Neural Precursor Cell Proliferation Is Disrupted Through Activation of the Aryl Hydrocarbon Receptor by 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin

Sarah E. Latchney,¹ Daniel T. Lioy,² Ellen C. Henry,¹ Thomas A. Gasiewicz,¹ Frederick G. Strathmann,³ Margot Mayer-Pröschel,³ and Lisa A. Opanashuk¹

Neurogenesis involves the proliferation of multipotent neuroepithelial stem cells followed by differentiation into lineage-restricted neural precursor cells (NPCs) during the embryonic period. Interestingly, these progenitor cells express robust levels of the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that regulates expression of genes important for growth regulation, and xenobiotic metabolism. Upon binding 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a pervasive environmental contaminant and potent AhR ligand, AhR, is activated and disrupts gene expression patterns to produce cellular toxicity. Because of its widespread distribution in the brain during critical proliferative phases of neurogenesis, it is conceivable that AhR participates in NPC expansion. Therefore, this study tested the hypothesis that AhR activation by TCDD disrupts signaling events that regulate NPC proliferation. The C17.2 NPC line served as a model system to (1) assess whether NPCs are targets for TCDD-induced neurotoxicity and (2) characterize the effects of TCDD on NPC proliferation. We demonstrated that C17.2 NPCs express an intact AhR signaling pathway that becomes transcriptionally active after TCDD exposure. ³H-thymidine and alamar blue reduction assays indicated that TCDD suppresses NPC proliferation in a concentration-dependent manner without the loss of cell viability. Cell cycle distribution analysis by flow cytometry revealed that TCDD-induced growth arrest results from an impaired G1 to S cell cycle transition. Moreover, TCDD exposure altered p27^{kip1} and cyclin D1 cell cycle regulatory protein expression levels consistent with a G1 phase arrest. Initial studies in primary NPCs isolated from the ventral forebrain of embryonic mice demonstrated that TCDD reduced cell proliferation through a G1 phase arrest, corroborating our findings in the C17.2 cell line. Together, these observations suggest that the inappropriate or sustained activation of AhR by TCDD during neurogenesis can interfere with signaling pathways that regulate neuroepithelial stem cell/NPC proliferation, which could adversely impact final cell number in the brain and lead to functional impairments.

Introduction

NEUROGENESIS IS A DYNAMIC PROCESS that commences with the proliferation of multipotent neuroepithelial stem cells (NSCs) and continues with their subsequent differentiation, migration, and integration into functional neuronal networks [1,2]. During the embryonic period, NSC expansion is followed by differentiation into neural precursor cells (NPCs) or glial progenitor cells with restricted phenotypic potential and limited capacity for self-renewal [1,2]. These lineage-restricted progenitors subsequently differentiate into neurons and glia, migrate to specific regions of the brain, and establish connections with other cells [1,2]. Disrupting any stage during neurogenesis such as NPC

proliferation could alter final cell numbers in the brain, resulting in neurological dysfunction.

The developing nervous system is highly susceptible to environmental insults because embryonic brain maturation is dictated by several waves of neurogenesis, which continue into adulthood in certain brain regions [3]. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin), a ubiquitous environmental contaminant belonging to a class of structurally related polyhalogenated aromatic hydrocarbons, has been associated with developmental neurotoxicity in humans and experimental animals [4–8]. However, the precise effects of TCDD on embryonic neurogenesis are largely unexplored. Because TCDD is highly lipophilic, it readily crosses the placenta and can be transferred to the developing offspring

¹Department of Environmental Medicine, ²Department of Biomedical Genetics, University of Rochester School of Medicine and Dentistry, Rochester, New York.

³The Vollum Institute, Oregon Health & Science University, Portland, Oregon.

during gestation [9–12]. Moreover, TCDD is resistant to degradation and not readily metabolized or excreted in exposed organisms [13]. Considering these properties, bioaccumulation could allow for low-dose exposures to reach hazardous levels in humans and animals, which could interfere with neurogenesis throughout the lifespan.

Although previous evidence suggests that prenatal TCDD exposure has been connected with neurodevelopmental deficits in humans and experimental animals, the cellular targets and molecular mechanisms of neurotoxicity are poorly understood. Neurobehavioral studies in rodents and primates after developmental TCDD exposure have revealed motor and cognitive deficits, suggesting abnormal brain development [14,15]. For example, maternal TCDD exposure has been linked to deficits in higher brain function in offspring, such as working memory in a radial arm maze [16] and operant response in running wheels or two-lever chambers [15,17]. Further, recent studies revealed that newborn head circumference, an indicator of fetal brain development, was negatively correlated with the concentration of TCDD in human breast milk [18,19]. Smaller head circumference during infancy has been associated with neurodevelopmental disabilities [20]. These observations suggest that TCDD exposure adversely impacts brain maturation and may be a risk factor for neurodevelopmental toxicity and disorders. Therefore, our laboratory is testing the hypothesis that neural progenitors are primary targets for TCDD actions during brain development.

TCDD exerts its toxicity via interaction with the aryl hydrocarbon receptor (AhR), an evolutionary-conserved, ligand-activated transcription factor that regulates expression of xenobiotic metabolizing enzymes and growth regulatory molecules [21]. In its ligand-free state, AhR resides in the cytoplasm bound to several chaperones. After binding TCDD, AhR dissociates from its chaperones [22,23], translocates to the nucleus, and dimerizes with the aryl hydrocarbon receptor translocator (ARNT). The AhR/ARNT complex then binds to aryl hydrocarbon response elements (AhREs) and modulates expression of genes involved in toxicity, adaptive responses, and cell cycle regulation [24]. The AhR is subsequently downregulated via proteasomal degradation [25]. Whereas information regarding AhR has been revealed in numerous TCDD exposure studies, the precise physiological roles of AhR are unclear.

Because AhR activation impacts growth and differentiation in various cell types [26,27], it is reasonable to postulate that exposure to TCDD disrupts these processes during neurogenesis. TCDD, via binding and activating AhR, has been shown to alter expression of genes involved in cell cycle regulation and apoptosis [28–31]. For example, AhREs are found in the promoter regions of growth regulatory molecules such as *p21^{cip1}*, *p27^{kip1}*, *HES1*, and *Bax* [28–32]. These genes have been shown to be upregulated after polyhalogenated aromatic hydrocarbon exposure, leading to perturbations in cell cycle control mechanisms in various cell types [28–31,33]. AhR activation by TCDD has also been shown to impact differentiation of keratinocytes, adipocytes, and mammary epithelial cells [34–37]. We previously demonstrated that TCDD exposure disrupted both proliferation and differentiation of cerebellar granule precursors (GNPs) [31,38]. Moreover, our findings that the cerebellum is smaller and granule neuron differentiation is abnormal in AhR deficient mice [31] suggest that the AhR is important during granule precursor maturation. Considering

that AhR and ARNT are expressed in the embryonic neuroepithelium during a peak window of neurogenesis on gestational day 10–13 in mouse embryos [39,40], it is plausible that AhR also participates in embryonic brain development.

Our present study tested the hypothesis that AhR plays a role in regulating NPC proliferation, which is dysregulated after inappropriate activation by dioxin. The multipotent C17.2 neural precursor cell line served as a model to explore the manner in which TCDD may interfere with NPC proliferation. C17.2 cells were originally derived from rapidly dividing neural progenitor cells of the mouse cerebellum and immortalized by the retroviral transfection of *v-myc* [41]. This NPC model expresses nestin, a marker for neural stem cells and progenitors, and responds to environmental cues resulting in terminally differentiated neurons or glia, thus fulfilling the properties of endogenous neural progenitors [42]. In this study, C17.2 NPCs were shown to express a functional AhR pathway that is responsive to TCDD. Our results suggested that TCDD interacts with the AhR to disrupt NPC proliferation by altering expression patterns of cell cycle regulatory proteins consistent with a G1 to S cell cycle phase arrest. We extended our studies to include primary NPCs derived from the ventral forebrain of mouse embryos. TCDD exposure in primary NPCs also diminished cell proliferation by inducing a G1 to S phase arrest, similar our observations in the C17.2 cells. Together, our findings from both the NPC cell line and primary NPCs provide insight into the cellular mechanisms of the toxic effects of TCDD on NPC proliferation, as well as possible roles for AhR in regulating neurogenesis.

Materials and Methods

Reagents

Mouse C17.2 neural precursor cells were a generous gift from Dr. Evan Snyder (UCSD). TCDD (Cambridge Isotopes) was solubilized in dimethyl sulfoxide (DMSO). DMSO, Triton X-100, phenylmethanesulfonyl fluoride, antiprotease cocktail, 35% bovine serum albumin (BSA) solution, putrescine, progesterone, insulin, transferrin, and sodium selenite were purchased from Sigma. Dulbecco's phosphate-buffered saline (DPBS), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), and L-glutamine were purchased from Mediatech. Dulbecco's modified of Eagle's medium (DMEM), Leibovitz's L-15 medium, calcium- and magnesium-free Hanks' balanced salt solution, DMEM/F12 (1:1 mixture), fetal bovine serum, horse serum, normal goat serum, penicillin/streptomycin (100 U/mL each), and EDTA were purchased from Gibco. Collagenase, deoxyribonuclease I, and papain were purchased from Worthington. Human plasma fibronectin was obtained from Millipore. Recombinant human epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were obtained from Peprotech.

C17.2 cell culture

C17.2 neural precursor cells were maintained in DMEM supplemented with 10% fetal bovine serum, 5% horse serum, 1% L-glutamine (2 mM), and 1% penicillin/streptomycin (100×stock) and maintained in a humidified atmosphere of 5% CO₂ at 37°C. This medium is referred to as the feeding medium. The medium was changed every 2–3 days. Cells were grown in culture dishes to subconfluence before analysis.

Primary neural precursor cell culture

Male and female C57BL/6J mice were obtained from Taconic Farms. Upon arrival, all mice were allowed to acclimate to their home cage for 2 weeks before initiation of experiments. 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. Once acclimated to their home cage, male and female mice were allowed to mate overnight. The day of the plug was designated as embryonic day 0.5 (E0.5). At E12.5, pregnant dams were anesthetized by CO₂, and embryos were removed via cesarean section and collected in a Petri dish containing Leibovitz's L-15 medium. The ventral forebrain was carefully dissected using a dissection microscope and transferred to calcium- and magnesium-free Hanks' balanced salt solution where the tissue were subjected to enzymatic dissociation using collagenase, deoxyribonuclease I, and papain. Cells were then mechanically dissociated using a sterile needle attached to a 1 mL syringe to create a single cell suspension. Cells were grown in DMEM/F12 (1:1 mixture) medium supplemented with 100 µg/mL transferrin, 25 µg/mL insulin, 10 mg/mL BSA, 100 µM putrescine, 20 nM progesterone, 30 nM sodium selenite, 5 mg/mL glucose, 2 mM L-glutamine, and 1% penicillin/streptomycin (100×stock), 20 ng/mL bFGF, and 20 ng/mL EGF. All cell culture dishes were pre-coated with fibronectin at 1.25 µg/cm². Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Immunoblot analysis

Cells were harvested for protein analysis in ice-cold PBS containing 0.1% Triton X-100, 1% phenylmethanesulfonyl fluoride, 1% EDTA, and 1% antiprotease cocktail. Total protein concentrations were determined by the microBCA assay (Pierce). Proteins (5–25 µg) were fractionated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (BioRad). Membranes were blocked with 5% powdered milk containing 0.2% Tween-20 and probed for AhR (1:2,000; Biomol), ARNT (1:2,000; Novus), cyclin D1 (1:1,000; Cell Signaling), p27^{kip1} (1:1,000; Santa Cruz), nestin (1:1,000, Developmental Studies Hybridoma Bank), or β-actin (1:2,000; Sigma) overnight at 4°C. Membranes were then probed with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories) for 1 h at room temperature. Proteins were observed with LumiGLO chemiluminescent substrate reagent (Kirkegaard & Perry Laboratories). Densitometric analysis was accomplished with ImageJ software (National Institutes of Health).

Immunocytochemistry

Cells were seeded in 8-well culture chamber slides (Lab-Tek, VWR). After exposure to vehicle (DMSO), 1 or 10 nM TCDD, the medium was removed and cells were rinsed with DPBS and then fixed with 4% paraformaldehyde at room temperature for 30 min. After fixation, cells were blocked in PBS containing 10% normal goat serum and 0.3% Triton X-100 at room temperature for 30 min. Cells were then incubated with an AhR antibody (1:800; Biomol) or nestin antibody

(1:100; Developmental Studies Hybridoma Bank) overnight at 4°C, washed, and incubated with the appropriate Alexa Fluor-conjugated secondary antibody (Molecular Probes) for 90 min at room temperature. Nuclei were subsequently stained Hoechst 333452 (1:2,000; Sigma) or DAPI (1:5,000, 1 mg/mL stock; Molecular Probes). Fluorescence was observed using a Nikon Eclipse TS100 inverted microscope and images were taken at 20× or 40× magnification with SPOT advanced software. Fluorescence was not detected in cultures incubated with a secondary antibody alone or with a non-specific IgG.

Electrophoretic mobility shift assay

C17.2 cells were grown to 90% confluence in 100 mm dishes (BD Falcon), exposed for 1 h to DMSO, 1 or 10 nM TCDD, and harvested in ice-cold PBS. Cells were centrifuged and pellets were resuspended in HEDG buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, and 10% glycerol) and NaCl was added to a final concentration of 0.4 M. Cells were then triturated using a 25-gauge needle and incubated on ice for 40 min, with periodic vortexing. After centrifugation at 100,000 g for 50 min, whole cell lysates from the supernatants were stored at –80°C. For gel shift assays, 10–20 µg of protein was used in HEDG buffer and equilibrated in 0.5 µg of herring sperm DNA and 20 mM DTT. About 2.5 × 10⁴–5.0 × 10⁴ cpm of radiolabeled ³²P-AhRE and unlabeled AhRE oligonucleotides were then added to the mixture for 20 min at room temperature. Protein-DNA complexes were resolved on a 4% native polyacrylamide gel. AhR/ARNT-AhRE bands were quantified using a PhosphorImager (Molecular Dynamics).

Transient transfections

C17.2 cells were seeded in 24-well plates (BD Falcon) at a density of 2 × 10⁴ cells per well. When the cells reached 60% confluency, 0.3 µg of the p2dLuc DNA plasmid was transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. As an internal control, 1 ng of *Renilla* luciferase (pRL-tk) was cotransfected along with p2dLuc. Twenty-four hours after transfection, cells were exposed to DMSO, or 1 nM or 4 nM TCDD for 24 h. Cells were harvested using 1× passive lysis buffer (Promega) and relative luciferase activity was measured using the Dual-Luciferase Reporter assay system (Promega). Normalized luciferase activity is reported as a ratio of the p2dLuc firefly luciferase to the *Renilla* luciferase.

³H-Thymidine incorporation

C17.2 cells were seeded in 96-well plates (BD Falcon) at a density of 2.5 × 10³ cells per well in the feeding medium. When they reached 50% confluency they were transferred to serum-free DMEM for 15 h. After serum starvation, cells were exposed to 3 µg/mL sonic hedgehog (Shh; a generous gift from Dr. Kenney, Memorial Sloan Kettering Cancer Center, NY), 25 ng/mL EGF, and 25 ng/mL insulin-like growth factor 1 (IGF-1; R&D Systems) with and without 0.1–10 nM TCDD for 24 h. For primary NPCs from mouse embryos, E12.5 ventral forebrain cells were seeded in 96-well plates previously coated with 1.25 µg/cm² fibronectin at a density of 5 × 10⁴ cells per well. Cells were grown with or without 20 ng/mL EGF and 20 ng/mL bFGF in the absence or presence of 1 nM

TCDD for 24 h. C17.2 and E12.5 cells were then labeled with 1 μ Ci of methyl- 3 H-thymidine (Perkin Elmer) during the last 6 and 12 h of incubation, respectively, and harvested onto filter paper using a Skatron cell harvester. After the filters dried, the amount of incorporated radioactivity was quantified by liquid scintillation counting.

Alamar blue reduction assay

Cells were seeded and treated as described for the 3 H-thymidine incorporation experiments. Alamar blue (BioSource) was added directly to the culture medium at 10% of the medium volume during the last 10 h of the 24 h exposure period. Absorbance was measured spectrophotometrically at 570 and 600 nm using a microplate reader.

Flow cytometry for cell cycle analysis

C17.2 and E12.5 ventral forebrain cells were seeded in 100 $\times$ 20 mm cell culture dishes (Corning, Inc.) and treated as described for the 3 H-thymidine incorporation experiments. Cells were then harvested by trypsinization 24 h after TCDD exposure, washed with PBS containing 0.5% BSA, fixed by the dropwise addition of ice-cold 70% ethanol, and incubated at least overnight at -20°C . After permeabilization, cells were treated with 1 mg/mL RNase A (Roche Diagnostics) and DNA was stained with 50 μ g/mL propidium iodide (Molecular Probes). Cells were acquired with a FACS Cantoll flow cytometer using a 488 nm argon blue laser (Becton Dickinson). A minimum of 50,000 events was collected per sample. Cell cycle analysis was performed using FlowJo software (Tree Star).

Cell survival assay

Neuron viability was assessed with the Live/Dead Cytotoxicity Kit (Molecular Probes). Calcein-AM and ethidium homodimer were used to distinguish dead and living cells. C17.2 and E12.5 ventral forebrain cells were seeded in 24-well plates (BD Falcon) at 2×10^4 cells per well and 3×10^5 cells per well, respectively, and then exposed to TCDD as described for the 3 H-thymidine incorporation experiments. After the 24 h exposure period, cells were rinsed with DPBS and incubated for 30 min at 37°C in serum-free DMEM containing ethidium homodimer and calcein-AM. Fluorescence was observed using a Nikon Eclipse Ti100 fluorescent microscope (20 $\times$ magnification). Approximately 800 cells from 3 randomly selected fields for each exposure condition were counted using Image-Pro Plus software Version 6.2 (Media Cybernetics) with the experimenter blinded to the treatment conditions. The percentage of live cells was determined as the ratio of calcein-AM-stained cells over the total cells stained for calcein-AM and ethidium homodimer. Cells grown in their standard feeding medium were used as positive control for calcein-AM staining. Cells treated with 70% methanol for 30 min were used as positive control for ethidium homodimer staining.

Statistical analyses

Data are expressed as means $\pm$ standard error of the mean from a minimum of 3 independent experiments. Sample sizes are indicated in the figure legends. Statistical analyses

were performed with either 1- or 2-way analysis of variance (ANOVA) where appropriate (GraphPad Prism 4.0). Tukey's or Dunnett's (for 1-way ANOVA), and Bonferroni's (for 2-way ANOVA) *post hoc* comparisons were used to compare select groups. The type of statistical test performed is indicated in the figure legends. *P* values of <0.05 were considered statistically significant.

Results

TCDD activates the AhR in C17.2 neural precursor cells

The first objective of the study was to confirm that C17.2 NPCs express an intact AhR pathway that is responsive to TCDD. To determine if C17.2 cells express the proteins that comprise an intact AhR pathway, several protein concentrations (5–25 μ g) of C17.2 lysate were analyzed for AhR and ARNT protein content by immunoblot analysis. These studies indicate that C17.2 cells robustly express AhR and ARNT proteins (Fig. 1A). Mouse hepatoma cell lysate (Hepa) served as a positive control.

To establish that TCDD activates AhR in C17.2 cells, cellular localization of AhR was determined by immunocytochemistry before and after TCDD exposure at specific time intervals. AhR was primarily localized to the cytoplasm in vehicle-treated cells, with minimal staining in the nucleus (Fig. 1B, top panels). There was no difference in AhR localization in the untreated cells compared to vehicle-treated cells (data not shown). After 1 and 10 nM TCDD exposure, AhR expression colocalized with the Hoechst 33342 nuclear stain within 5 min and remained in the nucleus after 60 min (Fig. 1B, bottom panels).

The DNA binding capacity of the AhR/ARNT complex to AhREs after TCDD exposure was next evaluated by an electrophoretic mobility shift assay (EMSA). EMSA analysis in cells exposed to vehicle (DMSO) exhibited no difference in the baseline levels of AhRE binding capability compared to cells not treated with vehicle (data not shown). After exposure to 1 and 10 nM TCDD for 1 h, there was a notable increase in AhR/ARNT binding with AhRE, respectively (Fig. 1C). Unlabeled AhRE-oligonucleotide confirmed the specificity of AhR/ARNT-AhRE binding. Mouse hepatoma cells (Hepa) exposed to vehicle or 10 nM TCDD served as negative and positive controls, respectively.

To confirm that the AhR/ARNT complex becomes transcriptionally active after AhRE binding, C17.2 cells were transiently transfected with a firefly luciferase plasmid containing 2 AhREs upstream of the luciferase promoter [43]. TCDD exposure elicited an increase in AhRE-driven luciferase activity after 24 h compared to the control (Fig. 1D), with 1 and 4 nM TCDD exposures inducing 2.3- and 2.75-fold increases in reporter activity, respectively, over the control. Together, these studies confirm that AhR becomes transcriptionally active after TCDD exposure in C17.2 neural precursor cells.

AhR protein levels decline after TCDD exposure

Several studies report that AhR levels rapidly decline after ligand binding [25,44]. AhR downregulation appears to be ubiquitin-mediated, as demonstrated by studies in which inhibition of the 26S proteasome results in enhanced AhR activity and the absence of ligand binding-induced reduction

FIG. 1. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) activates aryl hydrocarbon receptor (AhR) in C17.2 neural precursor cells. **(A)** C17.2 cell lysates (5–25 μ g) were examined for AhR and aryl hydrocarbon receptor translocator (ARNT) protein expression by immunoblot analyses. Mouse hepatoma cell lysate (Hepa) served as a positive control. Corresponding β -actin blots serve as loading controls. Results are representative of 3 separate experiments. **(B)** Images (20 $\times$) are of C17.2 cells exposed to a vehicle [dimethyl sulfoxide (DMSO)], 1 or 10 nM TCDD for 5 min (*left panel*) and 60 min (*right panel*), and then stained for AhR (red) and Hoechst nuclear stain (blue). *Arrowheads* indicate examples of cytoplasmic localization of AhR. *Arrows* are examples of nuclear localization of AhR. Results are representative of 4 separate experiments. **(C)** C17.2 cells were exposed to a vehicle (DMSO), 1 or 10 nM TCDD for 1 h. Whole-cell extracts were then incubated with 32 P-labeled aryl hydrocarbon response element (AhRE) oligonucleotides and resolved on a nondenaturing polyacrylamide gel. Unlabeled-AhRE oligos confirmed the specificity of the AhR/ARNT-AhRE band. Whole-cell extracts from mouse hepatoma cells (Hepa) treated with a vehicle (DMSO) or 10 nM TCDD served as a positive control. Results represent 3 separate experiments. **(D)** C17.2 cells were transiently transfected with a reporter plasmid (p2Dluc) containing 2 AhREs upstream of the luciferase reporter vector. After transfection, cells were exposed to a vehicle (DMSO) or TCDD for 24 h and assayed for reporter activity. Cells were also transfected a *Renilla* reporter as an internal control. Results represent means $\pm$ SEM from 4 independent experiments. Data were analyzed by 1-way ANOVA with Tukey's post-test (significantly different from DMSO, ** $P < 0.01$). Color images available online at www.liebertonline.com/scd.

of AhR protein levels [25,44,45]. To determine if AhR mRNA levels were altered after TCDD exposure, C17.2 cells were exposed to vehicle (DMSO) or TCDD for 6–48 h, and mRNA expression was evaluated. AhR mRNA transcript levels were not altered after TCDD exposure (data not shown). AhR protein levels were then evaluated after cells were exposed to vehicle, 1, or 10 nM TCDD and harvested at various time intervals for immunoblot analysis. Differences in AhR protein levels were not detected in vehicle-treated cells compared to untreated cells (data not shown). After TCDD exposure, there was a marked reduction in AhR protein as early as 4 h after TCDD treatment and was sustained

through 24 h postexposure (Fig. 2A). This result indicates that receptor levels may be diminished by post-translational mechanisms such as ubiquitination and degradation via the proteasome. Receptor levels returned to baseline levels 72 h after TCDD exposure and remained at baseline levels (Fig. 2B, C).

C17.2 cells respond to known NPC mitogens by increasing DNA synthesis and cell growth

To study the impact of TCDD on NPC proliferation, it was necessary to determine whether C17.2 progenitor cells

FIG. 2. AhR protein levels decline after TCDD exposure. C17.2 cell lysate (10 μ g) were examined for AhR protein content by immunoblot analyses. Cells were treated with vehicle (DMSO), 1 or 10 nM TCDD for 4–24 h (**A**), and 24–96 h (**B**) before protein isolation. Untreated mouse hepatoma cell lysate (Hepa) served as a positive control. Corresponding β -actin blots serve as loading controls. (**C**) Densitometry analysis of AhR levels after TCDD exposure for 24–96 h. Results represent means $\pm$ standard error of the mean (SEM) from 3 separate experiments and analyzed by 1-way ANOVA with Bonferroni's post-test for select comparisons (*significantly different from vehicle at respective time point, $P < 0.05$).

respond to mitogens in ways similar to their in vivo counterparts. One morphogen that is critical for ventral midline (floorplate) development and for stimulation of neuronal precursor proliferation is Shh [46–48]. In addition to Shh, molecules such as EGF and IGF-1 have been shown to regulate NPC proliferation [49–51]. Therefore, we evaluated the mitogenic potential of Shh, EGF, and IGF-1 in C17.2 precursor cells. Before assessing proliferation, C17.2 cells were serum-starved to synchronize them in the G_0/G_1 phase of the cell cycle. Cells were then exposed to vehicle, Shh, EGF, or IGF-1 for 24 h. DNA synthesis was evaluated by measuring 3 H-thymidine incorporation during the last 6 h of incubation. Cell growth was also examined by quantifying alamar blue reduction. The results demonstrated that Shh, EGF, and IGF-1 stimulated NPC proliferation by increasing DNA synthesis (Fig. 3A) and cell growth (Fig. 3B). Shh was the most efficacious mitogen, inducing an ~ 3 -fold increase in both DNA synthesis and cell growth compared to cells treated with a vehicle. Because C17.2 cells respond to Shh, EGF, and IGF-1 in a similar manner as native NPCs, it strengthens the use of the C17.2 cell line as an appropriate model to study NPC prolif-

FIG. 3. Mitogenic factors stimulate C17.2 DNA synthesis and cell growth without altering cell survival. After serum starvation, C17.2 cells were exposed to 3 μ g/mL sonic hedgehog (Shh), 25 ng/mL epidermal growth factor (EGF), or 25 ng/mL insulin-like growth factor 1 (IGF-1) for 24 h and were assessed for DNA synthesis by measuring 3 H-thymidine incorporation (**A**) and for cell growth by quantifying alamar blue reduction (**B**). Cell viability, as measured by the Live/Dead Cytotoxicity Kit (Molecular Probes), confirmed the mitogenic actions of Shh, EGF, and IGF-1 (**C**). Results are expressed as means $\pm$ SEM from 3 separate experiments, performed in triplicates. Data were analyzed by 1-way ANOVA with Dunnett's post-test (*significantly different from control, $P < 0.05$, ** $P < 0.01$).

eration. Cell survival was not enhanced after treatment with any of the mitogens, confirming that Shh, EGF, and IGF-1 function as mitogens to induce cells to re-enter the cell cycle, rather than as trophic factors to promote cell survival (Fig. 3C).

TCDD suppresses mitogen-induced DNA synthesis and cell growth in a concentration-dependent manner

To investigate the effects of AhR activation by TCDD on cell proliferation, C17.2 cells were exposed to Shh, EGF, or IGF-1 with or without 1 nM TCDD for 6 and 24 h, and then assayed for DNA synthesis and cell growth. TCDD did not significantly alter DNA synthesis or cell growth in quiescent cells at either time point (Fig. 4A–C). In contrast, when cells were stimulated to proliferate with Shh, EGF, or IGF-1, co-exposure to 1 nM TCDD for 6 and 24 h suppressed the mitogen-induced DNA synthesis (Fig. 4A, B) and cell growth (24 h only, Fig. 4C). TCDD did not significantly alter cell survival compared to the control (Fig. 4D).

To ascertain whether TCDD attenuated C17.2 proliferation in a concentration-dependent manner, cells were exposed to 0.1–10 nM TCDD in the presence or absence of Shh for 6 or 24 h. TCDD disrupted Shh-mediated proliferation in a concentration-dependent manner at both time points (Fig. 5A–C). C17.2 survival was only modestly reduced after exposure to 10 nM TCDD (Fig. 5D). Together, these observations indicate that TCDD reduces mitogenic actions in C17.2 cells, which leads to diminished growth rather than cell death.

TCDD causes an accumulation of C17.2 cells in the G₀/G₁ stage of the cell cycle

C17.2 cells were exposed to vehicle, Shh, EGF, or IGF-1, with or without 1 nM TCDD, for 24 h and were then subjected

to flow cytometric analysis to investigate alterations in cell cycle distribution. The Watson Pragmatic algorithm was used to determine the percentage of cells in the G₀/G₁, S, and G₂/M phases of the cell cycle based on PI fluorescence. When C17.2 cells were stimulated to proliferate in the presence of each of the 3 mitogens, there was a significant decrease in the percentage of cells in the G₀/G₁ phase, which coincided with a significant increase in the percentage of cells in S phase. This led to a significant decrease in the G₀/G₁:S ratio, signifying that these mitogens were effective in stimulating the cells past the G₁/S phase checkpoint and into mitosis (Table 1). As expected, exposure to 1 nM TCDD for 24 h did not significantly alter the cell cycle phase distribution of quiescent cells (Table 1). In contrast, TCDD caused a 20% increase ($P < 0.05$) in the percentage of cells in the G₀/G₁ phase and a 40% decrease ($P < 0.05$) in the percentage of cells in the S phase when cells were induced to proliferate in the presence of Shh, EGF, or IGF-1 (Table 1). TCDD did not elicit any change in the percentage of cells in the G₂/M phase. However, the G₀/G₁:S ratio was increased by ~50% in cycling cells, but not in noncycling cells, when exposed to TCDD. These results suggest that TCDD reduces NPC proliferation by causing an accumulation of cells in the G₀/G₁ phase, thereby interfering with the G₁ to S phase transition.

TCDD alters cell cycle regulatory protein expression consistent with G₀/G₁ phase arrest

Protein content for cell cycle regulators that govern the progression of a cell through the G₀/G₁ phase were examined to

FIG. 4. TCDD suppresses mitogen-induced DNA synthesis and cell growth without altering survival. C17.2 cells were exposed to a vehicle, Shh, EGF, or IGF-1, in the presence or absence of 1 nM TCDD for 6 or 24 h and assayed for DNA synthesis (A and B) and cell growth (24 h only, C). The reduced proliferation after TCDD exposure was not accompanied by a reduction in cell survival (D). Data represent means $\pm$ SEM of 3 independent experiments, done in triplicates. Data were analyzed by 2-way ANOVA with Bonferroni post-test for select comparisons (*significantly different from control without TCDD, $*P < 0.05$, $**P < 0.01$; #significantly different from the respective mitogen alone, $#P < 0.05$, $###P < 0.01$).

FIG. 5. TCDD disrupts Shh-mediated proliferation in a concentration-dependent manner without significantly altering cell survival. C17.2 cells were exposed to a vehicle or 3 μg/mL Shh with 0–10 nM TCDD for 6 or 24 h and assayed for DNA synthesis (**A** and **B**) and cell growth (**C**). Cell survival was quantified by the Live/Dead Cytotoxicity Kit (Molecular Probes) (**D**). Data represent means ± SEM of 3 independent experiments, each in triplicates. Data were analyzed by 2-way ANOVA with Bonferroni post-test for select comparisons (*significantly different from control without TCDD, * $P < 0.05$, ** $P < 0.01$; #significantly different from Shh exposure without TCDD, # $P < 0.05$, ## $P < 0.01$).

TABLE 1. 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN CAUSES AN ACCUMULATION OF CELLS IN THE G_0/G_1 STAGE OF THE CELL CYCLE

Treatment	Cell cycle phase (%)			
	G_0/G_1	S	G_2/M	$G_0/G_1:S$ ratio
Control+vehicle	88.8 ± 2.2	8.7 ± 1.5	4.2 ± 0.2	10.2
Control+TCDD	90.1 ± 1.4	8.1 ± 1.1	4.5 ± 0.3	11.1
Shh+vehicle	74.9 ± 5.5 ^a	15.6 ± 1.6 ^a	9.1 ± 0.4 ^a	4.8 ^a
Shh+TCDD	86.2 ± 1.5 ^b	8.4 ± 1.0 ^b	8.6 ± 1.4	10.3 ^b
EGF+vehicle	74.1 ± 4.4 ^a	14.3 ± 1.7 ^a	10.1 ± 0.8 ^a	5.2 ^a
EGF+TCDD	85.3 ± 1.9 ^b	8.9 ± 0.8 ^b	9.5 ± 1.3	9.6 ^b
IGF-1+vehicle	76.8 ± 2.9 ^a	15.2 ± 1.6 ^a	9.7 ± 0.8 ^a	5.1 ^a
IGF-1+TCDD	85.2 ± 1.9 ^b	7.9 ± 0.9 ^b	9.2 ± 1.3	10.8 ^b

C17.2 cells were exposed to a vehicle, Shh, EGF, or IGF-1, with or without 1 nM TCDD for 24 h. Twenty-four hours after exposure, C17.2 cells were harvested into a single cell suspension, permeabilized, and stained for PI. Cells were subsequently acquired on a FACS CantoII flow cytometer and data were analyzed using the Watson Pragmatic cell cycle algorithm to determine the percentage of cells in the G_0/G_1 , S, and G_2/M phases. Data represent means ± standard error of the mean from 4 independent experiments and analyzed by 2-way analysis of variance with Bonferroni post-test for select comparisons.

^aStatistically significant from control without TCDD.

^bStatistically different from respective mitogen alone. $P < 0.05$.

EGF, epidermal growth factor; IGF-1, insulin-like growth factor 1; Shh, sonic hedgehog; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

explore intracellular molecular signaling targets that may be responsible for the accumulation of cells in the G₀/G₁ phase of the cell cycle after TCDD exposure. In these experiments, C17.2 cells were exposed to a vehicle, Shh, EGF, or IGF-1 in the presence or absence of 1 nM TCDD for 6 and 24 h. Cyclin D1 and p27^{kip1} protein content was quantified by immunoblot analysis. In the absence of mitogens, TCDD alone did not significantly alter cyclin D1 expression (Fig. 6A) but significantly increased p27^{kip1} expression (Fig. 6B). After exposure to Shh, EGF, or IGF-1 at all time points, there was elevated cyclin D1 expression; coexposure to 1 nM TCDD significantly attenuated this effect (Fig. 6A). Cells exposed to 1 nM TCDD in the presence of a

mitogen expressed significantly more p27^{kip1} than cells exposed to the respective mitogen alone (Fig. 6B). Collectively, these results suggest that the upregulation of p27^{kip1} protein levels by TCDD is one possible mechanism for the G₀/G₁ phase arrest.

AhR is expressed and responsive to TCDD in neural precursor cells derived from ventral forebrain of mouse embryos

Our studies were extended to evaluate AhR expression activation by TCDD in primary NPCs isolated from the ventral forebrain of E12.5 mouse embryos. We isolated NPCs

FIG. 6. TCDD alters cyclin D1 and p27^{kip1} expression patterns consistent with G₀/G₁ phase arrest. C17.2 cells were exposed to a vehicle, Shh, EGF, or IGF-1, with or without 1 nM TCDD for 6 and 24 h. C17.2 lysates (10 μg) were analyzed for cyclin D1 (A) and p27^{kip1} (B) protein content by immunoblotting. Representative blots and densitometry analysis for each time points are shown. β-Actin served as loading controls. Results represent means ± SEM from 3 independent experiments and analyzed by 2-way ANOVA with Bonferroni post-test for select comparisons (*significantly different from control without TCDD, **P* < 0.05, ***P* < 0.01; #significantly different from the respective mitogen alone, #*P* < 0.05, ##*P* < 0.01).

FIG. 7. Primary neural precursor cells (NPCs) from the forebrain of mouse embryos express nestin and AhR. **(A, top)** E12.5 ventral forebrain NPCs were cultured in vitro for 24 h in the presence of EGF and basic fibroblast growth factor (bFGF). Cell lysates (5–40 μ g) were then examined for AhR and nestin protein expression by immunoblot analyses. Corresponding β -actin immunoblots serve as loading controls. Results are representative of 3 separate experiments. **(A, bottom)** Images (40 $\times$) are of primary cells immunostained for AhR (red), nestin (green), and DAPI (blue). Overlay images are shown. Results are representative of 3 separate experiments. **(B)** Images (40 $\times$) are of primary NPCs exposed to a vehicle (DMSO) or 1 nM TCDD for 4 h, then stained for AhR (red) and DAPI nuclear stain (blue). Overlay images are shown. *Arrowheads* indicate examples of cytoplasmic localization of AhR. *Arrows* are examples of nuclear localization of AhR. Results are representative from 3 separate experiments. Color images available online at www.liebertonline.com/scd.

at E12.5 because AhR and ARNT expression in the embryonic neuroepithelium peaks during E10–13 in mouse embryos [39,40]. Immunoblot analysis indicated that cultured NPCs robustly express AhR and nestin (Fig. 7A). In contrast to C17.2 cells, immunocytochemical studies revealed that AhR was localized in both the cytoplasm and nuclei of vehicle-treated cells (Fig. 7B), which is consistent with other studies reporting nuclear expression of AhR in the absence of a known exogenous ligand [52]. After TCDD, there was an increase in nuclear-localized AhR (Fig. 7B), demonstrating that AhR is responsive to TCDD.

TCDD reduced cell proliferation and leads to a G1 phase arrest in primary NPCs without altering survival

To investigate the effects of AhR activation by TCDD on cell proliferation, primary NPCs were cultured with or without EGF and bFGF in the absence or presence of 1 nM TCDD for 24 h then assayed for DNA synthesis. TCDD did not significantly alter DNA synthesis in cells grown without mitogenic factors (Fig. 8A). When cells were stimulated to proliferate with EGF and bFGF, coexposure to 1 nM TCDD for 24 h suppressed EGF- and bFGF-mediated proliferation (Fig. 8A) without the loss of cell viability (Fig. 8B).

Primary NPCs were next subjected to flow cytometric analysis to investigate if the reduced proliferation was also due to a G1 to S phase arrest that was observed in C17.2 cells. The percentage of cells in the G₀/G1, S, and G2/M phases of the cell cycle was determined in the same manner as for C17.2

cells. When E12.5 NPCs cells were stimulated to proliferate with EGF and bFGF, there was a significant decrease in the percentage of cells in the G₀/G1 phase, which coincided with a significant increase in the percentage of cells in S phase (Fig. 8C). As expected, exposure to 1 nM TCDD for 24 h did not significantly alter the cell cycle phase distribution in cells growing without EGF and bFGF. In the presence of TCDD there was a 10% increase ($P < 0.05$) in the percentage of cells in the G₀/G1 phase and an 8% decrease ($P < 0.05$) in the number of cells in the S phase when cells were induced to proliferate in the presence of EGF and bFGF (Fig. 8C). TCDD did not elicit any significant change in the percentage of cells in the G2/M phase. Our observations in primary NPCs provide additional evidence that TCDD reduces mitogenic actions in neural precursor cells by arresting cells in the G1 phase of the cell cycle.

Discussion

In this study TCDD served as an environmentally relevant chemical to test the hypothesis that inappropriate activation of AhR interferes with neurogenesis by disrupting signaling pathways important for NPC proliferation. The effects of AhR activation by TCDD on NPC proliferation were investigated in the C17.2 cell line model and in NPCs derived from the mouse embryonic forebrain. Whereas TCDD-induced G1 arrest in cell proliferation has been previously observed in the SK-N-SH neuroblastoma cell line [33], derived from a neuroendocrine tumor that originates from the neural crest of peripheral nervous system, this model is not reflective of a

FIG. 8. TCDD suppresses proliferation in primary NPCs forebrain without causing cell death. **(A)** E12.5 cells were grown in vitro without ("control") or with EGF and bFGF and incubated with a vehicle (DMSO) or 1 nM TCDD for 24 h and assessed for DNA synthesis by measuring ³H-thymidine incorporation. **(B)** Cell survival was quantified by the Live/Dead Cytotoxicity Kit (Molecular Probes) 24 h after TCDD exposure. **(C)** E12.5 NPCs were cultured in the presence or absence of 1 nM TCDD. Twenty-four hours after exposure, cells were harvested into a single cell suspension, permeabilized, and stained with propidium iodide. Cells were subsequently sorted on a FACS CantoII flow cytometer and data were analyzed using the Watson Pragmatic cell cycle algorithm to determine the percentage of cells in the G₀/G₁, S, and G₂/M phases. All data are expressed as means $\pm$ SE from 3 independent experiments and analyzed with 2-way ANOVA followed by Bonferroni *post hoc* for select comparisons (*statistically significant from control without TCDD, * $P < 0.05$, ** $P < 0.01$; #statistically significantly different from EGF + bFGF without TCDD, # $P < 0.05$).

neural precursor in central nervous system. In contrast, our study is the first to report that TCDD interferes with the proliferation of multipotent NPCs in 2 different cell models derived from the developing brain. Although C17.2 cells are derived from progenitor cells of a mouse cerebellum, they are predominately used as a model for multipotent stem cells [53–55]. C17.2 precursor cells possess distinct characteristics from cerebellar granule cell precursors [56] and have the unique ability to self-renew and generate both neurons and glia in vitro [57]. We determined that AhR was robustly expressed in C17.2 cells. Moreover, studies with TCDD demonstrated that C17.2 NPCs contain an intact and active AhR pathway, as evidenced by nuclear localization of AhR, AhRE binding, and an increase in AhRE-driven luciferase activity after TCDD exposure. C17.2 NPC proliferation was disrupted after TCDD exposure, suggesting that untimely or sustained AhR activation could impede neurogenesis. Studies in primary NPCs prepared from mouse embryos confirmed reduced cell proliferation and a G₁ phase arrest after dioxin exposure, which could have long-term consequences on final cell number in the brain. Collectively, our observations revealed that NPCs are novel targets for the neurotoxic actions of TCDD and suggest potential roles for AhR during neurogenesis. Moreover, studies confirming TCDD effects on proliferation in primary NPCs validate the C17.2 model for future mechanistic studies that require large numbers of cells, which are challenging to isolate in sufficient quantities from embryonic brain tissue.

Since neurogenesis is mediated by a tightly controlled spatiotemporal program that includes proliferation, differentia-

tion, and apoptosis [3,58], there are likely to be precise windows that may be vulnerable to environmental insult. For example, it has been shown that the nervous system is particularly sensitive to antimetabolic agents such as methylazoxymethanol when proliferation is actively occurring in a given region. However, when cell division ceases, the brain is more resilient [59]. Proliferative cells in the brains of experimental animals have also been shown known to be specifically targeted by exposure to other teratogens, such as ethanol [60,61], methyl mercury [62], and organophosphorous pesticides [63,64]. Because proliferating NSCs/NPCs robustly express AhR in vivo [39], the maturing brain is likely to be more susceptible to TCDD exposure during critical periods of embryonic development. In addition, we have previously demonstrated that the developing brain is also vulnerable to TCDD exposure during critical periods of postnatal neurogenesis [31]. For these reasons, TCDD can be added to the growing list of environmental contaminants that impair nervous system development, which could ultimately hinder proper brain function.

Our observations suggested that the susceptibility of NPCs to dioxin exposure may be dependent on the specific neurodevelopmental event occurring at the time of exposure. When cell cycle progression was initiated with growth factors, TCDD reduced the mitogenic effects in both the C17.2 cell line and primary NPCs. TCDD was found to impede proliferation in dividing NPCs by suppressing DNA synthesis and cell growth. Because NPC proliferation is disrupted after AhR activation by TCDD, it suggests that AhR may be involved in regulating NPC proliferation.

Further examination of the NPC growth arrest revealed that TCDD exposure interfered with the G₀/G₁ to S phase transition in mitogen-stimulated cells. One mechanism of G₀/G₁ phase arrest can be associated with a reduction in G₁ phase cyclins. Cyclin D proteins are G₁ phase regulators that act as mitogen sensors and their activation allows noncycling cells to reenter the cell cycle. A reduction in cyclin D levels can hinder the ability of cells to respond to extracellular mitogens and consequently inhibit cell cycle activity [65]. While TCDD did not alter cyclin D1 levels in quiescent C17.2 cells, our studies demonstrated that TCDD exposure in C17.2 cells dividing in the presence of a mitogen had significantly less cyclin D1 protein levels compared to nonexposed cells. Because cyclin D1 has not been identified as an AhR target gene, this observation suggests that while TCDD may not directly target cyclin D1, exposure may interfere with the ability of cells to respond to extracellular mitogens and hence their ability to initiate cell cycle progression. This finding is consistent with other reports demonstrating that TCDD interferes with the G₀/G₁ to S transition in proliferating, but not quiescent, cells by modulating expression of regulatory proteins controlling cell cycle progression [29].

An additional mechanism for G₀/G₁ phase arrest could involve enhanced levels of antimitogenic regulatory proteins such as the cdk2 inhibitor p27^{kip1} [28]. Cdk2 activity is necessary for entry into the S-phase and its inhibition by p27^{kip1} stalls the cell cycle in the G₀/G₁ phase. In addition to drug metabolizing enzymes such as cytochrome p450s, p27^{kip1} is known to be a direct target of AhR-mediated transcriptional regulation [21,28]. Therefore, directly targeting p27^{kip1} is a feasible mechanism by which inappropriate activation of AhR by exogenous ligands may interfere with the G₀/G₁ to S phase transition and consequently induce cell cycle arrest. Our studies demonstrated that exposure of quiescent, non-proliferating C17.2 cells to TCDD stimulated p27^{kip1} expression, which is in contrast to our cyclin D1 results. Cells exposed to TCDD in the presence of a mitogen also had significantly higher p27^{kip1} expression than cells exposed to the respective mitogen alone [65]. Because p27^{kip1} is an AhR target gene and its expression is elevated in nonproliferating cells, it is possible that AhR activation by TCDD may first directly upregulate p27^{kip1} and contribute to cyclin D1 down-regulation, which instigates G₀/G₁ phase arrest. Supporting this idea, studies in the chick embryonic spinal cord have demonstrated that forced expression of Cip/Kip cell cycle inhibitors in proliferating progenitor cells blocks cell cycle progression by antagonizing cyclin D1 activities, suggesting that an upregulation or overexpression of Cip/Kip inhibitors can subsequently downregulate cyclin D1 activity [66]. Taken together, our observations suggest that TCDD exposure may disrupt the dual regulation of p27^{kip1} and cyclin D1 to suppress NPC proliferation at the G₁ to S phase transition.

The principal hypothesis of our study was that TCDD targets NPCs by interfering with AhR-mediated signaling events that are vital for NPC development. Neurogenesis is a multifaceted process and NPC proliferation is crucial in setting the stage for proper nervous system development. As a result, disrupting NPC proliferation by TCDD may have adverse consequences on nervous system development and function. From our findings, we propose that dioxin exposure suppresses NPC cell proliferation, suggesting that perturbations of normal AhR-mediated signaling events may impinge

on regulatory mechanisms that exist to modulate neuronal development. However, additional studies are necessary to gain further understanding of the adverse impact of TCDD on NPCs during critical developmental periods in vivo, as well as to elucidate the biological roles of AhR during neurogenesis.

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Author Disclosure Statement

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References

- Lillien L. (1997). Neural development: instructions for neural diversity. *Curr Biol* 7:R168–R171.
- Lillien L. (1998). Progenitor cells: what do they know and when do they know it? *Curr Biol* 8:R872–R874.
- Rodier PM. (1994). Vulnerable periods and processes during central nervous system development. *Environ Health Perspect* 102 (Suppl. 2):121–124.
- Nakajima S, Y Saijo, S Kato, S Sasaki, A Uno, N Kanagami, H Hirakawa, T Hori, K Tobiishi, T Todaka, Y Nakamura, S Yanagiya, Y Sengoku, T Iida, F Sata and R Kishi. (2006). Effects of prenatal exposure to polychlorinated biphenyls and dioxins on mental and motor development in Japanese children at 6 months of age. *Environ Health Perspect* 114:773–778.
- Birnbaum LS. (1995). Developmental effects of dioxins and related endocrine disrupting chemicals. *Toxicol Lett* 82–83: 743–750.
- Birnbaum LS and J Tuomisto. (2000). Non-carcinogenic effects of TCDD in animals. *Food Addit Contam* 17:275–288.
- Kekeyama M and C Tohyama. (2003). Developmental neurotoxicity of dioxin and its related compounds. *Ind Health* 41:215–230.
- Rogan WJ and BC Gladen. (1992). Neurotoxicology of PCBs and related compounds. *Neurotoxicology* 13:27–35.
- Hurst CH, BD Abbott, MJ DeVito and LS Birnbaum. (1998). 2,3,7,8-Tetrachlorodibenzo-p-dioxin in pregnant Long Evans rats: disposition to maternal and embryo/fetal tissues. *Toxicol Sci* 45:129–136.
- Hurst CH, MJ DeVito, RW Setzer and LS Birnbaum. (2000). Acute administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in pregnant Long Evans rats: association of measured tissue concentrations with developmental effects. *Toxicol Sci* 53:411–420.
- Thomae TL, E Glover and CA Bradfield. (2004). A maternal AhR null genotype sensitizes embryos to chemical teratogenesis. *J Biol Chem* 279:30189–30194.
- Abbott BD, LS Birnbaum and JJ Diliberto. (1996). Rapid distribution of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to embryonic tissues in C57BL/6N mice and correlation with palatal uptake in vitro. *Toxicol Appl Pharmacol* 141:256–263.
- Boersma ER and CI Lanting. (2000). Environmental exposure to polychlorinated biphenyls (PCBs) and dioxins.

- Consequences for long term neurological and cognitive development of the child lactation. *Adv Exp Med Biol* 478: 271–287.
14. Thiel R, E Koch, B Ulbrich and I Chahoud. (1994). Peri- and postnatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin: effects on physiological development, reflexes, locomotor activity and learning behaviour in Wistar rats. *Arch Toxicol* 69:79–86.
 15. Markowski VP, C Cox, R Preston and B Weiss. (2002). Impaired cued delayed alternation behavior in adult rat offspring following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin on gestation day 15. *Neurotoxicol Teratol* 24:209–218.
 16. Seo BW, BE Powers, JJ Widholm and SL Schantz. (2000). Radial arm maze performance in rats following gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Neurotoxicol Teratol* 22:511–519.
 17. Markowski VP, G Zareba, S Stern, C Cox and B Weiss. (2001). Altered operant responding for motor reinforcement and the determination of benchmark doses following perinatal exposure to low-level 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Environ Health Perspect* 109:621–627.
 18. Nishijo M, K Tawara, H Nakagawa, R Honda, T Kido, H Nishijo and S Saito. (2008). 2,3,7,8-Tetrachlorodibenzo-p-dioxin in maternal breast milk and newborn head circumference. *J Expo Sci Environ Epidemiol* 18:246–251.
 19. Tawara K, M Nishijo, R Honda, S Maruzeni, T Seto, T Kido, S Saito and H Nakagawa. (2009). Effects of maternal dioxin exposure on newborn size at birth among Japanese mother-infant pairs. *Environ Health Prev Med* 14:88–95.
 20. Ivanovic DM, BP Leiva, HT Perez, MG Olivares, NS Diaz, MS Urrutia, AF Almagia, TD Toro, PT Miller, EO Bosch and CG Larrain. (2004). Head size and intelligence, learning, nutritional status and brain development. *Head, IQ, learning, nutrition and brain. Neuropsychologia* 42:1118–1131.
 21. Bock KW and C Kohle. (2006). Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. *Biochem Pharmacol* 72:393–404.
 22. Denison MS, A Pandini, SR Nagy, EP Baldwin and L Bonati. (2002). Ligand binding and activation of the Ah receptor. *Chem Biol Interact* 141:3–24.
 23. Denison MS and SR Nagy. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* 43: 309–334.
 24. Gu YZ, JB Hogenesch and CA Bradfield. (2000). The PAS superfamily: sensors of environmental and developmental signals. *Annu Rev Pharmacol Toxicol* 40:519–561.
 25. Pollenz RS. (2002). The mechanism of AH receptor protein down-regulation (degradation) and its impact on AH receptor-mediated gene regulation. *Chem Biol Interact* 141:41–61.
 26. Puga A, CR Tomlinson and Y Xia. (2005). Ah receptor signals cross-talk with multiple developmental pathways. *Biochem Pharmacol* 69:199–207.
 27. Puga A, Y Xia and C Elferink. (2002). Role of the aryl hydrocarbon receptor in cell cycle regulation. *Chem Biol Interact* 141:117–130.
 28. Kolluri SK, C Weiss, A Koff and M Gottlicher. (1999). p27(Kip1) induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells. *Genes Dev* 13:1742–1753.
 29. Barnes-Ellerbe S, KE Knudsen and A Puga. (2004). 2,3,7,8-Tetrachlorodibenzo-p-dioxin blocks androgen-dependent cell proliferation of LNCaP cells through modulation of pRB phosphorylation. *Mol Pharmacol* 66:502–511.
 30. Matikainen TM, T Moriyama, Y Morita, GI Perez, SJ Korsmeyer, DH Sherr and JL Tilly. (2002). Ligand activation of the aromatic hydrocarbon receptor transcription factor drives Bax-dependent apoptosis in developing fetal ovarian germ cells. *Endocrinology* 143:615–620.
 31. Collins LL, MA Williamson, BD Thompson, DP Dever, TA Gasiewicz and LA Opanashuk. (2008). 2,3,7,8-Tetrachlorodibenzo-p-dioxin exposure disrupts granule neuron precursor maturation in the developing mouse cerebellum. *Toxicol Sci* 103:125–136.
 32. Thomsen JS, S Kietz, A Strom and JA Gustafsson. (2004). HES-1, a novel target gene for the aryl hydrocarbon receptor. *Mol Pharmacol* 65:165–171.
 33. Jin DQ, JW Jung, YS Lee and JA Kim. (2004). 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits cell proliferation through arylhydrocarbon receptor-mediated G1 arrest in SK-N-SH human neuronal cells. *Neurosci Lett* 363:69–72.
 34. Hanlon PR, LG Ganem, YC Cho, M Yamamoto and CR Jefcoate. (2003). AhR- and ERK-dependent pathways function synergistically to mediate 2,3,7,8-tetrachlorodibenzo-p-dioxin suppression of peroxisome proliferator-activated receptor-gamma1 expression and subsequent adipocyte differentiation. *Toxicol Appl Pharmacol* 189:11–27.
 35. Ray SS and HI Swanson. (2003). Alteration of keratinocyte differentiation and senescence by the tumor promoter dioxin. *Toxicol Appl Pharmacol* 192:131–145.
 36. Lew BJ, LL Collins, MA O'Reilly and BP Lawrence. (2009). Activation of the aryl hydrocarbon receptor (AhR) during different critical windows in pregnancy alters mammary epithelial cell proliferation and differentiation. *Toxicol Sci* 111:151–162.
 37. Collins LL, BJ Lew and BP Lawrence. (2009). TCDD exposure disrupts mammary epithelial cell differentiation and function. *Reprod Toxicol* 28:11–17.
 38. Williamson MA, TA Gasiewicz and LA Opanashuk. (2005). Aryl hydrocarbon receptor expression and activity in cerebellar granule neuroblasts: implications for development and dioxin neurotoxicity. *Toxicol Sci* 83:340–348.
 39. Abbott BD, LS Birnbaum and GH Perdew. (1995). Developmental expression of two members of a new class of transcription factors: I. Expression of aryl hydrocarbon receptor in the C57BL/6N mouse embryo. *Dev Dyn* 204:133–143.
 40. Abbott BD and MR Probst. (1995). Developmental expression of two members of a new class of transcription factors: II. Expression of aryl hydrocarbon receptor nuclear translocator in the C57BL/6N mouse embryo. *Dev Dyn* 204:144–155.
 41. Ryder EF, EY Snyder and CL Cepko. (1990). Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer. *J Neurobiol* 21:356–375.
 42. Snyder EY, DL Deitcher, C Walsh, S Arnold-Aldea, EA Hartwig and CL Cepko. (1992). Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell* 68:33–51.
 43. Gasiewicz TA, AS Kende, G Rucci, B Whitney and JJ Willey. (1996). Analysis of structural requirements for Ah receptor antagonist activity: ellipticines, flavones, and related compounds. *Biochem Pharmacol* 52:1787–1803.
 44. Song Z and RS Pollenz. (2002). Ligand-dependent and independent modulation of aryl hydrocarbon receptor localization, degradation, and gene regulation. *Mol Pharmacol* 62:806–816.
 45. Ma Q and KT Baldwin. (2000). 2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor

- (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activator and DNA binding of AhR. *J Biol Chem* 275:8432–8438.
46. Wechsler-Reya RJ and MP Scott. (1999). Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* 22:103–114.
 47. Ruiz i Altaba A, V Palma and N Dahmane. (2002). Hedgehog-Gli signalling and the growth of the brain. *Nat Rev Neurosci* 3:24–33.
 48. Kenney AM and DH Rowitch. (2000). Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol Cell Biol* 20:9055–9067.
 49. Kitchens DL, EY Snyder and DI Gottlieb. (1994). FGF and EGF are mitogens for immortalized neural progenitors. *J Neurobiol* 25:797–807.
 50. Reynolds BA and S Weiss. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 175:1–13.
 51. Mairet-Coello G, A Tury and E DiCicco-Bloom. (2009). Insulin-like growth factor-1 promotes G(1)/S cell cycle progression through bidirectional regulation of cyclins and cyclin-dependent kinase inhibitors via the phosphatidylinositol 3-kinase/Akt pathway in developing rat cerebral cortex. *J Neurosci* 29:775–788.
 52. Chang CY and A Puga. (1998). Constitutive activation of the aromatic hydrocarbon receptor. *Mol Cell Biol* 18:525–535.
 53. Tamm C, J Duckworth, O Hermanson and S Ceccatelli. (2006). High susceptibility of neural stem cells to methylmercury toxicity: effects on cell survival and neuronal differentiation. *J Neurochem* 97:69–78.
 54. Tamm C, F Sabri and S Ceccatelli. (2008). Mitochondrial-mediated apoptosis in neural stem cells exposed to manganese. *Toxicol Sci* 101:310–320.
 55. Liu WG, XJ Wang, GQ Lu, B Li, G Wang and SD Chen. (2007). Dopaminergic regeneration by neurturin-overexpressing c17.2 neural stem cells in a rat model of Parkinson's disease. *Mol Neurodegener* 2:19.
 56. Mi R, Y Luo, J Cai, TL Limke, MS Rao and A Hoke. (2005). Immortalized neural stem cells differ from nonimmortalized cortical neurospheres and cerebellar granule cell progenitors. *Exp Neurol* 194:301–319.
 57. Muller FJ, EY Snyder and JF Loring. (2006). Gene therapy: can neural stem cells deliver? *Nat Rev Neurosci* 7:75–84.
 58. Rice D and S Barone, Jr. (2000). Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect* 108 Suppl 3:511–533.
 59. Sullivan-Jones P, SF Ali, B Gough and RR Holson. (1994). Postnatal methylazoxymethanol: sensitive periods and regional selectivity of effects. *Neurotoxicol Teratol* 16:631–637.
 60. Guerri C. (1998). Neuroanatomical and neurophysiological mechanisms involved in central nervous system dysfunctions induced by prenatal alcohol exposure. *Alcohol Clin Exp Res* 22:304–312.
 61. Miller MW. (1996). Limited ethanol exposure selectively alters the proliferation of precursor cells in the cerebral cortex. *Alcohol Clin Exp Res* 20:139–143.
 62. Burke K, Y Cheng, B Li, A Petrov, P Joshi, RF Berman, KR Reuhl and E DiCicco-Bloom. (2006). Methylmercury elicits rapid inhibition of cell proliferation in the developing brain and decreases cell cycle regulator, cyclin E. *Neurotoxicology* 27:970–981.
 63. Campbell CG, FJ Seidler and TA Slotkin. (1997). Chlorpyrifos interferes with cell development in rat brain regions. *Brain Res Bull* 43:179–189.
 64. Roy TS, JE Andrews, FJ Seidler and TA Slotkin. (1998). Chlorpyrifos elicits mitotic abnormalities and apoptosis in neuroepithelium of cultured rat embryos. *Teratology* 58: 62–68.
 65. Sherr CJ and JM Roberts. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 13:1501–1512.
 66. Gui H, S Li and MP Matise. (2007). A cell-autonomous requirement for Cip/Kip cyclin-kinase inhibitors in regulating neuronal cell cycle exit but not differentiation in the developing spinal cord. *Dev Biol* 301:14–26.

Address correspondence to:

Lisa A. Opanashuk, Ph.D.

Department of Environmental Medicine

University of Rochester School of Medicine and Dentistry

Box EHSC

Rochester, NY 14642

E-mail: lisa_opanashuk@urmc.rochester.edu

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