

SIRT1 Mediates Central Circadian Control in the SCN by a Mechanism that Decays with Aging

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SUMMARY

SIRT1 is a NAD⁺-dependent protein deacetylase that governs many physiological pathways, including circadian rhythm in peripheral tissues. Here, we show that SIRT1 in the brain governs central circadian control by activating the transcription of the two major circadian regulators, BMAL1 and CLOCK. This activation comprises an amplifying circadian loop involving SIRT1, PGC-1 α , and Nampt. In aged wild-type mice, SIRT1 levels in the suprachiasmatic nucleus are decreased, as are those of BMAL1 and PER2, giving rise to a longer intrinsic period, a more disrupted activity pattern, and an inability to adapt to changes in the light entrainment schedule. Young mice lacking brain SIRT1 phenocopy these aging-dependent circadian changes, whereas mice that overexpress SIRT1 in the brain are protected from the effects of aging. Our findings indicate that SIRT1 activates the central pacemaker to maintain robust circadian control in young animals, and a decay in this activity may play an important role in aging.

INTRODUCTION

In response to the daily 24 hr light-dark (LD) cycle, living organisms have developed an evolutionarily conserved program that allows appropriate physiology and behavior to be coordinated with the environment. To achieve circadian regulation, genes exist in cells for the generation of an oscillating transcriptional network that coordinates the expression of pathways involved in metabolism and physiology (Asher and Schibler, 2011). In mammals, systemic circadian regulation is accomplished through the central oscillator in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The SCN responds to LD cycles and coordinates rhythms of all aspects of circadian control, including locomotor activity, hormone secretion, body temperature maintenance, and feeding. Peripheral tissues, such as the liver, use the same circadian oscillatory machinery. SCN-driven processes are important for aligning peripheral tissues according to phase, but these tissues can also respond to feeding-fast cycles (Welsh et al., 2010). Importantly, SCN implantation is

able to restore circadian rhythms in SCN-lesioned animals or in nonrhythmic genetic models (Lehman et al., 1987; Ralph et al., 1990; Sujino et al., 2003). The restored period is determined by the genotype of the SCN donor, not the SCN-lesioned host, underscoring the dominating role of the SCN in circadian physiology (Lehman et al., 1987; Ralph et al., 1990; Sujino et al., 2003).

The link of circadian genes and circadian regulation to health has been recognized in many disease-associated fields, including sleep disorders, diabetes, and cancer. A number of pathologies can be triggered by circadian disruptions via genetic or environmental perturbations (Sahar and Sassone-Corsi, 2009; Takahashi et al., 2008), suggesting that proper maintenance of circadian control is crucial to maintaining robust health.

The molecular mechanism for oscillation in both SCN and peripheral tissues is generated by a transcriptional autoregulatory feedback loop (Bass and Takahashi, 2010; Dibner et al., 2010). The network involves the core transcriptional activators BMAL1 and CLOCK, which form a heterodimer to positively regulate the expression of target genes *Cryptochrome* (*Cry1* and *Cry2*) and *Period* (*Per1*, *Per2*, and *Per3*). When PER and CRY protein accumulation reaches critical levels, they translocate into the nucleus as dimers and repress the transcription activity of CLOCK-BMAL1. Orphan nuclear receptors REV-ERB α and REV-ERB β and ROR α proteins are also targets of CLOCK-BMAL1 and contribute to the transcriptional control of the *Bmal1* and *Clock* genes (Preitner et al., 2002; Sato et al., 2004). REV-ERB α and REV-ERB β act coordinately and, recently, were shown to be crucial in sustaining circadian behavior and metabolism (Bugge et al., 2012; Cho et al., 2012). Posttranslational events also regulate the molecular clock; i.e., the Skp1, Cullin1, F box protein (SCF)/ β -TrCP ubiquitin ligase complex targets PER and CRY proteins for degradation (Lamia et al., 2009). These transcriptional and posttranslational events ensure the fidelity of the circadian cycle.

The circadian machinery has recently been linked to the SIRT1 NAD-dependent deacetylase (Asher et al., 2008; Nakahata et al., 2008; Nakahata et al., 2009; Ramsey et al., 2009), illustrating one way in which circadian control is coupled with metabolism. SIRT1 is the mammalian homolog of yeast Sir2, an NAD-dependent protein deacetylase that is involved in aging, stress response, maintenance of genomic integrity, and energy metabolism (Finkel et al., 2009). SIRT1 mediates the salutary metabolic effects of caloric restriction, and its activity is critical in the maintenance of health (Guarente, 2012). Besides histones,

SIRT1 deacetylates a number of transcriptional regulatory proteins that govern major arms of metabolism; i.e., FOXOs, LXR, PPAR γ coactivator 1 α (PGC-1 α), HIF-1 α , and NF- κ B. SIRT1 is also able to mitigate neurodegenerative diseases in mouse model systems, such as Alzheimer's, Huntington's, and Parkinson's disease (Donmez et al., 2012; Donmez et al., 2010; Jeong et al., 2012; Jiang et al., 2012), and was recently demonstrated to couple diet and metabolism with mood and behavior (Libert et al., 2011). SIRT1 can also regulate POMC and NF-Y neurons in the hypothalamus for the regulation of feeding behavior (Ramadori et al., 2011; Ramadori et al., 2010; Satoh et al., 2010). Thus, its effects on the brain are pervasive.

In peripheral tissues such as the liver, it has been shown that SIRT1 can influence circadian rhythm in a cell-autonomous fashion by multiple mechanisms. It can deacetylate BMAL1 to affect its activity (Nakahata et al., 2008) or PER2 to alter its stability (Asher et al., 2008). Interestingly, one of the metabolic output targets of CLOCK-BMAL1 is Nampt, an enzyme required for the biosynthesis of the SIRT1-essential cofactor NAD⁺, which ensures the rhythmic accumulation of NAD⁺ and SIRT1 activity (Nakahata et al., 2009; Ramsey et al., 2009). Given that all studies on SIRT1 regulation of circadian rhythm to date have been conducted in these cell-autonomous systems, we were interested in investigating a role of SIRT1 in central circadian control of physiology and behavior. Here, we show that altering SIRT1 levels in the brain can exert moderate changes in the intrinsic circadian periods of mice. Moreover, SIRT1 appears to be at the center of aging-dependent decline in central circadian function. Our findings trace a circadian regulated loop in the brain involving Nampt, SIRT1, and PGC-1 α and may lead to strategies for the treatment of aging-dependent decline in circadian function.

RESULTS

Brain SIRT1 Regulates the Central Circadian Clock

We wished to test whether SIRT1 regulates the central clock in the brain and whether it could thus alter circadian behaviors in vivo. It is known that the suprachiasmatic nucleus (SCN) at the anterior hypothalamus is the central circadian pacemaker in the brains of mammals (Bass and Takahashi, 2010; Welsh et al., 2010). Our strategy involves knocking out and overexpressing SIRT1 in the brain by primarily using Nestin-Cre, given that an SCN-specific system is not available. Therefore, we cannot rule out the possibility that some of the phenotypes we describe below may have contributions from brain regions besides the SCN. All experiments use C57BL/6, wild-type (WT), and brain-specific SIRT1 knockout (BSKO) mice (Cohen et al., 2009) and transgenic lines that overexpress brain SIRT1 2-fold (Sir2d) (Bordone et al., 2007) or 10-fold (BSTG) (Firestein et al., 2008).

Western blots of punch-out biopsies of the SCN region in the anterior hypothalamus showed that SIRT1 protein was deleted in BSKO and appropriately overexpressed in transgenic mice (Figure 1A). RNA in situ hybridization of the SCN also showed that SIRT1 RNA was undetectable in SCN of BSKO mice and overexpressed in SCN of BSTG mice (Figure 1B). To determine whether SIRT1 activity in the SCN was affected in genetically altered mice, we carried out immunohistochemistry (IHC) using an antibody specific to acetylated Lys537 of BMAL1, a validated SIRT1

substrate in peripheral tissues (Nakahata et al., 2008). This assay revealed increased BMAL1 acetylation in SCN of BSKO in comparison to WT mice and decreased acetylation in SCN of BSTG mice (Figure 1C), even though we later show that total BMAL1 protein levels are lower in BSKO SCN and higher in BSTG SCN.

Activity assays for circadian period or actograms employed cohorts of eight to ten littermates, which were entrained on a 12 hr LD cycle and then placed in an all dark (DD) environment for 30 days. Their intrinsic periods were revealed by monitoring free-running activity on running wheels, which normally would occur during the DD cycle. Actograms show that WT mice had intrinsic periods of 23.6 hr (Figures 1D and 1E), which was close to the reported values for C57BL/6 mice (Valentinuzzi et al., 1997). In contrast, BSKO mice had an elongated period of 23.9 hr. We also scored the activity of animals, as indicated by the density of black ticks in the actograms, and found that BSKO mice showed reduced activity (Figure 1F), also indicated by the interrupted running pattern evident in the actograms.

Next, we tested the two SIRT1 overexpression strains and found effects that were reciprocal to the BSKO mice for both period and activity. The Sir2d mice had a period of 23.4 hr and the BSTG of 23.1 hr, in comparison to 23.7 hr for WT mice (Figures 1D and 1E). In addition, the activity level of BSTG mice was significantly higher than that of WT mice, whereas the Sir2d mice showed a trend in that direction (Figure 1F). The fact that SIRT1 BSKO and overexpressing mice showed opposite effects on period and activity in comparison to WT mice, along with the dosage-dependency of the effects of two overexpressing strains, strongly supports the notion that SIRT1 in the brain governs central circadian rhythm.

Brain SIRT1 Governs Aging-Dependent Decline in Circadian Function

The increase in intrinsic period from 23.6 to 23.9 hr in our young BSKO mice was similar to what was reported in aged (22 months) WT C57BL/6 mice animals (Valentinuzzi et al., 1997), raising the possibility that, like yeast Sir2p (Dang et al., 2009), SIRT1 function in the brain declines in aged mice. Critically, Valentinuzzi et al. (1997) also observed a decline in circadian function in aged mice, as measured by the ability to adapt to an abrupt change in the entrainment cycle. Young mice adapted to an abrupt advancement in the light cycle within 1–2 days, whereas aged mice took at least 8 days to adapt (Valentinuzzi et al., 1997). Thus, we tested young (6 months) or aged (21 months) WT, BSKO, and BSTG mice in this so-called “jet lag” experiment by advancing the light entrainment abruptly by 4 hr and following adaptation (Figure 2). Like Valentinuzzi et al. (1997), we also found that aged WT mice took much longer to adapt than young WT mice. Strikingly, young BSKO mice partially phenocopied aged WT mice, doubling the time required for re-entrainment (4.0 ± 0.6 days versus 2.1 ± 0.3 days) (Figure 2B). In 22-month-old BSKO mice, re-entrainment times were longer, but the percentage difference in re-entrainment times between WT and BSKO mice was reduced in comparison to young mice (Figure S2). More strikingly, we observed a partial rescue of the ability to adapt in 21-month-old BSTG mice in comparison to aged WT mice (4.0 ± 0.6 days versus 7.9 ± 0.3 days) (Figure 2B). This rescue was even evident in very old mice (30 months of

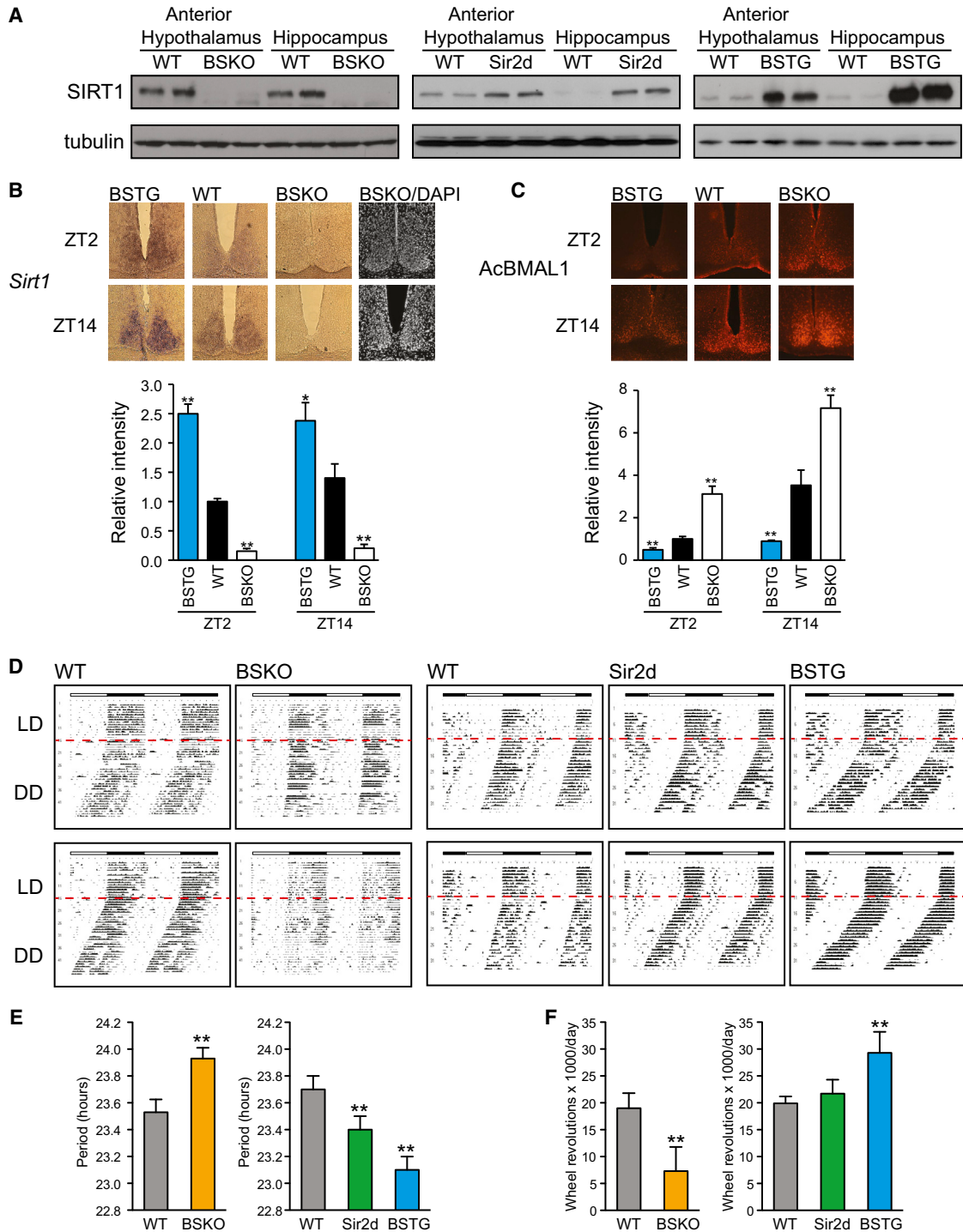


Figure 1. Circadian Phenotypes of Brain-Specific *Sirt1* Knockout and Transgenic Mice

(A) Immunoblot of SIRT1 in the anterior hypothalamus and hippocampus from the brain-specific *Sirt1* knockout (BSKO), *Sirt1* whole-body transgenic (Sir2d), and brain-specific *Sirt1* transgenic (BSTG) mice. Tubulin was probed as a loading control.

(B) Typical in situ hybridization images and relative signal intensities for *Sirt1* mRNA in the SCN. Sections were prepared from 3-month-old mice that were sacrificed at ZT2 and ZT14 (n ≥ 3 mice per genotype). Signal intensities were quantitated and are shown relative to WT mice (≥ 6 sections). DAPI-stained images were shown to indicate SCN in the BSKO sections.

(C) Typical immunohistochemical staining results and relative signal intensities for acetylated BMAL1-K537 in the SCN. Sections were prepared from 3-month-old mice that were sacrificed at ZT2 and ZT14 (n = 3 mice per genotype). Signal intensities of protein levels were quantitated and are shown relative to WT mice (six sections).

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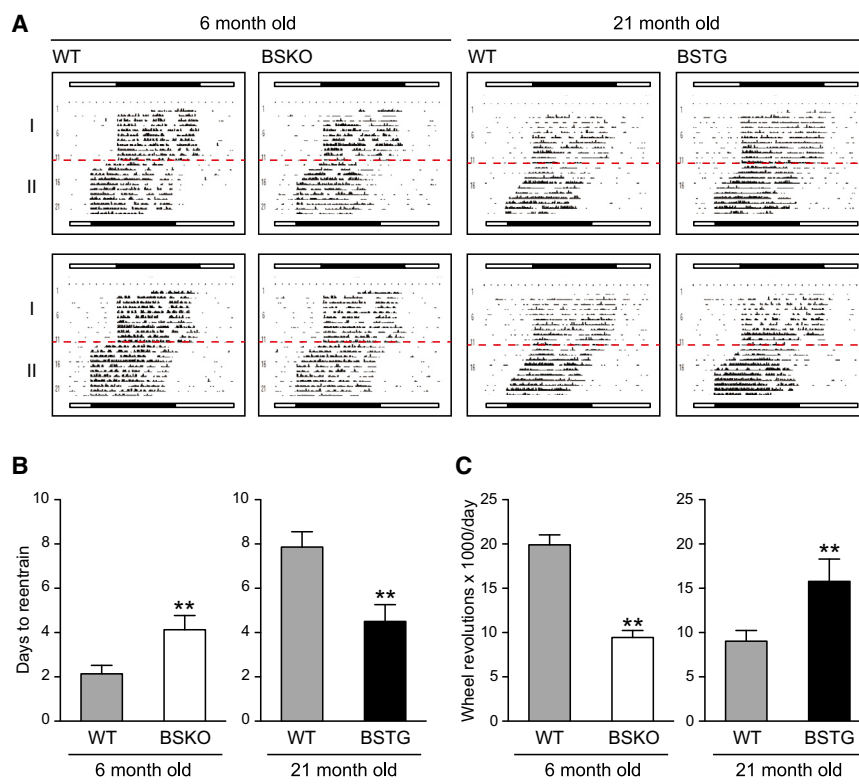


Figure 2. Aging-Induced Deficit in Re-entrainment Is Suppressed in Brain-Specific *Sirt1* Transgenic Mice

(A) A jet lag experiment depicting actograms of 6-month-old WT ($n = 7$) versus BSKO ($n = 7$) (left) mice and 21-month-old WT ($n = 6$) versus BSTG ($n = 7$) (right) mice subjected to a 4 hr phase advance. Red lines indicate the start of the new LD cycle.

(B) Days required for re-entrainment of animals.

(C) Total wheel revolutions per day of animals. Values in (B) and (C) represent the mean \pm SD. ** $p < 0.01$; t test.

See also Figures S1 and S2.

age) (Figure S1). Old BSTG mice also showed rescue of the aging-dependent decline in activity observed in WT mice, whereas young BSKO mice again showed less activity in comparison to young WT mice (Figure 2C). These findings indicate that a loss of SIRT1 in the brain partially mimics the jet lag phenotype of old mice, whereas SIRT1 overexpression rescues the defect. These findings are both consistent with the hypothesis that a loss of SIRT1 function in the brain is responsible for at least part of the decline in central circadian robustness in old animals.

SIRT1 Positively Regulates Circadian Genes in the SCN and Declines with Aging

Given that the SCN is the primary determinant of central circadian control in mammals (Bass and Takahashi, 2010; Welsh et al., 2010), we determined whether SIRT1 expression levels in the SCN could determine expression levels of circadian machinery components. Anterior hypothalamus samples that were enriched for SCN were collected at 4 hr time intervals. Quantitative real time PCR analysis of punch-out SNC biopsies revealed that all circadian-controlled genes tested, including the core transcription factor BMAL1 and CLOCK, were significantly downregulated in BSKO mice (Figure 3A). Conversely,

the expression of the circadian genes was markedly upregulated in BSTG mice (Figure 3A). The expression of these genes varied over Zeitgebers time (ZT) was circadian. To confirm that the expression differences in the real-time PCR assays reflected expression specifically in the SCN, we carried out in situ hybridization of *Bmal1* and *Per2* messenger RNA (mRNA) in coronal brain slices through the SCN. Deletion of *Sirt1* in BSKO mice resulted in a clear decrease in both *Bmal1* and *Per2* RNA levels in SCN, whereas SIRT1 overexpression in

BSTG mice increased the levels of both RNAs (Figure 3B). These findings are consistent with the idea that SIRT1 is a positive regulator of the *Bmal1* and *Clock* genes in the SCN.

Next, having established a functional link between SIRT1 and circadian gene expression, we addressed the possibility, which was raised above, that SIRT1 function declines in aged mice. Sections of brain were obtained by cryostat slicing to allow immunohistochemical staining in the SCN. In SCNs of WT C57BL/6 mice that were sacrificed at ZT4, and a significant decline in SIRT1 staining was observed in 22-month-old mice in comparison to 5-month-old mice (Figure 4A). Similarly, there was a large decline in the expression of the circadian proteins BMAL1 and PER2, which was consistent with the conclusion above that SIRT1 is a positive regulator of the genes encoding these proteins. Because there appears to be some variation in circadian behavior among different mouse strains, we repeated this analysis in young and aged 129sv mice and made very similar observations (Figure 4B). To be certain that these expression differences were not due to a circadian phase difference between genotypes, we repeated the experiment sampling more time points around the circadian period (ZT2, ZT8, ZT14, and ZT20). Again, the deletion of SIRT1 reduced BMAL1 and PER2 protein

(D) Actograms showing wheel-running activity in constant darkness after 2 weeks of LD entrainment. Representative results of 6-month-old male BSKO ($n = 10$) and the littermate WT control mice ($n = 10$) are shown in the left panel. Sir2d ($n = 6$) and BSTG ($n = 7$) compared to WT control mice ($n = 7$) are shown on the right. Red lines indicate the starting day for constant darkness.

(E) Bar graphs of innate periods of animals during the initial 3 weeks in constant darkness.

(F) Total wheel revolutions performed per day in animals.

Values in (B), (C), (E), and (F) represent the mean \pm SD. ** $p < 0.01$; t test.

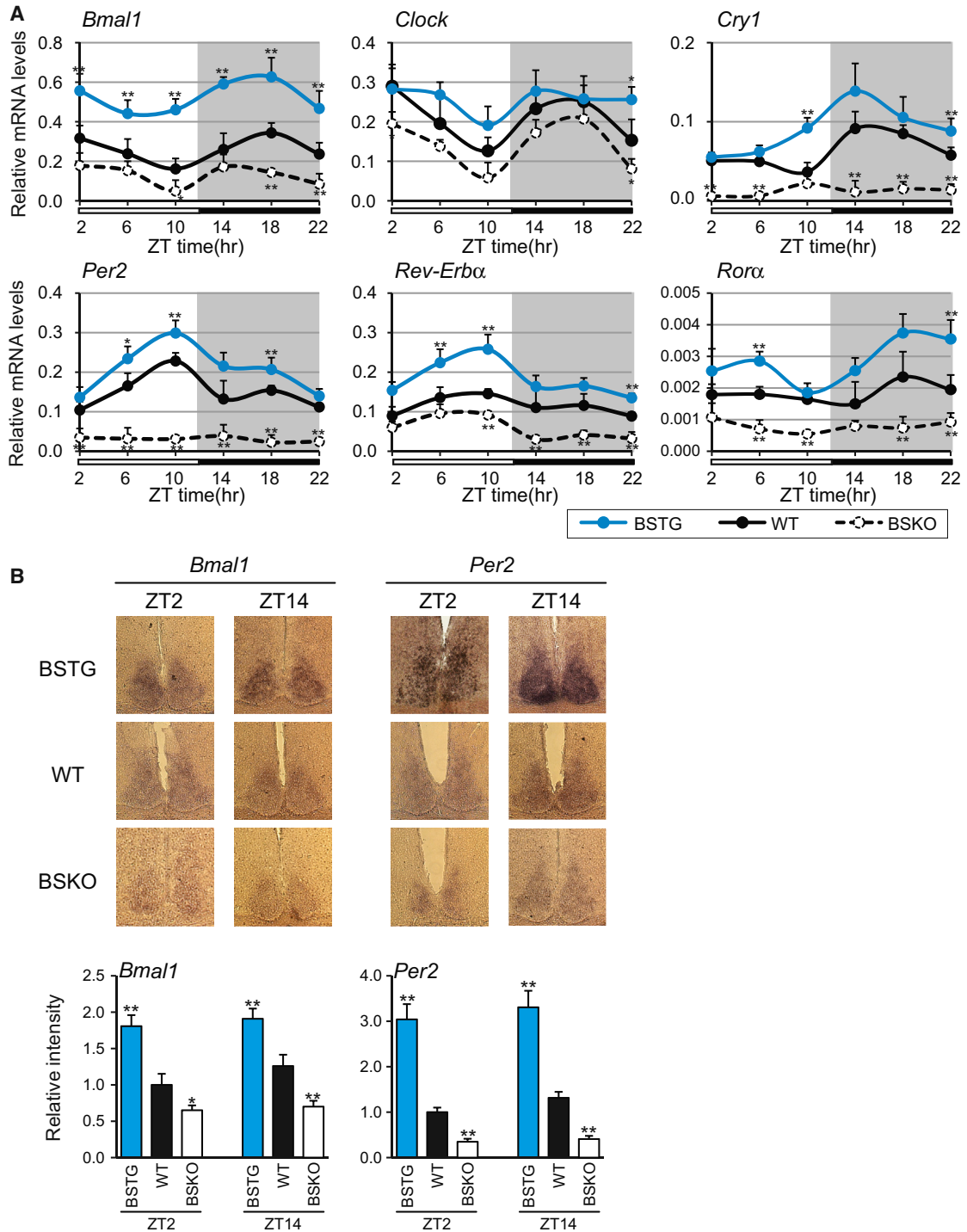


Figure 3. SIRT1 Upregulates Circadian Gene RNA Levels in the SCN

(A) Quantitative real-time PCR analysis of CLOCK-BMAL1-controlled genes. We sacrificed 6-month-old mice for brain samples at 4 hr intervals across the 12:12 hr LD cycle (n = 3–4 mice per genotype per time point). SCN-enriched anterior hypothalamus samples were later isolated for RNA preparation from frozen brain samples with needle-punch collection. Target gene expression levels are shown relative to the ribosomal protein reference gene *rpl19*.

(B) Typical in situ hybridization images and relative signal intensities for *Bmal1* and *Per2* mRNA in the SCN. Sections were prepared from 3-month-old mice that were sacrificed at ZT2 and ZT14 (n ≥ 3 mice per genotype). Signal intensities of protein levels were quantitated and are shown relative to WT mice (≥ 6 sections). Values in (A) and (B) represent the mean ± SD. *p < 0.05; **p < 0.01; t test.

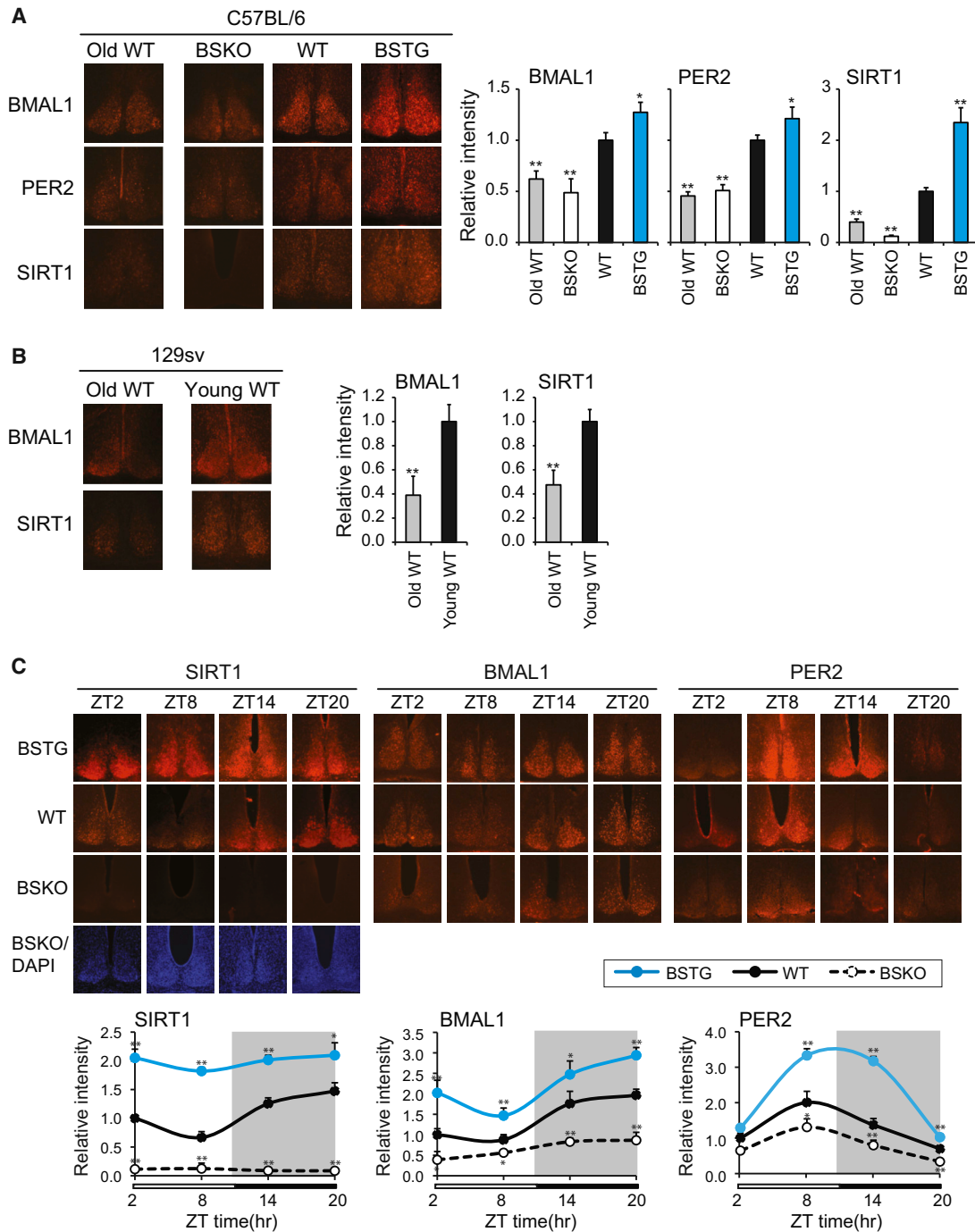


Figure 4. SIRT1 Upregulates Circadian Proteins in the SCN and Declines with Aging

(A) Typical immunohistochemical staining results and relative signal intensities for BMAL1, PER2, and SIRT1 proteins in the SCN. Sections were prepared from C57BL/6 background mice that were sacrificed at ZT4 ($n \geq 3$ mice per genotype). Aged WT mice (22 months) are compared to young BSKO, WT, and BSTG mice (5 months). Signal intensities of protein levels were quantitated and are shown relative to WT mice (≥ 6 sections).

(B) Typical immunohistochemical staining images and relative signal intensities of BMAL1 and SIRT1 proteins in the SCN. Sections were prepared from aged (21-month-old) or young (4-month-old) 129 sv background mice that were sacrificed at ZT4 ($n \geq 3$). Signal intensities of protein levels were quantitated and are shown relative to young animals (≥ 6 sections).

(C) Temporal analysis of BMAL1, PER2 and SIRT1 proteins in the SCN. SCN sections were prepared from 3 month-old mice that were sacrificed at the indicated time ($n \geq 3$ mice/genotype/time point). DAPI-stained images were shown to indicate SCN in the BSKO sections. Signal intensities of protein levels were quantitated and are shown relative to WT mice (≥ 6 sections).

Values in (A), (B), and (C) represent the mean \pm SD. * $p < 0.05$; ** $p < 0.01$; t test.

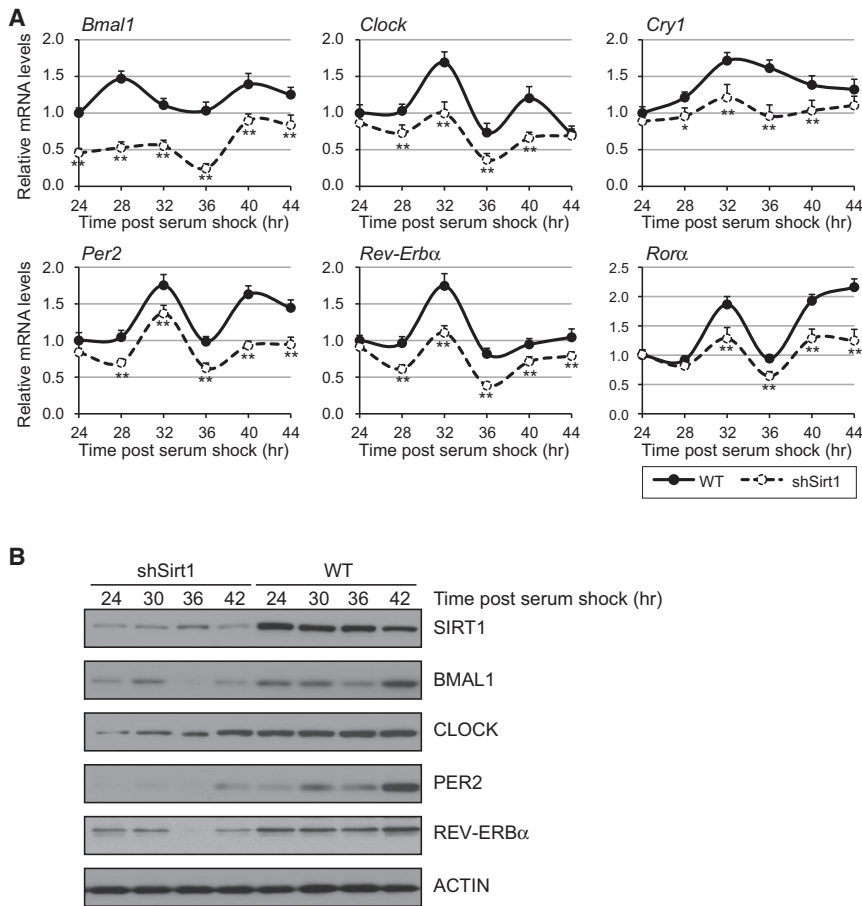


Figure 5. SIRT1 Regulates Endogenous Circadian Gene RNA and Protein Levels in Neuroblastoma N2a Cells

(A) Quantitative real-time PCR analysis of CLOCK-controlled genes. Mouse N2a cells (WT) and *Sirt1* shRNA knockdown cells were synchronized with 50% horse serum and time points were taken at 4 hr intervals. Results are shown relative to WT after normalization to ribosomal reference gene *rpl19*. Values represent the mean \pm SD. * $p < 0.05$; ** $p < 0.01$; t test.

(B) Immunoblots of SIRT1 and components of the circadian machinery. Time points were taken at 6 hr intervals after serum shock.

tion requires PGC-1 α (Liu et al., 2007). Moreover, PGC-1 α knockout mice show a similar shift in intrinsic period to the one observed in BSKO mice (Liu et al., 2007), and PGC-1 α is one of the most well-characterized SIRT1 substrates (Rodgers et al., 2005). Thus, it seemed possible that the mechanism by which SIRT1 regulates the circadian cycle in the SCN may involve PGC-1 α . We queried whether PGC-1 α knockdown in N2a cells would recapitulate the effect of SIRT1 knockdown on *Bmal1* expression level and its downstream circadian genes. Indeed, knockdown of PGC-1 α by shRNA resulted in a decreased level of circadian targets in both 24 and 36 hr time points after serum shock, similar to knocking down SIRT1 (Figures 6A and

levels in SCN in comparison to WT mice, whereas the overexpression of SIRT1 increased protein levels in a setting where we could observe the entire cycle of expression (Figure 4C).

SIRT1 Regulates Circadian Gene Expression in Neuronal Cells

To study the mechanism of how SIRT1 regulates expression of BMAL1 and CLOCK in SCN, we employed N2a murine neuroblastoma cells, which are frequently used to support *in vivo* studies. Importantly, we found that N2a cells can be synchronized by horse serum treatment for subsequent circadian studies; i.e., after horse serum shock, we found that numerous circadian transcripts (*Bmal1*, *Clock*, *Cry1*, *Per2*, *Rev-Erba*, and *Rora*) were regulated in a circadian manner (Figure 5A). Then, we examined the effect on the circadian regulation of SIRT1 depletion by small hairpin RNA (shRNA). Like that in the SCN, SIRT1 depletion reduced levels of all of these transcripts. Protein analysis confirmed that SIRT1 was knocked down and that levels of all the circadian proteins were also reduced (Figure 5B). These findings suggest that N2a cells faithfully recapitulate the regulation of circadian proteins by SIRT1 observed *in vivo*.

Next, we began to search for the mechanism by which SIRT1 regulates circadian gene expression. *Bmal1* transcription is positively regulated by the nuclear receptor ROR α , and this activa-

S3). Conversely, overexpression of PGC-1 α enhanced circadian gene expression in a manner similar to SIRT1 overexpression. It is noteworthy that the enforced expression of SIRT1 also drove higher expression of *Pgc-1 α* , suggesting that the activities of these two proteins are highly coupled in neurons.

To further support these findings, we tested whether SIRT1 could regulate a luciferase reporter driven by the *Bmal1* or *Per2* promoters (Nagoshi et al., 2004; Travnickova-Bendova et al., 2002). By transiently transfecting 250 or 500 ng of *Sirt1* plasmid in N2a cells followed by horse serum shock, we found that *Bmal1*- or *Per2*-luciferase were enhanced in a dose-dependent manner (Figure 6B). The effects of the transfection of a vector expressing PGC-1 α on *Bmal1*- and *Per2*-luciferase were very similar to that of SIRT1. Finally, the depletion of either SIRT1 or PGC-1 α caused a roughly 70% to 80% reduction in *Bmal1*- or *Per2*-luciferase (Figure 6B). Similar but milder effects were observed without serum shock (Figure S4). Altogether, these findings indicate that SIRT1 and PGC-1 α function in an interdependent manner to activate circadian genes in neurons.

Cooperative Binding of SIRT1 and PGC-1 α to the *Bmal1* Promoter

Given that both *in vivo* and cell-culture experiments indicated that SIRT1 activates the expression of circadian genes, including

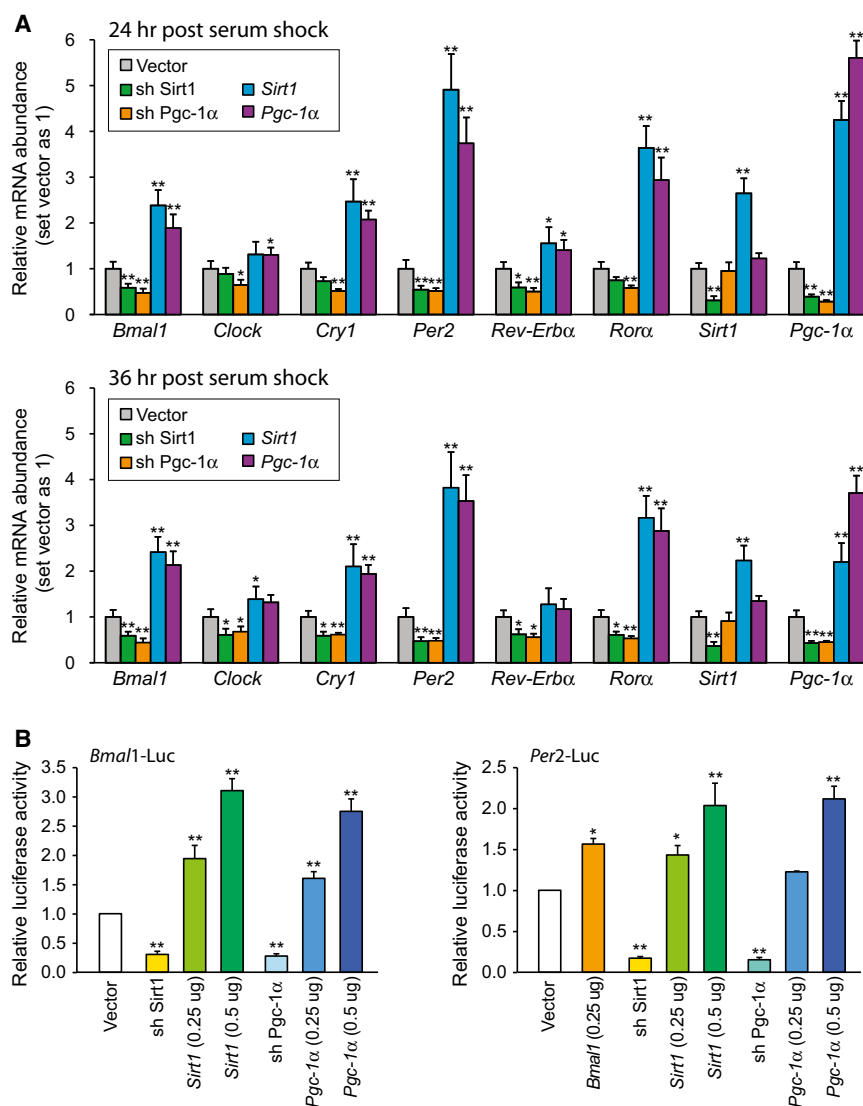


Figure 6. SIRT1 and PGC-1 α Mutually Regulate Circadian Gene Expression in N2a Cells

(A) Quantitative real-time PCR analysis of CLOCK-controlled genes. *Sirt1* or *Pgc-1 α* shRNA knockdown cells and transient *Sirt1* or *Pgc-1 α* overexpression cells were synchronized with 50% horse serum 24 or 36 hr before harvesting for total RNA. Results are shown relative to a vector control. (B) Reporter assays with *Bmal1*- or *Per2*-luciferase under the indicated knockdown or overexpression conditions. N2a cells were harvested for luciferase assays 24 hr after horse serum shock. Results are shown relative to a vector control.

Values in (A) and (B) represent the mean \pm SD. * p < 0.05; ** p < 0.01; t test.

See also Figures S3 and S4.

SIRT1 binds in close proximity to PGC-1 α . Remarkably, knockdown of PGC-1 α markedly reduced SIRT1 binding to the *Bmal1* promoter (as well as binding of itself), and knockdown of SIRT1 reduced binding of PGC-1 α (and itself) (Figure 7B). Furthermore, overexpressing either SIRT1 or PGC-1 α increased the occupancy on the *Bmal1* promoter, and, due to the overexpression of one protein (i.e., SIRT1), the enhanced binding was abolished by shRNA knockdown of the other (i.e., PGC-1 α) (Figures 7C and 7D). These studies show that neuronal SIRT1 and PGC-1 α bind cooperatively and in close proximity at the *Bmal1* promoter and suggest that the positive regulation of circadian genes occurs by the direct, cooperative action of SIRT1 and PGC-1 α at the *Bmal1* promoter.

Finally, to trace the SIRT1-mediated regulatory circuit back to the SCN, we measured *Sirt1*, *Pgc-1 α* , and *Nampt*

Bmal1, we hypothesized that it functioned at the *Bmal1* promoter. Thus, we conducted a chromatin immunoprecipitation (ChIP) assay in N2a cells to test whether SIRT1 binds to the ROR α -binding sites (RORE) at the proximal *Bmal1* promoter region. It has been demonstrated that PGC-1 α binds to these sites through a synergistic action with ROR α (Liu et al., 2007). Our ChIP results confirmed that PGC-1 α binds to this *Bmal1* promoter region spanning from -25 to $+114$, containing two RORE consensus sequences (Preitner et al., 2002) (Figure 7A). Interestingly, another set of primers revealed binding to the -698 to -502 region, but it is not clear whether this fragment has novel binding sites or whether it is scoring positive because of its linkage to the -25 to $+114$ sites. Importantly, primers in a control region in the *Bmal1* 3' untranslated region (3'UTR) showed no binding.

A second ChIP experiment was performed with SIRT1 antibodies, and it revealed similar binding at *Bmal1* promoter sites but not at the 3'UTR (Figure 7B). This finding indicates that

RNA levels in vivo, as shown in Figure 3A (Figure 7E). We found that all three genes were expressed in a phased, circadian manner, and SIRT1 positively regulated the level of *Pgc-1 α* transcription, as observed in N2a cells. This latter effect may have been due to a heightened PGC-1 α coactivation of FOXO at the *Pgc-1 α* promoter when the coactivator has been deacetylated by SIRT1 (Borniquel et al., 2010). All told, our in vivo and in vitro data suggest that an amplifying regulatory loop in the SCN comprising *Nampt*, SIRT1, and PGC-1 α drives the expression of circadian genes in an aging-sensitive fashion (Figure 7F).

DISCUSSION

In this report, we show that SIRT1 regulates central circadian control in the brains of mice to determine the period, activity levels, and ability to adjust to re-entrainment. SIRT1 directly activates the transcription of *Bmal1* via PGC-1 α to increase the amplitude of expression in the SCN of BMAL and other circadian

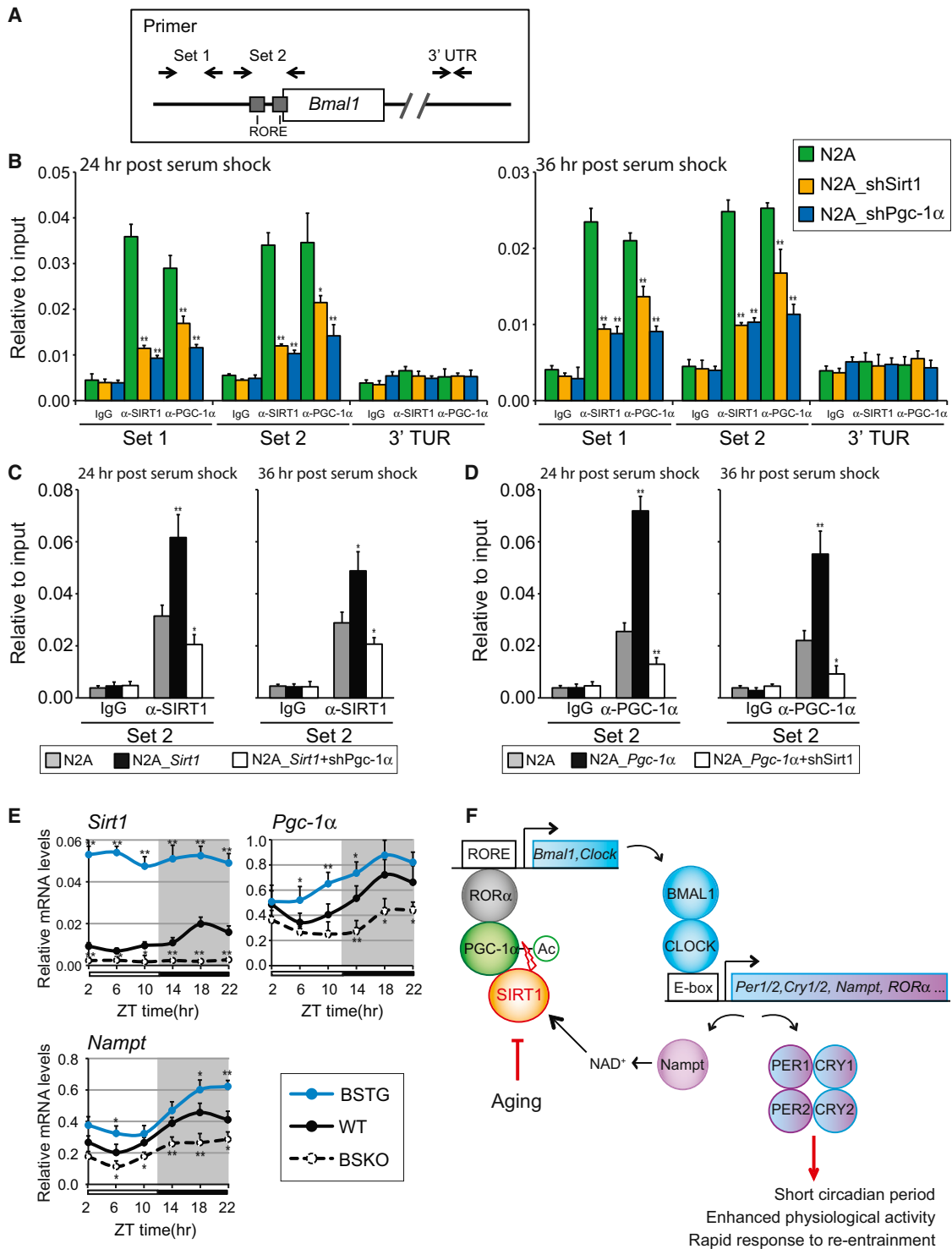


Figure 7. Cooperative Binding of SIRT1 and PGC-1α at the *Bmal1* Promoter

(A) A schematic view of primer locations. Primers that flank two RORE sequences (Set 2) and another upstream region (Set 1) at the *Bmal1* promoter are shown. Control primers were designed at the 3'UTR of the *Bmal1* gene (see Table S1).

(B–D) Chromatin immunoprecipitation (ChIP) assays in N2a cells. Cells were transfected with a vector control or shRNA knockdown constructs for SIRT1 or PGC-1α (B). Overexpression of SIRT1 (C) and PGC-1α (D) were ChIP assayed as in (B) with the indicated knockdown of PGC-1α or SIRT1, respectively. Rabbit IgG was used as an immunoprecipitation control. Assays were performed 24 or 36 hr after synchronization with 50% horse serum. ChIP results were analyzed by qPCR.

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regulatory proteins. The components of this amplifying loop, SIRT1 and PGC-1 α , and the NAD⁺ synthetic enzyme Nampt are circadian in the SCN. Thus, this regulatory loop influences the intrinsic period of mice, which is shortened in transgenic mice with increased SIRT1 levels in the brain and elongated in brain-specific SIRT1 knockout mice.

Moreover, our findings provide a molecular explanation of why robust central circadian control declines with aging. Aged mice exhibit a decrease in SIRT1 levels in the SCN and concomitant decreases in BMAL1 and other circadian regulatory proteins in this brain region. Thus, aged mice display an increase in their intrinsic period and an inability to adjust to abrupt light re-entrainment regimens, termed jet lag. Young mice lacking brain SIRT1 partially phenocopy old WT mice for period dilation and decline in ability to re-entrain. Critically, old mice overexpressing brain SIRT1 are partially protected from the aging-associated decline in ability to re-entrain. These findings suggest that SIRT1 may be a linchpin in the aging-sensitive decline of central circadian function.

Mechanistic Implications

Studies in liver cells and mouse embryo fibroblasts revealed two mechanisms by which SIRT1 impinges on the circadian machinery in peripheral tissues. In the first mechanism, SIRT1 deacetylated the CLOCK-BMAL1 target PER2 in order to facilitate its ubiquitination and degradation by the proteasome (Asher et al., 2008). Thus, SIRT1 functioned as a positive regulator of BMAL1 and circadian components (Asher et al., 2008), as we observed in the SCN. In the second study, SIRT1 was shown to deacetylate BMAL1 and histones at circadian gene promoters in order to facilitate repression by inhibitory components of the oscillator, thereby functioning as a negative regulator (Nakahata et al., 2008).

Our findings in SCN incorporate aspects of both of these mechanisms but also appear to have distinct features. Namely, SIRT1 appears to deacetylate Lys 537 of BMAL1 in the SCN, as observed in peripheral tissues by Nakahata et al. (2008), but the effect of SIRT1 on the amplitude of the central circadian clock is positive, as observed in peripheral tissues by Asher et al. (2008). Thus, we wondered whether an alternative mechanism in SCN might reconcile all of these observations. One clue was that the deletion of the validated SIRT1 substrate PGC-1 α in mice increased the intrinsic period from 23.5 to 24 hr (Liu et al., 2007), just as we observed for deleting SIRT1. Indeed, our studies in N2a neuroblastoma cells showed that SIRT1 and PGC-1 α cooperatively bound to the *Bmal1* promoter and functioned to activate transcription. Thus, we suggest that SIRT1 and PGC-1 α function together in SCN to drive *Bmal1* gene expression and the amplitude of the circadian machinery. We cannot rule out the possibility that deacetylation of BMAL1 by SIRT1 may play an additional role in regulating the central clock.

In the liver, the CLOCK-BMAL1 factor also drives circadian transcription of the NAD⁺ biosynthetic enzyme Nampt (Nakahata et al., 2009; Ramsey et al., 2009), which thus renders SIRT1 activity circadian. We find that SIRT1 and Nampt are also expressed in a circadian manner in the SCN, and SIRT1 activity appears necessary to drive normal PGC-1 α levels. The Nampt/SIRT1/PGC-1 α loop in the SCN is circadian most likely because it is driven by CLOCK-BMAL1, which themselves are subject to negative feedback by PER2, etc. We suggest that this Nampt-SIRT1/PGC-1 α loop is what amplifies the expression of BMAL1 and other circadian proteins and, thus, plays a critical role in the SCN central pacemaker (Figure 7). Consistent with this model, we observed that the expression of Nampt is reduced in SCN of aged mice (Figure S5), similar to what we found for SIRT1 and BMAL1.

Implications for Aging

Mutations in circadian genes have been associated with premature aging, cancer, and other health maladies in mice (Fu et al., 2002; Kondratov et al., 2006; Marcheva et al., 2010). Many studies in humans also indicate that severe disruptions in circadian patterns are deleterious to health (Gallego and Virshup, 2007; Sehgal and Mignot, 2011). Two recent studies indicate that, in rodents, having an intrinsic period close to 24 hr is strongly associated with a long lifespan (Libert et al., 2012; Wyse and Coogan, 2010). This was interpreted as an indication that animals with innate periods different from 24 hr were forced to re-entrain daily in the 12 hr light:12 hr dark (12:12 hr LD) cycle of the laboratory, and this imposed a life-shortening metabolic stress. However, most humans must re-entrain to daily changes in the diurnal cycle, which occur around the calendar. Were loss of SIRT1 and circadian proteins in the SCN to occur in aging humans, as we observe here in mice, it might trigger metabolic disruption and a decline in health due to an inability to re-entrain. Any intervention for maintaining expression of SIRT1 and circadian proteins in the SCN during aging would therefore be salutary. Given the central nature of this regulation, it will be important to determine whether the role of SIRT1 in the SCN is even more important for healthy aging than other functions of this sirtuin in other tissues.

Linking Circadian Control to Diet and Drugs

Our findings suggest that dietary and pharmacological interventions that affect SIRT1 activity may also impinge on central circadian control. Many studies indicate that calorie restriction increases SIRT1 levels, whereas a high-fat diet decreases SIRT1 levels in peripheral tissues. Were this to apply to the SCN, one would predict dietary effects on intrinsic periods and ability to adjust to changes in entrainment. For example, the progression through metabolic syndrome to diabetes might be expected to lengthen the intrinsic period and foster deterioration

(E) Oscillation of *Sirt1*, *Pgc-1 α* , and *Nampt* in the SCN. SCN-enriched samples were isolated from 6-month-old male mice at 4 hr intervals across the 12:12 LD cycle (n = 3–4 mice per genotype per time point). Target gene expression levels are shown relative to a ribosomal reference gene *rpl19*.

(F) A schematic model of SIRT1-mediated circadian gene activation in the SCN. The model depicts an oscillating loop that amplifies the expression of BMAL1, CLOCK, and their targets. Age-associated decline of SIRT1 results in circadian phenotypes, such as prolonged period and a defect in re-entrainment.

Values in (B), (C), and (E) represent the mean \pm SD. *p < 0.05; **p < 0.01; t test.

See also Figure S5.

in circadian function (Kohsaka et al., 2007). Conversely, calorie restriction or drugs that cause an increase in SIRT1 activity might have opposite effects. In this regard, it is noteworthy that resveratrol was recently reported to shorten the free-running circadian period in primates (Pifferi et al., 2011), which is consistent with our findings here. Several SIRT1 activators have been tested recently and show repressive activity on circadian expression in an osteosarcoma cell line and in the liver (Bellet et al., 2013), suggesting again that there are differences between central and peripheral circadian control. It will be interesting to test the *in vivo* effects of SIRT1 activator and inhibitor compounds on central circadian control.

It is also interesting to reconsider a role for melatonin, which is produced in the pineal gland, declines with aging, and can re-entrain the central circadian clock (Dibner et al., 2010; Kondratova and Kondratov, 2012; Sack et al., 1986). Melatonin was initially reported to increase the lifespan in C57BL/6 mice (Pierpaoli and Regelson, 1994), but this finding was later discounted because C57BL/6 mice were shown to have relatively low melatonin levels (Grace et al., 1999). Interestingly, melatonin was more recently reported to preserve SIRT1 expression in hippocampus of sleep-deprived rats (Chang et al., 2009). Thus, it may be of interest to investigate whether falling melatonin levels in normal aging play any role in the decline of SIRT1 in the SCN.

Conclusions

Sirtuins play many roles in adapting organisms to the two critical features of calorie restriction: metabolic reprogramming and stress resistance. Our findings indicate a function of SIRT1 in the brain in coupling metabolic processes faithfully to circadian control and maintaining central circadian function during aging. SIRT1 is part of a regulatory loop that is circadian and amplifies circadian gene expression. Our finding that SIRT1 in the SCN declines in aged animals helps explain the known aging-sensitive decline in circadian function and is consistent with the idea that the uncoupling of metabolic processes from a diurnal cycle may be central in the aging process. Therefore, strategies that can maintain SIRT1 function in the SCN may slow the onset and progression of diseases of aging.

EXPERIMENTAL PROCEDURES

Animals

SIRT1 BSKO mice were generated by crossing the *loxP*-flanked exon 4 *Sirt1* allele mice (Cheng et al., 2003) with the brain-specific Nestin-Cre-expressing mice (Cohen et al., 2009). For SIRT1 BSTG, a *Sirt1*^{STOP} strain that harbors a *loxP*-flanked transcriptional STOP element between the CAGGS promoter and the *Sirt1* complementary DNA (cDNA) was used to breed with Nestin-Cre mice for achieving a brain-specific excision of the STOP sequence (Firestein et al., 2008). A *Sir2d* strain was obtained from mice that were heterozygous for the *Sirt1*-KI transgene (Bordone et al., 2007). Additional details for strains are listed in Table S2. All mice were maintained in the C57BL/6 background and were housed in a standard animal maintenance facility under a 12:12 hr LD cycle. All animal procedures were performed in accordance with the MIT animal care committee. WT littermate controls were used throughout.

Plasmids

Constructs for *Bmal1*, *Per2*, and *Sirt1* probes for *in situ* hybridization were cloned into pCR-Blunt II TOPO backbone with the primers listed in Table S1. *Bmal1* (1.1 kb) and *Per2* (1.7 kb) promoter fragments were PCR amplified

from mouse genomic DNA and cloned into pGL3 firefly luciferase vector (Promega) as previously described (Nagoshi et al., 2004; Travnickova-Bendova et al., 2002; Table S1). pAd-Track *mSirt1* and pLKO *Pgc-1 α* for transient overexpression were kindly provided by P. Puigserver. *Pgc-1 α* shRNA constructs were purchased from Sigma-Aldrich (Table S1).

Cell Culture and Transfections

N2a cells that stably knock down for *Sirt1* with shRNA were as previously described (Libert et al., 2011; Table S1). For time point experiments, N2a cells at 5×10^5 cells per 10 cm dish density were seeded and grown for 5 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, and 25 mM HEPES (pH 7.2) and cultured at 37°C in a humidified incubator with 5% CO₂ until confluent. Then, cells were synchronized with 50% horse serum for 2 hr followed by replacement with low FBS (1%) containing DMEM for the subsequent 24 to 44 hr time points. For transient overexpression experiment, *Sirt1*, *Pgc-1 α* , and reporter constructs were transfected on day 4 and allowed for expression for 24 hr before serum shock. All transient transfections were carried out with FuGENE HD Transfection Reagent (Roche) according to the manufacturer's instructions.

Locomotor Activity

Wheel-running activity was assessed with wireless running-wheel systems (MED Associates, ENV-044) and recorded by a sensor hub (MED Associates, DIG-804) according to the manufacturer's instructions. At the indicated ages, male WT, SIRT1 BSKO, BSTG, and *sir2d* mice were used for the voluntary activity assays. First, mice were maintained at 12:12 hr LD cycle and recorded for their activities for at least 3 weeks followed by release into 12:12 hr DD free-running condition (Siepkha and Takahashi, 2005). We performed 4 hr phase shifts as previously described (Valentinuzzi et al., 1997). Actograms and a chi-square periodogram were analyzed and generated by wheel manger software (MED Associates, SOF-860) and online circadian software (www.circadian.org).

Tissue Collection and RNA Analysis by Quantitative Real-Time PCR

Brain samples were quickly harvested at the indicated time points and kept in OCT compound (Tissue-Tek) at -80°C until use. To obtain the SCN-enriched sample, we cut anterior hypothalamus at the optic chiasm level coronally into two 150 μm thick sections with a cryostat (Leica CM 1510S). Then, SCN was visualized under a phase contrast microscope and collected with a syringe needle. SCN samples from three to four mice were pooled for RNA extraction with an RNeasy Mini Kit (QIAGEN). cDNAs were generated with a RETROscript Kit (Ambion) according to the manufacturer's instructions. real-time PCR reactions were performed on a LightCycler 480 II (Roche) with iQ SYBR Green Supermix (Bio-Rad). The relative abundance of transcripts was calculated by normalizing to a ribosomal subunit gene, *rpl19*. Primers are listed in Table S1.

Immunohistochemistry

Mice were housed in all dark for 48 hr prior to the ZT collection experiments. Whole-brain samples were quickly harvested at the indicated time points under red dim light and fixed in 4% phosphate-buffered paraformaldehyde solution for overnight. After cryoprotected in 30% sucrose for 30 hr, fixed tissues were embedded in OCT (Tissue-Tek) and stored at -80°C until staining. Detailed methods can be found in the Extended Experimental Procedures.

In Situ Hybridization

Digoxigenin-labeled antisense and sense RNA probes were made with linearized plasmids as templates and transcribed with T7 or SP6 RNA polymerases, respectively (Roche). Brain sections were fixed in 4% paraformaldehyde permeabilized with 5 $\mu\text{g}/\text{ml}$ proteinase K for 10 min before an acetylation step in triethanolamine and acetic anhydride solution. Detailed methods can be found in the Extended Experimental Procedures.

Reporter Assays

N2a cells were seeded into six-well dishes at a 1.5×10^6 cells per well density 24 hr before transfections. *Sirt1* and *Pgc-1 α* overexpression constructs,

reporter constructs, and internal control Renilla-luciferase plasmid were transfected at the ratio of 0.5 (or 0.25):0.2:0.1 μg per well. Then, cells were synchronized with 50% horse serum for 2 hr followed by replacement with low FBS-containing DMEM for 24 hr before being harvested for luciferase assays. Cell lysis and luciferase measurements were performed according to the Promega Dual-Luciferase Reporter Assay System instructions.

ChIP Assays

N2a cells were cultured, transfected, and synchronized with 50% horse serum as described. Next, 24 or 36 hr after synchronization, cells were fixed in 1% formaldehyde and collected for chromatin preparation according to Simple-ChIP Enzymatic Chromatin IP Kit instructions (Cell Signaling Technology). Purified DNA was subjected to real-time PCR with the primers listed in Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.05.027>.

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