

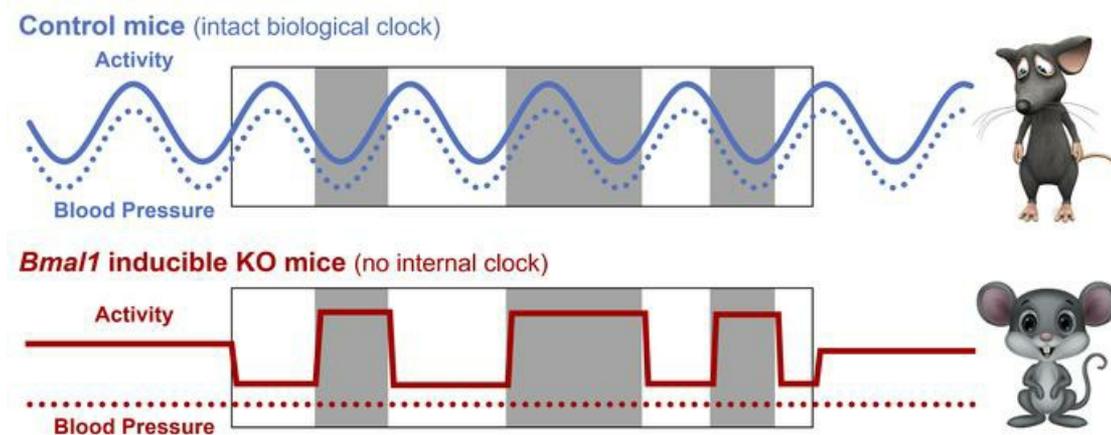
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JCI Insight. 2019. <https://doi.org/10.1172/jci.insight.125133>.

Research In-Press Preview Neuroscience

### Graphical abstract



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# **Bmal1 deletion in mice facilitates adaptation to disrupted light/dark conditions**

Guangrui Yang<sup>1,2\*\*</sup>, Lihong Chen<sup>3\*</sup>, Jiayang Zhang<sup>3</sup>, Baoyin Ren<sup>1</sup>, Garret A. FitzGerald<sup>2†</sup>

<sup>1</sup> School of Bioengineering, Dalian University of Technology, Dalian, Liaoning, 116024, China. <sup>2</sup> The Institute for Translational Medicine and Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA. <sup>3</sup> Advanced Institute for Medical Sciences, Dalian Medical University, Dalian, Liaoning, 116044, China.

\* These authors contributed equally to this work.

†Addresses for correspondence:

Guangrui Yang: School of Bioengineering, Room 615, 2 Linggong Rd, Ganjingzi District, Dalian, Liaoning, 116024, China Tel: +86-411-84706806. Email: [guangrui.yang@hotmail.com](mailto:guangrui.yang@hotmail.com) and

Garret A. FitzGerald: Institute for Translational Medicine and Therapeutics, Smilow Center for Translational Research, West Pavilion, 10th Floor, Room 116, 3400 Civic Center Boulevard, Building 421, Philadelphia, PA 19104-5158 Tel: +1-215-898-1185 | Fax: 215-573-9135. Email: [garret@upenn.edu](mailto:garret@upenn.edu)

## **Abstract**

Recently, by utilizing conventional and tamoxifen inducible *Bmal1* (Brain and muscle Arnt-like protein 1) knockout mice, we found that delaying the loss of circadian rhythms to adulthood attenuates the impact on general integrity and survival at least under 12h light/12h dark conditions (LD). To understand further the contribution of *Bmal1* in post-natal life under conditions of circadian disruption, we subjected inducible knockout mice (KO) and their littermate controls (Ctrl) to forced desynchrony protocols including cycles with non-24h periods, randomized light/dark cycles, and jet lag, and monitored their locomotor activity using radiotelemetry. Under these conditions, control mice cannot be entrained, as reflected by their maintenance of circadian behavior irrespective of schedules. By contrast, KO mice displayed higher activity levels in the dark phases of most cycles. Under a 3h light/3h dark regime, Ctrl mice displayed higher activity levels in the dark phases of all cycles although there were still obvious circadian rhythms, suggesting that an ultradian mechanism is also involved. Insulin sensitivity was markedly reduced by disrupted light schedules as expected in Ctrl mice, but not in the KOs. Thus, *Bmal1* deletion in

adult mice facilitates adaptation to new light/dark schedules and protects from insulin resistance induced by circadian disruption.

Keywords: Bmal1, circadian rhythm, desynchrony, behavior.

Conflict of interest: Dr. FitzGerald is a senior advisor to Calico Laboratories. His laboratory receives funding support from the Volkswagen Foundation, the American Heart Association and the NIH.

## Introduction

Chronic disruption of circadian rhythms may result in adverse effects in humans. For example, shift work has been associated with an increased burden of cardiometabolic disease, cancer and sleep disorders(1). Although other factors, such as socioeconomic status and behavioral covariates may confound such associations, circadian disruption in mice or in humans using forced desynchrony protocols results in analogous adverse phenotypes. Deletion of *Bmal1* (Brain and muscle Arnt-like protein 1), the only core clock gene whose loss results in complete absence of circadian rhythms, causes behavioral arrhythmicity concomitant with many adverse effects, including reduced body weight, impaired hair growth, abnormal bone calcification, eye pathologies, and shortened lifespan(2-4). Selective deletion of *Bmal1* in pancreas(5), liver(6), muscle(7) and adipose tissue(8) recapitulates discrete elements of the metabolic syndrome. This phenomenon is of increasing concern as circadian disruption becomes commonplace in modern life due to nocturnal use of illuminated phones, transmeridian flights and shift work.

Recently, utilizing tamoxifen (TAM) inducible *Bmal1* knockout mice, we found that while the mice lost behavioral, physiological and molecular circadian rhythms, there was an attenuated impact on general integrity and survival under regular light/dark conditions(9). However, the effect of *Bmal1* deletion in mice under disrupted light/dark schedules (DS) has not been evaluated. Thus, we have subjected inducible *Bmal1* knockout mice (KOs) and their littermate controls to a series of non-24h light/dark cycles (Figure 1), to determine whether the absence of a molecular clock in adulthood might facilitate adaptation to circadian disruption.

## Results

### Loss of circadian behavior

Locomotor activity was monitored using radiotelemetry or running wheels. Before TAM treatment, there was no behavioral difference between *Bmal1<sup>ff</sup>-EsrCre* mice and their littermate *Bmal1<sup>ff</sup>* controls under either 12h light/12h dark conditions (LD) or constant darkness (DD) conditions (Figure 2A). *Bmal1<sup>ff</sup>* mice treated with TAM (Figure 2A & B) and *Bmal1<sup>ff</sup>-EsrCre* mice

treated with corn oil (Figure 2B) still showed rhythmic behavior under DD, however, as expected, all *Bmal1<sup>ff</sup>-EsrCre* mice treated with TAM completely lost the rhythmicity after the treatment. It is noteworthy that TAM, but not corn oil, decreased locomotor activity (Figure 2B). Therefore, we chose TAM-treated *Cre* negative mice as controls for the following experiments to avoid possible confounding effects caused by the different ingredients of the gavage.

### **Adaptation to various light/dark schedules**

After DD, the mice were subjected to a series of abnormal light/dark schedules including L:D=10h:10h (Figure 3), L:D=8h:8h, (Figure 4), L:D=3h:3h (Figure 5), and a randomized light/dark condition (Figure 6) sequentially. Save for the exception of the L:D=3h:3h schedule, the behavioral results showed that KO mice were more adaptive to these disruptions than were Ctrl mice. This was reflected by calculation of the percentage of cycles that retained higher activity during the dark (active) phases (Figure 7).

Under these conditions, Ctrl mice could not be entrained, as reflected by their continuous circadian rhythmicity in behavior irrespective of non-24h light/dark periods. For instance, under L:D=10h:10h condition (8.3d, 10 cycles), Ctrl mice showed about 8 cycles instead of 10 cycles in behavior rhythmicity (Figure 3), while KO mice displayed higher activity levels in all dark phases. Similar results were also seen under L:D=8h:8h and randomized light/dark conditions. Interestingly, however, under L:D=3h:3h condition, Ctrl mice displayed higher activity levels mainly in dark phases, although there were still obvious circadian rhythms.

### **Dissociation between Behavior and blood pressure (BP) in KO mice.**

Systolic BP (SBP) mirrored behavior in Ctrl mice in that it failed to adapt to the disrupted schedules (Figure 3-6). Here, despite behavioral adaptation, SBP failed to retain rhythms in the KO mice exposed to the disrupted schedules (Figure 7).

### **Insulin tolerance test (ITT)**

Insulin sensitivity was markedly reduced by the abnormal schedules in Ctrl mice as expected. However, concordant with behavioral adaptation, the KO mice were indistinguishable from unchallenged mice with respect to insulin sensitivity (Figure 8).

### **Body weight and fat weight**

Body weight and epididymal fat weight were measured at the end of the experiments. No difference was found between Ctrl and KO under either LD or DS conditions (Figure 9).

## Discussion

Here we addressed the hypothesis that a functional molecular clock in adult mice would restrain their ability to adapt to circadian disruption. We utilized mice in which the only non-redundant core clock gene, *Bmal1* is deleted postnatally in a tamoxifen dependent manner. Previously we had shown that delaying such gene deletion until adulthood disrupted oscillatory gene expression, behavior and BP as in conventional *Bmal1* KOs but that the two KOs diverged considerably in their impact on non-cycling genes and various non-behavioral phenotypes. These observations may reflect a role for *Bmal1* independent of the core clock during development. Indeed, other core clock genes may also have “off target” effects. For example, *Per2* (Period 2), but not other clock genes, mediates a metabolic switch from fatty acid to glucose use in cardiomyocytes, and thus protects heart from ischemic injury in mice(10).

Here, the mice were subjected to a series of disrupted light schedules and a jet lag protocol. Generally, the KO mice adapted their behavior more readily to this circadian disruption than did the controls. Thus, while Ctrl mice could not be entrained under 10h:10h, 8h:8h or randomized light/dark regimes according to behavioral results, while the inducible KO mice displayed higher activity levels in most dark phases of these schedules. The exception was the 3h:3h light/dark regime in which the Ctrl mice showed adaptation, displaying higher activity in the dark than the light phases. Despite this, circadian rhythmicity was apparent suggesting an ultradian mechanism. Insulin sensitivity similarly was readily disrupted by circadian disruption in the Ctrl mice but not the *Bmal1* KOs. However, this phenotype was not accompanied by changes of body weight or epididymal fat weight, perhaps because the schedules in our study were insufficiently prolonged and/or disruptive to affect fat composition.

Surprisingly, SBP did not display a similar pattern in KOs despite their behavioral adaptation to disrupted schedules. While it mirrored behavior in Ctrl mice in that it failed

to adapt to these schedules. This suggests that BP is subject to more direct control by the clock than behavior at least under basal conditions. This is reminiscent of our previous findings of dissociation between behavior and BP regulation by the clock (11, 12). It's reported that cardiac function was compromised by mismatch between internal clock and external clock (13). It's worthwhile to see if the loss of the internal clock is protective of heart under such misaligned condition.

A loss of clock function may be beneficial in some settings in nature. Thus, reindeer(14) and the Svalbard ptarmigan(15), loose circadian behavior during winter and summer when the arctic sun neither rises nor sets. The molecular clock in the European hamster is arrested during hibernation. Despite continued expression of clock genes in the suprachiasmatic nucleus, they stop delivering rhythmic output signals(16).

In summary, molecular clocks in central and peripheral tissues serve to integrate and coordinate trans- tissue function and are intrinsic to cardio-metabolic and immunologic efficiency. Disruption of the molecular clock by deletion of *Bmal1* results in disease phenotypes that reflect this fundamental function. The adverse effects of circadian disruption are consequent to desynchronization between central and peripheral clocks and heterogeneity of entrainment kinetics between different organs, despite retention of circadian rhythmicity(17). Paradoxically, molecular clockworks, protective of our health under basal conditions, may undermine our ability readily to adapt to these disruptions, so common in modern life.

## **Methods**

### **Mice**

*Bmal1<sup>fl/fl</sup>* mice were obtained from Christopher A. Bradfield (University of Wisconsin, Madison, WI)(18). The floxed mice were crossed with a TAM (Sigma-Aldrich, MO)-inducible universal *Cre* (*EsrCre*) mice (Jackson lab, ME) to generate *Bmal1<sup>fl/fl</sup>-EsrCre* mice. Both flox and *Cre* mice were on a C57BL/6 background. To achieve global deletion of *Bmal1* (KO), each mouse was treated with 5mg TAM (in corn oil) per day by gavage for 5 consecutive days when they were 3-4 months old(9). Meantime, *Cre*- mice were also

treated with TAM as littermate controls (Ctrl). All mice in the study were male and single housed. They all had free access to food and water.

### **Telemetry recordings**

The radiotelemetry device (model No. TA11PA-C20, Data Sciences International, MN) was implanted through catheterization of the carotid artery as previously described(12). Following 1-week recovery, locomotor activity and blood pressure (BP) were continuously recorded throughout the studies.

### **Study design (Figure 1)**

Radiotelemetry-implanted mice (5 for each genotype) were housed under LD condition and then released into DD. 10 days thereafter, mice were treated with 5 doses of TAM and followed while maintained under DD for 2 more weeks. Mice were then subjected to series of disrupted light/dark regimes including: 1, L:D=10h:10h for 200h (10 cycles); 2, L:D=8h:8h dark for 144h (9 cycles); 3, L:D=3h:3h for 72h (12 cycles); 4, LD for 72h (3 cycles); 5, randomized light/dark (random numbers were picked at <https://www.random.org> with lower limit at 2 & upper limit at 24) for 360h (14 cycles); 6, LD for 72h (3 cycles); 7, jet lag including two 8-h delay shifts and one 8-h advance shift at 3-day intervals (data were not collected due to the running out of telemetry battery); 8, LD for 72h (3 cycles).

Meanwhile, additional mice (10 Ctrl, 8 KOs) without radiotelemetry implants were kept under normal LD condition or disrupted schedules (DS) as described above. At the end of the experiments, all mice were weighed and sacrificed for measurement of epididymal fat weight.

### **Running wheel**

Two independent sets of mice were used for activity recording using a running wheel system (Animal Activity Meter: OPTO-M4, Columbus Instruments, OH). For set 1, mice were kept under DD for 5 days, then gavaged with TAM or corn oil for 5 consecutive days. For set 2, mice were kept under regular LD for 7 days followed by L:D=3h:3h for 7 days.

### **ITT**

ITT was determined after the series of disrupted schedules. Mice were fasted for 4 hours before ITT. Blood from the tail vein was used for measurement of glucose level before (time 0min) and at 30, 60, 90, and 120 min after intraperitoneal injection of insulin (1 U/kg) (Eli Lilly, IN). Meanwhile, non-implanted Ctrl and KO mice kept under LD were included as controls for “normal” conditions.

### **Statistics**

All statistical tests were two-sided. Student’s *t*-test was used when a single variable was compared between two groups. Two-way ANOVA with Bonferroni’s posttest was used for weight and ITT. The cutoff for significance was  $P < 0.05$  (\*). In all figures with error bars, the graphs depict means  $\pm$  SEM.

### **Study approval**

Animal experiments performed in this study were approved by Dalian Medical University IACUC, or by the University of Pennsylvania IACUC.

### **Author contributions**

GY, LC, and GAF conceived and designed the research. GY, LC, JZ, and BR performed experiments. GY, LC analyzed the data. GY, LC, and GAF wrote the manuscript.

### **Acknowledgement**

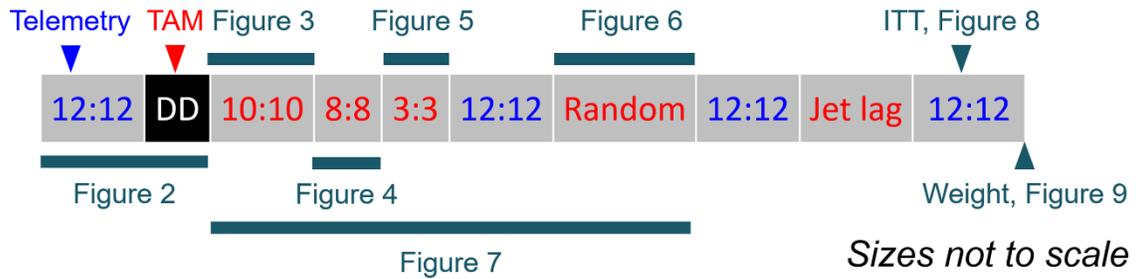
This work was supported by a grant from the NIH (U54TR001623), grants from the National Natural Science Foundation of China (81570643, 81670242 & 31871190), and a Merit Award from the American Heart Association. Dr. FitzGerald is the McNeil Professor of Translational Medicine and Therapeutics.

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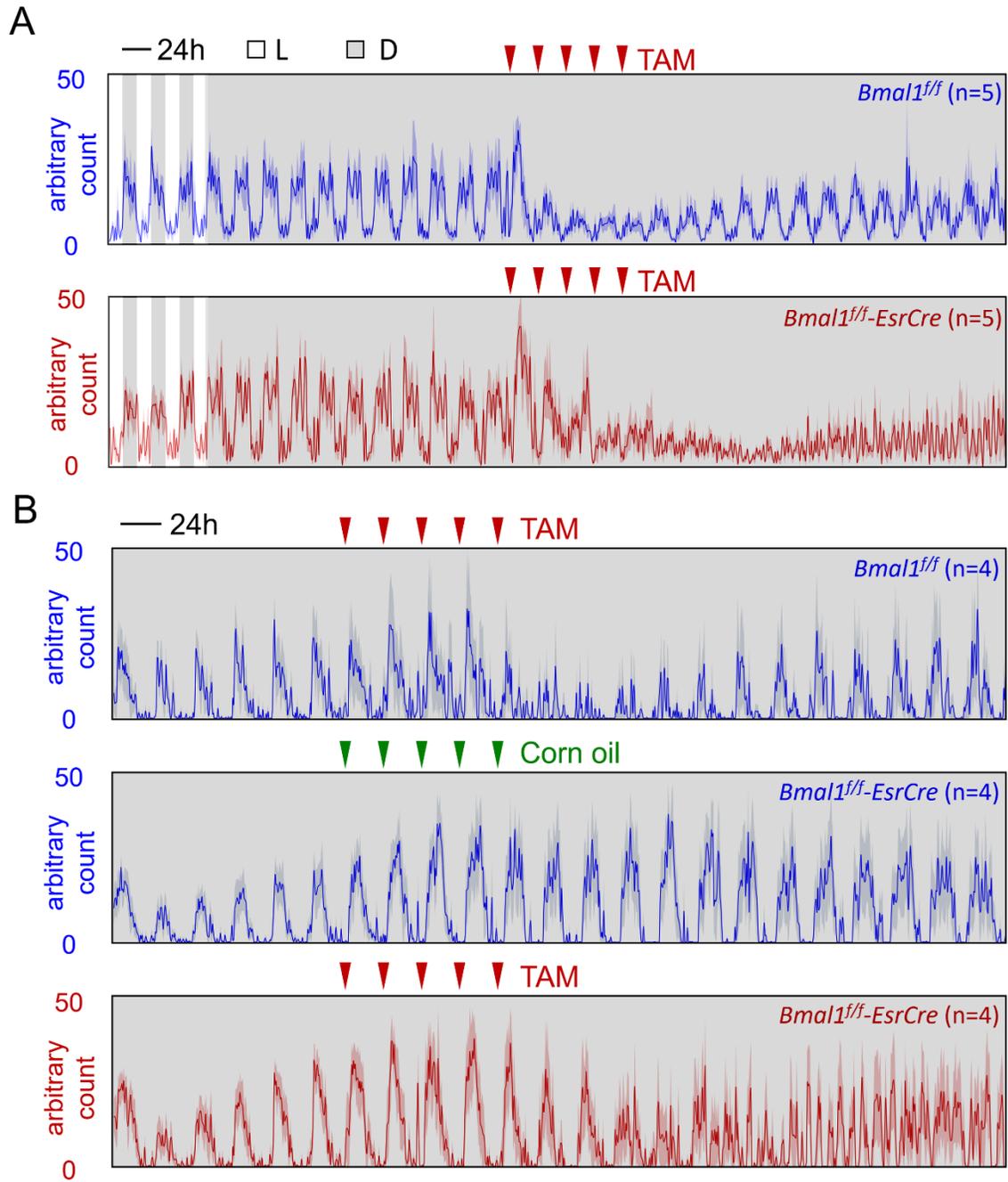
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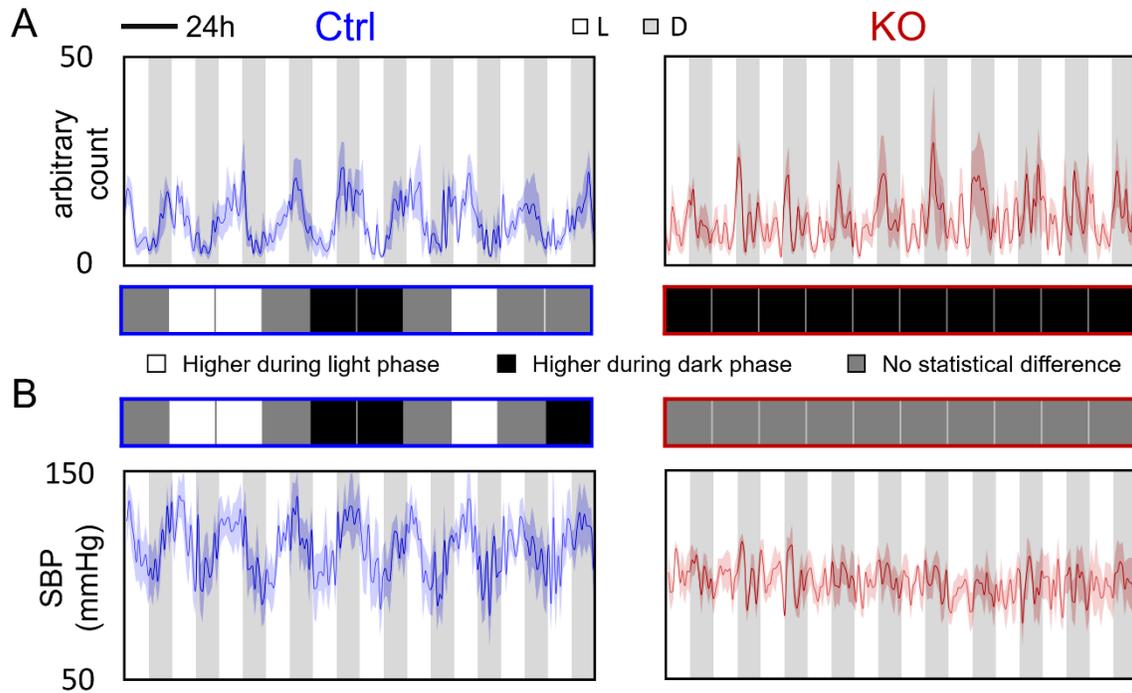
## Figures



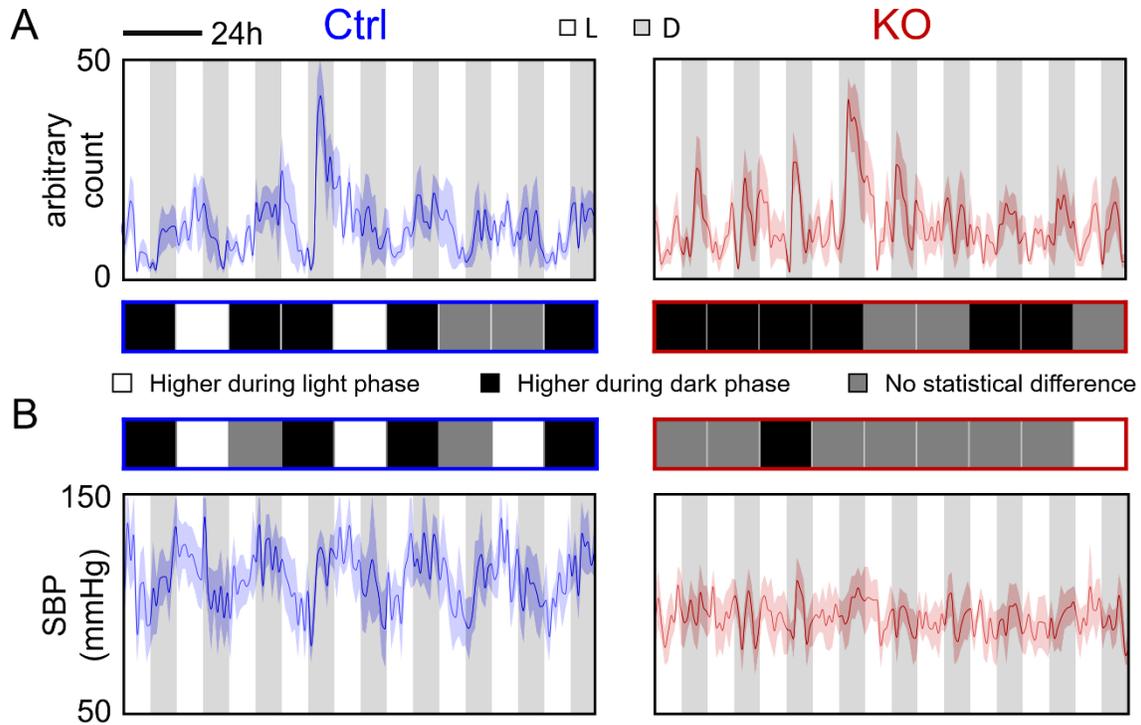
**Figure 1, Study design.** Telemetry-implanted mice were firstly maintained under 12h light/12h: dark (LD), and then released to constant darkness (DD), during which they were treated with TAM. 2-w later, mice were subjected to series of disrupted light/dark schedules including 10h light/10h dark, 8h light/8h dark, 3h light/3h dark, LD, randomized light/dark, LD, jet lag, and LD. ITT, body weight, and epididymal fat weight were measured after the series of schedules.



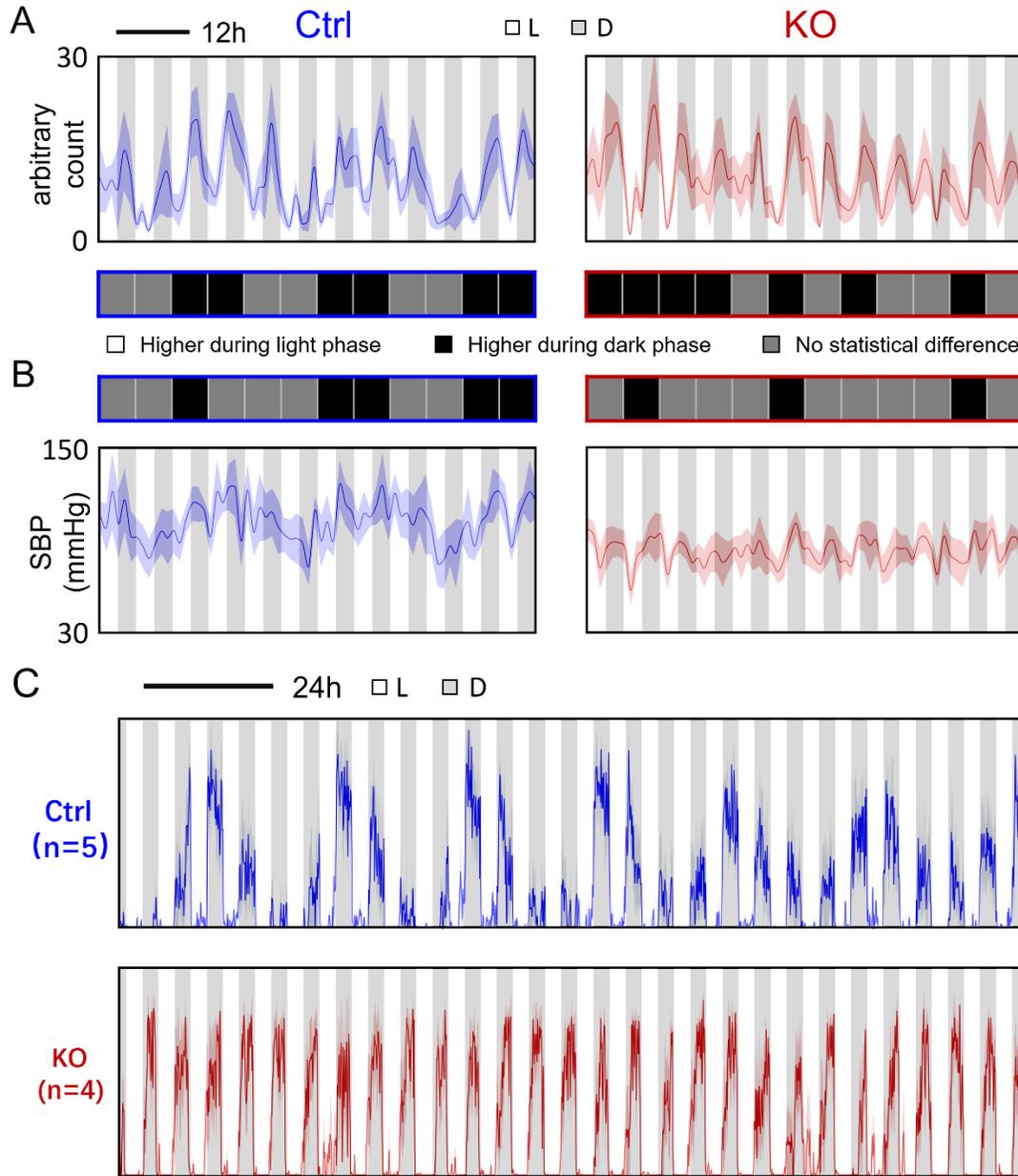
**Figure 2, Locomotor activity under constant darkness.** (A) Telemetry-implanted mice were kept under 12h light/12h dark followed by constant darkness for 1 month. 10 days after released to constant darkness, mice were treated with 5 doses of tamoxifen (TAM, red triangles). (B) Locomotor activity was recorded using running wheels. The activity level was depicted as average $\pm$ SEM (shade).



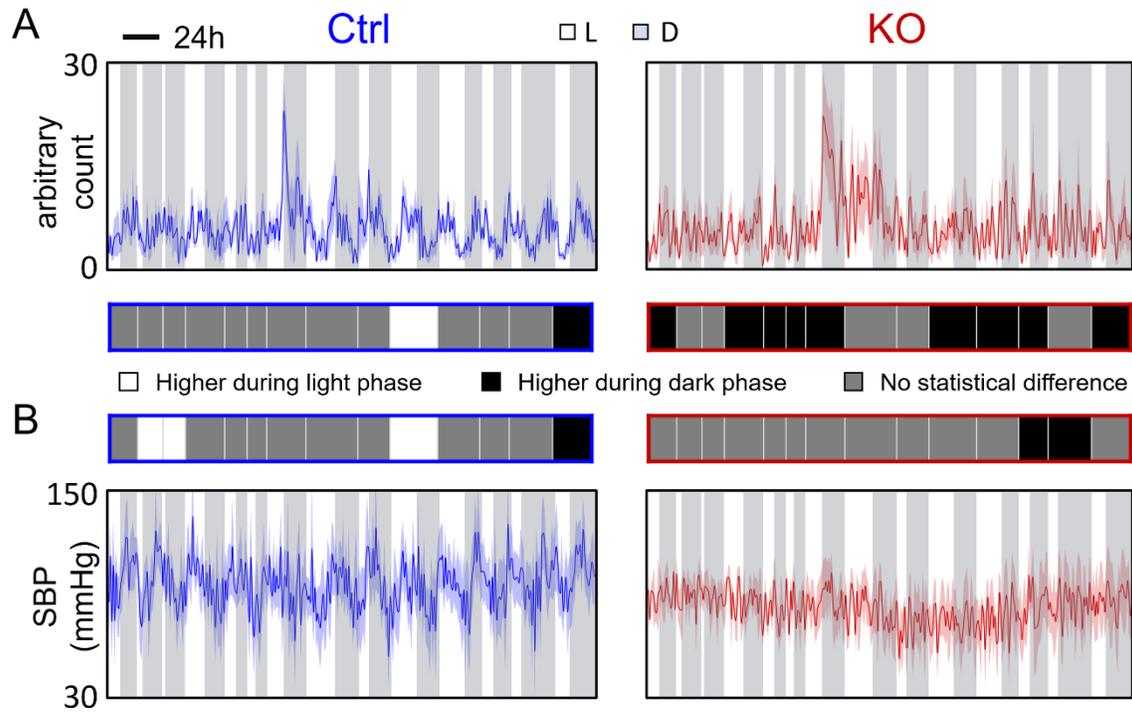
**Figure 3, Locomotor Activity and SBP (systolic blood pressure) under L:D=10h:10h condition.** Locomotor activity (A) and SBP (B) were measured using radiotelemetry. Their average levels between light phase and dark phase within each cycle were compared. The differences were shown in the middle (n=5 for each group, Student's *t*-test): white square, significantly higher activity level or SBP during light phase ( $P<0.05$ ); black square, significantly higher activity level or SBP during dark phase ( $P<0.05$ ); grey square, no statistical difference ( $P>0.05$ ) in activity level or SBP between light phase and dark phase.



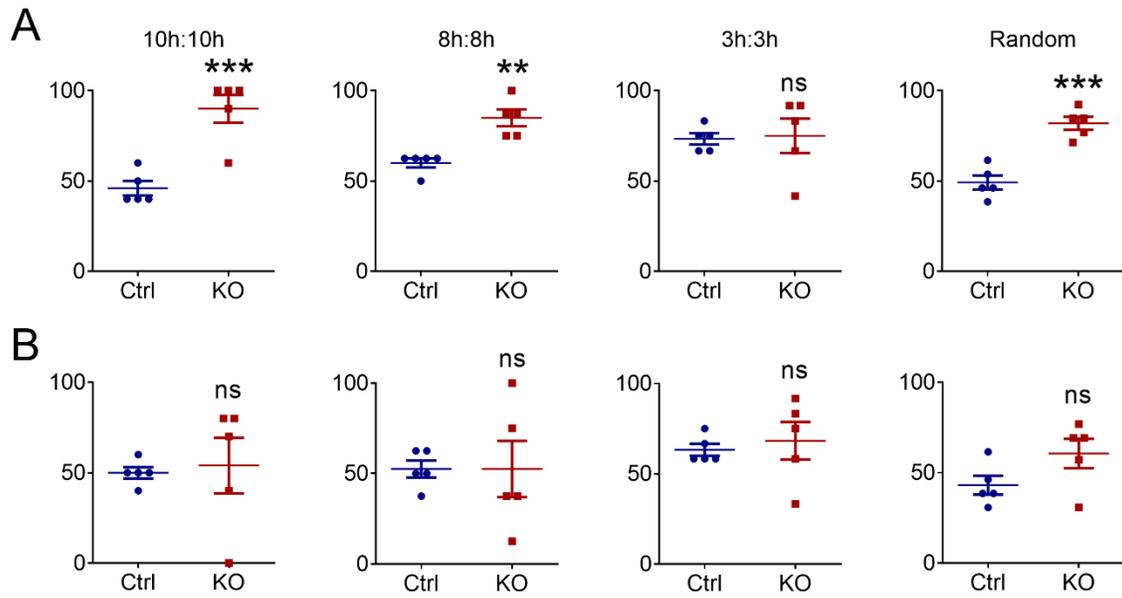
**Figure 4, Locomotor Activity and SBP (systolic blood pressure) under L:D=8h:8h condition.** Locomotor activity (A) and SBP (B) were measured using radiotelemetry. Their average levels between light phase and dark phase within each cycle were compared. The differences were shown in the middle (n=5 for each group, Student's *t*-test): white square, significantly higher activity level or SBP during light phase ( $P<0.05$ ); black square, significantly higher activity level or SBP during dark phase ( $P<0.05$ ); grey square, no statistical difference ( $P>0.05$ ) in activity level or SBP between light phase and dark phase.



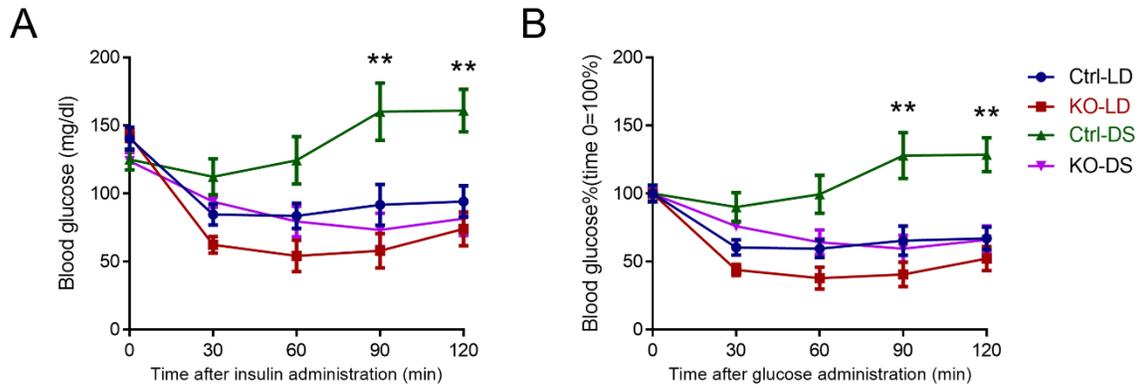
**Figure 5, Locomotor Activity and SBP (systolic blood pressure) under L:D=3h:3h condition.** Locomotor activity (A) and SBP (B) were measured using radiotelemetry. Their average levels between light phase and dark phase within each cycle were compared. The differences were shown in the middle (n=5 for each group, Student's *t*-test): white square, significantly higher activity level or SBP during light phase ( $P<0.05$ ); black square, significantly higher activity level or SBP during dark phase ( $P<0.05$ ); grey square, no statistical difference ( $P>0.05$ ) in activity level or SBP between light phase and dark phase. C, Wheel running activity under L:D=3h:3h condition. The activity level was depicted as average $\pm$ SEM (shade).



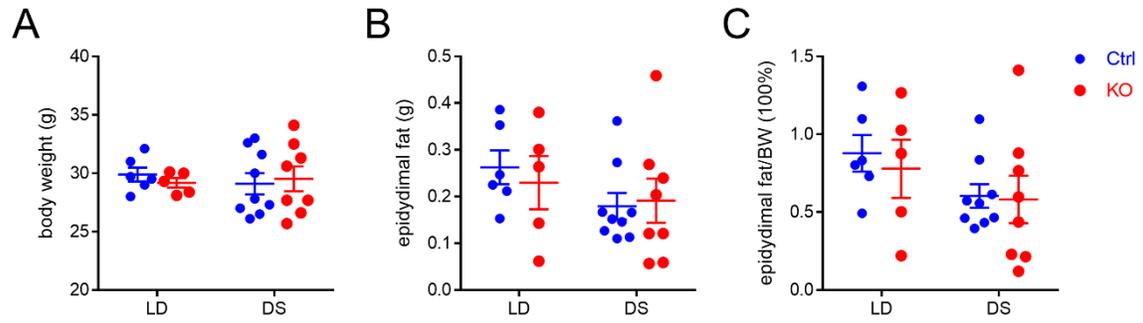
**Figure 6, Locomotor Activity and SBP (systolic blood pressure) under random light/dark conditions.** Locomotor activity (A) and SBP (B) were measured using radiotelemetry. Their average levels between light phase and dark phase within each cycle were compared. The differences were shown in the middle (n=5 for each group, Student's *t*-test): white square, significantly higher activity level or SBP during light phase ( $P < 0.05$ ); black square, significantly higher activity level or SBP during dark phase ( $P < 0.05$ ); grey square, no statistical difference ( $P > 0.05$ ) in activity level or SBP between light phase and dark phase.



**Figure 7, Percentage of cycles with higher activity level (A) and SBP (B) during dark phases (n=5 for each group, \*\*, p<0.01; \*\*\*, p<0.001; ns, not significant).**



**Figure 8, ITT (insulin tolerance test) in Ctrl and KO mice under regular light/dark condition (LD) or disrupted schedules (DS).** A, blood glucose levels (mg/dl) after insulin injection; B, ITT results were depicted as percentage. Basal blood glucose level of each group was defined as 100% (n=5 for each group, \*\*, p<0.01, Ctrl-DB vs. all other 3 groups, two-way ANOVA with Bonferroni's posttest).



**Figure 9, Body weight and epididymal fat weight of mice under regular light/dark condition (LD) or disrupted schedules (DS). A, body weight; B, epididymal fat weight; C, the ratio of epididymal fat weight to body weight. (n=5-9, two-way ANOVA with Bonferroni's posttest).**