nature portfolio

# Peer Review File

## A single-cell transcriptomic landscape of cadmium-hindered brain development in mice

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript investigates the effects of Cd on mouse brain development, using single-cell sequencing technology to obtain a wealth of information, which is beneficial for understanding the neurotoxicity mechanism of heavy metals. I have the following questions that the author needs to explain or discuss.

1. The Cd in the water standard cannot exceed 0.005mg/L, and 3mg/L was used in the experiment, which is 600 times higher. This is enough to cause neurotoxicity of Cd, and what I am wondering is what is the appropriate Cd concentration for mouse modeling? More than 10 times or 100 times the quoted health standards? Is there a special method or standard? Also, do we need to consider setting different Cd treatment concentrations?

2. Fig3, d, e, there is no marked statistical difference value, is there no difference? Similar issues also exist in Figures 7, 8, and 9.

3. Fig. 9J, Is there a difference in the total distance of mice swimming in the water maze experiment? Is it possible that the exercise ability of mice is affected, resulting in the difference between the Cd group and the Control group? Please explain.

4. Is there basic data for testing mice, such as body weight? The weight of the brain? Is there a difference in liver, kidney index, etc?

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors find that maternal cadmium exposure during pregnancy influenced neural behaviors of offspring mice. In addition, the authors used single-cell RNA-seq to map single-cell transcriptomic landscape in the brains of fetal mice with normal and maternal cadmium-exposed during pregnancy, which is a good research design. The research in this article still has some highlights, it reflects to the impact of maternal cadmium exposure during pregnancy on fetal brain cell subpopulations. I suggest to accept after major revision.
1. In summary, this research was superficial and not in-depth enough. The authors did not make full use of the results of single-cell RNA-seq to deeply explore the mechanism of the impact of cadmium exposure during pregnancy on fetal brain. Additionally, the existing evidence is insufficient to prove the relationship between changes in these cell subsets and neurobehavioral abnormalities. Please suggest to discuss the potential mechanism for Cd-hindered fetal brain development. Actually, cadmium rarely crosses the placental barrier. Placental injury may be the main mechanism for Cd-impaired fetal brain development.
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4. The sample capacity of each group using single cell RNA-Seq in the article should be emphasized in the “Results section”, although it is described in the “Methods section”.
5. When the author discovered that NPC and OPC changed greatly, why didn't they focus on them for further research? Subsequent analysis of other cell groups, as well as time series analysis of each cluster. This makes the entire study unfocused.
6. How is the data shown in the violin plot in Fig 4 related to the developmental abnormalities of NPC caused by cadmium exposure? Why are these results here? This is puzzling.
7. In the article, the author tried to use RT-qPCR to verify some results of single-cell RNA-seq, and the data were shown as negative. I think this method is inappropriate. The differential genes are in specific cells. Total RNA extraction and RT-qPCR cannot verify these differential genes.
8. What is the significance of pseudotime map of the cell clusters? The appearance of this part in the article is puzzling and has no connection with other results in the article.
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10. The authors should provide a visual summary of this study.
11. Please suggest to ask native English speakers and teachers to polish the manuscript.

**Responses to Reviewers' comments:**

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Generally, when making exposure standard, the lowest observed adverse level (LOAEL) or no observed adverse effect level (NOAEL) was first established in experiment animals, and a 10 to 100 uncertainty factor (UF) was adopted to make sure it is safe to human (Soisungwan Satarug, et al., Environ Health Perspect. 2010, PMID: 20123617; Heather R Schaefer, et al., Regul Toxicol Pharmacol. 2023, PMID: 37640100). Here, the maximum contaminant level of cadmium in drinking water is 0.005 mg/L, and the reference dose (Rfd) of cadmium in water for human exposure is $5\times10^{-4}$ mg/kg/day (U.S.EPA). The level of Cd in water 0.005 mg/L is the concentration that generates by NOAEL or LOAEL/UF. It would not cause any adverse effects to humans at this concentration based on previous studies. Thus, the LOAEL or NOAEL is generally considered instead of the maximum contaminant level and the Rfd when carry out animal experiments.

The concentrations of Cd for the mouse model were generally based on the following considerations. Firstly, the concentrations could be found in Cd-polluted areas in the real world or the human body. When it could be found in the real world, it needs to be studied. Secondly, the LOAEL and NOAEL values of Cd as previously reported in different animal models. It has been reported that it is 32.5 mg/L in rats (Diana Moroni-González, et al., Biol Trace Elem Res. 2023, PMID: 37955768; Victor Enrique Sarmiento-Ortega, et al., Biol Trace Elem Res. 2022, PMID:34846673). Thirdly, when studying the molecular mechanisms of Cd-induced effects, researchers always chose the concentrations that could cause adverse effects in target organs due to previous studies. 3 mg/L is the concentration that has been widely used due to the adverse effects on cognitive function found at this dose (Hao Wang, et al., Toxicol Sci. 2018, PMID: 29029324; Hao Wang, et al., Environ Toxicol. 2022, PMID: 34741586; Hao Wang, et al., Toxicol Sci. 2019, PMID: 31271426). Additionally, the peak blood Cd level result from 3 mg/L Cd exposure in mice has been confirmed reaching the blood Cd concentration in specific populations (Hao Wang, et al., Toxicol Sci. 2018, PMID: 29029324; Hao Wang et al., Environ Toxicol. 2022, PMID: 34741586; Aikaterini Sakellari, et al., Sci Total Environ. 2016, PMID: 27295597; Long-Lian Zhang, et al., Environ Res. 2015, PMID: 25836720). The internal exposure dose of Cd detected in the fetus's brain was lower than previously detected in human placenta tissues (María D Esteban-Vasallo, et al., Environ Health Perspect. 2012, PMID: 22591711; Long Xu, et al., Sci Total Environ. 2016, PMID: 26895036). It means that the concentration of 3 mg/L could be found in the real world and worth of study. We have added the reasons why we chose this concentration in the discussion part (Page 7, line 263-274).

It is a shortage of our study that we did not set different Cd concentrations in our study. The reason we just chose one concentration due to that the major purpose of our study here is to detect the mechanisms of how Cd exposure influences brain development and cognitive functions. Thus, we chose a previously widely studied concentration that caused brain injury. The dose-dependent effects are also worthy of study, and probably we will address them in the future.

2. Fig3, d, e, there is no marked statistical difference value, is there no difference? Similar issues also exist in Figures 7, 8, and 9.

There is no statistical difference Fig. 3d, e (Fig. 2d, e in the revised figure). Similar situations are in the previous Fig. 7b, f, i, j (Fig. 6 b, f, i, j in the revised figure), Fig. 8j (Fig. 7j in the revised figure), and the left panel of Fig. 9f (Fig. 8f in the revised figure). The statistic methods and sample numbers are described in the legend. To avoid misunderstanding, we have marked n.s. (no significant) in the revised Figures for those figures with no statistical differences.

3. Fig. 9J, Is there a difference in the total distance of mice swimming in the water maze experiment? Is it possible that the exercise ability of mice is affected, resulting in the difference between the Cd group and the Control group? Please explain.

We have provided the data of the average swimming speeds of mice in the Morris water maze in the revised supplementary data (Supplementary Fig. 9). There is no difference between the Control group and the Cd-exposed group. We have the same concern as the reviewer whether Cd exposure injured the exercise ability of mice because the brain is not the only target of Cd. The data of average swimming speed suggested that Cd exposure did not directly affect the exercise ability of mice at the concentration of 3 mg/L. Additionally, we also detected the $Ca^{2+}$ transients of cortical neurons in free-moving mice. The data provided direct evidence that the behavior changes were due to neural circuit dysfunction. This has been clarified in the revised manuscript (Page 6, line 243-246; Page 8, line 337-347).

4. Is there basic data for testing mice, such as body weight? The weight of the brain? Is there a difference in liver, kidney index, etc?

The data of body weight and brain weight have been provided in the revised supplementary data (Supplementary Fig. 1). There was no difference between the Control group and the Cd group (Page 3, line 89-91).

We have not detected the liver and kidney mass index. Although Cd exposure might affect the liver and kidney, it might not be severe enough to affect the liver and kidney mass index at the concentration we used. It has been reported that 50 mg/L Cd exposure in drinking water during pregnancy has no effect on the liver mass index of fetus in mice (Yi-Ting Fu, et al., Food Chem Toxicol. 2023, PMID: 37121429; Song-Jia Yi, et al., Sci Total Environ. 2021, PMID: 33677283). In rat model, there is also no effect on the liver, kidney and brain mass index of fetus when underwent 50 mg/L Cd exposure in drinking water during the pregnancy (B Sowa, et al., Arch Toxicol. 1985, PMID: 3994509). However, to fully explore it, more experiments

should be carried out. Here, we just focused on the brain.

Reviewer #2 (Remarks to the Author):
In this manuscript, the authors find that maternal cadmium exposure during pregnancy influenced neural behaviors of offspring mice. In addition, the authors used single-cell RNA-seq to map single-cell transcriptomic landscape in the brains of fetal mice with normal and maternal cadmium-exposed during pregnancy, which is a good research design. The research in this article still has some highlights, it reflects to the impact of maternal cadmium exposure during pregnancy on fetal brain cell subpopulations. I suggest to accept after major revision.

1. In summary, this research was superficial and not in-depth enough. The authors did not make full use of the results of single-cell RNA-seq to deeply explore the mechanism of the impact of cadmium exposure during pregnancy on fetal brain. Additionally, the existing evidence is insufficient to prove the relationship between changes in these cell subsets and neurobehavioral abnormalities. Please suggest to discuss the potential mechanism for Cd-hindered fetal brain development. Actually, cadmium rarely crosses the placental barrier. Placental injury may be the main mechanism for Cd-impaired fetal brain development.

We appreciate the suggestions very much. Although we did not explore the detailed molecular mechanisms of Cd exposure on fetal brain development, we uncovered it at the cellular level. Production of an appropriate number of distinct cell subtypes in the proper timing is crucial for the formation of the functional neocortex. Based on our data, the mechanisms of Cd-hindered fetal brain development might be due to the following reasons. First, Cd exposure hindered the differentiation of progenitor cells and the maturation of neurons. Second, Cd exposure caused cell subpopulation shifts. Third, these changes in cell subtypes finally impaired the functions of neural circuits and behaviors. This has been clarified in the discussion part (Page 7-8, line 292-295, 304-312; Page 8, line 323-346). We have not explored the detailed molecular mechanisms in our study. This is a shortage of our study.

We agree with the reviewer that Cd exposure might damage the placental barrier. It has been reported that Cd could pass the placental barrier and affect fetus development in previous studies (Xudong Dong, et al., Int J Womens Health. 2023, PMID: 36798790; Shiyong Zhu, et al., J Environ Sci (China). 2024, PMID: 38135421). We have detected the Cd concentration in the brain of the fetus, which provided direct evidence of internal Cd exposure concentration. The mechanisms of how Cd injures the placental barrier are important to understanding the development toxicity of Cd and are worth studying. However, to focus our study, we only investigated the effects and mechanisms of Cd on brain development here.

2. For methods section, what is the chosen basis for the dose of Cd? Why is fetal brain corrected on GD15.5?

The concentration chosen was based on previous studies. 3 mg/L is the concentration that has been widely studied due to its adverse effects on cognitive functions (Hao Wang, et al., Toxicol Sci. 2018, PMID: 29029324; Hao Wang, et al., Environ Toxicol. 2022, PMID: 34741586; Hao Wang, et al., Toxicol Sci. 2019, PMID: 31271426) and also it is closed to the concentration that

could be found in the real world (Jun-Pyo Myong, et al., PLoS One. 2014, PMID: 25383551; Long-Lian Zhang, et al., Environ Res. 2015, PMID: 25836720; Aikaterini Sakellari, et al., Sci Total Environ. 2016, PMID: 27295597; Luisa Torres-Sánchez, et al., Sci Total Environ. 2018, PMID: 2975842;).

During early corticogenesis, neuroepithelial cells (neural stem cells) initially self-amplify at E10.5. Then, these cells give rise directly to neurons by asymmetric neurogenic divisions, which predominate at E15-16. Thus, E15.5 is the time point that neuron differentiation reached the peak (S C Noctor, et al., Nature. 2001, PMID: 11217860; Denis Jabaudon, Nat Commun. 2017, PMID: 28671189; Siraj K Zahr, et al., Cell Death Differ. 2019, PMID: 31551564). At this time point, we can observe the outcomes of the changes of progenitor cells, and can also get a full view of the alterations of neurons using SnRNA-seq. That is the reason why E15.5 was chosen.

3. It is recommended to merge Fig 1 and Fig 2 and transfer some data in Fig 1 to the supplementary data. Such as Fig 1 D, E and F, these results are useless in the article.

Done accordingly. Fig. 1D revealed the differences between control and Cd-exposed mice. We kept it in the revised data.

4. The sample capacity of each group using single cell RNA-Seq in the article should be emphasized in the “Results section”, although it is described in the “Methods section”.

Done accordingly. See the revised manuscript Page 3, line 93-95.

5. When the author discovered that NPC and OPC changed greatly, why didn't they focus on them for further research? Subsequent analysis of other cell groups, as well as time series analysis of each cluster. This makes the entire study unfocused.

We appreciate the constructive suggestion. That is what we probably will do in our next work. In this study, we aimed to get a global profile of how Cd exposure influences the cell subpopulation distribution of all the cells in the brain. During brain development, the number of each cell subpopulation is strictly designed. Thus, cell subpopulation distribution shifts during brain development might cause significant influences on brain functions. Our data on neural circuit activity and neurobehavior of mice verified that the brain functions were affected due to Cd-induced cell subpopulation shift.

Additionally, neurons and glial cells are derived from NPCs and OPCs during brain development. The changes in neurons and neural circuits might be originated from progenitor cells. The time series analysis was used to demonstrate that the differentiation of NPCs and the maturation processes of differentiated cells were retarded. We have clarified this in the discussion part (Page 7, Line 296-305).

6. How is the data shown in the violin plot in Fig 4 related to the developmental abnormalities of NPC caused by cadmium exposure? Why are these results here? This is puzzling.

The reviewer referred to should be the previous Fig. 3 because there was no violin plot figure in the previous Fig. 4. The previous Fig. 3 (the revised Fig. 2) showed that the NPC subpopulations were significantly shifted in Cd-exposure mice. C4, C6, C9, and C16 were the predominate affected NPC subpopulations. The violin plot in Fig.3 presented the feature genes of these NPC subpopulations. These enriched genes, *Dlx1, Rnd3, Hmgb2, Mki67, Fabp7, Mt3, Tpx2*, and *Pcalf*, all play a critical role in the proliferation or cell subtype differentiation and maturation (John C Silbereis, et al., Neuron. 2014, PMID: 24507192; Emilie Pacary, et al., Nat Commun. 2013, PMID: 23535656; Zhao-Zhe Hao, et al., Nat Neurosci. 2022, PMID: 35637371; Cole J Ferguson, et al., Mol Cell. 2022, PMID: 34942119). Thus, these feature genes were indicators of the functions of these specific cell subpopulations. In that case, the readers could know what functions were influenced by Cd exposure instead of just knowing NPC subpopulations were altered. We have clarified it in the results part (Page 3, line 122-128). Additionally, we added discussion in our revised manuscript to clarify the meaning of this figure (Page7, line 292-295).

7. In the article, the author tried to use RT-qPCR to verify some results of single-cell RNA-seq, and the data were shown as negative. I think this method is inappropriate. The differential genes are in specific cells. Total RNA extraction and RT-qPCR cannot verify these differential genes.

There is a misunderstanding here. We agree with the reviewer that total RNA extraction and RT-qPCR cannot verify the differential genes found in SnRNA-seq. However, the purpose of RT-qPCR here was not to verify these differential genes. What we want to do here is to see whether the influences found by SnRNA-seq would be submerged by bulk analysis. By doing this, we want to highlight the importance and necessity of Single-cell technology in the study of Cd toxicity. We have discussed this in the discussion part (Page 7, line 280-295; Page 8, line 329-333).

8. What is the significance of pseudotime map of the cell clusters? The appearance of this part in the article is puzzling and has no connection with other results in the article.

The pseudotime map is one of the important evidences of the conclusion that Cd exposure hindered the differentiation of progenitor cells and maturation of differentiated cells. From cell subpopulation analysis, we found that Cd exposure increased the number of NPCs and decreased the number of excitatory neurons and interneurons. Because neurons are derived from NPCs, the reduction in the number of neurons might be due to the differentiation of NPCs being impeded. Thus, the results mean that Cd exposure hindered the normal differentiation of progenitor cells. These conclusions were in accordance with the results from the pseudotime map. In the pseudotime map analysis, we found that most of the cell subpopulations are impeded in an immature status. Additionally, these findings were further confirmed by the morphological analysis of neuronal spines in the revised Fig.6. We have discussed this in Page 7, line 301-305.

9. The author believes that maternal cadmium exposure leads to changes in fetal brain cell subpopulations, which may be the key cause of neurobehavior abnormalities in adult offspring mice. In fact, there is insufficient evidence for this.

We agree with the reviewer's opinion that our evidence about this in not enough. We have not yet uncovered some of the detailed mechanisms, especially molecular mechanisms. Here, we have revealed that cell subpopulation changes finally influenced the differentiation and maturation of neurons and the formation of neuronal circuits. The $Ca^{2+}$ transient changes of neuronal circuits in free moving mice are direct mechanisms and evidence of the cause of neurobehavior abnormalities (Page 8, line 341-347). Previous studies about Cd-toxicity have not yet revealed the direct relationship between neurobehavior changes and neuronal circuit activity. Thus, the neurobehavior abnormalities could not be directly attributed to neuronal function impairment. Our study filled the gap of this. This is also an innovation of our study.

10. The authors should provide a visual summary of this study.

Done accordingly. The revised Fig.9 shows the schematic summary of pregnant cadmium-hindered brain development in mice.

11. Please suggest to ask native English speakers and teachers to polish the manuscript.

Done accordingly. The manuscript was edited by AJE.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors of this manuscript have carefully answered my questions and made changes accordingly. Therefore, I feel that this manuscript can be accepted for publication.

Reviewer #2 (Remarks to the Author):

My concerns have been mainly responded. So I suggest to accept this manuscript.