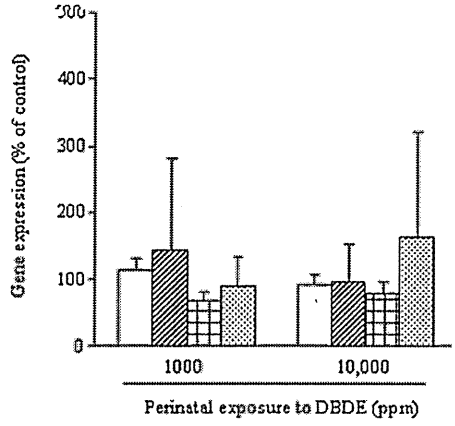

Fig. 1. Effects of perinatal exposure to DBDE on gene expressions of mouse innate pattern recognition receptors in lung tissues of RSV-infected offspring on day 1 post-infection. Amounts of mRNA of innate pattern recognition receptors were measured by real-time RT-PCR and normalized to the amount of mouse β-actin mRNA. The levels of gene expression of each molecule in DBDE-treated offspring mice were expressed as the percentages of mean of those in the Control group (n = 6). The data represent mean ± standard deviation of values of DBDE-treated at 1,000 ppm (n = 7), or 10,000 ppm (n = 6) offspring mice. Open bar, Toll-like receptor 3; shaded bar, Toll-like receptor 4; hatched bar, melanoma differentiation-associated gene-5; dotted bar, retinoic acid-inducible gene I.

that the US National Toxicology Program [1986] completed the carcinogenesis study using B6C3F1 mice exposed to DBDE at 50,000 ppm for 104 weeks. Teshima et al. [2008] reported that perinatal exposure to DBDE at 1,000 ppm, corresponded to the dose in this study, showed a weak immunomodulatory effect in offspring rat. Therefore, to evaluate a potential of DBDE as a developmental immunotoxicant, the much higher dosage, 10,000 ppm was used.

In the early phase of RSV infection in mice, the levels of TNF-α, IL-6, and IL-1β have been shown to increase locally in the lungs, which contribute to immunological host defenses against RSV infection, such as the reduction of pulmonary viral titers [Gordon and Read, 2002]. In this study, however, perinatal exposure to DBDE reduced significantly the levels of TNF-α and IL-6 in BALF on day 1 after infection, but increased significantly the level of IL-1β (Table II). Because viral titers were increased significantly by perinatal DBDE exposure on day 1 after infection (Table I), the perinatal exposure was probably responsible for the disorder of cytokine secretion against RSV infection in the early phase. Consequently, the primary immune response to RSV infection was disturbed, and viral titers increased in the lungs of offspring mice exposed to DBDE perinatally. It was shown already that perinatal DBDE exposure elevated significantly the level of IFN-γ on day 5 after infection [Watanabe et al., 2008b]. In this study, perinatal DBDE exposure increased significantly the level of IL-1β on day 1 after infection, suggesting that the increased IL-1β might activate T cells and affect the subsequent elevation of IFN-γ secretion.

Perinatal exposure to DBDE reduced significantly the levels of TNF-α and IL-6 in BALF on day 1 after infection, but the secretion of IL-1β increased significantly after DBDE exposure. The time point of 1 day after RSV infection corresponds to the phase from innate immunity to early induced immune response [Janeway et al., 2001]. In the early phase of RSV infection, TNF-α is produced mainly by macrophages and inhibits the progress of RSV infection both directly and indirectly [Neuzil et al., 1996]. IL-6 and IL-1β are produced from macrophages and activate T cells [Janeway et al., 2001]. Because production of IL-1β was reported to be regulated by caspase-1 and the nucleotide-oligomerization domain family [Franchi et al., 2009; Ichinohe et al., 2009] and the regulatory mechanism of IL-1β is different from those of TNF-α and IL-6, disorder of the signal transduction system for IL-1β may be involved in the unusual production of the cytokines. Although IL-12 is induced from macrophages [Janeway et al., 2001] and contributes to activation of NK cells, the levels of IL-12 were low on day 1 after infection in this study (Table II). NK cells are one of the key effectors of virus clearance, but it takes few days after RSV infection for NK cell to eliminate the virus [Hussell and Openshaw, 1998]. RSV infection does not induce significant levels of type I interferon [Hall et al., 1981]. Because macrophages are major effector cells for the clearance of initial infection of RSV [Gordon and Read, 2002], it is probable that DBDE exposure disordered the cytokine production by macrophages.

To reveal whether DBDE exposure damaged the function of TNF-α production in immune cells in lung tissues, ex vivo LPS stimulation tests were performed on bronchoalveolar lavage cells that were mainly the macrophage/monocyte-like cells (Table III). In DBDE-exposed offspring mice, the amount of LPS-induced TNF-α was increased rather than suppressed. These results suggest that DBDE does not damage cytokine production. Perinatal DBDE exposure caused probably some functional disorder of primary immune cells, like macrophages, responding to RSV infection. Because RSV infects predominantly epithelial cells in the respiratory tract, it is still possible that DBDE affected those cells remaining in lung tissues of offspring mice.

To investigate whether DBDE affected innate immunity, the levels of gene expression of innate pattern recognition receptors, which recognizes RSV and/or LPS [Haynes et al., 2001], were measured (Fig. 1). While no obvious change was observed in the gene expression of these molecules after DBDE exposure, involvement of DBDE in the processing of viral antigen in macrophages was not denied in innate immunity. It was reported that pentabrominated diphenyl ether (DE-71) induces respiratory burst, which is associated with the processing of viral antigen, in human granulocytes [Reistad and Mariussen, 2005]. DBDE may affect the processing of viral antigen and induce subsequently disorder of cytokine production in macrophages.

This study revealed that perinatal exposure to DBDE-induced disorder of the primary immune response to RSV infection in offspring mice. It is obvious that the disorder triggered the developmental immunotoxicity of

DBDE. However, we did not examine the effects of perinatal exposure to DBDE on cytotoxic T lymphocytes, which are the major effector cells in adaptive immune response and contribute to RSV clearance. Recent study in the rat has shown that perinatal exposure to DBDE decreased the populations of peripheral NK cells, and the proportions of splenic activated T and B cells in offspring [Teshima et al., 2008]. In the report, association between anti-thyroid activity and immune suppressive effects due to perinatal exposure to DBDE was discussed. However, DBDE might affect immune system directly in this study, because mouse was shown to be resistant to anti-thyroid effect [Watanabe et al., 2008a]. It is doubtful that DBDE affects only the primary immune response. Therefore, further studies on adaptive immune responses, including humoral immune response, are needed to illuminate the mechanism of action of DBDE on the developmental immunotoxicity.

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No effect of sustained systemic growth retardation on the distribution of Reelin-expressing interneurons in the neuron-producing hippocampal dentate gyrus in rats

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ABSTRACT

Reelin signaling plays a role in neuronal migration and positioning during brain development. To clarify the effect of systemic growth retardation on the distribution of Reelin-expressing interneurons in the hilus of the hippocampal dentate gyrus, pregnant rats were fed a synthetic diet with either a normal (20% casein) or low (10% casein) protein concentration from gestational day 10 to postnatal day (PND) 21 at weaning. Male offspring were immunohistochemically examined at PND 21 and on PND 77. Protein-restricted offspring displayed systemic growth retardation through PND 77 and had decreased absolute brain weights and an increased number of external granular cells in the cerebellar cortex, suggestive of retarded brain growth at weaning. However, maternal protein restriction did not change the cellular distribution of immunoreactivity for Reelin, Calbindin-D-28K, or glutamic acid decarboxylase 67 or of NeuN-positive postmitotic neurons in the dentate hilus either at PND 21 or PND 77, which suggests that the population of γ-aminobutyric acid-ergic interneurons involving synthesis of Reelin was not affected. Furthermore, as well as the distribution of hilar neurons expressing neurogenesis-related FoxG1, cell proliferation and apoptosis in the subgranular zone were unaffected through PND 77. These results suggest that systemic growth retardation caused by maternal protein restriction does not affect neuronal migration and postnatal neurogenesis of the dentate gyrus resulting in unaltered distribution of Reelin-synthesizing interneurons.

1. Introduction

In the hippocampal formation, neuronal subpopulations are known to produce Reelin from the embryonic period throughout adult life [1–5]. Reelin is a secreted extracellular matrix glycoprotein that plays a critical role in neuronal migration and positioning during brain development [2]. Its secretion is regulated by thyroid hormone [6]. Also, it has been suggested that Reelin release by γ-aminobutyric acid (GABA)ergic interneurons may regulate the migration and maturation of newborn granular cells in the dentate granular cell layer in adults [7]. Altered Reelin signaling has been reported in the dentate gyrus in some neurological disease conditions, such as depression and epilepsy [7,8]. Within the hippocampal formation, the dentate gyrus is a unique structure that can continue neurogenesis during postnatal life and that is a well-known target of developmental hypothyroidism [9].

Experimentally, developmental hypothyroidism leads to systemic growth retardation, neurological defects and impaired performance in a variety of behavioral learning tests [10,11]. The offspring of rats exposed to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) show impaired brain development, with aberrant neuronal migration and white matter hypoplasia involving limited axonal myelination and reduced oligodendrocytic distribution [12–14]. To detect a key molecular event reflecting the permanent disruption of neuronal development due to exposure to xenobiotic chemicals that can interfere with thyroid hormone signaling, we recently examined the change in the temporal distribution of Reelin-expressing cells in the dentate gyrus in the offspring of

Abbreviations: CA1, cornu ammonis 1; CA2, cornu ammonis 2; CA3, cornu ammonis 3; Calb-D-28K, Calbindin-D-28K; FoxG1, forkhead box G1; GABA, γ-aminobutyric acid; GAD67, glutamic acid decarboxylase 67; GD, gestational day; MMI, methimazole; NeuN, neuron-specific nuclear protein; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PND, postnatal day; PTU, 6-propyl-2-thiouracil; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; T_3, triiodothyronine.

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Table 1
Composition of normal and low protein diets.

	Normal protein diet (%)	Low protein diet (%)
Casein	20.00	10.00
L-Cystine	0.30	0.30
Corn starch	39.75	47.25
α-Corn starch	13.20	15.70
Sucrose	10.00	10.00
Soy oil	7.00	7.00
Cellulose	5.00	5.00
Mineral	3.50	3.50
Vitamin	1.00	1.00
Choline	0.25	0.25
tert-Butylhydroquinone	0.0014	0.0014

rats exposed to anti-thyroid agents during gestation and lactation [15]. As a result, an increase in GABAergic Reelin-synthesizing interneurons with immature phenotype that was sustained into the later stage at PND 77 was observed in the dentate hilus, which is suggestive of a compensatory mechanism for the impaired neurogenesis and mismigration caused by exposure to thyroid hormone-disrupting chemicals during neuronal development.

Systemic growth retardation of the offspring caused by maternal and/or offspring toxicity may cause a delay in brain development. In the above-mentioned study, we also observed a suppression of systemic growth in the offspring after exposure to anti-thyroid agents that caused apparent hypothyroidism in both dams and offspring [15,16]. It is possible that the sustained increase in Reelin-expressing cells is caused by the delayed brain growth accompanied by systemic growth retardation rather than the hypothyroidism-related impairment of thyroid hormone signaling in the brain. We also observed a sustained increase in Reelin-expressing cells even with mild hypothyroidism at a level that did not cause sustained body growth retardation through the later stage at PND 77. However, a slight, non-significant suppression of body growth was also detected in these animals at the end of developmental hypothyroidism on weaning. Because the high doses of chemicals in developmental toxicity studies sometimes cause systemic growth retardation of the offspring, any chemical-specific neurodevelopmental effects should be distinguished from those caused by systemic growth retardation resulting in delayed brain growth secondary to the systemic toxicity in dams and/or offspring.

In the present study, to clarify the effects of delayed brain growth on the distribution of Reelin-expressing interneurons in the dentate hilus, pregnant rats were fed a synthetic low-protein diet from the mid-gestation to the end of the lactation period to cause growth restriction in the offspring utilizing an intrauterine growth restriction model [17,18]. The distributional changes of GABAergic interneuron markers in the dentate hilus were investigated, as well as the effects on the neurogenesis of the subgranular zone in terms of cell proliferation and apoptosis.

2. Materials and methods

2.1. Animals, diets and experimental design

Pregnant Crl:CD®(SD) rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at gestational day (GD) 1 (appearance of vaginal plugs was designated as GD 0). Animals were housed individually in mesh cages in an air-conditioned animal room (temperature: 23 ± 2 °C; relative humidity: 45 ± 10%) with a 12-h light/dark cycle and were allowed *ad libitum* access to food and tap water. Animals were housed individually with their litter in plastic cages with wood chip bedding from GD 17 to postnatal day (PND) 21 (PND 0: the day of birth).

Pregnant rats were fed a CRF-1 basal diet (Oriental Yeast Co. Ltd.) from GD 1 to GD 10. Eight dams per group were then randomly divided into two groups and fed a synthetic diet with either a normal (20% casein) or a low (10% casein) protein concentration from GD 10 to PND 21. Compositions of the synthetic diets are shown in Table 1.

On PND 4, the litters were culled randomly, leaving four male and four female offspring per dam. On PND 21, 10 male and 10 female offspring per group from 8 dams (one or two males and one or two females per dam) were subjected to prepubertal necropsy.

The remaining animals were kept until PND 77. All offspring consumed the CRF-1 basal diet and tap water *ad libitum* from PND 21 onwards. On PND 77, 10 male and 10 female offspring per group from 8 dams (one or two males and one or two females per dam) were subjected to necropsy [16,19].

All animals used in the present study were weighed and killed by exsanguination from the abdominal aorta under deep anesthesia with ether. All procedures of this study were conducted in compliance with the "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee at BOZO Research Center Inc. All efforts were made to minimize animal suffering.

2.2. Histopathology, immunohistochemistry and apoptotic cell detection

The brains of the male offspring killed at PND 21 and PND 77 were fixed in Bouin's solution at room temperature overnight. Ten identical male animals from 8 dams (one or two males per dam) per group were subjected to analyses on histopathology, immunohistochemistry and apoptotic cell detection at each time point. Coronal slices at the positions of −3.0 and −3.5 mm from the bregma were prepared from the PND 21 and PND 77 brains, respectively. Brains were routinely processed for paraffin embedding, sectioned at 3 μm, and stained with hematoxylin and eosin for light microscopy.

For immunohistochemistry studies, the brain sections (3 μm in thickness) were incubated at 4 °C overnight with antibodies against Reelin (clone G10, mouse IgG_1, 1:1,000; Novus Biologicals, Inc., Littleton, CO, United States), neuron-specific nuclear protein (NeuN; clone A60, mouse IgG_1, 1:100, Millipore Corporation, Temecula, CA, United States), which specifically detects postmitotic neurons [20], calbindin-D-28K (Calb-D-28K; clone CB-955, mouse IgG_1, 1:500; Sigma Chemical Co., St. Louis, MO, United States), a Ca-binding protein that is expressed in the GABAergic interneurons [21], glutamic acid decarboxylase 67 (GAD67; clone 1G10.2, mouse IgG_{2a}, 1:50, Millipore Corporation), a GABA synthesizing enzyme that is expressed in GABAergic neurons [22], forkhead box G1 (FoxG1; rabbit IgG, 1:800; LifeSpan Bioscience, Inc., Seattle, WA, United States), a transcription factor that regulates neurogenesis in the embryonic telencephalon and the postnatal hippocampus [23,24], and proliferating cell nuclear antigen (PCNA; clone PC10, mouse IgG_{2a}, 1:200, Dako, Glostrup, Denmark). Antigen retrieval treatment was not performed for these antigens. To quench endogenous peroxidase, the slides were incubated in 0.3% hydrogen peroxide in absolute methanol for 30 min. Immunodetection was carried out using a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, United States) with 3,3′-diaminobenzidine/H_2O_2 as the chromogen, as previously described [25]. The sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

For evaluation of apoptosis in the subgranular zone of the dentate gyrus, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was applied to brain sections. Deparaffinized sections were treated with 20 μg/mL of proteinase K in phosphate buffered saline (PBS; pH 7.4) for 15 min at room temperature, and then incubated in 3.0% hydrogen peroxide in PBS for 5 min. Detection of apoptotic cells was carried out using the Apop Tag® *in situ* apoptosis detection kit (Millipore Corporation) according to the instructions provided by the manufacturer with 3,3′-diaminobenzidine/H_2O_2 as the chromogen.

2.3. Morphometry of immunolocalized cells and apoptotic cells

Reelin-, NeuN-, Calb-D-28K-, GAD67- or FoxG1-positive cells distributed in the hilus of the dentate gyrus were bilaterally counted and normalized for the number per area unit of the hilar area (polymorphic layer) as previously described [15]. In the subgranular zone of the dentate gyrus, apoptotic cells as detected by TUNEL method and proliferating cells as detected by nuclear immunoreactivity of PCNA were bilaterally counted and normalized for the length of the granular cell layer measured as previously described [15]. For quantitative measurement of each immunoreactive cellular component, digital photomicrographs at 100-fold magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a DP70 Digital Camera System (Olympus Optical Co.). Quantitative measurements were performed using the WinROOF image analysis software package (version 5.7, Mitani Corp., Fukui, Japan).

2.4. Statistical analysis

Maternal data regarding the body weight and food consumption during the animal study were analyzed using the individual animal as the experimental unit. Data for offspring regarding the body weight, food consumption, organ weight at necropsy, immunohistochemical data, and TUNEL-assay data, were analyzed using the litter as the experimental unit. Differences between the normal and low protein diet groups were evaluated using the following methods. An *F*-test for equal variance was used to determine if the variance was homogenous between the groups. If the variance was homogenous, numerical data were assessed using Student's *t*-test to compare between the normal and low protein diet groups. If a significant difference in variance was observed, the Aspin–Welch's *t*-test was used instead.

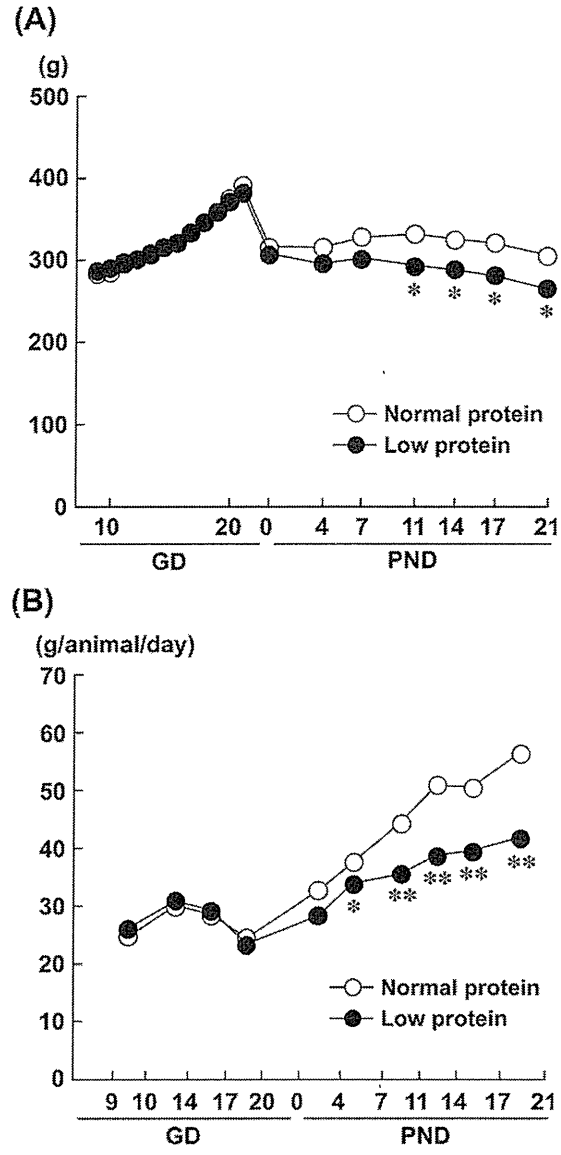

Fig. 1. Body weights and food consumption of dams fed either a normal or low protein diet from GD 10 to PND 21. (A) Body weight. (B) Food consumption. *,** Significantly different from the normal protein group by Student's or Aspin–Welch's *t*-test (*$P < 0.05$; **$P < 0.01$).

Data for offspring regarding the incidences and severity of histopathological lesions were analyzed using the individual animal as the experimental unit. The incidences of histopathological lesions were statistically compared using the Fisher's exact probability test. Severity of histopathological changes was compared using the Mann–Whitney's *U*-test.

3. Results

3.1. Effects on dams

During the gestational period, there were no statistically significant differences in body weight and food consumption between the normal and low protein diet groups (Fig. 1). During the lactation period, body weights of dams and food consumption per animal were significantly lower in the low protein diet group than in the normal protein diet group from PND 11 and PND 4 onwards, respectively (Fig. 1). Food consumption per body weight was also decreased from PND 11 to PND 21 (data not shown).

3.2. Effects on offspring growth

Throughout the lactation period, body weights of the offspring of both sexes were significantly lower in the low protein diet group than in the normal protein diet group (Table 2).

After weaning, body weights were significantly lower in the low protein group than in the normal protein diet group in both sexes through PND 77 (Table 2). Food consumption per animal was suppressed in the low protein group from PND 35 to PND 77 in males and from PND 56 to PND 77 in females (Table 3). Conversely, food consumption per body weight was increased in the low protein diet group from PND 35 to PND 63 and on PND 77 in males and from PND 35 to PND 49 in females.

3.3. Effects on organ weights of the offspring

At necropsy on PND 21, statistically significant decreases in the absolute brain and liver weights were observed in both sexes in the low protein diet group (Table 4). However, relative brain weights in both sexes and relative liver weights in males were significantly increased in the low protein diet group.

At necropsy on PND 77, statistically significant decreases in the absolute kidney weights in both sexes and liver and testes weights in males were observed in the low protein diet group. However, relative brain weights were significantly increased in males in the low protein diet group.

3.4. Histopathological changes

On histopathological analysis of the pups on PND 21, the grade of external granular cells remaining in the cerebellar cortex in the low protein group was significantly higher than in the normal protein group (Table 5).

On PND 77, there were no histopathological alterations in the brains of both low and normal protein diet groups.

3.5. Morphometry of immunolocalized cells in the dentate gyrus

On PND 21, Reelin expression was observed in the cytoplasm of neurons located within the dentate hilus (Fig. 2). Immunoreactivity for NeuN was observed exclusively in the nucleus of granular cells but not in cells located in the subgranular zone. NeuN-positive neurons were also observed within the dentate hilus, showing cytoplasmic immunoreactivity in addition to nuclear immunolocalization, as reported by others [26]. Calb-D-28K was expressed in both the cytoplasm and the nucleus in the granular cells and neurons within the dentate hilus. GAD67-expression was observed in the cytoplasm of the neurons located in the hilus and those sparsely distributed in the granular cell layer. FoxG1 expression was observed in the cytoplasm in neurons within the hilus and in granular cells, except for the cells located at the subgranular zone. There were no differences in the number of Reelin-, NeuN-, Calb-D-28K-, GAD67- or FoxG1-positive cells distributed in the hilus of the dentate gyrus between the normal and low protein diet groups (Fig. 2).

On PND 77, the cellular distribution of each molecule was similar to that observed on PND 21, while cells located in the subgranular zone were reduced, judging from the very sparse distribution of negative cells for NeuN and FoxG1 (Fig. 3). Within the dentate hilus, the number of positive cells for each molecule was lower on PND 77 than on PND 21. As on PND 21, there were no differences in the number of positive cells for any of the molecules in the dentate hilus between the normal and low protein diet groups (Fig. 3).

Table 2
Body weight of offspring after maternal protein restriction during the second half of gestation and lactation.

	Males		Females	
	Normal protein20%[c]	Low protein10%[c]	Normal protein20%[c]	Low protein 10%[c]
No. of offspring examined[a]	16	16	16	16
Body weight (g)				
PND 0	6.6 ± 0.6[b]	6.3 ± 0.4**	6.1 ± 0.4	5.9 ± 0.5*
PND 4	10.9 ± 1.4	9.0 ± 1.1**	10.4 ± 1.5	8.5 ± 1.0**
PND 7	17.5 ± 2.3	13.7 ± 1.9**	16.4 ± 2.6	12.4 ± 1.8**
PND 11	28.9 ± 3.6	21.4 ± 1.6**	27.0 ± 3.7	19.2 ± 2.3**
PND 14	38.4 ± 4.4	26.6 ± 1.5**	36.2 ± 3.9	24.5 ± 2.3**
PND 17	46.5 ± 5.5	31.6 ± 1.9**	43.9 ± 4.5	29.2 ± 2.5**
PND 21	61.4 ± 7.0	40.4 ± 2.4**	58.0 ± 6.1	38.0 ± 3.6**
PND 28	102.8 ± 10.9	75.4 ± 4.7**	90.1 ± 9.6	70.8 ± 4.1**
PND 35	174.5 ± 16.3	136.5 ± 8.1**	142.3 ± 12.3	121.4 ± 6.6**
PND 42	253.0 ± 19.6	203.1 ± 9.6**	181.3 ± 12.4	160.6 ± 8.4**
PND 49	322.8 ± 21.7	263.2 ± 13.6**	206.2 ± 13.6	187.5 ± 12.1**
PND 56	382.8 ± 22.1	318.6 ± 15.0**	228.7 ± 14.4	207.2 ± 15.1**
PND 63	429.4 ± 25.9	358.9 ± 20.5**	255.4 ± 19.1	231.1 ± 18.8**
PND 70	474.5 ± 28.0	398.3 ± 23.1**	276.8 ± 23.3	247.4 ± 20.4*
PND 77	508.3 ± 30.6	427.0 ± 26.9**	285.3 ± 22.5	254.9 ± 22.6*

*, **Significantly different from the normal protein group by Student's or Aspin–Welch's *t*-test ($P < 0.05$; $P < 0.01$).
Abbreviation: PND, postnatal day.
[a] Identical two male and two female offspring per dam ($n = 8$/group) were used for body weight measurement throughout the experiment. Statistical analysis was performed using the litter as the experimental unit.
[b] Mean ± SD.
[c] Casein level.

3.6. Apoptotic and proliferating cell indices in the dentate subgranular zone

On PND 21, there were very few TUNEL-positive apoptotic cells in the subgranular zone of the dentate gyrus, and there was no difference in the number per unit length between the normal and low protein diet groups (Fig. 4). There were no differences in the number of PCNA-positive cells in the subgranular zone per unit length between the normal and low protein diet groups (Fig. 4).

On PND 77, only one TUNEL-positive apoptotic cell was detected in one animal in the normal protein diet group and there were no apoptotic cells in any of the animals in the low protein diet group (Fig. 4). There were no differences in the PCNA-positive cell ratio in the subgranular zone between the normal and low protein diet groups, similar to PND 21 (Fig. 4).

4. Discussion

In the present study, maternal protein restriction did not change the cellular distribution of immunoreactivity for Reelin, Calb-D-28K, or GAD67 in the dentate hilus on either PND 21 or PND 77. This suggests that the populations of GABAergic interneurons involving Reelin-synthesizing ones were not affected by maternal protein restriction through PND 77. This result is in contrast with the increases in GABAergic interneurons with immature phenotype in the hilus facilitating Reelin synthesis and in NeuN-

Table 3
Food consumption of offspring after maternal protein restriction during the second half of gestation and lactation.

	Males		Females	
	Normal protein20%[c]	Low protein10%[c]	Normal protein20%[c]	Low protein10%[c]
No. of offspring examined[a]	16	16	16	16
Food consumption (g/animal/day)				
PND 35	19.6 ± 1.7[b]	17.1 ± 0.7**	16.6 ± 1.7	15.5 ± 0.9
PND 42	27.3 ± 2.0	23.4 ± 1.2**	19.9 ± 1.6	19.1 ± 1.1
PND 49	30.4 ± 2.2	25.9 ± 1.4**	20.2 ± 1.2	19.0 ± 1.2
PND 56	32.3 ± 1.9	28.0 ± 1.6**	20.6 ± 1.3	19.1 ± 1.4*
PND 63	31.6 ± 2.0	27.9 ± 1.8**	20.9 ± 1.5	19.2 ± 1.4*
PND 70	32.1 ± 2.1	27.9 ± 2.0**	22.2 ± 1.9	20.3 ± 1.3*
PND 77	31.3 ± 2.1	27.4 ± 2.4**	20.7 ± 1.9	18.9 ± 1.5*
Food consumption (g/kg body weight/day)				
PND 35	141.7 ± 5.1	161.4 ± 9.0**	143.1 ± 8.3	161.1 ± 6.4**
PND 42	127.6 ± 3.4	137.8 ± 5.7**	122.9 ± 7.8	135.0 ± 4.7**
PND 49	105.8 ± 5.2	111.0 ± 4.0**	104.3 ± 6.6	109.1 ± 4.3*
PND 56	91.5 ± 4.6	96.3 ± 4.6**	94.6 ± 4.7	96.6 ± 3.4
PND 63	77.9 ± 3.9	82.3 ± 4.1**	86.5 ± 3.9	87.7 ± 5.4
PND 70	71.1 ± 4.0	73.6 ± 3.7	83.3 ± 4.6	84.8 ± 3.2
PND 77	63.8 ± 3.2	66.3 ± 3.5*	73.6 ± 4.0	75.3 ± 3.9

*, **Significantly different from the normal protein group by Student's or Aspin–Welch's *t*-test ($P < 0.05$; $P < 0.01$).
Abbreviation: PND, postnatal day.
[a] Identical two male and two female offspring per dam ($n = 8$/group) were used for food consumption measurement after weaning. Statistical analysis was performed using the litter as the experimental unit.
[b] Mean ± SD.
[c] Casein level.

Table 4
Organ weights of offspring after maternal protein restriction during the second half of gestation and lactation.

	Males		Females	
	Normal protein20%[c]	Low protein10%[c]	Normal protein20%[c]	Low protein10%[c]
PND 21				
No. of offspring examined[a]	10	10	10	10
Brain				
(g)	1.55 ± 0.05[b]	1.43 ± 0.03**	1.45 ± 0.05	1.34 ± 0.09**
(g/100 g BW)	2.38 ± 0.23	3.63 ± 0.40**	2.57 ± 0.19	3.98 ± 0.68**
Liver				
(g)	2.56 ± 0.39	1.67 ± 0.35**	2.36 ± 0.27	1.43 ± 0.41**
(g/100 g BW)	3.88 ± 0.23	4.16 ± 0.37*	4.15 ± 0.22	4.06 ± 0.43
PND 77				
No. of offspring examined[a]	10	10	10	10
Brain				
(g)	2.05 ± 0.06	2.01 ± 0.07	1.88 ± 0.05	1.85 ± 0.08
(g/100 g BW)	0.42 ± 0.02	0.48 ± 0.04**	0.66 ± 0.09	0.72 ± 0.06
Liver				
(g)	18.30 ± 2.58	15.16 ± 1.89**	10.07 ± 2.05	9.51 ± 1.46
(g/100 g BW)	3.68 ± 0.30	3.57 ± 0.18	3.48 ± 0.21	3.67 ± 0.24
Kidneys				
(g)	3.17 ± 0.50	2.83 ± 0.26*	2.07 ± 0.27	1.85 ± 0.15*
(g/100 g BW)	0.64 ± 0.07	0.67 ± 0.05	0.71 ± 0.04	0.73 ± 0.05
Testes				
(g)	3.58 ± 0.30	3.20 ± 0.47*	n.a	n.a
(g/100 g BW)	0.72 ± 0.05	0.76 ± 0.09	n.a.	n.a.
Ovaries				
(mg)	n.a.	n.a	97.3 ± 17.6	83.9 ± 9.9
(mg/100 g BW)	n.a.	n.a.	33.9 ± 4.3	32.8 ± 5.1

*, **Significantly different from the normal protein group by Student's or Aspin–Welch's *t*-test ($P < 0.05$; $P < 0.01$).
Abbreviations: BW, body weight; PND, postnatal day.
[a] One or two offspring of each sex per dam ($n = 8$/group) were subjected to autopsy and following organ weight measurement at each time point. Statistical analysis was performed using the litter as the experimental unit, and mean values were estimated as a litter value when two offspring were examined from the same dam.
[b] Mean ± SD; n.a.: not applicable.
[c] Casein level.

positive postmitotic neurons that we observed after developmental hypothyroidism and that were sustained through PND 77, which is suggestive of a mature population of GABAergic interneurons [15]. We observed neither an increase in NeuN-positive neurons in the hilus nor changes in cell proliferation or apoptosis in the subgranular zone in offspring of any age as a result of maternal protein restriction. The latter is in contrast with the increased apoptosis and decreased proliferation observed on weaning after developmental hypothyroidism, which is suggestive of impaired neurogenesis [15]. We also observed no changes in the distribution of hilar FoxG1-expressing cells after maternal protein restriction. Considering the role of FoxG1 in the regulation of neurogenesis in the postnatal hippocampus [24], and the lack of alterations in apoptosis and proliferation in the subgranular zone, maternal protein restriction may not affect postnatal neurogenesis of the dentate gyrus, reflected by an unchanged distribution of GABAergic interneurons.

Developmental hypothyroidism results in systemic growth retardation of the offspring in rats [10]. We have detected a similar effect of developmental exposure to anti-thyroid agents in rats, in which treatment of dams with 12 ppm PTU and 200 ppm methimazole (MMI) in the drinking water from GD 10 to weaning induced apparent and equivalent effects on changes in serum thyroid-related hormones in the offspring at weaning [16]. Body weights of the offspring in the hypothyroid cases were 64.2–68.8% of the non-treated group at weaning (Table 6). In the present study, a similar magnitude of offspring body weight reduction was observed after maternal protein restriction, with values being 65.5–65.8% of those in the normal protein group (Table 6). In our previous study, developmental hypothyroidism caused only a weak reduction in the absolute brain weights on weaning, with values after treatment with 12 ppm PTU and 200 ppm MMI being 94.5–97.9% of untreated controls (Table 6). However, maternal protein restriction was shown to decrease offspring brain weights [27,28], and in the present study we also observed greater reductions in absolute brain weights (91.7–92.9%) than in the developmental hypothyroidism study (Table 6). We also observed an increase in external granular cells in the cerebellar cortex on weaning after maternal protein restriction, which is suggestive of a delay in brain growth caused by systemic growth retardation. Thus, delayed brain growth due to systemic growth retardation does not influence populations of Reelin-synthesizing GABAergic interneurons and neurogenesis at weaning. These results strongly suggest that developmental hypothyroidism-induced brain changes observed at weaning in our previous study were solely caused by insufficient thyroid hormone signaling in the developing brain, and that delayed brain growth secondary to systemic growth retardation induced by hypothyroidism was minimal and unrelated to the observed neuronal changes.

In our previous study [16], we also observed lower body weights sustained through PND 77 after developmental exposure to anti-thyroid agents, with values after treatment with 12 ppm PTU or

Table 5
Histopathological examination of the cerebellum of male offspring at PND 21.

	Normal protein 20%[c]	Low protein 10%[c]
No. of offspring examined[a]	10	10
Cerebellum		
Increase of external granular cells (±/+)[b]	1(1/0)	5(3/2)*

*Significantly different from the normal protein group by Mann–Whitney's *U*-test ($P < 0.05$).
[a] Statistical analysis was performed using the individual animal as the experimental unit.
[b] Grade of change: (±), minimal: (+), slight.
[c] Casein level.

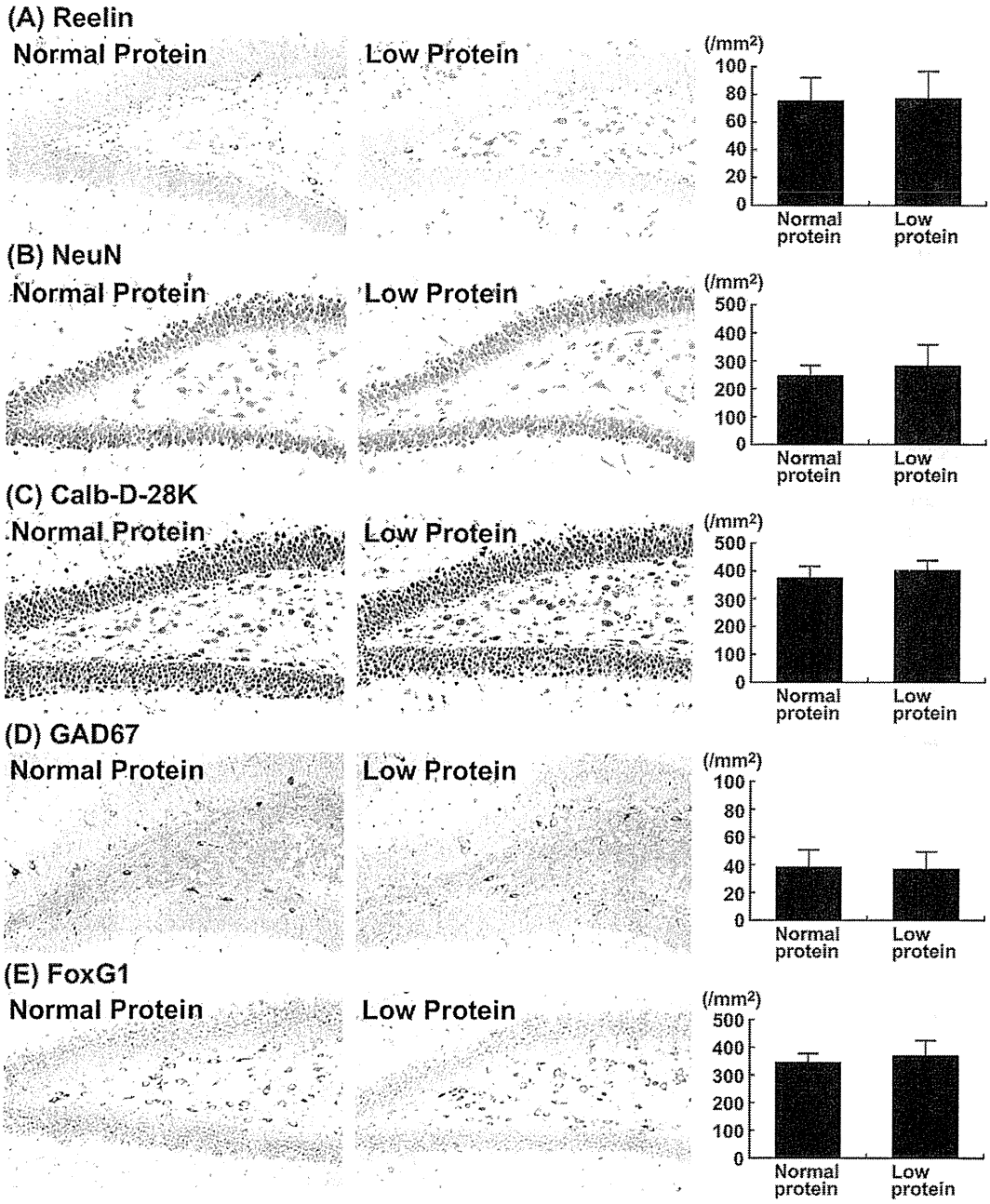

Fig. 2. Distribution of immunoreactive cells for Reelin, NeuN, Calb-D-28K, GAD67 and FoxG1 in the hilus of the hippocampal dentate gyrus in male offspring at PND 21 after maternal protein restriction from GD 10 to PND 21. All identical 10 male offspring from 8 dams (one or two animals per dam) were subjected to immunohistochemical analyses in each group. Statistical analysis was performed using the litter as the experimental unit, and litter mean values were subjected to analysis on two offspring samples from the same dam. (A) Reelin. (B) NeuN. (C) Calb-D-28K. (D) GAD67. (E) FoxG1. Representative image from the normal protein diet group (left) and from the low protein diet group (right). Magnification, 100× The graphs show the number of immunoreactive cells for each antigen/unit area (mm^2) of the hilus of the bilateral hemispheres at PND 21.

200 ppm MMI being 73.5–87.9% of untreated controls on PND 77 (Table 6). Maternal protein restriction during pregnancy and lactation causes a similar suppression of body growth sustained until the adult stage [29–31]. In the present study we observed a similar suppression in both sexes, with values in the low protein diet offspring being 84.0–89.3% of the normal protein diet offspring on PND 77 (Table 6). Interestingly, offspring from dams that were energy-restricted during lactation showed growth retardation at weaning but higher body weights at the adult stage than the control animals [31]. Although the mechanism is not clear, maternal protein restriction and developmental hypothyroidism may suppress body growth of the offspring until the adult stage by similar mechanisms. Male offspring in the hypothyroidism study showed apparent reductions in absolute brain weights on PND 77 after treatment with 12 ppm PTU or 200 ppm MMI (Table 6). This result is in contrast with the recovery in absolute weight on PND 77 after maternal protein restriction in the present study. White matter hypoplasia of the brain as a result of developmental hypothy-

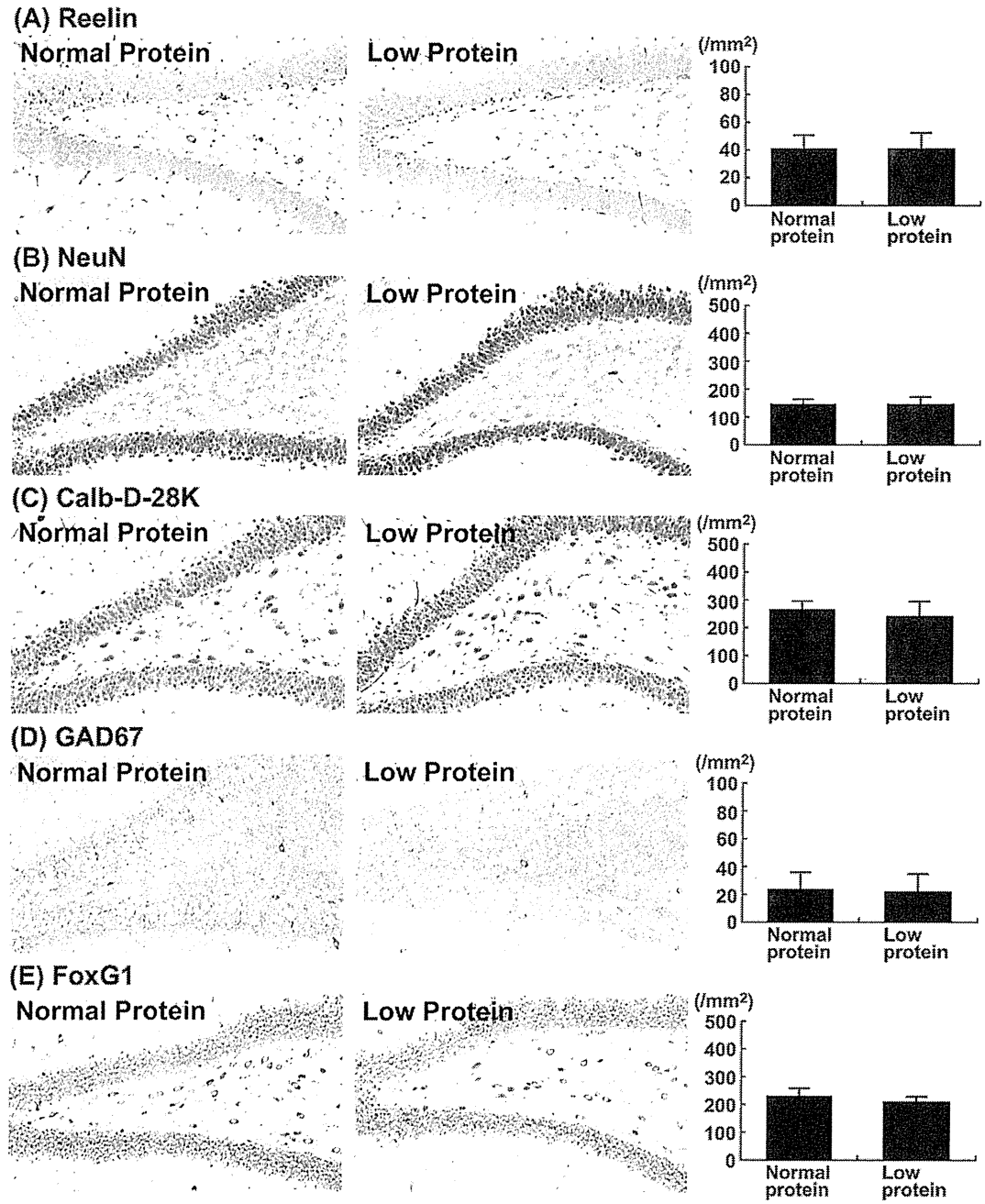

Fig. 3. Distribution of immunoreactive cells for Reelin, NeuN, Calb-D-28K, GAD67 and FoxG1 in the hilus of the hippocampal dentate gyrus in male offspring at PND 77 after maternal protein restriction from GD 10 to PND 21. All identical 10 male offspring from 8 dams (one or two animals per dam) were subjected to immunohistochemical analyses in each group. Statistical analysis was performed using the litter as the experimental unit, and litter mean values were subjected to analysis on two offspring samples from the same dam. (A) Reelin. (B) NeuN. (C) Calb-D-28K. (D) GAD67. (E) FoxG1. Representative image from the normal protein diet group (left) and from the low protein diet group (right). Magnification, 100×. The graphs show the number of immunoreactive cells for each antigen/unit area (mm^2) of the hilus of the bilateral hemispheres at PND 77.

roidism rather than delayed brain growth may be responsible for the reduction in absolute brain weight in the hypothyroidism cases [13,16]. These results suggest that the sustained systemic growth retardation allows recovery of brain growth through PND 77.

Developmental hypothyroidism resulted in a sustained increase in Reelin-synthesizing GABAergic interneurons at the later stage on PND 77 with increased population suggestive of immature phenotype [15]. This is in contrast with the unaffected distribution of GABAergic interneurons through PND 77 by maternal protein restriction. These results suggest that the sustained systemic growth retardation does not influence Reelin-synthesizing interneurons through PND 77. Maternal protein restriction during lactation results in elevation of serum triiodothyronine (T_3) levels due to an increase in thyroidal T_3 production in dams, and in continued higher serum concentrations of T_3 and thyroxine suggestive

Table 6
Comparison of body and brain weight data between the maternal protein restriction study and developmental hypothyroidism study.

	Maternal protein restriction	Developmental hypothyroidism[a]
Treatment	Fed diet with normal (20% casein) or low (10% casein) protein concentration between GD 10 and PND 21	Given PTU at 3 or 12 ppm or MMI at 200 ppm in the drinking water between GD 10 and PND 20
Weaning[b]		
Body weight		
Males	Low protein diet: 65.8%[c,**]	3 ppm PTU: 89.7%[d] 12 ppm PTU: 68.8%** 200 ppm MMI: 67.2%**
Females	Low protein diet: 65.5%**	3 ppm PTU: 86.3%** 12 ppm PTU: 65.0%** 200 ppm MMI: 64.2%**
Absolute brain weight		
Males	Low protein diet: 92.9%**	3 ppm PTU: 100.7% 12 ppm PTU: 97.9% 200 ppm MMI: 95.9%
Females	Low protein diet: 91.7%**	3 ppm PTU: 98.6% 12 ppm PTU: 95.2% 200 ppm MMI: 94.5%*
PND 77		
Body weight		
Males	Low protein diet: 84.0%**	3 ppm PTU: 99.7% 12 ppm PTU: 73.5%** 200 ppm MMI: 76.8%**
Females	Low protein diet: 89.3%*	3 ppm PTU: 87.9% 12 ppm PTU: 84.0%* 200 ppm MMI: 87.9%*
Absolute brain weight		
Males	Low protein diet: 98.5%	3 ppm PTU: 100.0% 12 ppm PTU: 90.5%** 200 ppm MMI: 92.4%**
Females	Low protein diet: 97.9%	3 ppm PTU: 101.6% 12 ppm PTU: 94.3% 200 ppm MMI: 96.9%

Abbreviations: GD, gestational day; MMI, methimazole; PND, postnatal day; PTU, 6-propyl-2-thiouracil.

[a] Published by Shibutani et al. [16].

[b] Offspring were weaned at PND 21 in the maternal protein restriction study and PND 20 in the developmental hypothyroidism study.

[c] Values were % ratios of the normal protein group with asterisks showing statistically significant difference by Student's or Aspin–Welch's *t*-test (**$P < 0.01$).

[d] Values were % ratios of the untreated group with asterisks showing statistically significant difference by Dunnett's test or Dunnett-type rank-sum test (*$P < 0.05$; **$P < 0.01$).

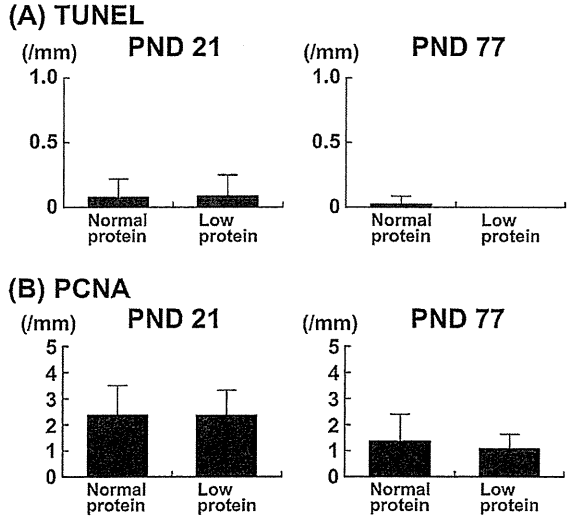

Fig. 4. Distribution of apoptotic cells and proliferating cells in the dentate subgranular zone of male offspring at both PND 21 and PND 77 after maternal protein restriction from GD 10 to PND 21. All identical male offspring (10 animals/group) as used in the immunohistochemical analyses on Reelin, NeuN, Calb-D-28K, GAD67 and FoxG1 at each time point were subjected to TUNEL-assay and PCNA-immunohistochemistry. Statistical analysis was performed using the litter as the experimental unit, and litter mean values were subjected to analysis on two offspring samples from the same dam. (A) Number of TUNEL-positive apoptotic cells/unit length (mm) of the subgranular zone of bilateral hemispheres at PND 21 and PND 77. (B) Number of PCNA-positive cells/unit length (mm) of the subgranular zone of bilateral hemispheres at PND 21 and PND 77.

of hyperthyroidism in the adult offspring [32,33]. Similar results were observed after maternal protein restriction during gestation and lactation as applied in the present study [34]. This observation is in contrast with the sustained but mild suppression of serum T_3 levels on PND 77 after developmental hypothyroidism in our previous study [16].

5. Conclusions

Maternal protein restriction resulted in systemic growth retardation sustained through PND 77 and retarded brain growth at weaning. However, it did not affect the distribution of Reelin-expressing GABAergic interneurons in the dentate hilus and postnatal neurogenesis in the subgranular zone through PND 77. These results suggest that systemic growth retardation accompanied by developmental hypothyroidism does not influence the impaired neuronal development caused by an insufficiency in thyroid hormone signaling in the brain. Because sustained increases in immature GABAergic interneurons synthesizing Reelin in the hilus could be a sign of a compensatory regulation for impaired neurogenesis and mismigration during neuronal development [15], monitoring of Reelin-expressing interneurons may provide a valuable tool for the detection of developmental neurotoxicants that can affect neurogenesis and migration. Importantly, Reelin-expressing immature cells are not affected by systemic growth retardation caused by systemic toxicity of dams and/or offspring.

Conflict of interest

All of the authors disclose that there are no conflicts of interest that could inappropriately influence the outcomes of the present study.

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臭素化難燃剤hexabromocyclododecane（HBCD）のラット周産期暴露における発達期免疫機能影響について

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Effects of perinatal exposure to the brominated flame-retardant hexabromocyclododecane (HBCD) on the developing immune system in rats

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To evaluate the developmental immunotoxicity of brominated flame retardant, hexabromocyclododecane (HBCD), maternal Sprague-Dawley rats were given HBCD at dietary concentrations of 0, 100, 1000, 10000 ppm from gestational day 10 to postnatal day 21 (postnatal week 3, PNW3). At PNW3 and PNW11, lymphocytes in the spleen, thymus, and peripheral blood of male pups were subjected to flow cytometric analyses for expression of surface markers (CD3, CD4, CD8a, CD25, CD45RA, CD71, and CD161 (NKRP1A)). The spleen and thymus weights, and number of white blood cells of two organs did not change between HBCD-exposed and control groups at PNW3 and PNW11. A significant decrease in thyroid hormone T3 and increase in serum albumin concentration were observed at PNW3 and lasted until PNW11. By flow cytometric analysis, the dramatic change was not observed in the population of the splenic and thymic T/B lymphocyte between the HBCD treated groups and control group. In the peripheral blood of BNW3 rats, the population of activated T cells was decreased and that of inactivated B cells was increased. And the population of NK cells in the spleen was decreased. All of these changes were mild in degree, and returned to the normal levels by PNW11. Production of anti-KLH IgG antibody after KLH immunization was reduced by the 10000 ppm HBCD treatment. These results suggest that developmental exposure to the highest dose of HBCD had a weak immunomodulatory effect at PNW3, and most of the immunomodulatory effect had recovered to normal levels by PNW11.

Keywords: hexabromocyclododecane, brominated flame retardant, rat, immunotoxicity, developmental toxicity

1．はじめに

難燃剤とは，プラスチック・ゴム・繊維・紙・木材などの可燃性の素材に添加してそれらを燃えにくくし，あるいは炎が広がらないようにする化合物であり，ハロゲン化合物などの有機系，金属水酸化物などの無機系に分類される．Hexabromocyclododecane（HBCD）は，ハロゲン系難燃剤の中でも臭素含有率が高いことから，少量で優れた難燃機能を有するとされ，日本において年間約3,000トンの需要量があり，その約８割が建設用の発泡系断熱材に，約２割がカーテンなどの繊維製品に利用されている．難分解性，高蓄積性であることから2004年９月化審法の第一種監視化学物質に指定された．長期毒性については不明な点が多いが，2008年Ema[1]らの２世代生殖毒性試験によりヒトに対する毒性は高くないと判断され，2009年５月の改正においても第一種監視化学物質のまま据え置かれている．

他方，免疫系は環境化学物質や薬物の有害影響に鋭敏に反応し，かつその健康影響はアレルギー，感染症，発がんなど多様な形で発現することが知られ，環境因子の影響を評価する際の重要な指標であることが指摘されてきた．また，胎児期から小児期にかけては生体組織・機

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能が発育段階にあり未熟であるために環境有害因子に対して感受性が高い時期（critical window）と考えられており，胎児期から小児期に受けた影響は生涯にわたって続く障害となる場合が少なくない．以上の点から，化学物質の発達期暴露による免疫影響についての検討は極めて重要なものであるが，一般毒性試験の中では実施されていないことから，免疫毒性試験評価手法の高度化，標準化が，国際的にも望まれている．

本報告では，対象化合物として本邦で使用頻度の高い臭素系難燃剤を取り上げ，胎児期から幼児期にかけてのラットに暴露した際の，胸腺，脾臓を中心にした免疫機能影響の評価研究を行った．HBCDを含むtetrabromobisphenol A（TBBPA），decabromodiphenyl ether(DBDE)等の臭素系難燃剤は，甲状腺機能阻害を有することが示唆されている化学物質でもある[2]．そこで，免疫影響について考察するにあたっては，甲状腺機能障害性についても考慮した．最後に，HBCDの免疫影響について，すでに報告したDBDEの免疫影響[3]との比較を加えた．

2．方法

動物は，各群10匹ずつの妊娠SD：IGSラットを用い，妊娠10日目から出産３週目まで，被験物質であるHBCDを，100ppm，1000ppm，10000 ppmの濃度で餌（大豆除去飼料（西川食））に混ぜ，親に自由摂取させた．大豆イソフラボンは代表的な植物エストロゲンであり，甲状腺ペルオキシダーゼの活性阻害作用によりヨウ素欠乏状態で甲状腺機能低下を来すとの報告があるため[4]，本実験では大豆成分の影響を避けるために被験物質投与期間は大豆除去食とした．出産３週目（PNW3）に離乳を行い，各群雌雄10匹ずつの児ラットの解剖を行なった．残りの児ラットについては，３週目からは，通常のCRF-1飼料を与えて11週まで飼育し，回復の程度を見る実験に供した．３週目，11週目の解剖時の免疫影響評価のための項目は，表１に示した通りである．病理組織学的検査のみ雌雄児ラットについて行い，他は雄児ラットについて行った．

(1)　血液学的検査：末梢血白血球数は，ラット後大動脈より採血した血液20μlをあらかじめ80μlの0.5% EDTA-2K溶液が入った1.5mlチューブに入れて混和し，多項目自動血球計数装置（M-2000，Sysmex corp）に供した．赤血球数（RBC），白血球数（WBC），血小板数（PLT），ヘモグロビン濃度（HGB），ヘマトクリット値（HCT），平均赤血球容積（MCV）平均赤血球血色素量（MCH）及び平均赤血球血色素濃度（MCHC）の測定を行なった．白血球百分比は，Wright染色した塗沫標本を作製し，杆状核好中球（Band），分葉核好中球（Seg），好酸球（Eosino），好塩基球（Baso），リンパ球（Lympho），単球（Mono）及び有核赤血球（Ebl）について血液細胞自動分析装置（Microx MEG50S, Sysmex）を用いて計測した．

表１　本実験で用いた免疫毒性のマーカー

(1)	末梢血白血球数，白血球百分比
(2)	胸腺，脾臓の重量
(3)	胸腺，脾臓の病理組織学的検査
(4)	体液性免疫： KLHに対する血中IgM, IgG抗体産生（ELISA） 脾臓，末梢血，リンパ節のフローサイトメトリー：B細胞数（CD45RA）
(5)	細胞性免疫 脾臓，末梢血，リンパ節のフローサイトメトリー：T細胞数（CD3）及び T細胞サブセット（CD4及びCD8a），調節性T細胞（CD4+CD25+）， 活性化T細胞（CD3+CD71+）
(6)	非特異的免疫 脾臓，末梢血のNK細胞数

(2)　胸腺，脾臓の重量：採血終了後，動物を放血死させ，免疫系器官である胸腺，脾臓の重量を測定した．重量測定後，臓器を２つにわけ，一方を病理組織学的検査用とし，残りをフローサイトメトリー用に供した．

(3)　病理組織学的検査：上記(2)で記した胸腺，脾臓の１部を，常法に従って中性緩衝ホルマリン液で固定を行い，薄切切片を作成し，ヘマトキシリン・エオジン染色を施した．

(4)　体液性免疫：抗体産生への影響を調べるために，解剖に供した児ラットとは別個体雄児ラットに，生後（PND）23日，33日及び43日にKLH（keyhole limpet hemocyanin）50μgをalum 1mgとともに腹腔内投与し，40及び50日目に採血し，500～500,000倍希釈し，KLHを固相抗原としたELISAにて，KLH特異的IgG及びIgM抗体価を測定した[5]．また，脾臓，末梢血，血液中Bリンパ球の割合を，PECy5標識抗CD45RA抗体処理によるフローサイトメトリーを用いて解析した．

(5)　細胞性免疫：細胞性免疫に関与する胸腺，脾臓，末梢血中Tリンパ球の割合の解析のため，フローサイトメトリーによる解析を行なった．全T細胞数は，FITC標識抗CD3抗体を用い，CD4，CD8 T細胞サブセットは，PECy5標識抗CD4抗体及びPE標識抗CD8a抗体を用い，調節性T細胞（CD4+CD25+）[6]については，PE標識抗CD25抗体を併用し，活性化T細胞（CD3+CD71+）については，PE標識抗CD71（トランスフェリン受容体）抗体を併用して解析を行なった[7]．

(6)　非特異的免疫：脾臓，末梢血中のNK細胞数の割合をFITC標識抗NKRP1A（CD161）抗体を用いてフローサイトメトリーで解析した．

(7)　フローサイトメトリー：上記(4)-(6)で示したリン

パ球サブポピュレーション解析は，脾臓，胸腺，末梢血細胞を３種の蛍光で標識した抗体を用い三重染色後，Facs Caliber（Becton Dickinson）を用いて行なった．動物実験は研究所の規定に準拠し，実験動物委員会の承認に基づき実施した．

３．研究結果

(1) HBCDの免疫担当細胞への影響

図１に，臭素化難燃剤HBCD親ラット投与による児雄ラットの体重，臓器重量変化の結果を示す．３週令時，11週令時共に，HBCD 100，1000，10000ppm投与群で対照群と比較して，体重，脾臓，胸腺重量に有意な差はみられなかった．なお，母動物の体重にも投与による影響は認められなかった．また，HBCD投与ラットの脾臓，胸腺細胞の白血球数についても，有意差はみられなかった．なお，肝臓重量は，1000, 10000ppm投与群の３週令において，有意な増加が認められたが，11週令においては，有意差は認められなかった．

次に表２，表３に，３週令ラットと11週令ラットの血液学的検査を行なった結果を示すが，末梢血白血球数（表２），白血球百分比（表３）ともに，３週令，11週令ラットにおいて，対照と比べて有意な変化はみられなかった．なお，３週令で，赤血球容積，11週令で血色素濃度の用量依存的な上昇が観察され（表２），また，３週令で，有核赤血球（Ebl）のわずかな上昇傾向がみられた（表３）．

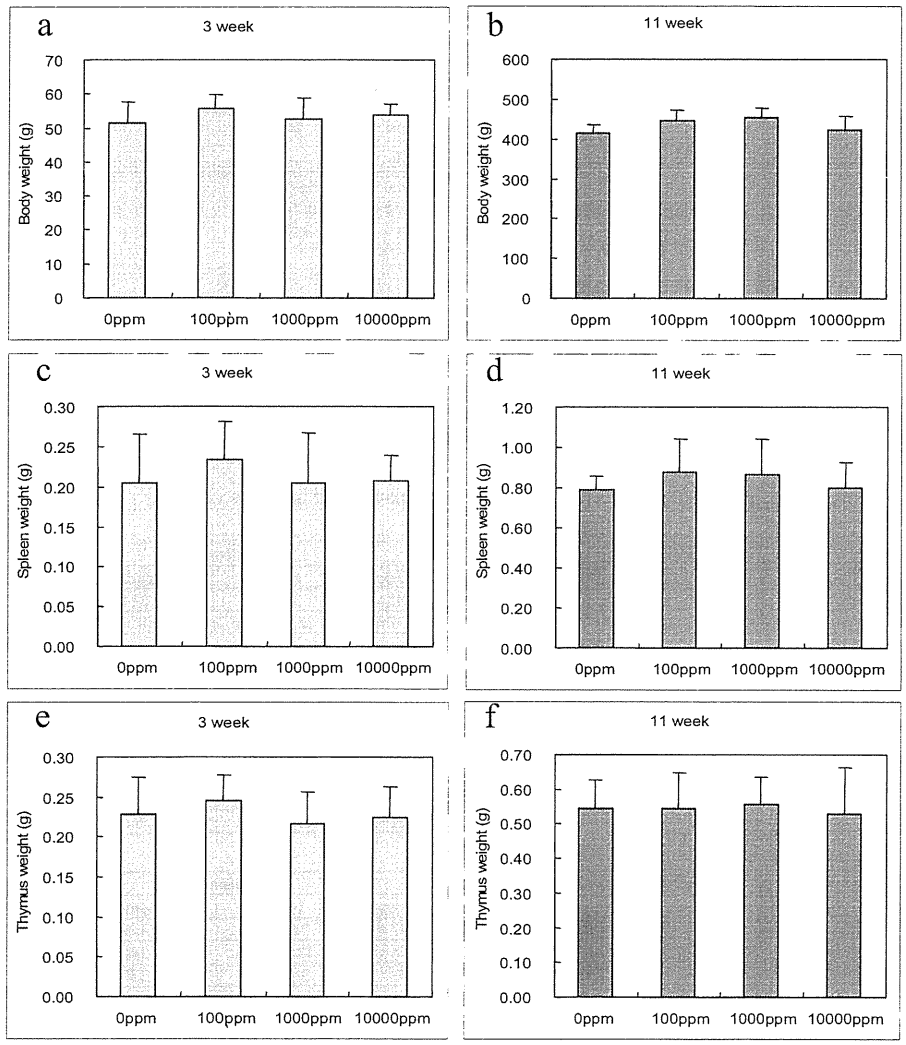

Fig. 1 Effect of perinatal exposure to HBCD on the body weight and the immune-related organs weight of offspring
Dams were fed *ad lib* HBCD-containing diet from gestational day 10 to PNW3. The body weight (a, b), spleen wight (c, d), and thymus weight (e, f) of the male offspring at PNW3 and PNW11, respectively. Means (n=10) ± SD are shown. There was no significant difference (Dunnett, $p<0.05$).

次いで，フローサイトメトリーによるリンパ球ポピュレーションの解析では，HBCD投与群と対照群との間に幾つかの項目で，リンパ球サブポピュレーションにおける変化が観察された．表４に，５%以上の有意差を持って変化のみられた項目につき，解析したリンパ球サブポピュレーションの割合を数値で表したものを示した．T細胞のサブポピュレーション解析では，脾臓リンパ球において，HBCD 100ppm, 10000ppm投与11週令時で，CD8+ T細胞の上昇が認められた．また，末梢血における活性化T細胞の割合の低下，並びに非活性化B細胞の

Table 2 Hematological changes in male rats perinatally exposed to HBCD

group	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	PLT	
PNW 3	x 10^2/ul	x 10^4/ul	g/dl	%	fL	pg	g/dL	x 10^4/ul	n
M-1 (control)	35.3 ± 11.3	327.7 ± 22.7	5.19 ± 0.69	20.00 ± 1.81	61.05 ± 4.23	15.85 ± 1.90	25.92 ± 1.93	118.92 ± 19.50	10
M-2 (HBCD 100 ppm)	30.9 ± 10.0	336.8 ± 30.2	5.83 ± 0.87	21.59 ± 1.85	64.25 ± 4.31	17.32 ± 2.32	26.85 ± 2.33	121.77 ± 11.28	10
M-3 (HBCD 1000 ppm)	47.5 ± 11.8 *	328.1 ± 24.0	5.76 ± 0.73	21.16 ± 2.07	64.50 ± 4.27	17.55 ± 1.77	27.17 ± 1.19	128.38 ± 11.06	10
M-4 (HBCD 1000 ppm)	39.6 ± 7.9	322.3 ± 23.7	5.98 ± 1.18	21.57 ± 3.24	66.71 ± 6.26 *	18.46 ± 2.67 *	27.55 ± 1.94	123.99 ± 22.54	10
PNW11									
M-1 (control)	82.1 ± 17.8	758.6 ± 63.6	14.27 ± 1.93	46.74 ± 3.79	61.64 ± 1.63	18.78 ± 2.01	30.49 ± 3.29	108.85 ± 9.88	10
M-2 (HBCD 100 ppm)	109.8 ± 30.8 *	786.5 ± 30.4	15.60 ± 0.59	48.03 ± 1.79	61.07 ± 1.23	19.85 ± 0.32	32.47 ± 0.50	111.32 ± 12.15	10
M-3 (HBCD 1000 ppm)	110.0 ± 29.3 *	815.1 ± 55.3 *	15.96 ± 0.82 *	49.29 ± 3.32	60.48 ± 1.16	19.59 ± 0.50	32.42 ± 0.82	117.26 ± 13.72	10
M-4 (HBCD 1000 ppm)	103.4 ± 34.1	766.4 ± 39.6	15.27 ± 0.56	46.42 ± 2.16	60.61 ± 1.30	19.94 ± 0.60	32.90 ± 0.46 *	123.97 ± 19.15 *	10

Values are mean ± SD of 10 rats. *<0.05 (Dunnett's-test)

Table 3 White blood cell classification in male rats perinatally exposed to HBCD

group	n	Lymp	Seg	Eosi	Mono	Band	Ebl
PNW3		%	%	%	%	%	n
M-1 (control)	9	78.88 ± 4.74	19.73 ± 4.77	0.61 ± 0.74	0.61 ± 0.49	0.0 ± 0.0	0.06 ± 0.17
M-2 (HBCD 100 ppm)	10	79.02 ± 3.18	19.63 ± 3.26	0.60 ± 0.46	0.75 ± 0.63	0.0 ± 0.0	0.48 ± 0.66
M-3 (HBCD 1000 ppm)	10	81.69 ± 3.81	17.21 ± 4.19	0.45 ± 0.37	0.65 ± 0.75	0.0 ± 0.0	0.26 ± 0.37
M-4 (HBCD 1000 ppm)	10	81.41 ± 4.06	17.42 ± 4.50	0.36 ± 0.50	0.81 ± 0.90	0.0 ± 0.0	1.00 ± 1.45
PNW11							
M-1 (control)	10	84.64 ± 5.46	13.51 ± 5.15	0.90 ± 0.94	0.95 ± 0.69	0.0 ± 0.0	0.05 ± 0.16
M-2 (HBCD 100 ppm)	10	84.27 ± 4.88	13.33 ± 4.67	1.50 ± 1.15	0.90 ± 0.70	0.0 ± 0.0	0.00 ± 0.00
M-3 (HBCD 1000 ppm)	10	87.56 ± 4.33	10.44 ± 3.79	1.05 ± 0.93	0.95 ± 0.64	0.0 ± 0.0	0.05 ± 0.16
M-4 (HBCD 1000 ppm)	10	86.44 ± 3.36	11.86 ± 3.34	0.75 ± 0.68	0.95 ± 0.50	0.0 ± 0.0	0.00 ± 0.00

Values are mean ± SD. There was no significant difference (Dunnett, $p<0.05$).

Table 4 Effects of HBCD on subpopulations of immune cells

T cell subpopulations

	Spleen3w				Spleen 11w				Note
HBCD:	0 ppm	100 ppm	1000 ppm	10000 ppm	0 ppm	100 ppm	1000 ppm	10000 ppm	
CD3(+) CD8a(+)	6.84 ± 1.3	8.15 ± 1.88	7.22 ± 1.52	6.6 ± 1.44	13.42 ± 2.71	18 ± 4.21*	15.2 ± 3.54	18.4 ± 4.58*	CD8 T cell
CD8a(+) CD4(−)	6.86 ± 0.95	8.12 ± 2.16	6.99 ± 1.42	6.43 ± 1.44	14.42 ± 2.23	18.54 ± 4.34*	16.85 ± 4.31	18.87 ± 4.82*	CD8

Activation of T/B cells

	Thymus 3w				Thymus 11w				Note
HBCD:	0 ppm	100 ppm	1000 ppm	10000 ppm	0 ppm	100 ppm	1000 ppm	10000 ppm	
CD3(+) CD71(+)	2.67 ± 0.87	2.46 ± 0.8	1.82 ± 0.55*	1.87 ± 1.15	0.92 ± 0.97	0.74 ± 0.51	1.02 ± 0.84	1.04 ± 0.7	active T cell
	Peripheral Blood 3w				Peripheral Blood 11w				Note
HBCD:	0 ppm	100 ppm	1000 ppm	10000 ppm	0 ppm	100 ppm	1000 ppm	10000 ppm	
CD3(+) CD71(+)	13.51 ± 3.47	14.01 ± 2.16	11.81 ± 1.96	10.4 ± 2.02*	1.45 ± 0.54	1.35 ± 0.6	1.27 ± 0.47	1.32 ± 0.24	active T cell
CD71(+) CD45RA(+)	20.79 ± 4.03	20.24 ± 2.97	23.08 ± 1.77	25.81 ± 2.84**	20.91 ± 5.79	19.65 ± 7.81	22.94 ± 6.7	21.22 ± 5.22	Inactive B cell

Treg, NK, NKT cells

	Spleen3w				Spleen 11w				Note
HBCD:	0 ppm	100 ppm	1000 ppm	10000 ppm	0 ppm	100 ppm	1000 ppm	10000 ppm	
NKRP1A(+) CD25(−)	11.36 ± 0.79	11.55 ± 1.19	11.25 ± 1.67	9.99 ± 1.11**	21.47 ± 2.47	21.47 ± 3.9	23.28 ± 4.29	20.77 ± 2.33	NK cell
NKRP1A(+) CD4(+)	6.47 ± 0.61	6.28 ± 0.81	6.4 ± 1.31	5.63 ± 0.81**	12.53 ± 1.88	12.89 ± 1.85	13.78 ± 2.66	13.09 ± 1.72	CD4NKT
NKRP1A(+) CD4(−)	5.75 ± 0.35	6.06 ± 1.09	5.65 ± 0.87	5.09 ± 0.76*	10.63 ± 1.63	9.97 ± 3.44	11.38 ± 2.47	9.44 ± 2.39	NK cell
	Thymus 3w				Thymus 11w				Note
HBCD:	0 ppm	100 ppm	1000 ppm	10000 ppm	0 ppm	100 ppm	1000 ppm	10000 ppm	
CD25(+) CD4(+)	7.7 ± 2.57	5.15 ± 0.94*	7.69 ± 1.27	7.85 ± 2.85	4.16 ± 1.09	3.98 ± 0.87	4.41 ± 0.76	4.32 ± 1.22	Treg
NKRP1A(+) CD4(−)	0.07 ± 0.03	0.07 ± 0.03	0.06 ± 0.02	0.07 ± 0.05	0.2 ± 0.04	0.2 ± 0.05	0.25 ± 0.09	0.27 ± 0.08*	NK cell
	Peripheral Blood 3w				Peripheral Blood 11w				Note
HBCD:	0 ppm	100 ppm	1000 ppm	10000 ppm	0 ppm	100 ppm	1000 ppm	10000 ppm	
NKRP1A(+) CD25(+)	0.12 ± 0.03	0.1 ± 0.03	0.09 ± 0.02	0.08 ± 0.04*	0.27 ± 0.07	0.23 ± 0.08	0.27 ± 0.07	0.25 ± 0.09	CD25NK

*$p<0.05$, **$p<0.01$

割合の上昇が，３週令時ラットHBCD 10000ppm投与群でみられた．さらに，NK細胞については，脾臓におけるNKRP1A（NK受容体）陽性細胞の割合の減少が，HBCD 10000ppm投与３週令ラットにおいて観察された．

HBCD投与による血液生化学的検査結果については，表５に示すが，３週令時10000ppmHBCD投与群において，甲状腺ホルモンT3の有意な減少及びTSHの有意な上昇が観察された．11週令時においてもT3の有意な低下が，1000, 10000ppm HBCD投与群でみられた．また，血清のアルブミン値の上昇が，11週令時の10000ppmHBCD投与群において観察された．

病理組織学的所見を表６に示す．３週令雄の1000ppm HBCD投与群において，貪食したマクロファージであるstarry-sky像が増加していたが用量依存性は認められなかった．その他は，顕著な変化は認められなかった．

投与ラットのKLHに対する抗体産生への影響を図２に示す．図には，KLHで２回免疫したラットから得た血清（PND40）のELISAで測定したKLH特異的IgG抗体価とHBCDの用量依存性を調べた結果を示しているが，HBCDの濃度が上昇するにつれ，抗体価の減少する傾向が得られ，HBCD 10000ppm投与群で，対照群と比較して有意な抗体価の減少が観察された．

４．考察

臭素化難燃剤HBCDの胎児期及び幼児期投与による児

Table 5　Serum levels of thyroid-related hormones of the offspring perinatally exposed to HBCD

	HBCD in diet (ppm)												
	0				100			1000			10000			
PNW3														
No. of offspring examined	10				10			10			10			
T3 (ng/ml)	1.09	±	0.11	[a]	1.13	±	0.12	1.06	±	0.08	0.93	±	0.10	**
T4 (ug/dl)	4.39	±	0.93		4.20	±	0.77	4.78	±	0.49	4.20	±	0.52	
TSH (ng/ml)	5.40	±	0.62		6.66	±	1.24	6.07	±	1.41	7.00	±	1.31	*
A/G ratio	2.17	±	0.43		2.34	±	0.59	2.08	±	0.34	1.93	±	0.70	[b]
albumin (g/dl)	3.55	±	0.18		3.62	±	0.25	3.62	±	0.16	3.84	±	0.15	[b], **
PNW11														
No. of offspring examined	10				10			10			10			
T3 (ng/ml)	0.96	±	0.06		0.93	±	0.07	0.88	±	0.05	**	0.89	±	0.06	**
T4 (ug/dl)	4.77	±	0.70		4.84	±	0.59	5.21	±	0.65		5.20	±	0.98	
TSH (ng/ml)	4.74	±	0.62		5.81	±	1.72	5.36	±	1.11		4.96	±	0.80	
A/G ratio	1.86	±	0.24		1.89	±	0.29	1.74	±	0.16		1.70	±	0.14	
albumin (g/dl)	3.53	±	0.32		3.63	±	0.29	3.82	±	0.34		4.00	±	0.39	**

[a] Mean ± SD.
[b] n=9
Abbreviations: HBCD, hexabromocyclododecane; PNW, postnatal week.
*, ** Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test (*p<0.05, **p<0.01).

Table 6　Histopathology of the thymus and spleen of male and female rats perinatally exposed to hexabromocyclododecane (HBCD)

	HBCD in diet (ppm)			
	0	100	1000	10000
HBCD 3				
No. of animals examined (male/female)	10/10	10/10	10/10	10/10
Thymus				
Increased starry sky appearance (±)[a]	0/0[b]	0/0	4*/0	1/0
Spleen				
Atrophy of white pulp (±)	0/0	0/0	0/0	1/0
Reduction in the number of white pulp (±)	0/0	0/0	0/0	1/0
HBCD 11				
No. of animals examined (male/female)	10/10	10/10	10/10	10/10
Thymus				
Increased starry sky appearance (±)	0/0	0/0	0/3	0/0
Reduction of cortical area (±)	1/2	0/0	1/1	4/3
Spleen				
No abnormalities detected	10/10	10/10	10/10	10/10

[a] Grade of change; ±, minimal.
[b] Total No. of animals with each finding.
* Significantly different from the controls by Fisher's exact probability test (*p < 0.05).

ラットの免疫系への影響を，リンパ球のポピュレーション，サブポピュレーション，NK細胞の割合の解析により検討した．その結果，胸腺，脾臓重量に対照群との間に差は認められなかったが，３週令の脾臓においてNK細胞の減少が観察され，末梢血の活性化Ｔ細胞の減少，非活性化Ｂ細胞の上昇も観察された．甲状腺機能障害性

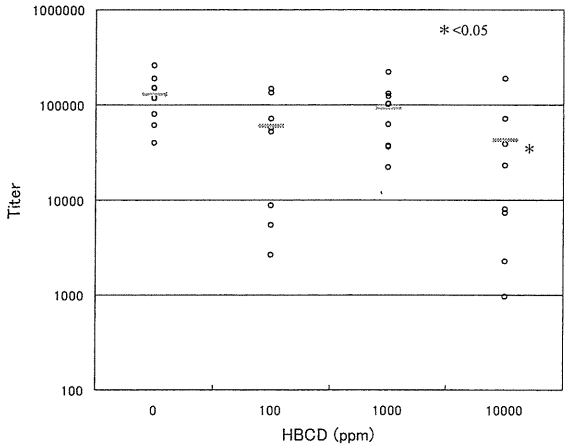

Fig. 2 Effect of HBCD on the antibody production of the offspring
The HBCD-exposed female offspring were challanged with 50μg of KLH with 1 mg of alam twice after ceasing exposure on postnatal day 23 and 33. Serum was obtained 1 week after final immunization, and the IgG titer to KLH was measured by ELISA. Open circles represent individual values, and gray lines indicate mean values. In the highest dose (10000 ppm), the antibody titer against KLH decreased in comparison with the control group significantly (Dunnett, $p<0.05$).

Table 7 Immunity-related influences exposed to HBCD(a) and DBDE (b)

a）HBCD	1000ppm	10000ppm
PNW3		
liver weight	↑	↑
spleen CD161+NK cell		↓
peripheral active T cell		↓
peripheral inactive B cell		↑
serum T3 level		↓
serum TSH level, albumin		↑
serum T3 level	↓	↓
serum albumin		↑
PNW3-7		
KLH-antibody production		↓

b）DBDE	100ppm	1000ppm
PNW3		
liver weight	↑	↑
spleen active T, B cell		↓
spleen CD4+ cell		↓
serum T3 level		↓
PNW11		
peripheral CD161+NK cell		↓
serum T4 level		↓

を有する薬物Propylthiouracil（PTU）及びMethimazole（MMI）[8,9]を用いた同様の実験では，Ｂ細胞の比率の低下等の大きなポピュレーション変化を伴う現象が観察されたが，それら抗甲状腺作用薬に比較してHBCDの影響は軽度であった．回復期の11週令においては，リンパ球ポピュレーションに有意な変化は観察されなかった．以上，HBCDは，軽度ではあるが，免疫担当細胞への影響が示唆されるデータが得られ，甲状腺ホルモンT3の低下と連動することから，これら影響は，甲状腺機能抑制と連関する可能性が考えられた．なお，血清アルブミンの上昇，肝臓の臓器重量の増加も観察されたことから，HBCDの甲状腺機能への影響は，HBCDが直接甲状腺機能を抑制している可能性と，肝重量が増加していたことから，肝臓の薬物代謝酵素誘導により甲状腺ホルモンが代謝されて血中濃度が減少する二次的影響の可能性の２つが考えられた．

HBCDの用量依存性が得られた免疫影響関連の結果をまとめたものを表７a)に示した．また，表７b)には，比較のために同じく用量依存性の免疫影響の得られたDBDEの結果[5]を示した．HBCD，DBDEともに，３週令で甲状腺機能抑制活性と連関すると思われる活性化Ｔ細胞群の低下がみられ，HBCDでは抗体産生の低下がみられた．NK細胞の割合の低下も両化合物でみられたが，HBCDの場合は３週令での抑制が11週令で回復しているのに比べ，DBDEの場合は11週令でも抑制が有意であるという違いがみられた．

以上，HBCDは，高濃度暴露において，幼児期ラットに対し免疫抑制影響を示すことが示唆された．また，DBDEも同様の免疫影響を示すが，HBCDの方がDBDEに比べ回復が早い傾向にあることが示された．

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Impaired oligodendroglial development by decabromodiphenyl ether in rat offspring after maternal exposure from mid-gestation through lactation

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ABSTRACT

Pregnant Sprague–Dawley rats were given diet containing decabromodiphenyl ether (DBDE) either at 0, 10, 100, or 1000 ppm from gestation day (GD) 10 until day 20 after delivery (PND 20). No significant alterations were observed in maternal and offspring reproductive parameters. At PND 20, serum triiodothyronine concentrations examined in males were slightly reduced at 1000 ppm (84.2% of the control value), and incidence of thyroid follicular cell hypertrophy was increased in both sexes with significant difference in males at 1000 ppm. Diffuse liver cell hypertrophy accompanying increased relative liver weight and increased cytoplasmic eosinophilia of the renal proximal tubules were observed in both sexes with significant difference from 10 ppm in males and females, respectively. At postnatal week 11, serum thyroxine concentrations examined in males were slightly reduced at 1000 ppm (85.9% of the control value), and the incidence of thyroid follicular cell hypertrophy was non-significantly increased from 10 ppm in males. There were reductions in the corpus callosum area and density of 2′,3′-cyclic nucleotide 3′-phosphodiesterase-immunoreactive oligodendrocytes in the cingulate deep cortex in males from 100 ppm. Conversely, NeuN-immunoreactive neuronal distribution in the hippocampal CA1 was unchanged. This suggests that developmental DBDE-exposure caused irreversible white matter hypoplasia targeting oligodendrocytes from 100 ppm, accompanied with developmental hypothyroidism. The lowest-observed-adverse-effect level of DBDE was determined to be 10 ppm (0.7–2.4 mg/kg-body weight-d).

1. Introduction

Thyroid hormones are required for normal brain development during the fetal and neonatal periods [1,2]. Developmental hypothyroidism during this period leads to growth retardation, neurological defects and impaired behavioral and learning abilities [3,4]. Rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil show brain retardation, resulting in impaired neuronal migration and white matter hypoplasia involving limited axonal myelination and decreased oligodendrocytic distribution [2,5–7]. Maternal serum thyroid hormone levels directly affect thyroid hormone levels in their fetuses [8]. In humans, mild or subclinical hypothyroidism is common in women and in the elderly and has been associated with an increased incidence of depression by lowering the threshold for the development of major depressive disorders [9] and other mood disorders [10]. In addition, mild hypothyroidism has been linked to a diminished response to standard psychiatric treatment and to cognitive dysfunction [10]. These findings suggest that even small changes in the mother's thyroid hormone status in early pregnancy may cause adverse effects on her child. Therefore there has been increased concern for thyroid hormone disrupting chemicals in the environment.

Brominated flame retardants (BFRs) are the most efficient flame retardants and are commonly used to protect a variety of commercial products such as computers, televisions, mobile phones, furniture, carpet, insulation boards and mattresses [11]. Many of these BFR compounds have highly lipophilic and persistent characteristics and are believed to have the highest potential for

Abbreviations: AGD, anogenital distance; BFR, brominated flame retardant; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CYP, cytochrome P450; DBDE, decabromodiphenyl ether; GD, gestation day; NeuN, neuron-specific nuclear protein; PBDEs, polybrominated diphenyl ethers; PND, postnatal day; PNW, postnatal week; T_3, triiodothyronine; T_4, thyroxine; TSH, thyroid-stimulating hormone; UGT, uridine diphosphate glucosyltransferase.

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