Brief Communications

Tuning the Period of the Mammalian Circadian Clock: Additive and Independent Effects of CK1ε^{Tau} and Fbxl3^{Afh} Mutations on Mouse Circadian Behavior and Molecular Pacemaking

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Circadian pacemaking in the suprachiasmatic nucleus (SCN) revolves around a transcriptional/posttranslational feedback loop in which period (*Per*) and cryptochrome (*Cry*) genes are negatively regulated by their protein products. Genetically specified differences in this oscillator underlie sleep and metabolic disorders, and dictate diurnal/nocturnal preference. A critical goal, therefore, is to identify mechanisms that generate circadian phenotypic diversity, through both single gene effects and gene interactions. The individual stabilities of PER or CRY proteins determine pacemaker period, and PER/CRY complexes have been proposed to afford mutual stabilization, although how PER and CRY proteins with contrasting stabilities interact is unknown. We therefore examined interactions between two mutations in male mice: *Fbxl3^{Afh}*, which lengthens period by stabilizing CRY, and *Csnk1ε^{tm1Asil}* (*CK1ε^{Tau}*), which destabilizes PER, thereby accelerating the clock. By intercrossing these mutants, we show that the stabilities of CRY and PER are independently regulated, contrary to the expectation of mutual stabilization. Segregation of wild-type and mutant alleles generated a spectrum of periods for rest-activity behavior and SCN bioluminescence rhythms. The mutations exerted independent, additive effects on circadian period, biased toward shorter periods determined by *CK1ε^{Tau}*. Notably, *Fbxl3^{Afh}* extended the duration of the nadir of the PER2-driven bioluminescence rhythm but *CK1ε^{Tau}* reversed this, indicating that despite maintained CRY expression, *CK1ε^{Tau}* truncated the interval of negative feedback. These results argue for independent, additive biochemical actions of PER and CRY in circadian control, and complement genome-wide epistatic analyses, seeking to decipher the multigenic control of circadian pacemaking.

Introduction

The sleep/wake cycle is the most dramatic manifestation of our circadian clockwork. It is accompanied by a complex temporal program of rhythmic gene expression, metabolism and physiology, coordinated by the suprachiasmatic nucleus (SCN), the principal brain pacemaker (Reppert and Weaver, 2002; Ko and Takahashi, 2006; Lamia et al., 2008). At the molecular level, the SCN and local pacemakers distributed across the brain and peripheral organs define circadian time via a transcriptional/posttranslational feedback loop in which period (*Per*) and cryptochrome (*Cry*) genes are negatively regulated by their protein products. Within the SCN, this cell-autonomous pacemaker is stabilized by interneuronal coupling (Maywood et al., 2006; Liu et al., 2007). In animal and human studies, disorders of sleep and metabolic and other functions have been associated with genetic modifications of this oscillator (Hastings et al., 2003; Allebrandt et al., 2010; Marcheva et al., 2010). Moreover, allelic variants of several circadian genes dictate diurnal/nocturnal preference (Roenneberg and Merrow, 2007; Viola et al., 2007), while the general population exhibits a broad range of sleeping patterns and tolerance to sleep deprivation (Roenneberg et al., 2007). Furthermore, misalignment between biological time and social routines (Wittmann et al., 2006) brings a significant cost to well-being. A critical goal for circadian biology, therefore, is to identify mechanisms that generate circadian phenotypic diversity, at the single gene level and through gene interactions (Shimomura et al., 2001).

PER and CRY are essential negative regulators within the clock, and mutations affecting the respective stability of PER and CRY alter circadian period. Stabilizing CRY1 and CRY2 by loss of function of the E3 ubiquitin ligase FBXL3 in the *Afh* (Godinho et al., 2007) and *Ovt* (Siepka et al., 2007) mutants lengthens period, whereas the *Tau* mutation of casein kinase (CK)1ε [*Csnk1ε^{tm1Asil}* (*CK1ε^{Tau}*)] destabilizes PER1 and PER2, thereby accelerating the clock (Lowrey et al., 2000; Meng et al., 2008). Considerable bio-

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chemical and cell biological data indicate that formation of heteromeric PER and CRY complexes affords mutual stabilization of the proteins, facilitating nuclear entry and mediation of transcriptional negative feedback (Reppert and Weaver, 2002). For example, CRY proteins stabilize phosphorylated PER, protecting it from ubiquitinylation and subsequent proteasomal degradation (Lee et al., 2001; Yagita et al., 2002), while PER in turn stabilizes CRY (Chen et al., 2009). A strong prediction, therefore, is that independent mutations affecting PER and CRY stability should exhibit epistatic interactions, with the long-period mutation preventing expression of the short-period phenotype, i.e., stabilizing CRY should protect PER from increased susceptibility to degradation: the wagon train goes as fast as the slowest wagon. This prediction is heavily reliant, however, on studies using recombinant proteins and has not been explicitly tested *in vivo*, in a dynamic context in which endogenous proteins control molecular pacemaking and behavioral rhythms. The *Fbxl3*^{Afh} and *CK1ε*^{Tau} lines were therefore crossed to generate mice with various combinations of mutant and wild-type alleles. The explicit hypothesis was that *Fbxl3*^{Afh} would be epistatic to *CK1ε*^{Tau} and maintain a long-period behavioral phenotype, regardless of CK1ε activity.

Materials and Methods

Studies conformed to the Animals (Scientific Procedures) Act (1986). Wild-type, *Fbxl3*$^{Afh/Afh}$, *Csk1ε*$^{Tau/Tau}$, and *Fbxl3*$^{Afh/Afh}$*::Csk1ε*$^{Tau/Tau}$ male mice on the PER2:LUC, C57BL/6 background (Yoo et al., 2004) were housed under 12 h:12 h light:dim red light (LD) with *ad libitum* food and water. For circadian recording, mice were caged individually within light-tight ventilated cabinets and transferred from LD to continuous dim red light (DD). Wheel-running behavior was analyzed using ClockLab software (ActiMetrics). SCN organotypic slice cultures from ~10-d-old pups (or adults for protein degradation studies) from all genotypes were prepared and bioluminescence emission recorded using photomultipliers and CCD cameras (Hamamatsu) as described previously (Maywood et al., 2006). Rhythmicity and period were assessed with BRASS software [A. Millar (University of Edinburgh, Edinburgh, UK) and M. Straume (University of Virginia, Charlottesville, VA)]. To assess the stability of PER and CRY in various genetic backgrounds, cycloheximide (CHX, 20 μg/ml) was added to SCNs at their peak of PER2:LUC expression [i.e., circadian time (CT) 12]. PER2 stability was monitored as the rate of degradation of PER2:LUC bioluminescence following CHX, and half-life was calculated (Prism, GraphPad) (Meng et al., 2008). To assess CRY stability, single SCNs were removed from the culture membrane either immediately after CHX treatment ($T = 0$) or 12 h later ($T = 12$), placed in 50 μl of 2× loading buffer, mixed, sonicated, and heated at 80°C (10 min). Following centrifugation at 13,000 × *g* (10 min), samples were loaded onto 4–12% Bis-Tris gel (NuPage) and, following electrophoresis, transferred onto nitrocellulose membrane. Membranes were blocked with 5% blocking agent in Tris-buffered saline containing 0.05% Tween 20 and incubated with anti-CRY1 serum (1:500 final dilution) raised against the murine peptide sequence SQEEDAQSVGPKVQRQSSN. Immunoreactive bands were visualized using anti-rabbit antisera and enhanced chemiluminescence detection (GE Healthcare). The absence of immunoreactivity in SCNs from *Cry1*$^{-/-}$ mice confirmed antiserum specificity. For each genotype, the mean signal intensity at $T = 0$ was normalized to 100% and the respective SCN signals at $T = 12$ were calculated. All data were compared by ANOVA with *post hoc* Bonferroni *t* test.

Results

PER and CRY stabilities in *Afh* and *Tau* intercrosses

In organotypic slices of mouse lung tissue treated with CHX at the circadian peak in PER2:LUC expression (CT12), the *Fbxl3*^{Afh} mutation significantly reduced CRY degradation over the subsequent 8 h (Godinho et al., 2007). A comparable reduction in the rate of CRY1 degradation was observed in the *Fbxl3*^{Afh} mutant SCN. In wild-type SCN slices treated with CHX at CT12, the CRY1 signal fell by 83.7 ± 3.5% over 12 h, compared to CT = 0 levels. In contrast, CRY1 in the mutant SCN declined by just 32.7 ± 7.6% (Fig. 1*a*). This stabilization was also observed in the presence of the *CK1ε*^{Tau} allele: the CRY1 signal fell by 37.0 ± 8.3% in intercrossed SCNs, which was not significantly different from the *Fbxl3*^{Afh} slices with a *CK1ε* wild-type background. Thus, *CK1ε*^{Tau} did not alter the biochemical effects of *Fbxl3*^{Afh} on CRY1. The half-life of PER2 protein in SCN slices, determined by monitoring the decline in PER2-driven bioluminescence following addition of CHX at CT12, was significantly shortened by ~27 min in *CK1ε*$^{Tau/Tau}$ SCN (2.13 ± 0.07 h vs 1.68 ± 0.04 h) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material; Fig. 1*c*). This is comparable to previous reports from primary fibroblast cultures and SCN slices (Meng et al., 2008). The *Fbxl3*^{Afh} allele had no significant effect, however, on PER2 in the *CK1ε* wild-type background. Importantly, the *CK1ε*^{Tau} allele was as effective in destabilizing PER2 in the *Fbxl3*^{Afh} homozygous background as it was in *Fbxl3*$^{+/+}$ SCN. Hence the destabilizing effect of the *CK1ε*^{Tau} allele on PER2 protein was unaffected by *Fbxl3*^{Afh}. Thus, PER and CRY proteins stabilities are determined independently: the *CK1ε*^{Tau} and *Fbxl3*^{Afh} alleles did not interact in their biochemical actions upon their respective targets.

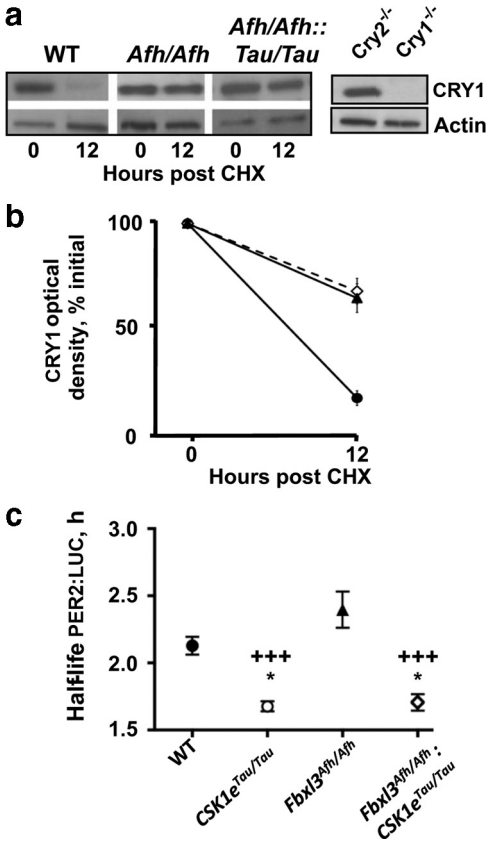

Figure 1. Independent control over PER and CRY protein stabilities by *CK1ε*^{Tau} and *Fbxl3*^{Afh} alleles. ***a***, Western blots for CRY1 in single representative SCN slices carrying either wild-type, *CK1ε*^{Tau}, or *Fbxl3*^{Afh} alleles, treated with CHX at CT12 and harvested immediately or after 12 h. Right, Blots from CRY2 and CRY1 null mice confirm specificity of antiserum; bottom panels, actin loading controls. ***b***, Group data (mean ± SEM, $n = 3–4$ SCNs per observation) reveal significant (ANOVA, $p < 0.01$) reduction in rate of CRY degradation in *Afh* mutant, with (diamond) and without (triangle) *Tau* mutation compared to wild type (circle). ***c***, Group data (mean ± SEM, $n = 6–8$ SCNs per genotype) for PER2 half-life across genotypes. *$p < 0.05$ significant versus wild-type control, $^{+++}p < 0.001$ versus *Afh* mutant.

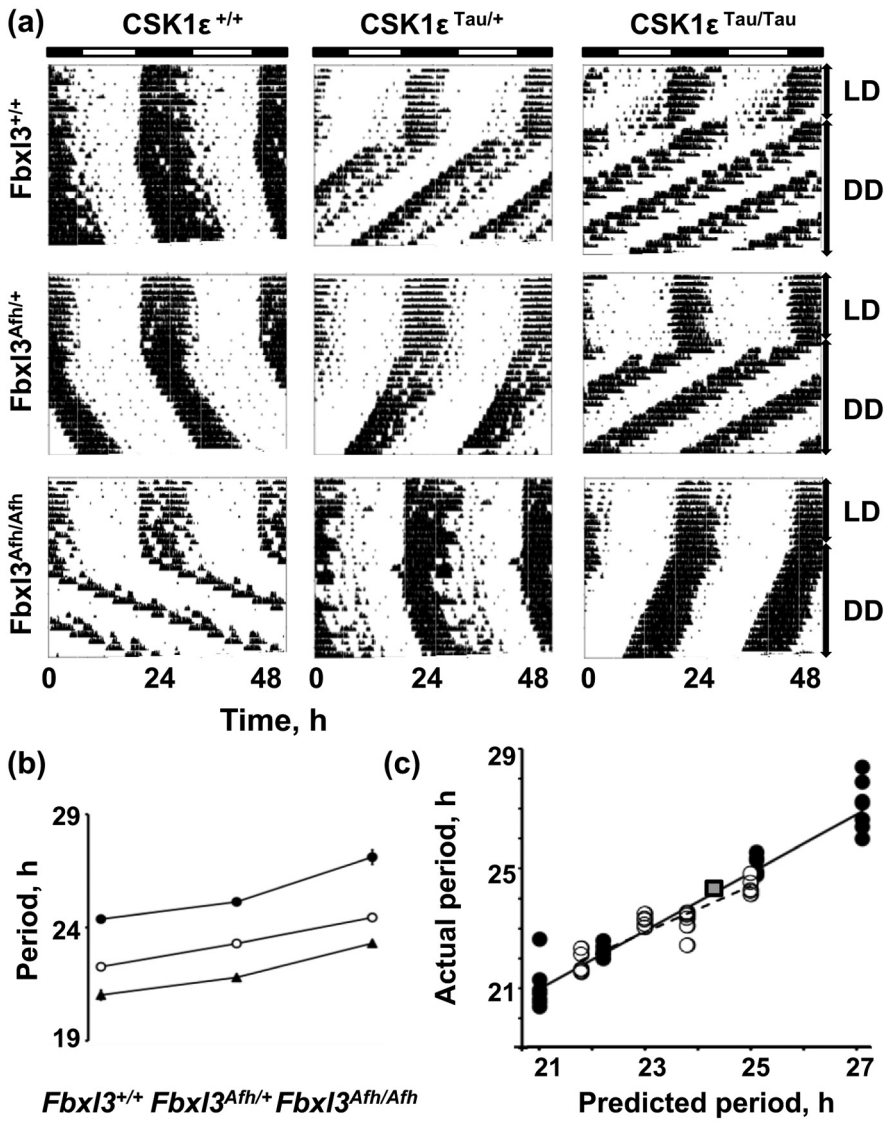

Figure 2. Tuning of the period of circadian behavioral rhythms *in vivo* by additive and independent effects of *CK1ε^Tau* and *Fbxl3^Afh*. ***a***, Representative actograms of mice carrying wild-type, *CK1ε^Tau* and *Fbxl3^Afh* alleles, initially held on LD and transferred to DD after 12 d. ***b***, Group data (mean ± SEM, $n = 6–11$, $N = 69$) to illustrate individual and combined effects of *CK1ε^Tau* and *Fbxl3^Afh* alleles on circadian period of rest/activity cycle. Closed circles, Wild type; open circles, *CK1ε^Tau/+*; closed triangles, *CK1ε^Tau/Tau*. ***c***, Predicted and observed effects of *CK1ε^Tau* and *Fbxl3^Afh* on circadian period of behavioral rhythms. The square symbol represents wild types, and the closed circles are the data from single gene mutants ("predicted" periods for these groups are the actual observed group mean). Open circles represent double mutant groups in which the predicted period was calculated on the basis of the single allele effects. The dotted regression line is the fit derived only from SCNs with complex genotypes; the solid regression line is calculated for the total population.

Behavioral rhythms in *Afh* and *Tau* intercrosses

Would increased stability of CRY in the *Fbxl3^Afh* background mask the acceleration due to *CK1ε^Tau*, or would decreased stability of PER by *CK1ε^Tau* compromise the action of *Fbxl3^Afh* on behavior? Under LD, the activity/rest cycle of wild types entrained to 24 h (Fig. 2*a*) with activity starting shortly after lights off (supplemental Table S1, available at www.jneurosci.org as supplemental material). Activity onset in *Fbxl3^Afh* homozygotes tended to be delayed, although all animals entrained. Heterozygote *CK1ε^Tau* mice also entrained to 24 h, but with a trend for activity onset to be advanced relative to wild types. In contrast, none of the *CK1ε^Tau/Tau* mice entrained to LD cycles; instead they exhibited relative coordination. This effect of *CK1ε^Tau/Tau* was rescued, however, by the *Fbxl3^Afh* allele. Thus, double homozygotes entrained to LD, becoming active shortly after lights off.

Under DD, the *CK1ε^Tau* allele shortened circadian period, while the *Fbxl3^Afh* allele dose-dependently lengthened it in respective heterozygote and homozygote single mutants (Fig. 2*a*,*b*). Importantly, the *Fbxl3^Afh* allele also progressively lengthened period in the short-period *CK1ε^Tau* homozygote background (Fig. 2*b*, lower line) and the *CK1ε^Tau* allele shortened circadian period in the *Fbxl3^Afh* homozygote background. There was no obvious effect of the combined alleles on amplitude or precision of the behavioral rhythm. Thus, combination of the two alleles generated a broad spectrum of stable circadian periods, and *prima facie*, the period of the behavioral rhythm was not solely determined by the stability of CRY proteins.

To quantify further the relative contributions of the two mutations, we calculated the individual effects of each allele in its respective heterozygous and homozygous conditions in wild-type backgrounds (i.e., *CK1ε^Tau/+* and *CK1ε^Tau/Tau* in *Fbxl3^+/+*, and *Fbxl3^Afh/+* and *Fbxl3^Afh/Afh* in *CK1ε^+/+*) and assuming no epistasis, predicted the period of the four complex genotypes containing mutant alleles at both loci (*CK1ε^Tau/+* and *CK1ε^Tau/Tau* in *Fbxl3^Afh/+* and *Fbxl3^Afh/Afh*). The correlation between predicted and observed periods for these four genotypes was highly significant ($r^2 = 0.85$, $p < 0.01$) (Fig. 2*c*). Thus, the period of the behavioral rhythm was determined by independent and additive contributions reflecting the individual stabilities of PER and CRY. Period lengthening due to *Afh*-dependent CRY stability did not prevent the expression of *Tau*-mediated PER instability, and vice versa.

SCN pacemaking in *Afh* and *Tau* intercrosses

The effects of single and double mutations on the period of PER2:LUC bioluminescence rhythms in SCN cultures (Yoo et al., 2004) were very similar to those seen for behavioral rhythms (Fig. 3*a*,*b*), the mean periods of SCN and behavioral rhythms being significantly correlated ($r^2 = 0.99$, $p < 0.01$) (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). The *Fbxl3^Afh* allele dose-dependently lengthened period, while the *CK1ε^Tau* allele shortened SCN period. In double-mutant slices, the presence of the *Fbxl3^Afh* allele progressively lengthened the period of *CK1ε^Tau* homozygotes (Fig. 3*b*, lower line). Critically, in the *Fbxl3^Afh* homozygous background, the *CK1ε^Tau* allele shortened circadian period (Fig. 3*b*, right hand points). Hence, double heterozygous and double homozygous mutant SCN exhibited periods similar to each other, but ~1.5–2 h shorter than wild type (Fig. 3*a*,*b*), revealing a bias toward *CK1ε^Tau* action.

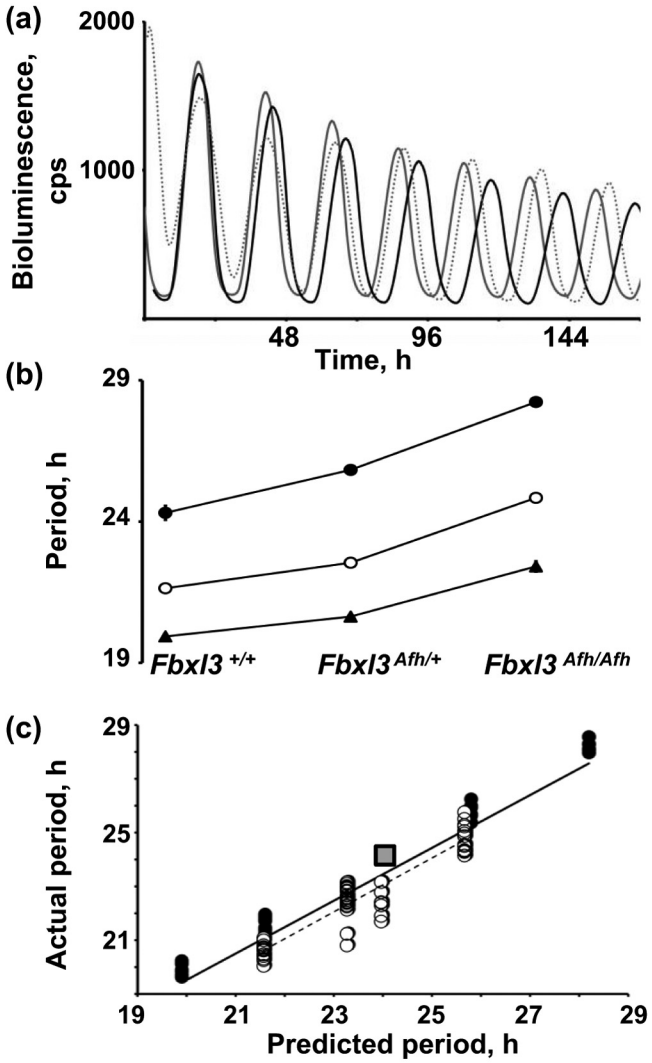

Figure 3. Tuning of the period of molecular pacemaking in the SCN by additive and independent effects of *CK1ε^Tau* and *Fbxl3^Afh*. ***a***, Representative recordings of bioluminescence rhythms from individual SCN slices: wild type (solid black), *CK1ε^Tau/+*::*Fbxl3^Afh/+* (solid gray), and *CK1ε^Tau/Tau*::*Fbxl3^Afh/Afh* (dashed gray). ***b***, Group data (mean ± SEM, $n = 6–20$, $N = 95$) illustrating individual and combined effects of *CK1ε^Tau* and *Fbxl3^Afh* on circadian period of SCN bioluminescence rhythms. Two-way ANOVA revealed significant ($p < 0.001$) effects of both mutations and an interaction. Closed circles, Wild type; open circles, *CK1ε^Tau/+*; closed triangles *CK1ε^Tau/Tau*. ***c***, Relationship between predicted and observed effects of *CK1ε^Tau* and *Fbxl3^Afh* alleles on SCN circadian period. The symbols are the same as in Figure 2*c*. The dotted regression line is derived only from SCNs with complex genotypes.

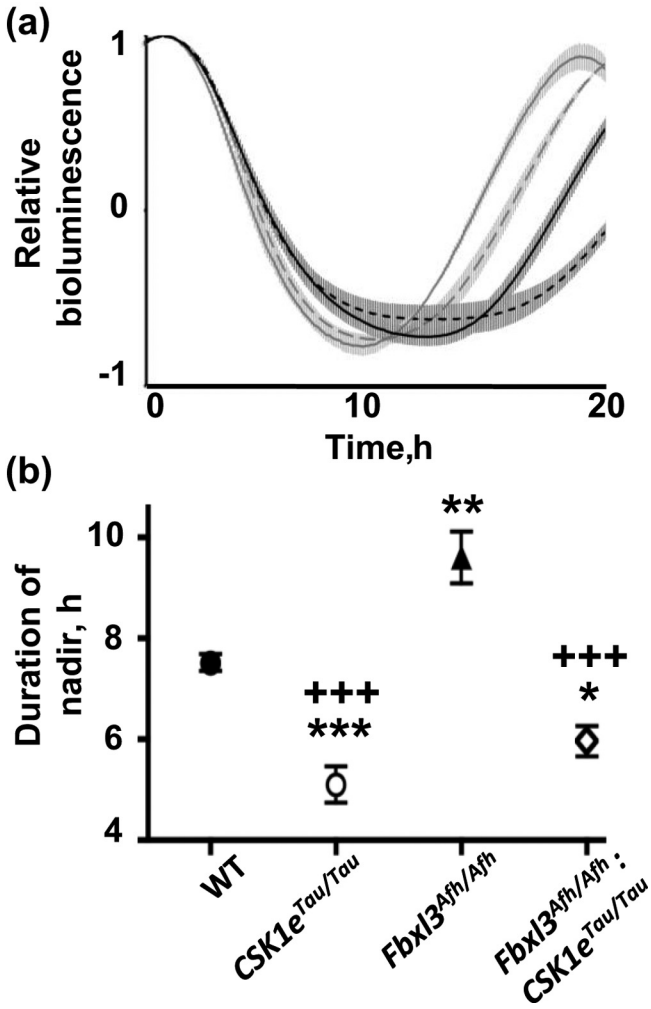

Figure 4. Circadian waveform for PER-driven bioluminescence from wild-type and single and double mutant SCN slices. ***a***, Normalized plots of bioluminescence curves from wild-type (solid black), *Afh* homozygous (dashed black), *Tau* homozygous (solid gray), and double homozygous (dashed gray) SCNs, aligned by peak expression. Curves represent mean (±SEM) of 5 slices per genotype. ***b***, Group data (mean ± SEM, $n = 5$ per genotype) for the duration of the interval of basal bioluminescence (duration of curve below level corresponding to 20% above nadir) from wild-type and single and double mutant SCN slices. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus wild type, $^{+++}p < 0.001$ versus *Afh* mutant (ANOVA and *post hoc* Bonferroni).

CCD imaging of wild-type and double homozygous mutant slices ($n = 3$ for both) confirmed that this additive effect on period was manifest in individual cells (data not shown) and subregions of the SCN, and that the amplitude and precision of regional pacemaking were equivalent in both genotypes (supplemental Table S2, available at www.jneurosci.org as supplemental material). As for behavior, we predicted the period of the complex genotypes containing mutant alleles at both loci, assuming no epistasis (Fig. 3*c*). The correlation between predicted and observed periods for the four intercrosses was highly significant ($r^2 = 0.88$, $p < 0.01$) and the regression slope was effectively 1 (0.9997). The fitted line ($y = 1.0x - 0.95$) showed, however, a consistent *Tau* bias, i.e., observed period was 0.95 h shorter than that predicted by additive allelic effects (Fig. 3*c*). Hence, the period of the pacemaker was determined independently and additively by the mutations: *Fbxl3^Afh* tempered acceleration by *CK1ε^Tau*, while *CK1ε^Tau* tempered deceleration by *Fbxl3^Afh*, and overall, *CK1ε^Tau*-determined PER stability was marginally predominant.

To identify how the combined alleles determined phenotypes, we examined the waveform of the circadian bioluminescence cycle. Acceleration of the SCN pacemaker by the *CK1ε^Tau* allele is associated with an earlier decline of PER levels (Dey et al., 2005; Meng et al., 2008), and thus earlier termination of the interval of negative feedback. Conversely, the *Afh* mutation prolongs the interval of negative feedback (Godinho et al., 2007). This interval (defined as the time between the two points on the PER2:LUC cycle 20% above the nadir) was significantly prolonged in *Afh* homozygous slices (wild type 7.52 ± 0.17 h, *Afh* 9.60 ± 0.40 h, $n = 5$) (Fig. 4*a*,*b*; supplemental Fig. S3, available at www.jneurosci.org as supplemental material). *Tau* homozygosity significantly shortened it (5.10 ± 0.35 h) but, unexpectedly, the duration of the interval was also significantly shortened in double homozygous mutant slices (5.96 ± 0.30 h). This was not significantly different from that in *Tau* slices. These changes in the

duration of the nadir of the cycle between genotypes explain the additive effects of the mutations on SCN period, and thereby, the behavioral rest/activity rhythm.

Discussion

By intercrossing mice with contrasting stabilities of PER and CRY, we show at the levels of molecular pacemaking in the SCN and behavioral rhythms in the animal that the tuning of circadian period is sensitive to the stability of both PER and CRY proteins, independently and additively. Thus, various combinations of wild-type and mutant alleles of *Fbxl3* and *CK1ε* generated a broad range of stable and precise periodicities and, in the context of the mutations used, the observed period of double-mutant SCN tended to be shorter than predicted by ~1 h, consistent with a more pronounced role for destabilized PER in setting clock period *in vivo*. This tuning of period was associated with changes in the duration of the phase of negative feedback.

Our original hypothesis was that *Fbxl3^{Afh}* would be epistatic to *CK1ε^{Tau}* and maintain a long-period, CRY-dependent phenotype, regardless of CK1ε activity. This hypothesis could only be tested if the biochemical effects of the mutations on their immediate targets were maintained in intercrossed backgrounds, and this was the case. Contrary to expectations of mutual stabilization in heteromeric complexes, however, the respective biochemical effects of the *Fbxl3^{Afh}* and *CK1ε^{Tau}* alleles upon the stabilities of CRY and PER were independent, one of the other: stabilized CRY did not protect PER from the destabilizing action of CK1ε^{Tau}. *In vivo*, therefore, the behavior of endogenous PER and CRY proteins did not conform to the "standard model" of mutual stabilization.

Independence of PER and CRY protein stabilities enabled us to explore their relative contributions to circadian periodicity: a problem difficult to dissect in recombinant overexpression studies in cell culture that do not recapitulate the biochemical dynamics of cellular pacemaking. Moreover, cell-based and whole-animal models have focused on whether rhythmic abundance *per se* of PER and/or CRY is necessary for clock progression (Lee et al., 2001; Fan et al., 2007; Chen et al., 2009), rather than consider how their relative stabilities might tune circadian period. A preeminent role for CRY proteins in sustaining rhythmic negative feedback in the clock has been proposed (Kume et al., 1999; Langmesser et al., 2008) and, consistent with this, loss-of-function mutations of FBXL3 ubiquitin ligase that compromise CRY degradation prolong the interval of negative feedback and extend circadian period (Godinho et al., 2007; Siepka et al., 2007). Recently, however, differential roles for PER and CRY within the feedback loop have been proposed (Chen et al., 2009), with rhythmic abundance of PER2 protein being identified as rate-limiting. Thus, PER2 acts as a scaffold to facilitate the negative feedback actions of CRY proteins such that "PER2 rhythms are far more critical than those of CRY for clock function . . ." (Chen et al., 2009). Thus, CRY needs to be present, but in contrast to PER proteins, it does not need to oscillate for pacemaking, a conclusion supported by studies using constitutive expression of CRY in cell culture (Lee et al., 2001; Fan et al., 2007).

A clear dichotomy of views exists, therefore, with regard to the primary determinant of circadian pacemaking. If the PER rhythm was the arbiter, then the *CK1ε^{Tau}* allele would set period, regardless of CRY stability. We found this was not the case. Conversely, if CRY alone regulated the clock, *Fbxl3^{Afh}* would set the pace, regardless of *CK1ε* genotype. Again, this was not the case. Clearly, the stability of both proteins contributes to pacemaker dynamics, although the mechanism(s) mediating these contributions is unclear. The independence and additivity of their respective effects may arise from independent and separable roles within negative feedback. For example, PER and CRY may interact with different components within the CLOCK::BMAL1 complex (Langmesser et al., 2008). Equally, they may be more effective at different phases of the cycle. For example, PER-mediated feedback may be important early in circadian night, with CRY contributing later in the cycle. In this simple, serial model, independent changes in the duration of one or the other process could affect overall period additively.

Alternatively, PER and CRY may affect different aspects of transcriptional control that have convergent and additive effects on overall circadian gene expression. The bioluminescence curves for *CK1ε$^{Tau/Tau}$* and *Fbxl3$^{Afh/Afh}$* slices revealed, in the latter, a marked extension of the interval of negative feedback, as reported by prolonged suppression of PER2:LUC activity. In the double homozygous condition, this interval was shorter. Thus, despite the stabilization of CRY, the prolongation of negative feedback did not occur, and overall circadian period was of intermediate duration. This suggests that as levels of PER decline, CRY becomes less potent as a negative regulator, consistent with the proposed role of PER cycles in driving the clock (Chen et al., 2009). Equally, as PER is differentially phosphorylated/degraded, the ability of existing CRY proteins to suppress CLOCK::BMAL1 complexes would be attenuated. This is supported by the absence of a significant difference in the nadir's duration in *CK1ε$^{Tau/Tau}$* and the double mutant.

In conclusion, our data reveal a surprising lack of epistasis between mutations that are known to impact on the stability of either PER or CRY proteins, and imply that *in vivo* there is an independent, additive contribution of these proteins to circadian timing. These results thereby challenge current models based on analyses of recombinant proteins in cell culture models. Moreover, they complement genome-wide epistatic analyses of multigenic regulation of circadian pacemaking (Shimomura et al., 2001) and recent systematic studies of the diversity of circadian phenotype ("chronotype") in human populations (Wittmann et al., 2006; Roenneberg and Merrow, 2007; Roenneberg et al., 2007).

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