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DISRUPTION OF CIRCADIAN RHYTHMICITY AND SUPRACHIASMATIC ACTION POTENTIAL FREQUENCY IN A MOUSE MODEL WITH CONSTITUTIVE ACTIVATION OF GLYCOGEN SYNTHASE KINASE 3

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Abstract—Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that has been implicated in psychiatric diseases, neurodevelopment, and circadian regulation. Both GSK3 isoforms, α and β, exhibit a 24-h variation of inhibitory phosphorylation within the suprachiasmatic nucleus (SCN), the primary circadian pacemaker. We examined the hypothesis that rhythmic GSK3 activity is critical for robust circadian rhythmicity using GSK3$\alpha^{21A/21A}/\beta^{9A/9A}$ knock-in mice with serine–alanine substitutions at the inhibitory phosphorylation sites, making both forms constitutively active. We monitored wheel-running locomotor activity of GSK3 knock-in mice and used loose-patch electrophysiology to examine the effect of chronic GSK3 activity on circadian behavior and SCN neuronal activity. Double transgenic GSK3α/β knock-in mice exhibit disrupted behavioral rhythmicity, including significantly decreased rhythmic amplitude, lengthened active period, and increased activity bouts per day. This behavioral disruption was dependent on chronic activation of both GSK3 isoforms and was not seen in single transgenic GSK3α or GSK3β knock-in mice. Underlying the behavioral changes, SCN neurons from double transgenic GSK3α/β knock-in mice exhibited significantly higher spike rates during the subjective night compared to those from wild-type controls, with no differences detected during the subjective day. These results suggest that constitutive activation of GSK3 results in the loss of the typical day/night variation of SCN neuronal activity. Together, these results implicate GSK3 activity as a critical regulator of circadian behavior and neurophysiological rhythms. Because GSK3 has been implicated in numerous pathologies, understanding how GSK3 modulates circadian rhythms and neurophysiological activity may lead to novel therapeutics for pathological disorders and circadian rhythm dysfunction.

Key words: glycogen synthase kinase 3, circadian rhythms, suprachiasmatic nucleus, electrophysiology.

INTRODUCTION

Circadian rhythms are endogenous 24-h physiological and behavioral rhythms that are present in nearly all living organisms, ranging from bacteria to mammals (Bell-Pedersen et al., 2005). Circadian disturbance in humans has been implicated in a number of pathologies including psychiatric disorders, cardiometabolic disease, inflammatory disease and cancer (Takahashi et al., 2008). In all mammals, daily physiological and behavioral rhythms are orchestrated by a primary circadian pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus (Welsh et al., 2010). SCN neurons generate characteristic, daily rhythms in electrical activity, exhibiting high activity during the day and low activity during the night (Inouye and Kawamura, 1979). The daily rhythm in the spontaneous firing rate (SFR) of SCN cells is important for synchronous output of the central pacemaker and is necessary for normal circadian behavior (Schwartz et al., 1987). At the molecular level, 24-h timing is driven by transcriptional/translational feedback loops of primary ''clock'' genes which are present in almost all cell types throughout the body (Takahashi et al., 2008). Post-translational modifications, such as phosphorylation, of the core clock components contribute to the precise timing and robustness of the primary feedback loop (Gallego and Virshup, 2007), but the roles of some kinases, such as glycogen synthase kinase 3 (GSK3), remain unclear.

GSK3 is a serine/threonine kinase that is able to phosphorylate nearly all of the circadian molecular clock components such as PER2, CLOCK, BMAL1, and REVERVBα (Iitaka et al., 2005; Wang et al., 2006; Spengler et al., 2009; Kurabayashi et al., 2010; Sahar et al., 2010). In addition, GSK3 is a therapeutic target of the mood-stabilizing agent, lithium (Klein and Melton, 1996), which can lengthen the period of behavioral and molecular rhythms of multiple organisms (LeSauter and Silver, 1993; Iwahana et al., 2004; Dokucu et al., 2005; Li et al., 2012). GSK3 has two isoforms, α and β, both of which are ubiquitously expressed throughout the

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Abbreviations: α-KI, GSK3$\alpha^{21A/21A}$ knock-in; β-KI, GSK3$\beta^{9A/9A}$ knock-in; CT, circadian time; C57, C57BL/6J; DD, constant dark; DKI, GSK3$\alpha^{21A/21A}/\beta^{9A/9A}$ knock-in; EGTA, ethylene glycol tetraacetic acid; GSK3, glycogen synthase kinase 3; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LD, 12:12 light–dark cycle; SCN, suprachiasmatic nucleus; SFR, spontaneous firing rate; WT, wild-type.

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brain (Woodgett, 1990). Unlike most kinases, GSK3 is by default active and can be inactivated by phosphorylation at serine-21 and serine-9 sites for α and β, respectively (Woodgett, 1990). Recent work has shown that GSK3 exhibits a daily rhythm in inhibitory phosphorylation within the SCN (Iwahana et al., 2004; Iitaka et al., 2005), yet little is known about what role this activity plays in overall circadian rhythmicity. In this study, we examine the function of rhythmic GSK3α/β phosphorylation using GSK3$\alpha^{21A/21A}$/$\beta^{9A/9A}$ (double knock-in, DKI) mice with serine–alanine substitutions at both inhibitory phosphorylation sites (McManus et al., 2005). With this model, we tested the hypothesis that rhythmic GSK3 activity is critical for generating robust circadian rhythms. Specifically, we measured the effect of chronic GSK3 activity on circadian wheel-running behavior in two different backgrounds of mice. We also examined the role of each GSK3 isoform individually using single transgenic animals (GSK3$\alpha^{21A/21A}$ knock-in (α-KI) and GSK3$\beta^{9A/9A}$ knock-in (β-KI)). Finally, we examined whether chronic GSK3 activity disrupts day/night differences in SCN neuronal output (i.e., action potential frequency) using loose-patch recordings of SCN neurons from DKI and wild-type (WT) mice during the subjective day and subjective night.

EXPERIMENTAL PROCEDURES

Animals and housing

Male, homozygous double transgenic GSK3$\alpha^{21A/21A}$/$\beta^{9A/9A}$ (McManus et al., 2005) mice (5–7 months old) on a mixed (C57BL/6 × Balb/c) background (kindly provided by Dario R. Alessi, Dundee, Scotland) or back-crossed at least 10 generations to C57BL/6J (C57) were compared to WT mice that were strain- and age-matched (generated within the colony or purchased from Jackson Laboratories, Bar Harbor, ME, USA). For single knock-in experiments, male, homozygous single transgenic, α-KI (8–11 months old) or β-KI (3–6 months old) on a C57 background were compared to WT mice that were strain- and age-matched. These serine–alanine substitutions resulted in the loss of phosphorylation of GSK3α and/or GSK3β within the SCN of the transgenic mice (Fig. 1).

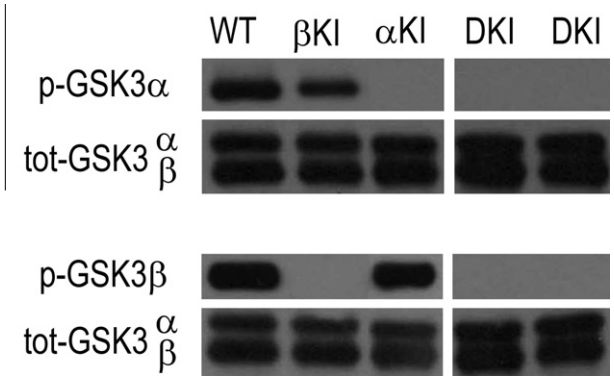

Fig. 1. Representative immunoblots of p-GSK3α/β in SCN tissue of WT, α-KI, β-KI, and DKI mice. Immunostaining for p-GSK3α S21 (top) and p-GSK3β S9 (bottom) showing loss of inhibitory phosphorylation of GSK3α, GSK3β, or both in SCN of α-KI, β-KI, or DKI mice, respectively. Total GSK3α/β staining shows loading control for each blot.

In addition, these mice develop normally and do not display any obvious behavioral or physiological phenotype. Mice were genotyped for GSK3α using the forward primer TTGAAGTGGCTGGTACTGGCTCTG and the reverse primer GTGTGCTCCAGAGTAGTACCTAGC and for GSK3β using the forward primer TCACTGGTCTAGGGGTGGTGGAAG and the reverse primer GGAGTCAGTGACAACACTTAACTT according to the specifications in (McManus et al., 2005). Mice were housed in individual wheel cages (Coulbourn Instruments, Whitehall, PA, USA) with standard rodent chow (#7917, Harlan Laboratories, Madison, WI, USA) and water provided *ad libitum*. All mice were maintained in a 12:12 light–dark (LD) cycle for at least 9 days before being placed into constant dark (DD). All handling of animals was done in accordance with the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH) guidelines.

Immunoblotting

In order to confirm the loss of phosphorylation of GSK3 in the SCN from the three transgenic models, the SCN was isolated from α-KI, β-KI, DKI and WT mice (2–3 months old) housed in a 12:12 LD cycle. Protein lysates were prepared and visualized using immunoblotting with an antibody to p-GSK3α S21 (1:750, Cell Signaling, Danvers, MA, USA) or p-GSK3β S9 only (1:500, Cell Signaling). Total GSK3α/β (1:750, Cell Signaling) staining was used on the same blot as a loading control.

Behavioral analysis

Wheel-running activity was recorded and analyzed using ClockLab software (Actimetrics, Wilmette, IL, USA). Actograms were generated using 6-min bins of activity and double plotted for ease of examination. Behavior was analyzed across 7–10 days for LD analysis, and 10 days of activity for DD analysis after the mice had been in constant conditions for 6–12 days. The activity levels were calculated using the batch analysis in ClockLab software. The free-running period (τ) and amplitude were determined by chi-squared (χ^2) periodogram analysis. Activity bout analyses were exported using the ''bout'' function in ClockLab, with a bout defined as a period where the activity level never fell below 3 counts/min for longer than 30 min. Due to the low levels of activity seen in DKI mice on the mixed background, the threshold was reduced to 1 count/min for the bout analyses in those experiments. The length of the active period (α) was measured as the time between onset and offset of activity. Activity onset was fit by eye, and activity offset was defined as the last point at which the activity in three of the previous six bins exceeded the mean activity level (Gorman and Yellon, 2010). In 2 of 16 DKI mice, activity levels were too low to reliably detect activity onset/offset, and these animals were excluded from the statistical analysis of α. Because there were no significant differences in the behavior of the WT groups age-matched to the either the α-KI or β-KI mice, the two WT groups were combined into one control group for analysis of the single KI behavior.

Slice preparation and electrophysiological recording

Mice were individually housed in DD for 3 weeks on running wheels and sacrificed at circadian time (CT) 4 and 16 (where CT 12 is conventionally defined as the onset of activity) by cervical dislocation and then enucleated with the aid of night-vision goggles. Brains were harvested, sectioned on a vibroslicer (Campden 7000SMZ, World Precision Instruments, Lafayette, IN, USA) in cold, oxygenated sucrose saline (in mM: 250 sucrose, 26 $NaHCO_3$, 1.25 Na_2HPO_4-$7H_2O$, 1.2 $MgSO_4$-$7H_2O$, 10 glucose, 2.5 $MgCl_2$, 3.5 KCl). Slices were