2,3,7,8-Tetrachlorodibenzo-\textit{p}-Dioxin Exposure Disrupts Granule Neuron Precursor Maturation in the Developing Mouse Cerebellum

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The widespread environmental contaminant 2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin (TCDD) has been linked to developmental neurotoxicity associated with abnormal cerebellar maturation in both humans and rodents. TCDD mediates toxicity via binding to the aryl hydrocarbon receptor (AhR), a transcription factor that regulates the expression of xenobiotic metabolizing enzymes and growth regulatory molecules. Our previous studies demonstrated that cerebellar granule neuron precursor cells (GNPs) express transcriptionally active AhR during critical developmental periods. TCDD exposure also impaired GNP proliferation and survival \textit{in vitro}. Therefore, this study tested the hypothesis that TCDD exposure disrupts cerebellar development by interfering with GNP differentiation. \textit{In vivo} experiments indicated that TCDD exposure on postnatal day (PND) 6 resulted in increased expression of a mitotic marker and increased thickness of the external granule layer (EGL) on PND10. Expression of the early differentiation marker TAG-1 was also more pronounced in postmitotic, premigratory granule neurons of the EGL, and increased apoptosis of GNPs was observed. On PND21, expression of the late GNP differentiation marker GABA\textsubscript{\textit{A}6} receptor (GABA\textsubscript{\textit{A}6}) and total estimated cell numbers were both reduced following exposure on PND6. Studies in unexposed adult AhR\textsuperscript{-/-} mice revealed lower GABA\textsubscript{\textit{A}6} levels and DNA content. \textit{In vitro} studies showed elevated expression of the early differentiation marker \textit{p27}\textsuperscript{KIP1} and the GABA\textsubscript{\textit{A}6} in GNPs following TCDD exposure, and the expression patterns of proteins related to granule cell neurite outgrowth, \textit{\beta}III-tubulin and polysialic acid neural cell adhesion molecule, were consistent with enhanced neuroblast differentiation. Together, our data suggest that TCDD disrupts a normal physiological role of AhR, resulting in compromised GNP maturation and neuroblast survival, which impacts final cell number in the cerebellum.

Key Words: neurogenesis; neurotoxicity; neural progenitor; differentiation; TCDD; cell cycle.

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observations raised the hypothesis that TCDD disrupts the process of GNP differentiation during cerebellar development.

Numerous dynamic cellular processes including, proliferation, differentiation, migration, and apoptosis are essential for proper cerebellar development (Altman and Bayer, 1997; Encha-Razavi and Sonigo, 2003; White and Barone, 2001). Following proliferation in the superficial external granular layer (EGL), which peaks between postnatal days (PND) 5–7, cerebellar granule neuroblasts accumulate p27/Kip1 (Miyazawa et al., 2000), become postmitotic, and migrate into the deep zone of the EGL. Postmitotic, premigratory, and migrating GNP s elaborate neurites and express the neuron-specific cytoskeletal protein, βIII-tubulin (Tuj1) (Przyborski and Cambray-Deakin, 1997), as well as TAG-1, a cell surface glycoprotein (Furley et al., 1990). Neuroblasts then migrate inward to form the internal granule cell layer (IGL) of the cerebellar cortex by PND21 (Hatten, 1999). In the IGL, granule neuroblasts terminally differentiate and undergo synaptogenesis, which is characterized by polysialic acid neural cell adhesion molecule (PSA-NCAM) and GABAR_A6 receptor (GABAR_A6) expression (Rutishauser and Landmesser, 1996; Zheng et al., 1993). Therefore, the adult cerebellar cortex has three highly defined layers, the molecular layer (ML), Purkinje layer, and IGL (Altman and Bayer, 1997). Disruption at any stage of this tightly regulated developmental process may alter subsequent cellular events and result in permanent deficits.

The proper timing and balance between cell proliferation and cell death is crucial in regulating the number and types of cells present during central nervous system development (Encha-Razavi and Sonigo, 2003). Two discrete waves of apoptosis occur during development of the rodent cerebellum. In the rat, the first occurs within the EGL and peaks at PND10–12, coinciding with the peak period of neuroblast migration from the EGL to the IGL (White and Barone, 2001). The second wave of apoptosis reportedly occurs in the IGL around PND21 (White and Barone, 2001), which is also a time of extensive synaptogenesis. Developmental apoptosis is essential for the establishment of final cell numbers and proper neuroanatomical connections in the mature brain.

This study tested the hypothesis that TCDD exposure interferes with cerebellar development by disrupting granule neuroblast maturation. We examined early and late GNP differentiation in mice following TCDD exposure during a critical period of neurogenesis that coincides with peak AhR expression. Cerebellar DNA content, bromodeoxyuridine (BrdU) incorporation, and terminal transferase deoxyuridine triphosphate nick-end labeling (TUNEL) were measured as indications of changes in cell proliferation/death and cell fate in the cerebellum following oral exposure to TCDD on PND6. Apoptosis was also estimated by examining expression of the proapoptotic Bax protein (Krajewska et al., 2002). Differentiation was investigated by monitoring neurite outgrowth and the temporally expressed developmental markers TAG-1, p27, βIII-tubulin, PSA-NCAM, and GABAR_A6. Our results suggest that TCDD interferes with granule neuroblast maturation. The dysregulation of GNP differentiation associated with TCDD exposure during neurogenesis appeared to reduce final cell number. Interestingly, AhR<sup>−/−</sup> mice also exhibited an abnormal cerebellar phenotype in the absence of TCDD exposure. Therefore, this study raises interesting questions regarding the molecular mechanism of TCDD neurotoxicity as well as the endogenous function of AhR in the cerebellum.

**MATERIALS AND METHODS**

**Reagents.** TCDD was obtained from Cambridge Isotopes (Cambridge, MA) and solubilized in dimethyl sulfoxide (DMSO). Triton X-100, bovine serum albumin (BSA), and DMSO were purchased from Sigma (St Louis, MO). Dulbecco’s phosphate buffered saline (DPBS) was purchased from Gibco (Grand Island, NY).

**Experimental animals.** C57BL/6J and AhR<sup>−/−</sup> mice (strain B6. 129-AhR<sup>fl/fl</sup>) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained on a 12-h light/dark cycle with food and water provided ad libitum and kept in accordance with the guidelines set by the University of Rochester University Committee on Animal Resources and the American Association for Laboratory Animal Science. Both male and female mice were analyzed in the following experiments, with no sex-specific differences in the effects of TCDD on cerebellar development observed.

**In vivo TCDD exposure.** Male and female mice were gavaged with 1.0 μg/kg TCDD dissolved in olive oil (vehicle) or with vehicle alone on PND6. Animals from at least three separate litters were subjected to each exposure condition. For all experiments, one male and one female pup per litter were analyzed for each exposure condition. Differences between males and females were not observed. A subset of animals was perfused via cardiac puncture, at the appropriate exposure endpoint, with saline followed by 4% paraformaldehyde. The tissue was then processed for immunohistochemical analysis. Separate animals were anesthetized with CO<sub>2</sub> on PND7, 8, 10, or 21, and cerebella were quickly removed and frozen at −80°C until they were processed for immunoblot analysis or DNA quantification.

**Quantification of DNA content.** DNA levels were measured using a dye-binding method (Labarca and Paigen, 1980) modified as follows. Tissues were homogenized briefly in 50mM sodium phosphate, 2M NaCl, 2mM ethylenediaminetetraacetic acid (pH 7.4) (Polytron PT 1200C, Brinkmann Instruments, Westbury, NY). Hoechst 33258 (Sigma) was added to samples at a final concentration of 1 μg/ml and samples were then read in a spectrophotometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 356 nm and an emission wavelength of 458 nm. The amount of DNA in each sample was extrapolated using linear regression with purified calf thymus DNA (Sigma) as a standard.

**Western blot analysis.** Cerebellar tissue was homogenized in PBS containing 0.1% Triton X-100 and antiprotease cocktail (Roche Molecular Biochemical, Manheim, Germany). Protein concentrations were determined by the microBCA assay (Pierce, Rockford, IL). Proteins (20–75 μg) were fractionated on 7% acrylamide gels and transferred to Immobilon PVDF membranes (BioRad, Hercules, CA). Membranes were blocked with 5% powdered milk containing 0.2% Tween-20 and probed with antibodies specific for p27 (1:2500; Santa Cruz, Plymouth Meeting, PA), GABAR_A6 (1:250; Chemicon, Temecula, CA), or Bax (1:5000; Chemicon, Temecula, CA), overnight at 4°C. Membranes were then probed with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000; Jackson Immunoresearch, Westgrove, PA) for 2 h, at room temperature. Proteins were visualized using a chemiluminescent substrate (Amersham Biosciences, Piscataway, NJ). All immunoblots were stripped and reprobed with anti-β-actin (1:5000; Sigma) to confirm equal protein loading. GABAR_A6, p27, and...
Bax proteins were not detected on blots that were incubated with either secondary antibodies alone or nonspecific IgG. Western blot band density was analyzed using SCION Image 4.0.2 or AlphaEaseFC 4.0 software.

**BrdU administration to analyze cell fate.** Mice were given a single injection of BrdU (50 mg/kg body weight; Sigma) 4 h prior to TCDD exposure on PND6, to label a subset of cells that were proliferating at this time point. This time point was chosen because it represents the peak proliferative phase during granule neuroblast development in the EGL. Four days later, the animals were perfused (as described above). Brains were then removed, and tissue was processed for immunohistochemical analysis.

**Immunohistochemistry.** Following perfusion (as above), whole brains were fixed in 4% paraformaldehyde overnight. Brains were transferred to 30% sucrose for 24 h. Cerebella were sectioned with a freezing, sliding microtome into 30-μm sagittal sections. Sections were rinsed in phosphate buffered saline containing 0.3% Triton X-100 (PBST). For tissue to be stained for BrdU, sections were incubated in 2M HCl for 60 min, rinsed, incubated in 3% hydrogen peroxide for 30 min, and rinsed again. All tissue was blocked in 10% normal goat serum in PBST for 1 h, and incubated with a monoclonal antibody against Tag-1/ID7 (1:400; Developed by Miyuki Yamamoto and obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health & Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA), a polyclonal antibody against phospho-Histone H3 (Ser 10; Upstate Cell Signaling Solutions), or a monoclonal antibody against BrdU (Sigma) overnight, at 4°C. After rinsing, sections were incubated in PBST containing the appropriate Alexa Fluor 488-conjugated secondary antibodies (1:1000; Molecular Probes, Eugene, OR) for 90 min at room temperature. Cell nuclei were subsequently stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Slides were coverslipped using anti-fade (Molecular Probes) mounting media. Fluorescence was visualized using a Nikon Eclipse 80i microscope. Images were obtained using SPOT Advanced software (Diagnostic Instruments, Inc.). Fluorescence was not detected in granule neuroblast cultures probed with secondary antibody alone. Cells positive for pH3 localized to the EGL were obtained using SPOT Advanced software (Diagnostic Instruments, Inc.). Fluorescence was visualized using a Nikon Eclipse 80i microscope. Images were obtained using SPOT Advanced software (Diagnostic Instruments, Inc.) for 90 min, at room temperature. Cell nuclei were subsequently stained with 1 μg/ml DAPI. Slides were coverslipped using anti-fade (Molecular Probes) mounting media. Fluorescence was visualized using a Nikon Eclipse 80i microscope. Images were obtained using SPOT Advanced software (Diagnostic Instruments, Inc.). Fluorescence was not detected in granule neuroblast cultures probed with secondary antibody alone.

**TUNEL staining.** The TUNEL assay for detection of apoptosis was accomplished with a commercially available in situ apoptosis detection kit (NeuroTACS II; R&D Systems, Minneapolis, MN). Following perfusion (as above) whole brains were fixed in 4% paraformaldehyde overnight. Brains were transferred to 30% sucrose for 24 h. Cerebella were sectioned sagittally at 10 μm on a cryostat (Microm HM500CM; Walldorf, Germany). Cryosections were dried for 2 h, at 45°C, on a slide warmer. Prior to labeling, sections were rehydrated in PBS. Tissue was then incubated with NeuroPore (R&D Systems, Minneapolis, MN) for 30 min in a 37°C humified chamber, to make the DNA accessible to the labeling enzyme. Sections were rinsed and immersed in labeling buffer (1× terminal deoxynucleotidyl transferase buffer) for 5 min, followed by incubation with labeling solution (R&D Systems, Minneapolis, MN) for 1 h in a 37°C humidified chamber. After labeling, sections were washed in PBS, at room temperature, prior to a 2-min incubation with DAB Working Solution (R&D Systems, Minneapolis, MN). Sections were then rinsed, counterstained, dehydrated, and coverslipped. Selected sections were incubated with DNase for 30 min, prior to TUNEL staining to serve as a positive control. Specificity of staining was determined by comparison with a negative control created by omitting the terminal transferase enzyme. TUNEL-positive cells were counted in four to six sections in folium III from three animals per exposure condition. The experimenter was blinded to the exposure groups.

**Quantification of layer thickness.** The EGL thickness was measured in DAPI-stained sagittal sections of the cerebellum. Images were captured using SPOT Advanced software (Diagnostic Instruments, Inc.) linked to a Nikon Eclipse 80i microscope. Layer thickness was analyzed using ImageQuant Software (Amersham Biosciences, Piscataway, NJ). Five randomly chosen fields of lobule III were analyzed in every section from three animals from each experimental group (four to six sections per animal). Values were reported as the mean ± SEM from the five fields measured.

**Reaggregates and monolayer cultures.** Primary granule cell cultures were prepared as previously described (Gao et al., 1991; Opanashuk et al., 2001). Briefly, cerebella were quickly dissected from PND5–6 C57BL/6J mice and the meninges removed prior to the addition of trypsin and DNase. Dissociated cells were then passed through a 30 μm nylon mesh ( Spectrum Laboratories, Rancho Dominguez, CA), and centrifuged through a 35%: 60% percoll gradient (Amersham Biosciences, Piscataway, NJ). Cells were preplated for 90 min in poly-n-lysine (0.1 mg/ml; Sigma) coated flasks. Cells for reaggregate cultures (see procedure for monolayer cultures below) were then resuspended in minimal essential medium (MEM) containing 10% horse serum, 5% fetal bovine serum, glucose (9 mg/ml), glutamine (292 mg/ml), and penicillin (0.1%) and plated in 96-well plates at a density of 3 × 10³ cells/well. After a 24-h reaggregation period, cells were transferred to serum-free media (SFM) that contained MEM, B27 and N2 supplements, glucose (9 mg/ml), glutamine (292 mg/ml), and penicillin (0.1%), and plated on high molecular weight poly-n-lysine coated plates (0.1 mg/ml). This time point is designated in vitro day 1 (DIV1). The characterization of these cultures, as previously described (Gao et al., 1991) by morphological and immunocytochemical criteria indicated that > 98% of these cells were of the granule neuron lineage. This culture model is conducive to examining early differentiation because cells retain their ability to proliferate as well as elaborate neurites (Gao et al., 1991).

**Immunocytochemistry.** After a 24-h reaggregation period, granule neuroblasts were plated into 12-well culture plates (VWR, Bridgeport, NJ) coated with 0.1 mg/ml poly-n-lysine. Cells were treated with DMSO or TCDD (0.1–10 μM) at the time of plating. After the incubation period, the medium was removed and cells were rinsed with DPBS, and then fixed with 4% paraformaldehyde for 30 min, at room temperature. After rinsing, neurons were incubated in PBST containing 10% BSA for 30 min, at room temperature. Reaggregates were then incubated overnight, at 4°C, in PBST containing PSA-NCAM antibody (undiluted; a generous gift from Dr Mark Noble, University of Rochester, Rochester, NY) or β III-tubulin antibody (1:100; Chemicon, Temecula, CA). After rinsing, cells were incubated in PBST containing Alexa Fluor 488 goat anti-mouse secondary antibody (1:1000; Molecular Probes) for 90 min, at room temperature. Cell nuclei were subsequently stained with 1 μg/ml DAPI. Fluorescence was visualized using a Nikon Eclipse TiS100 inverted microscope. Images were obtained using SPOT Advanced software (Diagnostic Instruments, Inc.). Fluorescence was not detected in granule neuroblast cultures probed with secondary antibody alone.

**In vitro cell counts.** Approximately 500 DAPI-stained neurons in each culture, located in the cell matrix surrounding reaggregates, were counted using a Nikon Eclipse T100 epifluorescence microscope (40× objective) with the experimenter blinded to the exposure groups. The numbers of PSA-NCAM–positive cells were counted in each culture. Values were expressed as a percentage of PSA-NCAM–positive neuroblasts relative to the total number of DAPI-stained cells in each culture. At least four cultures were evaluated for each experimental condition. Each granule neuroblast reaggregate culture was prepared from cells that were isolated from separate litters.

**Statistical analyses.** Data were expressed as mean ± SEM. Analysis of variance and the Fisher’s protected t test (Statview 5.0) was used for individual comparisons in PSA-NCAM–positive in vitro cell count data. Unpaired Student’s t-tests were performed to evaluate EGL thickness data, Western blot quantification data, and in vivo cell counts (GraphPad Prism 4.0). p values < 0.05 were considered statistically significant. Sample sizes are indicated in the figure legends.
RESULTS

Postnatal TCDD Exposure Elicits Increased Numbers of Granule Neuroblasts Expressing Phosphorylated Histone 3, a Marker of Mitosis, and Increased EGL Thickness

The cellular expression of phosphorylated histone 3 (pH3) protein was examined to determine if TCDD impacts GNP cell cycle regulation. pH3 has been associated with the G2/M phase of the cell cycle and has been routinely used to label mitotic cells (Juan et al., 1998). To determine whether mitosis was affected by TCDD, C57BL/6J mice were exposed to oil (vehicle) or TCDD (1 μg/kg) by oral gavage on PND6, and analyzed on PND10. TCDD exposure produced a 50% increase in the number of pH3-positive granule neuroblasts in the EGL on PND10 (Fig. 1A). Analysis of DAPI-stained sections also revealed that the EGL was approximately 30% thicker in TCDD-exposed mice (Fig. 1B). These results suggest that TCDD influences granule neuroblast proliferation following postnatal exposure during the GNP expansion phase, which peaks between PND5–7 (Altman and Bayer, 1997).

TCDD Exposure Modifies TAG-1 Expression Patterns in the Mouse Cerebellum

TAG-1 is a transient cell surface glycoprotein that belongs to the Ig family of adhesion molecules (Furley et al., 1990), which are known to play a pivotal role in neuroblast migration. TAG-1 is an early differentiation marker for GNPs and expression is predominantly localized to the cell soma and parallel fibers during the initial stages of axon outgrowth (Yamamoto et al., 1990). Because TAG-1 is transiently expressed by postmitotic cells residing in the premigratory inner EGL, it is thought to be functionally associated with the initial stages of axon outgrowth and interactions between GNPs and other developing cells (Furley et al., 1990). TCDD exposure resulted in more pronounced TAG-1 expression in cerebellar granule neuroblasts localized to the premigratory region of the EGL (Fig. 2). Moreover, the cellular density in the EGL appeared to increase following TCDD exposure as visualized when the cell nuclei were stained with DAPI (Fig. 2, upper right panel), which is consistent with our data suggesting increased numbers of mitotic cells and increased thickness of the EGL (Fig. 1). These observations support the hypothesis that TCDD exposure targets the mitotic precursor pool and promotes GNP differentiation.

TCDD Exposure Reduces the Number of GNPs in the ML on PND10

Along with differentiation, migration of GNPs from the EGL to the IGL is a critical event in establishing the layered structure and proper function of the mature cerebellum. The effects of TCDD on GNP fate following migration from the EGL were studied using BrdU incorporation to label and track a subpopulation of cells migrating through the

FIG. 1. pH3 expression in GNPs and EGL thickness are increased following TCDD exposure. Mice were exposed to oil (vehicle) or TCDD (1 μg/kg) by oral gavage on PND6 and tissue was harvested on PND10. (A) The upper photomicrographs show DAPI-stained sagittal cerebellar sections following exposure to oil (left) or TCDD (right). pH3-positive (green) staining is shown below. The graph indicates pH3-positive cell counts in folium III. Data represent mean ± SEM from four to six sections from three separate animals per exposure condition; *p < 0.001 versus OIL control. Scale bars: 100 μm. (B) Thickness of the EGL was measured in folium III in DAPI-stained cerebellar sections from three separate animals. Data represent mean ± SEM (n = 3); four to six sections were analyzed from each of three separate mice per exposure group; *p < 0.001 versus OIL control.
ML. BrdU-positive cells were not observed in the EGL following either vehicle or TCDD exposure (Fig. 3A). However, approximately 50% fewer BrdU-labeled cells were observed in the ML following TCDD exposure compared with controls (Fig. 3B). These findings suggest that TCDD could disrupt the timing of granule neuroblast migration from the EGL to the IGL. Alternatively, increased cell death among GNPs may result in substantial loss of cells prior to, or during, migration through the ML.

**GNP Apoptosis is Elevated in the Developing Cerebellum following TCDD Exposure**

To explore the possibility that GNPs are lost through increased apoptosis following TCDD exposure, expression of the proapoptotic protein Bax was measured. Bax protein levels were measured in cerebellar tissue from mice at PND7–10, a time period when apoptosis is normally elevated in the EGL (White and Barone, 2001). Immunoblot analyses demonstrated that Bax levels were significantly elevated in the cerebellum between PND8–10, following TCDD exposure on PND6 (Fig. 4). TUNEL staining was used to further measure programmed cell death among GNPs. TCDD exposure resulted in changes in the apoptotic program on PND10 (Fig. 5). Most of the apoptotic bodies were localized to the EGL in vehicle controls (Fig. 5A), and an equivalent number of TUNEL-positive cells were detected in the EGL in exposed tissue (Fig. 5B). However, in TCDD-exposed animals, there was an approximately fivefold increase in the number of TUNEL-positive granule neuroblasts in the cerebellar ML and IGL compared with controls (Fig. 5C). These data demonstrate that GNP cell death is increased and accompanied by alterations in spatial patterns of apoptosis following TCDD exposure during development of the cerebellum.

**DNA Content and GABAR_{A26} Protein Expression are Reduced in the Cerebellum on PND21 following Early Postnatal TCDD Exposure**

Previous studies have demonstrated that alterations in total DNA content are comparable to measurements derived from stereological cell counting, and can therefore serve as a surrogate method for estimating cell number within specific brain regions (Cheng et al., 2002). Exposure to TCDD on PND6 decreased DNA content by approximately 15% at PND21 (Fig. 6A). This observation suggests that TCDD exposure during the GNP expansion phase results in a long-term reduction in total cell number in the cerebellum. The final stage in postnatal cerebellar development involves granule neuron differentiation, which is characterized by the extension of neurites to form synapses with PCs and input fibers extending into the cerebellar cortex from the deep cerebellar nuclei. The EGL in mice disappears by PND15, but GNPs continue to migrate through the ML until the IGL is fully established at PND21. Expression of the GABAR_{A26} subunit is restricted to cerebellar granule neurons in the IGL during development, and expression is indicative of differentiation (Zheng et al., 1993). Therefore, to further determine whether GNP differentiation is disrupted following TCDD exposure, GABAR_{A26} protein levels were evaluated following exposure
to TCDD (1 μg/kg) on PND6. Immunoblot analyses of TCDD-exposed cerebellar tissue collected on PND21 revealed that GABARα6 protein levels were diminished by approximately 25% (Fig. 6B). This finding suggests that either late GNP differentiation is disrupted or there are fewer granule neurons present in the IGL, resulting in fewer differentiated neurons expressing GABARα6 protein.

**DNA Content and GABARα6 Expression are Reduced in the Adult AhR−/− Cerebellum**

In many cases, the AhR−/− mouse model has been a useful tool to determine whether the effects of TCDD exposure in a particular tissue or cell type are mediated through activation of AhR. Therefore we accomplished an initial analysis of cerebella from AhR−/− mice to determine whether defects exist due solely to the absence of AhR. Cerebellar tissue from adult (10 weeks old) AhR−/− and WT control mice was prepared for DNA content analysis. The lack of AhR was associated with a 30% reduction in DNA content compared with age-matched control mice (Fig. 7A). Furthermore, immunoblot analyses of cerebellar homogenates from adult AhR−/− mice revealed that GABARα6 protein levels were approximately 30% lower than those of WT controls (Fig. 7B). These data show that the lack of AhR results in an abnormal cerebellar phenotype. Therefore, use of this model to determine whether AhR is required for TCDD-mediated disruption of cerebellar development would result in data that are difficult to interpret.

**Early Differentiation Marker Expression is Increased following TCDD Exposure In vitro**

To determine the direct effects of TCDD on GNP differentiation, granule neuroblasts isolated from the cerebellum and cultured as reaggregates were exposed to DMSO or TCDD in vitro. The reaggregate culture model system is appropriate for examining early differentiation events such as cell cycle exit and neurite elaboration. The cell cycle regulatory protein p27 is a marker for postmitotic cells in the EGL and is
also an AhR target gene (Kolluri et al., 1999). Immunoblot analysis demonstrated a 40% increase in the expression of p27, an early marker of GNP differentiation and cell cycle exit (Miyazawa et al., 2000), compared with vehicle control cultures, beginning 2 h following TCDD exposure in vivo (Fig. 8). These observations suggest that TCDD exposure can directly modulate GNP differentiation.

**TCDD Promotes Neurite Outgrowth and Alters βIII-Tubulin Expression Patterns In vitro**

To further assess the direct impact of TCDD on granule neuron differentiation in vitro, neurite outgrowth was examined in reaggregate cultures exposed to vehicle (DMSO) or TCDD. Neurite sprouting was observed in cultures 4 h after TCDD exposure, but was not observed in control cultures (data not shown). After 8 h, TCDD-exposed reaggregate cultures showed signs of premature differentiation as evidenced by more pronounced neurite outgrowth and cytoskeletal polymerization compared with vehicle controls (Fig. 9A). These observations suggest that TCDD exposure can directly modulate GNP differentiation.

**TCDD Exposure In vitro Increases the Number of PSA-NCAM–Positive Granule Neuroblasts**

The direct effect of TCDD on later stages of GNP maturation was further examined in reaggregate cultures prepared from the cerebella of mice at PND5–6. PSA-NCAM is expressed by differentiating neurons and is thought to be important for morphoregulatory processes such as neurite outgrowth,
fasciculation, migration, and synaptogenesis (Durbec and Cremer, 2001). To determine the impact of TCDD on PSA-NCAM expression, granule neuroblast reaggregate cultures were exposed to DMSO or TCDD for three days. Cultures were then stained for PSA-NCAM, as a marker for differentiating neuroblasts (Durbec and Cremer, 2001), along with βIII-tubulin (Fig. 10A). The number of cells expressing PSA-NCAM was then counted, demonstrating a concentration-dependent increase in PSA-NCAM–positive cells following in vitro TCDD exposure (Fig. 10B). These results further suggest that GNP differentiation is stimulated by TCDD exposure.

**TCDD Exposure Increases Late Differentiation Marker Expression In vitro**

Previous studies have demonstrated that GABAR<sub>α<sub>6</sub></sub> is expressed in cerebellar granule neuron monolayer cultures containing 25mM KCl, which is required in order to mimic excitatory mossy fiber input (Gault and Siegel, 1997). This input is necessary for granule neurons to terminally differentiate. To further assess the possibility that TCDD promotes granule neuroblast differentiation, GABAR<sub>α<sub>6</sub></sub> expression was examined in granule neuron monolayer cultures exposed to DMSO or TCDD. TCDD-exposed neurons exhibited a 50% increase in GABAR<sub>α<sub>6</sub></sub> expression, compared with vehicle controls, after 6 days in culture (Fig. 11). Again, our findings support the idea that TCDD promotes granule neuroblast differentiation.

**DISCUSSION**

This investigation tested the hypothesis that TCDD exposure disrupts cerebellar granule cell neurogenesis. We define “neurogenesis” to be the production of neurons, which includes the molecular signaling events associated with precursor proliferation, differentiation, and death. Mice were exposed during the peak of GNP expansion to determine whether this is a period during which cells are vulnerable to TCDD toxicity. Our findings suggest that TCDD impacts both proliferating and differentiating GNPs. Moreover, studies from AhR<sup>−/−</sup> animals support a physiological role for AhR in cerebellar neuron development and/or maintenance.
AhR has been implicated in cell cycle regulation but TCDD actions on cell growth appear to be diverse and cell type specific (Puga et al., 2002). Our data suggest that TCDD exposure is associated with enhanced GNP mitotic activity. TCDD exposure increased the expression of pH3, a chromatin assembly factor associated with the G2/M phase of the cell cycle (Nowak and Corces, 2004), in GNPs within the EGL. Analysis of layer thickness also indicated a more substantial EGL in TCDD-exposed mice, suggesting that GNP numbers are elevated. These observations imply that TCDD is increasing the proliferative pool within the EGL rather than inducing mitotic arrest, suggesting distinct actions of TCDD in different cell types. Such aberrant GNP cell cycle activity could ultimately interfere with early precursor differentiation in the EGL. Because mice were exposed on PND6 and analyzed on PND10, it is difficult to reconcile the precise actions of TCDD on mitosis. Future studies that examine the impact of TCDD on proliferation at shorter time points following exposure will clarify its specific actions on GNP cell cycle activity and differentiation.

AhR activation has also been implicated in modulation of cellular differentiation. For example, neuroblastoma cells exhibited a neuronal phenotype following AhR overexpression (Akahoshi et al., 2006). TCDD has also been reported to impact differentiation in non-neural cells in a tissue specific manner (Hanlon et al., 2003; Ray and Swanson, 2003).
Therefore, we examined whether TCDD exposure influenced early GNP differentiation in vivo. TAG-1 is an early GNP differentiation marker that is transiently expressed and restricted to postmitotic cells in the premigratory EGL. The initial stages of axon outgrowth and interactions between GNPs and other developing cells are thought to be mediated by TAG-1 (Furley et al., 1990). Following TCDD exposure, TAG-1 protein expression was enhanced in the premigratory EGL compared with vehicle controls. These observations suggest that TCDD exposure induces differentiation and neurite outgrowth, supporting the hypothesis that TCDD promotes early stages of GNP differentiation. Abnormal differentiation of GNPs in the EGL could also influence migration into the IGL. When tracking a subpopulation of GNPs, which were labeled with BrdU 4 h prior to TCDD exposure on PND6, there were fewer migrating neuroblasts in the ML of exposed mice on PND10. However, increases in BrdU-positive cells were not readily detectable in the IGL of exposed animals, suggesting that the timing of migration was not altered. There were also increased numbers of TUNEL-positive cells in the ML, which suggests that apoptosis is increased in the migrating GNP population. Together these data suggest that GNPs have undergone programmed cell death in response to altered cell cycle control or abnormal differentiation events.

TCDD has been reported to interfere with apoptotic programs, but it is unclear whether these effects are primary or secondary toxic actions following exposure (Dong et al., 2002; Ivnitski et al., 2001). Programmed cell death is critical for eliminating neurons that fail to migrate, obtain proper trophic support, or complete synaptogenesis (Vogel, 2002; White and Barone, 2001). Approximately 56% of GNPs in the rodent cerebellum undergo apoptosis during early postnatal life (White and Barone, 2001). It has been proposed that elevated Bax expression is associated with developmental apoptosis, with cerebellar expression in the rat peaking around PND12, which would correspond approximately to PND10 in the mouse (Mooney and Miller, 2001). Our study indicates that cerebellar Bax expression was elevated between PND7–10 in mice exposed to TCDD on PND6, with maximal levels observed on PND8. As a direct target of AhR transcriptional regulation, the Bax promoter is activated by polycyclic aromatic hydrocarbons but not by halogenated aromatics, such as TCDD (Matikainen et al., 2001). Therefore, we favor the interpretation that apoptosis is a secondary event following AhR activation, as was previously reported by a group that examined TCDD neurotoxicity in the developing zebrafish midbrain (Dong et al., 2002). TCDD-mediated disruption of GNP proliferation or differentiation may force these cells to undergo apoptosis resulting from an absence of the appropriate trophic support. TCDD also altered spatiotemporal apoptotic patterns during GNP development in vivo. In control mice, apoptosis was restricted to the EGL on PND10, whereas TCDD exposure was associated with TUNEL-positive cells in the ML and IGL. The abnormal apoptotic pattern and increased Bax expression allude to a temporal shift in GNP development. These observations strengthen the possibility that TCDD interferes with granule neuron production following exposure, but the direct involvement of AhR requires clarification.

Our in vitro studies support the hypothesis that TCDD exposure stimulates differentiation at early and intermediate stages of GNP maturation. These observations are comparable with data indicating that TCDD exposure increased p27 expression and promoted differentiation in cultured keratinocytes (Ray and Swanson, 2003). The cell cycle inhibitor protein p27 is expressed in GNPs, serving as marker of cell cycle exit in the EGL (Miyazawa et al., 2000). TCDD exposure produced a rapid increase in levels of p27 protein in GNP reaggregate cultures designed to model early differentiation in the EGL (Gao et al., 1991). Because p27 is a direct target of AhR-mediated transcriptional regulation (Kolluri et al., 1999), it is a noteworthy candidate for further investigation toward defining the mechanisms of abnormal cell cycle activity during GNP differentiation following TCDD exposure. Furthermore, cultured GNPs exhibited enhanced neurite outgrowth within hours following TCDD exposure, and both βIII-tubulin and PSA-NCAM expression patterns indicated TCDD-mediated induction of a more mature neuronal phenotype.

Although AhR levels are highest during the GNP expansion phase, expression persists throughout later stages of granule neuron maturation (Williamson et al., 2005). Experiments using the in vitro monolayer model of terminal differentiation...
of GNPs in the IGL (Gault and Siegel, 1997) demonstrated that GABAR\textsubscript{A6} levels were elevated following TCDD exposure. This observed increase in GABAR\textsubscript{A6} protein may appear to contradict our \textit{in vivo} results of reduced expression at PND21. There are possible explanations to reconcile these differences. The culture system includes KCl to mimic excitatory input that is required for terminal differentiation of precursors into functional granule neurons, but it does not preserve cellular interactions within the cerebellum. Moreover, the TCDD insult in culture does not precisely mimic the temporal exposure paradigm in our animal studies, which target precursors at earlier stages. A more logical reason for this discrepancy in GABAR\textsubscript{A6} expression is that the impact of TCDD on GNP differentiation in the EGL could prevent cells from achieving the proper spatiotemporal context necessary for survival. Therefore, fewer mature granule cells would exist and/or express GABAR\textsubscript{A6} protein at later stages of development \textit{in vivo}. Alternatively, if mossy fiber input to the cerebellar cortex is perturbed, then terminal neuronal differentiation into a functional granule cell could be compromised. Future investigations are necessary to ascertain the impact of TCDD on terminal neuronal differentiation in the IGL. Nevertheless, our data support the concept that TCDD promotes GNP differentiation at various developmental stages, which could adversely impact the final cellular profiles in the mature cerebellum.

The central hypothesis of our research is that AhR participates in granule cell neurogenesis, which is disrupted by TCDD. This study suggests that an initial TCDD insult during the GNP expansion phase produces long-term alterations in cerebellar cell composition. Reduced cerebellar cell number, inferred from DNA content analysis, along with decreased expression of the late-stage differentiation marker GABAR\textsubscript{A6} on PND21, indicates that GNPs are lost earlier in development. Alternatively, lower GABAR\textsubscript{A6} expression could reflect compromised functional differentiation, which may also involve a TCDD-mediated impairment of critical signaling from Purkinje cells or mossy fibers that serve to integrate granule neurons into the circuitry. Interestingly, analysis of cerebella from adult AhR\textsuperscript{−/−} mice revealed a reduction in DNA content and GABAR\textsubscript{A6} expression. These observations suggest that an AhR deficiency leads to abnormal cerebellar cytoarchitecture and compromised granule neuron maturation or maintenance. Moreover, they raise interesting possibilities regarding physiological roles for AhR in neurogenesis and the manner in which TCDD may interfere with this process.

We postulate that TCDD exposure disrupts the intrinsic cellular program that controls granule cell neurogenesis through the inappropriate activation or suppression of AhR function. Our data suggest that early developmental insult leads to later-stage defects that may have long-term effects on formation of the cerebellum and ultimately on function. Based on defects observed in AhR\textsuperscript{−/−} mice, we propose that AhR signaling is involved in guiding neuronal maturation or survival in the cerebellum. Future studies are necessary to gain mechanistic insight into the adverse impact of TCDD on GNP maturation by investigating whether TCDD exposure leads to improper modulation of AhR-mediated signaling during critical developmental periods.

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### REFERENCES


