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Prenatal Low Dosage Dioxin (TCDD) Exposure Impairs Cochlear Function Resulting in Auditory Neuropathy

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Abstract

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a ubiquitous and persistent environmental contaminant, is a potent teratogen. Whereas developmental TCDD toxicity is mediated by the aryl hydrocarbon receptor (AhR), the normal function of the AhR is poorly understood. We tested whether dioxin exposure during a critical period of hair cell development disrupts cochlear function in three mouse strains, (C57BL/6, BalbC, and CBA) that contain high affinity AhR-b alleles. C57BL/6, BalbC, and CBA dams were exposed to 500 ng/kg TCDD or olive oil (vehicle) on embryonic day 12 by gavage. Cochlear function was analyzed at 1.5 months of age by measuring 1) auditory brainstem response (ABRs) to tone pips from 5.6 to 30 kHz, and 2) distortion-product otoacoustic emissions (DPOAEs) evoked by primaries with f_2 at the same frequency values. Cochlear threshold sensitivity following TCDD exposure was significantly elevated in both female and male mice in the C57BL/6 strain, carrying the Ahb-1 allele, but not significantly elevated in the BalbC or CBA strains, carrying the Ahb-2 allele. These ABR threshold deficits in mice carrying the Ahb-1 allele parallels the cleft palate incidence to higher TCDD exposures, suggesting that ABR testing could serve as a sensitive indicator of TCDD toxicity in at-risk children. Moreover, DPOAEs were not affected following TCDD exposure in any of the mouse strains, suggesting that following TCDD exposure mice with the Ahb-1 allele exhibit a mild auditory neuropathy. The causes of many auditory neuropathies are unknown, yet a developmental exposure to dioxin may be a risk factor for this condition.

Keywords

TCDD; Dioxin; cochlea; DPOAE; ABR; mouse; auditory neuropathy; High affinity Aryl hydrocarbon receptor; Ah b1 allele

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INTRODUCTION

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin), the most potent teratogen belonging to the halogenated aromatic hydrocarbon (HAH) family, is a ubiquitous and persistent environmental contaminant. Toxicity associated with TCDD exposure is mediated through the arylhydrocarbon receptor (AhR), a ligand-activated transcription factor whose normal function is not well characterized. When activated, the AhR translocates to the nucleus, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt), and interacts with xenobiotic response elements (XREs) in 5'-regulatory regions of target genes, which include xenobiotic metabolizing enzymes and growth regulatory molecules (Bock & Kohle, 2006; Wilson & Safe, 1998; Bock 2013). Bemis et al. (2005) developed a lacZ reporter mouse model driven by activation of the AhR, and found that exposure to TCDD on embryonic day 14 resulted in strong activation of the lacZ transgene in numerous tissues, including the inner ear otocyst. Interestingly, TCDD and other coplanar polychlorinated biphenyls (PCB) induced similar patterns of AhR activation in the inner ear otocyst. Moreover, prenatal exposures to PCB mixtures containing both non-coplanar and coplanar PCBs have caused auditory deficits in rats (Powers et al. 2006).

A known teratogenic effect of high doses of prenatal dioxin exposure is an increased incidence of cleft palate. In order to determine if cleft palate incidence segregates with the allelic binding affinities of the AhR, Powers et al. (2006) took advantage of the known mouse strain differences in AhR alleles, with the b alleles (Ahb-1, Ahb-2, Ahb-3) characterized as having a high binding affinity for dioxin, while the d allele (AhD) has a low binding affinity. (Poland et al.1994). In this study, the strain distribution of cleft palate formation, following a single prenatal TCDD high dose exposure of 30 µg/kg to the dam on embryonic day 10 (ED10), was examined in ten mouse strains containing either the high affinity b allele for AhR, or the lower affinity d allele (Poland & Glover, 1980). Of the five strains with the b allele, four of the strains, including c57Bl/6 (Ahb-1) showed a high occurrence of cleft palate formation, while the CBA strain (Ahb-2) and mouse strains carrying the d allele showed no incidence of cleft palate.

To examine potential role of prenatal TCDDs in mediating ototoxicity and if ototoxicity segregated with AhR alleles, pregnant mouse dams were given a single low dose (0.5 µg/kg) of TCDD to evaluate whether prenatal exposure (ED12) disrupted auditory system development and cochlear function. Using both auditory brainstem response (ABR) and distortion-product otoacoustic emissions (DPOAE) to assess cochlear function, we found that only the c57Bl/6 mouse strain, carrying the Ahb-1 allele, upon TCDD exposure had elevated ABR thresholds with no concomitant change in DPOAE responses or cochlear structure, whereas strains carrying the Ahb-2 allele were not affected. Elevated ABR thresholds, without concomitant DPOAE losses, is a phenotype reflective of auditory neuropathy (Menten et al. 2014; Ptak, 2000). Collectively our results suggest that a subclinical gestational TCDD exposure may a risk factor for and contribute to auditory neuropathy.

METHODS

Animals

All animal work was conducted under University of Rochester UCAR approved animal protocols in accordance with the AAALAC guidelines. Wildtype c57Bl/6, CBA mice and Balb/C mice, 6–8 weeks of age, were purchased from Jackson Laboratories for breeding. Males were removed the day after pairing, and the day of removal was considered embryonic day (ED) 0. Dams were assumed pregnant if a weight gain of greater than 1.5 grams was observed by ED 9. Fifty-eight c57Bl/6 mice (29 M:29F); 49 BalbC mice (24 M; 25F) and 76 DBA mice (38 M, 38F) were used in this study.

Dioxin Exposure

Pregnant c57Bl/6 dams, CBA dams and Balb/C dams were exposed to 500 ng/kg TCDD or olive oil (vehicle control) on ED 12, see Fig. 1, which corresponds to the day before hair cell progenitors become committed hair cells. TCDD or olive oil were absorbed onto a vanilla wafer and administered orally to dams.

Cochlear Function Analysis

Auditory nerve and outer hair cell function were assessed using auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs). Cochlear function was assessed in mice at 6 weeks of age and mice were sedated with ketamine (40 mg/kg ip) and acepromazine (1 mg/kg ip) prior to testing. Intelligent Hearing Systems (IHS) Smart EP and Smart DPOAE software were used for recording auditory data and subsequent analysis.

Auditory Brainstem Response (ABR) Testing

To obtain ABR measurements, needle electrodes were inserted subcutaneously: the ground electrode above the tail, the positive electrode at the vertex and the negative electrode behind the left ear, and the microphone was inserted in the animal's left ear canal. ABR potentials were evoked with 5 ms tone pips (0.5-ms rise-fall with a $\cos^2$ onset envelope, delivered at 35/s) at 6 log-spaced frequencies: 5.6, 8, 11.3, 16, 24.4 and 30 kHz. The response was amplified (10,000x), filtered (100 Hz-3kHz) and averaged using IHS Smart EP software. Sound level was decreased in 10 dB steps from 80 dB to 20 dB. Two waveforms per frequency per sound level were recorded at 512 averaged samples per waveform. Artifacts were rejected based on peak-to-peak amplitudes in response waveforms exceeding 15 μ V. Threshold was determined by visual inspection as the sound level at which peaks were no longer present or the two waveforms were no longer synchronous. Wave 1 amplitudes and peak latencies were also determined.

Distortion Product Otoacoustic Emissions (DPOAE) Testing

F_1 and f_2 primary tones for measurement of the $2f_1-f_2$ DPOAE were presented in a ratio of $f_2/f_1=1.2$ with unequal levels ($L_1=10\text{ dB}+L_2$). The microphone was inserted in the animal's left ear. In order to obtain moderate level DPOAE measurements, data was obtained at $L_1=70\text{ dB}$, $L_2=60\text{ dB}$ for each f_2 frequency, which corresponded to the same 6

log-spaced frequencies used in ABR testing; 5.6, 8, 11.3, 16, 24.4, and 30 kHz. Ear canal sound pressure waveforms were amplified and digitally sampled at 4 μ s intervals. Fast Fourier Transforms were computed from averaged waveforms and DPOAE amplitudes and noise floor sound levels were extracted. DPOAEs were rejected if amplitudes were less than 3 dB above the noise floor. DP-grams were assessed as a function of f_2 at $L_2=60$ dB.

Cochlear Histology

Mice were perfused intracardially with 4% paraformaldehyde at 8 weeks of age. Cochleae were removed and post-fixed overnight at 4° C followed by decalcification for 72 hours in 0.12 M EDTA at room temperature. Cochleae were then either prepared for plastic sectioning or microdissected for staining. Cochleae from three C57Bl/6 male animals that received TCDD exposure were compared with three C57Bl/6 male animals that received vehicle exposures.

Plastic Sectioning

Cochleae were removed from EDTA and rinsed in PBS. They were then osmicated for one hour with 1% OsO₄ in 0.1 PBS, followed by PBS rinses. Cochleae were then subjected to a series of ethanol dehydrations: in 70% EtOH, 90% EtOH, 95% EtOH and 100% EtOH. Dehydrations were followed by exchanges in 100% propylene oxide, 1:1 mixture of Araldite and propylene oxide, and an incubation in a 2:1 mixture of Araldite to propylene oxide overnight. Cochleae were then removed from the mixture and placed in coffin beds with fresh Araldite and baked at 60° C. All steps were performed at room temperature unless otherwise noted. Mid-modiolar sections of about 40 μ m thickness were cut using a diamond knife on a microtome. Sections were mounted on glass slides and imaged with a Zeiss Axioplan microscope outfitted with a Zeiss digital camera.

Microdissection and staining

Cochleae were removed from EDTA and rinsed in PBS. Sections were infiltrated with glycerol and mounted on glass slides and imaged with a Zeiss Axioplan microscope outfitted with a Zeiss digital camera.

Statistical Analysis

Statistical analysis was performed using Prism software (Graphpad, Inc.). Statistically significant differences between treated and control mice within each species, were determined via two-way ANOVA testing and Bonferroni post-hoc testing was used to determine significance at specific frequencies, for ABR thresholds, latencies and amplitudes, and DPOAE amplitudes (at $L_2=60$ dB). Statistical significance was determined at $p<0.01$.

RESULTS

TCDD (Dioxin) exposure and cochlear function

Mice of each strain were exposed to either vehicle or TCDD on embryonic day 12 (ED 12), and cochlear function was assessed on postnatal day (PN) day 45. Each group of animals came from at least three different dams, yet there were no significant differences in cochlear

function between litters so these groups were pooled. As shown in in Fig 2 (BDF) right panels, in all strains, exposure to TCDD had no significant effect on low-level DPOAE amplitudes in frequency regions (5.6–24.4 kHz), allowing us to surmise that outer hair cell function is normal in TCDD-treated mice.

While TCDD exposure does not affect outer hair cell function, inner hair cell function is differentially affected by TCDD exposure by AhR allele. Fig 2 (A,C,E) left panels show auditory brainstem response (ABR) thresholds which measures the afferent response to sound. TCDD exposure caused a 5–20 dB shift in ABR thresholds in the c57Bl/6 mouse strain at all frequencies, with frequencies 11.3–30 kHz significantly affected, as assessed by post-hoc analyses. Interestingly, the BalbC (Fig 2C) and CBA (Fig 2E) mice exposed to TCDD did not exhibit significant ABR threshold shifts. The auditory phenotype observed in the c57Bl/6 mouse with normal DPOAEs showed excellent outer hair cell function and increased ABR thresholds is indicative of a mild auditory neuropathy in the c57Bl/6 mouse exposed to TCDD. Wave 1 amplitudes were also assessed and these differences were directly comparable to ABR threshold differences in the C57Bl/6 strain carrying the Ah b-1 allele, and no differences in wave 1 amplitudes between TCDD and vehicle-treated animals carrying Ah b-2 alleles (Balb/C and CBA) were observed.

There were no sex differences on ABR responses observed between male and female mice treated with vehicle, yet there were sex differences on ABR responses between male and female mice treated with TCDD in specifically in the C57Bl/6 mouse strain carrying the Ah b-1 allele. In the c57Bl/6 strain, there was a greater threshold shift at the higher frequencies (16–30 kHz) in female mice and a greater threshold shift at lower frequencies (5.6–16 kHz) for male mice, though post-hoc testing revealed ABR thresholds at frequencies, 11.3–30 kHz were significantly different from vehicle-treated controls for both male and female mice (Fig 3A). Interestingly, in the BalbC mouse there were no significant sex differences observed (3B) and in the CBA strain, while there were no significant differences in ABR thresholds using post-hoc testing, there was a trend toward females being more affected at the high frequency regions and males affected at lower frequencies (Fig 3C). When the ABR response differences were averaged across frequency, only the TCDD-treated c57Bl/6 male and female mice exhibited ABR thresholds significantly different from vehicle-treated c57Bl/6 mice (Fig 4A). TCDD exposure in the c57Bl/6 strain, carrying the Ah b-1 receptor allele caused a 5–20 dB shift in auditory brainstem response (ABR) thresholds at all frequencies tested. Balb/C and CBA mice carrying the Ah b-2 subtype did exhibit small threshold shifts upon TCDD exposure, these shifts are not significant (Fig 3 B & C), and only c57Bl/6 strain carrying the Ah b-1 subtype of receptor shows significant threshold shifts (Fig 3A; $p < 0.01$). Fig. 3D show these shifts averaged for all 6 frequencies tested with male and female mice analyzed separately, further highlighting ABR threshold shifts across mouse strains carrying different AhR alleles.

Cochlear architecture

A light microscopic examination of cochleae in c57Bl/6 mice treated with TCDD revealed no qualitative structural abnormalities in the cochlea, (as shown in Fig. 4 for a male c57Bl/6 TCDD-treated mouse) including hair cells, supporting cells, or spiral ganglion cells where

the cell bodies of primary sensory neurons are located, when compared to vehicle treated C57Bl/6 males (n=3 animals/treatment group).

Interestingly, the cochlear structure of the c57Bl/6 mouse strain exposed to TCDD is qualitatively normal with no loss of inner hair cells, outer hair cells, or spiral ganglion cells, and overall architecture of the cochlear organ – ie, basilar membrane, supporting cells, spiral ligament, lateral wall, hair cells are also not affected by TCDD exposure when assessed at the light microscope level as shown in Fig. 4 A-C.

DISCUSSION

In this study, we show that c57Bl/6 mice, carrying the Ah b-1 subtype of the high-affinity Ah receptor, when prenatally exposed to TCDD exhibit significant ABR threshold shifts, with no concomitant change in DPOAE responses or cochlear structure. This phenotype is similar to a mild auditory neuropathy. The ototoxicity of this phenotype further segregates with the AhR locus as both Balb/C and the CBA mouse strains carrying the Ah b-2 subtype of the high-affinity receptor are not similarly affected.

Ototoxicity of TCDD, co-planar and non-coplanar PCBs

Like TCDD (dioxin) man-made polychlorinated biphenyls (PCBs) are ubiquitous persistent environmental pollutants, and have been classified as developmental neurotoxicants. TCDD and certain coplanar PCBs activate the AhR, whereas other non-coplanar PCBs (ie Aroclor 1254, PCB95) fail to activate the AhR in TCDD responsive mouse lines (Bemis et al. 2005). Developmental exposure to PCBs can result in cochlear impairments such as reduced DPOAE amplitudes and reduced transient evoked otoacoustic emissions in both rodents and humans (Trnovec et al. 2008, 2010; Jusko et al. 2014; Powers et al. 2006, 2009; Lasky 2002). Thyroid hormone or thyroxine is known to influence outer hair cells development and the loss of OAEs with PCB exposure could be due to reduced thyroxine during cochlear development. However, when polybrominated diphenyl esters (PBDE) that are similar in structure to PCBs and reduce thyroxine, were given daily to pregnant rat dams throughout both gestation and lactation, the rat pups exposed to PBDE did not exhibit DPOAE deficits (Poon et al. 2011). Interestingly, when rats were exposed to an environmental PCB mixture containing both coplanar AhR activating PCBs and non-coplanar PCBs, both DPOAEs and ABR thresholds were impaired. Perhaps the PCBs acting through the AhR caused the ABR losses, and the non-coplanar PCBs caused the DPOAE losses. Hexachlorobenzene is another dioxin-like compound widely distributed in the environment, and Hadjab et al. (2004) found that a 4-wk HCB treatment reduced circulating thyroxine, but did not cause DPOAE deficits but instead caused ABR threshold shifts as in our study.

Strain and AhR alleles

The incidence of ABR threshold increase in the different strains of mouse observed in the current study parallel the incidence of cleft palate in mice exposed prenatally to a higher dosage of TCDD (Poland and Glover, 1980). We found that the c57Bl/6 mouse strain (Ahb-1) was most sensitive to ototoxic effects due to a low dosage (30 µg/kg) TCDD prenatal exposure and the CBA mouse (Ahb-2) was not impaired. Similarly, when Thomae

et al. (2006) administered a higher dose of TCDD at embryonic day 9, nearly 100% of the c57Bl/6 offspring exhibited cleft palate, while a similar exposure in CBA mice only resulted in cleft palate in 8% of the offspring. Their finding lead to the conclusion that there must be an additional genetic basis for strain-dependent dioxin sensitivity, as both stains carried the high-affinity b allele. In pursuit of this unidentified locus, c57Bl/6 and CBA mice were crossed and linkage analysis on cleft palate incidence was performed to assess dioxin-induced teratogenic effects and found one locus segregating with dioxin-induced cleft palate (designated as *cmt1*) located on chromosome 3 (Thomae et al. 2006). Presumably, this locus is also required for ototoxic effects due to a lower dosage of TCDD.

Auditory neuropathy and TCDD exposure

The auditory phenotype observed in the c57Bl/6 mouse strain carrying the Ah b-1 allele and the *cmt-1* locus on chromosome 3 with subclinical exposure to TCDD, can be considered a mild auditory neuropathy due to the reduced ABR thresholds, intact DPOAEs, and normal cochlear structure. (Berlin et al. 2003; Ptok, 2000; Menten et al. 2014). Auditory neuropathy is know to cause severely delayed speech and difficulties hearing in background noise, and impaired temporal processing of sounds (Ptok, 2000). The causes of many auditory neuropathies are not known, yet our findings suggest that developmental exposure to TCDD may contribute and be a risk factor for this clinical condition. Moreover, given ototoxicity incidence to lower dosages of TCDD parallels the cleft palate incidence of higher TCDD exposures in mice, cochlear function testing could serve as a more sensitive-indicator of TCDD toxicity in at-risk children.

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Highlights

- Prenatal dioxin given to mice carrying high-affinity aryl hydrocarbon alleles
- DPOAEs and cochlear structure unaffected by prenatal dioxin exposure
- ABR thresholds significantly elevated in mice carrying the Ahb-1 allele
- ABR thresholds not significantly elevated in mice carrying the Ahb-2 allele
- Prenatal dioxin exposure may be a risk factor for auditory neuropathy

Figure 1. Time course of cochlear development and study design

Dams were exposed to either TCDD or vehicle on embryonic day 12, which is approximately 1.5 days before terminal mitosis of the hair cell-supporting cell precursor cell pool begins. In the mouse, hearing onset occurs at postnatal day 14, and we assessed cochlear function in the pups at postnatal day 45. We also assessed cochlear function in the adult dam that were exposed to TCDD and vehicle. In all instances, at least three different litters were exposed to vehicle and at least four different litters were exposed to TCDD.

Figure 2. Prenatal TCDD exposure resulted in “auditory neuropathy” phenotype in mice carrying the Ah b-1 locus

Figs. 2 (BDF) show DPOAE amplitudes to differing primary tones ($L_1=70\text{dB}$; $L_2=60\text{dB}$) with the f_2 primary frequency plotted. In all cases, the open symbols are the TCDD-exposed animals and the closed symbols are vehicle-exposed animals. There are no significant differences between the exposed and non-exposed DPOAEs magnitudes in any of the strains studied. (1-way ANOVA analysis, $p<0.01$). However, as shown in Figs. 2 (ACE) there are differences in the Auditory Brainstem Response (ABR) thresholds assayed at the same frequencies between animals treated with TCDD and vehicle controls. The differences

observed are only significant for the C57BL/6 strain (1-way ANOVA; $p < 0.01$) and at the frequencies indicated (11.3–30 kHz).

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Figure 3. C57BL/6 mouse strain carrying the high-affinity-b1 locus has reduced ABR thresholds with TCDD exposure

ABC. ABR change in threshold shift for each strain of mouse tested (ABR threshold with TCDD-ABR threshold with vehicle). While threshold shifts were observed for both C57BL/6 and Balb/C mice, the shifts were only significant for the C57BL/6 strain (1-way ANOVA, $p < .01$). **D.** Average ABR shift shows this same result plotted as the average threshold shift across all frequencies tested, separated by sex, showing that on average, C57BL/6 strain carrying the Ah b-1 locus show an average ~10 dB threshold shift, with the Balb/C and CBA strains, both carrying the Ah b-2 locus, exhibiting less than a 5 dB ABR

threshold shift which is not significantly different from 0 dB threshold shift. Open bars (male mice) and gray bars (female mice).

Figure 4. Cochlear structure is not affected in the C57BL/6 mouse strain subjected to prenatal TCDD exposure

A. Low-magnification image of an osmicated plastic section from a TCDD-exposed male C57BL/6 cochlea exhibiting normal basilar membranes, hair cells, and supporting cells, and lateral walls. B. Higher magnification (inset region in A) from a TCDD-exposed C57BL/6 cochlea showing that the normal lateral wall, and organ of Corti structure in TCDD-exposed animals. C. Fluorescent image of cells stained with DAPI of microdissected cochlear half-turn showing no losses of hair cells in the TCDD exposed C57BL/6 mouse.