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Original Article

# Methylmercury, $Mn^{2+}$ and $Pb^{2+}$ Exposure Promotes Premature Proliferation/Differentiation of Human Neural Stem Cells in Different Ways

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## ABSTRACT

**Background:** Methylmercury (MeHg), manganese ions ($Mn^{2+}$), and lead ions ($Pb^{2+}$) are ubiquitous environmental pollutants and may be neurotoxic especially during fetal development. We decided to explore the toxic mechanisms of MeHg (organic heavy metals), $Mn^{2+}$ (inorganic heavy metals) and $Mn^{2+}$ on the proliferation and differentiation of human neural stem cells (hNSCs). **Materials and Methods:** The proliferation and apoptosis of hNSCs were analyzed via CCK-8 method and flow cytometry under MeHg, $Mn^{2+}$ and $Pb^{2+}$, respectively. RNA-seq was used for analyzing proliferation/differentiation mechanism of MeHg, $Mn^{2+}$ and $Pb^{2+}$ stressing hNSCs. **Results:** Our experiment found that when hNSCs were exposed to below 0.5 nM MeHg, 5μM $Mn^{2+}$ and 10 μM $Pb^{2+}$, cell proliferation and differentiation were promoted. Apoptosis rates increased significantly when hNSCS were exposed to exceed 0.5 nM MeHg, 5μM $Mn^{2+}$ and 10 μM $Pb^{2+}$. RNA-seq results showed that metal ions altered the genes expression level and signaling pathways of hNSC differentiation and proliferation, but the regulatory mechanisms of MeHg, $Mn^{2+}$ and $Pb^{2+}$ were different. **Conclusion:** Our findings indicated that very low-dose metal exposure may deplete hNSC pool by making prematurely differentiated neurons increase, which may be the real cause of long-term nervous system disruption in adulthood, rather than higher metal doses will cause more direct toxicity during infant development.

**Keywords:** Methylmercury, Manganese ion, Lead ion, Neural stem cells, Cell differentiation.

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## INTRODUCTION

Neurodevelopmental Disabilities (NDD), such as learning disabilities, developmental delay, cerebral palsy, and cognitive dysfunction, are one of the main diseases that seriously affect the quality of the birth population and endanger the physical and mental health of children and adolescents.[1] At present, it is generally believed that genetic factors alone cannot fully explain the occurrence of NDD, and the developmental neurotoxicity (DNT) caused by a large number of industrial chemicals such as pesticides, heavy metals, and organic solvents in the environment is an important cause of the quiet spread of NDDs worldwide.[2,3] In the early stages of these nervous system developments, for example in the embryonic, fetal and neonatal stages, due to imperfect development of the placental barrier and the blood-brain barrier, the activity of metabolic enzymes in the body is low or deficient. At this point, it is very sensitive to toxic chemicals, especially some toxic metal ions such as lead and chromium, and exposure to very low doses can cause permanent irreversible damage.[4,5]

Neural stem cells (NSCs) exist in the nervous system which can self-renew and differentiate into neurons, astrocytes, and oligodendrocytes.[6] A moderate scale of NSCs is essential for the normal development of the nervous system. The abnormal proliferation or development of NSCs can cause severe neurological developmental defects.[7,8] Some factors can interfere with the differentiation and proliferation of nerves by affecting the normal proliferation or differentiation of NSCs, resulting in neurodevelopmental toxicity expressed by severe NDDs.[9] 0.4-0.9 μM $Pb^{2+}$ exposure changed the DNA methylation level in hNSCs, which changed the signal pathway of neural development and affected the differentiation of neurons.[10] Recent studies have confirmed that exposure of neural precursor cells to $Pb^{2+}$ can cause intracellular transcriptome disorders and abnormal RNA splicing, which can lead to neurological diseases.[11] Human umbilical cord blood derived NSCs (HUCB-NSC) in the early stage of differentiation were very sensitive to methylmercury chloride (MeHgCl), and their potential to differentiate into

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astrocytes was inhibited.[12] $Mn^{2+}$ can also affect the formation of nerves and become a potentially toxic metal ion.[13] These data all suggested that no matter how environmental chemicals enter the body or metabolize them, they may eventually interfere with the process of neural induction or generation by affecting the normal proliferation or differentiation of neural stem cells, resulting in neurodevelopmental toxicity.

However, the current research on the neurodevelopmental toxicity of toxic metal ions mainly focuses on offspring behavior, cell survival, apoptosis and other phenotypic effect indicators, and there are few systematic studies on the process of neural induction or generation.

Therefore, this project intends to select MeHg (organic heavy metals), $Pb^{2+}$ (inorganic heavy metals) and $Mn^{2+}$ that may have neurodevelopmental toxicity as the research objects, and explore their respective toxic mechanisms for the proliferation and differentiation of neural stem cells.

## MATERIALS AND METHODS

### Cell culture

Human NSCs were provided by Dr. Dan Lou (Shanghai Municipal Center for Disease Control and Prevention). Human NSCs were cultured in ReNcell NSC Maintenance Medium (SCM005) in a humidified atmosphere of 5% $CO_2$ at 37°C.

### Effects of $Pb^{2+}$, $Mn^{2+}$ and methylmercury (MeHg) on NSC morphology and proliferation

Human neural stem cells were inoculated into a 6-well cell culture plate at $1 \times 10^5$ cells/ml and cultured to 80% healing. Previous study have proved that low-dose MeHg has an adverse effect on rat neurogenesis,[14] so we set up 0.25nM, 0.5nM, 1nM and 2nM and a control group (0nm) to explore human neural stem cells *in vitro*. The amount of $Pb^{2+}$ is based on Chan's results,[15] that the $Pb^{2+}$ concentration of 50μM significantly inhibited the growth of adult rat hippocampal neural stem cells and had no effect on differentiation, but 10μM $Pb^{2+}$ had no significant influence on the proliferation and differentiation of adult rat hippocampal neural stem cells. Because of the differences in the cells used, based on this result, we determined to set the low-concentration group 5μM, 10μM and the high-concentration group 20μM, 40μM to compare and observe with the control group (0μM). The condition of $Mn^{2+}$ addition also was based on previous study,[16] neural stem cells were still dead under the condition of 50μM, so our initial experimental concentration was 20μM, but the cells still died quickly under this condition, so we finally determined to add 2.5μM, 5μM, 10μM and 20μM respectively compared with the control group.

Each concentration of the experimental group was set up in triplicate. After 12, 24, 48, 72, and 96 hr of exposure, the cell morphology was observed under a microscope, and the cell proliferation was measured by the Cell Counting Kit-8 (CCK-8) assay.

### Apoptosis assay

After 48 hr post-treatment with MeHg (0.25nM, 0.5nM, 1nM and 2nM), $Pb^{2+}$ (5μM, 10μM, 5μM and 10μM) and $Mn^{2+}$ (2.5μM, 5μM, 10μM and 20μM), respectively, the hNSC culture medium was removed. Pre-cooled PBS was used to wash the hNSCs for three times and 0.25% Trypsin-EDTA solution was used for digestion and collection of cells. Cells were centrifuged in the 15mL centrifuge tube and removed the supernatant. And then cells were resuspended in 1× binding buffer at a density of $1\times10^6$ cells/mL. Then, Annexin V-PE and 7-AAD (Keygen, KGA1015) were used to stain cells for 15 min at room temperature in the dark. Cells were analyzed for apoptosis using CytoFlex flow cytometer and software.

### QuantSeq 3′ mRNA sequencing and data analysis

Total RNAs were isolated from samples of different groups using PureLink RNA Mini Kit (Invitrogen) and submitted the isolated RNA to Beyotime Biotechnology. The subsequent QuantSeq 3' mRNA sequencing and data analysis was performed according to Lee *et al.*[17,18]

### Statistical Analyses

All experiments were performed independently at least three times. Data were expressed as the mean of three repeats ±Standard Deviation (SD). Data from NSC proliferation, GO analysis and KEGG pathway analysis were analyzed using one-way analysis of variance, followed by a student test. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed with the GraphPad Prism 6.0 program (www.graphpad.com).

## RESULTS

### MeHg, $Mn^{2+}$ and $Pb^{2+}$ affected proliferation and morphology of hNSCs

MeHg, $Mn^{2+}$ and $Pb^{2+}$ have been reported to affect proliferation or apoptosis of cells. We found that hNSC proliferation was increased when MeHg concentration increased from 0 to 0.5 nM. However, when the MeHg concentration exceeded 5 μM, NSC proliferation decreased. The proliferation of NSCs was found to increase with increasing concentration of $Pb^{2+}$ from 0 to 10 μM. However, when the concentration of $Pb^{2+}$ was 10 μM or more, the proliferation of NSCs decreased. In the phenomenon of $Mn^{2+}$, we discovered that though the proliferation of NSCs increased as the concentration of $Mn^{2+}$ increases from 0 to 5 μM, and when the concentration of $Mn^{2+}$ was above 5 μM, the proliferation of NSCs decreased, but when the concentration of $Mn^{2+}$ reached 20 μM, NSCs will rapidly apoptotic, which was a phenomenon that we have not observed in the first two (Figure 1). In addition,

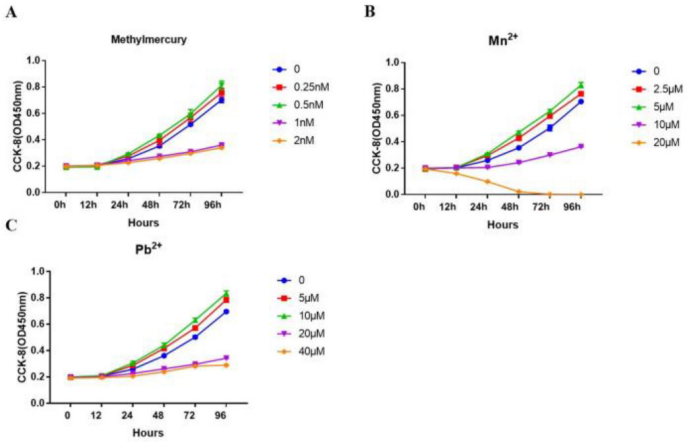


**Figure 1:** Effects of mental on NSC proliferation.
A: Changes in the proliferation number of neural stem cells in the 96-hr methylmercury exposure group; B: Changes in the proliferation number of neural stem cells in the 96-hr $Mn^{2+}$ exposure group; C: Changes in the proliferation number of neural stem cells in the 96-hr $Pb^{2+}$exposure group.

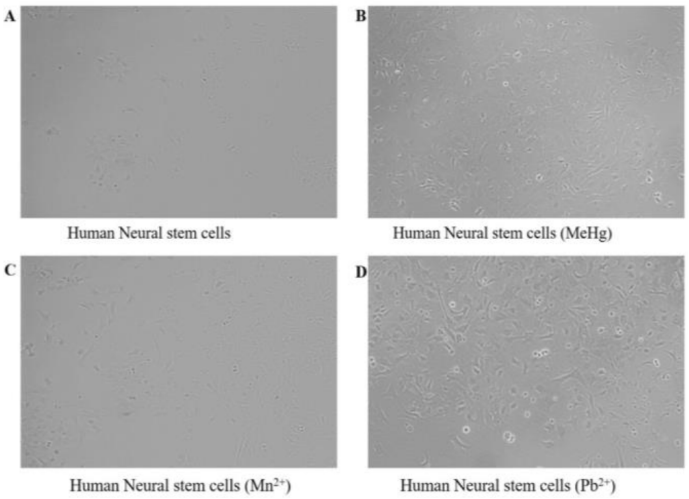


**Figure 2:** Morphology and differentiation of NSCs.
A: The untreated control group. B: 0.5 nM methylmercury treatment group. C: 5 μM $Mn^{2+}$ treatment group D: 10 μM $Pb^{2+}$ treatment group.

we also found that these three substances had no effect on the morphology of neural stem cells. (Figure 2).

## Effect of MeHg, $Mn^{2+}$ and $Pb^{2+}$ on hNSCs apoptosis

Flow cytometry was conducted to analyze the apoptosis rate of hNSCs after treatment with MeHg, $Mn^{2+}$ and $Pb^{2+}$ for 24 hr, respectively. As shown in Figure 3B, when the MeHg concentration exceeded 1 nM, apoptosis if hNSCs increased. There was a significantly increased hNSC apoptosis when the concentration of $Mn^{2+}$ was above 5 μM (Figure 3C) and concentration of $Pb^{2+}$ was 10 μM or more (Figure 3D).

## Differentially expressed genes under MeHg, $Mn^{2+}$ and $Pb^{2+}$ stress

Comparing control groups with MeHg-treated NSCs, there were 35,797 differential genes, of which 16,877 were up-regulated and

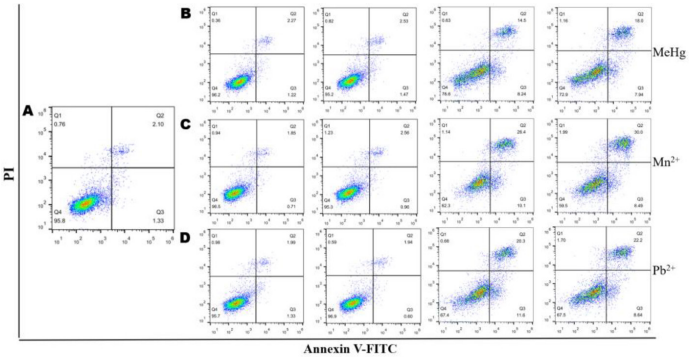


**Figure 3:** Apoptosis analysis of hNSCs.
A: The untreated control group; B: 0.25 nM, 0.5 nM, 1 nM and 2 nM MeHg treatment group; C: 2.5 μM, 5 μM, 10 μM and 20 μM $Mn^{2+}$ treatment group; D: 5 μM, 10 μM, 20 μM and 40 μM $Pb^{2+}$ treatment group.

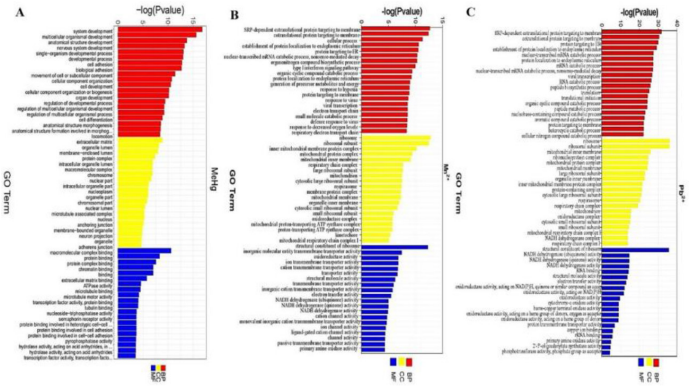


**Figure 4:** GO analysis of the differentially expressed genes.
A: 0.5 nM methylmercury enhances cell development and cell differentiation. B: 5 μM $Mn^{2+}$ enhances cellular process. C: 10 μM $Pb^{2+}$ enhances translation.

18,919 were down-regulated. Comparing NSCs treated with $Mn^{2+}$ with the NSCs of the control group, there were a total of 194437 differential genes, of which up-regulated 78,835, down-regulated 155602. $Pb^{2+}$ treated neural stem cells compared with the control group, a total of 71,250 differential genes, of which 19,518 were up-regulated and 51,732 were down-regulated.

## GO analysis

To classify and characterize the DEG functions and pathways, we performed a gene ontology (GO) classification and a functional annotation of molecular biological functions (MFs), cellular components (CCs), and biological processes (BPs) (Figure 4). Compared with the control group, the gene expression of the 1731 group of neural stem cells in the methylmercury group was significantly changed ($p<0.05$). Among them, 217 groups belonged to MF, 194 groups belonged to CC, and the remaining 1322 groups belonged to BP. A total of 12010 groups of genes in the $Mn^{2+}$ group changed significantly. Among them, 2074 groups belonged to MF, 1070 groups belonged to CC, and the remaining 8866 groups belonged to BP. In the $Pb^{2+}$ group, there were a total of 7274 groups of gene expression significantly changed, of which

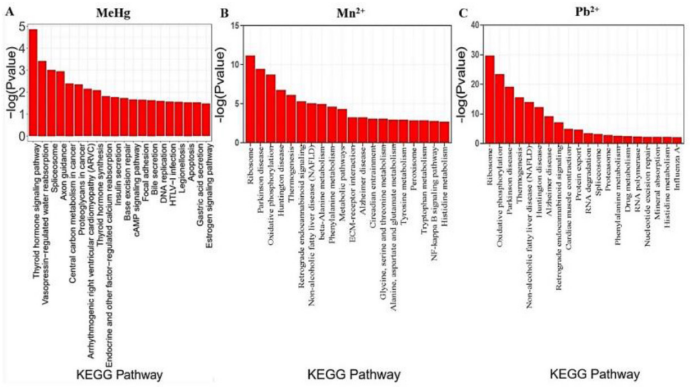


**Figure 5:** KEGG pathway analysis.
0.5 nM methylmercury activates thyroid hormone signaling pathway and endocrine and other factor–regulated calcium reabsorption. B: 5 μM $Mn^{2+}$ activates NF-kappaB signaling pathway. C: 10 μM $Pb^{2+}$ activates ribosome.

1144 groups belonged to MF, 730 groups belonged to CC, and the remaining 5400 groups belonged to BP.

### Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

To visualize the effects of MeHg, $Mn^{2+}$, and $Pb^{2+}$ on gene expression changes associated with specific cell signaling pathways, and to screen for potentially involved biological pathways, we performed KEGG pathway analysis. The results (Figure 5), including the significantly enriched KEGG signaling pathway ($p$<=0.05), the differential genes in the MeHg group are mainly involved in 29 types of metabolic pathways, the differential genes in the $Mn^{2+}$ group are mainly involved in 46 types of metabolic pathways, and the differential genes in the $Pb^{2+}$ group are mainly involved in 22 types of metabolic pathways.

## DISCUSSION

Studies have shown that methylmercury exposure may be a potential risk factor for mental illness and neurodegenerative diseases.[14] In previous studies, it was found that human umbilical cord blood derived neural stem cells (Human umbilical cord blood derived neural stem cells, HUCB-NSC) in the early stages of differentiation were very sensitive to MeHgCl, and the potential of cell differentiation is inhibited.[12] In this study, we found that when the MeHg concentration increased from 0 to 0.5 nM, the proliferation of neural stem cells increased. However, when the MeHg concentration exceeded 0.5 nM, we found that the proliferation rate of neural stem cells decreased, and adding 0.5 nM MeHg did not affected the morphology of nerve cells. The previous study found that the 0.5 nM MeHg group influenced cell differentiation and development. Exposure to 0.25 nM MeHg significantly increased the percentage of newborn neurons produced by mouse E12 cortical precursors labeled with βIII tubulin, that was, very low doses of MeHg can enhance premature neuronal differentiation,[19] the above observations were consistent with our conclusion that methylmercury can promote cell differentiation and development. Our findings of increased premature differentiation of neural stem cells at a dose of 0.5 nM MeHg may be the result of increased production of thyroid hormones (such as thyroid hormone $T_3$) due to activation of the thyroid hormone signaling pathway. Although it was not clear whether MeHg can increase the activity of thyroid hormones, it has been shown that thyroid hormones are the main physiological regulators of mammalian brain development,[20] and there were literatures that T3 promotes osteogenic differentiation at least partly through the activation of AMPK/p38 signaling pathway.[21] T3 *in vivo* treatment of animals has proved its key role in the participation of progenitor cells in lineage-specific differentiation,[22] MeHg exposure can affect the thyroid hormone signaling pathway in CGNs.[23] Therefore, the activation of the thyroid hormone signaling pathway may be a biological response induced by very low doses of MeHg. Premature proliferation of nerves can lead to depletion of the neural stem cell pool, resulting in reduced adult neurogenesis and impaired nerve function. Under the condition of 0.5 nM MeHg exposure, we speculated that the calcium reabsorption regulated by endocrine and other factors increased, the $Ca^{2+}$ channel was reported to be a MeHg target because it was located on the plasma membrane and was tightly regulated for various intracellular mediators.[24] This review by Orrenius S[25] stated that $Ca^{2+}$ plays a key role in central nervous system cell apoptosis, and It has been shown that $Ca^{2+}$ overload can cause necrosis, apoptotic or cell death. After exposure to MeHg, continuously increasing levels of $Ca^{2+}$ were found in various cells, and it has been reported that $Ca^{2+}$ channel blockers have protective effects *in vivo* and *in vitro*.[24,26,27] Importantly, low molar concentration of MeHg will cause a continuous increase in the intracellular cytoplasmic $Ca^{2+}$ concentration,[26,28] the above results are consistent with our speculation that MeHg can make intracellular $Ca^{2+}$ levels. In addition, $Ca^{2+}$ can be absorbed by mitochondria, thereby stimulating the production of ROS.[29] And there were literatures that the generation of ROS may be a mechanism that triggers the increase of $Ca^{2+}$ in MeHg neurotoxicity.[30] Therefore, this study can provide evidence for the other research that increased $Ca^{2+}$ may be one of the factors that MeHg causes neurological deficits.

There were documents showing that $Mn^{2+}$ interferes with cell copper regulation in the brain subventricular zone (SVZ) and rostral migratory stream (RMS), causing significant damage to adult neurogenesis.[13] In this study, we found some phenomena that when the $Mn^{2+}$ concentration increased to 5 μM, the proliferation number of neural stem cells will increased, but when the $Mn^{2+}$ concentration exceeded 5 μM, the proliferation number of neural stem cells will decrease. 5 μM $Mn^{2+}$ did not change the morphology of nerve cells. The previous study found that the 5 μM $Mn^{2+}$ group had an effect on the NF-κB signaling pathway in neural stem cells. Excessive $Mn^{2+}$ can cause the expression of NF-κB and TNF-α, which were related to brain inflammation in chickens *in vivo* and *in vitro* ($p$ <0.05), and cause changes and

destruction of brain tissue morphology and structure,[31] when exposed to $Mn^{2+}$ during larval development, $Mn^{2+}$ enhanced the inflammatory response in astrocytes through NF-κB signaling,[32] and there were studies showing that astrocytes becomed the key mediator of $Mn^{2+}$ neurotoxicity by enhancing the expression of inflammatory cytokines and chemokines,[33] therefore, extremely low doses of $Mn^{2+}$ induced biological reactions may activate the NF-κB signaling pathway. NF-κB was a key regulator of NSC proliferation, and the activation of the standardized NF-κB pathway can greatly increase the proliferation of NSC,[34] This may be one of the reasons why the 5μM $Mn^{2+}$ group can enhance cell proliferation and differentiation, and from this it is inferred that the activation of the NF-κB signaling pathway may be related to $Mn^{2+}$ exposure that can damage nerve function.

$Pb^{2+}$ can damage neurons and cause abnormal RNA splicing, which can lead to neurological diseases.[11] In this study, we found some phenomena, that was, when the $Pb^{2+}$ concentration increases to 10 μM, the proliferation of neural stem cells increases, but when the $Pb^{2+}$ concentration exceeds 10 μM, the proliferation of neural stem cells will decrease, and the addition of 10 μM $Pb^{2+}$ did not change the morphology of the nerve stem cells. 10 μM $Pb^{2+}$ exposure was strongly associated with Parkinson's and Alzheimer's disease ($p<0.05$), studies also showed that $Pb^{2+}$ may be involved in the pathogenesis of certain forms of Parkinson's disease in some way,[35] and $Pb^{2+}$ has a variety of effects on the release of dopamine from synaptic bodies,[36] the observation results mentioned above are consistent with our speculation that $Pb^{2+}$ was related to Parkinson's disease and Alzheimer's disease. Interestingly, the differential genes induced by 10 μM were significantly enriched in the ribosomal pathway. As we all know, ribosomes play an important role in intracellular protein translation, and more and more documents prove that protein translation plays an important role in the pathogenesis of Parkinson's disease (PD),[37] and studies have shown that ribosomal proteins (RPs) can also play an important role in ribosomal functions *in vitro*, while the function of ribosomal proteins is not related to protein biosynthesis, not just DNA replication, transcription, cell growth and proliferation, cancer, and aging development regulation.[38,39] Therefore, we suspected that one of the reasons for $Pb^{2+}$ to cause neurodegenerative diseases such as Parkinson's disease may be the accumulation of disease-related proteins (such as α-synuclein (SNCA), β-amyloid, etc.) due to excessive activation of the ribosomal pathway.

## CONCLUSION

In short, we believed that metal exposure in daily life was indeed closely related to NDD. Because we have found through analysis that the exposure of MeHg, $Mn^{2+}$, and $Pb^{2+}$ in the experiment has an effect on the proliferation and morphological changes of neural stem cells, and found that the expression of genes related to the proliferation and differentiation of neural stem cells has a significant impact, even though they produce this the way of influence was different. Therefore, based on the obtained data, we have speculated on the mechanism that caused the abnormal proliferation and differentiation of neural stem cells, which can provide ideas for further research in the future.

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We thanks Dr. Dan Lou (Shanghai Municipal Center for Disease Control & Prevention) for providing Human NSCs (ReNcell CX Immortalized cells SCC007, Millipore).

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**NDD:** Neurodevelopmental Disabilities; **DNT:** Developmental neurotoxicity; **NSC:** Neural stem cell; **HUCB-NSC:** Human umbilical cord blood derived neural stem cells; **MeHgCl:** Methylmercury chloride**; MeHg:** Methylmercury; **CCK-8:** Cell Counting Kit-8; **SD:** Standard deviation; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **GO:** Gene ontology; **MFs:** Molecular biological functions; **CCs:** Cellular components; **BPs:** Biological processes; **SVZ:** Subventricular zone; **RMS:** Rostral migratory stream; **PD:** Parkinson's disease; **RPs:** Ribosomal proteins; **SNCA:** α-synuclein.

## SUMMARY

In summary, we have observed experimentally that very small amounts of MeHg, $Mn^{2+}$, and $Pb^{2+}$ exposures influence the proliferation and morphological changes of neural stem cells, and through data analysis, we found that even if the three interfering factors have different ways of affecting neural stem cells, they all have a significant effect on the expression of genes associated with neural stem cell proliferation and differentiation. Therefore, based on the data obtained, we speculate on the mechanisms associated with their leading to abnormal proliferation and differentiation of neural stem cells.

### Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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