

Author's response to reviews

Title: Effect of dioxins on regulation of tyrosine hydroxylase gene expression by aryl hydrocarbon receptor: a neurotoxicology study

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Author's response to reviews: see over

Prof. Philippe Grandjean
Editors-in-Chief
The Environmental Health Editorial Team

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Dear Prof. Grandjean

We are returning herewith the manuscript entitled " Effect of dioxins on regulation of tyrosine hydroxylase gene expression by aryl hydrocarbon receptor: a neurotoxicology study" (MS: 5104883242583392) revised according to the reviewer's comments.

Here are revisions for the reviewer's comments:

>The statistical analysis of the Luciferase Assays is common, but should be a 1-way ANOVA rather than >multiple t-tests.

We designed the experiments, as shown in Fig.5, by setting the window size to about 100bp, truncated 5' end of TH-gene by about 100bp, then searched the point where the TCDD-responsibility goes steeply down. We thought the simple comparison of the TCDD-responses between before and after the truncation of 100bp is important for studying the transcriptional regulation activities of the truncated 100bp by avoiding the influences of the complex cross-interaction with the other region out of the window. In this sense, we employed t-test for comparing each 2 groups before and after the truncation, despite one-way ANOVA suits a comparison among many groups.

> The Abstract and Discussion are too long. Discussion needs to be focused on the issues germane to this >work and reduce speculation.

We deleted the sentences "Previously we reported that the tyrosine hydroxylase (TH) gene was hyperexpressed on AhR activation. TH is the rate-limiting enzyme of dopamine synthesis." and "Amounts of L-dopa and dopamine were elevated in N2a-R β cells exposed to TCDD." in the "Abstract" section.

We also deleted the sentences concerning speculation "On the other hand, it was reported that the transcriptional activity of AhR is cell-specific. AhR-regulated transcription involves many coactivators. For example, of these coactivators, FHL2 (four and half LIM domains protein 2) mediates AhR action in a cell-specific manner [45]. Although it remains unclear whether FHL2 participates in the selection of the AhR binding sequence, the binding sequence could be selected by a cell-specific coactivator acting together with AhR." in the "Discussion" section for focusing on the main story of this study.

> Figures 1-3 present information published in the 2006 EH publication. These should not be represented
> here as new information.

N2a-R β cells, newly cloned in this study, are different from N2a-R α cells that we used for exhibiting the activated AhR induced TH gene expression in N2a-R α cells in 2006EH. In 2006 EH, we showed that constitutively activated AhR up-regulated the TH gene expression but we could not mention the up-regulation of TH gene under dioxin exposure. By using of N2a-R β cells, we exhibited, in this study, AhR activated with exogenous ligand induced to elevate the TH mRNA expression (Fig.1) and the TH protein expression (Fig.3). Fig. 2 showed that the induction of TH gene was regulated by AhR under dioxin exposure. For clarifying this point, we described in the “Results” section as follows; “In a previous report, we demonstrated that N2a-R α cells show hyperexpression of the TH gene caused by AhR activation in a ligand-independent manner. For further investigation of neurotoxic phenotype including TH gene expression by ligands, we used another clone regarded as N2a-R β cells. N2a-R β cells express a large amount of AhR, which acts in a ligand-dependent manner.”

> Figure 5 is very difficult to interpret. The staining appears to be pretty weak and this should be explained
> in the figure legend. The dual-label (panel D) does not appear to be convincing to this reviewer as a
> stand-alone panel. It would be useful to have an inset for each of A, B, and C (rather than a stand-alone D)
> which shows the X400 image of the area under consideration. This would allow the reader to observe the
> different staining. Is the AhR ICC nuclear? One cannot tell in these images.

Although the staining of SNc was clearly distinguishable from the other areas and these results were reproducible, we replaced the panel A, B, and C, and inset the high magnified view in each panels. As shown in magnified view of panel A, AhR is located in cytoplasm rather than in nuclear because this staining was performed under condition without dioxin, according to the reviewer’s comments.

In addition, we replaced the color designations from “brown” and “red” to “light brown” and “light red” in the sentence of “Signals of AhR and TH proteins were developed using DAB/H₂O₂ (brown) and ALP-RED (red), respectively.” in “Figure legend” for improving the legibility of the stained areas.

> The Luciferase assay is unusual in that beta-galactosidase is used to determine transfection efficiency
> rather than a separate marker such as Renilla. The authors need to clarify the assay so that the reader can
> determine the degree to which the data can be interpreted to be valid.

As reviewer mentioned, Renilla is frequently used to determine transfection efficiency, recently. β -galactosidase is, however, used as much as Renilla. For example, β -galactosidase is used in the following recent literatures:

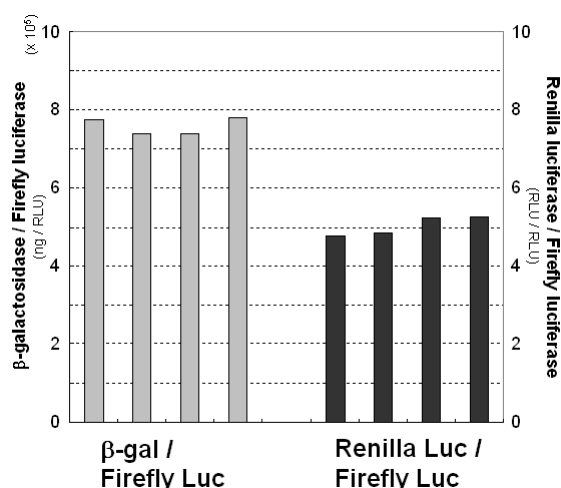
- (1) Flames N., et.al. (2009) Nature 458 885-889
- (2) Kang HJ., et.al. (2006) J.Biol. Chem., 281 14654-14662

In addition, we confirmed β -galactosidase is able to use as a control instead of Renilla. We have shown a result of preliminary experiment below.

As a transfection efficiency control, we construct two plasmids. One is a plasmid pcDNA/V5-His/LacZ (used in this study) expressing β -galactosidase and another is a plasmid pcDNA/V5-His/Ren expressing Renilla luciferase.

We co-transfected reporter vector containing TH promoter region and firefly luciferase gene, and control vectors above mentioned. We measured firefly luciferase activity of the cells in response to TCDD, and measured Renilla luciferase activity or β -galactosidase activity as a control.

Figure, as shown below, represents results of calculating Renilla luciferase activity and β -galactosidase activity for each firefly luciferase activities in four independent experiments.



Because relative β -galactosidase activities maintained equally constant as relative Renilla luciferase activity, we concluded β -galactosidase is used as a control of transfection efficiency.

Then, we added the sentences “Luciferase activity was normalized with β -galactosidase activity, based on the results of control experiments by using of β -galactosidase and Renilla Luc. (data not shown).” in the “Methods” section.

> TH is important for catecholamine synthesis, not just dopamine. Is the AhR co-localized with TH in >brainstem regions which use NE as the neurotransmitter?

In the brain stem region, TH is mainly detected in locus coeruleus of pons, but AhR is hardly detected. Although we did not perform IHC with NE (Norepinephrine), AhR did not seem to be co-localized with TH in the brain stem.

Certainly TH is a rate limiting enzyme of catecholamine, not only dopamine. In Table 1, we showed that amounts of L-DOPA and dopamine were increased by TCDD exposure but NE was not. Therefore, we concluded the increase of TH gene expression by AhR activation lead to the elevation of dopamine amount.

>What is the strength of this work being developed in an artificial cell line? Certainly, the Luciferase

>assays and EMSA are important contributions to the conclusion that AhR directly regulates TH

>expression. But, does TH mRNA increase in animals treated with TCDD? Is this brain region-specific?

>Are the regions among those in which AhR- and TH-expression overlaps? The authors weaken the

>significance of their work by avoiding this conversation.

As reviewer pointed out, we did not examine TH mRNA increase *in vivo*. In this study, we aimed to show that AhR directly regulates TH expression in the cells. It is the first step that we attempt to clarify the mechanism of TCDD toxicity in developing brain.

In order to specify that our finding is based on *in vitro* experiments, we changed the sentences to “In this study, we showed that TCDD not only increases TH gene expression but also the amounts of dopamine and L-dopa in TCDD-exposed murine neuroblastoma cells (Neuro2a) (Table 1). The result of these *in vitro* experiments indicates that TCDD can disturb the equilibrium of the dopamine system through abnormal dopamine synthesis resulting from abnormal TH gene expression.” in the “Discussion” section.

As we recognized the importance of evidences *in vivo*, we are carrying out investigation of TH mRNA increase in animal treated with TCDD at the moment. We would like to make clear that as soon as we can.

We hope all these corrections and revisions will be satisfactory. We also hope the revised version of the manuscript will be acceptable for publication in “Environmental Health: A Global Access Science Source”.

Sincerely yours,

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