

Author's response to reviews

Title: Over-expression of AhR (aryl hydrocarbon receptor) induces neural differentiation of Neuro2a cells: Neurotoxicology study

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Author's response to reviews: see over

Prof. David Ozonoff
Editors-in-Chief
Environmental Health: A Global Access Science Source

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Dear Prof. Onzonoff

We are very sorry for our delay to send you our revised manuscript. We are returning herewith a manuscript entitled "Over-expression of AhR (aryl hydrocarbon receptor) induces neural differentiation of Neuro2a cells: Neurotoxicology study" revised according to reviewers' comments.

Here are revisions for the reviews' comments:

Reviewer #1, Dr. Thomas Gasiewicz, University of Rochester

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>Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

>3. The rationale for using the Neuro2a cells is not defined. Why is this a valid model for studying neuronal differentiation or AhR-mediated neurotoxicity? What exactly do these cell represent in the brain? Additional consideration of this is needed here

To explain the reason of using Neuro2a, we added the sentences "The regulation of neurogenesis is key event for controlling brain development. To understand the regulatory mechanisms of neurogenesis, neuroblastoma is widely used as an experimental model. Neuroblastoma usually arises from the proliferation of neural crest-derived precursors resulting in defective differentiation *in vivo*. Neuroblastoma cells proliferate as undifferentiated cells in standard cell culture, but they have the potential to become neuronal precursors. Neuro2a cells are a murine neuroblastoma cell line that has proven to be a useful experimental model for studying some aspects of differentiation mechanism since its differentiation can be controlled by various conditions, such as low serum concentration, retinoic acid and cAMP " in the "Background" section.

>4. The authors draw conclusions regarding AhR activity following over-expression from a very limited data set, i.e. CYP mRNA expression). The studies should include XRE binding/transactivation analysis as well.

In order to confirm that the exogenous AhR is functional, we added the data of mRNA expression of other XRE-mediated genes, namely AHRR and GST. The data are shown in Figure 2C. We also added the sentences "we analyzed CYP1A1, GST and AhRR (AhR repressor) mRNA expression as functional markers of AhR. These genes were reported to be regulated by AhR binding to the XRE. The ligand-activated AhR binds to the XRE sequence in the promoter region of the CYP1A1 gene to activate its expression. As such, CYP1A1, GST and AhRR mRNA would be induced if functional AhR is expressed in N2a-R α ." are added in the "Results" section.

>5. It would also be useful to know the relative expression of AhR protein in both the untransfected and transfected cells. The finding that AhR may have transcriptional activity in the absence of known AhR ligand suggests an abnormal phenotype that may not be relevant to a neurotoxicity model. A comparison of relative AhR expression levels and activity in this model to those present in brain cells exposed to exogenous AhR ligands would be useful.

We added the data resulted from protein- level analysis of the expression of AhR in N2a-R α and N2a-Vc. The data are shown in Figure 2B. We also added the sentences “The expression of AhR in N2a-R α cells was reflected in the protein levels (Fig. 2B). Although immunoreactivity for AhR was not detected in the N2a-Vc and Neuro2a cells, the protein band corresponding to the molecular weight of AhR was clearly detected in N2a-R α .” in the “Results” section and added the procedure of Western Blot analysis in the “Methods” section.

>6. The authors claim that proliferations rates are reduced but fail to provide a compelling data set to support this contention. There may be other reasons for the decreased DNA content, i.e. loss in cell viability or apoptosis. Although the text indicates that there was no cell death or apoptosis, no data was presented and it is not clear exactly how this was determined. There is a need to modify the experimental design so that a more rigorous measurement of proliferation (as well as viability and apoptosis) is used.

Because we have clearer shown the decrease of the cell proliferation of N2a-R α , we showed the data of cell proliferation using Alamar Blue dye reduction analysis instead of DNA contents. The data are shown in Figure 5. We added the sentences “we measured cell proliferation rates by Alamar Blue dye reduction assay based on the resazurin reduction test. Cell proliferation was measured by quantitation of the reduction of the intracellular environment. The internal environment of the proliferating cell was attenuated compared with that of non-proliferating cells. Specifically, the ratios of NADP/NADPH, FADH/FAD, FMNH/FMN, and NADH/NAD, increase during proliferation. Alamar blue, which can be reduced by these metabolic intermediates, is useful in monitoring cell proliferation [37, 38]. If N2a-R α cells differentiate into neurons, cell growth would be reduced. The proliferation of N2a-R α cells decreased by approximately 60% after 7 days, compared to N2a-Vc (Fig. 5).” in the “Results” section and added the procedure of Alamar Blue dye reduction assay in the “Methods” section. We also analyzed the possibility of apoptosis in N2a-R α cells. After 7-day culture, caspase-3/7 activities were not far differed from N2a-Vc (data not shown).

>7. The TH mRNA data are interesting, but additional studies are needed to determine TH protein and functionality (i.e. TH protein, activity, dopamine measurements). The assessment of other markers of differentiation would also be useful.

We had additional evidence to suggest the dopaminergic differentiation of N2a-R α cells. In this study, we refer the up-regulation of TH gene expression based on AhR activation. In order to support the activation of the expression of TH, we analyzed the expression of other marker of dopaminergic differentiation, Nurr. The data are shown in Figure 6. We also added the sentences “Nurr-1 regulates the expressions of TH mRNA. It has been reported that over-expression of Nurr-1 induced neurite elongation and led to differentiation of neural stem cells to dopaminergic neurons.” and “The level of Nurr-1 mRNA in N2a-R α and N2a-Vc cells was also examined and higher levels were detected in N2a-R α compared to N2a-Vc.” in the “Results” section. Additionally, our primitive experiments of the dopamine detection bared positive data (data not shown).

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>Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

>1. The manuscript needs editing for grammar and syntax throughout.

We asked native speaker to read the proofs of this revised manuscript.

>2. Introduction: This is presented in a very superficial manner. There is a great deal of background

>and key references related to AhR expression, activity, and neurodevelopmental effects that are
>missing. While the Introduction need not be encyclopedic, additional information is needed here
>especially if the authors are suggesting that their system is a good model for developmental
neurotoxicity.

We added the sentences “The PAH receptor, namely the AhR, is expressed in various regions of the brain during the critical period of brain development. In particular, it has been reported that maximal expression of AhR protein was observed during P3 -10. This stage is considered to be the critical period for the growth and maturation of neuroblasts in the cerebellar granular cell layer. These findings imply that the AhR may be involved in the molecular processes of brain development, and may also be related to the developmental neurotoxicity of TCDD. However, it has been very difficult to directly examine the actual role of AhR in the pathway from TCDD to the function during brain development, because an experimental model has not yet been established to investigate AhR function in developing neurons.” in the “Background” section.

>3. P. 6, line 10 from bottom, “..AhR was decreased by about 50%..” . Actually, the figure indicates
>only about 30% decrease.

We correct, “AhR mRNA was attenuated by about 70% in N2a-R α cells”

>4. Discussion: There seems to be much focus on HES in the discussion, especially since no data on
>HES are presented. If AhR regulates HES through interaction with XREs, the expected result might
>be opposite to what the authors are reporting in this manuscript.

Because the function of Hes-1 was reported to be different in each cell type, the interaction of Hes-1 and AhR is a kind of speculation in N2a-R α . In order to understand physiological function of AhR in neural development, however, it is important fact that AhR is a member of bHLH family. Because other members of bHLH family transcriptional factor formed network and regulated their expressions mutually, it is likely that AhR play a role in the bHLH family network.

Reviewer #2, Dr. David Sherr, Boston University School of Public Health

>Major Compulsory Revisions (that the author must respond to before a decision on publication can
>be reached)

>The writing needs to be more clear.

>Figure 2: It is unclear if the PCR primers are derived from the rat AhR sequence since the AhR gene
>itself was cloned from rat brain. If the primers were in fact derived from the rat, do they cross-react
>with the murine AhR? If not, then the negative amplification shown in figure 2A is misinterpreted.

In order to determine whether both of the rat and mouse AhR genes can be amplified with the PCR primers, we performed RT-PCR with cDNAs prepared both from rat brain and mouse brain. The data are shown in Figure 2A. We added sentences “The AhR primers were designed for rat AhR, but also specifically recognize and amplify mouse AhR under identical PCR conditions (Fig 2A). AhR mRNA was not detected in either the original Neuro2a cells or N2a-Vc cells transfected with the vector that did not contain the insert. On the other hand, AhR mRNA was clearly detected (Fig. 2A) in N2a-R α . Since the primers for AhR amplify both rat and mouse AhR, these results indicate that N2a-R α expressed AhR mRNA at a high level and that N2a-Vc expression of AhR was below the detection limit.” in the “Results” section. We also added the procedure of extraction of tissue cDNA in the “Methods” section.

>Figure 4: Another interpretation of these data is that ectopic AhR expression induces cell death,
>thereby lowering the Hoechst signal. The authors should provide data demonstrating that

>AhR-transfected cells are as viable as cells transfected with the control plasmid. Similarly, a
>sentence in the “Conclusion” section on page 2 states that, “Activated AhR may disrupt the
>irregular differentiation occurring rather than cell death.” No data are provided on cell viability.

In order to clarify the cell proliferation and viability, we measured the redox activity based on mitochondrial respiratory chain of living cells using Alamar Blue dye reduction assay. When Alamar Blue is uptaken in living cells, it is reduced by mitochondrial activity and its wavelength of absorption changes to be measurable. We have shown the data of living-cell proliferation using Alamar Blue dye reduction analysis instead of DNA contents. The data are shown in Figure 5. We added the sentence “we measured cell proliferation rates by Alamar Blue dye reduction assay based on the resazurin reduction test. Cell proliferation was measured by quantitation of the reduction of the intracellular environment. The internal environment of the proliferating cell was attenuated compared with that of non-proliferating cells. Specifically, the ratios of NADP/NADPH, FADH/FAD, FMNH/FMN, and NADH/NAD, increase during proliferation. Alamar blue, which can be reduced by these metabolic intermediates, is useful in monitoring cell proliferation. If N2a-R α cells differentiate into neurons, cell growth would be reduced. The proliferation of N2a-R α cells decreased by approximately 60% after 7 days, compared to N2a-Vc (Fig. 5).” in “Results” section and added the procedure of Alamar Blue dye reduction assay in “Methods” section. We also analyzed the possibility of apoptosis in N2a-R α cells. After 7-day culture, caspase-3/7 activities were not far differed from N2a-Vc (data not shown).

>The authors frequently refer to conditions as being “ligand-less” . The authors do not consider the
>possibility that there is an endogenous ligand that is activating the AhR.

We analyzed the conditioned medium of N2a-R α cells not induced CYP1A1 mRNA expression in Hepa1c1c7, which is a cell line express AhR and responsive to AhR-mediated ligand (data not shown). However, we rewrite the sentence to “without the application of ligand to the culture medium” to avoid misunderstanding.

>The statement, “AhR-mediated pathways are known to work essentially for the action of TCDD in
>the liver or reproductive organs” significantly understates the breadth of research on the AhR. For
>example, the AhR has been shown by many investigators to play a role in the development and
>function of the immune system. Similarly, several environmental AhR ligands are well known to be
>carcinogenic in many organs. In general, the literature is replete with examples of how the AhR and
>its environmental ligands influence development and function of many organ systems.

We added the sentences “Activation of the AhR by dioxins results in the induction of xenobiotic metabolism and successive toxic responses, including hepatocellular damage, immune system disorders, reproductive system disorders, teratogenesis and carcinogenesis. In the immune system, activated AhR suppresses a variety of B-cell mediated responses through aberrations in bone marrow B-cell development. In the reproductive system, activated AhR causes depletion of fetal oocytes by apoptosis. In response to unknown developmental cues, in addition to those stated above, the AhR can influence normal development.” In the “ Discussion” section.

>No description is given for what statistical tests were used. For data presented in Figures 2, 3, 4, 5,
>were statistics performed with replicates from 1 experiment? If so, each value is not an independent
>event and statistics cannot be performed. It is the experiment that must be replicated, not just the
>number of wells/samples/replicates in any one experiment. A minimum of 3 independent
>experiments must be performed before statistical analysis can be applied.

We perform the each experiment at least three independent trials before statistical analysis. On Figure1 and 2, presented data were representative of triplicate experiments. We added sentences “
Statistics: All values are presented as the mean \pm standard error of the mean (SEM). All experiments

were replicated at least three times. Data were analyzed using Student's T-test with the Exel software package." in the "Method" section. We also added the sentence "Representative data are presented from triplicate experiments" in the "figure legends".

>Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

>At the end of paragraph 2 on page 9, what is meant by "formationText for this section." ? I assume it is a typo.

>There are many typos throughout the paper.

We asked native speaker to read the proofs of this revised manuscript.

>What is meant by "ontogenesis" on page 3.

"Ontogenesis" means the development of individual.

>What is meant by "...is functional as ligand-binding AhR" on page 6?

AhR is thought to be a ligand-activated transcriptional factor originally. But, the transfected AhR in N2a-R α is shown to be activated without the addition of the ligand. Then we concluded that the AhR in N2a-R α act as if a ligand-binding or ligand-activated AhR

I hope that revised manuscript is now acceptable for publication.

Thank you very much for your cooperation and patience.

Sincerely yours,

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