POSTNATAL TCDD EXPOSURE INTERFERES WITH NEURONAL MIGRATION, DIFFERENTIATION, AND SURVIVAL DURING CEREBELLAR DEVELOPMENT BY DISRUPTING GENE EXPRESSION PATTERNS.

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Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a ubiquitous and persistent environmental contaminant that exerts developmental toxicity via binding to the aryl hydrocarbon receptor (AhR), a ligand activated transcription factor [1]. AhR is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) superfamily. These transcription factors are known to play important roles in a variety of cellular processes including neuronal development, cell fate determination, and differentiation [2, 3]. TCDD, through interaction with the AhR, is also known to regulate the expression of drug metabolizing enzymes, inflammatory mediators, and cell cycle regulatory proteins by binding to dioxin/AhR responsive elements (DRE/AhRE) in promoter regions of target genes [4]. Whereas developmental TCDD exposure is known to result in adverse neurological outcomes, a direct role for the AhR in TCDD-mediated developmental neurotoxicity has not been evaluated. More importantly, the cellular targets and molecular mechanisms of TCDD neurotoxicity during brain development are largely unknown. Our lab recently reported that AhR is robustly expressed and transcriptionally-active in mouse cerebellar granule neuroblasts during a critical period of development [5]. Therefore, it is feasible to hypothesize that TCDD exposure disrupts the spatiotemporal expression of differentiation factors and interferes with neuroblast maturation in the cerebellum.

Several observations are consistent with the notion that TCDD exposure disrupts cerebellar development. Epidemiological studies have suggested that accidental developmental exposures to PCB/dioxin mixtures resulted in delayed motor development, higher incidences of hypotonia, and increased activity levels [6-8]. These findings are supported by studies in rats demonstrating that perinatal TCDD exposure impeded development of the righting reflex and impaired rotarod performance [9], behaviors that normally depend on proper cerebellar development and function[10]. Developmental TCDD exposure was also shown to modulate the cerebellar expression profile of Sp1, a transcription factor involved in growth and differentiation[12], but the biological significance requires clarification. Furthermore, indigo (a putative endogenous AhR ligand) stimulated DRE binding in cerebellar granule neurons [11]. Together these observations provide support that the developing cerebellum is a site of action for developmental TCDD neurotoxicity.

The first three weeks of postnatal cerebellar development in the rodent represents a vulnerable phase for toxic insult because of the dynamic ongoing programs of neurogenesis, differentiation, apoptosis, and synaptogenesis[13]. This phase of rodent brain maturation corresponds to neuroanatomical development from the late fetal period to 1.5 years in humans. In rodents, cerebellar granule neurons arise from a distinct germinal zone, the external granule cell layer (EGL). Following extensive proliferation in the superficial zone of the EGL on postnatal days (PND) 5-8, the cerebellar granule neuron precursors (CGNP) become post mitotic while migrating into the deep zone of the EGL. CGNP then receive molecular cues to migrate, between PND 8-10, along Bergman glial cell fibers through the molecular and Purkinje cell layers into the internal granule cell layer (IGL) of the cerebellar cortex. The EGL disappears by P21, after CGNP migrate into the IGL. Granule neuroblasts undergo a final maturation process in the IGL and establish synapses, leaving the adult cerebellar cortex with three highly defined layers: molecular (ML), purkinje (PL), and internal granule cell layers. Throughout postnatal development, there are also periods of apoptosis, which help to coordinate cerebellar cytoarchitecture[14, 15]. Furthermore, a tightly regulated spatiotemporal program of gene expression orchestrates the specific cellular events associated with neuronal differentiation[16]. Our recent study indicated that AhR is robustly expressed between PND6-10, a critical period of granule neuroblast

development. Additionally, TCDD reduced CGNP proliferation *in vitro*, suggesting that exposure could modify neuroblast differentiation. These observations lead to the hypothesis that TCDD exposure interferes with cerebellar development by prematurely instigating granule neuroblast maturation. One goal of this study was to determine the impact of dioxin exposure on the expression of genes that are critical for granule neuron development. The second objective was to characterize the effects of postnatal TCDD exposure on CGNP differentiation, migration, and normal programmed cell death (PCD) The findings from this study support the hypothesis that TCDD precociously activates the granule neuroblast differentiation program. Additionally, TCDD exposure disrupted CGNP migration and increased PCD. This study also raises interesting questions regarding the molecular mechanism of TCDD neurotoxicity and the endogenous function of AhR during cerebellar granule neuroblast maturation. Future studies are obviously required to determine the functional outcomes related to abnormal cerebellar development following TCDD exposure.

Results and Discussion

TCDD modifies gene expression patterns during cerebellar granule neuroblast maturation. Two separate approaches were implemented to test the hypothesis that TCDD exposure modifies the genetic differentiation program to interfere with CGNP maturation. First, gene expression profiling experiments were accomplished in freshly isolated CGNP 8 hours following *in vivo* exposure to 1.0 μg/kg TCDD on postnatal day (PND) 6, a time period when AhR expression is highest during postnatal cerebellar development [5]. This time point also corresponds to the peak period of CGNP proliferation [13]. To determine the direct effects of TCDD on CGNP differentiation, gene expression patterns were also analyzed in granule neuroblasts that were isolated on PND6. One day following isolation, cultured CGNP were exposed to vehicle, 1nM, or 10 nM TCDD *in vitro* and RNA was prepared after 6 hours, and then subjected to gene profiling analyses. Results from both experimental paradigms indicated that approximately 45-52% of the genes modulated by TCDD play a role in granule neuroblast proliferation, differentiation, migration, and synaptogenesis. These studies also suggest that TCDD exposure interferes with the expression of molecules that regulate transcription, cell cycle activity, and programmed cell death (PCD). Altered expression of relevant candidate genes (i.e., Zipro-1; Sox4, Laminin Receptor, Ephrin B1, and p55CDC) was confirmed by RT-PCR. Based on these gene expression fingerprints, TCDD exposure appears to be associated with accelerated maturation of CGNP. Therefore, additional studies tested this hypothesis by investigating CGNP development in mice following TCDD exposure.

Postnatal TCDD Exposure interferes with cerebellar layer formation. During rodent cerebellar development, the EGL rapidly expands between PND 5-8 due to CGNP proliferation then wanes and eventually disappears by PND21 following neuroblast migration through the ML into the IGL[13]. To determine if TCDD altered the progression of granule neuroblast development, C57/B6 mice were exposed to vehicle or TCDD (1.0 μg/kg) by gavage on PND6 and perfused four days later. AhR is at peak expression throughout this time period. Following TCDD exposure, the EGL was significantly reduced in thickness, by approximately 50% (p<0.01) on PND10 (Fig.1). This observation was accompanied by a 25% increase the ML thickness, as compared to vehicle controls (p<0.01). These data indicate that there is an early disappearance of the EGL following TCDD exposure and further support the contention that TCDD precociously activates CGNP differentiation and migration during development.

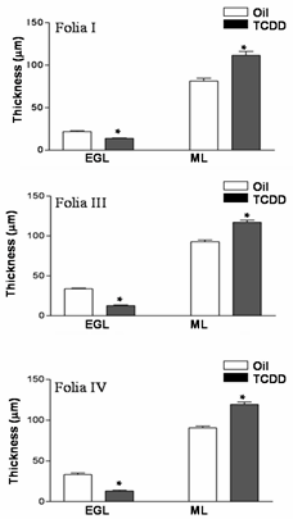

Fig. 1. TCDD promotes early disappearance of the EGL and a premature increase in ML thickness during cerebellar development (p< 0.01 compared to oil controls).

Postnatal TCDD exposure disrupts cerebellar granule neuroblast cell cycle regulation, differentiation, migration, and normal patterns of apoptosis. To further characterize the effects of TCDD on the progression of CGNP differentiation, C57/B6 mice were injected with 50mg/kg bromodeoxyuridine (BrdU) on PND6 to label cells during S-phase of the cell cycle for studying cell fate following TCDD exposure. Mice were then exposed to vehicle or TCDD (1 μg/kg) by gavage four hours later. On PND10, animals were perfused and cerebellar tissue was processed for immunohistochemistry (IHC) to examine the impact of TCDD on CGNP development. The cellular expression of phosphorylated histone 3 (pH3) protein was examined to determine if TCDD impacts CGNP cell cycle regulation. Because pH3 is associated with the G2/M phase of the cell cycle, it is routinely used as a marker to identify mitotic cells [17]. TCDD exposure produced a 50% increase in the number of pH3-positive granule neuroblasts in the EGL following TCDD treatment. These results suggest that TCDD may interfere with granule neuroblast cell cycle activity following postnatal exposure. It is possible that TCDD exposure was associated with CGNP mitotic arrest suggesting that differentiation or migration of this subpopulation could be altered. TAG-1 expression was evaluated by IHC to further examine whether TCDD exposure modifies the genetic program of granule neuroblast differentiation and/or migration. TAG-1, a protein associated with migration of CGNP, is normally expressed in postmitotic cells localized in the premigratory inner EGL [18]. TCDD exposure generated uncharacteristic TAG-1 staining patterns in CGNP that reside in the inner premigratory EGL. Granule neuroblasts appeared to have more mature neurite outgrowth and enhanced levels of TAG-1 protein following TCDD. These observations are consistent with the hypothesis that TCDD exposure accelerates the onset of CGNP differentiation. Furthermore, the altered TAG-1 staining patterns suggests cellular maturation states that could adversely impact granule neuroblast migration through the ML, which could temporally disrupt synaptogenesis or lead to unscheduled PCD if cells fail to reach the IGL in a timely manner. To examine the migration patterns of cells that were labeled during S-phase on PND6, cerebellar tissue was processed for IHC detection of BrdU on PND10. BrdU positive cells were not observed in the EGL following either vehicle or TCDD treatment. However, approximately 50% fewer migrating granule neuroblasts were observed in the ML following TCDD exposure compared to controls ($p<0.05$). These findings suggest that TCDD temporally disrupts granule neuroblast migration from the EGL to the IGL during a critical period of cerebellar development. Cerebellar granule neurons may have reached the IGL more quickly or have undergone apoptotic death prior to reaching their final position. To determine if TCDD exposure on PND6 increased programmed cell death (PCD) associated with CGNP development, Bax (a pro-apoptotic protein) expression was examined between PND7-10, a time period when apoptosis normally peaks in the EGL [14]. Immunoblot analyses demonstrated that Bax levels were significantly elevated in the cerebellum between PND 7-10 compared to vehicle controls ($p<0.05$ on PND8; $p<0.001$on PND10) following TCDD exposure on PND6. These results suggest that TCDD exposure is associated with increased programmed cell death in the developing cerebellum. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining further confirmed that TCDD exposure on PND6 altered PCD patterns during CGNP development on PND 10. Most of the apoptotic bodies were localized to the EGL in vehicle controls. In the EGL, an equivalent number of TUNEL positive cells were detected following exposure to either vehicle or TCDD (Fig.2). However, in TCDD-exposed animals, there was approximately a five-fold increase in the number of TUNEL positive granule neuroblasts in the cerebellar ML and IGL compared to controls ($p<0.001$). These observations suggest that increased and uncharacteristic patterns of PCD occur following TCDD exposure during a critical period of cerebellar development.

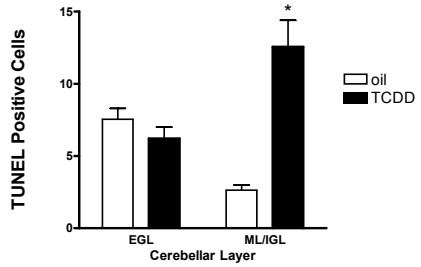

Fig. 2. TCDD disrupts normal PCD patterns and increases apoptosis in the cerebellum during development (p<0.001 compared to oil controls)

Conclusions.

This study characterized the impact of *in vivo* TCDD exposure on granule neuroblast maturation at the cellular level during a vulnerable phase of cerebellar development. TCDD exposure was shown to modify the

expression of genes that regulate cell cycle activity, neuronal migration, synaptogenesis, and apoptosis. These observations were consistent with the hypothesis that TCDD accelerates the onset of granule neuroblast differentiation. At the morphological level, TCDD exposure interfered with granule neuroblast migration and enhanced PCD. Additional studies are necessary to elucidate the mechanism by which TCDD interferes with granule neuroblast differentiation, and the role of AhR in this process. However, it is conceivable that the observed increased PCD levels and abnormal apoptotic patterns following TCDD exposure could result from desynchronization of normal molecular cues that regulate CGNP differentiation during a critical developmental period. Abnormal development of CGNP could ultimately impair synaptogenesis and disrupt intercellular communication, particularly with Purkinje cells, which are the sole output neurons from the cerebellar cortex. Therefore, TCDD could interfere with cerebellar neurochemical signaling and give rise to neurobehavioral abnormalities.

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