2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN PHASE ADVANCES THE DEER MOUSE (*Peromyscus maniculatus)* CIRCADIAN RHYTHM BY ALTERING EXPRESSION OF CLOCK PROTEINS

Joseph D. Miller, Daam Settachan*, Lynn T Frame* and Richard L. Dickerson*

Department of Pharmacology, Texas Tech University Health Sciences Center, 3601 4th Street, Lubbock, Texas, USA
*and The Institute of Environmental and Human Health, Texas Tech University / Texas Tech University Health Sciences Center, Lubbock, Texas, USA.

Introduction

Halogenated aromatic hydrocarbons (HAHs) are ubiquitous environmental pollutants found in parts per trillion concentrations in many wildlife and in human tissues. These compounds are strongly lipophilic, degraded very slowly and are progressively concentrated in higher levels of the food chain. HAHs exhibit a wide variety of toxic effects, including carcinogenicity, teratogenicity, weight loss, atrophy of the thymus, loss of immune and thyroid function, hepatotoxicity, endocrine disturbances, and sleep disruption (1). The most potent of the HAHs is dioxin, otherwise known as 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD; for review see 2).

Many of the effects of TCDD depend on binding to a particular site on the aryl hydrocarbon (Ah) receptor, a cytosolic ligand-activated transcription factor, which has been identified in many tissues from a large number of animal species (3). The Ah receptor and its dimer partner ARNT both possess a basic helix-loop-helix (bHLH) DNA-binding domain (4). In addition, the Ah receptor and ARNT exhibit a 300 amino acid region called the PAS (PER, ARNT, SIM) domain, highly homologous to *Drosophila melanogaster sim* and *per* genes, which are critical for neuronal development and circadian rhythmicity, respectively. Both the HLH and PAS domains are critical for formation of fully functional heterodimers composed of the Ah receptor and ARNT, or PER and SIM (5, 6). The PAS domain contains the ligand-binding domain for TCDD in the Ah receptor, but TCDD is unable to bind to ARNT or SIM. More recently, other members of the PAS domain-containing family have been characterized, including three mammalian homologs of *per*, an essential gene for circadian rhythmicity in rodents named *clock*, and its dimer partner, *bmal1* (7, 8, 9, 10, 11, 12,13,14,15).

The suprachiasmatic nucleus (SCN) of the hypothalamus is necessary and sufficient for the generation and maintenance of circadian rhythms in mammals (for recent reviews see 16, 17). One gene has been shown to be necessary for clock function (*clock*; 18). Members of a second gene family (*mper1-mper3* are the known homologues) are the mammalian versions of the Drosophila *per* gene, which is essential for circadian rhythmicity in that species. All three of the *per* homologues are known to vary in abundance in the SCN in a circadian rhythm (9, 10, 8, 7, 12). The ability of TCDD to bind with high affinity to the PAS domain of the Ah receptor, in conjunction with the presence of PAS domains in all known clock proteins, suggests that TCDD and perhaps other HAH's may have direct effects on the biological clock in the SCN and this hypothesis is supported by the data contained within this paper.

Material and Methods

Male adult deer mice are maintained in our behavioral facility in constant darkness with food andwater supplied ad libitum. TCDD was administered by gavage at 10 μg/kg under dim red light at CT14. Each mouse served as its own control (2 week pre-drug baseline) and data was taken for 2weeks following drug administration. Locomotor activity was automatically recorded throughout the course of all experiments for each separately housed animal. The index of locomotor activity is interruption of infrared beams as the animal moves about the cage (San Diego Instruments).

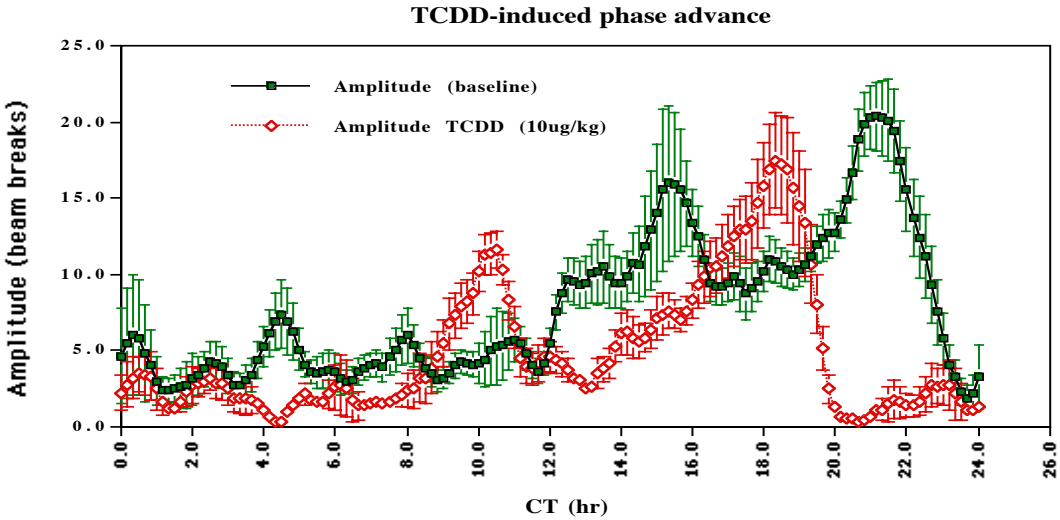
Western analysis of clock proteins was conducted by exposing 48 male deer mice to 10 μg/kg TCDD and 48 to vehicle beginning at Zeitgeber time 18. 6 treated and 6 control mice were euthanized at 3 hour intervals for 24 hours. The SCN was removed, homogenized in Tris-EDTA-sucrose buffer, and S14 supernatant prepared. 30 μg of 14,000 g supernatant protein was used for electrophoretic analysis. SDS/PAGE (10%) resolved proteins between five and 100 kilodaltons (kDa), via a Novex system. The proteins were then transferred onto Immobilon-P membrane using standard published protocols (Millipore, Bedford, MA). The immobilized proteins were detected using enhanced chemiluminescence (ECL) (Amersham). Protein loading on the Western blot membranes was visualized and documented after Ponceau staining. Differences in amounts of immunoreactive proteins involved in clock function were quantitated by densitometry of exposed film.

Results and Discussion

Three deer mice (Peromyscus maniculatus; 2-3 months old) were maintained in constant darkness for 18 days and were each subsequently administered TCDD (10 ug/kg by gavage) at CT14 on day 19. Phase advances (2.6 ± 0.2 hr) were seen in all three mice. All three mice showed some fragmentation of the rest/activity cycle following TCDD, consistent with previous observations of motor activity during the usual sleep period (Frame, unpublished data, 1999). Strong circadian peaks (24.2-24.8 hr) continued to be present following TCDD. One mouse exhibited a substantial increase in period (23.3 to 24.8 hr following TCDD) in addition to a phase advance (2.7 hr), suggesting a long-lasting effect of TCDD on period. Period lengthening has been predicted to result from persistent reduction in PER (19), consistent with our Western results (see below). The following figure displays the average circadian waveform before and after TCDD (10 ug/kg). The y-axis represents motor activity in terms of infra-red beam breaks/10 min interval ± S.E. and the x axis is in circadian time (CT) with activity onset at CT12. It is apparent that the waveform is strongly phase-advanced under TCDD, with activity bouts now occurring during the usual rest portion of the cycle, in agreement with previous unpublished observations (Frame, 1999). Careful inspection also reveals extended periods of inactivity during the usual activity period, following TCDD. In conjunction with a general decrease in amplitude under TCDD, these data suggest that the circadian rhythm is not only phase advanced under TCDD, but shows some signs of fragmentation; that is, reduced consolidation of rest and activity to their appropriate phases of occurrence.

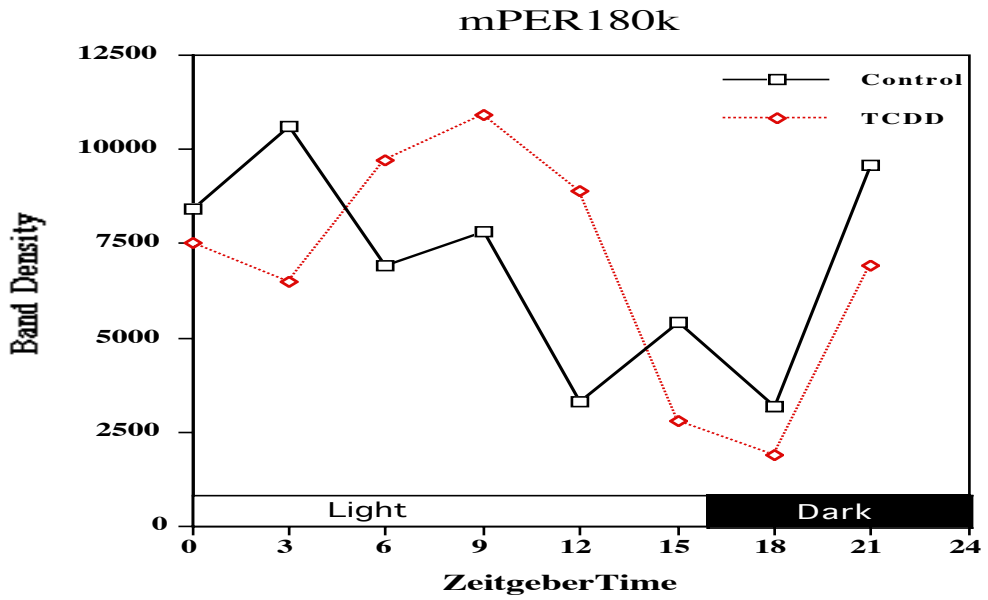
No difference in presumed CLOCK abundance (96 Kda band; 13) was observed in the CT14 vs. CT4 conditions, (data not shown), in agreement with other observations that *clock* message fails to exhibit a circadian rhythm (20). However, TCDD treatment at CT10 produced about a 1.5 fold increase in abundance of presumed CLOCK protein vs. the corresponding vehicle control measured at CT6, consistent with our observations (see below) that TCDD reduces mPER1 protein around ZT18 the previous night, releasing CLOCK from negative feedback. mPER1, and

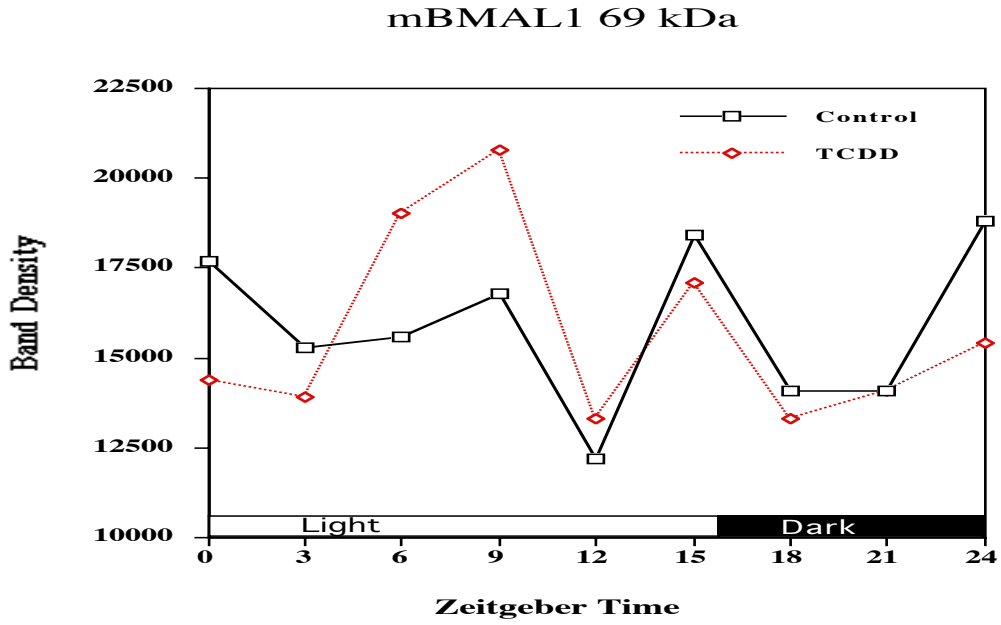
BMAL1 antibodies also labeled bands of the appropriate molecular weights in different Westerns (not shown).



The graph displayed below shows the amount of mPER protein (180 kDa) from a Western blot prepared as described above, with groups of three mice sacrificed every three hr over a 24 hr span in a 16:8 L/D cycle. Zeitgeber time (ZT; with ZT0=lights on) is displayed on the x axis and band density (NIH Image) on the y-axis. All groups of mice received either vehicle or TCDD (10 ug/Kg) by gavage at ZT18. It is apparent that the circadian rhythm in mPER is phase-delayed by TCDD administration. The second graph displayed below is a probing of the previous western blot with anti-BMAL1 and shows the amount of mBMAL1 protein (69 kDa). There is no strong evidence for a BMAL1 circadian rhythm in these initial data, although there is a general decline in BMAL1 over the light phase. Interestingly, BMAL1 does decline relative to control following TCDD at ZT18, as did mPER in the previous graph. A reduction in BMAL1 should cause a reduction in mPER1 by current theory. that reduction in mPER1 should eventually result in a rebound increase in BMAL1 at later time points, as seen in this graph from ZT3 to ZT12. Likewise, an increase in BMAL1 (in conjunction with the apparent small elevation in CLOCK) should increase expression of mPER1 over roughly the same time interval, as was also seen in the previous graph.

The data presented in this paper show that TCDD exposure can exert pronounced effects upon the proteins that form the circadian clock in mammal. This results in a phase advance of several hours at relatively low concentrations of TCDD. Such a phase advance can manifest itself has sleep disturbances in humans or a nocturnal to diurnal shift in wildlife. In wildife, the end result may well be increased predation. In humans, sleep disturbances result in decreased productivity and increased accident rates.


mPER180k
12500
10000
7500
5000
2500
0
Band Density
Control
TCDD
Light
Dark
0
3
6
9
12
15
18
21
24
ZeitgeberTime


mBMAL1 69 kDa
22500
20000
17500
15000
12500
10000
Band Density
Control
TCDD
Light
Dark
0
3
6
9
12
15
18
21
24
Zeitgeber Time

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