light–dark cycle. Another study has suggested that alteration of GFAP expression might reflect activity of astrocytes (41), indicating the possibility that circadian rhythms of GFAP expression are correlated with those of astroglial functions. Glutamate transporter EAAT2 has also been proposed as a candidate for regulation by astrocyte circadian rhythms (4). Thus, although further investigation may be necessary, the signal cascades described in our study might plausibly participate in regulation of such clock-controlled gene expression through *Per1* induction.

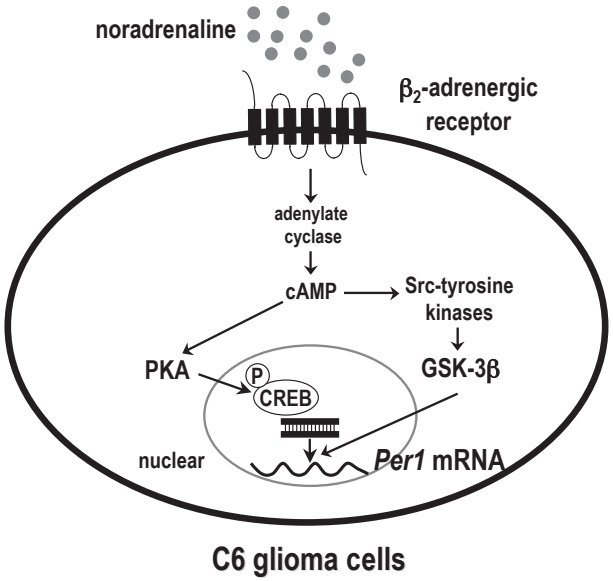

Fig. 7. Schematic illustration of NA – β_2-adrenergic receptor stimulation–triggered induction of *Per1* mRNA expression in C6 cells.

In conclusion, we observed two distinct signal transduction system–mediated clock gene *Per1* expressions in C6 cells. The mechanism behind how circadian-rhythm generation in astroglial cells affects such rhythms in neurons is thus not well understood, but at the least, the SCN astroglial component is thought to be crucial for operation of circadian patterns of SCN neuronal activity. Thus, alteration of glial functions including intracellular Ca^{2+} concentration, and expression of gliotransmitters, their receptors, or transporters via NA-mediated circadian rhythms in astrocytes might affect transmission to generate rhythms between SCN astrocytes and neurons. Therefore, our finding has the potential to contribute to enhanced understanding of circadian-rhythm generation in SCN neurons, as well as perhaps systemic circadian rhythms.

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References

1 Dunlap J. Circadian rhythms: an end in the beginning. Science. 1998;280:1548–1549.
2 Sassone-Corsi P. Molecular clocks: mastering time by gene regulation. Nature. 1998;392:871–874.
3 King DP, Takahashi JS. Molecular genetics of circadian rhythms in mammals. Annu Rev Neurosci. 2000;23:713–742.
4 Spanagel R, Pendyala G, Abarca C, Zghoul T, Sanchis-Segura C, Magnne MC, et al. The clock gene *Per2* influences the glutamatergic system and modulates alcohol consumption. Nature Med. 2005;11:35–42.
5 Cai W, Rambaud J, Teboul M, Masse I, Benoit G, Gustafsson JA, et al. Expression levels of estrogen receptor β are modulated by components of the molecular clock. Mol Cell Biol. 2008; 28:784–793.
6 Shinohara K, Honma S, Katsuno Y, Abe H, Honma K. Two distinct oscillators in the rat suprachiasmatic nucleus in vitro. Proc Natl Acad Sci U S A. 1995;92:7396–7400.
7 Guldner FH. Numbers of neurons and astroglial cells in the suprachiasmatic nucleus of male and female rats. Exp Brain Res. 1983;50:373–376.
8 Prosser RA, Edgar DM, Heller HC, Miller JD. A possible glial role in the mammalian circadian clock. Brain Res. 1994;643: 296–301.
9 Prolo LM, Takahashi JS, Herzog ED. Circadian rhythm generation and entrainment in astrocytes. J Neurosci. 2005;25:404–408.
10 Akiyama M, Kouzu Y, Takahashi S, Wakamatsu H, Moriya T, Maetani M, et al. Inhibition of light- or glutamate-induced *mPer1* expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms. J Neurosci. 1999; 19:1115–1121.
11 Hannibal J, Jamen F, Nielsen HS, Journot L, Brabet P, Fahrenkrug J. Dissociation between light-induced phase shift of the circadian rhythm and clock gene expression in mice lacking the pituitary adenylate cyclase activating polypeptide type 1 receptor. J Neurosci. 2001;21:4883–4890.
12 Varcoe TJ, Kennaway DJ. Activation of 5HT2C receptors acutely induces Per1 gene expression in the rat SCN in vitro. Brain Res. 2008;1209:19–28.
13 Cagampang FRA, Okamura H, Inouye ST. Circadian rhythms of norepinephrine in the rat suprachiasmatic nucleus. Neurosci Lett. 1994;173:185–188.
14 Semba J, Toru M, Mataga N. Twenty-four hour rhythms of norepinephrine and serotonin in nucleus suprachiasmaticus, raphe nuclei, and locus coeruleus in the rat. Sleep. 1984;7:211–218.
15 Vacher CM, Frétier P, Créminon C, Seif I, De Maeyer E, Calas A, et al. Monoaminergic control of vasopressin and VIP expression in the mouse suprachiasmatic nucleus. J Neurosci Res. 2003; 71:791–801.
16 Terazono H, Mutoh T, Yamaguchi S, Kobayashi M, Akiyama M, Udo R, et al. Adrenergic regulation of clock gene expression in mouse liver. Proc Natl Acad Sci U S A. 2003;100:6795–6800.
17 Takata M, Burioka N, Ohdo S, Fukuoka Y, Miyata M, Endo M, et al. β2-adrennoceptor agonists induce the mammalian clock gene, h*Per1*, mRNA in cultured human bronchial epithelium cells in vitro. Chronobiol Int. 2005;22:777–783.
18 Hertz L, Chen Y, Gibbs ME, Zang P, Peng L. Astrocytic adrenoceptors: a major drug target in neurological and psychiatric disorders? Curr Drug Targets CNS Neurol Disord. 2004;3:239–267.
19 Morioka N, Abdin JM, Morita K, Kitayama T, Nakata Y, Dohi T. The regulation of glycine transporter GLYT1 is mainly mediated by protein kinase Cα in C6 glioma cells. Neurochem Int. 2008; 53:248–254.
20 Fujioka A, Takashima N, Shigeyoshi Y. Circadian rhythm generation in a glioma cell line. Biochem Biophys Res Commun. 2006; 346:169–174.
21 Morioka N, Tanabe H, Inoue A, Dohi T, Nakata Y. Noradrenaline reduces the ATP-stimulated phosphorylation of p38 MAP kinase via β-adrenergic receptors-cAMP-protein kinase A-dependent mechanism in cultured rat spinal microglia. Neurochem Int. 2009;55:226–234.
22 Yagita K, Okamura H. Forskolin induces circadian gene expression of *rPer1*, *rPer2* and *dbp* in mammalian rat-1 fibroblasts. FEBS Lett. 2000;465:79–82.
23 Best JL, Amezcua CA, Mayr B, Flechner L, Murawsky CM, Emerson B, et al. Identification of small-molecule antagonists that inhibit an activator: coactivator interaction. Proc Natl Acad Sci U S A. 2004;101:17622–17627.
24 Lesort M, Jope RS, Johnson GVW. Insulin transiently increases tau phosphorylation: involvement of glycogen synthase kinase-3β and Fyn tyrosine kinase. J Neurochem. 1999;72:576–584.
25 Iitaka C, Miyazaki K, Akaike T, Ishida N. A role for glycogen synthase-3β in the mammalian circadian clock. J Biol Chem. 2005;280:29397–29402.
26 Bhat RV, Shanley J, Correll MP, Fieles WE, Keith RA, Scott CW, et al. Regulation and localization of tyrosine^{216} phosphorylation of glycogen synthase kinase-3β in cellular and animal models of neuronal degeneration. Proc Natl Acad Sci U S A. 2000;97: 11074–11079.
27 Yeh WL, Lu DY, Lee J, Fu WM. Leptin induces migration and invasion of glioma cells through MMP-13 production. Glia. 2009;57:454–464.
28 Gumz ML, Stow LR, Lynch IJ, Greenlee MM, Rudin A, Cain BD, et al. The circadian clock protein Period 1 regulates expression of the renal epithelial sodium channel in mice. J Clin Invest. 2009;119:2433–2434.
29 Akiyama M, Minami Y, Kuriyama K, Shibata S. MAP kinase-dependent induction of clock gene expression by α_1-adrenergic receptor activation. FEBS Lett. 2003;542:109–114.
30 Daaka Y, Luttrell LM, Lefkowitz RJ. Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. Nature. 1997;390:88–91.
31 Benovic JL. Novel β_2-adrenergic receptor signaling pathways. J Allergy Clin Immunol. 2002;110:S229–S235.
32 Akiyama M, Minami Y, Nakajima T, Moriya T, Shibata S. Calcium and pituitary adenylate cyclase-activating polypeptide induce expression of circadian clock gene *mPer1* in the mouse cerebellar granule cell culture. J Neurochem. 2001;78:499–508.
33 Hinoi E, Ueshima T, Hojo H, Iemata M, Takarada T, Yoneda Y. Upregulation of *per* mRNA expression by parathyroid hormone through a protein kinase A-CREB-dependent mechanism in chondrocytes. J Biol Chem. 2006;281:23632–23642.
34 He Q, Wu G, Lapointe MC. Isoproterenol and cAMP regulation of the human brain natriuretic peptide gene involves Src and Rac. Am J Physiol Endocrinol Metab. 2000;278:E1115–E1123.
35 Kronfeld I, Kazimirsky G, Lorenzo PS, Garfield SH, Blumberg PM, Brodie C. Phosphorylation of protein kinase Cδ on distinct

tyrosine residues regulates specific cellular functions. J Biol Chem. 2000;275:35491–35498.

36 Tsuchioka M, Takebayashi M, Hisaoka K, Maeda N, Nakata Y. Serotonin (5HT) induces glial cell line-derived neurotrophic factor (GDNF) mRNA expression via the transactivation of fibroblast growth factor receptor 2 (FGFR2) in rat C6 glioma cells. J Neurochem. 2008;106:244–257.

37 Takahashi-Yanaga F, Shiraishi F, Hirata M, Miwa Y, Morimoto S, Sasaguri T. Glycogen synthase kinase-3β is tyrosine-phosphorylated by MEK1 in human skin fibroblasts. Biochem Biophys Res Commun. 2000;316:411–415.

38 Menon B, Johnson JN, Ross RS, Singh M, Singh K. Glycogen synthase kinase-3β plays a pro-apoptotic role in β-adrenergic receptor-stimulated apoptosis in adult rat ventricular myocytes: role of β1 integrins. J Mol Cell Cardiol. 2007;42:653–661.

39 Moriya T, Yoshinobu Y, Kouzu Y, Katoh A, Gomi H, Ikeda M, et al. Involvement of glial fibrillary acidic protein (GFAP) expressed in astroglial cells in circadian rhythm under constant lighting conditions in mice. J Neurosci Res. 2000;60:212–218.

40 Lindley J, Deurveilher S, Rusak B, Semba K. Transforming growth factor-β and glial fibrillary acidic protein in the hamster circadian system: daily profile and cellular localization. Brain Res. 2008;1197:94–105.

41 Hsiao HY, Mak OT, Yang CS, Liu YP, Fang KM, Tzeng SF. TNF-α/IFN-γ-induced iNOS expression increased by prostaglandin E_2 in rat primary astrocytes via EP2-evoked cAMP/PKA and intracellular calcium signaling. Glia. 2007;55:214–223.