



Published in final edited form as:

J Biol Rhythms. 2015 April ; 30(2): 155–160. doi:10.1177/0748730415573167.

Circadian Rhythmicity of Active GSK3 Isoforms Modulates Molecular Clock Gene Rhythms in the Suprachiasmatic Nucleus

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Abstract

The suprachiasmatic nucleus (SCN) drives and synchronizes daily rhythms at the cellular level via transcriptional-translational feedback loops comprised of clock genes such as *Bmal1* and *Period* (*Per*). Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase, phosphorylates at least five core clock proteins and shows diurnal variation in phosphorylation state (inactivation) of the GSK3 β isoform. Whether phosphorylation of the other primary isoform (GSK3 α) varies across the subjective day-night cycle is unknown. The purpose of this study was to determine if the endogenous rhythm of GSK3 (α and β) phosphorylation is critical for rhythmic BMAL1 expression and normal amplitude and periodicity of the molecular clock in the SCN. Significant circadian rhythmicity of phosphorylated GSK3 (α and β) was observed in the SCN from wild-type mice housed in constant darkness for two weeks. Importantly, chronic activation of both GSK3 isoforms impaired rhythmicity of the GSK3 target BMAL1. Furthermore, chronic pharmacological inhibition of GSK3 with 20 μ M CHIR-99021 enhanced the amplitude and shortened the period of PER2::luciferase rhythms in organotypic SCN slice cultures. These results support the model that GSK3 activity status is regulated by the circadian clock and that GSK3 feeds back to regulate the molecular clock amplitude in the SCN.

At the cellular level, 24-hour timing is maintained by transcriptional-translational feedback loops resulting in rhythmic expression of core “clock genes,” in which the activators (CLOCK and BMAL1) are expressed in anti-phase to the suppressors (PERs and CRYs; (Partch et al., 2014 for review). A secondary feedback loop consists of CLOCK-BMAL1 activation of a nuclear orphan receptor REV-ERB α which feeds back to transcriptionally repress *Bmal1*. The length of the molecular clock cycle can be regulated by post-translational events including phosphorylation by kinases such as casein kinase I enzymes (Partch et al., 2014). We know much less about how other kinases, such as glycogen synthase kinase 3 (GSK3), regulate the period and/or amplitude of the molecular clock, especially in neurons of the central circadian pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus. Given that dysregulated GSK3 signaling has been implicated in psychiatric and neurological disorders (Hooper et al., 2008; Li and Jope, 2010; Duncan, M J,

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2013), and many therapeutic drugs target GSK3 (*e.g.*, lithium, valproate, risperidone, etc; Li and Jope, 2010), important questions to address are how GSK3 activity varies over the course of the day and how the molecular clock is affected by these rhythms.

A key feature of GSK3 is that it is active in its default state and that its two isoforms are inactivated by phosphorylation (at Ser-21 for GSK3 α [S21-GSK3 α] and Ser-9 for GSK3 β [S9-GSK3 β]). This critically important phosphorylation state balance of S9-GSK3 β changes across the light-dark cycle in the SCN (Iwahana et al., 2004; Iitaka et al., 2005). In some brain regions, the two distinct GSK3 isoforms can have functional differences, such as differences in substrate recognition, axonal growth, regulation of synaptic plasticity, and formation of senile plaques in an Alzheimer's mouse model (Castaño et al., 2010; Soutar et al., 2010; Hurtado et al., 2012; Shahab et al., 2014). Therefore, it is important to determine whether phosphorylation of S21-GSK α also varies across the subjective day-night cycle in the SCN. Because rhythmic phosphorylation of S9-GSK3 β is dependent upon the molecular clock in peripheral tissues such as the heart (Durgan and Young, 2010), we hypothesized that rhythmic phosphorylation of GSK3 α and GSK3 β persists in constant darkness in the SCN.

Using Western blot analysis (as in Paul et al., 2012), we investigated p-GSK3 α and p-GSK3 β levels in mice housed in constant darkness (DD) for 14-20 days. Both p-GSK3 α and p-GSK3 β (as a percentage of total GSK3 α or GSK3 β , respectively) displayed a statistically significant rhythm ($n = 27$ /time course; $p < 0.05$; Figure 1A, C, E) during DD conditions. Interestingly, p-GSK3 α peaked during the late subjective night (circadian time or CT phase, 20.94 ± 0.47 , where CT 12 refers to activity onset; mesor, 1.02 ± 0.51 ; amplitude, 0.41 ± 0.08). In contrast, p-GSK3 β peaked during the early subjective day (circadian time or CT phase, 2.93 ± 1.45 ; mesor, 1.51 ± 0.16 ; amplitude, 0.61 ± 0.22). There were no significant differences in total GSK3 α or GSK3 β expression with respect to time (cosinor non-linear regression, $p > 0.05$; Figure 1A, D, F), consistent with previous reports in the SCN and liver from mice housed in LD (Iitaka et al., 2005). The persistence of rhythmic GSK3 α/β inactivation in the absence of light cues provides evidence for intrinsic clock regulation of GSK3 inactivation state.

Previous evidence suggests that GSK3 directly phosphorylates at least five core clock proteins: PER2, CRY2, CLOCK, BMAL1, and REVERB α) (Kaladchibachi et al., 2007; Spengler et al., 2009; Kurabayashi et al., 2010; Sahar et al., 2010). Given that phosphorylation of BMAL1 by GSK3 β impacts *Bmal1* translation and protein stability *in vitro* (Yin et al., 2006; Sahar et al., 2010; Valnegri et al., 2011), we tested the initial hypothesis that the time-dependent balance of phosphorylated to de-phosphorylated GSK3 is critical for BMAL1 expression rhythms in the SCN. Specifically, p-GSK3 α and p-GSK3 β rhythms were eliminated using a double transgenic mouse model of chronic GSK3 activity (GSK3-KI mice) in which two serine-alanine mutations (GSK3 α ^{S21A/S21A} and GSK3 β ^{S9A/S9A}) render both isoforms of GSK3 constitutively active (but at endogenous levels; McManus et al., 2005; Paul et al., 2012). Wheel running rhythms of these mice have decreased rhythmic amplitude, lengthened alpha (active period), increased activity bouts per day, and increased SCN excitability at night compared to wild-type (WT) controls (Paul et al., 2012). This circadian phenotype was not observed in mice bearing single KI mutations

(GSK3 α ^{S21A/S21A} or GSK3 β ^{S9A/S9A}), likely due to functional redundancy between the GSK3 isoforms, and therefore, only mice with both α and β isoform mutations were investigated in the current studies. Using Western blot analysis of isolated SCN from individual animals, we quantified BMAL1 expression (as a percentage of α -Tubulin expression) over a 24-h period in isolated SCN after GSK3-KI or WT control mice were housed in DD for at least 2 weeks.

Cosinor non-linear regression revealed a significant BMAL1 expression rhythm in WT mice ($n = 27$ /time course; cosinor nonlinear regression analysis; $p < 0.05$ for SCN) with peak BMAL1 expression in the subjective night (mesor, 0.82 ± 0.08 ; amplitude, -0.30 ± 0.11 ; phase, 14.85 ± 10.61 ; Figure 2). In GSK3-KI mice, however, BMAL1 expression did not exhibit a significant 24-h rhythm ($n = 25$ /time course; as determined by cosinor nonlinear regression analysis, $p = 0.91$; Figure 2). There were no significant differences in α -Tubulin expression levels with respect to time or genotype ($p > 0.05$). These results indicate that constitutive GSK3 activation disrupts circadian expression of BMAL1 in the central pacemaker. Based on phosphorylation status, we predict that GSK3 β activity is highest in WT SCN neurons at approximately CT16, when BMAL1 protein levels peak. GSK3 α activity likely peaks around CT9, a time at which BMAL1 protein levels are increasing. These observations are consistent with the conceptual model that GSK3-mediated phosphorylation is an early event, which primes BMAL1 for subsequent degradation via ubiquitin/proteosomal degradation (Figure 2; Sahar et al., 2010). The possibility also remains that chronic GSK3 activation influences BMAL1 protein levels indirectly, through phosphorylation of other clock components (e.g., REV-ERB α , which in turn represses *Bmal1* transcription).

While it is appreciated that *Bmal1* is a necessary molecular clock component for behavioral rhythmicity (Bunger et al., 2000), whether or not BMAL1 protein level rhythmicity is necessary for a functional pacemaker is unclear (Shi et al., 2010). In order to test the idea that GSK3 activation rhythms regulate molecular clock amplitude and periodicity, we used the *Period2^{Luciferase}* (*Per2^{Luc}*) mouse model as a read-out of real-time molecular clock function (Yoo et al., 2004). Specifically, a selective GSK3 inhibitor (20 μ M CHIR-99021) was chronically applied to the media of organotypic tissue cultures of SCN from *mPer2^{Luc}+/−* reporter mice (Yoo et al., 2004; Besing et al., 2012). This compound inhibits both GSK3 isoforms by competing with ATP for the ATP-binding site of the kinase (Cline et al., 2002; Ring et al., 2003). Application of this small molecule GSK3 inhibitor nearly doubled the amplitude of the chi-squared periodogram analysis and significantly shortened the period length of SCN explant PER2::LUC rhythms ($n = 9$ /group; t-tests, $p < 0.05$; Figure 3A,B), suggesting that chronic GSK3 inhibition enhances the amplitude and speeds up the periodicity of the molecular clock. These results are consistent with previous *in vitro* studies that show that molecular clock amplitude is enhanced and period is shortened by pharmacological GSK3 inhibition in fibroblasts and mammalian cell lines (Hirota et al., 2008; Vougiannopoulou et al., 2008; Li et al., 2012). On the other hand, molecular clock amplitude is reduced by GSK3 over-expression (Sahar et al., 2010; Li et al., 2012). Although it is well documented that the GSK3 inhibitor lithium lengthens the period of circadian rhythms (Mason and Biello, 1992; Abe et al., 2000; Hirota et al., 2008; Mohawk et

al., 2009; Osland et al., 2011), our results with a more selective GSK3 inhibitor raise the possibility that lithium-induced effects may be due to nonspecific targets, perhaps via inositol phosphatase inhibition (Quiroz et al., 2004).

The present study shows that both GSK3 isoforms are rhythmically inactivated in the SCN in constant conditions consistent with previous evidence in LD (Iwahana et al., 2004; Iitaka et al., 2005). These results support the model that GSK3 activity is clock-controlled (Durgan and Young, 2010) potentially regulated by rhythmic phosphorylation of AKT (Panda et al., 2002; Durgan and Young, 2010), a primary upstream kinase of GSK3 (Hur and Zhou, 2010). In addition, our data suggests that GSK3 feeds back to impact molecular clock function in SCN neurons. Specifically, when both GSK3 isoforms were constitutively activated, BMAL1 rhythms within the SCN became arrhythmic. It is interesting that BMAL1 levels do not fall below baseline levels in mice with chronic GSK3 activity. Future studies should investigate whether there is a pool of BMAL1 that is resistant to degradation via the GSK3-E3-ubiquitin pathway and whether chronic GSK3 activation affects transcriptional clock gene rhythms and/or cellular compartmentalization of clock proteins. Our study also found that disruption of GSK3 activation rhythms with chronic pharmacological inhibition nearly doubled PER2::LUC rhythms in SCN organotypic cultures. Investigation of the effects of phase-specific inhibition of GSK3 on the molecular clock will be important to address in the future. Taken together, our results support the conceptual model that disruption of GSK3 activity rhythms impacts the molecular clock such that chronic activation impairs rhythmicity of some molecular clock components (such as BMAL1; Figure 2), while chronic inhibition enhances rhythmicity of the molecular clock (as indicated by real-time PER2::LUC rhythms).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants R00GM086683 and R01NS082413 to K.L.G., F31NS086282 to J.R.P., F31NS084683 to LMH, R01HL123574 to M.E.Y. and T32NS061788 – Training Program in the Neurobiology of Cognition and Cognitive Disorders. We thank Rita M. Cowell for technical assistance.

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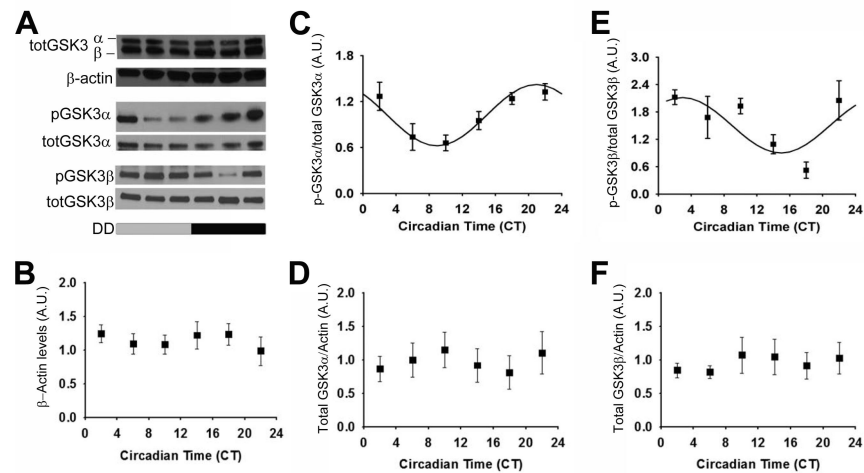


Figure 1. Rhythmic phosphorylation of GSK3 in the SCN persists in constant darkness
 (A) Representative western blots of total GSK3 α/β re-blotted for beta-actin (top); p-GSK3 α re-blotted for GSK3 α (middle); p-GSK3 β re-blotted for GSK3 β (bottom). Quantification (mean \pm SEM per CT bin) of (B) beta-actin levels, (C) p-GSK3 α to total GSK3 α ratio, (D) total GSK3 α (normalized to beta-actin), (E) p-GSK3 β to total GSK3 β ratio, and (F) total GSK3 β (normalized to beta-actin) when sampled at various times across DD. Cosinor non-linear regression: (C) $R^2 = 0.53$, $F(2, 24) = 13.73$, $p < 0.05$, $N = 27$ /time course; (D) $R^2 = 0.25$, $F(2, 24) = 3.96$, $p < 0.05$; $N = 27$ /time course; (E) $R^2 = 0.21$, $F(2, 22) = 2.91$, $p > 0.05$; $N = 26$ /time course.

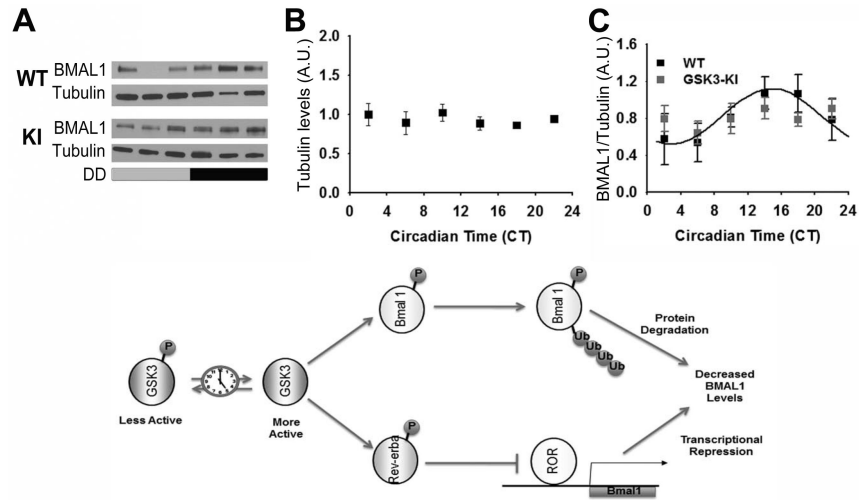


Figure 2. Rhythmic BMAL1 expression in mice with chronic GSK3 activation
 (A) Representative western blots of BMAL1 re-blotting for alpha-tubulin. Quantification (mean ± SEM per CT bin) of (B) alpha-tubulin levels and (C) BMAL1 levels (normalized to alpha-tubulin) when sampled across DD for WT (black; N = 27/time course) and GSK3-KI (gray; N = 25/time course) mice. Cosinor non-linear regression: WT, $R^2 = 0.23$, $F(2, 24) = 3.50$, $p < 0.05$; GSK3-KI, $R^2 = 0.01$, $F(2, 22) = 0.57$, $p = 0.91$. (Bottom) Schematic representing hypothetical model for the interrelationship between GSK3 and the circadian clock. Circadian clock dependent regulation of GSK3 results in time-of-day-dependent oscillations in target proteins, including BMAL1 and REV-ERB α . Phosphorylation of BMAL1 primes this clock component for ubiquitination and subsequent degradation, while phosphorylation of REV-ERB α promotes nuclear translocation and inhibition of ROR-mediated *Bmal1* transcription. Increased BMAL1 protein degradation coupled with diminished *Bmal1* transcription will result in diminished BMAL1 protein levels and therefore attenuated circadian clock output.

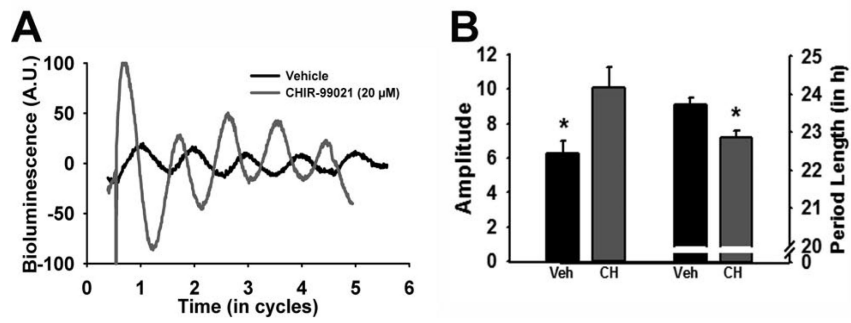


Figure 3. GSK3 inhibition modulates the molecular clock

(A) Representative bioluminescent traces from PER2::LUC organotypic SCN cultures treated with vehicle (black) or CHIR-99021 (20 μM; gray). (B) Bar graph (mean ± SEM) of amplitude (left; $t(16) = 2.68$; $*p < 0.05$) and period length (right; $t(16) = -3.83$, $*p < 0.05$).