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CIRCADIAN CLOCK DISRUPTION IN THE MOUSE OVARY IN RESPONSE TO 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN

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Abstract

Activation of the aryl hydrocarbon receptor (AhR) by the highly toxic, prototypical ligand, 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) or other dioxin-like compounds compromises ovarian function by altering follicle maturation and steroid synthesis. Although alteration of transcription after nuclear translocation and heterodimerization of AhR with its binding partner, aryl hydrocarbon nuclear transporter (ARNT), is often cited as a primary mechanism for mediating the toxic effects of dioxins, recent evidence indicates that crosstalk between AhR and several other signaling pathways also occurs. Like the circadian clock genes, AhR is a member of the basic helix-loop-helix, Per-ARNT-SIM (bHLH-PAS) domain family of proteins. Thus, these studies tested the hypothesis that TCDD can act to alter circadian clock regulation in the ovary. Adult female c57bl6/J mice entrained to a typical 12 h light/12 h dark cycle were exposed to a single 1 µg/kg dose of TCDD by gavage. Six days after exposure, animals were released into constant darkness and ovaries were collected every 4 h over a 24 h period. Quantitative real-time PCR and immunoblot analysis demonstrated that TCDD exposure alters expression of the canonical clock genes, *Bmal1* and *Per2* in the ovary. AhR transcript and protein, which displayed a circadian pattern of expression in the ovaries of control mice, were also altered after TCDD treatment. Immunohistochemistry studies revealed co-localization of AhR with BMAL1 in various ovarian cell types. Furthermore, co-immunoprecipitation demonstrated time-of-day dependent interactions of AhR with BMAL1 that were enhanced after TCDD treatment. Collectively these studies suggest that crosstalk between classical AhR signaling and the molecular circadian clockworks may be responsible for altered ovarian function after TCDD exposure.

Keywords

AhR; Circadian Rhythm; BMAL1; PER2; Ovary

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1. Introduction

Dioxins and dioxin-like compounds are toxicants, detrimental to biological systems, that are produced as unwanted by-products of industrial processes (Kulkarni et al., 2008). The most potent of the dioxins, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is formed from combustion processes, during chlorine bleaching of paper-products and in the production of pesticides and herbicides. The resistance of TCDD to metabolism, as well as its lipophilic chemical nature, contributes to bioaccumulation in animals and humans, as well as biomagnification within the ecosystem. Its half-life can be more than 10 years in humans (Aylward et al., 2005). Human TCDD exposure at low levels commonly occurs through the consumption of contaminated meats and dairy products (Travis and Hattemer-Frey, 1991). Toxic effects of TCDD include teratogenesis, immune impairment, carcinogenesis, and developmental and reproductive dysfunction (Birnbaum, 1994; Pohjanvirta et al., 1994).

TCDD exposure causes compromised ovarian function through alteration of follicle development, alteration of steroidogenesis and inhibition of ovulation (Petroff et al., 2001). A single acute exposure to TCDD causes estrous cycle irregularity and a reduction in the ovulation rate in adult, cycling rats (Li et al., 1995a). TCDD alters levels of both gonadotropins and ovarian steroid hormones, and reduces ovulation in the gonadotropin-primed immature rat model (Li et al., 1995b). Effects of TCDD on ovarian function are likely mediated indirectly through the hypothalamic-pituitary axis, as well as directly at the level of the ovary (Hernandez-Ochoa et al., 2009; Miller et al., 2004; Petroff et al., 2001).

TCDD acts primarily through high affinity binding and activation of the aryl hydrocarbon receptor (AhR) (Beischlag et al., 2008). In the absence of ligand, AhR resides in the cell cytoplasm bound to heat shock protein 90, p23 and Ara9 (Petrulis and Perdew, 2002). Binding to TCDD or other ligands induces a conformational change in AhR, revealing a nuclear localization signal and promoting translocation to the nucleus, where AhR is released to partner with aryl hydrocarbon nuclear transporter (ARNT); the AhR/ARNT heterodimer acts as a transcription factor at specific promoter sites identified as dioxin response elements (DRE) to regulate the expression of target genes (Denison et al., 1989). Of particular toxicological relevance is the activation of various xenobiotic metabolizing enzymes, including the P450 enzymes CYP1A1, 1A2 and 1B1 (Kleiner et al., 2004; Nebert et al., 2004; Nebert et al., 1993). Although transcriptional activation of target genes is a major mechanism for AhR-dependent functions, mounting evidence demonstrates that numerous other pathways may be influenced by activation of AhR (Pocar et al., 2005). TCDD activates protein tyrosine kinases leading to changes in protein phosphorylation (Enan and Matsumura, 1995), and mobilizes internal calcium stores (Puga et al., 1997) in a transcriptionally-independent manner. AhR cross-talk with steroid hormone pathways, growth factor signaling and the cell cycle highlight the diverse and complex actions of AhR in the cell (Pocar et al., 2005; Puga et al., 2009).

AhR is a founding member of the PER-ARNT-SIM (PAS) domain family of transcriptional regulators. Typically, PAS domain-containing proteins act as environmental and/or physiological sensors to direct changes through protein-protein interactions and ultimately through alteration of gene expression. An analysis of intron/exon splice patterns of PAS domain-containing proteins reveals significant similarities between the canonical circadian clock gene, BMAL1 (brain and muscle ARNT-like) and AhR (Yu *et al.*, 1999). Typically, BMAL1 forms a heterodimer with CLOCK (Circadian Locomotor Output Cycles Kaput) to drive circadian rhythms. However, PAS domain proteins are notoriously promiscuous, forming various alternative pairs (Hogenesch et al., 1998; Probst et al., 1997). BMAL1 interacts with AhR *in vitro* (Hogenesch *et al.*, 1997) and *in vivo* (Xu et al., 2010). Recent evidence suggests that there are reciprocal interactions between AhR signaling and the

circadian clock. Activation of AhR inhibits the clock gene *Per1* (Garrett and Gasiewicz, 2006; Xu et al., 2010), and AhR signaling is modified when *Per1* is suppressed (Qu et al., 2007, 2009; Qu et al., 2010).

The molecular components of a functional circadian clock are present in the mammalian ovary (Fahrenkrug et al., 2006; Karman and Tischkau, 2006). Ovarian clock gene expression is regulated by gonadotropins and ovarian steroids (He et al., 2007b; Karman and Tischkau, 2006; Nakamura et al., 2010; Petrulis and Perdew, 2002; Tischkau et al., 2011). Although the function of the clockworks in the ovary remains ambiguous, circadian control of steroidogenesis and cell proliferation are apparent (He et al., 2007a, 2007b; Ratajczak et al., 2009). Recently, our laboratory has demonstrated that activation of AhR with TCDD or β -naphthoflavone (BNF) alters circadian rhythms in the central clock in the suprachiasmatic nucleus, as well as in the liver (Mukai et al., 2008; Mukai and Tischkau, 2007; Xu et al., 2010). This study was designed to examine the hypothesis that alteration of circadian rhythmicity contributes to the mechanism by which TCDD exerts its effects on the ovary.

2. Materials and Methods

2.1 Animals and TCDD Exposure

Adult female c57bl6/J mice (2 months of age) were obtained from Jackson Labs and housed 4 per cage under 12 h of light and 12 h of darkness, in light-tight chambers, for at least two weeks to entrain their circadian rhythms. Under these lighting conditions, denoted as LD, zeitgeber time 0 (ZT0) represents the time of lights-on in the colony. The corresponding time of lights-off is denoted as ZT 12. Room temperature and humidity were regulated at 22°C and 39%, respectively. Feed and water was available *ad libitum*. Animal use was in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and was approved by the Institutional Animal Care and Use Committee. All mice exhibited at least 3 consecutive regular estrous cycles, as determined by vaginal swabbing, prior to their inclusion in the experiment (Karman and Tischkau, 2006). Mice without regular estrous cycles were excluded from the experiment. Animals were orally dosed with 1 μ g/kg of body weight (BW) of TCDD or vehicle (corn oil) during their lights-on period and released into constant darkness (DD) 6 days after treatment (Mukai et al., 2008). Under DD conditions, animals express their endogenous circadian rhythm, denoted as circadian time (CT), where CT is arbitrarily set as the onset of light in the previous light dark cycle. Thus CT0 and ZT0 are the same time of day. After 48 h in constant darkness, animals were killed by decapitation every 4 h over a 24-h period (n=4 per group) starting at CT0. Ovaries were snap frozen in liquid nitrogen for immunoblot, co-immunoprecipitation or mRNA analysis.

2.2 Quantitative Real-time RT-PCR

Total ovarian RNA was extracted using TRI-reagent (Sigma, St. Louis, MO), according to the manufacturer's protocol. Concentration, quality and purity of RNA samples were determined with the Nanodrop ND-1000 UV spectrophotometer, and by gel electrophoresis. RT reactions and PCR were performed as previously described (Karman and Tischkau, 2006). Intron-spanning primers for AhR, *Per2* and *BMAL1* were chosen using Primer Express 3 software and have been published previously (Mukai et al., 2008; Mukai and Tischkau, 2007). Transcript levels were determined using the relative standard curve method for calculations. Standard curves were derived from 3 sets of pooled RNA samples using a range of RNA concentrations between 2 μ g and 62.5 ng. The standard curve equation for *AhR* was $y = -1.4068x + 42.221$ ($R^2 = 0.9755$, $E = 1.64$, where E = the efficiency of doubling in each cycle); for *Bmal1* was $y = -1.22x + 33.52$ ($R^2 = 0.9975$, $E = 1.76$); and for *Per2* was $y = -1.0587x + 35.958$ ($R^2 = 0.9924$, $E = 1.94$). Relative amounts of *AhR*, *Bmal1* and *Per2* were determined according to the equation: relative amount = $2^{-(Ct - \text{Intercept})/[\text{slope}]}$. Relative amount

was converted to percent of the maximum amount for each experiment. Data are presented as the mean $\pm$ SEM.

2.3 Immunoblot

Ovarian tissues were homogenized in Tissue Protein Extraction Reagent (Pierce, Rockford, IL) with protease inhibitor cocktail (Sigma, St. Louis, MO) on ice using cold glass beads and a bead beater. Protein was quantified using the Micro BCA protein Assay (Pierce, Rockford, IL). Samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes as previously described (Karman and Tischkau, 2006). Blots were blocked in non-fat dry milk, incubated with primary antibody solution overnight at 4°C (PER2 affinity purified 5 μ g/ml; ADI, San Antonio, TX; BMAL1 affinity purified 5 μ g/ml; ABR, Golden, CO; AhR 1 μ g/ml, Biomol, Plymouth Meeting, PA). After exposure to secondary antibody, membranes were washed and developed using chemiluminescent substrate according to the manufacturer's protocol (Pierce). Densitometry using the digital imaging software Scion Image for Windows 4.0.3 (Scion Corporation) provided quantitative analysis of data. The relative density was normalized against alpha tubulin. Data were plotted as fold change from the lowest point.

2.4 Co-immunoprecipitation

Tissue lysates were prepared from whole ovaries as described above, centrifuged at 14,000 $\times$ g for 5 min and then quantitated with the BCA kit. Soluble proteins (500 μ g) were incubated on a rotating platform with 2 μ g of goat anti-AhR antibody (Santa Cruz) or rabbit anti-BMAL1 antibody (ABR) at 4°C overnight, followed by incubation with immobilized protein A/G gel slurry (Pierce) at room temperature for 2 h with gentle mixing. Beads were collected by brief centrifugation and washed five times with 0.5 ml of immunoprecipitation (IP) buffer (25 mM Tris and 150 mM NaCl, pH 7.2). The complex-bound gel was washed with 0.5 ml of water and centrifuged for 3 min at 2500 $\times$ g, the supernatant was discarded, laemmli buffer was added and samples boiled for 5 min at 95°C. Proteins were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h. The membrane, containing protein pulled down by either anti-AhR or anti-BMAL1 antibody, was incubated with the alternate antibody, washed with TBST and incubated with HRP-conjugated secondary antibody (Jackson ImmunoResearch) for 1 h at room temperature.

2.5 Immunohistochemistry

Despite repeated attempts, the AhR antibody failed to show specific staining in the mouse ovary. Therefore, adult female Sprague-Dawley rats were used for these experiments. Housing was exactly as described for the mice. Rat ovaries were fixed in 10% neutral buffered formalin for 48 h at room temperature. After paraffin embedding, 4 μ m sections were cut. After deparaffinization, antigen retrieval, and blocking in normal goat serum, slides were incubated overnight in primary antibody, AhR (Biomol) or BMAL1 (ABR). Primary antibodies were used at 10 μ g/ml. Sections were washed and incubated with an HRP-labeled secondary antibody for 1 h (Vector Laboratories, Burlingame, CA). The peroxidase antibody complex was visualized using aminoethyl carbazole substrate (Zymed, San Francisco, CA). Control experiments included omission of the primary antibody and preabsorption with the peptide specific to the primary antibody. Sections were counterstained with hematoxylin.

2.6 Statistical Analysis

Results from qPCR and immunoblot studies were subjected to statistical analysis using two-way analysis of variance. Tukey's *post-hoc* analysis was applied when the analysis of variance indicated that statistically significant differences were present. A *p* value of 0.05 or less was considered statistically significant.

3. Results

3.1 TCDD alters circadian expression of circadian clock genes in the mouse ovary

Six days following administration of a single, low dose (1 µg/kg) of TCDD (or corn oil vehicle for the control group) by gavage, mice were placed into DD for 48 h to allow expression of their endogenous circadian rhythm. Starting at 48 h after placement into DD, mice were sacrificed and ovaries were collected every 4 h for 24 h. The status of the circadian clock was determined by examining 24-h profiles of transcripts and proteins for the canonical clock genes *Bmal1* and *Per2*. Control animals displayed a robust circadian variation in the levels of transcripts and proteins for both *Bmal1* and *Per2* (Fig. 1). *Bmal1* transcripts were maximal at CT0 with a 7.23 ± 0.14 fold oscillation between peak and trough; the oscillation for the BMAL1 protein was 4.24 ± 0.37 fold, with a peak also occurring at CT0 (Fig. 1a–b). The *Per2* transcript rhythm in control animals was out-of-phase with the *Bmal1* rhythm, as expected. *Per2* transcripts displayed a 4.79 ± 0.21 fold change, with a peak at CT12; the *Per2* protein oscillation was comparable to the transcript oscillation at 4.04 ± 0.31 fold with peak levels observed with the typical 4 h delay, at CT16 (Fig. 1c–d).

Bmal1 and *Per2* levels were altered after TCDD treatment. Peak levels of *Bmal1* transcripts were significantly suppressed in TCDD-treated mice; the amplitude of the change was reduced from 7.23 ± 0.14 fold to 2.40 ± 0.11 fold (Fig 1a). Peak transcript levels were observed at CT4, although there was no significant difference between CT4 and CT0. The amplitude of the BMAL1 protein oscillation was not altered by TCDD treatment, although the phase of the rhythm appeared to be shifted by 4 h. After TCDD treatment the time-of-peak *Per2* transcript levels shifted from CT12 to CT16, and the amplitude of the oscillation was not significantly changed (Fig. 1c). *Per2* protein oscillations were similarly altered such that the time-of-peak was delayed to occur 4–8 h later.

3.2 TCDD alters circadian rhythms of AhR in the mouse ovary

AhR transcript and protein were expressed with a circadian rhythm in the mouse ovary under control conditions (Fig. 2). Transcript levels for AhR were maximal at CT8 and minimal at CT0, with a 3.70 ± 0.22 fold difference between peak and trough values. The amplitude of the AhR protein oscillation was 3.98 ± 0.38 fold, with a peak at CT12 and minimal expression levels at CT0. Circadian expression of AhR transcript and protein was altered significantly by TCDD treatment. There was no oscillation in AhR transcript levels after TCDD treatment (Fig. 2a). The oscillation in AhR protein was retained upon TCDD treatment with a 3.15 ± 0.45 fold amplitude, although protein expression patterns were dramatically altered (Fig. 2b). Peak protein levels shifted from CT12 in controls to CT20 in TCDD-treated mice.

3.3 AhR interactions with BMAL1 are enhanced by TCDD treatment

Previously, we demonstrated that AhR could form a heterodimer with BMAL1 to alter *Per1* expression in the liver. To determine whether AhR and BMAL1 interact in the ovary, we first examined the expression patterns of both proteins in the rat ovary. Immunohistochemistry demonstrates that AhR and BMAL1 expression occurs in a similar pattern in the ovary (Fig. 3). Both BMAL1 (left column) and AhR (right column) are present in developing follicles at all stages of development, in the ovarian stroma, and in luteal cells.

Co-immunoprecipitation demonstrates that AhR can interact with BMAL1 in the ovary and that this interaction is enhanced after TCDD treatment (Fig. 4). After pull-down with the AhR antibody, ovarian extracts were run on a gel and probed for BMAL1 (Fig. 4a). In control samples, the band for BMAL1 was stronger at CT4 than at CT20. The BMAL1 band was enhanced in CT4 samples obtained from TCDD treated mice. AhR was also found in samples after a pull-down with the BMAL1 antibody (Fig. 4b). Furthermore, specificity of the interaction was demonstrated by showing that neither the nuclear BMAL1 protein nor AhR protein interacts with the primarily cytoplasmic calbindin protein and there were no bands in the NRS controls.

4. Discussion

The results presented in this manuscript clearly demonstrate that the circadian expression pattern of AhR is altered in the ovaries of adult female mice after exposure to a low dose of TCDD. Circadian patterns of expression of the canonical clock genes, *Bmal1* and *Per2*, are also altered after TCDD exposure. TCDD enhanced interactions between AhR and BMAL1, suggesting a potential mechanism for altered circadian rhythms. Thus, these data invoke a novel mechanism for dioxin action downstream of AhR activation and highlight the importance of crosstalk between AhR signaling and the molecular circadian clockworks in ovarian function.

To our knowledge, this is the first study to examine an alteration in expression of circadian rhythms in the ovary after TCDD treatment. Circadian expression patterns of AhR mRNA and protein, as well as the canonical clock genes *Bmal1* and *Per2*, were observed in the mouse ovary. It is not surprising that the clock genes were expressed with circadian periodicity in the mouse ovary, as previous studies indicated that these genes displayed diurnal rhythmicity in the rat ovary (Fahrenkrug et al., 2006; Karman and Tischkau, 2006; Nakamura et al., 2010). Similarly, the circadian expression of AhR is in relative agreement with previous studies demonstrating that AhR protein oscillates in the liver, lungs, and thymus of rats (Richardson et al., 1998). *AhR* transcripts are also clearly rhythmic in the rat liver, although the time of peak relative to the light schedule (14L:10D) was not reported (Huang et al., 2002). In a previous study from our laboratory, *AhR* transcripts were rhythmic in both suprachiasmatic nucleus (SCN) and liver under LD and DD (Mukai et al., 2008). Peak mRNA expression of *AhR* in both SCN and liver under LD occurred at ZT12 and ZT8, respectively. Collectively, the available data suggest that AhR expression is under the control of the circadian clock.

The data presented in this manuscript clearly indicate that a single, low dose of TCDD is sufficient to alter expression of the canonical clock genes, BMAL1 and *Per2*, in the mouse ovary. Other literature on dysregulation of circadian rhythms after AhR activation is limited. Doses of TCDD 1000 times higher than used in the current study cause altered behavioral rhythmicity in mice (Frame et al., 2004; Miller et al., 1999), which is associated with a concomitant divergence in the expression pattern of PER1 protein in the SCN of these mice. The SCN clock has a diminished ability to respond to nocturnal light after TCDD exposure, an event dependent on the clock gene *Per1* (Mukai et al., 2008; Tischkau et al., 2003). AhR activation subsequent to TCDD administration significantly altered the phase, period and amplitude of circadian rhythms in the numbers of antigenically and functionally defined hematopoietic progenitor cell classes in bone marrow. TCDD-induced changes were associated with dampening of the rhythms of the clock genes *Per1* and *Per2*, which may imply a direct crosstalk between AhR signaling and the core circadian clock (Garrett and Gasiewicz, 2006). Previous studies in our own laboratory also demonstrated phase alteration and dampening of *Per1* and BMAL1 rhythms in the mouse liver after a single low dose of TCDD (Mukai et al., 2008; Xu et al., 2010). Consistent with those results, the *Bmal1* mRNA

rhythm was dampened in this study; however, only the phase of the Per2 rhythm was changed. The collective data indicate that TCDD alters circadian rhythms by acting directly on the primary clock in the SCN and by acting on clocks located in peripheral tissues.

TCDD also affected the circadian expression of AhR in the mouse ovary. Although AhR mRNA was clearly rhythmic in the control animals with peak levels at CT8, the rhythm was abolished after TCDD treatment. TCDD did not ablate the rhythm in AhR protein, which peaked at CT12 in control animals. Rather, the peak was shifted 12 h out of phase after TCDD treatment. The reason for this discrepancy between effects on AhR mRNA and protein is not clear at this time. However, the data do suggest that posttranslational modifications and/or alteration in protein degradation may also be involved in circadian regulation of the AhR protein. The altered rhythmicity in AhR may be a consequence of altered expression of the circadian genes. Silencing of Per2 significantly alters AhR and ARNT expression in response to TCDD treatment (Qu et al., 2009). AhR/ARNT and the core circadian clock elements are PAS proteins capable of promiscuous interactions. Thus, it is reasonable to hypothesize that clock disruption might be a common feature underlying alteration in biological processes consequent to dioxin exposure.

Structural similarity between AhR and the canonical circadian clock elements, particularly BMAL1 (Takahata et al., 2000), suggests the possibility that AhR and/or ARNT may be involved in the regulation of circadian rhythmicity. Protein-protein interactions regulated by PAS domain-containing proteins are central to both circadian clock regulation and to AhR signaling (McIntosh et al., 2010). PAS domain proteins, however, are notoriously promiscuous. Heterodimers can form between many alternative family members, providing ample opportunity for diverse functionality and crosstalk. Not surprisingly given the multiplicity of PAS domain interactions, it seems that the two pathways are intertwined in a relatively complex manner. Activation of AhR leads to alteration of the clockworks (Garrett and Gasiewicz, 2006; Mukai et al., 2008; Mukai and Tischkau, 2007; Xu et al., 2010), and reciprocally, the clock modulates the activity of AhR (Qu et al., 2007, 2009; Qu et al., 2010). In the present study, TCDD administration increased physical interactions between AhR and Bmal1, suggesting that these two PAS domain proteins can form a functional heterodimer in the ovary. Although we did not examine functional consequences of AhR/BMAL1 complexes in the ovary, our previous data suggest that this heterodimer can repress Per1 (Xu et al., 2010). Inhibition of Per1 would dampen the robustness of core clock and slow its progression. Per1 rhythms in liver are attenuated and phase delayed after TCDD exposure (Xu et al., 2010), which is compelling evidence that AhR slows and weakens circadian rhythmicity.

Conversely, disruption of the clockworks, specifically in the presence of aberrant Per1 expression, precipitates changes in AhR signaling. Production of the AhR target genes are significantly enhanced after Per1 knockdown using siRNA, or in animals bearing mutated Per1 genes (Qu et al., 2007, 2009; Qu et al., 2010). Although the molecular events underlying the heightened AhR activity remains unknown, it is tempting to speculate that PER1 may physically interact with the AhR/ARNT heterodimer to function as a transcriptional repressor, similar to the effects of PER1 on CLOCK/BMAL1. The aggregate data paint a complex picture of reciprocal interactions between AhR signaling, which is enhanced by TCDD treatment as in the current study, and the molecular clockworks.

Whether the effects of TCDD are mediated within the ovary itself or through interactions of TCDD at other sites in the hypothalamic-pituitary-ovarian axis remains an open question. TCDD is known to alter hypothalamic and pituitary production of gonadotropic hormones, leading to inhibition of ovulation (Li et al., 1995a, 1995b). Ovarian clock gene expression is also influenced by the gonadotropins (Karman and Tischkau, 2006; Nakamura et al., 2010).

However, interactions of AhR and Bmal1 specifically within the ovary were enhanced by TCDD treatment, suggesting direct action of TCDD on the ovary. Furthermore, the consequences of TCDD-induced alteration in circadian rhythms in the ovary remain unknown. However, both the circadian clock and AhR regulate cell cycle progression, and that AhR is important for follicle development in the ovary (Borgs et al., 2009; Hernandez-Ochoa et al., 2009). Thus, disruption of circadian clock function after TCDD may alter follicular development. Future studies will investigate how interactions of AhR and clock genes affect ovarian function.

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Fig. 1. Effects of TCDD on circadian rhythms of Bmal1 and Per2 in the mouse ovary

A) Bmal1 transcript rhythms are dampened after TCDD treatment (n=3–6 per timepoint). Bmal1 transcripts are significantly lower at CT0 and CT20 in TCDD-treated mice ($p<0.01$; ANOVA with Tukey's post-hoc analysis). B) The BMAL1 protein rhythm was altered by TCDD (n=3–6 per timepoint). Blots are representative of 3–6 independent samples per timepoint. BMAL1 is decreased at CT0 and increased at CT16 compared to control. ($p<0.01$; ANOVA with Tukey's post-hoc analysis). C) Time of peak expression of Per2 mRNA is altered in TCDD treated mouse ovaries. Per2 transcripts are significantly higher at CT12 and CT16 in TCDD-treated mice ($p<0.01$; ANOVA with Tukey's post-hoc analysis). D) The PER2 protein rhythm was altered by TCDD (n=3–6 per timepoint). Blots are representative of 3–6 independent samples per timepoint. PER2 is decreased at CT0 and increased at CT12 compared to control. ($p<0.01$; ANOVA with Tukey's post-hoc analysis).

Fig. 2. Effects of TCDD on circadian rhythms of AhR in the mouse ovary

A) AhR transcript rhythms are dampened after TCDD treatment (n=3–6 per timepoint). AhR transcripts are significantly lower at CT8 and higher at CT0 in TCDD-treated mice ($p < 0.01$; ANOVA with Tukey's post-hoc analysis). B) The AhR protein rhythm was altered by TCDD (n=3–6 per timepoint). Blots are representative of 3–6 independent samples per timepoint. AhR is decreased at CT0 and increased at CT4, 8 and 12 compared to control. ($p < 0.01$; ANOVA with Tukey's post-hoc analysis).

Fig. 3. Immunohistochemistry of AhR and BMAL1 in the rat ovary

Staining of consecutive sections shows similarity in localization of AhR and BMAL1 in structures of the rat ovary.

Fig. 4. Interactions of AhR and BMAL1 are enhanced by TCDD treatment

A) BMAL1 immunoreactivity in protein lysates immunoprecipitated with an AhR antibody is increased at CT4 compared to CT20 in untreated controls and increased after TCDD treatment at CT4. BMAL1 was not co-immunoprecipitated with a calbindin (Calb) antibody or when normal rabbit serum was used for precipitation (NRS). B) AhR immunoreactivity in protein lysates immunoprecipitated with a BMAL1 antibody is increased at CT4 in TCDD-treated mice. Blots is representative of 3 experiments.