Preliminary Evidence in Support of a Role for the AhR in Neural Development: Implication for TCDD-Induced Neurotoxicity

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Childhood exposure to environmental contaminants can lead to developmental abnormalities and pathologies later in life. One environmental pollutant implicated in the etiology of a range of developmental deficits and other biological impairments is the polyhalogenated aromatic hydrocarbon (PAH) congener 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). While TCDD is not deliberately manufactured today, it is an inadvertent byproduct of many industrial processes, including the incineration of various chlorine-containing wastes, the manufacturing of pesticides, and the bleaching of pulp and paper products. In addition to its continued widespread production, TCDD is a highly stable molecule, with a half-life in the environment of up to 10 years. Furthermore, due to its extremely low solubility in water and relative insusceptibility to metabolic breakdown, TCDD bioaccumulates in the adipose tissue of animals with high body fat to body mass ratios. Thus, while there are varied incidents of mass exposure to high levels of TCDD in the human population through work-related accidents and the use of contaminated pesticides, the primary and more insidious mode of chronic human exposure to TCDD is through the consumption of animal products, especially dairy, beef, and fatty fish that are at the top of the food chain.

Due to the prevalence and persistence of TCDD in the environment, risk factors associated with exposure have been the subject of major public health concern. Accidental TCDD exposure in humans elicits a variety of responses, including liver damage, endocrine disruption, carcinogenesis, teratogenesis, behavioral and cognitive perturbations, and severe skin condition known as chloracne. However, widespread contact with high levels of TCDD in the human population is infrequent. Furthermore, exposure rarely occurs in isolation of other contaminants, making it difficult to attribute ensuing health problems exclusively to TCDD toxicity. To address this problem, animal models have been a useful tool in examining the effects of TCDD exposure and the underlying mechanisms by which TCDD exerts its effects.

Early exposure to TCDD in mice and rats leads to a broad spectrum of developmental abnormalities. Gestational exposure to TCDD has been linked to lowered birth weight and increased mortality rates in offspring. Multiple organ systems also acquire altered developmental phenotypes following perinatal exposure to TCDD, including delayed tooth development, delayed bone ossification, delayed bone formation, thyroid hyperplasia and atrophy, altered prostate and seminal vesicle development, and delayed opening of the vaginal canal. In post-natal mice, a single oral dose of TCDD decreases the long-term reconstitution activity of hematopoietic stem cells and alters the relative proportions of developing B-lymphocyte subpopulations in a dose-dependent manner. Furthermore, treatment of proliferating keratinocytes in culture with as little as 1nM TCDD leads to an accelerated period of proliferation followed by the early onset of markers associated with epidermal keratinocyte differentiation.

Early exposure to TCDD also leads to neurodevelopmental abnormalities at markedly lower exposure levels as compared to other tissues. Male and female rats exposed to TCDD during critical periods of cortical development show a reversal of sex-specific cortical lateralization, with males acquiring left hemisphere dominance and females acquiring right hemisphere dominance. Both male and female rats show an overall decrease in cortical thickness. Prenatal TCDD exposure also abolishes sex-specific expression patterns of the γ-aminobutyric acid (GABA) synthesizing enzyme GAD67 in the preoptic area and anteroventral periventricular nucleus. Furthermore, in the raphe nuclei of mice exposed to TCDD in utero and via lactation, a 50% decrease in serotonin-positive cells is seen as compared to control mice. Taken together, these findings suggest that TCDD may act to deregulate points of control necessary for proper development in a variety of organ systems and cell types.

Although there may be several unidentified cellular targets for TCDD, the toxicological effects are most potently and primarily mediated by the aryl hydrocarbon receptor (AhR) signaling pathway. AhR is an evolutionarily conserved ligand-activated transcription factor that belongs to a family of proteins containing basic-helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) homology domains. Several studies point to a role for PAS proteins in the detection of changes in the extracellular environment and in the coordination of cellular responses to those changes. Consistent with this role for PAS proteins, AhR has been shown to mediate a pleiotropic response in multiple cell populations to coplanar xenobiotics, including many polychlorinated biphenyl and PAH congeners.
AhR may participate in the program of cell-cycle progression positioned directly upstream of the hes-1 gene, suggesting that factor Mash promotes cell-cycle arrest and acquisition of a Hes protein levels and an increase in the activity of bHLH-proteins and interacting with components of the proliferative state by antagonizing the activity of pro-neuronal particularly during neural development.

While a role for AhR in the detection and in the mediation of cellular responses to environmental contaminants like TCDD is well-established, it is unlikely that this would be the sole function of AhR. The expression and highly conserved morphology of AhR across animal species is likely the result of a function uniquely fulfilled by AhR in the innate physiology of the cell. Indeed, several lines of evidence point to a role for AhR in regulating the activities of signaling cascades involved in cell-cycling and development. Furthermore, the AhR pathway interacts with several steroid hormone signaling cascades, including the estrogen receptor, androgen receptor, and thyroid hormone receptor. For instance, AhR binds to inhibitory XREs in the regulatory regions of ER-responsive genes. A native role for AhR in cellular physiology is also supported by the discovery of endogenous ligands capable of activating the AhR pathway. Recent studies in yeast systems demonstrate that indirubin and indigo, two indol-containing molecules present in human urine, can bind to and activate the transcriptional activity of AhR at physiologically relevant levels. Moreover, both indirubin and indigo have EC50 values comparable to or lower than that of TCDD, with indirubin being approximately fifty times more potent a ligand to AhR.

In addition to mechanistic evidence in support of a dynamic role for AhR in native cellular processes, numerous studies implicate transcription factors that contain the bHLH-domain in the regulation of key pathways during development.

Cellular development consists of three principle stages, including proliferation, determination, and differentiation, and the genetic programs of cells through each stage must be tightly orchestrated in order to achieve proper development of the whole organism. bHLH-containing proteins have been shown to mediate the timing and passaging of a diverse range of cell populations through each developmental stage, particularly during neural development. For instance, in corticogenesis the expression of the bHLH-proteins Id and Hes has been shown to maintain cortical progenitor cells in a proliferative state by antagonizing the activity of pro-neuronal bHLH-proteins and interacting with components of the cell-cycle machinery. Conversely, a decrease in Id and Hes protein levels and an increase in the activity of bHLH-factor Mash promotes cell-cycle arrest and acquisition of a corticoneuron phenotype. Interestingly, TCDD has been shown to induce the expression of Hes protein via an XRE positioned directly upstream of the hes-1 gene, suggesting that AhR may participate in the program of cell-cycle progression and neural cell lineage determination.

Similar patterns of regulation by bHLH-proteins in neurodevelopment are seen in cerebellar granule neurogenesis. In mammals, development of cerebellar granule neurons begins in the dorsal portion of the metencephalon known as the rhombic lip. At embryonic day 13 (E13) in mice, a subpopulation of cells from the anterior rhombic lip migrate in a rostromedial direction over the surface of the developing cerebellum. Between postnatal days 5 and 7 (P5 and P7), two distinct zones within the EGL are observable. The contributions of each layer to cerebogenesis are different. The outer EGL is a secondary zone of neurogenesis comprised of mitotic precursor cells while the inner EGL consists mainly of post-mitotic cells that express the cytoskeletal protein βIII-tubulin, an early marker of neuronal differentiation. Cells in the outer EGL proliferate from approximately P0 to P15 with peak proliferation occurring from P3 to P10. After P10 the outer EGL begins to decrease in size as the cells migrate into the inner EGL. By P21, most EGL cells have migrated through the molecular layer and Purkinje cell layers towards the inner granule cell layer (IGL). Once in the IGL, cerebellar granule neurons undergo terminal differentiation marked by the expression of the GABA receptor subunit 6.

Two bHLH-proteins identified as critical to regulating this program of cerebellar granule neurogenesis are the transcription factors Math1 and NeuroD. Math1 is the earliest known marker for cells in the rhombic lip that are destined for the EGL. Studies using Math1-/- mice report a markedly reduced anterior rhombic lip and a lack of EGL formation, demonstrating an essential role for Math1 in the earliest stages of granule neuron development. Conversely, distinct outer and inner EGL layers fail to form in animals genetically engineered to overexpress Math1. Markers of early granule neuron differentiation such as βIII-tubulin that localize to the inner granule cell layer (IGL). Once in the IGL, cerebellar granule neurons undergo terminal differentiation marked by the expression of the GABA receptor subunit 6.

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C17.2 cells acquire the phenotypic markers of neurons and glia, in particular to the region of engraftment, including terminally differentiated granule neurons in the IGL. Furthermore, lesioned animals that are implanted with C17.2 cells regain varying degrees of functionality associated with the region of neural ablation, suggesting that C17.2 cells also integrate functionally into local neural networks. Thus, C17.2 cells represent a cell population that is in an early stage of neural development, a necessary property for the examination of a role for AhR in progressive stages of granule neuron maturation. Furthermore, the C17.2 cell line can be maintained in vitro in distinct developmental stages for prolonged periods of time, allowing for the direct examination of AhR activity in sequential phases of neural development.

To gain additional insight into a potential role for AhR in neuronal maturation, studies were designed to evaluate the activity of the AhR pathway in C17.2 cells; more specifically to see whether C17.2 cells express a functional AhR pathway, and if so, whether AhR expression is differentially regulated during neuronal development. The findings of this study provide preliminary evidence in support of a role for AhR in neuronal maturation.

The C17.2 cell line was a generous gift of Dr. Evan Y. Snyder (Harvard Medical School). Briefly, the C17.2 cell line was originated from proliferating EGL cells of a P4 CD-1 x C57BL/6J hybrid mouse. Isolated cells were immortalized by retroviral transfection with the avian oncogene v-myc. Cells were maintained either in serum-containing or serum-free medium referred to here as “feeding medium” and “defined medium,” respectively. Protein concentrations of cell extracts were determined using a microBCA assay as specified by the manufacturer. All immunoblot and immunocytochemical analyses were performed using protein-specific primary antibodies complexed to biotinylated-secondary antibodies.

AhR and ARNT proteins are expressed in C17.2 cells

To determine whether C17.2 cells express a functional AhR pathway, C17.2 cell lysate was analyzed for AhR and ARNT protein content. C17.2 cells were grown in feeding medium and harvested two to three days after plating for immunoblot analysis. Both AhR and ARNT proteins were detected in C17.2 cell lysates. Hepa1c1c7 cell lysate served as a positive control. Corresponding β-actin blot confirms that decreasing AhR and ARNT signals correlate to decreasing amounts of total protein loaded (Figure 2).

AhR undergoes nuclear localization upon TCDD exposure

Previous studies report that in a variety of cell types, unbound AhR resides in the cytosol in a multimeric complex with hsp90, XAP2, and p23. Upon binding to a ligand, AhR dissociates from the complex and undergoes translocation to the nucleus where it heterodimerizes with ARNT. To determine the responsiveness of AhR to TCDD in the C17.2 cell line, cells were grown in feeding medium and fixed 5, 10, 15, 30, and 60-minutes following exposure to DMSO vehicle, 1nM TCDD, or 10nM TCDD. Immunocytochemical analysis revealed that in DMSO treated cells, AhR remained localized primarily to the cytosol, with minimal staining in the nucleus. Differences in the subcellular location of AhR were not observed in basal

Reduced IGL in NeuroD-/- mice. Thus, bHLH-proteins are critical to the proper development of many cell types, including cerebellar granule neurons.

Interestingly, rat offspring exposed to TCDD during gestation exhibit altered in behaviors associated with cerebellar function, including decreased performance on rotordor testing, increased motor reflexivity, and increased activity when placed in a novel environment. Furthermore, the expression of AhR and ARNT mRNA in mature cerebellar neurons is exclusive to granule cells. AhR and ARNT mRNA restriction to granule neurons in the cerebellum suggests a unique role for the AhR pathway in granule neuron physiology. However, despite mounting evidence in support of a role for AhR in the development of multiple cell populations, including the specification of GABAergic motorneuron subpopulations in C. elegans and the effects of TCDD on behaviors which are contingent upon proper cerebrogenesis, a role for AhR in neurodevelopment remains largely unexplored. For instance, while it has been shown that AhR and ARNT mRNA are expressed in various brain regions, no studies to date characterize the expression of AhR and ARNT mRNA and the activity of the AhR pathway in the central nervous system (CNS). Furthermore, while previous studies have demonstrated that AhR and ARNT mRNA are expressed in the adult cerebellum, cerebellar dysfunction due to TCDD toxicity is associated with perinatal exposure. If AhR is the primary mediator of TCDD toxicity and TCDD alters normal development of the cerebellum as evidenced by changes in behaviors that are dependent upon proper cerebrogenesis, then it stands to reason that AhR may play a role in development of the cerebellum. This line of reasoning, together with previous data from our lab demonstrating that cells isolated from the mouse EGL express AhR and ARNT proteins, raised the central hypothesis that the AhR pathway plays a role in the developmental program of cerebellar granule neurons.

This hypothesis was explored using the C17.2 cell line. C17.2 cells provide an attractive in vitro model for studying a role for AhR in cerebellar granule development for several reasons. Most importantly, C17.2 cells originate from the EGL, a zone of rapidly proliferating precursor cells that, in addition to giving rise to terminally differentiated cerebellar granule neurons, may also give rise to other neural populations. C17.2 cells maintain the ability to differentiate down several neural lineages as evidenced by studies in which C17.2 cells are grafted into various regions of the mouse and rat brains.
AhR binds to XREs upon TCDD exposure

Upon binding an agonist, AhR translocates to the nucleus, heterodimerizes with ARNT, and the transcriptionally active AhR/ARNT complex then binds to XREs positioned upstream of target genes. To resolve the XRE binding activity of AhR/ARNT in the C17.2 cell line, cells were grown in feeding medium and treated with DMSO, 1nM TCDD, or 10nM TCDD. Cells were harvested 1 hour following treatment for preparation of total cell extracts. EMSA analysis revealed no difference in the baseline levels of XRE binding in cells treated with DMSO vehicle as compared to basal conditions (data not shown). However, a 2.1 ± 0.5 and 2.5 ± 0.8 fold increase in AhR/ARNT-XRE binding was observed in cells treated with 1nM and 10nM TCDD, respectively, as compared to DMSO conditions. Unlabeled XRE-oligonucleotide band confirmed specificity of AhR/ARNT-XRE band (Figure 4).

AhR protein levels downregulate following TCDD exposure

Several reports demonstrate that AhR levels rapidly downregulate following ligand binding. The downregulation of AhR appears to be ubiquitin-mediated as evidenced by studies in which inhibition of the 26S proteasome results in an attenuation of AhR protein depletion and enhanced AhR activity. To determine if AhR levels downregulate in C17.2 cells exposed to ligand, cells were treated with DMSO vehicle, 1nM TCDD, or 10nM TCDD and harvested 4, 8, and 24 hours later for immunoblot analysis. In cells treated with DMSO vehicle, a pronounced signal from the AhR band was seen at all time points examined. Differences in AhR protein levels were not observed in DMSO treated cells as compared to basal conditions (data not shown). However, a marked reduction in AhR levels was observed in cells exposed to 1nM and 10nM TCDD as compared to DMSO treated cells at all time points examined. Corresponding b-actin blot is provided to confirm relative amounts of total protein loaded (Figure 5).

TCDD exposure induces the expression of COX-2 protein

The transcriptionally active AhR/ARNT heterodimer, once bound to the XRE of target genes, induces the expression of such proteins as COX-2, the first enzyme in the metabolic pathway that converts arachidonic acid to a group of molecules collectively known as eicosanoids. To determine the ability of AhR in C17.2 cells to induce the expression of proteins known to be directly regulated by the AhR pathway, cells were treated with DMSO vehicle, 1nM TCDD, or 10nM TCDD and harvested 24 and 48 hours later for immunoblot analysis of COX-2 protein levels. Differences in COX-2 protein levels in DMSO treated cells versus basal conditions were not detected (data not shown). However, a 1.12 ± 0.09 and 1.16 ± 0.14 fold increase in COX-2 protein levels was observed in cells treated with 1nM or 10nM TCDD, respectively, as compared to DMSO conditions beginning 48 hours following exposure. In addition, AhR protein levels were downregulated in cells treated with 1nM and 10nM TCDD as compared to DMSO-treated cells, indicating that AhR-mediated signal transduction was altered upon TCDD exposure. Differences in COX-
protein levels were not observed before 48 hours in cells treated with DMSO vehicle versus TCDD (data not shown). Corresponding b-actin blot is provided to confirm relative amounts of total protein loaded (Figure 6).

**C17.2 cells can differentiate in a fully defined, serum-free medium into at least two morphologically distinct cell populations**

When maintained in feeding medium, C17.2 cells rapidly proliferate as evidenced by their ability to grow to confluence in culture. Prior to reaching confluence, there are two general morphologies observable in the overall population of cells. Both subpopulations assume an irregularly shaped cytosolic domain, with a large nuclear compartment located near the center of the cell. However, one subpopulation of cells is flat and more rounded with multiple wide, flat, and short extensions protruding in all directions. A second subpopulation of cells acquires a more elongated morphology.

The standard feeding medium for C17.2 cells consists of a total of 15% serum (10% FBS and 5% HS). Serum contains a large number of unknown factors including proteins, peptides, cofactors, and other unidentified, biologically active compounds that are likely to have a profound influence on the physiology and morphology of C17.2 cells. In order to identify a medium in which the factors that influence the developmental program of C17.2 cells in culture could be more precisely controlled for, cells were plated in the previously described and widely used Bottenstein and Sato medium, referred to here as defined medium. Previous studies report that when maintained in this fully-defined medium, the C17.2 cell line remains viable for extended periods of time. Furthermore, C17.2 cells maintained in defined medium have been shown to exit the cell-cycle, as evidenced by previous studies that report DNA content remains constant in C17.2 cells maintained in defined medium.

To begin to define the differentiative capacity of C17.2 in culture, cells were plated in feeding medium for 24 hours and then replaced in defined medium. A subpopulation of cells maintained in defined medium alone spontaneously acquired a neuronal-like morphology beginning 4 days after treatment; the soma of a minority of cells became small and circular with 2 to 3 neurites of varying lengths protruding in opposite directions. The soma contains a large, centrally located nucleus and scanty cytoplasm. Furthermore, cells assuming a neuronal-like morphology tend to do so in clusters, leaving large expanses of the plate surface void of similarly appearing cells. The majority of cells however (approximately 70%) acquire morphology that is consistent with glial cells, maintaining a large, flat, and generally polygonal cytoplasmic domain with multiple large and flat protrusions. These cells also maintain a large, centrally located nucleus. Furthermore, cells acquiring the glial-like morphology tend to do so in clusters, leaving large expanses of the plate surface void of similarly appearing cells. The majority of cells however (approximately 70%) acquire morphology that is consistent with glial cells, maintaining a large, flat, and generally polygonal cytoplasmic domain with multiple large and flat protrusions. These cells also maintain a large, centrally located nucleus. Furthermore, cells acquiring the glial-like morphology tend to do so in clusters, leaving large expanses of the plate surface void of similarly appearing cells. The majority of cells however (approximately 70%) acquire morphology that is consistent with glial cells, maintaining a large, flat, and generally polygonal cytoplasmic domain with multiple large and flat protrusions. These cells also maintain a large, centrally located nucleus. Furthermore, cells acquiring the glial-like morphology tend to do so in clusters, leaving large expanses of the plate surface void of similarly appearing cells. The majority of cells however (approximately 70%) acquire morphology that is consistent with glial cells, maintaining a large, flat, and generally polygonal cytoplasmic domain with multiple large and flat protrusions. These cells also maintain a large, centrally located nucleus. Furthermore, cells acquiring the glial-like morphology tend to do so in clusters, leaving large expanses of the plate surface void of similarly appearing cells.
The figure provided depicts the morphology of C17.2 cells grown in defined medium for 7 days (Figure 7).

C17.2 cells undergo neuronal differentiation in culture

Prior to differentiating toward a neuronal or glial lineage, proliferating neural precursor cells express the intermediate filament nestin, a marker that is universally expressed in dividing neural stem cells. Upon cell-cycle arrest, individual neural precursors can differentiate toward a neuronal or glial cell fate. Cells pushed to actively differentiate down a neuronal lineage will express the microtubule subunit βIII-tubulin, an early marker of neuronal differentiation. Similarly, cells pushed to actively differentiate down a glial lineage will express GFAP, an early marker of glial differentiation. To further characterize the differentiative capacity and phenotypic traits of C17.2 cells in culture, cells were stained for nestin, βIII-tubulin, and GFAP proteins. Co-labeling with Hoechst nuclear stain revealed that when grown in feeding medium for 4 days, all cells express nestin protein, but not βIII-tubulin or GFAP proteins. While a majority of cells (approximately 95%) maintain nestin expression when kept in defined medium for 4 days, a minority of cells gain expression of βIII-tubulin protein. C17.2 cells that show expression of βIII-tubulin protein also exhibit morphology that is consistent with a neuronal phenotype (Figure 8). Cells did not, however, acquire expression of GFAP protein (data not shown). These data demonstrate that individual C17.2 cells can spontaneously differentiate in defined medium from a pluripotent neural precursor cell into a post-mitotic neuronal cell.

The expression of AhR protein is downregulated in post-mitotic C17.2 cells

To evaluate a role for AhR in neuronal development, AhR protein levels were assessed in C17.2 cells at sequential stages of neuronal development. C17.2 cells were grown in feeding medium or defined medium and harvested after 2 to 6 days for immunoblot analysis of AhR protein content. A marked decrease in AhR protein levels was observed at all time points in cells maintained in defined medium as compared to those maintained in feeding medium. In contrast, β-actin protein levels increased in cells maintained in defined medium. AhR levels were normalized to the corresponding β-actin blot (Figure 9).

Because of the heterogeneous expression of βIII-tubulin protein in cells maintained in defined medium, it was necessary to determine whether AhR protein downregulation is the result of a uniform downregulation in post-mitotic C17.2 cells or is the result of downregulation in a subpopulation of cells. Cells were kept in feeding medium or defined medium and fixed after 6 days in culture. Immunocytochemical analysis revealed that AhR protein is downregulated uniformly in all cells maintained in defined medium. Cells maintained in feeding medium served as a positive control (Figure 10). These data demonstrate that AhR protein levels are uniformly downregulated in non-dividing C17.2 cells as compared to AhR protein levels in mitotic C17.2 cells.

TCDD does not alter the proportion of C17.2 cells that express βIII-tubulin protein

Previous studies have demonstrated in several cell populations that TCDD can alter the expression of markers associated with distinct phases of cellular development. To determine whether the developmental program of C17.2 cells is altered upon exposure to TCDD, cells were treated with DMSO vehicle, 1nM TCDD, or 10nM TCDD following a 4-hour incubation in defined medium. Cells were fixed 6 days later for immunocytochemical analysis of βIII-tubulin expression. Differences in the percentage of βIII-tubulin positive cells present in untreated cultures or those treated with DMSO vehicle were not detected (data not shown). Similarly, differences were not detected after TCDD treatment (Table 1). Furthermore, the number of βIII-tubulin positive cells in samples treated with 1nM or 10nM TCDD were well within the range of C17.2 cells that normally express βIII-tubulin protein after 6 days in defined medium (approximately 25 to
Immunocytochemical analysis of AhR protein distribution in C17.2 cells

Figure 8: ICC analysis of nestin protein (A)(B) and βIII-tubulin protein (C)(D) expressions in C17.2 cells maintained in feeding medium (A)(C) or D.M. for 4-days (B)(D). Cells were fixed and then labeled with nestin or βIII-tubulin primary antibodies and Hoechst nuclear stain. A phase contrast image of field D is provided (E). Arrows designate corresponding βIII-tubulin positive cells in fields E and D. Results are representative of 3 separate experiments. Bar, 1μm. [original magnification 20x]

Figure 9: AhR protein content in C17.2 cells grown in F.M. or D.M. Immunoblot analyses of AhR protein levels in C17.2 cells maintained in F.M. or D.M. for 2 to 6-days. Lanes contain 10μg of protein isolated from C17.2 cells. Untreated Hepa1c1c7 cells were used as a positive control. Corresponding β-actin blot is provided (A). Bar chart of AhR protein levels normalized to β-actin is provided. Results were generated with Scion Image software (B).

Figure 10: Immunocytochemical analysis of AhR protein distribution in C17.2 cells maintained in F.M. (A) or D.M. (B) for 6-days. Cells were fixed and then labeled with AhR primary antibody and Hoechst nuclear stain. Bar, 1μM. [original magnification 20x]. Results are representative of 2 separate experiments.
confluence and, as previously reported, the DNA content remains constant over time.\(^\text{80}\) Furthermore, a subpopulation of C17.2 cells differentiates into post-mitotic, neuronal-like cells as evidenced by the appearance of βIII-tubulin protein beginning 4 days after plating in defined medium. While a subpopulation of C17.2 cells assume a neuronal morphology and acquire expression of βIII-tubulin, the majority of C17.2 cells assume a glial-like morphology with a large, flat, and irregularly-shaped cytosolic domain and large nucleus. Based on morphological analysis and a lack of βIII-tubulin staining in these cells, it is unlikely that this subpopulation is developing toward a neuronal lineage. While C17.2 cells were devoid of GFAP protein expression, previous studies have reported that the majority of C17.2 cells plated in defined medium acquire GFAP staining, suggesting that this subpopulation may be fated for an astrocyte lineage.\(^\text{86,104}\)

Fate decision processes are mediated by a complex set of extracellular and intracellular factors that together alter the genetic program of a cell in order to produce a novel regulatory state.\(^\text{105}\) The passaging of cells through successive regulatory states ultimately leads to lineage commitment and differentiation. It is likely that C17.2 cell expression of GFAP protein is dependent upon the balance and timing of multiple extracellular factors, including trophic factors and cell-cell contact. Without the appropriate cellular environment, C17.2 cells may not acquire the expression of GFAP protein. Cells assuming a glial-like morphology may be fated for a glial lineage, while not yet achieving a regulatory state in which GFAP protein is expressed. Alternatively, cells that acquire morphology consistent with glial cells may not yet be committed to a glial or neuronal state, as evidenced by the continued expression of nestin protein and lack of βIII-tubulin and GFAP proteins. Supplementing defined media with trophic factors known to promote a neuronal lineage, such as the brain-derived neurotrophic factor (BDNF),\(^\text{106,107}\) may help to determine whether cells that are devoid of GFAP and βIII-tubulin staining are committed to a glial lineage or maintain the capacity to differentiate towards a neuronal fate.

Interestingly, AhR protein levels are differentially regulated in proliferating and non-dividing C17.2 cells. AhR protein declines in C17.2 cells that are maintained in defined medium as compared to feeding medium, suggesting that AhR levels are downregulated in non-dividing cells relative to proliferating cells. Non-proliferating populations are not, however, devoid of AhR protein content. While AhR protein levels are reduced, immunoblot analysis confirmed the presence of AhR protein in cells maintained in defined medium. Sustained expression of AhR in non-dividing neural populations may reflect a function for AhR in mediating adaptive cellular responses to environmental contaminants, which is consistent with its role in detection and mediation of cellular responses to environmental factors.\(^\text{44}\) Similarly, the continued expression of AhR in non-dividing C17.2 cells could reflect a role for AhR in intercellular communication processes.\(^\text{108}\) In support of this possibility, AhR has been shown to interact with several extracellular signaling molecules including hormone-receptor pathways\(^\text{44}\) and circulating indigoids.\(^\text{109}\) AhR may regulate physiological processes dependent upon these extracellular factors in neural cells.

While this study did not aim to define a finite role for AhR in neural development, the findings suggest that AhR may participate in the transition from proliferation to cell-cycle arrest in C17.2 cells. AhR protein is expressed in all cells when maintained in feeding medium and is downregulated uniformly in cells plated in defined medium. However, despite the continued uniform expression patterns of AhR protein in C17.2 cells, the non-proliferating cell population is comprised of at least two distinct cell types. This suggests that AhR may not function in the determination of lineage commitment or the differentiation process of a particular subpopulation of neural cells. In light of the synchrony between AhR protein downregulation and the transition of C17.2 cells from proliferation to cell-cycle arrest, it is more likely that AhR may function to regulate components of the cell-cycle machinery in C17.2 cells. Therefore, AhR may aid in neurodevelopment by modulating the proliferation of neural precursor cells, since cell-cycle arrest is a prerequisite to neuronal differentiation.

In support of a role for AhR in regulating the program of cell-cycle progression, previous studies have identified the gene encoding the p27kip1 cyclin/cyclin-dependent kinase inhibitor as a direct target of the AhR pathway.\(^\text{110}\) AhR has also been shown to mediate the transcription of the c-myc gene, a potent proliferative factor, by forming a transcriptionally active complex with the NF-κB subunit RelA.\(^\text{111}\) Interestingly, COX-2 activity has also been implicated in mediating the program of cell-cycle progression in a variety of cell types.\(^\text{64,112}\) While this study does not provide direct evidence in support of COX-2 as a mediator of cell-cycle progression in C17.2 cells, it demonstrates that COX-2 protein levels are increased in cells exposed to TCDD, possibly by an AhR-mediated event. It has also been previously shown that TCDD alters the transition from proliferation to cell-cycle arrest in a variety of cell populations via the AhR pathway.\(^\text{113}\) The ability of TCDD to alter the program of cell-cycle progression is likely the result of a pleiotropic response directed in part by the AhR pathway.\(^\text{114}\) AhR-mediated alterations in COX-2 activity in cells exposed to TCDD may be one component of this complex genetic response.\(^\text{115-117}\)

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<tr>
<th>DMSO Vehicle</th>
<th>Percentage of βIII-tubulin Positive Cells ± S.E.M.</th>
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<tr>
<td></td>
<td>29.40 ± 1.48%</td>
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<tr>
<td>1 nM TCDD</td>
<td>29.59 ± 1.91%</td>
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<td>10 nM TCDD</td>
<td>30.61 ± 2.12%</td>
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Table 1. The number of C17.2 cells that express βIII-tubulin protein after 6-days of exposure to DMSO vehicle, 1nM TCDD, or 10nM TCDD in defined medium. Cell counts are represented as the percentage of βIII-tubulin positive cells relative to the overall population ± S.E.M. Results represent 4 separate populations of cells per treatment group within one experiment.
of exposure in defined medium. If TCDD alters the timing of C17.2 cell progression from the proliferative phase of cellular development to the cell-cycle arrest phase as it has been shown in keratinocytes, then the proportion of cells expressing βIII-tubulin may be altered as a result of a prolonged or shortened proliferation phase. However, defined medium alone is a potent inhibitor of cell-cycle progression. Consequently, any influence TCDD may exert on the transition of C17.2 cells from proliferation to cell-cycle arrest may be masked by the anti-mitogenic effects of the media. To determine whether TCDD alters the developmental program of C17.2 cells, the anti-proliferative effects of the defined medium will likely have to be balanced with the activity of a mitogenic factor. The development of culture conditions in which C17.2 cells can be maintained in a state of proliferation by factors controlled for by the experimenter will allow for an accurate assessment of the effects of TCDD on the developmental program of C17.2 cells. Previous studies have demonstrated that C17.2 cells can be made to proliferate in defined medium by the application of epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF). Culturing C17.2 cells in defined medium supplemented with EGF or bFGF may allow for the detection of an alteration in the program of C17.2 cell-cycle progression and development upon exposure to TCDD.

This study showed that C17.2 cells express a functional AhR pathway at an early stage of neural development and that AhR protein levels are differentially regulated according to the phase of cell-cycle progression and development. This, in combination with the finding that individual C17.2 cells maintain the capacity of EGL cells to differentiate into neuronal-like cells, supports the notion that the C17.2 cell line can serve as a relevant in vitro model of AhR involvement in normal neurodevelopment. The generation of AhR knockout cells in combination with specific inhibitors to AhR will help to address the question of whether AhR is necessary to the developmental program of C17.2 cells. Furthermore, the generation of culture conditions in which whole populations of C17.2 cells can be guided predominantly towards a neuronal or glial fate will allow for the direct analysis of AhR activity during the development of specific neuronal lineages.

This study also provided preliminary evidence suggesting that TCDD does not alter the developmental program of C17.2 cells in defined medium. Supplementing the defined medium with mitogenic factors to C17.2 cells will allow for a more accurate assessment of whether TCDD alters C17.2 cell development. However, based on the expression of a functional AhR pathway in C17.2 cells, this study supports the notion that neural precursor populations may be direct targets of TCDD-mediated developmental toxicity.

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