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## Behavioral Rhythmicity of Mice Lacking AhR and Attenuation of Light-induced Phase Shift by 2,3,7,8-Tetracholorodibenzo-*p*-dioxin

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### Abstract

Transcription factors belonging to the Per/Arnt/Sim (PAS) domain family are highly conserved and many are involved in circadian rhythm regulation. One member of this family, aryl hydrocarbon receptor (AhR), is an orphan receptor whose physiological role is unknown. Recent findings have led to the hypothesis that AhR has a role in circadian rhythm, which is the focus of the present investigation. First, time-of-day dependent mRNA expression of *AhR* and its signaling target, cytochrome p4501A1 (*Cyp1a1*) was determined in C57BL/6J mice by quantitative RT-PCR. Circadian expression of *AhR* and *Cyp1a1* was observed both in the suprachiasmatic nucleus (SCN) and liver. Next, the circadian phenotype of mice lacking AhR (AhRKO) was investigated using behavioral monitoring. Intact AhRKO mice had robust circadian rhythmicity with a similar *tau* under constant conditions compared to wild-type mice, but a significant difference in *tau* was observed between genotypes in ovariectomized female mice. Time to re-entrainment following 6-h advances or delays of the light/dark cycle was not significantly different between genotypes. However, mice exposed to the AhR agonist 2,3,7,8-tetracholorodibenzo-*p*-dioxin (TCDD, 1 µg/kg BW) displayed decreased phase shifts in response to light and had altered expression of *Per1* and *Bmal1*. These results suggest that chronic activation of AhR may affect the ability of the circadian timekeeping system to adjust to alterations in environmental lighting by affecting canonical clock genes. Further studies are necessary to decipher the mechanism of how AhR agonists could disrupt light-induced phase shifts. If AhR does have a role in circadian rhythm, it may share redundant roles with other PAS domain proteins and/or the role of AhR may not be exhibited in the behavioral activity rhythm, but could be important elsewhere in the peripheral circadian system.

### Keywords

dioxin toxicity; bHLH-PAS; light entrainment; period; drinking; wheel-running; mice

### INTRODUCTION

Many proteins involved in the clockwork mechanism, including CLOCK (circadian locomotor output cycles kaput), BMAL1 (brain and muscle, ARNT-like) and NPAS2 (neuronal PAS protein 2), belong to the basic helix-loop-helix/Per-ARNT-SIM (bHLH/PAS) domain family.

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Another bHLH/PAS domain protein, aryl hydrocarbon receptor (AhR), has been extensively studied for its role in mediating the toxicity of environmental contaminants, such as the halogenated aromatic hydrocarbons. Among them is the prototypical dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin).

TCDD is formed as a by-product of combustion processes, during chlorine bleaching of paper-products and in the production of pesticides and herbicides. Human and animal exposure occurs at low levels primarily through the consumption of contaminated food such as fish, meat and dairy products (Travis and Hattemer-Frey 1991). Due to the lipophilicity of TCDD and its slow rate of metabolism, it bioaccumulates in animals and humans and biomagnifies in the environment. It is not readily metabolized *in vivo* and its half-life can be as long as several to more than 10 yrs in humans (Aylward et al. 2005). Toxic effects of TCDD include teratogenesis, immune impairment, carcinogenesis, and developmental and reproductive dysfunction (Birnbau 1994; Pohjanvirta and Tuomisto 1994).

AhR activation by TCDD leads to nuclear translocation of the TCDD bound AhR followed by its dimerization with another PAS domain-dependent transcription factor, the aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR:ARNT heterodimer binds to xenobiotic response elements (XREs; also known as dioxin response elements, DREs) to induce a battery of AhR responsive genes including those that are involved in xenobiotic metabolism (Nebert et al. 1993). Endogenous ligands for AhR have been sought for decades (Bjeldanes et al. 1991; McMillan and Bradfield 2007; Rannug et al. 1995; Song et al. 2002). However, they remain elusive and thus, the physiological role of AhR remains unknown.

AhR sequences have been identified in early vertebrates and possible homologs have been suggested even in *C. elegans* (Hahn et al. 1997). Mice lacking AhR show abnormal liver development, ductus venosus retention, immune system impairment, and abnormal ovarian follicular development (Benedict et al. 2000; Fernandez-Salguero et al. 1995; Lahvis et al. 2005; Schmidt et al. 1996). The evolutionary conservation of AhR and abnormal phenotype of AhR null mice strongly suggests the importance of AhR in normal physiological function. Recently, 55 tryptophan photoproducts have been suggested as potential endogenous AhR ligands, and AhR may have a role in the regulation of biological rhythms (Rannug and Fritsche 2006). The tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole (FICZ) blocks glutamate-induced phase shifts in SCN slices (Mukai and Tischkau 2007), lending credence to this hypothesis.

Daily rhythms of AhR protein expression have been reported in liver, lung and thymus (Richardson et al. 1998). In addition, diurnal expression of *AhR* mRNA, as well as its downstream target gene, cytochrome P4501A1 (*Cyp1a1*), occurs in liver and pituitary (Huang et al. 2002). Thus, AhR related genes exhibit a circadian rhythm, similar to many other 'clock' genes. However, previous studies were done only under light/darkness (LD) conditions. We determined expression patterns of these genes both under LD and constant darkness (DD) conditions to reveal their endogenous rhythms in SCN and liver. We also examined circadian behavior in *AhR* null (*AhRKO*) mice. Finally, we tested the effect of TCDD on light-regulation of circadian rhythm. Our results demonstrate that *AhR* and its signaling targets maintain rhythmicity under DD. *AhRKO* mice overall have robust rhythmicity under LD, DD and LL conditions and adjust well to jetlag-like light entrainment shifts. However, exposure to TCDD attenuates light-induced phase shifts, suggesting interactions between activation of AhR signaling and the circadian system.

## MATERIALS AND METHODS

### Animals

Unless noted otherwise, 6-week-old wild-type male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used. For activity measurements in AhRKO, founding breeders on a C57BL/6J background (Schmidt et al. 1996) were bred at the University of Illinois, Urbana-Champaign. Genotypes of pups resulting from *AhR*<sup>+/-</sup> male/female crosses were determined by PCR (Benedict et al. 2000). WT siblings were used as controls in these experiments. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### Tissue Collection

Male C57BL/6J mice were housed 3–4 per cage in a light-tight chamber, provided with feed and water *ad libitum*, and entrained for at least 2 weeks under controlled lighting (12L:12D), temperature (22°C), and humidity (39%). Fluorescent light bulbs (13W, company, Sylvania, Danvers, MA) generated 400–500 lux of light to the cages. All other experiments described herein were done under these environmental conditions. Animals were decapitated; liver and SCN were collected at 4 h intervals starting at ZT0 (zeitgeber time, lights on at ZT0, lights off at ZT12). For DD collection, animals were placed in darkness for 48 h prior to collection at 4 h intervals. Circadian time (CT) was reckoned from the previous light/dark cycle. SCN were carefully punched from a 500 µm brain slice using a 1 mm diameter sample corer (Fine Science Tools, Foster City, CA). Tissues were snap-frozen and stored at –80°C until use.

For investigating clock gene expression in AhRKO and in TCDD-exposed mice, two time-points, CT8 and CT20, were chosen based on the time when clear out of phase-relationship of *Per1* and *Bmal1* was observed under DD conditions. In this experiment, after 2 weeks of entrainment under LD, the animals were orally dosed with 1 µg/kg BW of TCDD or vehicle (corn oil) during their lights-on period and released into DD 6 days after treatment. Both liver and SCN were collected on the third day in DD.

### Real-time Quantitative PCR

Total RNA was isolated using TRIzol (GIBCO BRL, MD) for liver and RNAqueous (Ambion, Austin, TX) for SCN, and qPCR was performed as previously described (Mukai and Tischkau 2007). Primers were designed across two exons to inhibit amplification of genomic DNA using Primer Express 3 (PE Applied Biosystems, UK). Specific primer sequences used were: forward 5'-TTCTTAGGCTCAGCGTCAGCTA-3' and reverse 3'-GCAAATCCTGCCAGTCTCTGAT-3' for *AhR*; forward 5'-CTACAAGCCAACATTTCTATCAGATGA-3' and reverse 5'-GGTCACATCCTACGACAAACAAA-3' for *Bmal1*; and as previously published (Mukai and Tischkau 2007) for *Cyp1a1* and *Per1*. The C<sub>t</sub> (threshold cycle) value was obtained, and relative RNA amount was calculated using the relative standard curve method (Applied Biosystems User Bulletin 2). Serial dilutions of pooled RNA, representative of all times of day, were used to generate standard curves. Linear regression analysis produced an equation for determining doubling efficiency and expression of each transcript. Internal standards were not used because many 'housekeeping-genes' exhibit circadian variation.

### Activity Recording

WT and AhRKO mice were housed individually in 30 × 19 × 12.5 cm cages (Ancare, Bellmore, NY) equipped with a water bottle attached to a lick sensor (Mini Mitter, Bend, OR). Some females were ovariectomized two weeks before initiation of activity recording to remove the

effect of endogenous estrogens on circadian rhythm. The initial intent of ovariectomy was to allow pooling of data from males and females without confounding effects of estrous cyclicity. Other females and all males remained intact. Lick counts were recorded in 1-min bins using VitalView (Mini Mitter, Bend, OR). After entrainment to 12L:12D schedule, animals were moved into DD for 18–21 days and subsequently to LL for 14 days to measure the effect of lighting changes on their circadian period (*tau*).

For 6-h shift experiments, animals were provided with 4.5-inch diameter activity wheels. Wheel revolutions were recorded upon the circuit closure of hermetically sealed reed switch (Hermetic Switch, Inc., Chickasha, OK), by a neodymium magnet (Magnetic Energies, Inc.; San Antonio, TX) attached to each wheel (Tischkau et al. 2003). After entrainment to a 12L:12D schedule, the light schedule was advanced by 6 h. After 14 days, the cycle was delayed by 6 h. For data analysis, lick and wheel-running counts in 1-min bins were recalculated into 10-min bins by ClockLab (Actimetrics, Evanston, IL). *Tau* was calculated from activity onset of the last 10 days in DD and LL. Number of days to re-entrainment to a new light schedule was estimated by visual analysis of the actograms. Advance shifts were assessed by activity onset; activity offset was used for delay shifts to account for masking by light.

### TCDD and Light Exposure

After entrainment in LD, animals were orally dosed with 1  $\mu\text{g}/\text{kg}$  BW of TCDD or vehicle (corn oil) during their lights-on period. They were subsequently placed into DD for 8 days; light pulse experiments were performed to measure phase-resetting after light exposure. Each animal was exposed to light (400 lux) for 30 min starting at CT16, as determined by adding a factor of  $24/\tau$  to the predicted onset of activity. *Tau* was calculated from the slope of a regression line drawn through the onset of activity 5 days prior to the treatment. Light exposure occurred in a separate lit chamber; wheel-running activity data were not collected during the treatment. After 30 min, the cage was returned to DD and collection of activity data was reinitiated. *Tau* after light treatment was calculated using the activity onset 4–8 days after light exposure.

### Statistics

Statistical analysis was performed by one way ANOVA followed by Tukey's test for pairwise comparisons or by Student's t-test using SYSTAT (SSI, Richmond, CA). The *p* values were two-sided and considered statistically significant at  $p < 0.05$ . All data are shown as mean  $\pm$  SEM.

## RESULTS

### Circadian expression of AhR and Cyp1a1 in SCN and in liver

To characterize daily expression patterns of *AhR* and one of its signaling target genes, we measured mRNA levels for *AhR* and *Cyp1a1* by real-time RT-PCR. Expression of *Per1* and *Bmal1* served as positive controls. Fig. 1 is double-plotted at CT(ZT)0 and CT(ZT)20 for easier visualization of rhythms. Results show a 24 h oscillation for *AhR* under both LD and DD, although they were less robust compared to *Bmal1* and *Per1*.

In SCN (Fig. 1, left panel), *AhR* displayed a peak at ZT12 ( $2.39 \pm 0.2$  fold vs. trough expression at ZT0,  $p < 0.0001$ ) under LD. After 48 h in DD, *AhR* expression peaked 4 h earlier at CT8 ( $1.91 \pm 0.3$  fold vs. trough at CT16,  $p < 0.01$ ). Levels of *Cyp1a1* were weakly rhythmic in the SCN. Peak expression of *Cyp1a1* was delayed compared to *AhR*; levels were highest at ZT20 ( $1.78 \pm 0.1$  fold vs. trough at ZT4,  $p = 0.01$ ) in LD and at CT12 ( $1.91 \pm 0.1$  fold vs. trough at CT0,  $p < 0.01$ ) in DD. *Per1* levels peaked at ZT4 ( $5.00 \pm 0.3$  fold vs. trough at ZT12,  $p < 0.0001$ ) in LD and at CT8 ( $2.31 \pm 0.2$  fold vs. trough at CT16,  $p < 0.01$ ) in DD. *Bmal1* was

characteristically out-of-phase with *Per1*, peaking at ZT20 ( $4.46 \pm 0.4$  fold vs. trough at ZT12,  $p < 0.0001$ ) in LD and at CT0 ( $3.52 \pm 0.1$  fold vs. trough at CT8,  $p < 0.0001$ ) in DD.

In liver (Fig. 1, right panel), *AhR* peaked at ZT8 ( $2.02 \pm 0.1$  fold vs. trough at ZT0,  $p < 0.0001$ ) under LD; *Cyp1a1* peaked at ZT20 ( $1.87 \pm 0.2$  fold vs. trough at ZT16,  $p < 0.001$ ), *Per1* peaked at ZT12 ( $16.95 \pm 0.2$  fold vs. trough at ZT0,  $p < 0.0001$ ) and *Bmal1* peaked at ZT20 ( $19.32 \pm 0.5$  fold vs. trough at ZT8,  $p < 0.0001$ ). Expression patterns were different under DD conditions. Under DD, *AhR* peaked 8 h earlier at CT0 ( $2.35 \pm 0.04$  fold vs. trough at CT12,  $p < 0.0001$ ). The *Cyp1a1* oscillation had a higher amplitude in DD than in LD, with a peak at CT12 ( $2.58 \pm 0.1$  fold vs. trough at CT4,  $p < 0.0001$ ). *Per1* had a reduced amplitude compared to LD but still peaked at CT12 ( $7.46 \pm 0.8$  fold vs. trough at CT4,  $p < 0.0001$ ) and *Bmal1* peaked at CT0 ( $27.16 \pm 0.8$  fold vs. trough at CT8,  $p < 0.0001$ ) under DD.

### Circadian activity rhythm of AhRKO mice

There were no apparent abnormalities in the circadian rhythm of AhRKO mice (Fig. 2). The circadian period of AhRKO males was not significantly different from that of WT males under both DD and LL conditions (Fig. 2G). Some females were ovariectomized to remove the effect of endogenous estrogens on circadian rhythmicity in order to pool the data with intact males. However, differences between these two groups were observed, therefore the data are shown separately. Data from intact females were also added as a comparison.

Drinking activity of intact females showed estrous cycle-dependent changes (Fig. 2B&E). Changes of activity rhythms caused by estrous cycle have been previously reported in various rodent species (Fitzgerald and Zucker 1976; Gerall et al. 1973; Morin et al. 1977). In this study, some mice showed clear estrous cycle-dependent changes (5 out of 7 and 4 out of 7 in WT and AhRKO, respectively). Of those that showed cyclicity, WT females had a cycle of 3–4 days whereas AhRKO had longer cycles of about 5–6 days. However, the circadian period under DD and LL was not significantly different between intact WT and AhRKO females (Fig. 2H).

The only group that showed significant difference ( $p < 0.05$ ) in circadian period between WT and AhRKO was ovariectomized females (Fig. 2I). AhRKO mice in this group had shorter *tau* ( $23.72 \pm 0.03$ h) compared to WTs ( $23.85 \pm 0.03$  h). *Tau* under LL was similar ( $25.35 \pm 0.06$  and  $25.46 \pm 0.11$  h, WT and AhRKO, respectively). Overall drinking activity appeared to be reduced in ovariectomized (Fig. 2C) compared to intact females (Fig. 2B), or males (Fig. 2A) as indicated on the actogram.

### Light entrainment shifts of AhRKO mice

To determine whether mice lacking AhR can re-entrain to new light conditions, animals were subjected to 6-h advance and delay shifts (Fig. 3). Wheel-running activity was used to measure days until re-entrainment after 6-h shifts. This change was made because significant drinking during the light phase in the previous study made analysis of activity onset difficult and forced the removal of some samples. We also wanted to determine whether activity differences could be observed using a different behavior. Clear differences between drinking (Fig. 2A&D) and wheel-running activity (Fig. 3A) are present in the actograms; however, no significant differences in wheel-running activity were observed between genotypes.

The time needed for mice to entrain to a 6-h advance shift varied from 2–10 days. There were no significant differences between WT ( $6.00 \pm 0.75$  days) and AhRKO mice ( $7.09 \pm 0.55$  days; Fig. 3B), although 3 out of 10 WT re-entrained as fast as 2–4 days, whereas no AhRKO (n=11) re-entrained that quickly. Activity offset was used to measure days to re-entrainment to delay shifts to avoid the effect of masking by light. There was no significant difference between the genotypes ( $2.87 \pm 0.44$  and  $2.58 \pm 0.42$  days; WT and AhRKO, respectively; Fig. 3C).



### Effect of TCDD exposure on light-pulse induced phase shifts

To study whether exposure to dioxins affects the response to nocturnal light exposure, circadian period and phase shifting was assessed in TCDD- and vehicle-treated mice. Rhythms in TCDD-treated mice under DD were as robust as in vehicle-treated mice (Fig. 4A). *Tau* was not altered by TCDD treatment before light exposure ( $23.70 \pm 0.04$  and  $23.71 \pm 0.05$  h, vehicle- and TCDD-treated) or after light exposure ( $23.68 \pm 0.04$  and  $23.72 \pm 0.04$  h, vehicle- and TCDD-treated) (Fig. 4B). However, the amplitude of phase delay after light exposure was significantly reduced in TCDD-treated ( $-1.69 \pm 0.09$  h) compared to vehicle-treated mice ( $-2.20 \pm 0.16$  h,  $p < 0.01$ ; Fig. 4C).

### Per1 and Bmal1 expression in AhRKO and TCDD-treated mice

To determine whether deletion of AhR or TCDD exposure affect clock gene expression, we examined *Per1* and *Bmal1* levels in SCN and liver of AhRKO and TCDD-treated mice at two timepoints, selected based on relatively high and low levels of expression of these genes under DD as in Fig. 1. *Per1* and *Bmal1* levels were not changed in the SCN of AhRKO mice, compared to controls at both CT8 and CT20 (Fig. 5A&C). However, *Per1* levels at CT8 were decreased 27% ( $p < 0.05$ ) in the SCN of TCDD- vs. vehicle-treated mice (Fig. 5A); TCDD had no effect on *Per1* in the SCN at CT20. *Bmal1* levels in the SCN were not changed at CT8 with TCDD treatment (Fig. 5C); at CT20 SCN levels of *Bmal1* were increased 21% ( $p < 0.05$ ) in TCDD-treated mice.

Effects of AhR deletion and TCDD treatment were more prominent in the liver (Fig. 5B, D). At CT8, levels of *Per1* were increased 44% ( $p < 0.05$ ) in AhRKO vs. WT control, but substantially decreased (51% vs. control,  $p < 0.01$ ) in TCDD treated mice (Fig. 5B). At CT20, *Per1* was not changed in AhRKO, but was increased 129% by TCDD exposure. *Bmal1* levels were unchanged at CT8, but were increased 52% ( $p < 0.05$ ) in AhRKO at CT20, compared to control at respective timepoints (Fig. 5D). With TCDD treatment, *Bmal1* levels were increased 633% ( $p < 0.001$ ) at CT8, but decreased by 56% ( $p < 0.05$ ) at CT20.

## DISCUSSION

Previous studies indicate that AhR protein expression exhibits a similar oscillatory pattern in the liver, lungs, and thymus of rats (Richardson et al. 1998), with a peak approximately at ZT5 under LD. *AhR* mRNA (Huang et al. 2002) expression was also clearly rhythmic in rat liver, although the time of peak relative to the light schedule (14L:10D) was not reported. However, whether expression of *AhR* or its signaling targets are under control of the endogenous circadian oscillator has remained an open question. Whereas AhR and its dimerization partner ARNT are present in the SCN and other brain regions (Petersen et al. 2000), circadian variation in the SCN has not, to our knowledge, been reported. Our results demonstrate circadian variation of *AhR* transcripts in both SCN and liver under LD and DD, although the overall amplitude of oscillation was smaller than the clock genes, *Per1* and *Bmal1*.

Peak mRNA expression of *AhR* in both SCN and liver under LD occurred at ZT12 and ZT8, respectively. Although apparently contradictory to a previous report (Richardson et al. 1998), because the protein expression peak in the liver in that report (ZT6) precedes the mRNA expression peak in the current study, differences may simply be related to species, rat versus mouse. Expression of the AhR target gene, *Cyp1a1*, was out-of-phase with *AhR* (4–8 h delay). Under DD, peak expression levels of both *AhR* and *Cyp1a1* were advanced. Whether this reflects shortening of the circadian period under DD conditions, or direct effects of light on expression patterns of these genes, remains to be explored.

Interestingly, the amplitude of *Cyp1a1* increased in DD compared to in LD, especially in liver. Although absolute values could not be compared between LD and DD because the PCR was performed at different times, it is possible that *Cyp1a1* expression is increased during the day, resulting in higher basal expression (CT4 under DD). Increased trough expression could result in attenuated amplitude of oscillation. UV light exposure upregulates down-stream target genes of AhR not only in skin but also in liver (Goerz et al. 1996), providing a potential mechanism for increased baseline *Cyp1a1* expression during the day. Additional experiments that examine AhR signaling in response to light exposure are required to test this hypothesis. Expression patterns of clock genes *Per1* and *Bmal1* are similar to previous reports (Karman and Tischkau 2006; Oishi et al. 1998).

AhRKO mice had no apparent abnormalities in behavioral circadian rhythmicity. Day/night differences in *Per1* and *Bmal1* transcripts followed the same pattern in AhRKO mice compared to controls. AhRKO mice re-entrained to a new light schedule similar to wild-type controls. However, in 6-h advance shift experiments, there was tendency for AhRKO mice to take longer to adjust to the new light schedule. An increased sample size may produce significant differences between genotypes. The only difference observed was the circadian period between AhRKO and WT of ovariectomized females. Although the explanation for this remains obscure, potential interactions between AhR and estrogen receptor (Klinge et al. 2000; Ohtake et al. 2003) may prove to be important. Estrogen replacement in ovariectomized females would help to clarify whether the changes observed are estrogen-dependent.

Robust behavioral rhythmicity in AhRKO mice, nevertheless, does not substantiate immediate rejection of the hypothesis that AhR has a role in circadian rhythm. Robust rhythmicity is observed in mice bearing single mutations of known clock genes (Dudley et al. 2003; van der Horst et al. 1999; Zheng et al. 2001). In fact, deletion of a single clock gene rarely causes complete disruption of rhythmicity (Dudley et al. 2003; van der Horst et al. 1999; Zheng et al. 2001), with the exception of severe disruption observed in *Bmal1* knockouts (Bunger et al. 2000). Conversely, deletion of two clock genes often results in clear behavioral arrhythmicity (Bae et al. 2001; Oster et al. 2002; van der Horst et al. 1999). Robust rhythmicity in *Clock* null mice (Debruyne et al. 2006) occurs because NPAS2 can functionally substitute for CLOCK (Debruyne et al. 2007). Similarly, a protein that shares redundancy with AhR may compensate for the complete absence of AhR. Therefore, it would be interesting to study the circadian phenotype of *AhR* mutant or AhRKO mice that have deletion of another clock gene such as *Clock* or *Npas2*.

TCDD affects circadian hormone rhythms (Jones et al. 1987; Pohjanvirta et al. 1989; Yellon et al. 2000) and feeding behaviors (Kelling et al. 1985; Seefeld et al. 1984). US Vietnam war veterans have sleep disorders that are dependent on TCDD exposure from the defoliant, Agent Orange (Liu et al. 2004). TCDD alters circadian expression of clock genes in murine hematopoietic stem and progenitor cells (Garrett and Gasiewicz 2006), in SCN of deer mice (Miller et al. 1999) and C57BL6 mice (Filipski et al. 2004). Oral TCDD exposure (10 µg/kg BW) has reportedly caused phase advances of behavior rhythm in deer mice (Miller et al. 1999), as well as behavioral splitting and arrhythmicity in mice with a high dose of TCDD (Frame et al. 2004).

However, we did not observe these phase advancing or arrhythmic effects in our behavioral studies. C57BL/6J mice exposed to TCDD showed robust rhythmicity in DD for at least 3 weeks following exposure. Transient shifts of wheel-running activity immediately after TCDD exposure were not apparent. Because many previous reports are available only in abstract form (Frame et al. 2004; Li et al. 2004; Miller et al. 1999), it is difficult to identify the precise cause of these discrepancies, but differences in TCDD dose and species/strain of animal used may contribute.

TCDD treated mice showed a small but significant reduction in light-induced phase resetting at CT16. This result is consistent with the inhibition of glutamate-induced phase shifts in brain slices preincubated with the AhR agonist, 6-formylindolo[3,2-b]carbazole (FICZ) (Mukai and Tischkau 2007). Several factors may contribute to the difference between complete inhibition of the phase shift *in vitro* and partial inhibition of the phase shift *in vivo* by AhR agonists. First, it may be due to a reduced ability of TCDD to reach the SCN when administered systemically *in vivo* (Pohjanvirta et al. 1990). Although *Cyp1a1* induction was observed after incubation of SCN 2.2 with FICZ, this was never observed in SCN *in vivo* with FICZ administered *i.p.* (Mukai and Tischkau 2007) or with TCDD administered *p.o.* (unpublished data; M. Mukai and S. Tischkau). Direct application of AhR agonists onto the SCN or into the third ventricle *in vivo* could be used to circumvent this problem. Second, the inhibitory effect of an AhR agonist on the phase shift at the level of the SCN firing rate rhythm may not translate efficiently to other mechanisms that could be involved in driving the wheel-running activity rhythm. Altered *Per1* and *Bmal1* mRNA expression at CT8 and CT20 with TCDD exposure suggest some changes in the clockworks. A phase-response-curve for light-induced phase shifts in TCDD treated animals will be necessary to determine whether these animals actually respond less to light overall, or whether their phase response curve is shifted, thus resulting in decreased response at CT16, the time-point selected in this study. The use of AhRKO mice is planned for future studies.

The mechanism that underlies the attenuation of the light response in TCDD-treated animals requires further investigation. However, immediate metabolism of endogenous ligands by the highly induced xenobiotic metabolic enzymes in certain peripheral tissues may also be important. Moreover, because AhR will be constantly occupied by TCDD, the cyclic pattern of AhR signaling, which is generated physiologically by binding of putative endogenous ligands, such as tryptophan photoproducts, will be disrupted. This may lead to an aberrant light signal.

It is also possible that activation of AhR by TCDD blocks light-induced phase shifts by interfering with induction of *Per1* or *Per2*. Interrelationships between AhR signaling and the *Per* genes have been demonstrated; TCDD-induced AhR signaling is altered in the mammary glands of *Per1<sup>ldc</sup>* and *Per1<sup>ldc</sup>/Per2<sup>ldc</sup>* (Qu et al. 2007). The physiological significance of these interactions requires further investigation.

Recent discoveries of new PAS domain proteins and their roles in circadian rhythm raise questions regarding whether this ancient family of proteins has evolved as a central mediator of circadian timing among different organisms and tissues. For example, NPAS2, a peripheral replacement for CLOCK in some tissues, is important in the food-entrainable oscillator. *Npas2* mouse mutants cannot adapt well to restricted feeding during daylight, leading ultimately to death (Dudley et al. 2003). *AhR* is expressed abundantly in various tissues, including the SCN (Petersen et al. 2000), other parts of the brain and the rest of the body (Abbott et al. 1995; Carver et al. 1994; Roman and Peterson 1998). It is possible that, like *Npas2*, *AhR* has evolved to have a specific role in the circadian control of peripheral clocks in a whole organismic system. In this study, both AhR deletion and TCDD exposure had greater effect on *Per1* and *Bmal1* mRNA expression in the peripheral clock, liver, vs. the master clock, SCN. In contrast, to the minimal effect observed in the SCN, the day/night pattern of both *Per1* and *Bmal1* were strikingly reversed after TCDD treatment in the liver. Determining whether the differences between tissues are due to distribution of TCDD or different responsiveness to TCDD will require further investigation. Although more complete investigation is necessary, it is possible that TCDD exposure caused a phase shift in the molecular clockworks in the liver.



In today's society where people live under schedules that ignore the natural photoperiod provided by sunlight, such as in night-shift workers and international travelers who experience jetlag, the ability to adjust the circadian rhythm is important. Chronic disruption of circadian rhythm manifests as various physiological problems that may lead to more serious diseases, such as sleep and eating disorders and cancer (Haus and Smolensky 2006). Xenobiotics that activate AhR are ubiquitous in the environment. Although the AhR-mediated toxicity of TCDD has been studied for decades, mechanisms remain ambiguous. Symptoms associated with dioxin exposure, such as sleep disruption, suggest a relationship with the circadian timing system. Therefore, further investigation on the inhibitory effect of dioxins on light regulation of circadian rhythm is necessary to understand potential novel mechanisms of action of TCDD.

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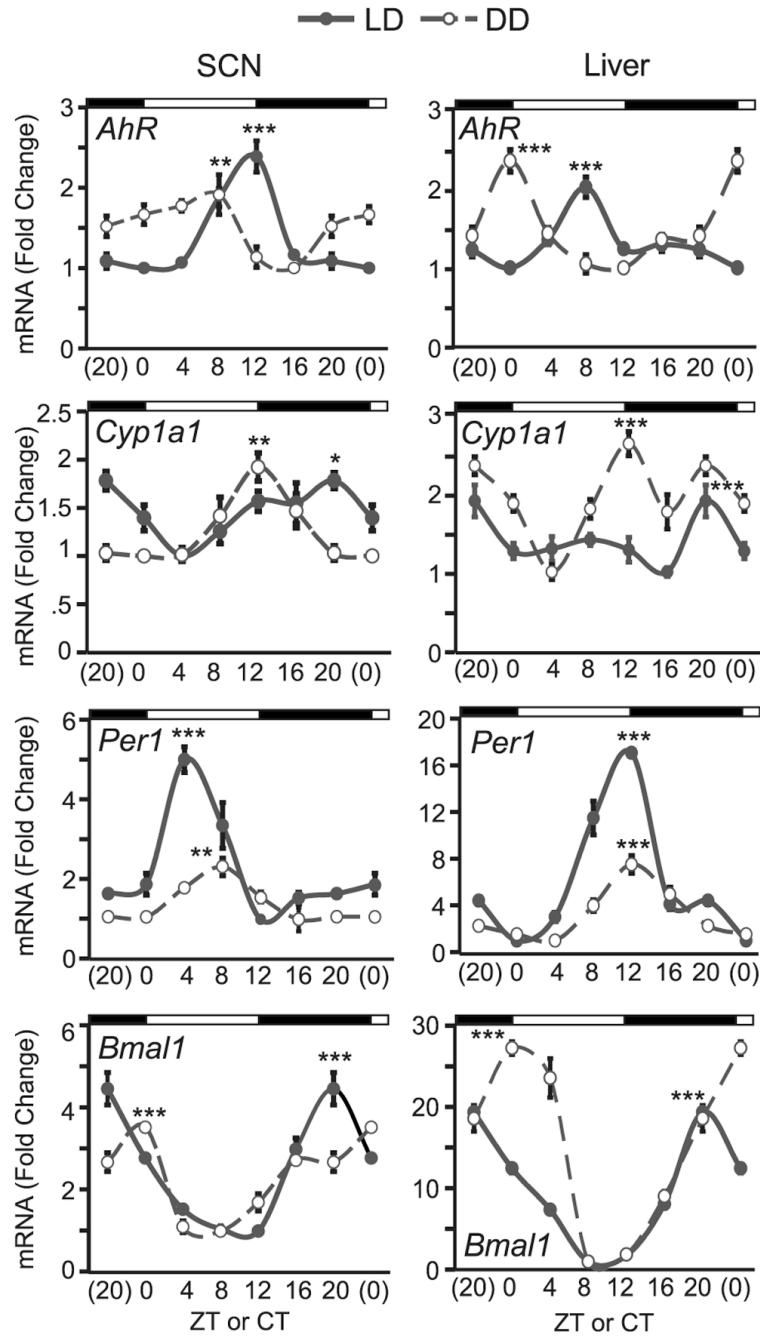
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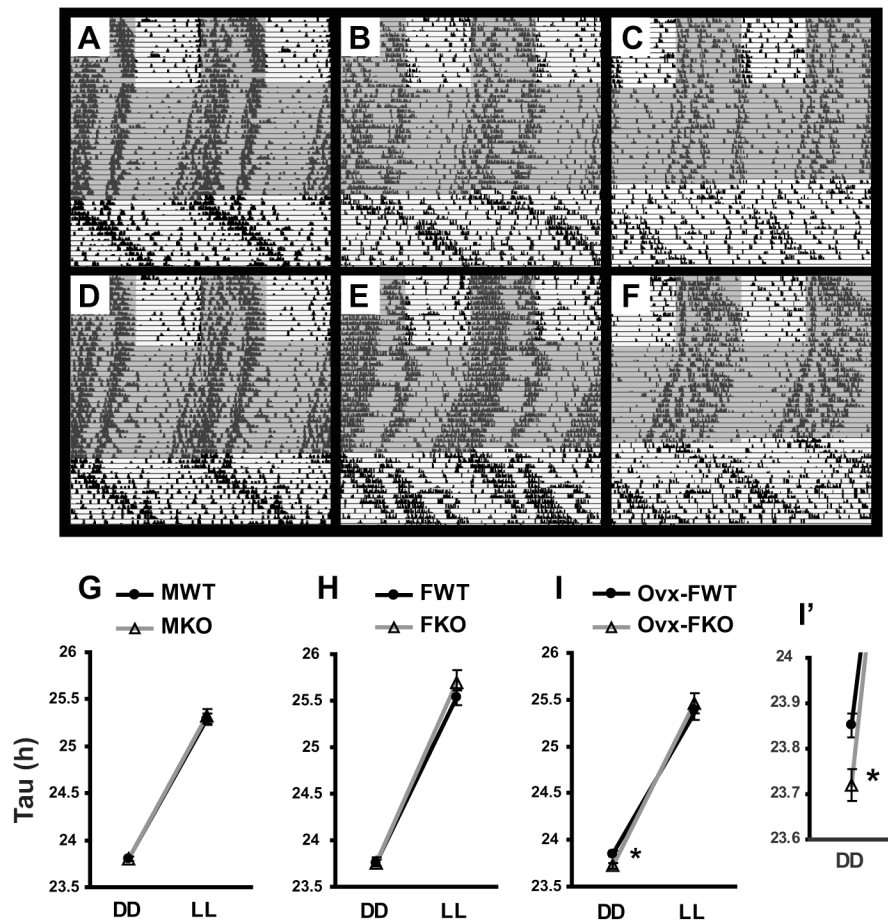
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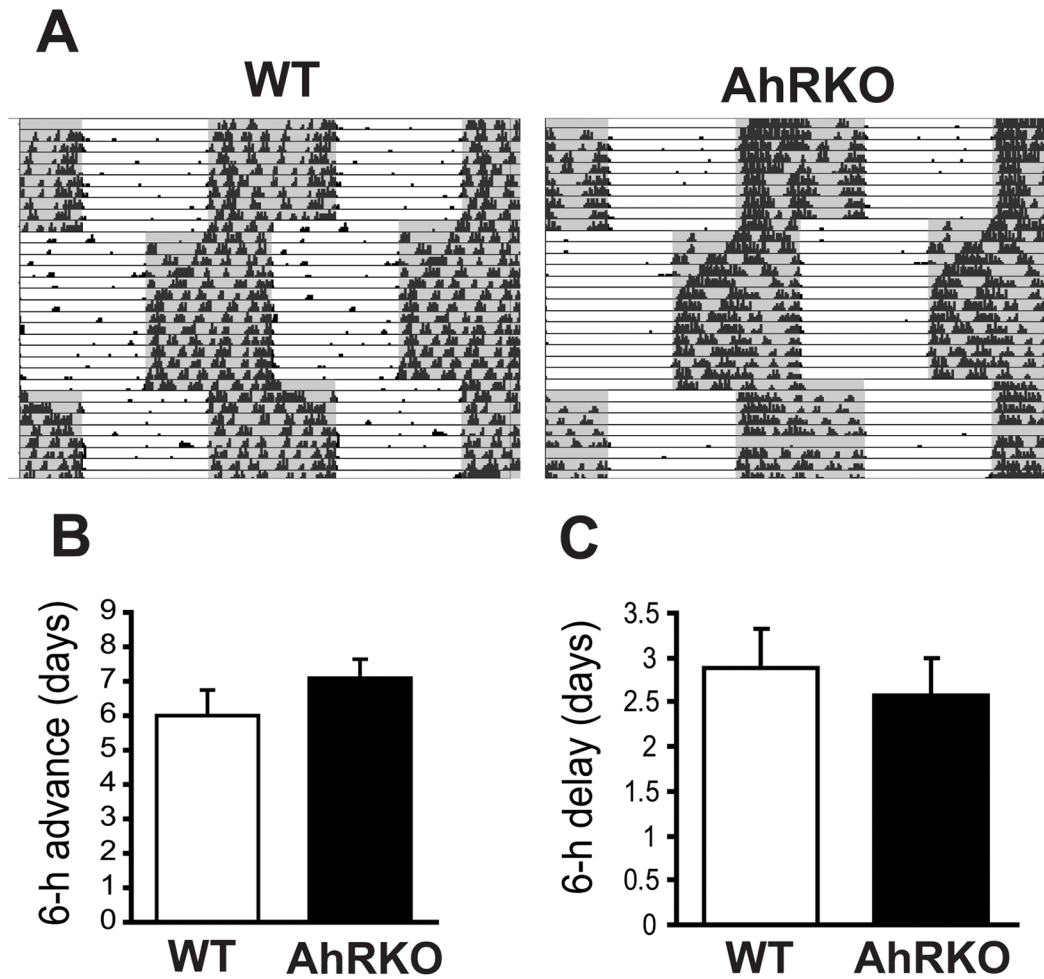
**Fig. 1. Circadian mRNA levels of *AhR*- and clock-related genes in C57BL/6/J SCN and liver**  
 Samples were collected every 4 hours under light/darkness (L12:D12, solid line) and constant darkness (DD, dotted line) schedules. Real time RT-PCR was used to measure *AhR*, *Cyp1a1* and *Per1*, *Bmal1* (top to bottom). Results are shown as mean fold-change to lowest (trough) level  $\pm$ SEM in SCN (left panel) and liver (right panel). Note that data at CT(ZT)0 and CT(ZT)20 are double plotted simply for easier visualization of the rhythm. The DD data was obtained from mice entrained under same lighting schedule as LD and released into DD for 48 hrs.  $n=3-4$ /group, \*  $p<0.05$ , \*\*  $p<0.01$ , and \*\*\*  $p<0.001$  by ANOVA in peak vs. trough expression comparison.



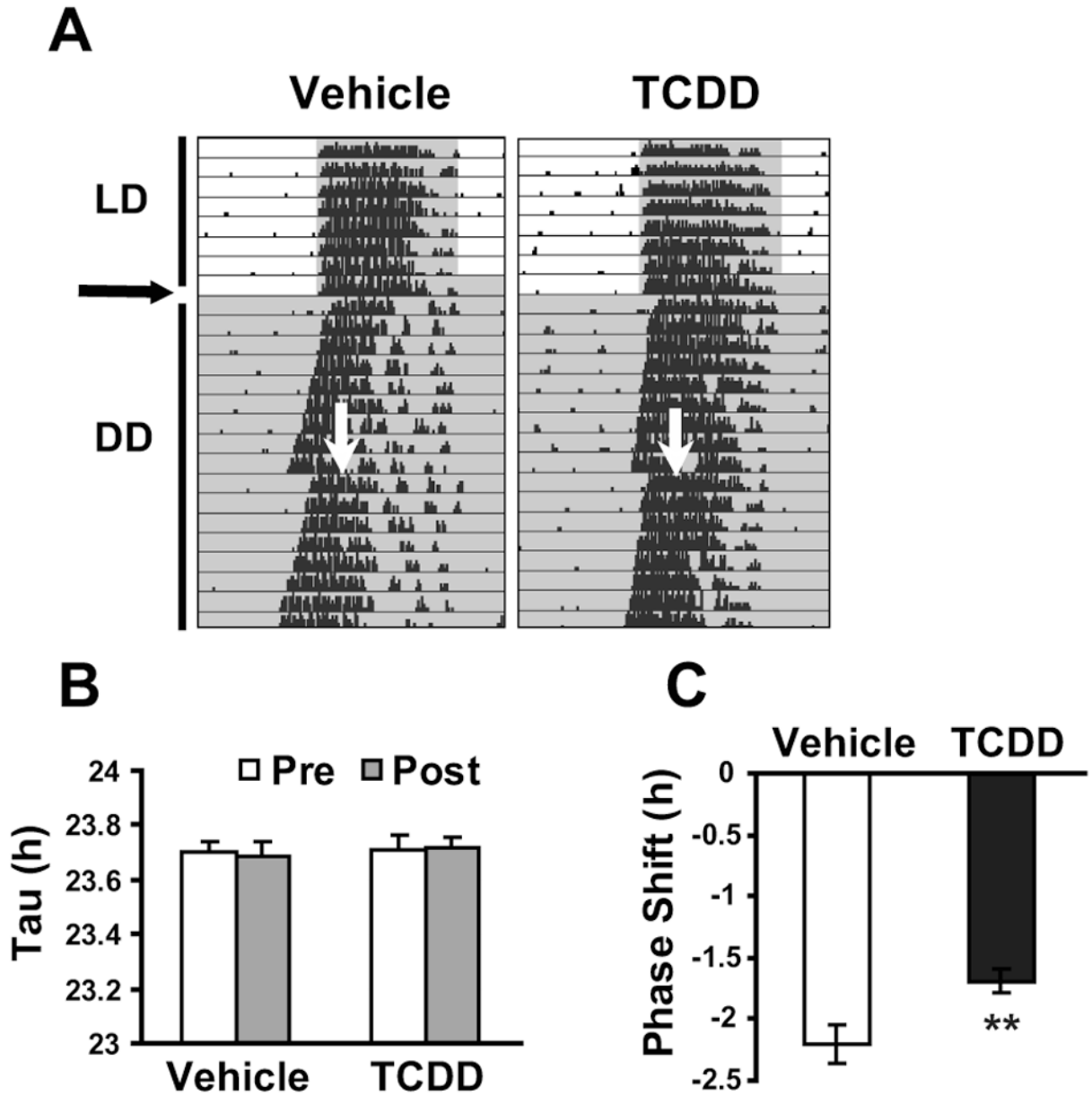


**Fig. 2. Double-plotted actogram and circadian periods of drinking activity in wild-type (WT) and AhR knockout (AhRKO) mice under constant light conditions**

Mice were entrained to 12L:12D schedule for 2 weeks, then placed in constant darkness (DD, gray background) for about 3 weeks and then placed constant light (LL, white background) for 2 weeks. Representative actogram of intact male (A,D), intact female (B,E), and ovariectomized female (C, F); WT (A,B,C) and AhRKO (D,E,F). Tau values of intact male (G), intact female (H), and ovariectomized female (I) under constant dark (DD) and constant light (LL). (I') Higher magnification of (I) at DD.  $n=5-11/\text{group}$ , \*  $p<0.05$  by ANOVA.

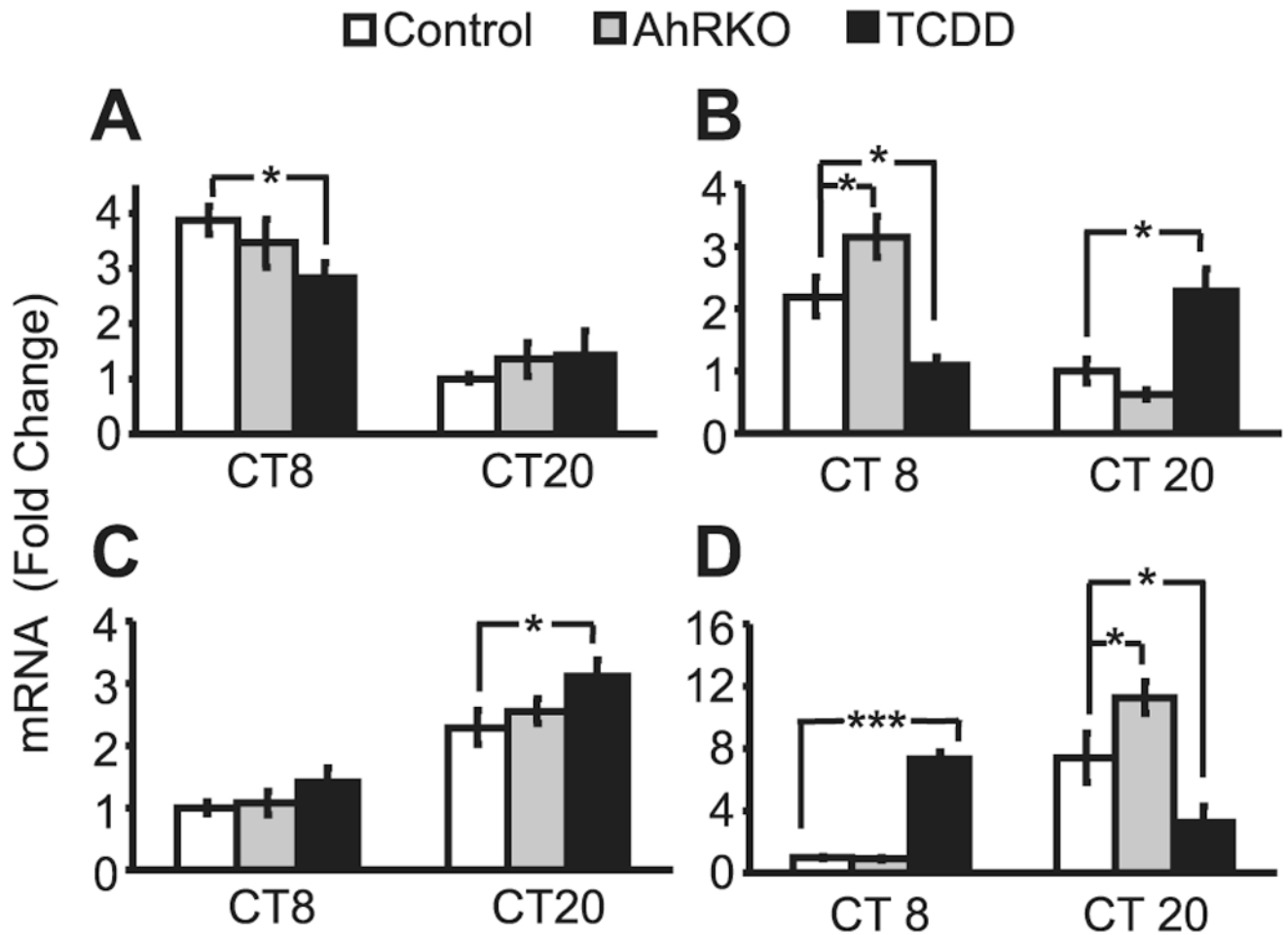


**Fig. 3. Re-entrainment of wild-type and AhR knockout (AhRKO) mice to 6-h jetlag shifts**  
 (A) Double plotted actogram measuring wheel-running activity during 6-h shifts. Days it took to re-entrain to new light schedule after 6-h advance shift (B) and delay shift (C). n=10–11/group



**Fig. 4. Effect of TCDD on light-induced phase shifts**

(A) Representative actogram of wheel-running activity. Mice were entrained in 12L:12D schedule and then 1 $\mu$ g/kg BW of TCDD or vehicle was orally exposed at horizontal arrow. Subsequently animals were placed under DD and given a 30-min light pulse at vertical arrow. (B) Circadian period pre- and post-light exposure at CT16–16.5 in vehicle and TCDD-treated mice. (C) Phase shift after light exposure at CT16–16.5 of vehicle and TCDD treated mice. n=9–10/group, \*\*p<0.01 by t-test.



**Fig. 5. Expression of *Per1* and *Bmal1* mRNA in SCN and liver of AhRKO and TCDD-treated mice**  
 SCN and liver were collected at CT8 and CT20 during the third day in DD, from control (WT, vehicle), AhRKO (vehicle), and TCDD-treated mice. Real time RT-PCR was used to measure mRNA expression of *Per1* (A,B) and *Bmal1* (C, D) in SCN (A, C) and liver (B, D). Results are shown in mean fold-change compared to lowest control level  $\pm$ SEM.  $n=4$  per group. \* $p<0.05$ , \*\* $p<0.01$  by ANOVA.