A Role for Glycogen Synthase Kinase-3β in the Mammalian Circadian Clock*§

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The Drosophila shaggy gene product is a mammalian glycogen synthase kinase-3β (GSK-3β) homologue that contributes to the circadian clock of the Drosophila through TIMELESS phosphorylation, and it regulates nuclear translocation of the PERIOD/TIMELESS heterodimer. We found that mammalian GSK-3β is expressed in the suprachiasmatic nucleus and liver of mice and that GSK-3β phosphorylation exhibits robust circadian oscillation. Rhythmic GSK-3β phosphorylation is also observed in serum-shocked NIH3T3 cells. Exposing serum-shocked NIH3T3 cells to lithium chloride, a specific inhibitor of GSK-3β, increases GSK-3β phosphorylation and delays the phase of rhythmic clock gene expression. On the other hand, GSK-3β overexpression advances the phase of clock gene expression. We also found that GSK-3β interacts with PERIOD2 (PER2) in vitro and in vivo. Recombinant GSK-3β can phosphorylate PER2 in vitro. GSK-3β promotes the nuclear translocation of PER2 in COS1 cells. The present data suggest that GSK-3β plays important roles in mammalian circadian clock.

The behavior and physiology of most organisms show circadian, 24-h rhythmicity (1). Negative feedback loops in circadian genes are thought to control circadian rhythmicity in all organisms from bacteria to mammals (2–6). Oscillating molecules that control their own circadian expression seem to be important for generating circadian rhythms (7, 8). The transcriptional factors of the mammalian clock are the CLOCK and BMAL1 proteins that contain the PAS domain (9, 10), whereas the negative factors include the PERIOD proteins PER1 and PER2 and the CRYPTOCHROME proteins CRY1 and CRY2 (9, 11, 12).

The circadian timing system in mammals is hierarchical with the main clock being located in the suprachiasmatic nucleus (SCN) of the hypothalamus (2). The master clock synchronizes circadian oscillators in peripheral tissues through humoral and adrenergic outputs (13–17). The molecular oscillators are operative in cell lines because serum shock can induce circadian gene expression in cultured fibroblasts (18, 19).

The stability and the activity of circadian molecules are dependent on several factors such as kinase. Positional cloning has revealed that the tau locus (that shortens circadian rhythms) in hamsters is encoded by casein kinase 1ε (CK1ε) (20), a homologue of the Drosophila clock gene double-time. The double-time gene product phosphorylates PER and causes protein degradation in Drosophila (21). CK1ε phosphorylates PER1, PER2, and PER3, then renders them unstable in mammals (22–24). The novel clock gene, shaggy, was recently discovered in Drosophila (25). The gene product SHAGGY (SGG) has serine/threonine kinase activity, and it affects phosphorylation of the Drosophila clock protein TIMELESS (TIM). The overexpression of SGG shortens, whereas reduced expression lengthens, the period of circadian locomotor activity, indicating that SGG activity plays an intrinsic role in circadian rhythmicity of Drosophila. The overexpression of SGG in vivo converts hypophosphorylated TIM to a hyperphosphorylated protein, and SGG might phosphorylate TIM in vitro. The proposed ortholog of shaggy in mammals is glycogen synthase kinase-3β (GSK-3β), the activity of which is down-regulated through phosphorylation of an NH2-terminal serine residues (Ser-9) (26, 27). It is a serine/threonine kinase that plays a key role in many signaling pathway (28–30). However, whether GSK-3β can affect the mammalian circadian clock has not yet been determined. Thus, we characterized the role of GSK-3β activity in the mammalian circadian clock.

MATERIALS AND METHODS

Animals—ICR mice were entrained in a 12 h light:12 h dark cycle for at least 2 weeks. At selected times, mice were killed, and SCN and livers were dissected and frozen on dry ice.

Plasmid Construction—To generate eukaryotic expression constructs of rGSK-3β (a gift from Dr. K. Ishiguro (31)), the cDNA encoding PCR-amplified fragments were cloned into pFLAG-CMV (Sigma). The expression constructs encoding full-length rPER2, amino-terminal of rPER2 (amino acids 1–512) and carboxyl-terminal of rPER2 (amino acids 512–1257) have been described previously (32). The rPER2 fragments (amino acids 1–171, 172–319, 320–512, and 372–512) were amplified by PCR and cloned into pFLAG-CMV·Full-length mCry1 and mCry2 (provided by Dr. T. Todo) were cloned into

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1 The abbreviations used are: SCN, suprachiasmatic nucleus; CK1ε, casein kinase 1ε; GSK-3β, glycogen synthase kinase-3β; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase gene; siRNA, short interfering RNA; ZT, Zeitgeber time.
At time 0, the medium was changed to DMEM containing 50% horse serum and a mixture of penicillin and streptomycin in a humidified incubator at 37 °C and 5% CO2. COS1 cells were transfected using Lipofectamine PLUS (Invitrogen) or FuGENE 6 (Roche Diagnostics) according to the protocols supplied by the manufacturers. NIH3T3 cells transfected with Polyfect (Qiagen). The cells were washed twice with phosphate buffered saline (PBS) after 24 h post-transfection and suspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40) containing Complete protease inhibitor mixture (Roche Diagnostics). Lysates were incubated for 30 min on ice then clarified by centrifugation at 12,000 × g for 5 min at 4 °C. Western Blotting—The amount of protein was determined using DC protein assay (Bio-Rad), and the same amounts of proteins were normalized for total protein. The protein samples were boiled in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad). Nonspecific binding was blocked with 3% dry milk in PBS and proteins were probed with anti-hPER1, anti-rPER2 (provided by Dr. T. Nagase), anti-Xpress (Invitrogen), anti-V5 (Invitrogen), and anti-FLAG (SIGMA), all diluted 1:5000; anti-PSK-3β (Transduction Laboratories) diluted 1:2500; or anti-phospho-PSK-3β (Cell Signaling) diluted 1:1000. Immunoprecipitation—Transfected COS1 cells were lysed as described above. A slurry of antibody and protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) was added to clarified supernatants. After an overnight incubation at 4 °C, immuno complexes were washed twice with lysis buffer, mixed in SDS sample buffer, and analyzed by Western blotting. To generate the data shown in Fig. 4C, fresh mouse brains (0.5 g) at ZT2 and ZT14 were dissected and then homogenized in 1 ml of lysis buffer (20 mM Tris-HCl (pH 8.0), 0.4 mM NaCl, 1% Triton X-100, 5% glycerol) containing phosphatase inhibitor mixture 1 (Sigma) and Complete protease inhibitor mixture. The resulting homogenate was separated by centrifugation and the supernatants were immunoprecipitated using anti-rPER2 antiserum. Serum Shock and Northern Blotting—Two days after seeding ~5 × 105 cells/cm 2 Petri dish (Iwaki), confluent NIH3T3 cells were serum-shocked by a 24-h incubation in serum-free medium as described (18). At time 0, the medium was changed to DMEM containing 50% horse serum (Invitrogen), and 2 h later, this medium was replaced with DMEM containing 10% fetal bovine serum, penicillin, and streptomycin. At the indicated times, the cells were washed twice with PBS, and total RNA was extracted using ISOGEN reagent (Nippon Gene). The Northern blotting proceeded as described (15). Samples were normalized by comparison with the amount of GAPDH mRNA. Kinase Assay—rPER2 and mCLOCK were expressed in COS1 cells, and the cells were sonicated in lysis buffer. Proteins were immunoprecipitated by incubating the cell lysates with the appropriate antibodies and protein A/G-agarose beads overnight at 4 °C. The beads were washed twice with lysis buffer and once with kinase reaction buffer containing 25 ng of recombinant GST-3β (Upstate Biotechnology) and then with 100 μM ATP, 10 μCi of [γ-32P]ATP (Amersham Biosciences) for 15 min at 30 °C. To exclude the effect of endogenous CK1 in COS1 cells, we added 400 μM LiCl. Total protein prepared at the indicated times was Western blotted and probed for anti-phospho-PSK-3β (pPSK-3β) and anti-PSK-3β antibodies. B, the expression or phosphorylation of PSK-3β is quantified by ImageJ software and plotted as PSK-3β/PSK-3β signal ratios. The highest ratio was arbitrarily established as 100% and results are represented as percent ± S.E. (n = 3). Western Blotting—The amount of protein was determined using DC protein assay (Bio-Rad), and the same amounts of proteins were normalized for total protein. The protein samples were boiled in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad). Nonspecific binding was blocked with 3% dry milk in PBS and proteins were probed with anti-hPER1, anti-rPER2 (provided by Dr. T. Nagase), anti-Xpress (Invitrogen), anti-V5 (Invitrogen), and anti-FLAG (SIGMA), all diluted 1:5000; anti-PSK-3β (Transduction Laboratories) diluted 1:2500; or anti-phospho-PSK-3β (Cell Signaling) diluted 1:1000. Immunoprecipitation—Transfected COS1 cells were lysed as described above. A slurry of antibody and protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) was added to clarified supernatants. 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Changes of GSK-3β phosphorylation differed between the SCN and the liver. The phosphorylation of GSK-3β in the SCN reached a nadir at ZT14 and peaked at ZT2 (Fig. 1B). In contrast, the phosphorylation level of GSK-3β in the liver was minimal at ZT20 and reached maximum at ZT11. In cultured fibroblasts, serum-rich medium triggers the rhythmicity of several genes (proteins), the activity of which also oscillates in vivo (18, 19). We examined whether GSK-3β phosphorylation undergoes circadian changes in serum-shocked mouse fibroblast NIH3T3 cells. We then recorded the profile of GSK-3β expression and phosphorylation for 52 h following serum shock. The total level of GSK-3β protein did not appear to undergo circadian changes, but the phosphorylation level of GSK-3β changed in a circadian manner like that in the SCN and the liver (Fig. 2).

Lithium Chloride (LiCl) specifically inhibits GSK-3β, and it can cause increased phosphorylation of GSK-3β on Ser-9 (33, 34). We therefore examined GSK-3β expression and phosphorylation in the presence of 20 mM LiCl, which inhibited GSK-3β activity with 80–90% inhibition (35, 36). The levels of total GSK-3β expression were similar to those in the absence of LiCl, but GSK-3β phosphorylation was elevated during the 52 h following serum shock (Fig. 2). Inhibition of GSK-3β Activity Delayed the Phase of Clock Gene Expression in Serum-shocked NIH3T3 Cells—To determine whether GSK-3β affects the circadian oscillation of core clock components, we examined mPER2, BMAL1, and DBP mRNA rhythmic expression in serum-shocked NIH3T3 cells in the presence of LiCl. After transient exposure to 50% serum, the expression levels of various genes oscillated with a period length of about 24 h in confluent cells (18, 19). The expression of mPER2 mRNA peaked at 23.56 ± 0.64 h in the absence of LiCl (Fig. 3, A, upper panel, and B, solid line). The expression of mPER2 mRNA peaked in the cells at 25.80 ± 0.46 h in the presence of 10 mM LiCl with a phase delay of 2.2 h (Fig. 3, A, center panel, and B, dashed black line). Furthermore, when cells were incubated with 20 mM LiCl, mPER2 mRNA expression peaked at 28.04 ± 0.18 h with a 4.4-h phase delay (Fig. 3, A, lower panel, and B, dashed gray line). The expression of BMAL1 and DBP mRNA was also delayed like mPER2 when LiCl was added (supplemental Fig. 1A). These results indicate that the inhibition of GSK-3β activity delayed the mRNA expression of core clock components in serum-shocked NIH3T3 cells.

In the presence of LiCl, the expression levels of mPER2, but not of BMAL1 or DBP, dose-dependently increased (supplemental Fig. 2). The mPER2 expression levels in cells incubated in 5, 10, 15, or 20 mM LiCl were 1.2-, 1.5-, 1.8-, and 2.3-fold increased, respectively, as compared with control (absence of LiCl).

Overexpression of rGSK-3β Advanced the Phase of mPER2, BMAL1, and DBP mRNA Expression in Serum-shocked NIH3T3 Cells—Inhibitory experiments alone were insufficient to confirm that GSK-3β affects the circadian clock. We therefore tested the effects of rGSK-3β gene overexpression to the phase of the clock gene expressions in NIH3T3 cells transfected with rGSK-3β, mPER2, BMAL1, and DBP mRNA expression peaked at 22.25 ± 0.05, 34.23 ± 0.33, and 20.02 ± 0.18 h, respectively, in cells overexpressing rGSK-3β (Fig. 3, C and D, and supplemental Fig. 1B). The phase of all three genes was advanced by 1.4 h in cells overexpressing rGSK-3β compared with vector control. The results demonstrated that rGSK-3β overexpression advanced the mRNA phase of core oscillators in serum-shocked NIH3T3 cells.

GSK-3β Physically Interacts with PER2 In Vitro and in Vivo—We examined the possible physical interactions of GSK-3β with each component of the mammalian circadian feedback loop to search for specific GSK-3β targets. We co-transfected hPER1, hPER2, hCRY1, hCRY2, hClock, or hBMAL1 with rGSK-3β in COS1 cells and 24 h later immunoprecipitated cell lysates with anti-GSK-3β antibody and immunooblotted them against appropriate antibodies. Fig. 4A shows that rPER2 co-precipitated with rGSK3β. To confirm the interaction between rPER2 and

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**Fig. 3.** Inhibition and overexpression of GSK-3β activity, respectively, delayed and advanced the phase. A, cells were shifted to medium containing 50% horse serum at time 0 and incubated for 2 h, then the medium was replaced with that containing 0, 10, or 20 mM LiCl. Total RNA was prepared at indicated times, and relative levels of mPER2 and GAPDH mRNA were determined by Northern blotting. B, levels of mPER2 mRNA were quantified with a BAS2500 imaging analyzer and plotted as mPER2/GAPDH mRNA signal ratio. The value at time = 2 h was set to 100%. C, twenty-four hours after cells were transfected with rGSK-3β or empty vector, cells were shifted to medium containing 50% horse serum at time 0 and incubated for 2 h, and then the medium was replaced. Total RNA prepared at the indicated times was analyzed by Northern blotting. Expression profiles of mPER2 and GAPDH mRNA are shown. D, signals obtained from Northern blots to detect mPER2 mRNA were quantified and normalized to those obtained for GAPDH mRNA. The value at time = 2 h was set to 100%.
immunoprecipitated with anti-GSK-3β or vector in COS1 cells and lysed 24 h later. Crude lysates were Western blotted (Input) and then immunoprecipitated with anti-GSK-3β antibody and immunoblotted with the appropriate antibodies. B, rGSK-3β or vector and rPER2 were co-expressed in COS1 cells. At 24 h post-transfection, crude cell lysates were Western blotted (Input) and then immunoprecipitated with anti-rPER2 and probed with anti-GSK-3β antibodies. C, brain extracts from ZT2 and ZT14 were immunoprecipitated (IP) with antibody against PER2 and then Western blotted (WB) and probed for GSK-3β. Crude lysates were Western blotted (Lysate) and probed for anti-phospho-GSK-3β (pGSK-3β) and anti-GSK-3β antibodies.

rGSK3β, cell lysates immunoprecipitated with anti-rPER2 antisemur were blotted against anti-GSK-3β antibody. Fig. 4B shows that rGSK-3β co-precipitated with rPER2.

To examine interactions between PER2 and GSK-3β in vivo, we immunoprecipitated brain extracts with anti-rPER2 antisemur and then probed the resulting immune complexes for GSK-3β by Western blotting (Fig. 4C). We focused on the periods before (ZT2) and during (ZT14) the expected PER2 peaks. As shown in Fig. 4C, GSK-3β co-precipitated with PER2 at ZT14. The data suggested that immunoprecipitated GSK-3β binding to PER2 was more abundant at ZT14 than at ZT2.

Identification of the rGSK-3β Binding Site in rPER2—To determine the specific region of rPER2 required for rGSK-3β binding, we constructed various fragments of rPER2 (Fig. 5A) and analyzed the binding activity of rPER2 mutants with rGSK-3β. We co-transfected rPER2 fragments with rGSK-3β in COS1 cells. At 24 h post-transfection, cell lysates were immunoprecipitated with tagged GSK-3β antibody and then immunoblotted PER2 fragments against appropriate antibodies. As shown in Fig. 5B, the amino-terminal fragments of rPER2 (amino acids 1–512, 1–319, and 1–171), PASA region of rPER2 (amino acids 172–319), and PASB region of rPER2 (amino acids 320–512) co-immunoprecipitated with rGSK-3β (Fig. 5B, lanes 2, 4, 5, 6, and 7). In contrast, the carboxyl-terminal fragments of rPER2 (amino acids 512–1257) and NES region of rPER2 (amino acids 372–512) did not co-immunoprecipitate (Fig. 5B, lanes 3 and 8). These results indicate that rGSK-3β binds to at least amino acids residues 1–372 on rPER2. This region is not overlapped with CRY or CK1ε binding sites (23, 32).

Phosphorylation of rPER2 in Vitro by GSK-3β—To investigate whether GSK-3β phosphorylates rPER2, recombinant GSK-3β was assayed for kinase activity using immunoprecipitated rPER2 from transfected COS1 cells as substrates. Because PER2 was phosphorylated by endogenous CK1 in cultured cells (37), we added CK1–7, a specific inhibitor of CK1 to reduce the phosphorylation by co-precipitating endogenous CK1. Under these conditions, rPER2 was phosphorylated by GSK-3β (Fig. 6A, lanes 4 and 5). The phosphorylation of rPER2 was decreased to 62.7% in the presence of 20 mM LiCl, which inhibits GSK-3β (Fig. 6B, lanes 5 and 6). Immunoprecipitated mCLOCK was used as a negative control. mCLOCK was not phosphorylated by GSK-3β (Fig. 6A, lanes 7 and 8) as expected. These results suggest that GSK-3β phosphorylates rPER2.

Regulation of rPER2 Nuclear Localization—When expressed alone, PER1 was shown to localize predominantly in the nuclei of transfected cells (23), and the localization was regulated by CK1ε. However, PER2 predominantly localized in the cytoplasm when PER2 alone was expressed (23). In Drosophila, the SGG-dependent phosphorylation of TIM promotes nuclear localization of the PER/TIM complex (25). Thus, we speculated...
that rPER2 phosphorylation by GSK-3β promotes the nuclear entry of rPER2. To test this hypothesis, we co-transfected rPER2 with rGSK-3β, and the localization of rPER2 was examined by immunocytochemistry. In the presence of rGSK-3β, rPER2 was nuclear in 47.3% of the cells as shown in MOCK (Fig. 7, B and C). To confirm whether GSK-3β promotes PER2 nuclear entry, we attempted to eliminate GSK-3β expression using siRNA. When a siRNA targeting GSK-3β was transiently transfected into COS1 cells (Fig. 7A), the abundance of rGSK-3β protein was reduced to 50%. After adding GSK-3β siRNA, rPER2 became predominantly cytoplasmic (nuclear in 28.6% of the cells) (Fig. 7, B and C). Moreover, when we reduce GSK-3β activity using 20 mM LiCl, rPER2 was cytoplasmic (nuclear in 14.4% of the cells) as expected (supplemental Fig. 3, A, lower panel, and B). These results suggest that GSK-3β promotes the nuclear translocation of PER2 in mammals.

**DISCUSSION**

The oscillation of clock gene products appears to be an important regulator of processes controlled by the circadian clock in a variety of organisms. In *Drosophila*, PER and TIM accumulate, become increasingly phosphorylated, and degrade during the daily cycle. Martinek *et al.* (25) originally showed that *Drosophila* SGG, a protein with kinase activity, regulates *Drosophila* circadian rhythm. The closest mammalian homologue of *Drosophila* SGG is GSK-3β, which functions in several distinct signaling pathways. However, no role for GSK-3β in circadian rhythmicity has been described.

We found here that the phosphorylation level of GSK-3β at Ser-9 in total SCN and liver extracts varies over the circadian cycle but not the total amount of GSK-3β protein (Fig. 1). Yet the actual kinase that is responsible for this oscillation remains unknown (30). In the SCN, levels of GSK-3β phosphorylation are highest at ZT2 and lowest at ZT14. The level of PER2 in the nuclear exhibits circadian rhythms in the SCN and peaks at ZT12–14 (38). These data suggest that GSK-3β regulates the nuclear entry of PER2 in the SCN by activation through GSK-3β dephosphorylation around ZT14. Moreover, the phase angle of circadian phosphorylation of GSK-3β at Ser-9 differs between the SCN and the liver, with levels of phosphorylation in the SCN being maximal and minimal earlier during the day. This suggests that the rhythmic phosphorylation of GSK-3β at Ser-9 is regulated by master clock mechanisms, while the peripheral clock might be controlled by systemic signals of which emission is governed by the SCN. The phase of peripheral oscillations for *Per* gene expression was 3–9 h delayed compared with the SCN (39). Several observations suggest significant differences in clock gene expression between the SCN and the liver.

The present study showed that GSK-3β contributes to the mammalian circadian clock in a similar way with in *Drosophila*. Increased GSK-3β function results in a phase advance whereas decreased function causes substantial phase delay of the molecular oscillator in serum-shocked NIH3T3 cells (Fig. 3). These data suggest that these effects might change the period of circadian clock in NIH3T3 cells. Our findings suggest that GSK-3β might have a role for rhythm generation through PER2 nuclear localization in mammals.

We found that the peak levels of *mPer2* mRNA in serum-shocked NIH3T3 cells increased dose-dependently in the presence of LiCl (supplemental Fig. 2). This suggests that LiCl affects the CLOCK/BMAL1-mediated transactivation of *Per2*.

**FIG. 7. RNA interference-mediated knockdown of rGSK-3β inhibited the nuclear entry of rPER2.** Three hours after rPer2 was co-transfected with rGSK-3β in COS1 cells, a 25-base pair siRNA targeting GSK-3β was transfected. Thirty-six hours post-transfection, cells were examined by Western blotting (A) or immunocytochemistry (B) as described above. A, Western blot analysis of the expression of GSK-3β. siRNA is efficient in knocking down GSK-3β. B, representative micrographs illustrating subcellular localization of rPER2. C, quantitation of the experiments illustrated above. Black bars, predominantly nuclear localization; dark gray bars, equal localization in cytosol and nucleus; light gray bars, predominantly cytoplasmic localization. Each bar is the result of at least three independent experiments (± S.E.) in which 50–100 cells were counted.
However, DBP mRNA was also expressed under the direct transcriptional control of CLOCK/BMAL1 (40, 41), but LiCl at different doses did not alter the peak levels of DBP mRNA (supplemental Fig. 1). These data suggest that GSK-3β is involved in a central clock component (Per2) rather than an output pathway (DBP).

Our data showed that GSK-3β could phosphorylate PER2 (Fig. 6). Motif Scanner protein phosphorylation identified six potential sites for GSK-3β in mammalian PER2. Four of the six predicted sites are located near sequences encoding three putative NES (42) or NLS.

Pull-down assays demonstrated that GSK-3β binds the amino-terminal region of PER2 including a PAS region (Fig. 5) that is neither a CK1ε nor CRY binding site (23, 32). This suggests that PER2 forms a quaternary complex with GSK-3β, CK1ε, and CRY. Our in vivo pull-down assays showed that GSK-3β binds PER2 at ZT14 to ZT2 in the brain. As phosphorylation of GSK-3β is low at ZT14 in the brain, dephosphorylation of Ser-9 for GSK-3β might be required for the binding to PER2, but there might be another possibility that pull-down is more efficient at ZT14 than ZT2 because the level of PER2 is higher at ZT14.

We showed that rPER2 accumulates in the nucleus when rGSK3β is co-expressed (Fig. 7). Studies indicate that GSK-3β promotes the nuclear translocation of PER2. This viewpoint holds that GSK-3β expression decreased by GSK-3β siRNA or activity reduced by LiCl would tend to retard nuclear transfer and thus length the period. Although others have suggested that PER plays an important role in the nuclear translocation of CRY (32, 43), our study suggests that GSK-3β might be key to PER2 nuclear localization.

The free-running period length of the circadian rhythm increases slightly in mice given a daily dose of LiCl in water (44). If the target of LiCl was GSK-3β in that study, then our data are consistent with their findings because the effects of lithium might be explained as action on GSK-3β that regulates the circadian clock.

In conclusion, our data suggest that GSK-3β functions as a component of the mammalian circadian clock. The binding of GSK-3β to PER2 appears to promote nuclear entry at a specific time in a day (Fig. 8). The establishment of GSK-3β regulation in the molecular circadian clock represents an important first step toward understanding mammalian circadian regulation.

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