Protein phosphatase PHLPP1 controls the light-induced resetting of the circadian clock

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The pleckstrin homology domain leucine-rich repeat protein phosphatase 1 (PHLPP1) differentially attenuates Akt, PKC, and ERK1/2 signaling, thereby controlling the duration and amplitude of responses evoked by these kinases. PHLPP1 is expressed in the mammalian central clock, the suprachiasmatic nucleus, where it oscillates in a circadian fashion. To explore the role of PHLPP1 in vivo, we have generated mice with a targeted deletion of the *PHLPP1* gene. Here we show that *PHLPP1*-null mice, although displaying normal circadian rhythmicity, have a drastically impaired capacity to stabilize the circadian period after light-induced resetting, producing a large phase shift after light resetting. Our findings reveal that PHLPP1 exerts a previously unappreciated role in circadian control, governing the consolidation of circadian periodicity after resetting.

Per | period | after-effect | suprachiasmatic nucleus | phase shift

Essential properties of the mammalian circadian clock are a self-sustained oscillation under constant conditions and the dynamic resetting induced by light. These properties of the clock are necessary for humans and other mammals where they control cyclical activities as disparate as the sleep/wake cycle, metabolism, and cardiac function (1–3). The fine tuning of the endogenous clock by exogenous cues (zeitgebers) can be especially apparent when one travels across time zones or is transferred to a night shift working schedule (4). Circadian rhythms in mammals are driven by the central pacemaker, which is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (5). Light administration during the subjective night activates neurons of the retino-hypothalamic tract that target the SCN, and thereby resets behavioral rhythmicity. If mice are exposed to a light stimulus in the early night the clock delays, whereas it advances if light is given at late night (5, 6), underscoring the adaptability of the clock to changing lighting environment. Although there have been remarkable advances in deciphering the molecular organization of the circadian clock (1, 7), understanding of the molecular pathways involved in light-induced clock resetting is still incomplete (5).

PHLPP1 and PHLPP2 (pleckstrin homology domain leucine-rich repeats protein phosphatases 1 and 2) are Ser/Thr protein phosphatases that have been implicated in the regulation of various signaling pathways. These phosphatases negatively regulate Akt and protein kinase C (PKC) by direct dephosphorylation (8–10). In addition, a splice variant isoform of PHLPP1 (PHLPP1β, also known as suprachiasmatic nucleus circadian oscillatory protein, SCOP, see ref. 11) has been implicated in cognitive processes and its degradation appears to be required for long-term memory formation (12). PHLPP1 is thought to be involved in long-term memory formation by inhibiting ERK1/2 via direct binding to and inhibition of K-Ras, a small GTPase operating upstream of ERK1/2 (13). Interestingly, PHLPP1 (SCOP) is expressed in a circadian manner in the SCN, peaking in the subjective night (11). Its role in circadian function, however, remains obscure.

Here, we report the generation of *PHLPP1*-deficient mice by homologous recombination. These *PHLPP1*$^{-/-}$ mice display a delayed shortening of circadian period length (*tau*) after light-induced phase shift. The delayed shortenings result in a large phase shift in *PHLPP1*$^{-/-}$ mice following light resetting tasks. Considering the previously published role of PHLPP1 in cognitive function (12), the results presented here implicate PHLPP1 in SCN neuronal plasticity, where it appears to fine tune the clock, adapting it specifically to changes in external cues. Our results indicate that the phosphatase PHLPP1 plays a critical role in the consolidation of circadian periodicity after resetting.

Results

***PHLPP1*$^{-/-}$ Mice Show a Normal Free-Running *tau*.** To elucidate the physiological functions of PHLPP1, mice were generated in which the *PHLPP1* gene was ablated by homologous recombination (Fig. 1 *A*–*C*). The mutant mice show no detectable levels of the PHLPP1 mRNA and protein (Fig. 1 *D* and *E*). Importantly, PHLPP2 expression levels in *PHLPP1*$^{-/-}$ versus the *PHLPP1*$^{+/+}$ mice were equivalent (Fig. 1*F*), a notion that confirmed previous PHLPP1 interference experiments (10). These animals develop normally and show no gross anatomical defects (Fig. S1 *A* and *B*). Daily activities (running wheel revolutions under light–dark cycle) do not differ between in *PHLPP1*$^{-/-}$ and *PHLPP1*$^{+/+}$ mice (Fig. S1*C*). The circadian expression of PHLPP1 in the SCN (11) prompted us to study the circadian behavior of the mutant mice. To determine whether *PHLPP1*$^{-/-}$ mice had normal circadian rhythmicity, we first analyzed *tau* under constant conditions. After entrainment on a LD 12:12 cycle for more than 3 weeks, *PHLPP1*$^{-/-}$ and wild-type (*PHLPP1*$^{+/+}$) littermate mice were moved into constant darkness (DD, free running) starting at the time of lights "off," zeitgeber time (ZT) 12. This day was defined as day 1. To measure *tau*, two different detection systems were used, passive (pyroelectric) infrared sensors (Fig. 2, *Top*) and running wheels (Fig. S1*D*), obtaining equivalent results. *Tau* length was measured from day 2 to day 21. In both systems, there was no difference in *tau* between genotypes (Fig. S1*E*) ($P > 0.05$ Welch's *t* test).

Late *tau* Shortening Produces a Large Phase Resetting in *PHLPP1*-Null Mice. To compare the ability of PHLPP1-null mice versus wild-type littermates to adapt to light-induced resetting of the clock phase, we administered a short-light pulse to the animals during

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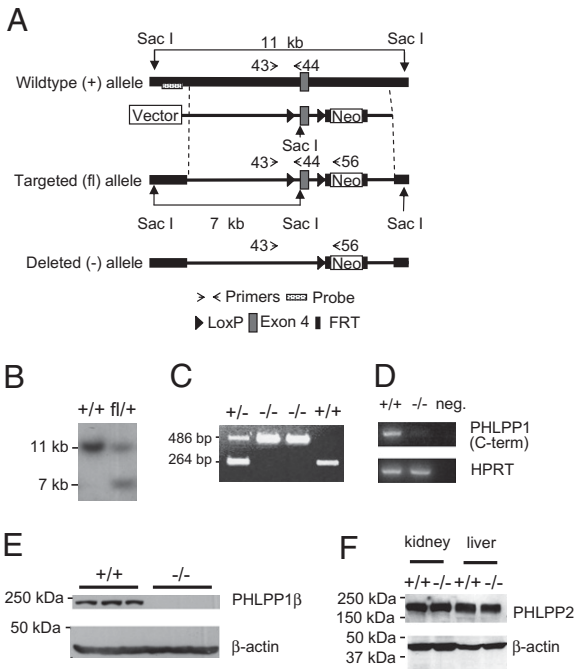

Fig. 1. Generation of *PHLPP1*$^{-/-}$ mice. (*A*) Targeting strategy to disrupt the PHLPP1 gene. Shown are the wild-type allele, the targeting vector, the targeted (floxed) allele, and the deleted allele generated by Cre-mediated recombination of the floxed allele. The DNA probe used for screening the Southern blots is marked by a dotted box, and the PCR primers used for genotyping are indicated by small arrows. (*B*) Southern blot analysis of SacI-digested DNA from embryonic stem cells used to generate chimeric mice. The probe labels an 11-kb fragment in the wild-type cells and a 7-kb fragment in cells in which homologous recombination has occurred. (*C*) Genotyping PCR results from a cross between heterozygous PHLPP1 mutant mice. The PHLPP1 WT (+) allele gives a 264-bp PCR product whereas the deleted allele (–) produces a 486-bp fragment. (*D*) Lack of PHLPP1 transcript expression in *PHLPP1*$^{-/-}$ mice. Total RNA isolated from *PHLPP1*$^{+/+}$ and *PHLPP1*$^{-/-}$ mouse brain was used as templates for the RT-PCR analysis. The PHLPP1-specific primers used were located in exons 16 and 17, respectively. The hypoxanthine phosphoribosyltransferase 1 (HPRT) primers were used as controls for RT-PCR reactions. For a negative control, the cDNA template was omitted in the reaction (the lane labeled "neg"). (*E*) Western blot of PHLPP1 protein in brain lysates derived from PHLPP1$^{+/+}$ and PHLPP1$^{-/-}$ mice. β-Actin serves as a loading control. (*F*) Western blot of PHLPP2 protein in liver and kidney whole cell lysates derived from PHLPP1$^{+/+}$ and PHLPP1$^{-/-}$ mice. β-Actin serves as a loading control.

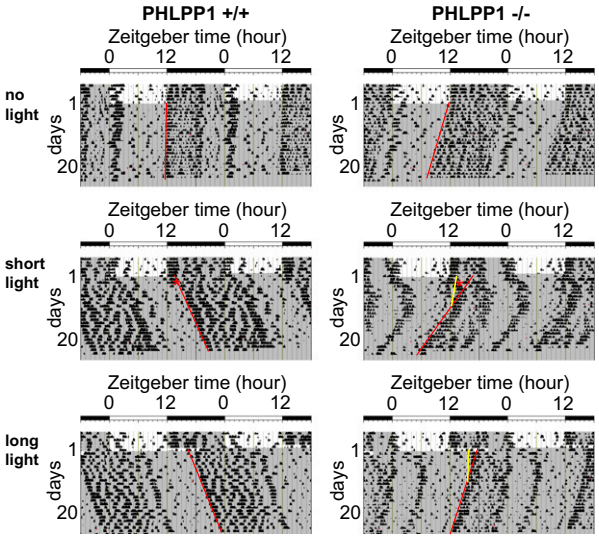

Fig. 2. Differential light-induced phase shifts in PHLPP1-null mice. Double-plotted activity records of wild-type mice (*PHLPP1*$^{+/+}$) and PHLPP1-deficient mice (*PHLPP1*$^{-/-}$). Mice were entrained in 12:12 light–dark (LD) cycles and then placed in constant darkness (DD) from the light off (ZT12), on day 1. After 50 h in DD (CT14, day 3), no light pulse (no light; *Top*), or a 30-min light pulse (short light; *Middle*) was administered to *PHLPP1*$^{+/+}$ and *PHLPP1*$^{-/-}$ mice. A separate group of mice were entrained and moved to constant darkness following 8 h of light prolongation (long light; *Bottom*) on the last day (day 1) of LD cycle. Locomotor activities were monitored by infrared sensors and are expressed in the histogram. Periods of darkness are indicated by gray backgrounds. Short-light pulses are denoted by asterisks. Red regression lines estimate the phase shift from days 10 to 21. Yellow regression lines estimate the phase shifts occurring several days after light pulses (short-light task = days 4–10; long-light task = days 2–10; lines are shown for phase shifted *PHLPP1*$^{-/-}$ only).

the subjective night. Mice were exposed to a 30-min light pulse of ~200 lx during the second early subjective night (CT14) in DD (50 h following entry into DD following entrainment) (Fig. 2, *Middle*). Our results demonstrate that several cycles after the resetting task, the circadian period shortens spontaneously in the *PHLPP1*$^{-/-}$ mice (Fig. 2, *Middle Right*). Two regression lines were made, one for the first several activity cycles after light manipulation [early phase (E), day 4 to day 10] and one for the following activity cycles [late phase (L), day 10 to day 21].

Tau values were compared from early-phase and late-phase days in each lighting task (i.e., "no light" and "short light"). When no light was administered (no-light group), the estimated phases are located around light offset (Fig. S2*A*). No statistical difference between the two genotypes was observed (repeated measure ANOVA; $P > 0.05$) (Fig. S2*A*, *Upper*). Interestingly, in the short-light exposed group, only the value from late-phase days (L) of *PHLPP1*$^{-/-}$ mice showed a larger phase angle to light off (repeated measure ANOVA; $P < 0.005$) (Fig. S2*A*, *Lower*). This data suggests that the delayed phase change was solely responsible for the larger phase delays in short-light-exposed *PHLPP1*$^{-/-}$mice.

Behavioral rhythms from early-phase days (E) and late-phase days (L) were also tested for differences in the phase shifts. Calculations from the early-phase groups, showed no difference in the phase shift between *PHLPP1*$^{+/+}$ and *PHLPP1*$^{-/-}$ mice (two-way ANOVA; $P > 0.05$) (Fig. 3*A*, *Upper*). However, by late-phase days, *PHLPP1*$^{-/-}$ mice phase delayed more than wild-type animals in response to the short-light tasks (two-way ANOVA, $P < 0.05$). The short-light task induced a more pronounced phase delay (~3 h) in *PHLPP*$^{-/-}$ compared to *PHLPP1*$^{+/+}$ mice (Fisher's PLSD $P < 0.005$) (Fig. 3*A*, *Lower*). These results indicate that PHLPP1 contributes to light-induced clock resetting. During the first several days, there was no phase difference between *PHLPP1*$^{+/+}$ and *PHLPP1*$^{-/-}$ mice, but *PHLPP1*$^{-/-}$ mice ultimately showed a larger phase shift than *PHLPP1*$^{+/+}$ mice after 21 days of consecutive recording due exclusively to the *tau* shortening during the late-phase days.

A large period change several days after resetting induced a drastic phase change in *PHLPP1*$^{-/-}$ mice (Fig. 2, *Middle Right*), even though the initial phase shift was equivalent in both genotypes (Fig. 3*A*, *Upper*). To address this further, we next estimated the *tau* length during early-phase days and late-phase days. The *tau* of *PHLPP1*$^{+/+}$ mice was extended by the phase delay (Fig. 3*B*, *Left*; $P < 0.05$ Welch's *t* test). However, no differences between the early-phase days and the late-phase days in both no-light and short-light groups were observed. The unchanged *tau* is reflected by the lack of change in phase delay (Fig. 3*A*).

In contrast, there was no *tau* extension induced by short-light exposure in *PHLPP1*$^{-/-}$ mice (Fig. 3*B*, *Right*). Furthermore, the *tau* shortened in the late-phase period (compare E and L, Fig. 3*B*, *Right*; $P < 0.01$ paired *t* test). This drastic and late *tau* change in *PHLPP1*$^{-/-}$ mice is reflected in the final phase delay (Fig. 3*A*).

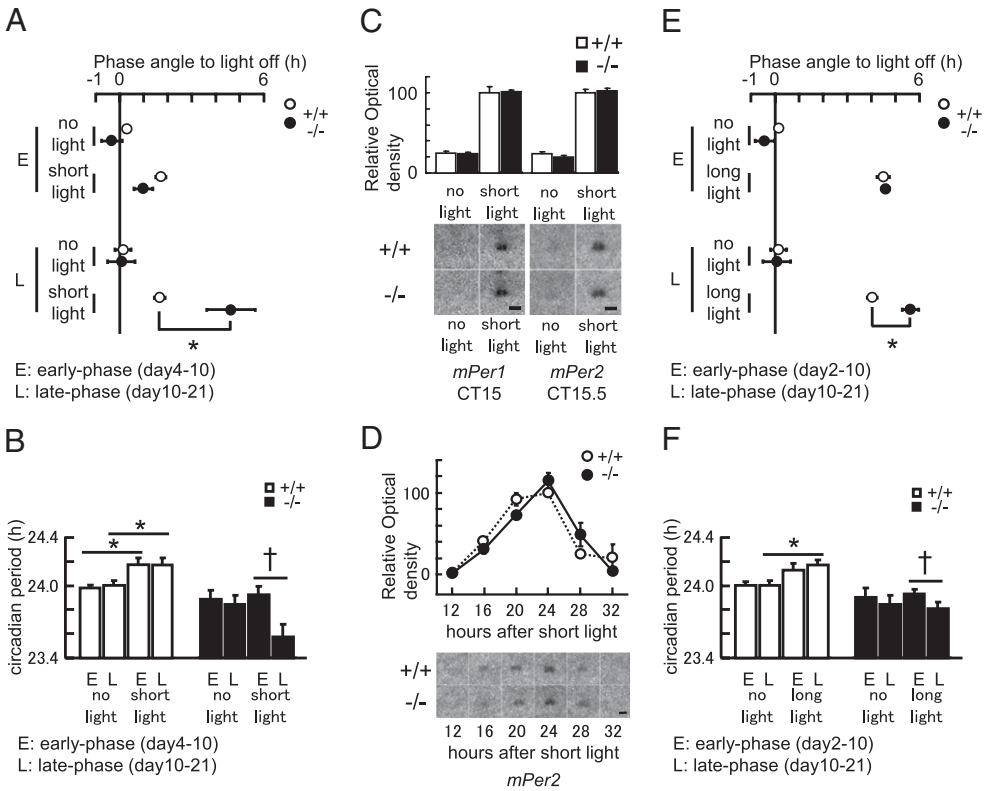

Fig. 3. Light task effects on the phase and circadian period of *PHLPP1*$^{-/-}$ mice. (*A*) Quantification of short-light task-induced changes in activity rhythm phases [no light; $^{+/+}$ (n = 6): $^{-/-}$ (n = 6), short light; $^{+/+}$ (n = 8]): $^{-/-}$ (n = 8)] from days 4 to 10 (E, early phase) and days 10 to 21 (L, late phase). Extrapolated activity onsets of early phase and late phase are indicated by open circles (*PHLPP1*$^{+/+}$) and filled circles (*PHLPP1*$^{-/-}$) (mean ± SEM). *P < 0.005 (Fisher's PLSD). (*B*) Circadian period during days 4–10 (E, early phase) and from days 10 to 21 (L, late phase) are indicated by open bars (*PHLPP1*$^{+/+}$) and filled bars (*PHLPP1*$^{-/-}$) (mean ± SEM). *P < 0.05 (Welch's t test), †P < 0.01 (paired t test). (*C*) Acute expression of m*Per1* and m*Per2* in the SCN by short-light tasks. *Upper*: quantified values of m*Per1* [$^{+/+}$ (n = 3), $^{-/-}$ (n = 3)] and m*Per2* [$^{+/+}$ (n = 4), $^{-/-}$ (n = 4)]. Short-light induced RNA levels of *PHLPP1*$^{+/+}$ are adjusted to 100. Normalized RNA levels are indicated by open bars (*PHLPP1*$^{+/+}$) and filled bars (*PHLPP1*$^{-/-}$) (mean ± SEM). After short-light exposure (CT14–CT14.5, 200 lx) mice were returned to DD and killed 30 min later (CT15) for m*Per1* and 60 min later (CT15.5) for m*Per2*. (*Lower*) representative films (Scale bar, 0.5 mm.) (*D*) m*Per2* expression rhythms in the SCN after short-light tasks. *Upper*: quantified values of m*Per2* [$^{+/+}$ (n = 3), $^{-/-}$ (n = 3)]. Peak level of RNA in *PHLPP1*$^{+/+}$ mice is adjusted to 100. Normalized RNA levels are indicated by open circles (*PHLPP1*$^{+/+}$) and filled circles (*PHLPP1*$^{-/-}$) (mean ± SEM). After short-light exposure (CT14–CT14.5, 200 lx) mice were returned to DD and killed 12–32 h later from the short-light onset. (*Lower*) representative films (Scale bar, 0.5 mm.) (*E*) Quantification of long-light task-induced changes in activity rhythm phases [no light; $^{+/+}$ (n = 6): $^{-/-}$ (n = 6), long light; $^{+/+}$ (n = 14): $^{-/-}$ (n = 14)] days 2–10 (E, early phase) and days 10–21 (L, late phase). Extrapolated activity onsets of the early phase and late phase are indicated by open circles (*PHLPP1*$^{+/+}$) and filled circles (*PHLPP1*$^{-/-}$) (mean ± SEM). *P < 0.0005 (Fisher's PLSD). (*F*) Circadian periods of activities days 2–10 (E, early phase) and from days 10 to 21 (L, late phase) are indicated by open bars (*PHLPP1*$^{+/+}$) and filled bars (*PHLPP1*$^{-/-}$) (mean ± SEM). *P < 0.05 (Welch's t test), †P < 0.005 (paired t test).

Quantified *tau* analysis revealed a twofold effect caused by the *PHLPP1* mutation. Finally, there is a loss of acute *tau* extension by phase delay lighting conditions but, there is a "late effect" revealed by a shortening of *tau* after several days in DD, which induced the large phase resetting in *PHLPP1*$^{-/-}$ mice.

Intact Response of the SCN to Resetting Light in *PHLPP1*$^{-/-}$ Mice. Due to the normal initial behavioral shifts of *PHLPP1*$^{-/-}$ mice, we analyzed light responsiveness of their SCN. The response to light of the SCN appeared to be normal in the mutant mice. Specifically, we analyzed the expression of the light-inducible clock genes, m*Per1* and m*Per2* (14, 15) and found the kinetics of induction and the final expression levels to be equivalent in wild-type and mutant mice. All mice were exposed to a 30-min light pulse (short light, ~200 lx, CT14–14.5). This treatment induced a robust induction of m*Per1* at CT15 in the SCN, 30 min following the light pulse. m*Per2* was also induced, peaking at CT15.5, 60 min after the light pulse (Fig. 3*C*).

Next, we examined the circadian m*Per2* expression in the SCN during the first cycle after resetting light. Mice were exposed to short light (~200 lx) at CT14–14.5 (30 min) and sampled every 4 h from 12 h to 32 h after the beginning of light pulse. Using this approach, m*Per2* expression was found to be lower at 12-h and 32-h time points and higher at 20-h and 24-h time points in both *PHLPP1*$^{+/+}$ and *PHLPP1*$^{-/-}$ mice. Although *PHLPP1*$^{-/-}$ mice showed a larger phase delay (~3 h), there was no phase difference between *PHLPP1*$^{+/+}$ and *PHLPP1*$^{-/-}$ animals (Fig. 3*D*; two-way ANOVA $P > 0.05$). The magnitude and profile of light-induced gene expression and subsequent gene expression rhythms in the SCN were equivalent in both genotypes.

Long-Light Treatment Induces Large Phase Delays in *PHLPP1*$^{-/-}$ Mice by Changing the Period Length. Because two distinct light tasks, short light (30 min) and long light (~8–12 h), play differential roles in clock resetting (16–18), we tested whether long-light tasks similarly affected the period length of *PHLPP1*$^{-/-}$ mice. Mice were exposed to 8 h of light (~200 lx) from the last day of LD 12-h cycle (ZT12 of day 1) (Fig. 2, *Lower*) (16). Our results show that several cycles after the resetting task, the circadian period again shortened spontaneously in the *PHLPP1*$^{-/-}$ mice similar to the *tau* shortening after the short-light task (Fig. 2, *Lower Right*). Two regression lines were made (early phase, day 2 to day 10, late phase, day 10 to day 21) and the values from early-phase and late-phase days were compared for each task (no light

and long light). Without light exposure (no-light group) there was no statistical difference in estimated phases (repeated measure ANOVA; $P > 0.05$) (Fig. S2*B*, *Upper*). In the long-light exposed group, only the *tau* from the late-phase days (days 10–21) of *PHLPP1*$^{-/-}$ mice behavior showed a larger phase angle to lights off (repeated measure ANOVA; $P < 0.005$) (Fig. S2*B*, *Lower*). This suggests that similarly to the short-light exposure, a delayed change in *tau* after long-light exposure produced the ultimate phase delay observed in *PHLPP1*$^{-/-}$ animals.

The *tau* values from the early-phase and late-phase days were subsequently examined. During the early-phase, there was no difference in the phase shift between *PHLPP1*$^{+/+}$ and *PHLPP1*$^{-/-}$ mice (two-way ANOVA; $P > 0.05$) (Fig. 3*E*, *Upper*), although long-light exposure delayed activity phase extensively. During the late-phase period, *PHLPP1*$^{-/-}$ mice showed a delayed phase compared to wild-type animals (two-way ANOVA, $P < 0.05$). The long-light task induced a more pronounced phase delay (~1.5 h) in *PHLPP*$^{-/-}$ mice when compared to their wild-type controls (Fisher's PLSD, $P < 0.005$) (Fig. 3*E*, *Lower*). These results indicate that PHLPP1 contributes not only to short light but also to long-light-induced clock resetting.

We next examined the effect of long-light exposure on *tau* (Fig. 3*F*). The *tau* of *PHLPP1*$^{+/+}$ mice was extended by long light significantly during the late phase (Fig. 3*F*, *Left*; $P < 0.05$ Welch's *t* test). There was no difference, however, between early-phase and late-phase *tau*, with or without long light in these animals (Fig. 3*F*, *Left*). In contrast, long-light exposure did not induce *tau* extension in *PHLPP1*$^{-/-}$ mice (Fig. 3*F*, *Right*), similar to the effect elicited by the short-light task. In addition, the *tau* of *PHLPP1*$^{-/-}$ mice in the long-light group shortened in the early-phase (compare E and L, Fig. 3*F*, *Right*; $P < 0.005$ paired *t* test). This late but drastic *tau* shortening is reflected in the final phase shift in *PHLPP1*$^{-/-}$ mice (Fig. 3*E*).

Finally, it is noteworthy that *tau* length in *PHLPP1*$^{-/-}$ mice was shorter after short-light exposure than after long-light exposure (repeated measure ANOVA, $P < 0.05$; Fisher's PLSD, $P < 0.05$) (Fig. S3). Interestingly, this indicates that the length of the light stimulus may have an impact on the molecular determinants of *tau* stability.

Impairment of Shortening of Circadian Period by Phase Advancing Light in *PHLPP1*$^{-/-}$ Mice. Although phase delaying light extends the circadian period, phase advancing light typically shortens the circadian period (19). We next examined the circadian period after phase advance in *PHLPP1*$^{-/-}$ mice. Mice were exposed to 4 h of light (~200 lx) at the last day of LD 12-h cycle (ZT20 to ZT24 of day 1) (Fig. S4*A*). In contrast to phase delaying tasks, there was no difference in phase advance behavior between *PHLPP1*$^{-/-}$ and *PHLPP1*$^{+/+}$ mice (Fig. S4*B*; $P > 0.05$ two-way ANOVA). Phase advancing tasks shorten *tau* in *PHLPP1*$^{+/+}$ animals both in early phase (day 2 to day 10) and late phase (day 10 to day 21) (Fig. S4*C*; $P < 0.01$ Welch's *t* test). Interestingly, similar to phase delay experiments (Figs. 2 and 3), *tau* was not affected in *PHLPP1*$^{-/-}$ mice (Fig. S4*C*; $P > 0.05$ Welch's *t* test). Differences in the circadian period between early phase and late phase was not observed in *PHLPP1*$^{-/-}$ mice (Fig. S4*C*; $P > 0.05$ Welch's *t* test).

Discussion

A comprehensive understanding of the molecular pathways by which light is able to induce clock resetting is still lacking. In this study we show that the Ser-Thr phosphatase PHLPP1 is critical for the control of clock resetting. Our results indicate that light-induced acute phase shift is unchanged in *PHLPP1*$^{-/-}$ mice, but that the *tau* length is drastically modified, a property that increases the final phase delay. This model shows that resetting light differentially regulates acute clock resetting, causing the subsequent period change (Fig. 4). Therefore, resetting light can

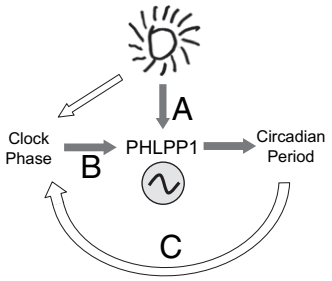

Fig. 4. Model depicting how PHLPP1 affects the circadian system. Light pulse-induced phase shift of circadian rhythms and period change. Light pulse directly (*A*) or via clock resetting (*B*) controls circadian period after the phase shift. Irregular and delayed circadian period change in *PHLPP1*$^{-/-}$ affects the magnitude of resetting (*C*).

directly (Fig. 4*A*) or indirectly (Fig. 4*B*) (via phase resetting) control *tau*. In this process, PHLPP1 appears to act as fine regulator of *tau* after resetting, ascribing to PHLPP1 an in vivo role unappreciated to date (Fig. 4). Importantly, PHLPP1 has been implicated in memory formation (12), via the attenuation of MAPK signaling. PHLPP1 also contributes to the duration and amplitude of responses of other kinases implicated in neuronal plasticity, including PKC and Akt (20, 21). It is tempting to speculate that the circadian phenotype of the PHLPP1-null mice described here could be parallel to the function of PHLPP1 in memory formation. This intriguing possibility is supported by the fact that PKC and MAPKs are important for circadian function (22, 23) and that PHLPP1 itself oscillates in the SCN (11). Our findings support a scenario in which PHLPP1 may operate in controlling the consolidation of circadian periodicity after resetting. Whereas this particular function may be conceptually different from a classical view of "memory," our results indicate that some common molecular pathways may contribute to the plasticity required for circadian *tau* maintenance.

Our study reveals that ablation of *PHLPP1* in the mouse has dual effects on *tau* length. One effect is indicated as an "acute effect," wherein there is absence of *tau* extension (shortening) after phase delay (advance)-inducing light exposure. Another effect is the "late effect," wherein there is a delayed shortening of *tau*, which subsequently is expressed as a large phase delay in *PHLPP1*$^{-/-}$ mice during the late-phase period after light exposure (Fig. 4*C*).

After light-induced phase shifting, *tau* length can change in subsequent days. This plasticity of *tau* has been referred to as the "after-effect" (19). Light pulses that cause a phase delay in the clock are typically followed by a lengthening of *tau* whereas light that triggers a phase advance subsequently shortens the *tau*. Congenital factors that define *tau* include species differences (19), different substrains within species (i.e., specific mouse strains) (24) and clock gene mutations (7). The determining factors responsible for after effects have remained elusive. In our studies, the lengths of *tau* that accompany phase delay (advance) shifts are longer (shorter) than when they are not accompanied by phase shifts in *PHLPP1*$^{+/+}$ mice (Fig. 3 *B* and *F* and Fig. S4). This response to light in *PHLPP1*$^{+/+}$ mice is compatible with canonical "after effects of phase shifts" that occur under free running conditions (19). Typically there is extension of *tau* by phase delay. Conversely, there is a shortening of *tau* by phase advance. The loss of acute *tau* extension and shortening in *PHLPP1*$^{-/-}$ mice (Fig. 3 *B* and *F* and Fig. S4) indicates a specific contribution of *PHLPP1* to the after effect. Lighting cycles that deviate from 24 h (T cycles) also induce after effects. Shorter (<24 h) and longer (>24 h) lighting cycles shorten and lengthen behavioral *tau*, respectively. Interestingly, these lighting tasks have reversed effects on the SCN. For example, SCN isolated from animals entrained to short lighting cycle shows long period

and vice versa (25, 26). Although the molecular basis for this discrepancy are still undetermined, *PHLPP1* appears to operate as an after effect controlling gene, and thereby is expected to play a central role in this complex phenomenon.

Interestingly, the late-phase *tau* shortening may indicate that the function of PHLPP1 is to stabilize the period after phase shift. Thus, the *PHLPP1*$^{-/-}$ mice show a very unique response which is demonstrated by a delayed change in *tau* after resetting. This phenotype is different (although maybe related to) the "delayed disappearance" of rhythmicity in *Clock* (27–29) and *Per2* (30) mutant mice that occurs after ~2 weeks in DD. Perhaps PHLPP1 may also regulate the phosphorylation state of some circadian regulators, including CLOCK and PER2, the phosphorylation of which has been shown to be critical for normal circadian function (31, 32). Furthermore, PKC and MAPK, (PHLPP1-regulated kinases) have been shown to be important for clock resetting (22, 23). Future studies will establish whether PHLPP1 physiologically dephosphorylates any components of the clock machinery. Interestingly, evidence of multiple targets of PHLPP1 as a phosphatase exists in vivo (8–10, 13). Involvement of these and/or other novel pathways requiring PHLPP1 function may clarify how PHLPP1 contributes to *tau* stability.

In the context of this study it is notable that short-light (30 min) and long-light (~8–12 h) treatments play different roles in clock resetting (18). For example, mice with mutations in the clock gene, *Per1*, or in the inhibitor of DNA-binding 2 (*Id2*) gene show phase delays that are larger than wild-type littermates after long-light tasks, an effect that is absent when animals are exposed to short light (16, 17, 33). In the present study, short light and long light had differential impact on the behavioral response in *PHLPP1*$^{-/-}$ mice. Interestingly, short light shortened *tau* more than long light in *PHLPP1*$^{-/-}$ mice (Fig. S3), a situation opposite to what is observed in the *Per1* or *Id2* mutant animals. Although the after effects were different in these mouse models, collectively, they demonstrate that the stimulus produced by short light versus long light has different effects on *tau* stability. That *PHLPP1* also plays a role in the differential response to short and long lights implicates it in SCN neuronal plasticity.

When activity rhythm is reset by a light pulse, changes in the SCN program of gene expression typically occur within the first hour or two after the light pulse (34). Experiments using in vitro real time monitoring systems have shown that activation of NMDA receptors induces this rapid SCN phase resetting as demonstrated by the change in circadian *Per1* promoter activity (35). However, what maintains *tau* length in free-running conditions? Although the molecular feedback loops of the circadian clock are necessary for *tau* maintenance in free-running conditions (1, 7), how these contribute to the plasticity required for such maintenance is yet unclear. Our data indicate that PHLPP1 may occupy a privileged position in the mechanism by which rhythmicity is stabilized in the SCN in the absence of zeitgeber cues. As PHLPP1 has already been implicated in neuronal plasticity (12), the data presented here ushers in a unique perspective on the role of this phosphatase in long-lasting plasticity in the circadian system. Its role in *tau* maintenance indicates that it may be central to the fine tuning necessary in the central pacemaker for long-term synaptic plasticity, especially in response to changing stimuli.

Materials and Methods

Gene Targeting and Generation of Homozygous Null Mice. The targeting exon (exon 4) of PHLPP1 was subcloned in between two LoxP sites on the pFlox-FRT vector. A SacI restriction site was introduced into the targeting vector for detection of homologous recombination events by Southern blot analysis. For negative selection, sequences encoding diphtheria toxin (DT) were amplified using PCR and subcloned into the vector. The neomycin selection cassette (*neo*^{r}) is flanked by two FRT sites, which allow deletion of the *neo*^{r} gene via Flp-mediated recombination in mice. The targeting vector was electroporated into 129/Sv ES cells and the cells were subjected to positive and negative selection on the basis of neomycin and DT sensitivity (Fig. 1*A*). Genomic DNA was isolated from the ES cells and digested with SacI, and relevant products were detected by Southern blot using the probe as marked on the diagram. The wild-type allele generates an 11-kb fragment whereas the targeted knockout allele gives a 7-kb fragment. ES cells with a recombinant allele were injected into C57BL/6 blastocysts and transplanted into pseudopregnant C57BL/6 mice to generate chimeric pups. PHLPP1 fl/+ chimeras were bred with Protamine-Cre mice (129 background; 129-Tg(Prm-cre)58Og/J; The Jackson Laboratory) to generate fl/+, Protamine-Cre/+ males (Fig. 1*B*). Breeding of these mice to wild-type mice resulted in recombination of the LoxP sites and deletion of exon 4 in the male gametes, yielding PHLPP1$^{+/-}$ mice. Genotyping PCR was performed using the following primers: FP43 (forward primer): 5′-TAG GAG AGA CTA GTG ACA TC-3′, RP44 (reverse primer 1): 5′-TGA GCT TAT ACG CTG TGA TGC-3′, and RP56 (reverse primer 2): 5′-AGC CGA TTG TCT GTT GTG C-3′ (Fig. 1*A*). Primer pair FP43/RP44 generates a 264-bp product from the wild-type allele and a 336-bp product from the floxed allele. Primer pair FP43/RP56 generates a 486-bp product from the deleted allele (Fig. 1*C*). RNA from mouse brain samples was isolated using a Qiagen RNEasy lipid tissue kit, and RT-PCR was performed using a Qiagen OneStep RT-PCR kit and the following primers: (forward) 5′-TCT GTC GAA ATG GGA AGC CAC TGT C-3′ and (reverse) 5′-TGT ACC ACC ACA GCA CTG ATG C-3′. Protein extracts from *PHLPP1*$^{+/+}$ and *PHLPP1*$^{-/-}$ brains, livers, and kidneys were subjected to Western blot analysis using the PHLPP antibodies from Bethyl Laboratories (PHLPP1; cat. no. A300-659A, PHLPP2; cat. no. A300-661A) and the β-actin antibody from Sigma (cat. no. A2228).

Animals and Behavioral Rhythm Monitoring. Animal protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the the University of California, Irvine and University of California, San Diego.

PHLPP1$^{+/+}$ and *PHLPP1*$^{-/-}$ mice were bred and housed under 12-h light (fluorescent, Sylvania 25 w no. 4100K, ~200 lx) /dark (LD) cycles. Locomotor activity was detected using running wheels (33) and passive (pyroelectric) infrared sensors (PU-2201; EK Japan). Running wheels and passive infrared sensors were used for *tau* analysis and passive infrared sensors were used for resetting analysis. Locomotion data were collected using the VitalView data acquisition system (Mini-Mitter) using a sampling interval of 5 min. Actograms were acquired using Actiview Biological Rhythm Analysis software (Mini-Mitter). Circadian period and phase shift of the activity rhythms were analyzed by Clocklab software (Actimetrics).

In Situ Hybridization. In situ hybridization histochemistry using free-floating sections was performed according to methods detailed previously (14). We used ^{33}P-radiolabeled cRNA probes for m*Per1* and m*Per2* for the in situ hybridization studies. *mPer1* (75-411 NM_011065) fragment was obtained by RT-PCR and subcloned into pCR-BluntII-TOPO vector (Invitrogen). m*Per1* and m*Per2* (33) cDNA containing vectors were linearized with restriction enzymes and used as templates for standard ^{33}P-labeled cRNA synthesis (14).

Statistical Analysis. The effects of genotypes, lighting tasks, and number of days after the light task on the behavioral rhythms were tested by two-way or repeated measure ANOVA. A post hoc Fisher test was used for the comparison between the values. The effects of light tasks to *tau* were tested by Welch's or paired *t* test.

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