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Effects of the *Duper* Mutation on Circadian Responses to Light

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Abstract The circadian mutation *duper* in Syrian hamsters shortens the free-running circadian period (τ_{DD}) by 2 hours when expressed on a *tau* mutant (τ_{ss}) background and by 1 hour on a wild-type background. We have examined the effects of this mutation on phase response curves and entrainment. In contrast to wild types, duper hamsters entrained to 14L:10D with a positive phase angle. Super duper hamsters (expressing *duper* on a τ_{ss} background) showed weak entrainment, while τ_{ss} animals either completely failed to entrain or showed sporadic entrainment with episodes of relative coordination. As previously reported, wild-type and τ_{ss} hamsters show low amplitude resetting in response to 15-minute light pulses after short-term (10 days) exposure to DD. In contrast, super duper hamsters show high amplitude resetting. This effect is attributable to the *duper* allele, as hamsters carrying *duper* on a wild-type background also show large phase shifts. Duper mutants that were born and raised in DD also showed high amplitude resetting in response to 15-minute light pulses, indicating that the effect of the mutation on PRC amplitude is not an aftereffect of entrainment to 14L:10D. Hamsters that are heterozygous for *duper* do not show amplified resetting curves, indicating that for this property, as for determination of free-running period, the mutant allele is recessive. In a modified Aschoff type II protocol, super duper and duper hamsters show large phase shifts as soon as the second day of DD. Despite the amplification of the PRC in super duper hamsters, the induction of *Period1* gene expression in the SCN by light is no greater in these mutants than in wild-type animals. *Period2* expression in the SCN did not differ between super duper and wild-type hamsters exposed to light at CT15, but albumin site D-binding protein (*Dbp*) mRNA showed higher basal levels and greater light induction in the SCN of super duper compared to wild-type animals. These results indicate that the *duper* mutation alters the amplitude of the circadian oscillator and further distinguish it from the *tau* mutation.

Key words circadian, phase response curve, tau mutation, hamster, Period gene, Dbp

The *tau* (τ_{ss}, or "super short") mutation in Syrian hamsters shortens the free-running circadian period in constant darkness (τ_{DD}) to 20 hours. Homozygous *tau* mutant hamsters generally fail to entrain to light cycles presented with a period (T) of 24 hours (Ralph and Menaker, 1988). These animals carry 2 copies of an allele of *casein kinase 1*ε that increases the phosphorylation rate of PER2, resulting in a shortening of its

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nuclear residence time (Lowrey et al., 2000; Dey et al., 2005; Meng et al., 2008). The phase response curve (PRC) to light pulses is altered by the *tau* mutation, but the effects are subtle, and the specific changes reported by different researchers vary. Grosse et al. (1995) found an increase only in the amplitude of phase advances during the late subjective night in *tau* mutants presented with 15-minute light pulses after maintenance for 7 to 10 days in constant dim red light. Shimomura and Menaker (1994) found no change in the PRC after 7 days in DD but observed a striking amplification of both advances and delays after 7 weeks. Gradual amplification of the PRC is observed in several species upon protracted exposure to DD, however (Refinetti, 2007). Thus, the effect of the *tau* mutation on PRC amplitude may be to accelerate or exaggerate a process that occurs in wild types.

The newly discovered *duper* mutation arose on the *tau* background (Monecke et al., 2011). These super duper hamsters experience a 2-hour shortening of τ_{DD} but show no changes in the coding sequence of either CK1ε or CK1δ. Through backcrosses to wild-type hamsters, we isolated the *duper* mutation on the wild-type background and found that it shortens τ_{DD} by 0.9 hours (Monecke et al., 2011). Hamsters that are heterozygous for *duper* exhibited no shortening of τ_{DD}, indicating that the mutant allele is recessive for this trait.

In the present experiments, we explored the impact of the *duper* mutation on the entrained phase and the PRC. Phase shifts induced by light and other cues are believed to result from transcriptional activation of core clock genes (particularly *Per1*) in the suprachiasmatic nucleus of the hypothalamus (SCN), which constitutes a master circadian pacemaker (Akiyama et al., 1999; Wakamatsu et al., 2001; Yamamoto et al., 2001; Yan and Silver, 2004). We compared light induction of clock gene expression in the SCN of wild-type and super duper hamsters. Our results dissociate changes in τ_{DD} from alterations in the PRC and indicate that amplification of phase shifts in *duper* does not arise from increased induction of *Period1* gene expression by light.

MATERIALS AND METHODS

The provenance and conditions of maintenance of hamsters were identical to those described by Monecke et al. (2011). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts at Amherst and conform to all US federal animal welfare requirements.

Construction of Phase Response Curves

The free-running period of wheel-running rhythms before and after light pulses was calculated by linear regression using ClockLab software (Actimetrics, Evanston, IL) and evaluated by χ^2 periodogram analysis when appropriate. ClockLab (Actimetrics) emulates the traditional actogram, representing time of day on the abscissa and day of the record on the ordinate. The slope of the regression fitted through activity onsets is the reciprocal of the period, which is calculated by the program. Data reported here reflect the mean and standard error of these period estimates among individuals. Calculations of the period using the acrophase, rather than the onset, of activity as a phase reference point provided similar values and standard errors and correspond to center of gravity estimates used by others (Daan and Oklejewicz, 2003).

A standard Aschoff type I protocol (Aschoff, 1965) was used to construct a PRC. Phase shifts were induced by removing the cage containing the animal from the dark room on the 10th or 11th day of DD to an adjacent room and exposing it to white fluorescent light (intensity of 4 μeinstein/m^2/sec) for 15 minutes. Animals were then returned to DD, and phase was assessed from linear regression fit to activity onset over at least 10 cycles, omitting any transient cycles observed immediately after the day of light presentation. Light intensity thresholds and effects of the *duper* mutation on parametric effects of light will be reported elsewhere.

In order to determine whether the rearing of mutant hamsters in a 14L:10D photoperiod affected the amplitude of phase shifts, 7 hamsters that had been born and raised in DD were exposed to successive 15-minute light pulses at intervals of several weeks beginning at the age of 8 weeks. Each light exposure was followed by at least 2 weeks of DD.

In order to assess the phase angle of entrainment, and to determine whether differences between duper or super duper hamsters and other strains reflect an effect of this mutation on the rate at which the PRC changes upon transfer to constant darkness, we conducted an additional experiment using a modified Aschoff type II protocol. Fifty-eight hamsters (25 duper, 12 super short, 6 super duper, and 15 wild types) that had been born and raised in 14L:10D were placed in running wheels in DD in order to assess τ_{DD}. Activity of these animals was next monitored in 14L:10D for at least 12 days in order to assess entrainment and calculate phase angle, where appropriate. In order to evaluate the contribution of masking to the phase of activity onset, these animals were then transferred to

DD late in the dark phase of the 14L:10D cycle. Recording of their free-running locomotor activity in DD for the next 10 days also served to obtain a baseline for subsequent phases of this experiment, in which light was presented at specific phases of the free run. The hamsters were next returned to 14L:10D for at least 15 days. Afterward, they were returned to DD and given a 15-minute light pulse targeted for the early to mid-subjective night on the second free-running cycle. Ten days later, these hamsters were transferred to 14L:10D, and entrainment was assessed once again for 3 weeks. Finally, the hamsters were returned to DD and given another light pulse, targeted at the middle to late subjective night on the second free-running cycle. Phase of activity onset was assessed over the next 10 days of DD.

Effects of Light on Gene Expression in the SCN

The PRCs obtained for wild-type and super duper hamsters differed greatly in amplitude. In order to determine whether this difference was associated with an effect of the mutation on the induction by light of transcription of core clock or clock-controlled genes in the SCN, wild-type and *super duper* hamsters that had been maintained in 14L:10D for a minimum of 3 weeks after initial assessment of τ_{DD} were returned to DD for 10 days. Experimental hamsters were exposed to light for 15 minutes at CT15 using the same procedure as described above for the generation of PRCs. Hamsters were then returned to darkness for 30 minutes or 2 hours. Control animals received a sham light pulse consisting of movement of the cage without exposure to light. Hamsters were rapidly decapitated, and brains were immediately frozen on dry ice. They were stored at –80 °C until sectioning at 20 µm, followed by in situ hybridization for *per1*, *per2*, or *dbp*. In situ hybridization histochemistry was performed as previously described (Tong et al., 2004; Guo et al., 2006). Paraformaldehyde-fixed, deaminated, dehydrated, and delipidated sections were hybridized using [^{35}S]-UTP–labeled antisense and sense cRNA probes. For *haPer1*, AF249882 nt 215 to 1336, homologous to AF02292 nt 337 to 1120, approximately 758 bp in pBluescript II vector (Stratagene, La Jolla, CA), was cut with *ApaI* or *BstXI* to generate antisense or sense cRNA, respectively (Yamamoto et al., 2001). Template for *haPer2* (homologous to AF035830(m) nt 841-1620, 780 bp) was cut with *BstXI* or *ApaI* to generate antisense or sense probes, respectively (Hamada et al., 2001). Klenow fragment was used to blunt 3′ overhangs of both *haPer1* and *haPer2* templates. In order to generate a homologous probe for *dbp*, total RNA was isolated from Syrian hamster hypothalamus and reverse transcribed using oligo dT and the Ambion Retroscript kit (Austin, TX). Specific *dbp* primers were used to clone a 375-bp template that was verified by sequencing as homologous to nt 867 to 1152 of *Mus musculus dbp* (NM_016974.3) and nt 847 to 1152 of *Rattus norvegicus dbp* (NM_012543.2). It was linearized with *Spe1* and *NcoI* in order to transcribe antisense and sense probes, respectively.

Sections were hybridized with antisense or sense probes (4×10^4 cpm probe/µL hybridization buffer) overnight at 57 °C, as previously described (Tong et al., 2004). After posthybridization rinses and RNase A digestion, sections were exposed to Kodak Biomax MR film (Rochester, NY). Background was subtracted, images were calibrated using standards, and density over the rostral, middle, and caudal SCN was quantified using a Dage MTI72 CCD camera (Michigan City, IN) and National Institutes of Health (NIH) Image software (Bethesda, MD). Peak hybridization intensity was calculated and used for statistical comparisons between groups.

Statistical Evaluations

SuperAnova (Abacus Concepts, Berkeley, CA) was used to evaluate effects of genotype and time and their interaction on gene expression patterns. JMP statistical software (SAS Institute, Cary, NC) was used to evaluate entrainment patterns, with the Dunnett test to compare mutant groups with the wild types when overall effects were found by ANOVA.

RESULTS

Entrainment to 14L:10D

As expected, the onset of wheel running in wild-type hamsters was approximately coincident with the onset of darkness (Table 1; Fig. 1A). The phase extrapolated from the free run in DD was about 40 minutes earlier than the time of activity onset during entrainment, indicating negative masking. Although duper hamsters also adopted a 24-hour period in 14L:10D, wheel running preceded lights-off by 2.42 ± 0.23 hours ($p < 0.005$ v. wild type) (Table 1; Fig. 1B). The phase of the oscillation based on extrapolation of running onset during the DD free run was about 1 hour later than activity onset in 14L:10D. χ^2 periodograms showed that the period of both wild-type and duper hamsters was almost exactly 24 hours during exposure to 14L:10D,

Table 1. Assessment of phase, period, and amplitude of circadian rhythms in wild-type and mutant hamsters exposed to light:dark cycles and constant darkness.

	Wild Type	Duper	Super Duper	τ_{ss}
Time of onset relative to lights-off, h	–0.09 ± 0.23	2.42 ± 0.23	2.49 ± 0.70	–2.85 ± 3.22[b]
Period during 14L:10D,[a] h	24.02 ± 0.09	24.04 ± 0.05	24.14 ± 0.08	21.99 ± 0.91[f]
Amplitude in 14L:10D[a]	783 ± 111	855 ± 64	397 ± 43[e]	285 ± 14[e]
Phase difference (14L:10D v. DD), h	–0.71 ± 0.32	0.95 ± 0.45	–10.05 ± 3.05	2.72 ± 2.67
Period in DD,[a] h	24.00 ± 0.11	23.05 ± 0.10[c]	17.75 ± 0.13[c]	19.83 ± 0.10[c]
Amplitude in DD[a]	666 ± 56	947 ± 71[d]	333 ± 44[d]	541 ± 66

Data are presented as mean ± SEM; $n = 6$ to 9 hamsters per group.
[a]Calculated by χ^2 periodogram.
[b]Includes only τ_{ss} hamsters with χ^2 periodogram peaks near 24 hours.
[c]$p < 0.0001$ versus wild type; Dunnett test. [d]$p \leq 0.01$ versus wild type. [e]$p \leq 0.002$ versus wild type. [f]$p = 0.03$ versus wild type.

and the peak was of high amplitude and statistically significant in all cases.

In contrast, the pattern of activity onsets in super duper hamsters maintained in 14L:10D was variable both within and between animals. These hamsters typically showed activity bouts during the middle to late portion of the light phase on several successive cycles, with more robust running at or shortly after the time of lights-off, suggestive of masking. Activity onset in super duper hamsters led the light:dark transition by several hours (Table 1; Fig. 1C; Suppl. Fig. S1A-F). Super duper hamsters occasionally broke entrainment or showed relative coordination. Although χ^2 periodogram analysis also indicated a principal and statistically significant peak near 24 hours in most super duper hamsters, the amplitude was significantly lower than in wild-type and duper animals ($p = 0.002$). The phase of running onset of super duper hamsters inferred from the free run did not correspond as closely or consistently to the time of activity onset in 14L:10D as in wild-type or duper animals. As previously reported (Ralph and Menaker, 1988), τ_{ss} hamsters failed to entrain to 14L:10D, but free-running activity bouts that fell during the light phase were often less robust than those during the night as a result of masking (Fig. 1D). χ^2 periodograms indicated that the period of many *tau* mutant hamsters maintained in 14L:10D did not match 24 hours, was variable between individuals, and did not differ significantly from the subsequent τ_{DD} ($T = 0.334$, $p > 0.1$) (Table 1).

Aschoff Type I Phase Response Curves

As previously reported, 15-minute light pulses presented during the subjective night induced phase shifts of less than 3 hours in wild-type hamsters (Fig. 2A and 2B; Fig. 3A). Also in agreement with earlier studies (Shimomura et al., 1998; Grosse et al., 1995), τ_{ss} hamsters showed only a small amplification of the PRC (Fig. 3B). In contrast, presentation of 15-minute light pulses to super duper hamsters during the subjective night of days 10 to 11 of DD provoked large phase shifts (Fig. 2C and 2D). The phase response curve of super duper mutants is clearly type 0, with a crossover around CT16 and shifts of as much as 12 circadian hours (Fig. 3C).

The amplified PRC of super duper hamsters was associated with the shortening of τ_{DD} to 18 hours. However, high amplitude resetting might be attributable to the *duper* allele and unrelated to the extremely short τ_{DD}. In order to examine this, we constructed a PRC in hamsters expressing *duper* on the wild-type background, whose free-running period is approximately 23 hours (Monecke et al., 2011). Like super duper hamsters, duper animals showed a high amplitude resetting curve (Fig. 2E and 2F; Fig. 3D), except that phase advances were generally smaller than delays.

We wished to examine the effect of prior exposure to 14L:10D on the PRC of duper hamsters. Thus, we constructed a PRC for duper mutants born and raised in DD. Up to six 15-minute light pulses were presented at 2- to 3-week intervals to 7 such individuals. The resulting curve was of high amplitude and was similar to that compiled by giving single pulses on days 10 to 11 of DD to duper mutants that had been born in 14L:10D (Fig. 3E). The only difference between the responses of duper mutants that had been dark reared and those that were raised in 14L:10D was that there was less evidence of asymmetry in the former group: the delay and advance portions of the curve were approximately equal.

In previous work (Monecke et al., 2011), we found that hamsters carrying one copy of *duper* on a wild-type background showed a τ_{DD} similar to that of the wild type. Consistent with this, the PRC of *duper* heterozygotes (Fig. 3F) lacked the high amplitude phase shifts typical of *duper* homozygotes.

As previously reported, wild-type hamsters completed phase delays within one cycle, while advancing shifts occur with transient cycles (Fig. 2A and 2B). Duper and super duper hamsters exposed to light during the late subjective night also advanced phase to reach steady state after several transient cycles. The

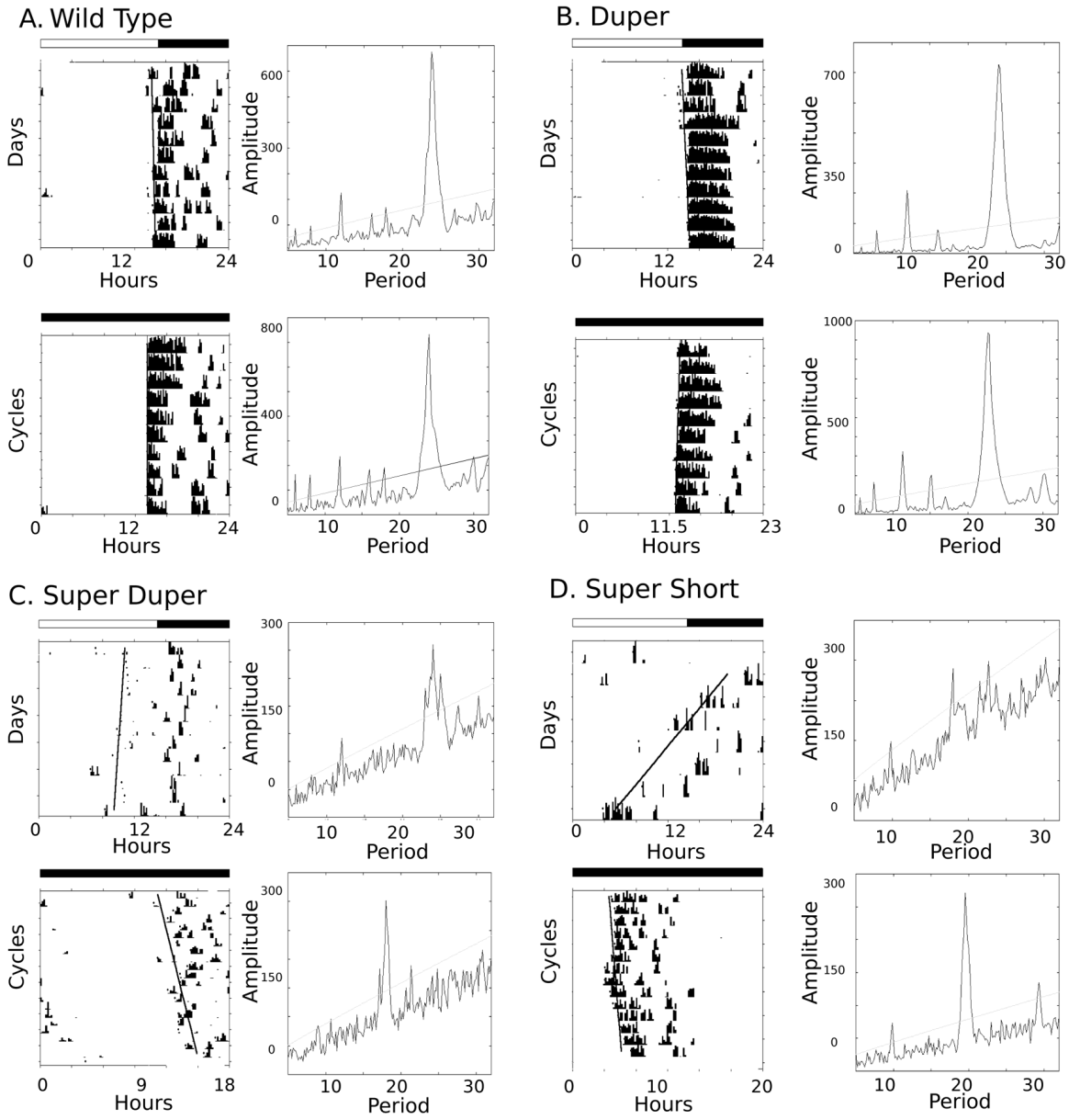

Figure 1. Assessment of entrainment of locomotor activity rhythms to 14L:10D in representative (A) wild-type, (B) duper, (C) super duper, and (D) τ_{ss} hamsters. Records in 14L:10D (top left of each panel; white and black bars at top of record indicate light and dark phases) are plotted modulo 24 hours. The record in DD (lower left of each panel) is continuous with the actogram of activity in 14L:10D to permit assessment of effects of the light cycle on the phase of free-running activity onset, but activity in DD is plotted modulo τ_{DD} using period calculated by χ^2 periodogram analysis. Regression lines fit to activity onsets in 14L:10D and DD are indicated. The χ^2 periodograms for activity during light:dark and DD exposure are shown to the right of the actograms in both 14L:10D and free-running conditions. Wild-type and duper animals invariably entrained, but the phase angle differed between these groups: activity onset approximately coincided with lights-off in wild types and anticipated light offset by over 2 hours in the *duper* mutants (see Table 1). Note that the phase of activity in the ensuing DD is close to the time of onset in the preceding 14L:10D in A and B. Super duper animals often showed transient entrainment; activity is negatively masked by light, but the principal significant component of the χ^2 periodogram is close to 24 hours, and the phase of the ensuing free run is consistently related to positive phase angle of activity onset in the preceding 14L:10D cycle. Additional examples are shown in Supplementary Figure S1. *Tau* mutants generally failed to entrain to 14L:10D; unlike the other genotypes, periodograms of τ_{ss} animals failed to show a single statistically significant 24-hour component, and phase angle of the free run in DD was not consistently related to time of lights-off in the preceding 14L:10D condition.

high amplitude phase delays of super duper and duper hamsters afforded the opportunity to assess whether the presence or absence of transients in the approach to the steady state might be a function of the numbers of hours shifted. Phase delays as large as 10 hours were accomplished by duper mutants without evidence of

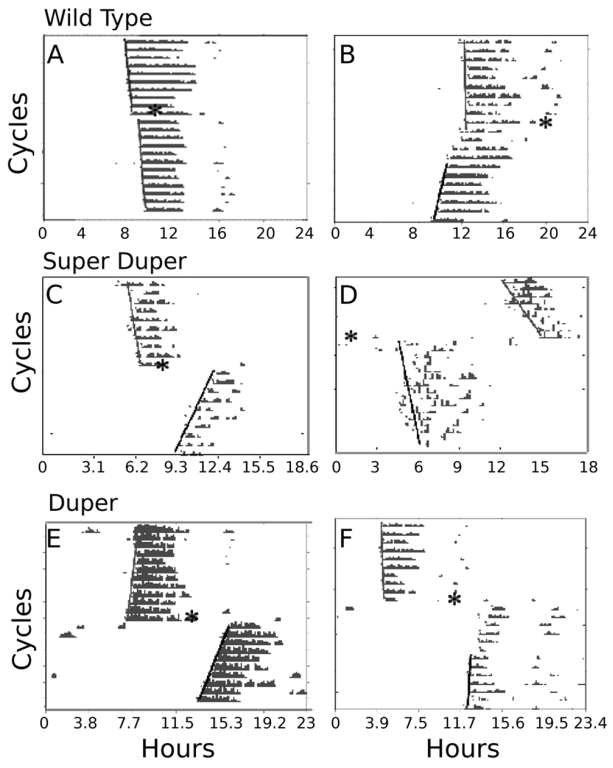

Figure 2. Actograms depicting locomotor activity in representative hamsters induced to shift circadian phase by presentation of 15-minute light pulses (indicated by the asterisk) on days 10 to 11 of DD. (A, B) Activity records of wild-type hamsters showing phase delay or advance after light pulse in the early or late subjective night, respectively. Note that the phase shifted by less than 3 hours and that transients occurred only in the course of phase advances. (C, D) Activity records of 2 representative super duper hamsters, plotted modulo τ_{DD} (approximately 18 hours) to facilitate visual inspection. These hamsters were exposed to light in the early to midsubjective night and exhibited large phase delays. (E, F) Activity records of 2 duper hamsters exposed to light in the midsubjective night. Note that the *duper* allele is sufficient to produce high amplitude phase shifts, even on the wild-type genetic background. Note that the hamster shown in E experienced a delay of 9.3 circadian hours with no evidence of transients. The hamster shown in F showed several transient cycles, initially overshooting the steady state phase before stabilizing with a delay of about 10.5 circadian hours.

transients (Fig. 2E). Phase advances, and delays in excess of 10 hours, often included transient cycles before the phase stabilized (Fig. 2F).

Phase shifts triggered by light pulses were associated with small and inconsistent changes in τ_{DD}. The *tau* response curves (Beersma et al., 1999; Daan, 2000) for wild-type, τ_{ss}, super duper, duper, and duper heterozygote hamsters are plotted in Figure 4. τ_{DD} rarely changed by more than 30 minutes in hamsters of any strain following delivery of a light pulse to elicit a phase shift. With the exception of the super duper hamsters, the average change of τ_{DD} was greatest in the late subjective night. There was no apparent difference between strains in the *tau* response and only a weak positive correlation between the phase shift (negative or positive) and the change in τ_{DD}. Phase delays were not associated with reductions in free-running period.

Aschoff Type II PRC

Protracted exposure to DD increases the amplitude of the PRC in several rodent species (Refinetti, 2007), and this effect is greater in *tau* mutant than in wild-type hamsters (Shimomura and Menaker, 1994). Thus, the effect of the *duper* mutation may be merely to accelerate amplification of the PRC. In order to determine whether strong resetting characteristics of duper hamsters appear quickly after transfer to DD, we performed a modified type II protocol (Aschoff, 1965). To minimize difficulties in assessment of the phase of the light pulse presented by differential masking (Table 1; Fig. 1), hamsters were exposed to light for 15 minutes on the second free-running cycle after transfer to DD.

Duper hamsters showed markedly greater phase shifts in response to light than did wild types (Fig. 5). Thus, the results of the Aschoff type I and type II experiments were comparable, suggesting that the results of the type I procedure (Fig. 2) do not reflect an acceleration in duper mutants of a process whereby protracted DD exposure amplifies the PRC of τ_{ss} hamsters.

Light-Induced Gene Expression in the SCN

In order to determine whether the dramatic differences between super duper and wild-type hamsters in phase resetting arise from increases in initial molecular responses of the pacemaker to light pulses, we performed in situ hybridization to examine induction of *Per1*, *Per2*, and *Dbp* expression in the SCN of animals exposed to 15-minute pulses at CT15. Control animals were subjected to cage movement, but no light pulse was presented. In contrast to the behavioral responses, induction of *Per1* mRNA in the SCN was equivalent in super duper and wild-type hamsters at intervals of 30 minutes and 2 hours after the light pulse (Fig. 6A). There was a main effect of treatment ($p = 0.001$) but no influence of genotype or genotype × treatment interaction. *Per2* was not significantly affected by light pulses in either the wild-type or the super duper hamsters at either survival time (Fig. 6B). In contrast, basal expression of *dbp* at CT15 was higher in the SCN of super duper than wild-type hamsters. There was a significant effect of genotype ($p < 0.0001$) and a genotype × treatment interaction ($p = 0.02$) on levels of *dbp* mRNA

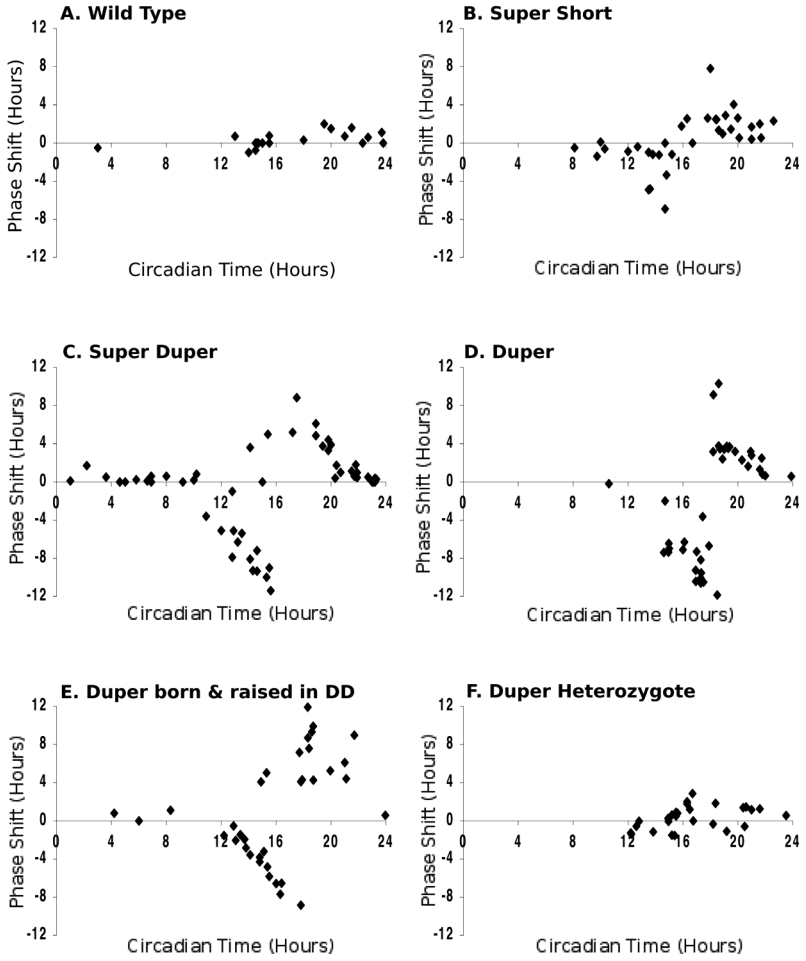

Figure 3. PRC of (A) wt, (B) τ_{ss}, (C) super duper, and (D) duper hamsters exposed to a single 15-minute light pulse at the indicated circadian time on days 10 to 11 of DD. All hamsters had been maintained in 14L:10D for at least 3 weeks prior to transfer to constant darkness. (E) PRC of 7 hamsters that were born to mothers moved to DD on day 12 of gestation. These animals were placed in running wheel cages at 8 weeks of age and presented with 15-minute light pulses on up to 6 occasions, each separated by at least 2 weeks. (F) PRC of hamsters that were heterozygous for the *duper* mutation on the wild-type background.

(Fig. 6C). Light pulses elicited a significant and sustained rise in *Dbp* expression in the SCN of super duper hamsters ($p < 0.002$ at 2 hours) but had no significant effect on *dbp* mRNA levels in wild-type animals.

DISCUSSION

Whether expressed on a *tau* mutant or a wild-type background, the *duper* mutation produces a marked increase in PRC amplitude. The effects of this newly discovered mutation on light responses differ sharply from those of the more thoroughly studied *tau* mutation and presumably reflect the difference between the gain of function of CK1ε and the effects of the new mutation on the as yet unknown molecular function of *duper*. Although the *duper* mutation affects circadian period, its impact on entrainment and phase resetting cannot be attributed to its effect on τ_{DD} or a change in the action of light to induce *Per1* expression in the SCN. While its function remains to be established, *duper* regulates basal and light-regulated expression of the clock-controlled gene, *Dbp*, in the circadian pacemaker.

Type 0 PRCs have rarely been reported in mammals. Circadian period mutants and clock gene knockouts provide exceptions to this generalization. *Clock* mutant mice show high amplitude resetting, provided that 6-hour light pulses are presented around CT18 (Vitaterna et al., 2006). Jud et al. (2010) found that mice deficient in both *Per1* and *Rev-erbα* experience a shortened free-running period and an amplified PRC. The similarity between these double knockout mice (Jud et al., 2010) and duper hamsters suggests that a single base change at a locus yet to be identified may replicate the effects of knocking out 2 major clock genes. Grosse et al. (1995) found that τ_{ss} hamsters show low amplitude resetting in the early subjective night but mean shifts of up to 5 hours at CT16 to CT18. Most of their *tau* mutants showed 3-hour phase advances in response to light presented between CT16 and CT22, but 2 animals experienced phase advances of 10 to 11 hours. These animals had been maintained on T20 cycles prior to construction of the PRC. Shimomura and Menaker (1994) found that *tau* mutant hamsters given 1-hour light pulses after 7 free-running cycles in DD showed small phase shifts comparable to wild types. After 49 cycles, however, they exhibited high amplitude resetting. The increase in the amplitude of phase delays among *tau* mutants in response to 1-hour light pulses given at CT14 began after 14 cycles in DD and accelerated markedly after 35 cycles. Phase advances at CT18 changed little over 49 cycles in DD and remained similar to those in wild-type hamsters. Our data on τ_{ss} hamsters are in agreement with these observations. Minor discrepancies with earlier studies

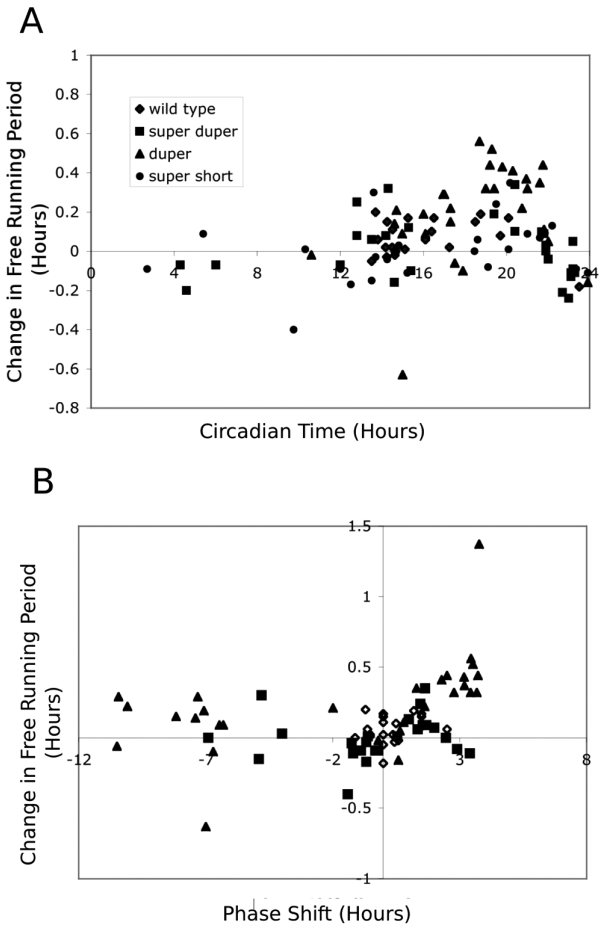

Figure 4. (A) *Tau* response curves of individual wild-type (diamonds), *super duper* (squares), *duper* (triangles), and τ_{ss} (circles) hamsters induced to shift phase by presentation of a 15-minute light pulse on days 10 to 11 of DD at the phases indicated. (B) Relationship between phase shifts and change of τ_{DD} in the same animals.

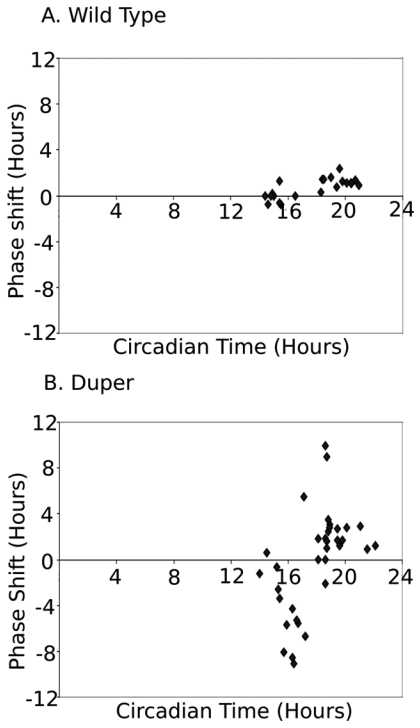

Figure 5. Aschoff type II PRC in (A) wild-type and (B) duper hamsters. Animals were exposed to 15-minute light pulses on the second day after transfer from 14L:10D to DD. Phase shifts were comparable to those elicited by light pulses given on days 10 to 11 of DD (Fig. 2), demonstrating that strong resetting is apparent in duper hamsters within 2 days of transfer to constant darkness.

may be related to the T cycle to which animals were exposed prior to transfer to DD, to the use of dim red light, or to the length of the light pulse.

The effects of the *duper* mutation on phase resetting differ qualitatively from those of the *tau* mutation. Not only are the shifts much larger (Fig. 2), but strong resetting is also apparent within 2 days of transfer of *duper* mutants to DD (Fig. 5). Refinetti (2007) reported a gradual increase in PRC amplitude in 4 rodent species as duration of DD exposure increased, but in no instance were type 0 curves produced. While it is possible that PRC amplitude increases at different rates in *duper* and *tau* mutant hamsters, reflecting a process that happens even in wild types, the present results favor an alternative interpretation. The *duper* mutation confers a marked increase in the phase shifting effects of light above that in τ_{ss} or wild-type hamsters, independent of effects of DD exposure. The relationship of this effect to the change in free-running period and circadian organization is unknown.

The differences between strains in their activity pattern in 14L:10D (Fig. 1) may be interpreted with reference to their PRCs. χ^2 periodograms indicate that unlike *tau* mutant hamsters, super duper animals consistently adopt a locomotor activity period close to 24 hours when exposed to T24. Although this could be attributable to increased masking in the super duper mutants, their behavior in a 3.5L:3.5D cycle does not support a greater responsiveness to masking effects of light (Bittman, unpublished data). Furthermore, the phase of their free run in DD, while variable, is more consistently related to their activity onset in the preceding 14L:10D cycle than is that of

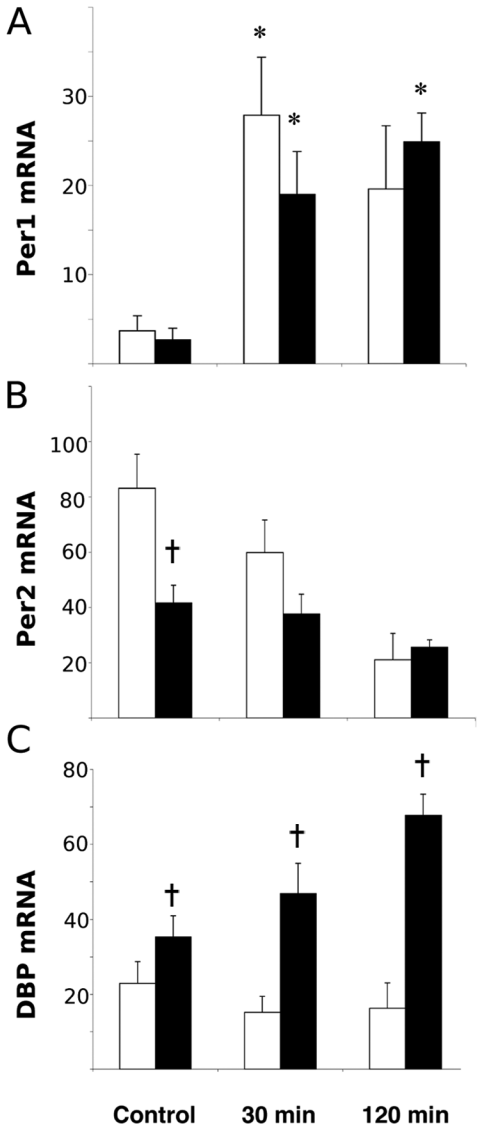

Figure 6. Effects of 15-minute light pulses on expression of (A) *haPer1*, (B) *haPer2*, and (C) *haDbp* in the SCN of wild-type (open bars) or super duper (filled bars) Syrian hamsters. In situ hybridization was performed in groups of hamsters subjected to cage movement (control) or light pulses at CT15 on day 10 of DD. Control hamsters were rapidly decapitated 30 minutes after cage movement; light-exposed hamsters were killed 30 minutes or 2 hours after the stimulus, as indicated. Film density (arbitrary units) used to assess mRNA levels is represented on the ordinate. *$p \leq 0.02$ versus sham control of same genotype. †$p < 0.05$ versus wild type.

tau mutants (Table 1). Finally, the locomotor period of super duper animals matches that of a wider range of T cycles than does that of wild types, and their phase angle is linearly related to the value of T (Bittman, unpublished data). Taken together, the data indicate that the effect of the *duper* mutation to amplify the PRC results in an expansion of the limits of entrainment.

The present experiments clearly dissociate the amplitude of the PRC from the free-running period. Even though super duper and duper animals have free-running periods that are less than and greater than those of *tau* mutants, respectively, they both show higher amplitude resetting than do the τ_{ss} animals. The amplification of phase shifts in duper hamsters is more pronounced in the early subjective night, while the high amplitude PRC of super duper animals is more symmetrical (Fig. 2). This may be a contribution of aftereffects of prior exposure to 14L:10D, to which duper hamsters entrain by phase delays. Duper hamsters born and raised in DD have a symmetrical PRC, with high amplitude shifts in response to light pulses presented either in the early or the late subjective night (Fig. 2E).

Shimomura and Menaker (1994) proposed that the similarity of the PRC of τ_{ss} and wild-type hamsters reflects a proportionate change in clock speed across the circadian cycle rather than a selective acceleration at any particular phase. In contrast, Dey et al. (2005) found that the *tau* mutation accelerates the clearance of PER2, suggesting a selective effect on the early subjective night. The active zone of the PRC appears to be shorter in the super duper than in τ_{ss} hamsters, with few phase shifts observed when light pulses are presented later than CT20. It remains to be determined whether the effect of the *duper* mutation to shorten τ_{DD} reflects a disproportionate effect on molecular events that occur during the later subjective night.

It has been suggested that the amplitude of the PRC is more closely related to α than to τ_{DD}. Shimomura and Menaker (1994) suggested that the amplitude of the PRC is suppressed when hamsters entrain with a phase angle such that light falls 6 to 9 hours after activity onset. Our observations are consistent with this idea, in that the positive phase angle of duper and super duper hamsters in 14L:10D makes it unlikely that the late subjective night is illuminated. The hypothesis that lengthening of α increases resetting amplitude is supported by mathematical modeling (Oda et al., 2000). The speculation that the PRC is the compound response of E and M oscillators (Pittendrigh and Daan, 1976; Daan and Berde, 1978) is consistent with the idea that α reflects pacemaker organization. Not all of our data support this idea, however. We found that the τ_{ss} hamsters had a shorter α than wild types but showed similar or slightly larger phase shifts. More important, the striking amplification of the PRC in super duper hamsters was associated with a still shorter α. Although decompression of α may be correlated with increased amplitude of phase shifts in wild-type Syrian hamsters, this

is not the case in several other species (Refinetti, 2007). Shimomura et al. (1998) also speculated that α/ρ might be distorted in hamsters exposed to T cycles and that changes in PRC amplitude in *tau* mutants may be related to such a change. We find no clear relationship between the magnitude of phase shifts and the α/ρ ratio.

Shimomura et al. (1998) and Grosse et al. (1995) both assessed light induction of *c-fos* expression in wild-type and τ_{ss} hamsters. Despite differences in phase shifting effects in *tau* mutant and wild-type animals, light pulses had similar effects on *c-fos* mRNA and the number of cell nuclei that stained positively for c-Fos protein in the SCN. Their experiments were performed shortly before the critical role of *Period* gene expression was recognized. Although *c-fos* expression may modulate phase-shifting responses, it is unlikely to be a state variable in the oscillation (Wollnik et al., 1995; Honrado et al., 1996). In contrast, *Per1* and *Per2* play critical roles in the core of the transcriptional-translational feedback loop, and clock-controlled genes including *Dbp* provide a window on the output of the core oscillator. Theoretically, increases in the amplitude of the PRC could be explained by a reduction in the range of values through which the state variables change as the limit cycle progresses, such that the perturbation induced by a zeitgeber triggers an excursion across a larger number of isochrons (Winfree, 1980; Peterson, 1980; Johnson et al., 2003; Vitaterna et al., 2006). Alternatively, increases in PRC amplitude might result from enhanced induction of *Period* gene expression by light pulses in the absence of any change in limit cycle amplitude. Thus, we examined the relationship between phase shift amplitude and the induction of *Per1*, *Per2*, and *Dbp* expression in the SCN of wild-type hamsters and super duper mutants.

Despite the striking difference in the amplitude of the behavioral phase shifts, we observed similar induction of *Per1* expression by light pulses at CT15 in super duper and wild-type hamsters. The induction of *Per1* transcription is believed to initiate phase shifts, leading to subsequent induction of *Per2* and perhaps *Cry1* and *Cry2* (Shearman et al. 1997; Shigeyoshi et al., 1997; Yamamoto et al., 2001; Reddy et al., 2001; Masubuchi et al., 2005). Our finding implies that the *duper* mutation affects the response pathway distal to the initial response. We found no consistent change in *per2* mRNA in the SCN of either mutant or wild-type hamsters within 2 hours of the light pulse. In contrast, *Dbp* expression in the *super duper* mutants was higher both in hamsters not exposed to light and in animals killed 30 minutes or 2 hours after the light pulse at CT15. *Dbp* transcription is activated by CLOCK/BMAL1 heterodimers, and DBP can activate the *mPer1* expression promoter (Yamaguchi et al., 2000), possibly acting as a feedback on the central clock. Indeed, *Dbp*-deficient mice show a shortened circadian period (Lopez-Molina et al., 1997). DBP may compete with the transcription factor E4BP4 for a common response element in promoters of a variety of clock-controlled and perhaps some of the core clock genes (Mitsui et al., 2001). Our findings suggest that changes in *Dbp* expression in super duper hamsters may contribute to the enhancement of light-induced phase shifts, even though both basal levels of *Per1* expression and the immediate *Per1* response are comparable to those of wild-type animals. Furthermore, the duper mutant may exhibit global changes in the phase map of clock-controlled gene expression. Indeed, the phase and amplitude of *Dbp* expression are altered in some peripheral organs of super duper and duper mutants (Krug, Blegen, and Bittman, unpublished data). We found that phase delays of greater than 10 hours in duper mutants may be accompanied by transients (Fig. 3F). Although it is unclear whether such large phase delays might be equally regarded as advances (shifts of close to 180°), the perspective that transients arise downstream from the pacemaker (Chandrashekaran et al., 1967; Best et al., 1999; Yamazaki et al., 2000) suggests that the *duper* mutation may precipitate unusual relationships between the master oscillator and its slaves. Effects of the *duper* mutation on other core clock genes, clock-controlled genes, and physiological measures remain to be explored in both the pacemaker and the periphery.

We conclude that the *duper* mutation reduces the amplitude of the circadian oscillator without markedly altering the induction of *Per1* expression by light pulses. The specific changes in the core clock loop responsible for both the shortening of period and the enhancement of phase resetting remain to be determined.

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CONFLICT OF INTEREST STATEMENT

The author(s) have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

NOTE

Supplementary material for this article is available on the *Journal of Biological Rhythms* website at http://jbr.sagepub.com/supplemental.

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