Original Article

Gene expression profiles in the dorsal root ganglia of methylmercury-exposed rats

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ABSTRACT — Methylmercury (MeHg) exposure is known to induce neurodegeneration in both the central nervous system (CNS) and peripheral nervous system (PNS). Molecular mechanisms of MeHg-induced neurotoxicity have been well investigated in the CNS, however, it remains unclear in the PNS. In the present study, comprehensive gene expression analysis was performed by analyzing MeHg-exposed adult rat dorsal root ganglion (DRG) by DNA microarray. Methylmercuric chloride (6.7 mg/kg/day) was administered to nine-week-old male Wistar rats for five days, followed by two days without administration; this cycle was repeated once. Rats were anesthetized at 7 or 14 days after commencement of MeHg exposure, and their DRGs were removed and homogenized to make total RNA samples. DNA microarray data from Day 7 samples identified 100 out of 18,513 detected genes as annotated genes with more than two-fold upregulated or downregulated expression compared with controls. Database for Annotation, Visualization, and Integrated Discovery (DAVID) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses suggested strong involvement of immune activation and inflammation pathways in rat DRG exposed to MeHg, and some genes overlapped with previously reported genes affected by MeHg exposure in the cerebellum. The present results suggest that MeHg-induced neurotoxicity is associated with immune activation and inflammatory responses in rat DRG.

Key words: Methylmercury, Dorsal root ganglion, DNA microarray, Immune activation, Inflammation

INTRODUCTION

Methylmercury (MeHg) is a well-known pollutant that causes severe neuropathological changes in the cerebral cortex, cerebellum, and dorsal root ganglion (DRG) (McAlpine and Araki, 1958; Eto and Takeuchi, 1978; Takeuchi *et al.*, 1978; Eto, 1997). As the principal neuronal and behavioral impairments elicited by MeHg exposure are caused by neural degeneration in some parts of the brain, most efforts to clarify the mechanisms underlying MeHg neurotoxicity have been performed in the central nervous system (CNS) of rodents and primates (Eto *et al.*, 2002; Costa *et al.*, 2004; Farina *et al.*, 2011; Patel and Reynolds, 2013; Antunes Dos Santos *et al.*, 2016; Unoki *et al.*, 2018). Therefore, despite observations of peripher-

al nerve impairment in humans (Eto and Takeuchi, 1978; Takeuchi *et al.*, 1978; Eto, 1997), mechanisms underlying MeHg-induced neurotoxicity in the peripheral nervous system (PNS) have not been thoroughly investigated.

One of the effective approaches to investigate molecular mechanisms of tissue and cellular responses to MeHg is comprehensive gene expression analysis using DNA microarray or next-generation sequencing. Indeed, comprehensive analyses of changes in gene expression influenced by MeHg exposure were previously performed in mouse cerebellum (Hwang *et al.*, 2011), zebrafish brain (Richter *et al.*, 2011), largemouth bass brain (Richter *et al.*, 2014), and human neuroblastoma cells such as SH-SY5Y and IMR-32 in a culture system (Hwang and Naganuma, 2006; Toyama *et al.*, 2011). These data pro-

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vided a lot of information about the molecular mechanisms underlying MeHg-induced neurotoxicity; however, comprehensive gene expression analysis of MeHg-exposed PNS tissue, such as DRG, has not been performed. A comparison of results from the CNS and PNS would be both informative and valuable.

In the present study, we performed DNA microarray analysis of adult rat DRG after exposure to MeHg to examine potential molecular mechanisms underlying MeHg-induced neurotoxicity in rat DRG.

MATERIALS AND METHODS

Animals and MeHg administration

MeHg administration was performed as previously reported (Shinoda *et al.*, 2019). Briefly, nine-week-old male Wistar rats (Tokyo Laboratory Animals Science, Tokyo, Japan) were housed in cages under a 12/12-hr light-dark cycle, with *ad libitum* access to water and food. Methylmercuric chloride (Sigma-Aldrich, Tokyo, Japan) was dissolved in MilliQ water to a concentration of 2 mg/mL. The MeHg solution was administered orally using a gastric tube as described previously (Qu *et al.*, 2019) at a daily dosage of 6.7 mg/kg for 5 days, followed by no administration for 2 days (to obtain Day 7 samples). This cycle was repeated once to obtain Day 14 samples. Age-matched rats not administered treatment were used as controls. We prepared four rats for each group (control, Day 7, and Day 14). All experimental protocols were evaluated and approved by the Regulations for Animal Research at Tokyo University of Pharmacy and Life Sciences. All efforts were made to minimize the number of animals used and their suffering.

RNA isolation

RNA isolation was performed as previously reported with some modifications (Shinoda *et al.*, 2016). The lumbar spinal cord of deeply anesthetized control, Day 7, and Day 14 rats (four per group) were quickly excised and DRGs were removed in cold PBS. At least 6 DRGs were collected from each individual and mixed as an individual sample. Total RNA was extracted from DRGs using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA quantity and quality were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), as recommended.

RNA amplification, labeling, and hybridization

RNA amplification, labeling, and subsequent hybridization methods were performed as described previously

with some modifications (Yoshikawa *et al.*, 2010). Total RNA was amplified and labeled with Cyanine 3 (Cy3) using a Low-Input Quick Amp Labeling Kit, One-Color (Agilent Technologies) according to the manufacturer's instructions. Briefly, total RNA was reversed transcribed into double-strand cDNA using a poly dT-T7 promoter primer. Primer, template RNA, and quality-control transcripts of known concentration and quality were first denatured at 65°C for 10 min and then incubated for 2 hr at 40°C with 5 × first strand buffer containing 0.1 M DTT, 10 mM dNTP mix, and AffinityScript RNase Block Mix. The AffinityScript enzyme was inactivated at 70°C for 15 min. cDNA products were then used as templates for *in vitro* transcription to generate fluorescent cRNA. cDNA products were mixed with a transcription master mix in the presence of T7 RNA polymerase and Cy3 labeled-CTP, and incubated at 40°C for 2 hr. Labeled cRNAs were purified using Qiagen's RNeasy mini spin columns and eluted in 30 µL of nuclease-free water. After amplification and labeling, cRNA quantity and Cy3 incorporation were determined using a Nanodrop ND-1000 spectrophotometer and Agilent Bioanalyzer. For each hybridization, 0.60 µg of Cy3-labeled cRNA were fragmented and hybridized at 65°C for 17 hr to an Agilent SurePrint G3 Rat Gene Expression v2 8 × 60K Microarray (Design ID: 074036). After washing, microarrays were scanned using an Agilent DNA microarray scanner.

Microarray analysis

DNA microarray analysis was performed as previously reported (Furuichi *et al.*, 2011; Watanabe *et al.*, 2017). Intensity values of each scanned feature were quantified using Agilent Feature Extraction software version 11.5.1.1, which performs background subtractions. We only used features that were flagged as no errors ("detected") and excluded features that were not positive, not significant, not uniform, not above background, saturated, or population outliers ("not detected" or "compromised"). Normalization was performed using Agilent GeneSpring software version 14.8 (per chip: normalization to 75 percentile shift). There are a total of 45,598 probes on the Agilent SurePrint G3 Rat Gene Expression v2 8×60K Microarray (Design ID: 074036) without control probes. Four individual samples from each group (control, Day 7, and Day 14; 12 samples total) were analyzed, and statistical significance was detected using one-way ANOVA with *post hoc* Tukey Honest Significant Difference test. The Benjamini Hochberg False Discovery Rate was used for the correction of multiple comparisons.

Functional and pathway analyses

Functional and pathway analyses were performed as previously described (Sadakata *et al.*, 2017). We used The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (<https://david.ncifcrf.gov/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg/pathway.html>) to analyze DNA microarray data. The list of differentially expressed genes with fold change > 2 and $p < 0.05$ was analyzed for common functions of altered genes using KEGG pathway and gene ontology (GO) terms. Enrichment analysis was performed using Fisher's exact test. For GO term analysis, % scores (analyzed genes involved in each GO term divided by total genes analyzed) were calculated; GO terms with a score of more than 5% are shown in each table.

RESULTS AND DISCUSSION

Using total RNA samples extracted from DRGs in control and MeHg exposed rats, we identified 18,513 genes on the microarray having raw signals with the flag value "detected" in at least three samples from each group. Cluster analysis of differentially regulated genes ($p < 0.05$) showed a specific pattern of gene expression attributable to the treatment (Fig. 1). Compared with controls, a total of 982 and 9,706 genes were identified as sig-

nificantly different in Day 7 and Day 14 samples, respectively. In the present study, genes from Day 7 samples that were differentially expressed compared with the control were used for further analysis, as there was no observable neuronal cell death in DRG at this stage (Shinoda *et al.*, 2019). Therefore, genes differentially expressed in this stage must be related to the subsequent initiation of neuronal death.

All genes that showed more than two-fold upregulation or downregulation compared with the control are listed in Table 1. A total of 117 genes containing 100 annotated and 17 non-annotated genes were identified. The number of downregulated genes was markedly small compared with upregulated genes. Previously, gene expression profiling of MeHg-exposed mouse cerebellum was analyzed adult C57BL/6 mice subcutaneously injected with 10 mg/kg/day MeHg for 7 days using DNA microarray (Hwang *et al.*, 2011). Their results indicated that the expression of 21 genes was increased, while that of 11 genes was decreased in MeHg-exposed mice compared with controls. Compared with our present data, some of the same genes such as *Gfap*, *Ccl12*, *Fcgr2b*, *Ccl7*, *Timp1*, and *Cd14* were increased in both ours and the previous one (Hwang *et al.*, 2011). In addition, expression of similar families of genes were also increased in both studies, such as *Fcgr3a*, *Ms4a6a*, *Bcl3*, *Clec4a3* and *Chi3l1* in our study and *Fcgr2b*, *Ms4a6d*, *Bcl2a1b*, *Clec7a* and *Chi3l* in the previous study, respectively. Therefore, there may be similar mechanisms for MeHg-induced neuronal death in both the cerebellum and DRG. In contrast, gene profiling of MeHg-exposed human neuroblastoma, SH-SY5Y, and IMR-32 cells (Hwang and Naganuma, 2006; Toyama *et al.*, 2011), showed no cross-correlation with the present study. Therefore, MeHg-induced neural toxicity might employ different mechanisms in a heterogeneous *in vivo* environment and homogeneous *in vitro* environment. Conceivably, mechanisms underlying MeHg neurotoxicity may be different between species, tissues, cell types, experimental conditions, or other factors.

All genes in the Day 7 sample that exhibited more than two-fold upregulation or downregulation compared with the control were applied to DAVID pathway analysis and confirmed by KEGG pathway analysis. In Tables 2-4, the number of counts represents the number of genes included in the related term. KEGG-pathway analysis indicated that 19 significant ($p < 0.05$) pathways responded to MeHg (Table 2). This result contains seven terms related to infection (tuberculosis, staphylococcus aureus infection, malaria, pertussis, leishmaniasis, Chagas disease, and legionellosis), four terms related to immune response (cytokine-cytokine receptor interaction, chemokine sign-

Fig. 1. Cluster analysis of gene expression in control (Ctrl), Day 7, and Day 14 samples (calculated from the beginning of MeHg exposure) of dorsal root ganglia in MeHg-exposed rats. Red and green represent upregulated and downregulated genes, respectively.

Table 1. MeHg responsive genes (fold change > 2.0).

Gene Symbol	Gene Name	Fold Change	<i>p</i> value	Genbank
Increased				
<i>Cxcl14</i>	chemokine (C-X-C motif) ligand 14	20.2	2.75E-04	NM_001013137
<i>Gfap</i>	glial fibrillary acidic protein	7.98	3.66E-04	NM_017009
<i>H19</i>	H19, imprinted maternally expressed transcript (non-protein coding)	6.76	2.50E-04	NR_027324
<i>Cxcl10</i>	chemokine (C-X-C motif) ligand 10	6.34	1.53E-02	NM_139089
<i>Slpi</i>	secretory leukocyte peptidase inhibitor	5.91	1.11E-02	NM_053372
<i>Slpil3</i>	antileukoproteinase-like 3	5.18	1.32E-02	NM_001008873
<i>Fcgr3a</i>	Fc fragment of IgG, low affinity IIIa, receptor	5.09	2.86E-03	NM_207603
<i>Cxcl13</i>	chemokine (C-X-C motif) ligand 13	4.75	4.64E-03	NM_001017496
<i>Fcnb</i>	ficolin B	4.72	3.11E-03	NM_053634
<i>Ccl12</i>	chemokine (C-C motif) ligand 12	4.68	1.28E-02	NM_001105822
<i>Mt2A</i>	metallothionein 2A	4.39	1.02E-03	NM_001137564
<i>Defa5</i>	defensin, alpha 5, Paneth cell-specific	4.35	1.11E-03	NM_173329
<i>Socs3</i>	suppressor of cytokine signaling 3	4.34	2.39E-04	XM_008768398
		4.21	1.13E-03	U50353
<i>Np4</i>	defensin NP-4 precursor	3.95	1.08E-03	NM_173299
<i>Fcgr2b</i>	Fc fragment of IgG, low affinity IIb, receptor	3.91	3.40E-03	NM_175756
<i>Il1rn</i>	interleukin 1 receptor antagonist	3.86	2.77E-03	NM_022194
<i>RatNP-3b</i>	defensin RatNP-3 precursor	3.66	2.64E-03	NM_001079898
<i>Fgf2</i>	fibroblast growth factor 2	3.43	4.05E-03	M22427
<i>Siglec1</i>	sialic acid binding Ig-like lectin 1, sialoadhesin	3.38	1.23E-02	NM_001107777
<i>Col7a1</i>	collagen, type VII, alpha 1	3.37	1.04E-02	NM_001106858
<i>C3</i>	complement component 3	3.28	1.65E-02	NM_016994
<i>Gbp2</i>	guanylate binding protein 2, interferon-inducible	3.18	9.18E-03	NM_133624
<i>Ccl7</i>	chemokine (C-C motif) ligand 7	3.13	2.38E-04	NM_001007612
		3.11	4.59E-03	XM_006250150
<i>C4a</i>	complement component 4A (Rodgers blood group)	3.09	2.45E-03	NM_031504
<i>Plac8</i>	placenta-specific 8	3.01	1.30E-02	NM_001108353
<i>Inmt</i>	indolethylamine N-methyltransferase	3.01	5.46E-04	NM_001109022
		2.99	4.81E-03	XM_006250150
<i>Adams4</i>	ADAM metalloproteinase with thrombospondin type 1 motif, 4	2.96	2.37E-04	NM_023959
<i>Ms4a6a</i>	membrane-spanning 4-domains, subfamily A, member 6A	2.95	2.42E-02	XM_001075502
<i>Ucn2</i>	urocortin 2	2.94	4.82E-02	NM_133385
<i>Defa10</i>	defensin alpha 10	2.94	4.25E-03	NM_001033074
<i>LOC103691468</i>	uncharacterized LOC103691468	2.91	2.15E-02	XR_590927
<i>Gpr84</i>	G protein-coupled receptor 84	2.89	3.19E-03	NM_001109509
<i>A2m</i>	alpha-2-macroglobulin	2.89	5.75E-03	NM_012488
<i>C4b</i>	complement component 4B (Chido blood group)	2.88	1.96E-03	NM_001002805
<i>Napsa</i>	napsin A aspartic peptidase	2.85	5.96E-03	NM_031670
<i>Cclcf1</i>	cardiotrophin-like cytokine factor 1	2.83	2.47E-03	NM_207615
<i>Ccl11</i>	chemokine (C-C motif) ligand 11	2.79	7.57E-03	NM_019205
<i>Ifitm3</i>	interferon induced transmembrane protein 3	2.79	2.24E-03	NM_001136124
<i>S100a8</i>	S100 calcium binding protein A8	2.79	1.69E-03	NM_053822
<i>Atf3</i>	activating transcription factor 3	2.76	1.06E-03	NM_012912
<i>Sbsn</i>	suprabasin	2.75	5.91E-03	NM_001044231
<i>Timp1</i>	TIMP metalloproteinase inhibitor 1	2.75	2.15E-03	NM_053819
<i>Gpr171</i>	G protein-coupled receptor 171	2.73	8.11E-04	NM_001109510
		2.72	2.36E-03	XM_223483
<i>Upp1</i>	uridine phosphorylase 1	2.67	2.83E-03	NM_001030025
		2.62	2.01E-02	XM_006225974
<i>Cebpd</i>	CCAAT/enhancer binding protein (C/EBP), delta	2.61	2.50E-04	NM_013154

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Table1. (Continued).

Gene Symbol	Gene Name	Fold Change	p value	Genbank
<i>Tlr12</i>	toll-like receptor 12	2.58	2.75E-02	XM_008764148
		2.57	1.07E-02	XM_221091
		2.55	3.14E-03	XM_001054983
<i>S100a9</i>	S100 calcium binding protein A9	2.53	3.11E-03	NM_053587
		2.52	4.48E-03	XM_008774527
<i>LOC102550593</i>	uncharacterized LOC102550593	2.51	7.77E-04	XR_591063
<i>Myo1g</i>	myosin 1G	2.51	5.23E-03	NM_001134843
<i>Bcl3</i>	B-cell CLL/lymphoma 3	2.5	2.15E-04	NM_001109422
<i>Icam1</i>	intercellular adhesion molecule 1	2.48	4.86E-04	NM_012967
<i>Batf3</i>	basic leucine zipper transcription factor, ATF-like 3	2.48	3.76E-03	XM_008769874
<i>Camp</i>	cathelicidin antimicrobial peptide	2.47	8.74E-03	NM_001100724
<i>Akr1cl</i>	aldo-keto reductase family 1, member C-like	2.47	6.56E-03	NM_001109900
<i>LOC684871</i>	similar to Protein C8orf4 (Thyroid cancer protein 1) (TC-1)	2.43	8.64E-04	NM_001115043
<i>Lst1</i>	leukocyte specific transcript 1	2.43	1.76E-03	NM_022634
		2.43	4.47E-02	XM_006237321
<i>Dmbt1</i>	deleted in malignant brain tumors 1	2.38	2.44E-03	NM_022849
		2.38	8.09E-03	XM_006241766
<i>Rac2</i>	ras-related C3 botulinum toxin substrate 2	2.37	2.57E-03	NM_001008384
<i>Runx2</i>	runt-related transcription factor 2	2.37	2.26E-03	NM_001278483
<i>Slco2a1</i>	solute carrier organic anion transporter family, member 2a1	2.35	2.23E-03	NM_022667
<i>Fos</i>	FBJ osteosarcoma oncogene	2.34	4.24E-03	NM_022197
<i>Cd14</i>	CD14 molecule	2.32	2.01E-02	NM_021744
<i>Thbs2</i>	thrombospondin 2	2.31	1.91E-04	NM_001169138
<i>Junb</i>	jun B proto-oncogene	2.3	6.88E-04	NM_021836
		2.3	1.84E-02	XM_008760706
<i>Scube1</i>	signal peptide, CUB domain, EGF-like 1	2.29	1.55E-03	NM_001134884
		2.29	2.24E-02	XM_006247739
<i>LOC103690369</i>	leukocyte immunoglobulin-like receptor subfamily B member 4	2.28	8.73E-03	XM_008774415
<i>Rgs16</i>	regulator of G-protein signaling 16	2.27	3.01E-04	XM_006250012
<i>Crlf1</i>	cytokine receptor-like factor 1	2.23	2.93E-03	NM_001106074
<i>Ripk3</i>	receptor-interacting serine-threonine kinase 3	2.23	4.95E-03	NM_139342
<i>Lsp1</i>	lymphocyte-specific protein 1	2.23	2.96E-03	NM_001025420
<i>Cd68</i>	Cd68 molecule	2.22	8.85E-03	NM_001031638
<i>Fcrlg</i>	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	2.21	5.29E-03	NM_001131001
<i>Clec4a3</i>	C-type lectin domain family 4, member A3	2.21	1.25E-02	NM_001005891
<i>Il1b</i>	interleukin 1 beta	2.2	3.33E-03	NM_031512
<i>Gbp5</i>	guanylate binding protein 5	2.2	1.70E-02	NM_001108569
<i>Mmp19</i>	matrix metalloproteinase 19	2.2	4.88E-03	NM_001107159
		2.19	3.60E-03	FQ220235
<i>Cd3g</i>	CD3 molecule, gamma	2.15	3.41E-04	NM_001077646
<i>LOC100911413</i>	deleted in malignant brain tumors 1 protein-like	2.15	7.98E-03	XM_006223692
<i>Cldn14</i>	claudin 14	2.15	3.41E-02	NM_001013429
<i>Chi3l1</i>	chitinase 3-like 1 (cartilage glycoprotein-39)	2.15	1.30E-02	NM_053560
		2.14	3.73E-02	XM_006224276
<i>Col16a1</i>	collagen, type XVI, alpha 1	2.1	4.11E-04	NM_001302967
<i>Igtp</i>	interferon gamma induced GTPase	2.1	7.63E-03	NM_001008765
<i>Empl</i>	epithelial membrane protein 1	2.08	2.04E-02	NM_012843
<i>Mt1a</i>	metallothionein 1a	2.07	4.96E-03	NM_138826
<i>Egr2</i>	early growth response 2	2.06	2.57E-03	NM_053633
<i>Arid5a</i>	AT rich interactive domain 5A (Mrfl like)	2.05	1.09E-03	NM_001034934

Table 1. (Continued).

Gene Symbol	Gene Name	Fold Change	<i>p</i> value	Genbank
<i>Slamf9</i>	SLAM family member 9	2.04	1.17E-02	NM_001105971
<i>Phf11b</i>	PHD finger protein 11B	2.04	1.21E-02	NM_001014235
<i>Gas7</i>	growth arrest specific 7	2.04	3.46E-04	NM_053484
<i>C3ar1</i>	complement component 3a receptor 1	2.04	4.65E-03	NM_032060
<i>Fam150b</i>	family with sequence similarity 150, member B	2.03	3.89E-03	NM_001109372
		2.03	4.01E-02	FQ217794
		2.03	2.17E-03	FQ217918
<i>Glpr1</i>	GLI pathogenesis-related 1	2.02	1.22E-02	NM_001011987
<i>Cyth4</i>	cytohesin 4	2.01	2.90E-03	NM_001130577
<i>Lyz2</i>	lysozyme 2	2	1.82E-02	NM_012771
<i>Igsf10</i>	immunoglobulin superfamily, member 10	2	9.23E-04	NM_198768
<i>Maff</i>	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog F	2	7.28E-04	NM_001130573
<i>Ptpn7</i>	protein tyrosine phosphatase, non-receptor type 7	2	3.46E-04	NM_145683
Decreased				
<i>LOC102552721</i>	uncharacterized LOC102552721	-2.02	1.37E-02	XR_345109
		-2.15	8.23E-03	DY472854
<i>LOC102556699</i>	uncharacterized LOC102556699	-2.21	2.14E-02	XM_008774562
<i>Atp2b3</i>	ATPase, Ca++ transporting, plasma membrane 3	-2.57	7.76E-03	NM_133288

Table 2. KEGG-pathways related to MeHg responsive genes.

Term	Count	%	<i>p</i> value	Fold Enrichment
TNF signaling pathway	8	8.6	3.06E-06	12.1
Tuberculosis	8	8.6	8.67E-05	7.25
Cytokine-cytokine receptor interaction	8	8.6	1.52E-04	6.63
Staphylococcus aureus infection	7	7.53	6.51E-07	21.37
Chemokine signaling pathway	7	7.53	5.72E-04	6.52
Osteoclast differentiation	6	6.45	9.38E-04	7.67
MAPK signaling pathway	6	6.45	1.75E-02	3.85
Malaria	5	5.38	3.90E-04	13.97
Pertussis	5	5.38	8.77E-04	11.29
Phagosome	5	5.38	2.70E-02	4.27
Leishmaniasis	4	4.3	8.77E-03	9.16
Complement and coagulation cascades	4	4.3	8.77E-03	9.16
Salivary secretion	4	4.3	1.02E-02	8.68
Rheumatoid arthritis	4	4.3	1.60E-02	7.33
Toll-like receptor signaling pathway	4	4.3	1.96E-02	6.8
Natural killer cell mediated cytotoxicity	4	4.3	2.01E-02	6.73
Chagas disease (American trypanosomiasis)	4	4.3	2.53E-02	6.16
Cytosolic DNA-sensing pathway	3	3.23	4.34E-02	8.83
Legionellosis	3	3.23	4.48E-02	8.68

aling pathway, toll-like receptor signaling pathway, and natural killer cell-mediated cytotoxicity), and two terms related to inflammatory response (tumor necrosis factor signaling pathway and rheumatoid arthritis). These results suggest that infection-associated-like immune and inflammatory responses were induced in MeHg-exposed DRG. A similar tendency was also shown for GO terms of biological processes (Table 3). Fourteen out of 26 GO

terms identified for the response to MeHg were related to immune and inflammatory responses (Fig. 2; inflammatory response, response to lipopolysaccharide, defense response to bacterium, innate immune response, cellular response to lipopolysaccharide, neutrophil chemotaxis, cellular response to interferon-gamma, immune response, defense response to Gram-positive bacterium, cellular response to interleukin-1, cellular response to tumor

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Table 3. GO term (Biological Process) related to MeHg responsive genes.

Term	Count	%	<i>p</i> value	Fold Enrichment
inflammatory response	14	15.05	1.33E-09	9.84
response to lipopolysaccharide	12	12.9	8.25E-08	8.95
positive regulation of transcription from RNA polymerase II promoter	12	12.9	8.71E-03	2.45
defense response to bacterium	11	11.83	6.81E-10	17.27
innate immune response	10	10.75	3.93E-06	8
positive regulation of cell proliferation	10	10.75	6.12E-04	4.15
cellular response to lipopolysaccharide	9	9.68	1.23E-06	11.25
positive regulation of ERK1 and ERK2 cascade	9	9.68	2.76E-06	10.1
positive regulation of angiogenesis	8	8.6	1.34E-06	14.27
neutrophil chemotaxis	7	7.53	3.11E-07	25.19
cellular response to interferon-gamma	7	7.53	4.22E-07	23.95
negative regulation of endopeptidase activity	7	7.53	7.22E-05	9.87
immune response	7	7.53	1.67E-03	5.47
response to drug	7	7.53	3.90E-02	2.77
defense response to Gram-positive bacterium	6	6.45	7.49E-05	13.61
cellular response to interleukin-1	6	6.45	1.01E-04	12.78
cellular response to tumor necrosis factor	6	6.45	4.21E-04	9.42
response to organic cyclic compound	6	6.45	9.41E-03	4.6
transcription from RNA polymerase II promoter	6	6.45	4.05E-02	3.15
chemokine-mediated signaling pathway	5	5.38	1.52E-04	18.31
positive regulation of inflammatory response	5	5.38	2.25E-04	16.57
chemotaxis	5	5.38	2.39E-04	16.31
wound healing	5	5.38	3.02E-03	8.28
regulation of cell shape	5	5.38	3.02E-03	8.28
response to glucocorticoid	5	5.38	3.66E-03	7.85
response to ethanol	5	5.38	1.33E-02	5.41

Fig. 2. Schematic of mechanisms of MeHg-induced neuronal cell death in rat DRG. GO terms for biological processes related to immune and inflammatory responses are depicted.

necrosis factor, chemokine-mediated signaling pathway, positive regulation of inflammatory response and chemotaxis). Indeed, most of the MeHg-responsive genes that overlapped between the present study and previous studies, such as *Ccl12*, *Ccl17*, *Timp1*, and *Cd14* (Hwang *et al.*, 2011), were highly associated with inflammation. In addition, inflammatory responses induced by MeHg have been reported in several species, tissues, and cell types both *in vivo* (Cambier *et al.*, 2012; da Silva *et al.*, 2012; Nøstbakken *et al.*, 2012) and *in vitro* (David *et al.*, 2017; Yamamoto *et al.*, 2017; Crowe *et al.*, 2018).

GO terms (molecular function) were also investigated by DAVID analysis (Table 4). The results indicated that the higher order in the table contains cytokine and chemokine activities. Detailed gene components of identified terms are shown in Table 5. Several principal cytokine and chemokine ligands were induced by MeHg exposure. Cytokines and chemokines are well known for their roles in immune responses and activation of inflammation (Turner *et al.*, 2014). In fact, microglial activation and/or macrophage invasion induced by MeHg exposure

Table 4. GO term (Molecular Function) related to MeHg responsive genes.

Term	Count	%	<i>p</i> value	Fold Enrichment
protein heterodimerization activity	9	9.68	3.20E-03	3.59
cytokine activity	7	7.53	6.97E-05	9.9
endopeptidase inhibitor activity	6	6.45	1.98E-07	44.4
chemokine activity	6	6.45	5.12E-07	37
heparin binding	6	6.45	4.80E-04	9.12
transcription regulatory region DNA binding	6	6.45	4.07E-03	5.62
RNA polymerase II core promoter proximal region sequence-specific DNA binding	6	6.45	1.89E-02	3.85
carbohydrate binding	5	5.38	5.99E-03	6.81
GTPase activity	5	5.38	1.17E-02	5.61
transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	5	5.38	2.65E-02	4.37

Table 5. Genes included in cytokine and chemokine activities.

Gene Name	Genbank
Cytokine activity	
C-X-C motif chemokine ligand 10 (<i>Cxcl10</i>)	NM_139089
TIMP metalloproteinase inhibitor 1 (<i>Timp1</i>)	NM_053819
cardiotrophin-like cytokine factor 1 (<i>Clcf1</i>)	NM_207615
cytokine receptor-like factor 1 (<i>Crlf1</i>)	NM_001106074
fibroblast growth factor 2 (<i>Fgf2</i>)	NM_019305
interleukin 1 beta (<i>Il1b</i>)	NM_031512
interleukin 1 receptor antagonist (<i>Il1rn</i>)	NM_022194
Chemokine activity	
C-C motif chemokine ligand 11 (<i>Ccl11</i>)	NM_019205
C-C motif chemokine ligand 7 (<i>Ccl7</i>)	NM_001007612
C-X-C motif chemokine ligand 10 (<i>Cxcl10</i>)	NM_139089
C-X-C motif chemokine ligand 13 (<i>Cxcl13</i>)	NM_001017496
C-X-C motif chemokine ligand 14 (<i>Cxcl14</i>)	NM_001013137
C-C motif chemokine ligand 12 (<i>Ccl12</i>)	NM_001105822

were previously reported in rat cerebellum, common marmoset, and mouse cerebral cortex (Sakamoto *et al.*, 2008; Fujimura *et al.*, 2009; Yamamoto *et al.*, 2012). Therefore, we hypothesized that MeHg-induced cytokines and chemokines in rat DRG attract activation/invasion of microglia and macrophages. This hypothesis is strongly supported by our previous results showing activation and increased numbers of microglia and macrophages in DRG after MeHg exposure (Shinoda *et al.*, 2019).

In the present study, we investigated gene expression responses to MeHg exposure using rat DRG. Many genes detected in our experimental conditions showed significant association with immune activation and induction of inflammation. However, further investigations are required to clarify the origin of involved cytokines and

chemokines, the cause of microglial activation and macrophage invasion (whether the cause or result of neural cell death in MeHg-exposed rat DRG), and related molecular mechanisms of MeHg-induced neurotoxicity.

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

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