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Original Article

Increased Cellular Distribution of Vimentin and Ret in the Cingulum of Rat Offspring After Developmental Exposure to Decabromodiphenyl Ether or 1,2,5,6,9,10-Hexabromocyclododecane

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Abstract: To determine effects of developmental exposure to brominated flame retardants (BFRs), weak thyroid hormone disruptors, on white matter development, white matter-specific global gene expression analysis was performed using microdissection techniques and microarrays in male rats exposed maternally to decabromodiphenyl ether (DBDE), one of the representative BFRs, at 10, 100 or 1000 ppm. Based on previous gene expression profiles of developmental hypothyroidism and DBDE-exposed cases, vimentin$^+$ immature astrocytes and ret proto-oncogene (Ret)$^+$ oligodendrocytes were immunohistochemically examined after developmental exposure to representative BFRs, i.e., DBDE, 1,2,5,6,9,10-hexabromocyclododecane (HBCD; 100, 1000 or 10,000 ppm) and tetrabromobisphenol A (TBBPA; 100, 1000 or 10,000 ppm). Vimentin$^+$ and Ret$^+$ cell populations increased at ≥ 100 ppm and ≥ 10 ppm DBDE, respectively. Vimentin$^+$ and Ret$^+$ cells increased at ≥ 1000 ppm HBCD, with no effect of TBBPA. The highest dose of DBDE and HBCD revealed subtle fluctuations in serum thyroid-related hormone concentrations. Thus, DBDE and HBCD may exert direct effects on glial cell development at ≥ middle doses. At high doses, hypothyroidism may additionally be an inducing mechanism, although its contribution is rather minor. (DOI: 10.1293/tox.26.119; J Toxicol Pathol 2013; 26: 119–129)

Key words: BFRs, glial development, vimentin, Ret, hypothyroidism, rat

Introduction

Thyroid hormones (THs) are essential for normal fetal and neonatal brain development, control neuronal and glial proliferation in definitive brain regions and regulate neuronal migration and differentiation[1–3]. Experimentally, developmental hypothyroidism leads to growth retardation, neurological defects and impaired performance in a variety of behavioral learning actions[4,5]. Rat offspring exposed maternally to anti-thyroid agents show brain growth impairment associated with neuronal mismigration and white matter hypoplasia involving limited axonal myelination and decreased oligodendroglial distribution[2,6]. The outcome of this type of impairment is permanent, resulting in apparent structural and functional abnormalities.

Some environmental chemicals are thought to potentiate a TH-disrupting effect that may lead to abnormal brain development[7]. To evaluate the impact of developmentally exposed TH-disrupting chemicals on brain growth, we have established morphometric analysis methods of brain development in terms of neuronal migration and white matter development using a rat developmental hypothyroidism model[8]. We also have recently reported the molecules showing fluctuations in expression by microdissected region-specific microarray analysis and following immunohistochemical analysis in each of the hippocampal cornu ammonis and white matter after developmental hypothyroidism in rats[9–11]. With regard to molecules in the white matter responding to developmental hypothyroidism, we found vimentin$^+$ immature astrocytes and ret proto-oncogene (Ret)$^+$ oligodendrocytes in the cingulum[11].

Brominated flame retardants (BFRs), some of which are environmental contaminants used in plastics, textiles, electronic circuitry and other materials to prevent fires,

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are known to be weak TH disruptors. Therefore, there is a growing concern regarding the developmental neurotoxicity of these chemicals[12]. Among the variety of BFRs, decabromodiphenyl ethers (DBDE), 1,2,5,6,9,10-hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) are the most widely used BFRs throughout the world and have been detected in human blood and breast milk[13]. DBDE, HBCD and TBBPA have been investigated in relation to hypothyroidism and neurotoxic effects. Among these chemicals, developmental exposure to DBDE resulted in a decrease in serum TH levels at the adult stage in mice and rats[14, 15], showing *in vivo* evidence of neurotoxicity involving spontaneous locomotor behavior and synaptogenesis[14, 16, 17]. Regarding HBCD, developmental exposure showed impairment in learning and memory and aberrant spontaneous behavior[18]. Also, HBCD inhibited the uptake of neurotransmitters, particularly dopamine and glutamate, into synaptosomes[19]. In the case of TBBPA, the possibility of hypothyroidism and neurotoxicity has been suggested to be low. In a two-generation reproductive toxicity study, TBBPA did not induce effects on neurodevelopmental end points[20]. On the other hand, a one-generation reproductive study of TBBPA showed neurobehavioral effects in offspring[21], and *in vitro* studies showed antagonistic activity on TH receptors and inhibition of synaptic neurotransmitter uptake[19, 22].

We have recently reported the effects of developmental exposure to DBDE, TBBPA and HBCD on white matter development by histomorphometric assessment using rats in association with thyroid parameters[23, 24]. Our results suggested that maternal exposure to DBDE or HBCD through diet caused irreversible white matter hypoplasia at the highest doses in offspring as examined in males, as well as the induction of mild developmental hypothyroidism as judged by fluctuations in the serum concentrations of thyroid-related hormones at the end of developmental exposure[23, 24]. On the other hand, we have also found white matter hypoplasia at the middle dose with DBDE without accompanying fluctuations in serum TH concentrations, suggesting a direct effect on the brain[24]. In another study, we also found that neuronal development was affected by all of these BFRs, with DBDE and TBBPA appearing to have direct effects on the brain[25].

In the present study, to elucidate whether TH-disrupting chemicals, such as BFRs, affect hypothyroidism-related white matter development after developmental exposure, we performed cerebral white matter-specific global gene expression analysis using microarrays in developmentally DBDE-exposed rat offspring and compared this with the profiles in the developmental hypothyroidism model using anti-thyroid agents as previously reported[11]. Molecules showing commonly altered expression in animals between DBDE and anti-thyroid agents were analyzed for immunohistochemical distribution in the cerebral white matter using the same previously published study samples of DBDE, TBBPA and HBCD[23, 24]. DBDE study samples also were analyzed for the immunohistochemical distribution of the other candidate molecules obtained from DBDE microarray analysis.

Materials and Methods

Chemicals and animals

DBDE (CAS No. 1163-19-5, purity: >98%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TBBPA (CAS No. 79-94-7, purity: >98%) and HBCD (CAS No. 3194-55-6, purity: >95%) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Pregnant CD® (SD) IGS rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) at gestational day (GD) 3 (the day when vaginal plugs were observed was designated as GD 0). Animals were individually housed in polycarbonate cages (SK-Clean, 41.5 cm × 26 cm × 17.5 cm; CLEA Japan, Inc., Tokyo, Japan) with wood chip bedding (Sankyo Labo Service Corp., Tokyo, Japan) and maintained in a climate-controlled animal room (24 ± 1°C, relative humidity: 55 ± 5%) with a 12-h light/dark cycle. A soy-free diet (Oriental Yeast Co., Ltd., Tokyo, Japan) was chosen as the basal diet for maternal animals to eliminate possible phytoestrogen effects[26]. Animals received food and water *ad libitum* throughout the experimental period, including a 1-week acclimation period.

Experimental design

Exposure studies of DBDE, HBCD and TBBPA were individually performed, and dams were randomly divided into four groups including untreated controls[23, 24]. The highest dose of each chemical was determined with a preliminary dose-finding study by estimating the dose range that causes changes in thyroid weights and histopathological findings of thyroid glands in dams but does not affect pregnancy, implantation or delivery. For DBDE, 8 dams per group were provided with the soy-free diet containing 0 (control), 10, 100 or 1000 ppm of DBDE from GD 10 to postnatal day (PND) 20 (PND 0: the day of delivery). For TBBPA or HBCD, 8 dams per group in the TBBPA study and 10 dams per group in the HBCD study were provided with the soy-free diet containing 0 (control), 100, 1000 or 10,000 ppm of the compound from GD 10 to PND 20. In all studies, litters were culled randomly on PND 2, leaving 4 male and 4 female offspring. On PND 20, 20 male and 20 female offspring (at least one male and one female per dam) per group were euthanized and subjected to prepubertal necropsy.

All animals used in the present study were killed by exsanguination from the abdominal aorta under deep anesthesia. The protocols were reviewed in terms of animal welfare and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Preparation of tissue specimens and microdissection

For microarray analysis in developmentally DBDE-exposed animals, the whole brain of male offspring was immediately removed at prepubertal necropsy on PND 20 (n = 4/group, 1 pup/litter) and fixed with methacarn solution

for 2 h at 4°C[27]. Coronal brain slices taken at –3.5 mm from the bregma were dehydrated and embedded in paraffin. Embedded tissues were stored at 4°C until tissue sectioning for microdissection.

According to the method described previously[11], regions of the corpus callosum (CC) and external capsule in 20-μm-thick serial sections were subjected to laser microbeam microdissection (Leica Microsystems GmbH). Forty sections from each animal were used for microdissection, and microdissected samples were individually stored in 1.5 ml tubes at –80°C until total RNA extraction.

RNA preparation, amplification and microarray analysis

Total RNA extraction from microdissected regions, quantitation of RNA yield and RNA amplification were performed using methods described elsewhere[28].

For microarray analysis, second-round-amplified biotin-labeled antisense RNAs were subjected to hybridization with a GeneChip® Rat Genome 230 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA).

Gene selection and normalization of expression data were performed using GeneSpring® software 7.2 (Silicon Genetics, Redwood City, CA, USA). Per chip normalization was performed according to a method described elsewhere[28]. Genes with expression changes of at least 2-fold in magnitude caused by DBDE exposure as compared with those of untreated controls were selected. Using these gene expression data, we further selected genes showing commonly fluctuated expressions with previously reported genes responding to developmental hypothyroidism in an experimental induction model using anti-thyroid agents consisting of groups of 3 or 12 ppm propylthiouracil and 200 ppm methimazole[11].

Immunohistochemistry

To evaluate the immunohistochemical distribution of molecules showing commonly altered expression between DBDE and anti-thyroid agents, brains of male pups obtained at PND 20 were fixed in Bouin's solution at room temperature overnight in all BFRs studies of DBDE, TBBPA and HBCD. Five animals (1 pup/litter) for each group were used for immunohistochemistry using antibodies against vimentin (mouse monoclonal antibody, 1:200, Millipore Corporation, Billerica, MA, USA) and Ret (rabbit polyclonal antibody, 1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Furthermore, to evaluate the immunohistochemical distribution of molecules whose transcript levels fluctuated in microarray analysis due to DBDE exposure, antibodies against neuregulin 1 (Nrg1, also named as heregulin; mouse monoclonal antibody, 1:40, Exalpha Biologicals, Inc., Watertown, MA, USA), Crk (mouse monoclonal antibody, 1:2000, BD Biosciences, Franklin Lakes, NJ, USA) and Claudin 11 (Cld11, also named as oligodendrocyte specific protein; rabbit polyclonal antibody, 1:200, Novus Biologicals, LLC, Littleton, CO, USA) were used in the DBDE study.

For antigen retrieval, the sections were heated in 10 mM citrate buffer by microwave for 10 min before incubation with anti-vimentin and anti-Nrg1 antibodies. Immunodetection was carried out using a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3'-diaminobenzidine/H_2O_2 for the chromogen as described elsewhere[32]. Sections were then counterstained with hematoxylin and coverslipped for microscopic examination. Non-immunized sera were substituted for the primary antibody as negative controls for immunoreactivity.

Morphometry of immunolocalized cells

The number of immunoreactive cells was quantitatively measured by vimentin and Ret expression in white matter at the cingulum of the bilateral sides by blind trial for the treatment conditions according to the method and equipment described previously[11]. The values were normalized and expressed as those in the unit area (mm^2).

To evaluate immunoreactivity of Nrg1, Crk and Cld11 in the white matter, staining intensity was scored as 0 (none), 1 (minimal), 2 (slight), 3 (moderate) or 4 (strong) by observation at 40-fold magnification.

Statistical analysis

Data for offspring were analyzed using the litter as the experimental unit. Numerical data were assessed by one-way analysis of variance or the Kruskal-Wallis test following Bartlett's test. Statistically significant differences were analyzed by Dunnett's multiple comparison test for comparison with the untreated control group. For grading immunohistochemical findings, scores of Nrg1, Crk and Cld11 expression were compared between the untreated control group and each DBDE-exposed group using the Mann-Whitney's *U*-test.

Results

Microarray analysis in developmentally DBDE-exposed rats

Figure 1 shows a Venn diagram of genes showing altered expression in microdissected cerebral white matter in combination or individually in each DBDE exposure group. Numerous genes were found to be up- or downregulated commonly in DBDE-exposed groups. One hundred forty-five genes (129 genes upregulated; 16 genes downregulated) were identified showing altered expression commonly among all DBDE groups, and 893 genes (669 genes upregulated; 224 genes downregulated) were identified as showing altered expression commonly between 100 and 1000 ppm DBDE groups (Fig. 1 and Supplementary Tables 1–4: on-line only). Twelve genes (11 genes upregulated; 1 gene downregulated) were found to be brain development-related among those that commonly fluctuated in expression between all DBDE groups, and 70 genes (52 genes upregulated; 18 genes downregulated) were also identified as those related to brain development in a group of genes that commonly fluctuated between 100 and 1000 ppm DBDE groups (Supplementary Table 5: on-line only).

Comparison of the microarray data between the studies of DBDE and anti-thyroid agents

The global gene-expression profile of the cerebral white matter in the DBDE study was compared with that of the study using anti-thyroid agents[11]. Figure 2 shows a Venn diagram of genes with commonly altered expression between the studies of DBDE and anti-thyroid agents. Among upregulated genes, 4 genes were detected as those showing common fluctuation in all DBDE and anti-thyroid agents groups (Table 1). Forty-two genes were found to be upregulated commonly in 100 and 1000 ppm DBDE and all anti-thyroid agents groups. Thirty-three genes were upregulated commonly in 1000 ppm DBDE and all anti-thyroid agents groups. Downregulated genes were not observed commonly in all DBDE exposure groups and all anti-thyroid agents groups. Two downregulated genes were commonly observed in 100 and 1000 ppm DBDE and all anti-thyroid agents groups. Also, another two downregulated genes were commonly observed in 1000 ppm DBDE and all anti-thyroid agents groups.

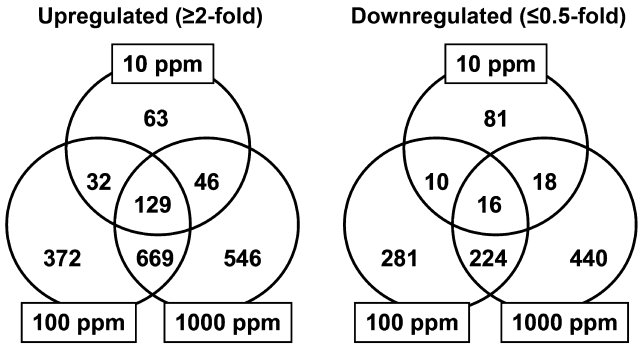

Fig. 1. Venn diagram of genes with altered expression in microarray analysis in response to maternal exposure to DBDE. (Left) Upregulated genes (≥ 2-fold). (Right) Downregulated genes (≤ 0.5-fold).

Vimentin and Ret in the cerebral white matter of BFR-exposed animals

Microarray analysis showed that vimentin and Ret commonly upregulated transcript levels between anti-thyroid agents and DBDE (Table 1). The immunohistochemical distributions of vimentin$^+$ or Ret$^+$ cells were found to be increased in the cingulum in our previous study of developmental hypothyroidism by maternal exposure to anti-thyroid agents[11]. We, therefore, evaluated the cellular distribution of these molecules in the white matter of BFR-exposed animals.

Vimentin$^+$ cells were scarcely distributed in the white matter tissue of untreated control animals (Fig. 3). With DBDE, the distribution of vimentin$^+$ cells was mainly observed in the cingulum and increased dose dependently with statistical significance at 100 and 1000 ppm as compared with the untreated controls (Fig. 3). In HBCD-exposed animals, vimentin$^+$ cells showed a similar distribution to the DBDE-exposed animals, showing a statistically significant increased distribution at 1000 and 10,000 ppm as compared with the untreated controls (Fig. 3). TBBPA-exposed animals did not show a significant change in the number of positive cells compared with the untreated control group at all doses (Fig. 3).

With regard to Ret, immunoreactive cells were sparsely observed in oligodendrocytes of the white matter (Fig.

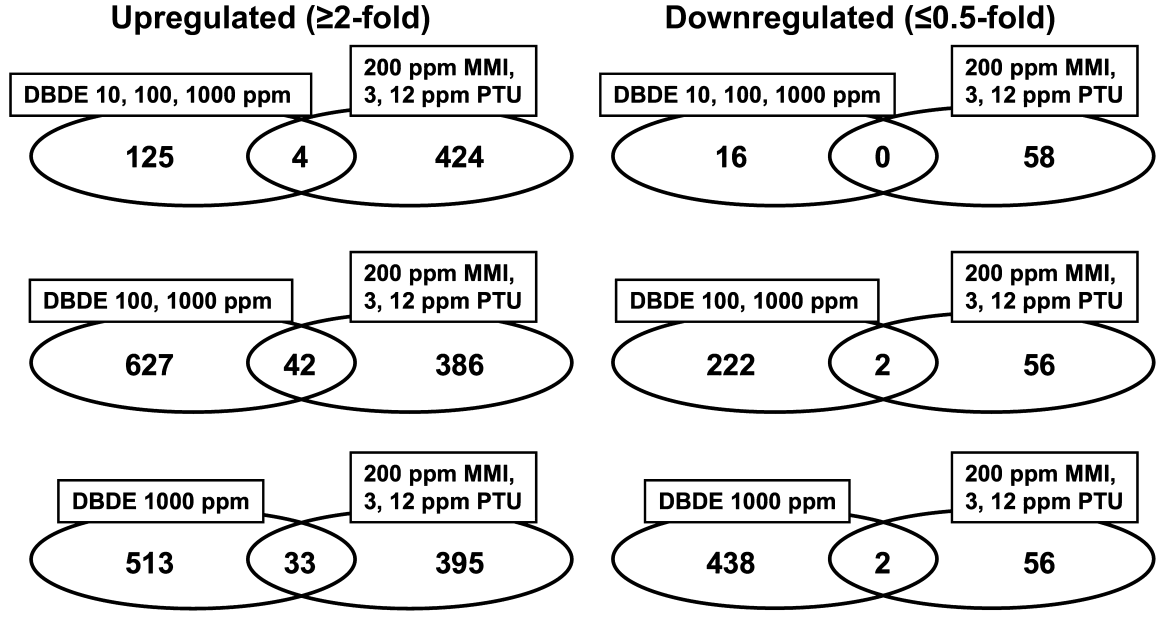

Fig. 2. Venn diagram of genes with altered expression in microarray analysis between the studies of DBDE and anti-thyroid agents, propylthiouracil (PTU) and methimazole (MMI). (Left) Upregulated genes (≥ 2-fold). (Right) Downregulated genes (≤ 0.5-fold).

Table 1. List of Genes with Commonly Altered Expression Between the Studies of DBDE and Anti-thyroid Agents (≥ 2-fold, ≤ 0.5-fold)

Accession no.	Gene title	Symbol	DBDE (ppm)			MMI (ppm)	PTU (ppm)	
			10	100	1000	200	3	12
DBDE 10, 100 and 1000 ppm, MMI 200 ppm and PTU 3 and 6 ppm commonly upregulated (4 genes)								
NM_031502	Amylase 2, pancreatic	Amy2	6.73	3.92	6.36	2.96	3.94	2.25
BE096287	EST	–	2.44	2.01	3.87	2.11	3.55	4.26
XM_002729411	Similar to Nkrp1f protein	LOC689809	2.33	3.38	2.48	2.56	3.11	2.65
AI237240	EST	–	2.22	2.42	2.03	3.15	3.50	3.18
DBDE 10, 100 and 1000 ppm, MMI 200 ppm and PTU 3 and 6 ppm commonly downregulated (0 genes)								
DBDE 100 and 1000 ppm, MMI 200 ppm and PTU 3 and 6 ppm commonly upregulated (42 genes)								
XM_001055507	Outer dense fiber of sperm tails 3B	Odf3b	1.55	5.87	29.88	3.91	8.63	7.91
NM_031686	Sodium channel, voltage-gated, type VII, alpha	Scn7a	0.89	7.34	11.60	4.31	42.69	17.08
XM_001060951	Coiled-coil domain containing 113	Ccdc113	1.33	2.08	11.31	8.32	20.95	18.68
NM_001106595	SET and MYND domain containing 1	Smyd1	1.13	6.35	9.10	8.21	9.71	13.66
XR_086009	Similar to CG8138-PA	LOC685158	0.92	2.13	7.07	3.26	5.09	4.73
NM_022213	Phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	Pik3r3	0.86	2.82	6.50	2.70	4.10	2.74
BF545930	EST	–	0.89	3.20	6.27	13.86	21.20	30.60
NM_001109289	Similar to RIKEN cDNA 1700012B09	RGD1561795	1.05	2.20	5.59	3.16	4.22	7.64
NM_001109024	DnaJ (Hsp40) homolog, subfamily C, member 30	Dnajc30	1.49	2.12	4.77	3.74	5.49	6.54
NM_031538	CD8a molecule	Cd8a	0.98	2.40	4.70	2.30	3.40	3.95
NM_001108066	AT rich interactive domain 3A	Arid3a	1.05	3.93	4.62	2.65	2.13	3.69
BF288845	EST	–	1.86	4.18	3.88	2.11	4.43	4.53
NM_001110099	Ret proto-oncogene	Ret	1.67	4.48	3.67	2.89	5.01	4.39
NM_001011947	Retinoic acid induced 14	Rai14	1.35	2.11	3.51	3.30	2.34	3.44
NM_012524	CCAAT/enhancer binding protein (C/EBP), alpha	Cebpa	0.95	2.63	3.36	2.49	2.52	2.01
NM_001106319	Cysteinyl-tRNA synthetase	Cars	1.15	4.23	3.31	2.35	4.23	4.16
NM_012849	Gastrin	Gast	1.10	3.30	3.26	4.40	5.81	4.33
NM_012715	Adrenomedullin	Adm	1.22	2.01	3.24	3.79	4.10	4.17
NM_001108463	Radial spokehead-like 2	Rshl2	1.75	2.87	3.22	3.81	5.27	6.01
NM_001010970	Amylase, alpha 1A	Amy1a	0.39	23.87	3.21	46.70	64.86	102.80
BM385125	EST	–	0.73	3.06	3.17	2.87	2.56	3.67
NM_001108238	Ankyrin repeat domain 12	Ankrd12	0.86	3.47	3.12	2.93	5.71	5.91
AI013206	EST	–	1.69	2.81	3.08	3.14	4.09	5.20
NM_001010970	Amylase, alpha 1A	Amy1a	1.20	8.40	3.08	8.37	11.41	17.62
NM_031140	Vimentin	Vim	1.65	2.82	2.99	2.11	6.01	4.27
NM_001109530	SAM pointed domain containing ets transcription factor	Spdef	1.22	2.09	2.96	2.20	2.99	3.43
AW142608	EST	–	1.01	2.76	2.75	2.73	6.42	4.92
NM_212459	ADP-ribosylation factor-like 9	Arl9	0.36	2.13	2.74	2.63	2.27	3.59
BF412962	EST	–	1.60	4.74	2.67	3.02	3.91	3.52
BF387484	EST	–	0.93	2.48	2.66	2.04	2.82	3.03
BF404369	EST	–	0.99	2.13	2.66	2.08	2.12	3.31
XM_002727399	Paired immunoglobin-like type 2 receptor alpha	Pilra	1.23	2.06	2.63	2.26	2.56	2.46
NM_080768	Tachykinin receptor 2	Tacr2	1.42	2.37	2.60	3.60	2.91	7.34
BF413876	EST	–	0.77	2.35	2.58	2.51	2.31	3.81
BF418099	EST	–	1.20	2.52	2.53	3.59	5.41	6.52
NM_001014008	Asporin	Aspn	1.08	3.61	2.46	3.89	8.18	5.50
NM_080581	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Abcc3	0.95	2.37	2.45	2.79	2.34	4.04
XM_001077495	Nuclear receptor co-repressor 1	Ncor1	0.69	2.28	2.32	2.67	2.01	2.97
NM_031017	cAMP responsive element binding protein 1	Creb1	1.21	2.01	2.17	2.19	2.81	2.86
NM_001107780	Hydroxyacid oxidase (glycolate oxidase) 1	Hao1	1.93	2.54	2.16	2.26	2.02	2.90
NM_053810	Synaptosomal-associated protein 29	Snap29	1.50	2.94	2.10	2.26	2.26	3.00
BE119364	EST	–	1.08	2.17	2.02	2.94	5.40	6.04
DBDE 100 and 1000 ppm, MMI 200 ppm and PTU 3 and 6 ppm commonly downregulated (2 genes)								
NM_019154	Amelogenin X chromosome	Amelx	1.22	0.28	0.13	0.31	0.07	0.09
NM_013107	Bone morphogenetic protein 6	Bmp6	1.60	0.48	0.43	0.23	0.38	0.25
DBDE 1000 ppm, MMI 200 ppm and PTU 3 and 6 ppm commonly upregulated (33 genes)								
NM_001015027	cAMP responsive element binding protein-like 2	Crebl2	1.02	0.90	14.38	5.02	10.61	11.53
XM_002724862	Similar to hypothetical protein FLJ23074	RGD1566400	1.23	1.70	4.36	4.23	18.89	10.14
NM_001014087	Coiled-coil domain containing 67	Ccdc67	1.28	1.59	4.34	3.50	15.54	8.25
XM_002724609	Family with sequence similarity 183, member B	Fam183b	1.12	1.03	3.60	5.34	10.84	11.19
NM_001134933	Cysteine-rich intestinal protein	Crip	0.90	1.67	3.38	3.91	10.16	8.74
NM_001033655	Dynein, axonemal, heavy chain 1	Dnah1	1.09	0.99	3.22	3.25	5.73	5.99
NM_001105991	ATPase, H+ transporting, lysosomal V1 subunit G3	Atp6v1g3	0.80	1.53	3.11	2.94	5.01	4.82
AI548601	EST	–	1.03	1.94	3.05	2.33	3.61	4.10
AI535351	EST	–	0.90	1.65	2.85	2.83	4.55	2.74

Table 1. Continued.

Accession no.	Gene title	Symbol	DBDE (ppm)			MMI (ppm)	PTU (ppm)	
			10	100	1000	200	3	12
NM_001004263	Integrin, beta 6	Itgb6	1.23	1.09	2.84	2.26	3.84	3.82
NM_001108055	Adenylate kinase 7	Ak7	1.00	1.26	2.83	2.88	7.39	6.24
BF409110	EST	–	1.39	1.09	2.76	2.59	5.90	5.61
NM_001107702	Fc receptor-like S, scavenger receptor	Fcrls	0.92	1.01	2.65	4.44	12.50	10.53
XM_001058430	Ribosomal protein L3-like	Rpl3l	0.76	1.69	2.62	2.31	2.05	3.52
AI231422	EST	–	0.34	1.16	2.59	2.67	8.39	6.24
NM_173094	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2	1.10	0.65	2.52	3.88	11.91	9.03
XM_001073839	Coiled-coil domain containing 30	Ccdc30	1.16	1.45	2.47	2.10	4.58	4.01
BF419834	EST	–	0.79	0.89	2.43	3.04	2.56	3.33
NM_001110155	RGD1565611	RGD1565611	1.16	0.71	2.37	2.64	5.17	4.74
AI043711	EST	–	0.64	1.33	2.34	2.22	2.78	2.60
BF406167	EST	–	1.32	1.13	2.33	4.69	4.51	7.33
NM_012840	Cytochrome c, testis	Cyct	0.86	1.95	2.32	3.49	3.54	3.60
AA859744	EST	–	1.20	0.82	2.29	2.62	3.28	3.60
NM_053293	Glutathione S-transferase theta 1	Gstt1	1.42	1.22	2.22	4.06	5.33	6.31
AA899303	EST	–	1.72	1.70	2.19	4.90	7.40	7.29
XM_001058720	Coiled-coil domain containing 74A	Ccdc74a	1.02	1.62	2.19	4.47	10.75	11.26
NM_001109074	Similar to novel protein	RGD1565283	0.79	1.39	2.19	3.48	9.39	7.58
BE096723	EST	–	0.99	0.95	2.18	2.26	2.62	3.57
NM_001106825	Hypothetical LOC300751	RGD1311874	0.77	1.66	2.16	2.81	2.33	3.99
NM_001005900	Hematopoietic cell signal transducer	Hcst	1.52	1.37	2.06	2.02	2.12	3.72
XM_001066515	Similar to hypothetical protein FLJ22527	RGD1305311	1.10	1.24	2.06	2.04	4.46	4.00
BF400248	EST	–	1.74	1.53	2.02	5.15	6.50	7.96
AA964532	EST	–	1.74	1.53	2.02	5.15	6.50	7.96
DBDE 1000 ppm, MMI 200 ppm and PTU 3 and 6 ppm commonly downregulated (2 genes)								
BG374305	EST	–	1.12	0.60	0.31	0.42	0.17	0.17
AT003256	EST	–	1.25	0.80	0.44	0.44	0.11	0.15

DBDE, decabromodiphenyl ether; MMI, methimazole; PTU, propylthiouracil; EST, expressed sequence tag.

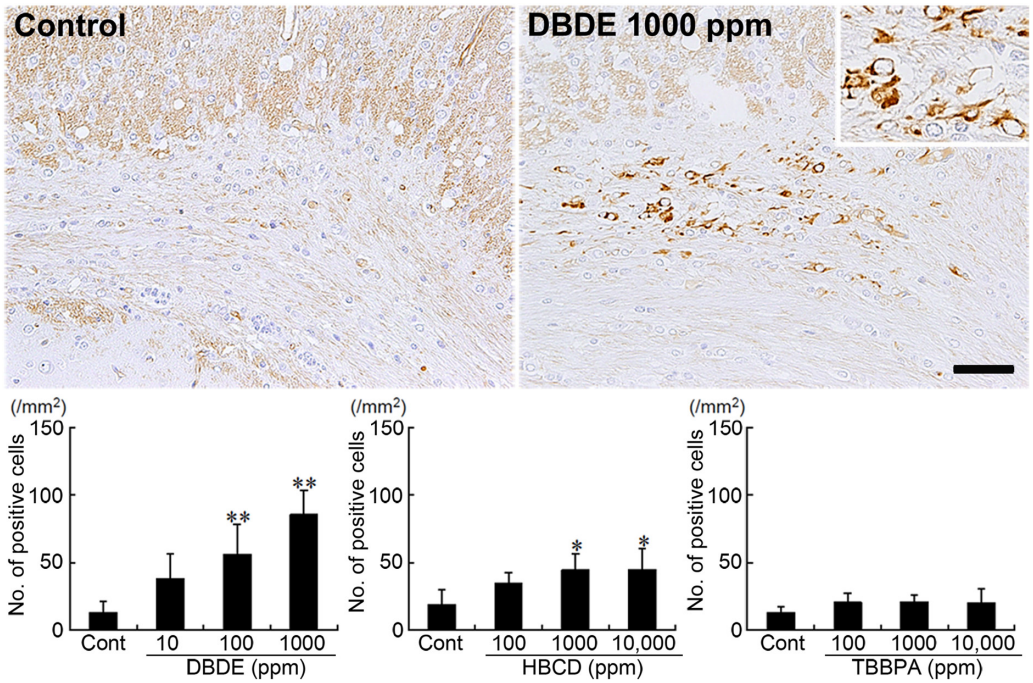

Fig. 3. Immunohistochemical distributions of vimentin$^+$ cells in the white matter tissue of BFR-exposed offspring. Untreated control animal (left) and 1000 ppm DBDE-exposed animal (right). 200× magnification (inset: 400× magnification). Bar = 50 µm. Graph shows the mean number of positive cells within the cingulum at 100× magnification (n = 5/group). Values are expressed as means + SD. * $P < 0.05$ vs. untreated controls, ** $P < 0.01$ vs. untreated controls.

4). DBDE exposure showed that distribution of Ret^+ cells was mainly observed in the cingulum and increased dose dependently with statistical significance in the number of positive cells in all dose groups as compared with untreated controls (Fig. 4). In HBCD-exposed animals, Ret^+ cells also increased, showing statistical significance at 1000 and 10,000 ppm groups as compared with untreated controls (Fig. 4). TBBPA-exposed animals did not show a significant change in the number of positive cells compared with the untreated control group at all doses (Fig. 4).

Immunohistochemical staining scores of molecules in the cerebral white matter of DBDE-exposed animals

Among genes selected from microarray analysis, we additionally examined immunohistochemical staining scores in molecules showing diffuse immunoreactivity in the white matter without specific cellular localization in DBDE-exposed animals. Immunoreactivity of Nrg1, Crk and Cld11 in the cerebral white matter was examined. Cld11 was found to show common downregulation between DBDE (100 and 1000 ppm) and all anti-thyroid agents (Table 1). Nrg1 and Crk showed upregulation at 100 and 1000 ppm DBDE (Supplementary Table 5). Because the 100 and 1000 ppm DBDE groups showed white matter hypoplasia targeting oligodendrocytes by morphological analysis, these molecules were selected[24].

Nrg1, Crk and Cld11 showed diffuse immunoreactivity in the white matter suggestive of the myelin sheaths (Fig. 5A–C). The immunoreactivity of Nrg1 or Crk showed a statistically significant increase at 1000 ppm as compared with untreated controls (Fig. 5A and B). The immunoreactivity of Cld11 showed a significant decrease at 10 ppm DBDE as compared with untreated controls. While statistically nonsignificant, 100 and 1000 ppm DBDE also tended to decrease the intensity scores of Cld11 (Fig. 5C).

Discussion

In our previous study[24], maternal exposure to DBDE induced white matter hypoplasia targeting oligodendrocytes beginning at 100 ppm in rat offspring accompanied by mild hypothyroidism at least at 1000 ppm. Using the same study samples, in the present study, we obtained a global gene expression profile of DBDE-responding genes in the cerebral white matter tissue consisting of the CC and external capsule collected using a microdissection technique. We further compared this expression profile with that responding to developmental hypothyroidism in rats using anti-thyroid agents[11]. As a result, there was a population of genes with commonly fluctuating expression between hypothyroidism and DBDE exposure, suggesting a similar mechanism to developmental hypothyroidism of action on the cerebral white matter by DBDE, while the gene expression changes observed only after DBDE exposure might be the direct brain effect of DBDE. Among genes showing commonly altered expression between hypothyroidism and DBDE exposure,

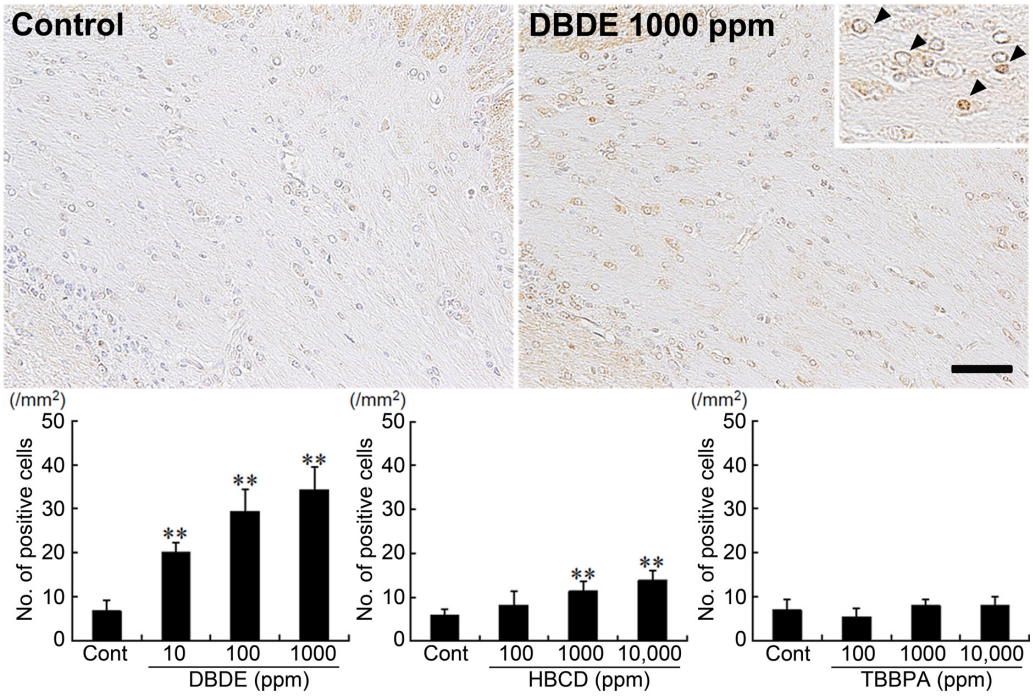

Fig. 4. Immunohistochemical distributions of Ret^+ cells in the white matter tissue of BFR-exposed offspring. Untreated control animal (left), 1000 ppm DBDE-exposed animal (right). 200× magnification (inset: 400× magnification, Ret^+ cells are indicated with arrowheads). Bar = 50 µm. Graph shows the mean number of positive cells within the cingulum at 100× magnification (n = 5/group). Values are expressed as means + SD. ** $P < 0.01$ vs. untreated controls.

vimentin and Ret were found to show an increased immunoreactive cell distribution after developmental hypothyroidism[11].

In the present study, increased distributions of vimentin$^+$ cells were observed in the cingulum of offspring maternally exposed to DBDE or HBCD at both middle and high doses on PND 20. These changes were well in accordance with the development of white matter hypoplasia detected at PND 77 by quantitative histomorphometric analysis in these cases, while a hypoplastic change was evident only at the high dose in HBCD-exposed cases[23, 24]. On the other hand, TBBPA-exposed offspring did not show changes in the number of vimentin$^+$ cells in the present study, in accordance with the lack of the development of white matter hypoplasia as reported in our previous study[23]. We previously found a similar immunohistochemical localization of vimentin and glial fibrillary acidic protein (GFAP) in the cingulum after developmental hypothyroidism[11]. Vimentin is expressed in immature astrocytes during development[29], and GFAP is expressed in both immature and mature astrocytes[30]. Therefore, vimentin$^+$ cells that appeared in response to DBDE or HBCD exposure may be immature astrocytes as seen in developmental hypothyroidism. While the reason for the increase in vimentin$^+$ cells in the cingulum as a result of exposure to DBDE or HBCD in the present study is not clear, we previously reported frequent induction of subcortical band heterotopia in the corpus callosum, manifested by the appearance of aberrant cortical tissue in this anatomical area, in hypothyroid animals[8]. While the anatomical location of this heterotopic tissue was close to the cingulum accumulating immature astrocytes in the hypothyroid cases, suggestive of an etiological relation between the two, we did not observe heterotopic tissue with DBDE and HBCD. As another possibility, the increased immature astrocytes may

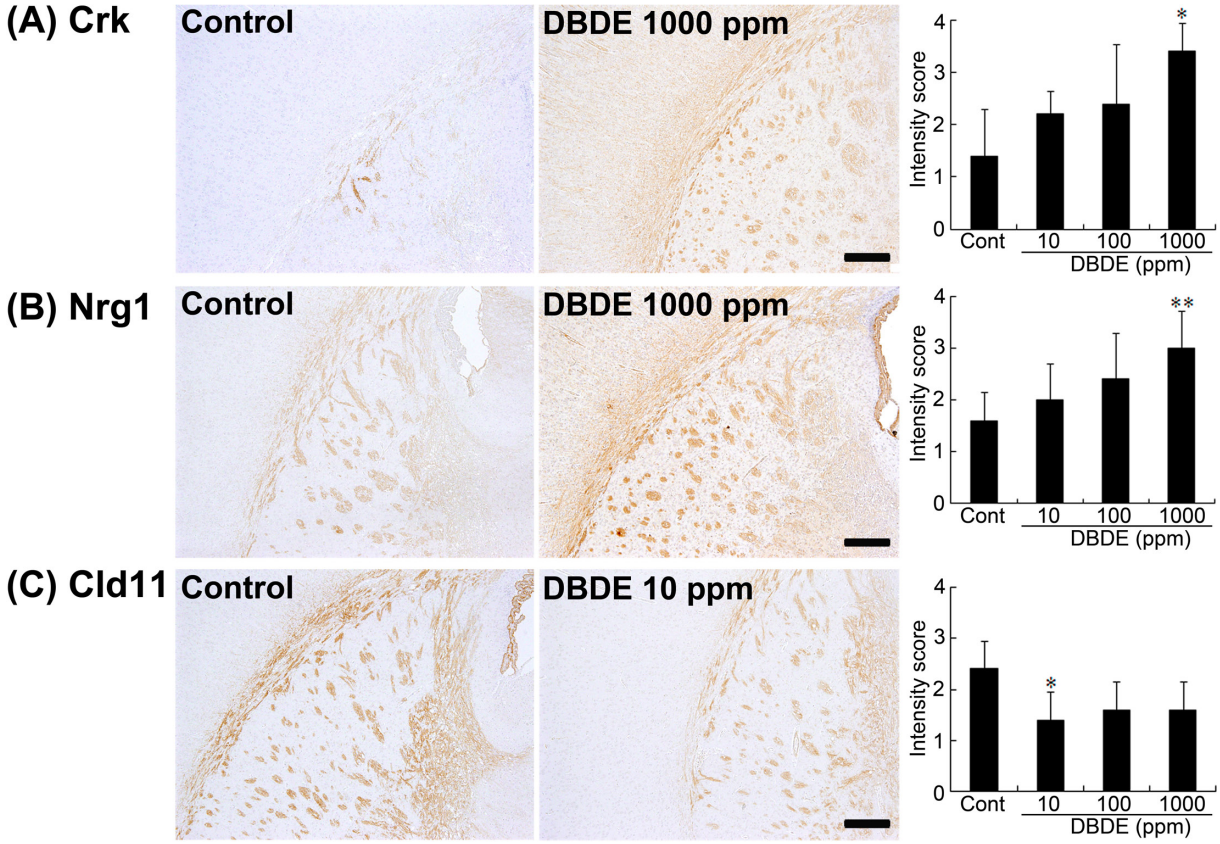

Fig. 5. Immunohistochemical distributions of Crk, Nrg1 and Cld11 in the white matter tissue of DBDE-exposed offspring. (A) Crk immunoreactivity in the myelin sheath of the external capsule, internal capsule and fimbria of the hippocampus. Untreated control animal (left), 1000 ppm DBDE-exposed animal (right). 40× magnification. Bar = 250 µm. Graph shows the mean intensity score of immunoreactivity at 40× magnification (n = 5/group). Values are expressed as means + SD. * $P < 0.05$ vs. untreated controls. (B) Nrg1 immunoreactivity in the myelin sheath of the external capsule, internal capsule and fimbria of the hippocampus. Untreated control animal (left), 1000 ppm DBDE-exposed animal (right). 40× magnification. Bar = 250 µm. Graph shows the mean intensity score of immunoreactivity at 40× magnification (n = 5/group). Values are expressed as means + SD. ** $P < 0.01$ vs. untreated controls. (C) Cld11 immunoreactivity in the myelin sheath of the external capsule, internal capsule and fimbria of the hippocampus. Untreated control animal (left), 10 ppm DBDE-exposed animal (right). 40× magnification. Bar = 250 µm. Graph shows the mean intensity score of immunoreactivity at 40× magnification (n = 5/group). Values are expressed as means + SD. * $P < 0.05$ vs. untreated controls.

simply be a reactive change in response to reduced oligodendrocytes as a result of exposure to DBDE or HBCD[23, 24].

In the present study, we found an increase of Ret^+ oligodendrocytes in the cingulum of offspring at all doses of DBDE and the middle and high doses of HBCD on PND 20, while TBBPA-exposed cases did not show any distribution changes of Ret^+ oligodendrocytes. Ret is a receptor protein-tyrosine kinase of glial cell line-derived neurotrophic factor (GDNF)[31]. It induces cell death in the absence of its ligand[32]. Because we did not find an increase in GDNF transcript levels in DBDE-exposed cases in microarray analysis in the present study, facilitation of apoptosis due to the increase of ligand-free Ret may be responsible for induction of white matter hypoplasia in DBDE and HBCD cases, as suggested in our previous hypothyroidism cases[11].

In the present study, Cld11 was found to be commonly downregulated in the white matter in the microarray analysis between developmental hypothyroidism and DBDE exposure. We, therefore, examined the immunohistochemical staining intensity of Cld11 in DBDE-exposed offspring. Cld11 is a four-transmembrane protein expressed in oligodendrocytes[33]. *In vitro* study has shown that Cld11 overexpression results in induction of oligodendrocyte proliferation[34]. This result indicates that the decrease in Cld11 immunoreactivity in the white matter may cause white matter hypoplasia in DBDE-exposed offspring[24].

Among genes showing altered expression by microarray analysis in DBDE study, Crk and Nrg1 showed an increase in immunoreactive intensity in the white matter of DBDE-exposed offspring at 1000 ppm in the present study. Crk is an adaptor molecule associated with the tyrosine phosphorylated Dab1 in the reelin pathway regulating the migration and maturation of newborn granule cells in the hippocampal dentate gyrus[35]. Nrg1 is a trophic factor that regulates neuronal migration, axonal pathfinding, neurotransmission and synaptic plasticity in the central nervous system[36]. The increased intensity of both Crk and Nrg1 only at 1000 ppm DBDE suggested that these molecules could be considered to be less sensitive biomarkers for detection of DBDE-induced effects than vimentin and Ret. Also, immunohistochemical expression of these molecules could not be measured quantitatively.

Both thyrotoxic effects[14, 15] and developmental neurobehavioral effects[14, 16] have been reported in mice and rats after exposure to DBDE. In our previous study, DBDE at 1000 ppm was suggested to cause developmental hypothyroidism in the same samples as used in the present study [24]. Zhang *et al.*[37] recently reported the tissue distribution, including the brain, of DBDE and its debrominated metabolites in suckling rat pups after prenatal and/or postnatal exposure, suggesting a possibility of direct effects of DBDE on the glial population changes. In the present study, we observed an increase in $vimentin^+$ immature astrocytes after exposure to DBDE beginning at 100 ppm (7.0–22.8 mg/kg body weight/day) and an increase in Ret^+ oligodendrocytes after exposure to DBDE beginning at 10 ppm (0.7–2.4 mg/kg body weight/day) on PND 20. We also observed that only around 6% of total genes showing altered expression due to DBDE exposure commonly fluctuated with developmental hypothyroidism due to exposure to anti-thyroid agents. These results suggest primarily a direct effect of DBDE on glial population changes accompanied by subtle hypothyroidism-related effects at 1000 ppm. The European food safety authority (EFSA) reported that average adult consumers were exposed to between 0.35 and 2.82 ng/kg body weight/day of DBDE via diet and between 0.045 and 7 ng/kg body weight/day via house dust[38]. Breast-fed infants were exposed to between 1.44 and 19.95 ng/kg body weight/day of DBDE via human milk[38]. In the present study, the treatment doses of DBDE were approximately $\times 10^6$ higher than the estimated daily intake level. In our previous study, effects on neural development were also observed after exposure to DBDE at 100 ppm and higher on PND 20[25]. There is a study suggesting a disruption of TH-mediated transcription by DBDE due to interference with the thyroid receptor-DNA binding domain[39]. These results suggest the potential of DBDE to suppress TH action in the brain; however, the effect may appear at extremely high doses as compared with human exposure levels.

We have previously shown a decrease in serum triiodothyronine (T_3) concentration and increase in serum thyroid-stimulating hormone concentration caused by HBCD at 10,000 ppm on PND 20 in the same samples as used in the present study; however, the magnitude of changes was rather mild[23]. As a hypothyroidism-related effect, we have previously reported reduction of the oligodendrocyte distribution at 10,000 ppm (803.2–2231.3 mg/kg body weight/day)[23]. We also observed effects on neuronal development caused by HBCD, at least at 10,000 ppm, similar to the effect on the number of oligodendrocytes in relation to developmental hypothyroidism[25]. In the present study, however, we detected increases in $vimentin^+$ immature astrocytes and Ret^+ oligodendrocytes at 1000 ppm (80.7–212.9 mg/kg body weight/day) and higher on PND 20, suggesting possible direct action on the developing brain. The EFSA reported that average adult consumers were exposed to between 0.09 and 0.99 ng/kg body weight/day of HBCD via diet and between 2.4 and 6 ng/kg body weight/day via house dust and that breast-fed infants were exposed to between 0.90 and 213 ng/kg body weight/day of HBCD via human milk[40]. In the present study, the treatment doses of HBCD were approximately $\times 10^7$ higher than the estimated daily intake level. There are studies that have shown a direct action of HBCD on TH receptors or competing potential of HBCD with thyroxin (T_4) for binding to transthyretin, a T_4 plasma transporter protein[41, 42]. Therefore, there is a possibility that HBCD affects brain development directly, although the effect may appear at extremely high doses as compared with human exposure levels.

With regard to TBBPA, we have shown no obvious thyrotoxic changes in the offspring except for a non-dose-related decrease in serum T_3 concentrations at 100 and 1000 ppm on PND 20 using the same samples as used in the present study[23]. While a direct effect on neuronal development

was suggested in our previous study[25], we did not detect any changes in the number of vimentin$^+$ immature astrocytes and Ret$^+$ oligodendrocytes as a result of developmental TBBPA exposure in the present study.

In conclusion, by means of microarray analysis of DBDE-exposed offspring, we found increased expression of vimentin and Ret in the cerebral white matter as observed with cases of developmental hypothyroidism[11]. By immunohistochemical analysis of these molecules in offspring developmentally exposed to BFRs, we found increases in vimentin$^+$ immature astrocytes at $\geq$ 100 ppm for DBDE and $\geq$ 1000 ppm for HBCD and in Ret$^+$ oligodendrocytes at $\geq$ 10 ppm for DBDE and $\geq$ 1000 ppm for HBCD. TBBPA did not show an effect on either vimentin$^+$ or Ret$^+$ cells. A direct effect of DBDE and HBCD on glial cell development may be considered under the present experimental conditions. At the highest dose of DBDE as well as that of HBCD, hypothyroidism may additionally be an inducing mechanism, although its contribution is rather minor. The observed brain effects occurred at levels extremely higher than the human exposure level of these compounds. The results from this study suggest that vimentin$^+$ and Ret$^+$ cells in the cerebral white matter may be used for assessment of the effects of developmental neurotoxicants mimicking TH-disrupting chemicals.

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