The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Toxicomics Report

Alteration of gene levels in fetal brain by prenatal exposure to methylmercury, copper, and their combination

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(Received May 22, 2024; Accepted May 24, 2024)

ABSTRACT — Methylmercury (MeHg), a potent neurotoxin, poses substantial risks to prenatal brain development by crossing the placental barrier. In our daily lives, we are exposed to various environmental metals simultaneously with MeHg. Therefore, the combined exposure effects of these metals and MeHg should be investigated. Hence, this study examined the combined fetal exposure effects of MeHg and copper (Cu), an essential element. Gene expression changes in the fetal brains of mice exposed to MeHg, Cu, or both were examined through RNA-seq analysis. Our results showed that the number of variable genes exposed to combined MeHg and Cu increased compared with that in single exposure. Most of them were gene variations specific to combined exposure. Gene Ontology biological process analysis revealed the amplified effects on GABAergic interneurons in the cerebral cortex under combined exposure. IPA pathway analysis indicated considerable variations in pathways related to oxidative stress, neuronal development, and energy metabolism, including the activation of NRF2-mediated oxidative stress response and the suppression of mitochondrial fatty acid beta-oxidation. These findings highlighted the complexity and enhanced risks of combined MeHg and Cu exposure. Therefore, neurodevelopmental effects were more severe and multifaceted than those caused by individual exposures. This research highlighted the importance of understanding the mechanisms of the combined exposure effects of MeHg.

Key words: Methylmercury, Combined exposure, Exposome, Prenatal exposure, RNA-sequencing analysis

INTRODUCTION

Methylmercury (MeHg), a potent neurotoxin found in contaminated seafood, can cross the placental barrier (Kajiwara *et al*., 1996). Therefore, prenatal exposure to MeHg poses a substantial risk to prenatal development (Rice and Barone, 2000). Humans were exposed to other toxic metals, pesticides, persistent organic pollutants,

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and other chemicals through diet, air, and water (Shetty *et al*., 2023). These mutual exposures may interact with MeHg, thereby enhancing or weakening its toxic effects. Therefore, the combined effects of MeHg and other environmental exposures should be explored.

We have previously reported that exposure to MeHg, along with other metals such as copper (Cu), can increase the adverse effects of MeHg, especially by consuming important protective nucleophiles such as supersulfide in cells; consequently, it leads to more damage to cell proteins and increased mercury concentrations in the brain. Furthermore, when pregnant mice are exposed to MeHg and Cu, mercury levels found in the offspring increase (Akiyama *et al*., 2022). This finding suggests that this combined exposure can be even more dangerous during pregnancy.

RNA-sequencing (RNA-seq) analysis and DNA microarray are widely used for transcriptomic profiling to identify genes that are differentially expressed in response to chemical exposure (Shockley and Dunnick, 2022; Mellingen *et al*., 2022; Shinoda *et al*., 2019; Wang *et al*., 2023; Hwang and Naganuma, 2006). By comparing exposed and unexposed samples, researchers can pinpoint specific genes and gain insights into affected molecular pathways and biological processes. Pathway and network analysis of these genes reveals disrupted biological pathways, providing a holistic view of impacted cellular processes like metabolism, DNA repair, and stress response. Thus, RNA-seq is a valuable and versatile tool for investigating the biological effects of chemical exposure, offering detailed insights into the molecular interactions between chemicals and biological systems.

In the present study, changes in gene expression in the brains of next-generation mice were examined through RNA-seq analysis to investigate the effects of combined Cu exposure on fetal brain during exposure to MeHg.

MATERIAL AND METHODS

Materials

Methylmercury chloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Copper (II) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents and chemicals were of the highest grades available.

Animals and treatment

Pregnant mice were purchased from CLEA Japan, Inc. and used after a certain period of rearing in a breeding room. The rearing room was air-conditioned and maintained at a room temperature of 24 ± 1°C and a humidity of 55 ± 5%, with a light period of 14 hr and a dark period of 10 hr. All animals were fed a solid diet as a sample. All animals were fed ad libitum with solid feed as a sample and 5 μm filtered tap water as drinking water. Each animal experiment was conducted according to the animal experiment handling regulations of the University of Tsukuba. Pregnant mice on day 15 of gestation were orally exposed to MeHg (5 mg/kg) and Cu (30 mg/kg) alternately every 12 hr for a total of three doses. These mice were dissected under anesthesia to collect tissue, as well as fetuses, 72 hr after the first MeHg dose. For RNA-sequencing, post-exposure fetuses were dissected, and the extracted brains were stored at -80°C.

RNA-sequencing Analysis

RNA sequence analysis was performed by Department of Sports Medicine Analysis (Organization for Open Facility Initiatives) in University of Tsukuba. For primary analysis, the obtained FASTQ files were trimmed the adapter checked the quality using Trim Galore, and subsequently were mapped to murine reference genomes using HITSAT2 (Kim *et al*., 2019). Gene expressions were quantified using StringTie (Shumate *et al*., 2022) after converting the alignment results to BAM files using SAM tools. For secondary analysis, such as differential gene expressions, Gene Ontology (GO) pathway analysis was performed using iDEP (Ge *et al*., 2018). After ID conversion and the default filter (1.5 counts per million in at least 4 sample). Differentially expressed genes were analyzed with DESeq2 package using a threshold of false discovery rate (FDR) < 0.1 or 0.05 and fold-change > 1.1 or 1.5. The canonical pathway analysis was conducted on the gene list with $p < 0.05$ and fold-change > 1.1 using the Ingenuity Pathway Analysis software (Qiagen, CA, USA).

Statistical Analysis

Statistical analysis was performed on iDEP. Data were visualized using the Python (version 3.8.3), matplotlib (version 3.5.0), UpSetPlot (version 0.9.0), and seaborn (version 0.11.2) packages and GraphPad Prism software (version 10.0.3, GraphPad Software Inc.).

RESULTS

Comparisons of differential gene expression

RNA sequencing was performed to investigate the effects of prenatal MeHg or Cu alone and their combined exposure on gene expression in the fetal brain. A Volcano plot was created with all the differentially expressed genes (Fig. 1A-C). With the DESeq2 package, we iden-

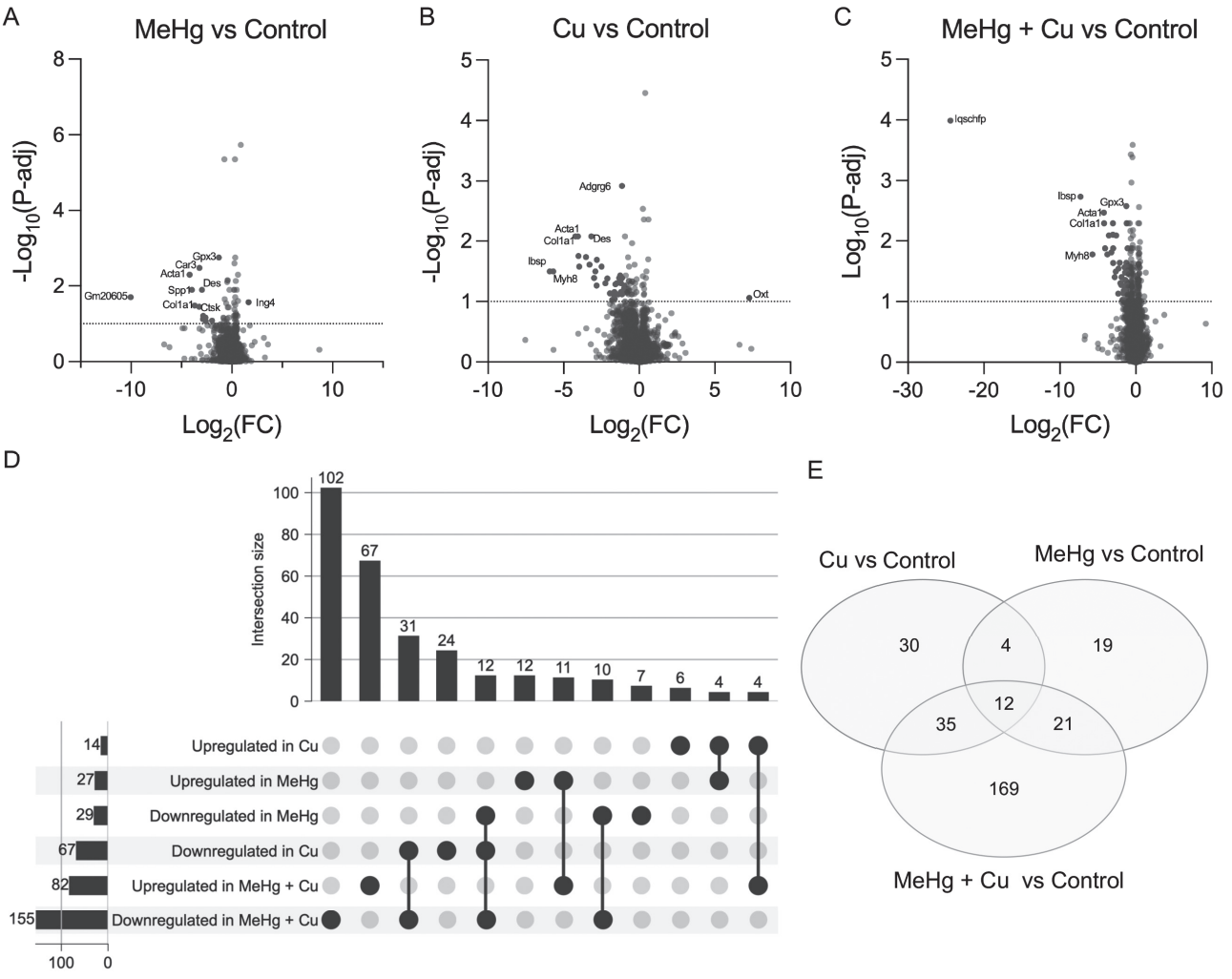

Fig. 1. Fetal brain genes altered by prenatal MeHg or Cu alone and their combined exposure in mice. (A-C) Volcano plot with DEGs. The plots, which are colored red and annotated with gene names are significantly up/downregulated and changed more than Log2 (FC) ≥ 1. The dashed line shows the Log10 (FDR) = 1.0. (D) The set plot shows the intersection of significantly changed genes among the genes. (E) Venn diagram of differentially expressed genes.

tified differentially expressed genes. The altered genes with high physiological significance (fold change ≥ 1; dashed line shows the log10 [FDR] = 1.3) are listed in Table 1-3. Single MeHg exposure significantly downregulated the expression of 29 genes, such as *Gm20605*, *Fap*, *Ctsk*, *Gpx3*, and *Col1a1*, and upregulated the gene expression of 27, such as *Ing4* compared with those of the control group (Table 1). Single Cu exposure significantly downregulated the expression of 67 genes, such as *Col5a1*, *Col3a1*, *Gxylt2*, *Col6a1*, and *Adgrg6*, and upregulated the expression of 14 genes, such as *Oxt* compared with those in the control group (Table 2). Additionally, MeHg and Cu co-exposure strongly affected the gene expression in the brain; specifically, the expression of 155 genes, such as *Iqschfp*, *Pcdhgb2*, *Mmp9*, *Anxa1*, and *Agtr2*, was downregulated, and the expression of 82 genes, specifically *Dio3*, were upregulated (Table 3). Furthermore, 12 genes were shared among the single MeHg exposure, single Cu exposure, and combined MeHg + Cu exposure. In addition, 35 genes for single Cu exposure and 21 genes for single MeHg exposure were each shared in the combined MeHg and Cu exposure. Of the 237 genes that varied in the combined MeHg and Cu exposure, 169 were specific to combined exposure

(Fig. 1D and E).

Functional enrichment analysis

A gene set enrichment analysis was performed on the basis of Gene Ontology (GO) term analysis with iDEP to determine the molecular functions of significantly regulated genes. The GO biological process terms enriched in DEGs are shown in Table 4-6. Although the three groups shared a common regulation of GABAergic neuron development, the altered processes involved in the regulation of interneuron migration, glutamate release, and grooming behavior were only observed in the combined exposure group. The distance among the terms was measured on the basis of the percentage of overlapped genes and used to construct a hierarchical clustering tree (Fig. 2A-C). The plots showed that single exposure-altered genes were associated with one to three related themes: cerebral cortex GABAergic interneuron development, migration, and differentiation (Fig. 2A and B). Combined exposure-altered genes were linked to six related themes: cerebral cortex GABAergic interneuron development, migration, differentiation, and interneuron migration from the subpallium to the cortex, interneuron migration, and cerebral cortex neuron differentiation (Fig. 2C). Ingenuity pathway analysis (IPA) was also used to examine the effect of changes in pathway activation or inhibition, which was presented as a positive or negative Z-score, respectively. The pathways with the highest change in activation or inhibition in the combined exposure group are shown in Fig. 2D and E. The activated canonical pathways included the SUMOylation of chromatin organization proteins, endocannabinoid developing neuron pathway, NRF2-mediated oxidative stress response, and GDNF family ligand–receptor interactions (Fig. 2D). The inhibited canonical pathways included intra-Golgi and retrograde Golgi-to-ER traffic, sphingolipid metabolism, fatty acid β-oxidation I, glycogen metabolism, syndecan interactions, interleukin-6 family signaling, mitochondrial fatty acid beta-oxidation, and

Table 1. The differential gene expressions in MeHg vs Control.

Gene symbol	Log2 (FC)	-Log10 (FDR)
Gm20605	-10.0347	1.704189
Acta1	-4.17622	2.29698
Spp1	-3.97309	1.895628
Col1a1	-3.69229	1.480387
Ctsk	-3.23011	1.452859
Car3	-3.20369	2.476857
Des	-2.96871	1.895628
Col1a2	-2.87487	1.121253
Tpm2	-2.84065	1.213132
Fap	-2.6142	1.161249
Ttn	-2.58318	1.068783
Sgms2	-1.95755	1.084549
Gpx3	-1.28544	2.748082
Ing4	1.660706	1.569853

The significantly changed (Log_2 (FC) ≥1) genes were listed.

Table 2. The differential gene expressions in Cu vs Control.

Gene symbol	Log2 (FC)	-Log10 (FDR)	Gene symbol	Log2 (FC)	-Log10 (FDR)
Ibsp	-5.93273	1.497852	*H19*	-1.73521	1.001377
Myh8	-5.68553	1.497852	*Ldb3*	-1.68948	1.119405
Col1a1	-4.2229	2.077592	*Col5a1*	-1.64773	1.154434
Acta1	-4.0646	2.077592	*Col3a1*	-1.63293	1.042198
Spp1	-4.01868	1.752883	*Duxbl2*	-1.57946	1.285383
Aspn	-3.98255	1.575136	*Duxbl3*	-1.49769	1.30286
Ctsk	-3.51831	1.736279	*Alpl*	-1.38061	1.119405
Col1a2	-3.28376	1.608799	*Pygm*	-1.31782	1.3876
Des	-3.17705	2.077592	*Col6a2*	-1.26524	1.429281
Dnm3os	-2.99368	1.3876	*Gxylt2*	-1.2147	1.30286
Ttn	-2.90542	1.497852	*Slc13a5*	-1.20074	1.154434
Tpm2	-2.81855	1.263027	*Col6a1*	-1.16772	1.381274
Car3	-2.81551	1.688416	*Pgm5*	-1.13772	1.119405
Nrk	-2.49431	1.575136	*Prss35*	-1.13598	1.3876
Pgam2	-2.23357	1.30286	*Adgrg6*	-1.13459	2.91902
Sgms2	-2.10559	1.381274	*Adamtsl3*	-1.01787	1.154434
Ccn4	-1.94463	1.136949	*Ahnak*	-1.00493	1.210334
Msx2	-1.79688	1.026175	*Oxt*	7.278647	1.059649

The significantly changed (Log_2 (FC) ≥1) gene expression was listed.

Table 3. The differential gene expressions in HeMg + Cu vs Control.

Gene symbol	Log2 (FC)	-Log10 (FDR)	Gene symbol	Log2 (FC)	-Log10 (FDR)
Iqschfp	-24.4223	3.991644	*Syne4*	-1.37594	1.357806
Ibsp	-7.28445	2.734674	*Ell2*	-1.10866	1.317441
Gpx3	-1.23608	2.583303	*Mmp9*	-2.01436	1.305319
Acta1	-4.22152	2.474251	*Pygm*	-1.17902	1.304818
Col1a1	-4.17648	2.288426	*Myl4*	-2.01023	1.300925
Car3	-3.03175	2.288426	*Gm42890*	-1.91474	1.273242
Pdlim3	-1.2062	2.288426	*Nfatc1*	-1.07546	1.262004
Des	-3.01665	2.100205	*Col24a1*	-1.52476	1.254131
Ctsk	-3.54491	2.089003	*Col11a2*	-1.6156	1.177815
Pgam2	-2.59831	2.089003	*Wif1*	-1.57699	1.177815
Aspn	-4.03606	1.878572	*Cd44*	-1.27188	1.177815
Ttn	-2.99214	1.878572	*Pcdhgb2*	-2.32623	1.131169
Stbd1	-1.09447	1.878572	*Ldb3*	-1.58749	1.131169
Col1a2	-3.22453	1.811479	*Anxa1*	-1.29944	1.126086
Spp1	-3.74691	1.778725	*Pgm5*	-1.0608	1.111086
Myh8	-5.70592	1.778725	*Slc13a5*	-1.09752	1.110534
Col6a2	-1.25455	1.639543	*Gemin8*	1.128838	1.086632
Sgms2	-2.13311	1.639543	*Col14a1*	-1.77175	1.027558
Tpm2	-2.96985	1.636064	*Twist1*	-1.36389	1.026806
Alpl	-1.49572	1.566528	*Col5a1*	-1.46871	1.025071
Hapln1	-1.12368	1.566528	*Ccn4*	-1.73832	1.025071
Nrk	-2.28944	1.543365	*Rcn3*	-1.1541	1.021775
Tnxb	-1.25901	1.543365	*Cd109*	-1.459	1.021775
Fap	-2.65009	1.493478	*Agtr2*	-1.28826	1.016336
Prss35	-1.08385	1.480706	*Ttpa*	-1.03503	1.01145
Lgals1	-1.40809	1.476515	*Copz2*	-1.03483	1.007914
Gm26541	1.310103	1.476515	*H19*	-1.57897	1.002973
Cd59a	-1.00215	1.462396	*Tmem119*	-1.57706	1.002973
Capn6	-1.88585	1.402925	*Dio3*	1.081582	1.002973
Dnm3os	-2.7677	1.402925			

The significantly changed (Log_2 (FC) ≥1) gene expression was listed.

reactive oxygen species (ROS) detoxification (Fig. 2E).

DISCUSSION

This study evaluated the co-exposure of MeHg and Cu in the fetal brain based on changes in gene expression. The number of the altered genes was increased to 238 in the combined exposure group compared with 56 in the MeHg-alone group and 81 in the Cu-alone group. This finding suggested that the effect of exposure was enhanced by the combined exposure compared with that of each single exposure. Furthermore, 102 of the genes that varied with combined exposure were specific to the combined exposure (Fig. 1D and E). These results indicated that the effect of combined exposure was not a simple additive effect of exposure to MeHg alone and Cu alone. Notably, markedly downregulated by combined exposure, Iqschfp (Iqcj and Schip1 fusion protein) participates in neurological development, particularly in the axon guidance and maintenance of neuronal polarity (Elsaid *et al*., 2018; Klingler *et al*., 2015).

The GO biological process term-enriched analysis showed common effects on cerebral cortex GABAergic interneuron in each exposure group. Further clustering analysis revealed that the number of themes associated with GABAergic interneurons ranged from 1 to 3 for single exposures and 6 for combined exposures. These results suggested that the effects on GABAergic interneurons were magnified by combined exposure. The cerebral cortex is the primary effect site in mice exposed to methylmercury. Further, MeHg inhibits glutamate decarboxylase and GABA-transaminase activities; as a result, GABAergic signaling is impaired (Basu *et al*., 2010). We further examined the effect on canonical pathways via

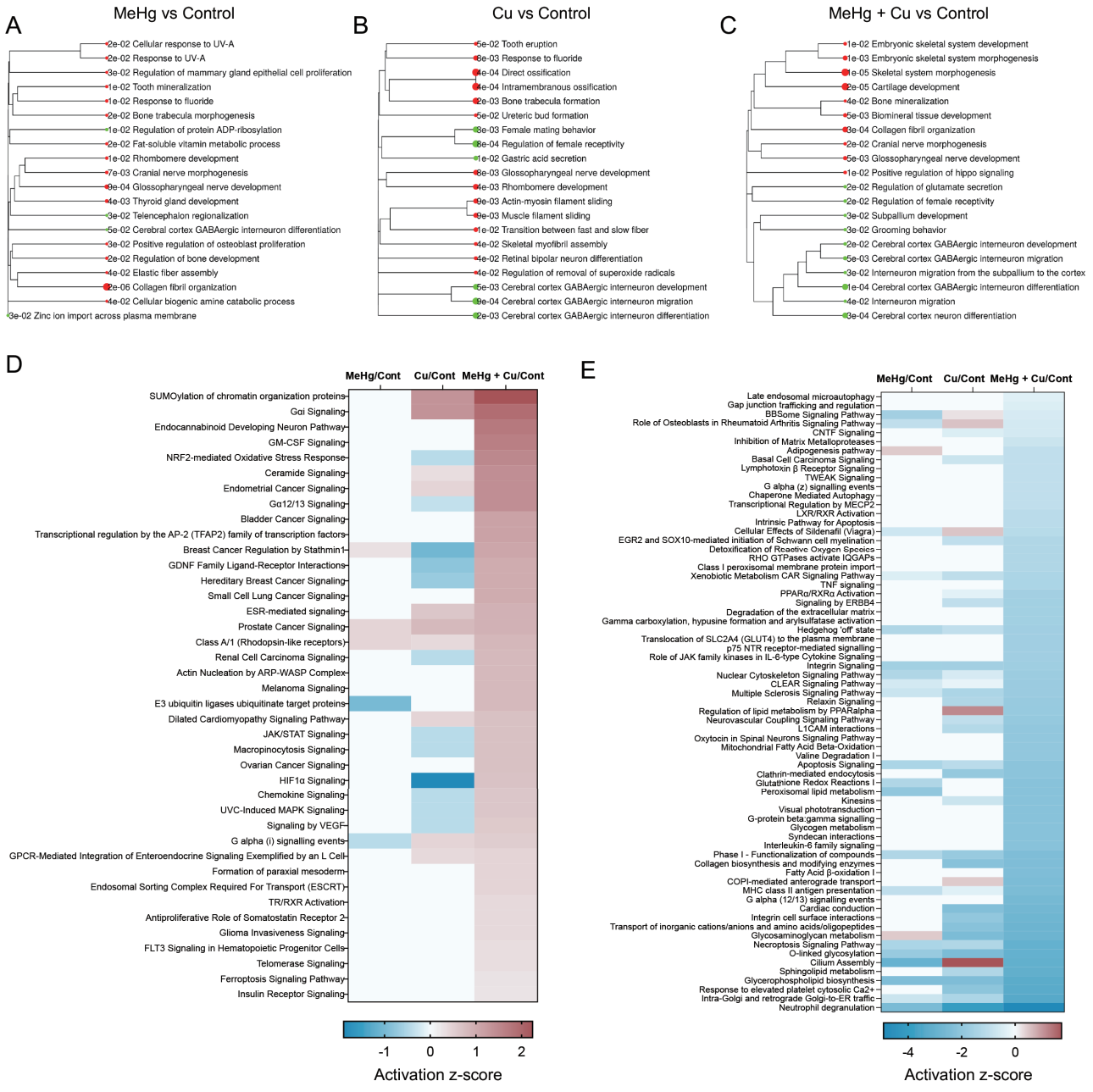

Fig. 2. Functional enrichment analysis of fetal brain genes altered by prenatal MeHg or Cu alone and their combined exposure in mice. (A-C) Hierarchical tree graphs of over-represented gene ontology terms for genes in (A) MeHg single exposure, (B) Cu single exposure, (C) MeHg and Cu co-exposure. Green and red represents up- and down-regulated pathways. (D and E) The heat map shows the highest change in Z-scores for activation (D) or inhibition (E) of the canonical pathway in the combined exposure group among each exposure group.

Table 4. Enriched GO Biological Process terms in HeMg vs Control.

GSEA analysis: MgHg vs Control Pathways	NES	adj.Pval
Collagen fibril organization	-0.8322	1.70E-06
Glossopharyngeal nerve development	-0.9847	9.00E-04
Thyroid gland development	-0.843	4.20E-03
Cranial nerve morphogenesis	-0.8391	7.00E-03
Response to fluoride	-0.9672	1.10E-02
Rhombomere development	-0.9435	1.20E-02
Tooth mineralization	-0.8393	1.30E-02
Regulation of bone development	-0.9344	1.60E-02
Response to UV-A	-0.8967	1.70E-02
Fat-soluble vitamin metabolic process	-0.8077	1.80E-02
Bone trabecula morphogenesis	-0.8666	1.90E-02
Cellular response to UV-A	-0.9347	2.30E-02
Regulation of mammary gland epithelial cell proliferation	-0.8127	2.80E-02
Positive regulation of osteoblast proliferation	-0.8626	3.20E-02
Elastic fiber assembly	-0.8652	3.50E-02
Cellular biogenic amine catabolic process	-0.8516	4.00E-02
Regulation of protein ADP-ribosylation	0.8999	1.30E-02
Zinc ion import across plasma membrane	0.8912	2.60E-02
Telencephalon regionalization	0.8063	3.00E-02
Cerebral cortex GABAergic interneuron differentiation	0.8257	4.60E-02

Normalized enrichment scores (NES) of the top 20 enriched genes sets. adj.pval, adjusted *p* value.

Table 5. Enriched GO Biological Process terms in Cu vs Control.

GSEA analysis: Cu vs Control Pathways	NES	adj.Pval
Intramembranous ossification	-0.9518	4.20E-04
Direct ossification	-0.9518	4.20E-04
Bone trabecula formation	-0.9154	1.50E-03
Rhombomere development	-0.9392	3.60E-03
Glossopharyngeal nerve development	-0.9598	7.60E-03
Response to fluoride	-0.9587	8.10E-03
Muscle filament sliding	-0.9084	9.40E-03
Actin-myosin filament sliding	-0.9084	9.40E-03
Transition between fast and slow fiber	-0.9091	1.50E-02
Retinal bipolar neuron differentiation	-0.9179	3.90E-02
Skeletal myofibril assembly	-0.9286	4.00E-02
Regulation of removal of superoxide radicals	-0.9145	4.40E-02
Tooth eruption	-0.9254	4.60E-02
Ureteric bud formation	-0.9234	4.70E-02
Regulation of female receptivity	0.9389	8.40E-04
Cerebral cortex GABAergic interneuron migration	0.9538	9.50E-04
Cerebral cortex GABAergic interneuron differentiation	0.9003	1.90E-03
Female mating behavior	0.9151	2.50E-03
Cerebral cortex GABAergic interneuron development	0.9124	4.80E-03
Gastric acid secretion	0.9096	1.50E-02

Normalized enrichment scores (NES) of the top 20 enriched genes sets. adj.pval, adjusted *p* value.

Table 6. Enriched GO Biological Process terms in HeMg + Cu vs Control.

GSEA analysis: MgHg + Cu vs Control Pathways	NES	adj.Pval
Skeletal system morphogenesis	-0.6863	1.10E-05
Cartilage development	-0.6932	1.80E-05
Collagen fibril organization	-0.861	3.30E-04
Embryonic skeletal system morphogenesis	-0.789	1.30E-03
Glossopharyngeal nerve development	-0.981	4.80E-03
Biomineral tissue development	-0.6782	4.80E-03
Positive regulation of hippo signaling	-0.9516	1.30E-02
Embryonic skeletal system development	-0.7142	1.50E-02
Cranial nerve morphogenesis	-0.8899	1.70E-02
Bone mineralization	-0.6991	4.30E-02
Cerebral cortex GABAergic interneuron differentiation	0.949	1.50E-04
Cerebral cortex neuron differentiation	0.8133	3.30E-04
Cerebral cortex GABAergic interneuron migration	0.9692	5.40E-03
Cerebral cortex GABAergic interneuron development	0.9349	1.70E-02
Regulation of female receptivity	0.9056	2.10E-02
Regulation of glutamate secretion	0.7476	2.10E-02
Grooming behavior	0.7876	2.60E-02
Interneuron migration from the subpallium to the cortex	0.8585	3.20E-02
Subpallium development	0.7409	3.20E-02
Interneuron migration	0.8546	4.00E-02

Normalized enrichment scores (NES) of the top 20 enriched genes sets. adj.pval, adjusted *p* value.

IPA for a more detailed prediction of effects. IPA showed that the combined exposures changed several canonical pathways compared with single exposures. Variable pathways included the activation of NRF2-mediated oxidative stress response and the suppression of ROS detoxification. These findings suggested that the combined exposure increased the effects on pathways related to oxidative stress in the brain. The endocannabinoid developing neuron pathway was identified as the pathway significantly activated by combined exposure. During early neurodevelopment stages, the endocannabinoid system regulates the migration, morphogenesis, and formation of appropriate connections in the local circuits of interneuron progenitor cells (Song *et al*., 2021). This finding supported the increased effects on GABAergic interneurons because of the combined exposure observed in the GO analysis. Conversely, mitochondrial fatty acid beta-oxidation and glycogen metabolism were identified as a markedly suppressed pathway. Therefore, it might affect energy metabolism.

The concept of exposome, which relates total environmental exposures to health effects, has been established; the effects of combined exposure on health have been the focus of much attention (Vermeulen *et al*., 2020). In the real world, we are exposed to a complex of different environmental chemicals. Therefore, the biological effects of combined exposure rather than single exposure to MeHg should be investigated. However, the mechanisms underlying these interactions are complex and not fully understood. In this study, the effects of combined fetal exposure to Cu and MeHg on the fetal brain were evaluated in terms of genetic changes via comprehensive analysis by RNAseq. These findings would help elucidate the mechanisms of the combined exposure effects of MeHg.

ACKNOWLEDGMENTS

This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grants (18H05293 to K.Y. and 23K06102 to M.A.) and JST FOREST Program (Grant Number JPMJFR221A to M.A.) We would like to thank Wordvice (https://wordvice.jp/) for editing this manuscript.

Conflict of interest---- The authors declare that there is no conflict of interest.

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