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ORIGINAL ARTICLE

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Deconstructing circadian disruption: Assessing the contribution of reduced peripheral oscillator amplitude on obesity and glucose intolerance in mice

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Abstract

Disturbing the circadian regulation of physiology by disruption of the rhythmic environment is associated with adverse health outcomes but the underlying mechanisms are unknown. Here, the response of central and peripheral circadian clocks to an advance or delay of the light-dark cycle was determined in mice. This identified transient damping of peripheral clocks as a consequence of an advanced light-dark cycle. Similar depression of peripheral rhythm amplitude was observed in mice exposed to repeated phase shifts. To assess the metabolic consequences of such peripheral amplitude depression in isolation, temporally chimeric mice lacking a functional central clock (*Vgat-Cre*$^+$ *Bmal1*$^{fl/fl}$) were housed in the absence of environmental rhythmicity. In vivo PER2::LUC bioluminescence imaging of anesthetized and freely moving mice revealed that this resulted in a state of peripheral amplitude depression, similar in severity to that observed transiently following an advance of the light-dark cycle. Surprisingly, our mice did not show alterations in body mass or glucose tolerance in males or females on regular or high-fat diets. Overall, our results identify transient damping of peripheral rhythm amplitude as a consequence of exposure to an advanced light-dark cycle but chronic damping of peripheral clocks in isolation is insufficient to induce adverse metabolic outcomes in mice.

KEYWORDS

external misalignment, internal desynchrony, internal misalignment, metabolism, peripheral oscillator, rhythm amplitude, suprachiasmatic nucleus

1 | INTRODUCTION

Daily rhythms in the physiology and behavior of mammals are controlled by endogenous circadian clocks located throughout the body. At the molecular level, circadian clocks are driven by cell-autonomous transcription-translation feedback loops. In mammals, these loops consist of a positive limb (BMAL1, CLOCK, and NPAS2) which drives expression of a negative limb (PERIOD1-3, CRYPTOCHROME1-2).[1] Negative-limb proteins subsequently inhibit the transcriptional activity of proteins of the positive limb.[1] Global disruption of this transcription-translation feedback loop by mutation of essential

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core clock genes (eg, *Bmal1-KO* mice[2]) results in loss of behavioral and physiological rhythmicity.

The circadian system of mammals consists of a hierarchy of clocks located in (nearly) all cells of the body.[3-5] The suprachiasmatic nuclei (SCN) contain the master clock which controls the timing of behavioral rhythms and is synchronized (entrained) to environmental time by light signals from the eyes. Synchrony between cellular clocks located in all cells of the body is maintained by rhythmic timing signals (zeitgebers) such as rhythms in body temperature, hormones, and food intake.[3,4] The timing and amplitude of these internal rhythms depends on rhythmic zeitgebers emanating from the SCN and rhythmic changes in the environment (eg, light-dark cycle). Physiological rhythms in tissues throughout the body are controlled by the interaction of local cellular clocks and systemic rhythms in physiology and behavior.[3,5,6]

Disruption of circadian regulation is associated with adverse health outcomes in human shiftworkers.[7-9] Similarly, circadian disruption induced in rodents by chronic shifting of the light-dark cycle, exposure to light at night or altered food timing is associated with adverse health outcomes which include increased mortality, obesity, and disrupted glucose regulation.[10-17] Disruption of circadian regulation by disturbances of the encountered rhythmic environment results in a multitude of changes to different components of the circadian timing system of human shiftworkers and rodents exposed to circadian disruption protocols. Because of this complexity, the critical mechanisms underlying the adverse health effects of circadian disruption induced by environmental disturbances have not been identified.

Here, in vivo PER2::LUC bioluminescence imaging was used to describe how organ-level rhythmicity is affected by phase shifts of the environmental light-dark cycle. This identified transient states of external misalignment, internal misalignment, and depression of peripheral rhythm amplitude as consequences of exposure to both single and repeated phase shifts of the environmental light-dark cycle. Since the metabolic consequences of peripheral amplitude depression have not previously been assessed in isolation, we developed a genetic mouse model to induce similar peripheral amplitude depression chronically in the absence of internal and external misalignment. Housing chimeric mice lacking a functional central clock (*Vgat-Cre*$^+$ *Bmal1*$^{fl/fl}$)[18] in constant darkness allowed us to assess the metabolic consequences of chronic reduction in the amplitude of peripheral rhythms. Surprisingly, chronic depression of peripheral rhythms' amplitude in combination with the absence of systemic rhythmicity did not cause changes in body mass or glucose tolerance. Overall, our results show that advancing the phase of the environmental light-dark cycle causes a transient state of peripheral rhythm damping but that chronic depression of peripheral rhythms' amplitude is insufficient, in isolation, to induce a metabolic phenotype in mice.

2 | METHODS

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Massachusetts Medical School and/or Smith College. All mice used in this study were generated by crossing previously described genotypes.[18-21] The response to a single shift of the light-dark cycle was assessed in wild-type and *Per2::Luc/+* mice exposed to an 8 hours phase advance or 8 hours phase delay of a 12-hour light/12-hour dark lighting cycle. The response to repeated shifts was assessed in mice lacking a functional central clock (*Vgat-Cre*$^+$ *Bmal1*$^{fl/fl}$) and their Cre-negative controls by exposing these mice weekly to an 8-hour phase advance followed 4 days later by an 8 hours phase delay of the light-dark cycle. General locomotor activity was measured using a passive infrared detector placed above each cage. In vivo PER2::LUC bioluminescence measurements were performed in anesthetized mice injected with 0.25 mg luciferin according to previously established procedures[22,23] or in freely moving mice using an Actimetrics In Vivo Luminometer. Mice measured in the In Vivo Luminometer were shaved and implanted subcutaneously with an osmotic minipump filled with CycLuc1 3 days before the start of the 7-day bioluminescence recording session, with the transfer to constant darkness coinciding with the start of recordings. The relative amplitude of peripheral PER2::LUC bioluminescence rhythms was determined by expressing the absolute amplitude of the rhythm as a percentage of the daily average PER2::LUC bioluminescence. Mice used for body mass and i.p. glucose tolerance test measurements were housed in constant darkness throughout their life. Extended methodological details are available in the Methods S1.

3 | RESULTS

3.1 | Re-entrainment following a single shift of the light-dark cycle

Following a shift of the light-dark cycle, the state of different clocks in the hierarchical circadian system of mice was determined by measuring the timing of activity and the PER2::LUC bioluminescence rhythms of peripheral organs. In the first cohort (wild-type and *Per2::Luc/+* mice), the timing of general locomotor activity was monitored in mice that were exposed to an 8-hour phase advance followed 20 days later by an 8-hour delay of the light-dark cycle (Figure 1; Figure S1). In response to a phase advance, the timing of general locomotor activity was out of phase with the environmental light-dark cycle followed by a gradual re-entrainment of the behavioral rhythm (Figure 1A,B). A similar pattern was observed following an 8-hour delay of

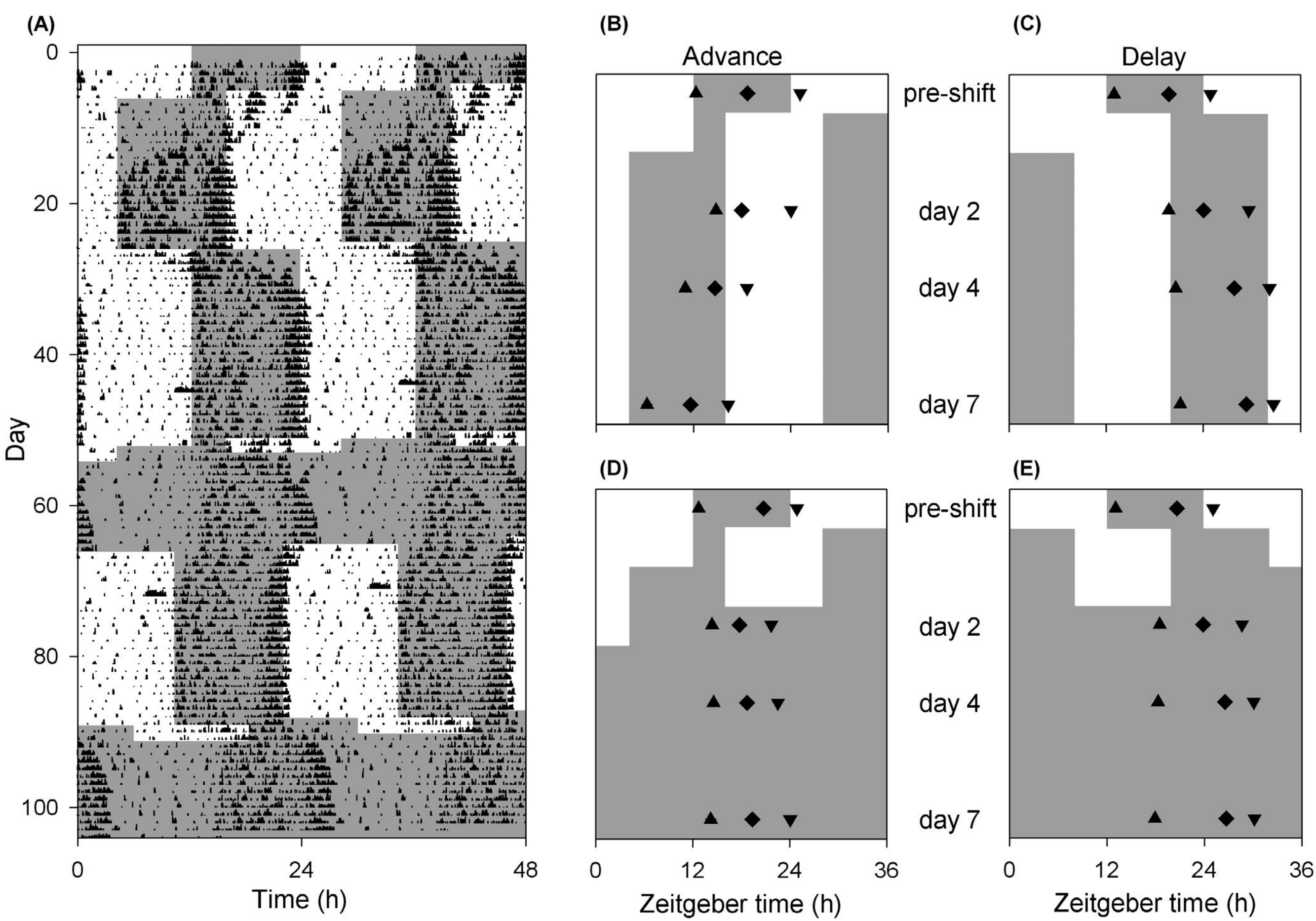
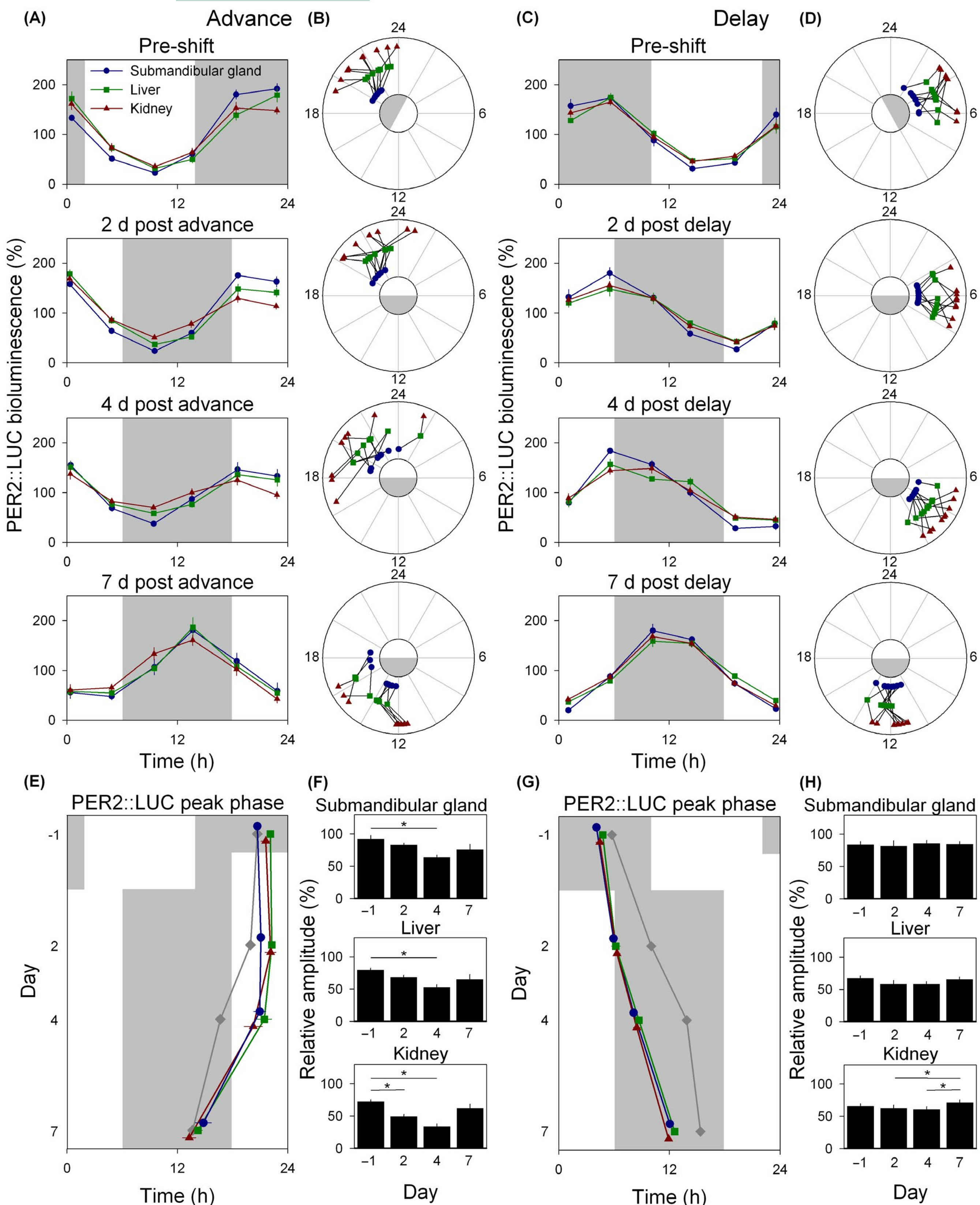


FIGURE 1 Behavioral re-entrainment in response to advances and delays of the light-dark cycle. A, Representative actogram of a mouse being exposed to repeated shifts of the light-dark cycle. B-E, The timing of activity quantified by the onset (upward triangles), midpoint (circle), and offset (downward triangles) of general locomotor activity on day −1, 2, 4, and 7 following a LD-cycle advance (B), delay (C), advance with release in constant darkness (D), and delay with release in constant darkness (E). Graphs represent mean ± SEM. SEM bars fall within the symbols for the means. Light and dark phases are indicated by the white and gray background, respectively

the light-dark cycle although re-entrainment occurred significantly faster (Figure 1C; Figure S1C,G). Subsequently, mice were released into constant darkness following 2 cycles of a shifted light-dark cycle to assess whether SCN phase was re-entrained to the shifted light-dark cycle (Figure 1D,E). The change in the timing of the activity onset showed that mice exposed to an 8-hour delay of the light-dark cycle already completed most of the shift (−5.80 hour shift, one-sample t test: $t_{13} = 10.74$, $P < .0001$, Figure 1E), while two cycles of exposure to an 8-hour advance did not advance, but instead resulted in a small phase delay of the timing of activity onsets (−1.84 hour shift, $t_{13} = 4.47$, $P = .0006$, Figure 1D). All responses to light-dark cycle shifts were similar in *Per2::Luc/+* and wild-type mice.

The response of peripheral rhythms to an environmental shift was determined in a separate cohort of *Per2::Luc/+* mice that were littermates of the mice studied above. These mice were exposed to either an 8-hour phase advance or an 8-hour delay of the light-dark cycle. The rhythmicity of peripheral clocks in the submandibular gland, liver, and kidneys was assessed by measuring in vivo PER2::LUC bioluminescence at six timepoints throughout the day before, as well as 2, 4, and 7 days after shifting the light-dark cycle. These measurements showed that rhythmicity persisted in all three organs on each of the measurement days following the shifted light-dark cycle (Rayleigh tests: $P < .005$, Figure 2A-D) and that the response to a shifted light-dark cycle was consistent in all three peripheral tissues (Figure 2E,G). In response to an 8-hour delay of the light-dark cycle, peripheral clocks in all three peripheral organs gradually re-entrained to the shifted environmental rhythm at a consistent rate of ~1 h/d (Figure 2G), resulting in a transient state of misalignment with both the environment and the quickly resetting SCN (Figure 1C,E). Surprisingly, peripheral re-entrainment following an 8-hour advance of the light-dark cycle did not show a similar gradual pattern. The phase of all three recorded peripheral clocks remained unchanged 2 and 4 days after the light-dark cycle shift, but peripheral rhythms were nearly fully re-entrained after 7 days (Figure 2E). The rapid re-entrainment rate between



day 4 and 7 was preceded by a depression of the relative amplitude of the PER2::LUC bioluminescence rhythms on day 4 after advancing the light-dark cycle (submandibular gland: $F_{3,22.31} = 5.398$, $P = .0060$; liver: $F_{3,22.75} = 7.416$, $P = .0012$; kidney: $F_{3,20.98} = 13.89$, $P < .0001$; Figure 2F). A similar depression of the peripheral amplitude was not

FIGURE 2 Re-entrainment of peripheral clocks in response to an advanced or delayed light-dark cycle. A and C, Average PER2::LUC bioluminescence of three organs at different times of day on day −1, 2, 4, and 7 following an 8-h phase advance (A) or an 8-h delay (C) of the light-dark cycle. Bioluminescence values are expressed as a percentage of the average bioluminescence of each organ observed on day −1. B and D, Phase plots indicating the time of peak PER2::LUC bioluminescence of different organs in individual mice on day −1, 2, 4, and 7 following an 8 h phase advance (B) or delay (D) of the light-dark cycle. Organs within the same animal are connected with a black line. E and G, The average PER2::LUC bioluminescence peak phase of different organs on day −1, 2, 4, and 7 following an 8-h advance (E) or delay (G) of the light-dark cycle. Dark gray lines indicate the midpoint of behavioral activity of a different cohort of mice, reported in Figure 1. These data are replotted to facilitate comparisons between central and peripheral phase. F and H, The relative amplitude of peripheral PER2::LUC bioluminescence rhythms following an 8-h advance (F) or delay (H) of the light-dark cycle. Graphs represent mean ± SEM. Light and dark phases are indicated by the white and gray background, respectively

observed following a delay of the light-dark cycle (submandibular gland: $F_{3,24} = 0.1887$, $P = .9031$; liver: $F_{3,24} = 1.372$, $P = .2753$; kidney: $F_{3,24} = 4.915$, $P = .0084$; Figure 2H).

3.2 | Circadian responses to chronic shifting of the light-dark cycle

The response of the circadian system to chronic circadian disruption by shifting of environmental rhythms was assessed in *Per2::Luc/+* mice (*No-Cre Bmal1$^{fl/fl}$*) exposed each week to an 8-hour phase advance followed 4 days later by an 8-hour phase delay (Figure 3A). Exposure to this repeated shifting of environmental rhythmicity resulted in a state of chronic circadian disruption in which the timing of running wheel behavior was consistently misaligned with the environmental light-dark cycle (Figure 3B). The timing of activity onsets advanced by ~1 h/d following phase advances of the light-dark cycle. Following phase delays of the light-dark cycle, the onset of activity was determined by the negative masking effects of light. Nevertheless, the week-to-week consistency of the timing of activity on each day of the week showed that the timing of the circadian clock regulating activity was consistently delayed by phase delays of the light-dark cycle. The daily timing of food intake maintained its regular phase relation to the timing of activity and was also consistently out of phase with the environmental light-dark cycle (Figure S2C).

The behavior of peripheral clocks in response to chronic environmental disruption was assessed in mice following repeated environmental phase shifts by measuring peripheral PER2::LUC bioluminescence rhythmicity on the last day of the advanced, as well as the last day of the delayed, light-dark cycle. The relative amplitude of peripheral PER2::LUC bioluminescence rhythms was between that observed "normally" (eg, during stable light-dark entrainment) and that observed 4 days following a single 8-hour phase advance (submandibular gland: $F_{3,21.01} = 6.409$, $P = .0030$; liver: $F_{3,21.73} = 13.59$, $P < .0001$; kidney: $F_{3,21.33} = 17.40$, $P < .0001$; Figure 3C). Surprisingly, no consistent differences were observed in the relative amplitude of the PER2::LUC bioluminescence rhythms between the last day of the advanced and the last day of the delayed light-dark cycle (submandibular gland: $F_{1,9} = 3.889$, $P = .0801$; liver: $F_{1,9} = 3.116$, $P = .1114$; kidney: $F_{1,9} = 0.2853$, $P = .6062$). In line with our observation that peripheral phase remained unchanged during the first 4 days following a single 8-hour phase advance of the light-dark cycle (Figure 2E), the peak phase of the PER2::LUC bioluminescence rhythms measured on the last day of the advanced and on the last day of the delayed light-dark cycle did not differ (submandibular gland: $F_{1,9} = 0.2558$, $P = .6252$; liver: $F_{1,9} = 0.4702$, $P = .5102$; kidney: $F_{1,9} = 2.266$, $P = .1665$; Figure S2E). The observed time of peak during both the advanced and delayed light-dark cycle (Figure S2E) was similar to that seen in mice entrained to a stable light-dark cycle aligned with the delayed light-dark cycle (Figure 2E,G).

3.3 | Peripheral amplitude depression in mice lacking a functional central clock housed in constant darkness

A chronic state of peripheral clock amplitude depression was induced in mice by using a temporally chimeric mouse line (*Vgat-Cre$^+$ Bmal1$^{fl/fl}$*)[18] in which the endogenous clock in GABAergic neurons (including the SCN) is disrupted, while cellular clocks in peripheral organs remain effectively wild type. These mice are behaviorally arrhythmic when housed in the absence of environmental rhythmicity (Figure S3).[18] While daily rhythmicity in behavior can be induced by exposing these mice to a light-dark cycle, they become arrhythmic immediately upon release into constant darkness.[18] Repeated exposure to a weekly phase advance and delay revealed that the behavior of these mice rapidly re-aligned with the shifted light-dark cycle (Figure S2A-C) while peripheral PER2::LUC bioluminescence rhythms were disrupted (low relative amplitude and wide phase dispersal) with the response to light-dark cycle phase shifts being different between organs (Figure S2D,E). Based on previous measurements of in vivo and ex vivo peripheral clock amplitude in mice with disrupted SCN rhythmicity,[22,24,25] we expected that mice without a functional central clock would exhibit continued peripheral rhythmicity, albeit with a reduced amplitude, when housed in the absence of environmental rhythms. Indeed, in mice housed in constant

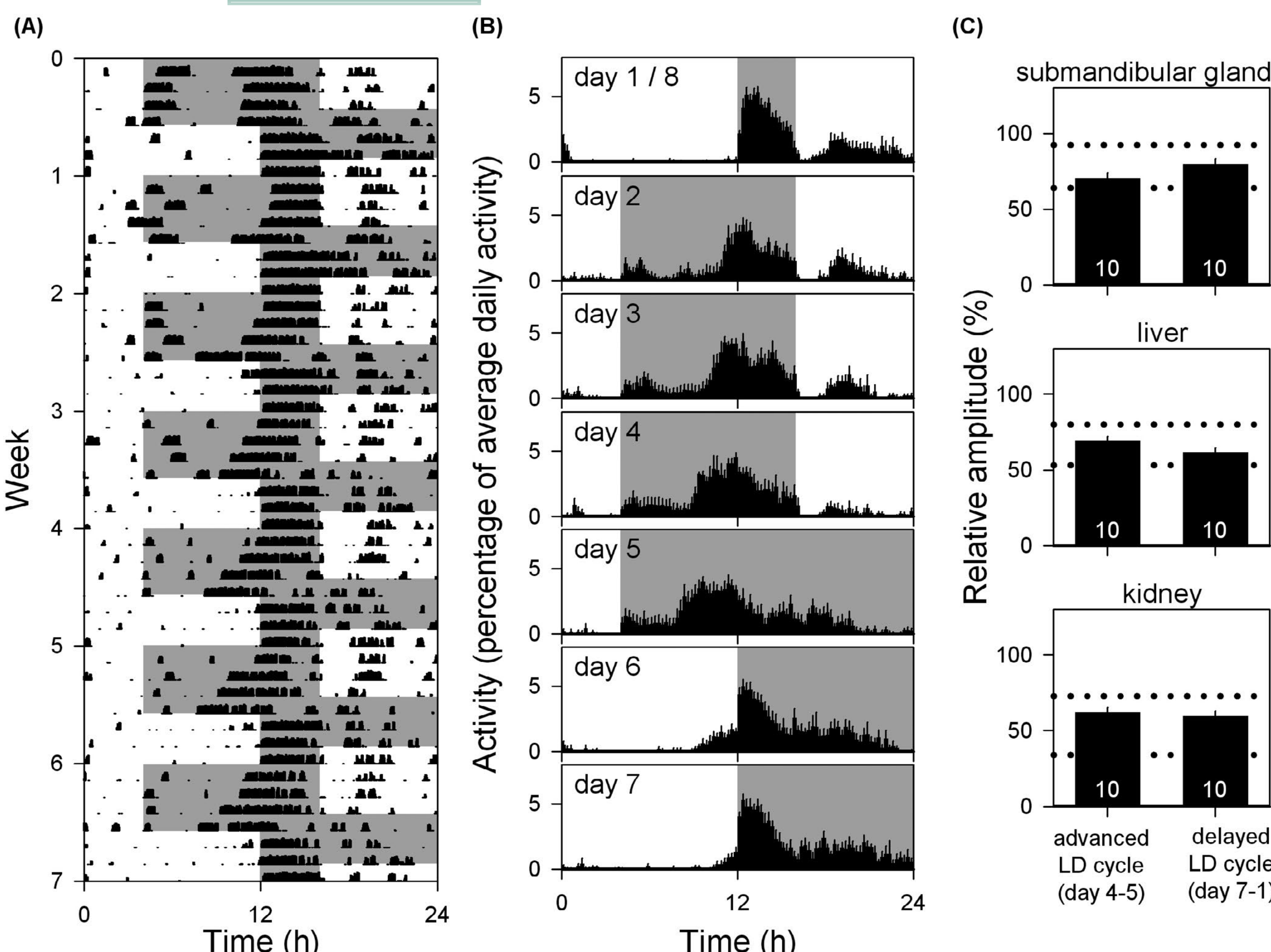


FIGURE 3 Response to repeated phase shifting consisting of an advance and a delay of the light-dark cycle each week. A, Representative actogram of running wheel activity from a mouse being exposed to an 8-h phase advance and 8-h phase delay of the light-dark cycle each week. B, Average activity profile of mice exposed to chronic 8-h advances and 8-h delays of the light-dark cycle. C, Relative amplitude of peripheral PER2::LUC bioluminescence rhythms of mice exposed to chronic 8-h advances and 8-h delays of the light-dark cycle. Peripheral rhythmicity was measured on the last day of the advanced and delayed light-dark. The upper and lower dotted lines represent the relative amplitude of peripheral PER2::LUC bioluminescence rhythmicity observed in mice exposed to a single 8-h phase advance of the light-dark cycle (from Figure 2F) on day −1 and 4 postshift, respectively. Graphs represent mean ± SEM. Light and dark phases are indicated by the white and gray background, respectively. The number of animals measured during each condition is indicated at the base of each bar

darkness throughout their life, PER2::LUC bioluminescence rhythms in the submandibular gland, liver, and kidney had a reduced amplitude in mice lacking a functional central clock, compared to Cre-negative *Bmal1*$^{fl/fl}$ controls (submandibular gland: $t_{21} = 3.75$, $P = .0012$; liver: $t_{21} = 4.93$, $P < .0001$; kidney: $t_{21} = 4.23$, $P = .0004$; Figure 4A; Figures S4 and S5). A similar depression of peripheral rhythms was not observed in mice housed in a 12-hour light/12-hour dark lighting cycle (submandibular gland: $t_{10} = 0.87$, $P = .4059$; liver: $t_{10} = 1.20$, $P = .2600$; kidney: $t_{10} = 1.17$, $P = .2718$; Figure 4B). Interpretation of these results is, however, complicated by the anesthesia required to perform these measurements, which might potentially induce rhythmicity in otherwise desynchronized tissues.

To exclude the potential caveat that anesthesia might induce peripheral rhythmicity and establish whether within-animal phase alignment is maintained in mice lacking a functional central clock in the absence of environmental rhythmicity, whole-body PER2::LUC bioluminescence rhythmicity was measured in a separate cohort of freely moving mice. These mice were not subjected to repeated anesthesia between bioluminescence measurements. Mice lacking functional clocks throughout the body (*Bmal1*$^{-/-}$), lacking a functional central clock (*Vgat-Cre*$^{+}$ *Bmal1*$^{fl/fl}$) or with a fully functional circadian system (*No-Cre Bmal1*$^{fl/fl}$) were exposed to a 12-hour light/12-hour dark lighting cycle before being released into constant darkness. Whole-body PER2::LUC bioluminescence was recorded for 7 days in

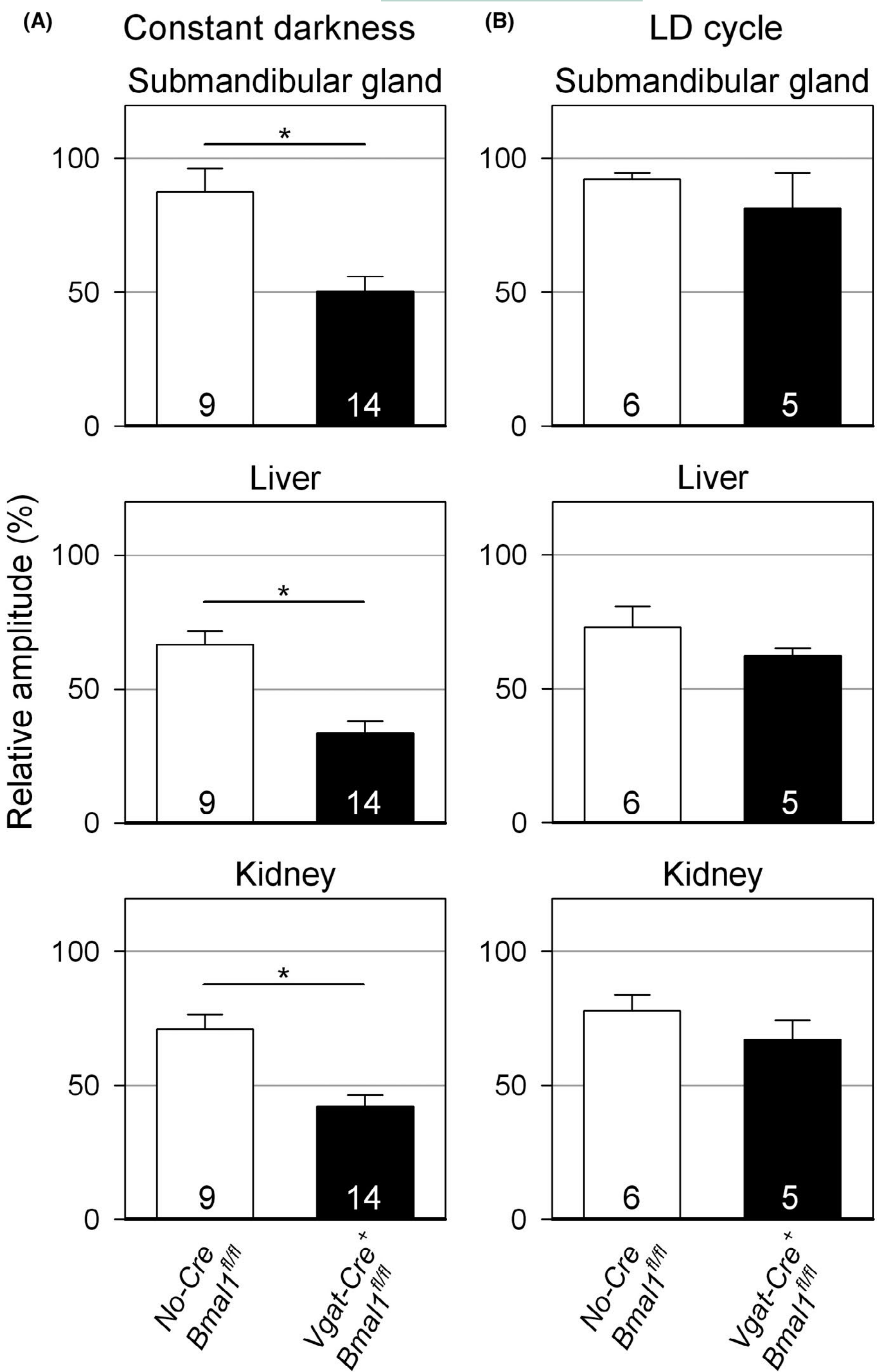


FIGURE 4 Reduced peripheral PER2::LUC bioluminescence rhythms' amplitude in the absence of both central-clock and environmental rhythmicity. A, In the absence of a light-dark cycle, the relative amplitude of peripheral PER2::LUC bioluminescence rhythms in three peripheral organs was significantly reduced in mice without a functional central clock (*Vgat-Cre*$^{+}$ *Bmal1*$^{fl/fl}$) compared to control mice (*No-Cre Bmal1*$^{fl/fl}$). B, When housed in a 12-h light/12-h dark lighting cycle, the relative amplitude of peripheral PER2::LUC bioluminescence rhythms was statistically indistinguishable between mice without a functional central clock and clock-proficient controls. Values represent mean ± SEM. The number of animals per genotype is indicated at the base of each bar

constant darkness. In the absence of environmental timing signals, mice lacking functional clocks throughout their body became arrhythmic immediately, while Cre-negative control mice maintained high-amplitude bioluminescence rhythms throughout the 7-day measurement period (Figure 5A). Because the reduction in relative amplitude of peripheral PER2::LUC bioluminescence rhythms during the 7-day measurement was not significantly different in mice lacking a functional central clock compared to Cre-negative controls ($F_{2,206.7}$ = 1.533, P = .2184), the average amplitude of peripheral rhythms was compared over the whole 7-day measurement period. Peripheral PER2::LUC bioluminescence rhythmicity in mice lacking a functional central clock was depressed in amplitude and less robust compared to controls, but not fully absent as seen in whole-body Bmal1-KO mice (relative amplitude: $F_{2,22.1}$ = 22.95, P < .0001, Figure 5B; signal to noise: $F_{2,24}$ = 25.90, P < .0001, Figure 5C). Overall, our comparison of in vivo PER2::LUC bioluminescence rhythms between mice lacking a functional central clock and both control groups showed that, in the absence of environmental and central-clock rhythmicity, peripheral organ-level rhythms were chronically depressed in amplitude, to a level comparable to that observed transiently following an 8 hours advance of the light-dark cycle (Figure 2F).

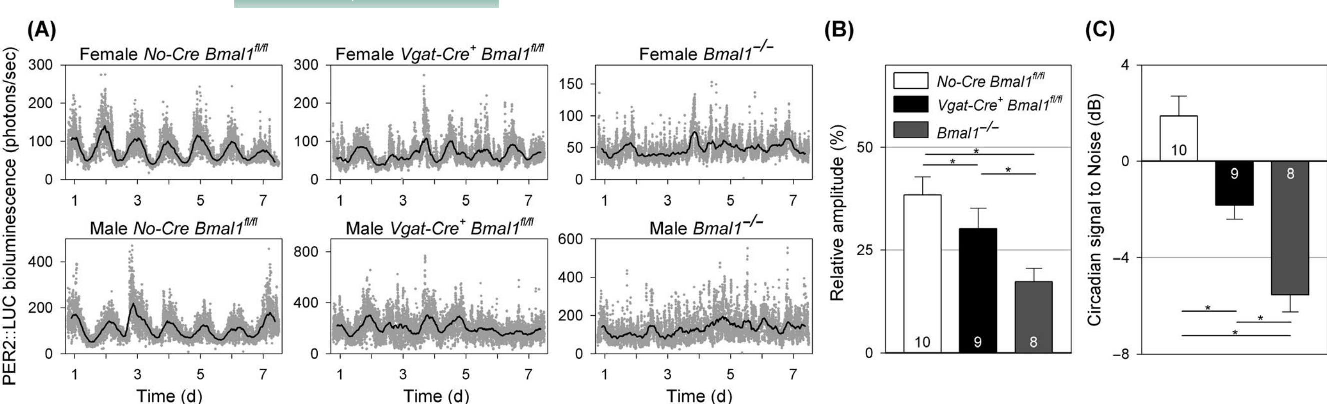


FIGURE 5 Whole-body PER2::LUC bioluminescence rhythms in freely moving mice. Mice either lacked a functional central clock (*Vgat-Cre*$^+$ *Bmal1*$^{fl/fl}$), had disrupted clocks throughout the body (*Bmal1*$^{-/-}$) or had an effectively wild-type circadian system (*No-Cre Bmal1*$^{fl/fl}$). A, Representative examples of 7-d whole-body bioluminescence traces of female and male mice. Black lines represent the 6-h running average. B, The relative amplitude of PER2::LUC bioluminescence rhythmicity, showing depression of amplitude in mice lacking a functional central circadian clock and further reduction in mice lacking functional clocks throughout the body. C, The circadian signal to noncircadian noise strength detected using Wavelet analysis. Higher values indicate a stronger circadian signal relative to the strength of the noncircadian noise. Values are mean ± SEM. The number of animals per genotype is indicated within each bar

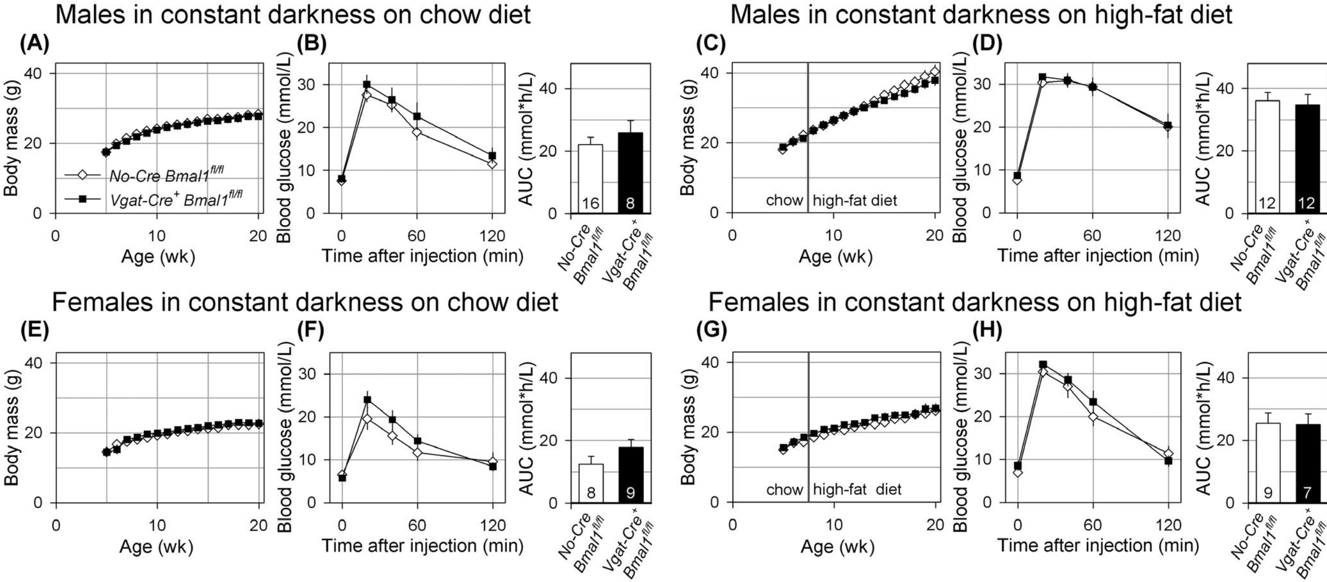


FIGURE 6 Mice without a functional central clock do not show an adverse metabolic phenotype when housed in the absence of environmental rhythmicity throughout their lives. Body mass and glucose tolerance were compared between mice without a functional central clock (*Vgat-Cre*$^+$ *Bmal1*$^{fl/fl}$) and their littermate controls (*No-Cre Bmal1*$^{fl/fl}$). In the absence of environmental rhythmicity, the absence of a functional central clock did not result in obesity or glucose intolerance in male mice fed a regular chow diet (A, B), male mice fed a high-fat diet (C, D), female mice fed a regular chow diet (E, F) or females fed a high-fat diet (G, H). High-fat diet exposure started at 7 wk of age. Graphs are mean ± SEM. Sample size for glucose tolerance tests is indicated at the base of the bar plots. AUC, area under the curve

3.4 | Mice lacking a functional central clock do not become obese or glucose intolerance

The chronic depression of peripheral clock amplitude in mice lacking a functional central clock when housed in constant darkness (Figures 4 and 5) provides us with a unique opportunity to assess whether chronic depression of peripheral clock amplitude, in the absence of internal and external misalignment, is sufficient to generate the adverse health outcomes associated with disruption of environmental rhythmicity. In other words, if reduction in the amplitude of peripheral rhythms following advances of the light-dark cycle (Figures 2 and 3) is in itself a causal factor in driving the adverse health outcomes observed following repeated exposure to phase shifts of the light-dark cycle,[13,16] then chronic depression of peripheral rhythms' amplitude in this genetic

model would similarly be expected to result in adverse health outcomes. To test this conjecture, mice lacking a functional central clock and their Cre-negative controls were housed in constant darkness throughout their life in mixed-genotype groups. Weekly weighing from 5 to 20 weeks of age revealed that the absence of a functional central clock did not result in body mass differences in male and female mice fed regular chow (males: $F_{13,286}$ = 0.7167, P = .7466, Figure 6A; females: $F_{13,195}$ = 0.6938, P = .7681, Figure 6E) or a high-fat diet (males: $F_{13,286}$ = 1.461, P = .1313, Figure 6C; females: $F_{13,182}$ = 0.5669, P = .8780, Figure 6G). Similarly, no differences in glucose tolerance were observed between genotypes in these same mice on either normal chow (AUC: males: $F_{1,21.92}$ = 0.4049, P = .5311, Figure 6B; females: $F_{1,13.26}$ = 2.106, P = .1699, Figure 6F) or a high-fat diet (AUC: males: $F_{1,21.7}$ = 0.0881, P = .7695, Figure 6D; females: $F_{1,12.46}$ = 0.4074, P = .5349, Figure 6H). To preclude the possibility that housing with clock-proficient control mice obscured potential adverse metabolic outcomes of a disrupted central clock, a separate cohort of chow-fed mice were moved to constant darkness at birth and housed individually after weaning. Again, no differences in body mass (males: $F_{13,336}$ = 1.022, P = .4292, Figure S6A; females: $F_{13,247}$ = 0.1786, P = .9994, Figure S6D) or glucose tolerance (males: $F_{1,20.84}$ = 0.8413, P = .3695, Figure S6C; females: $F_{1,11.88}$ = 1.733, P = .2129, Figure S6F) were observed between genotypes. The individual housing also allowed the assessment of food intake, which did not differ significantly between genotypes (males: $F_{1,20.76}$ = 0.7946, P = .3829, Figure S6B; females: $F_{1,15.69}$ = 0.7369, P = .4036, Figure S6E). Overall, these results show that chronic depression of peripheral rhythms' amplitude in this genetic model of central-clock disruption did not result in elevated body mass and/or glucose intolerance.

4 | DISCUSSION

Disturbance of the circadian regulation of physiology and behavior by disruption of the rhythmic environment has been associated with adverse health outcomes in both humans and rodents.[7,10,13,26,27] The present study is part of our effort to identify mechanisms responsible for the adverse health consequences associated with circadian disruption.[23,26,28-34] With this aim in mind, we first described the behavior of different components of the circadian system in mice following single or repeated phase shifts of the environmental light-dark cycle (Figures 1-3). This identified external and internal misalignment as well as depression of peripheral amplitude as consequences of disturbances of the rhythmic environment. To reveal mechanisms responsible for the adverse metabolic consequences of circadian disruption, we aim to test whether individual changes to the organization of the circadian system are, in isolation, sufficient to induce an adverse metabolic phenotype. The effects of external and internal misalignment have previously been assessed in isolation.[23,35] Therefore, the current study was designed to assess the metabolic consequences of depression of peripheral rhythms' amplitude. With this goal in mind, a genetic mouse model lacking systemic rhythmicity was developed, resulting in a similar degree of peripheral amplitude depression to that observed following exposure of wild-type mice to an advancing light-dark cycle (Figures 4 and 5). Surprisingly, these mice did not show changes in body mass or glucose tolerance (Figure 6).

The behavior of different components of the hierarchical circadian system of mice in response to environmental disruption was first studied by assessing responses to a single 8-hour phase advance or delay of the light-dark cycle. The behavior of the central clock was assessed by measuring the timing of locomotor activity upon release into constant darkness, while the behavior of peripheral clocks was described using in vivo bioluminescence imaging. In line with previous reports,[36] the SCN re-entrained quickly (<2 days) in response to an 8-hour delay of the light-dark cycle, followed by a gradual re-entrainment of peripheral clocks over a period of ~7 days. Previous studies assessing the phase of the SCN in response to an advanced light-dark cycle resulted in contradictory outcomes depending on the method used.[36,37] Here, using the timing of locomotor behavior upon release into constant darkness following a phase shift to infer SCN phase, we observed that an 8-hour advance of the light-dark cycle did not result in an immediate shift of the SCN. This agrees with previous results obtained using a different in vivo approach.[37] Our in vivo within-animal assessment of peripheral clock behavior following a phase advance revealed that peripheral clocks show a long-latency but abrupt re-entrainment following a transient reduction of peripheral PER2::LUC bioluminescence rhythm amplitude. This finding is in line with a previous assessment of peripheral clock-gene transcript rhythmicity following an advanced light-dark cycle, which showed transient reduction of *Per1* and *Dbp* transcript rhythm amplitude in liver.[37] The reduction in organ-level amplitude of PER2::LUC bioluminescence rhythms was likely a consequence of misalignment of individual cellular clocks, although our measurements do not allow us to exclude the possibility that the amplitude reduction occurred at the level of individual cellular clocks. Overall, the present study shows that an advance of the light-dark cycle results in three major alterations in the organization of the hierarchical mammalian circadian clock system: external misalignment between the phase of endogenous clocks and the environment, internal misalignment between the SCN and peripheral clocks as well as a transient damping of peripheral PER2::LUC bioluminescence rhythms.

Our assessment of peripheral rhythmicity in mice exposed to repeated advances and delays of the light-dark cycle

confirms the findings following a single phase advance. Chronic exposure to an 8-hour phase advance and delay each week results in chronic misalignment between the environmental light-dark cycle and internal circadian phase as well as between the timing of activity (and food) and peripheral clock phase. Similar to our observation following a single phase advance of the light-dark cycle exposure to chronic shifting of the light-dark cycle also resulted in depression of the amplitude of peripheral PER2::LUC bioluminescence rhythms.

Since the circadian regulation of physiology is dependent on the interacting influences of central and peripheral clocks,[3-5] disturbance of the phase relationships between different clocks in the body has been thought to drive adverse health outcomes.[31] The multitude of changes at different levels of the circadian system resulting from exposure to circadian disruption protocols (eg, shiftwork, jetlag) has complicated the search for the mechanisms responsible for the adverse health effects of circadian disruption. The present study identified a transient reduction in organ-level amplitude as well as internal and external misalignment as major changes in response to shifting of environmental light-dark cycles. The metabolic consequences of external and internal misalignment have previously been assessed in isolation,[23,35] and therefore, the present study aimed to assess the metabolic consequences of chronic organ-level amplitude depression, in isolation from the misalignments that normally occur with circadian disruption. Housing temporally chimeric mice lacking a functional central clock (*Vgat-Cre*$^{+}$ *Bmal1*$^{fl/fl}$) in the absence of environmental rhythmicity resulted in a chronically reduced peripheral organ-level rhythm amplitude similar in severity to that observed transiently following a light-dark cycle phase advance. In line with previous studies,[22,24] the lifelong absence of both environmental and SCN timing cues did not result in the complete disappearance of organ-level rhythms but resulted in a level of rhythmicity between that of clock-proficient controls and of mice lacking functional clocks throughout the body. Exposure of mice lacking a functional central clock to a light-dark cycle led to high-amplitude peripheral rhythms, likely driven by daily rhythmicity in activity,[18] food intake (Figure S2C) and associated body temperature changes induced by the masking effects of light. The observation that peripheral rhythmicity is maintained with reduced amplitude validates our temporally chimeric *Vgat-Cre*$^{+}$ *Bmal1*$^{fl/fl}$ mice housed in constant darkness as a model to study the metabolic consequences of reductions in the amplitude of peripheral organ-level rhythmicity. We note that this model also lacks centrally driven rhythms, whose influence on metabolic responses might strongly interact with the effects of peripheral amplitude depression. Development of complementary models will be needed to separate these factors.

Studies assessing transcript rhythms in the blood of humans exposed to simulated shiftwork conditions have identified a reduced amplitude as one of the consequences of circadian disruption which might be responsible for its adverse metabolic consequences.[38,39] In line with this hypothesis, disruption of the SCN's control of systemic rhythmicity by SCN lesion or exposure to constant light has previously been shown to result in metabolic abnormalities.[12,17] Surprisingly, the present study shows that lifelong absence of the synchronizing influences of both environmental and SCN-controlled timing cues, resulting in a depression of peripheral rhythm amplitude, did not result in genotype differences in body mass or glucose tolerance in both female and male mice fed either a regular or high-fat diet. Given that a high-fat diet resulted in obesity and reduced glucose tolerance in both genotypes, this absence of genotype differences in the development of an adverse metabolic phenotype was not the result of a general inability of our *Vgat-Cre*$^{+}$ *Bmal1*$^{fl/fl}$ mice to gain weight. The absence of a metabolic phenotype seemingly contradicts with the recent report by Kolbe et al that a different temporally chimeric mouse line with disrupted SCN rhythmicity (*Syt10*$^{Cre/Cre}$ *Bmal1*$^{-/fl}$ mice housed in constant darkness) exhibits an adverse obesity and glucose tolerance phenotype.[40] Assessment of transcript rhythmicity suggests, however, that the specific genetic strategy chosen to disrupt SCN rhythmicity in these mice resulted in a (nearly) complete depression of peripheral rhythms' amplitude, although peripheral rhythmicity was not assessed at the level of individual animals.[40] When combined with our findings, these results suggest that although a complete damping of peripheral rhythmicity might result in an adverse metabolic phenotype, chronic amplitude depression to an extent similar to that observed transiently following shiftwork or jetlag is insufficient to make mice obese and/or glucose intolerant.

In order to identify the mechanisms responsible for the adverse health effects of circadian disruption, future studies will have to account for the complexities presented by the hierarchical organization of the mammalian circadian system.[3,4] Circadian disruption in modern 24/7 societies can take many forms and be associated with a plethora of changes to both the internal organization of the circadian system (eg, internal misalignment, peripheral amplitude depression) and its interactions with the environment (eg, external misalignment with light and food) that might be transient or chronic.[26,28-34] The present study assessed the metabolic consequences of a specific aspect of circadian disruption (ie, mice with chronically depressed peripheral amplitude lacking both central and environmental timing inputs) that, by design, did not recapitulate all aspects of circadian disruption induced by phase shifting environmental rhythms. In order to identify the key features of disruption of circadian regulation responsible for its adverse health consequences in modern 24/7 societies, future studies will have to test the contributions of specific circadian changes associated with disruptions in the real world. Since real-world disruption of circadian regulation is a complex

phenomenon it might be necessary to study the interactions between different disruption characteristics and assess additional ones such as differences between transient and chronic disruption. Recent advances in the development of temporally chimeric animal models,[23,41-45] imaging techniques in freely moving animals[46,47] and high-throughput omics approaches[38,48-50] will enable the development of new paradigms to further test the involvement of specific aspects of circadian disruption. The proximate knowledge gained from such mechanistic studies will enable the formulation of strategies to remedy the adverse health consequences inherent to modern 24/7 societies.

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AUTHOR CONTRIBUTIONS

VvdV, MEH, and DRW designed research. VvdV and BMB conducted experiments. VvdV and BMB analyzed data. VvdV wrote the paper with input from all other authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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