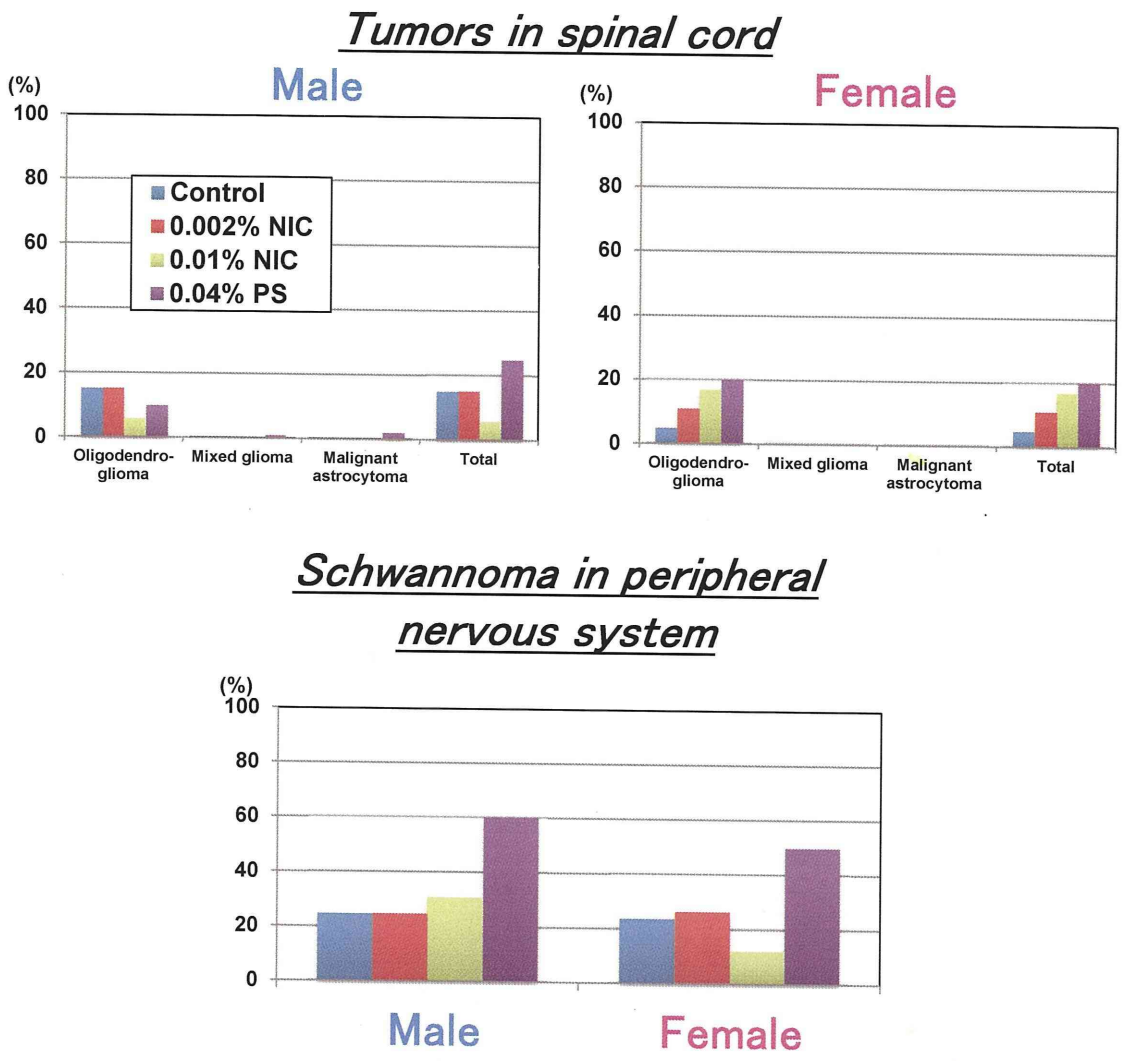

Fig. 6. Incidence of neural tumors (Spinal cord and peripheral nervous system)

Table 1. Water consumption and test chemical intakes of dams

Group	No. of dam	Water consumption (g/rat/day)	Intake of test chemical (mg/kg b.w./day)
Control	5	58.0	0
Nicotine 0.002%	5	41.7	5.0
Nicotine 0.01%	5	28.3	18.5
Nicotine 0.025–0.05%	5	7.0	15.8
Propane sultone 0.04%	5	45.4	103.3

Table 2. Final body and organ weights of dams

	Control	Nicotine 0.002%	Nicotine 0.01%	Nicotine 0.025–0.05%	Propane sultone 0.04%
No. of animals	5	5	5	5	5
Body Weight(g)	201.7 ± 9.0	184.9 ± 7.4 *	164.2 ± 3.9**	145.4 ± 13.9**	187.6 ± 8.5
Absolute					
Brain(g)	1.76 ± 0.03	1.75 ± 0.04	1.74 ± 0.02	1.76 ± 0.01	1.74 ± 0.02
Lungs(g)	0.73 ± 0.06	0.70 ± 0.04	0.68 ± 0.04	0.65 ± 0.02	0.71 ± 0.03
Spleen(g)	0.47 ± 0.03	0.43 ± 0.02	0.37 ± 0.02**	0.37 ± 0.07**	0.43 ± 0.04
Liver(g)	9.34 ± 0.48	8.39 ± 0.53*	7.17 ± 0.31**	4.50 ± 0.30**	8.12 ± 0.58**
Kidneys(g)	1.55 ± 0.06	1.50 ± 0.12	1.46 ± 0.06	1.21 ± 0.07**	1.49 ± 0.09
Relative Brain(%)	0.88 ± 0.03	0.95 ± 0.04	1.06 ± 0.03*	1.22 ± 0.13**	0.93 ± 0.04
Lungs(%)	0.36 ± 0.02	0.38 ± 0.01	0.42 ± 0.02*	0.45 ± 0.06**	0.38 ± 0.002
Spleen(%)	0.24 ± 0.01	0.23 ± 0.004	0.22 ± 0.004	0.25 ± 0.03	0.23 ± 0.01
Liver(%)	4.63 ± 0.16	4.53 ± 0.17	4.37 ± 0.17	3.11 ± 0.20**	4.32 ± 0.14*
Kidneys(%)	0.77 ± 0.02	0.81 ± 0.04	0.89 ± 0.02**	0.84 ± 0.05*	0.80 ± 0.02

Each value represents the mean±S.D.

*, **: Significantly different from the control at $p<0.05$ and $p<0.01$, respectively

Table 3. Water consumption and test chemicals intake of offspring

Group		No. of rats	Water consumption (g/rat/day)	Intakes of test chemicals (mg/kg b.w/day)
Male	Control	19	26.5	—
	0.002% NIC	20	20.6	1.9
	0.01% NIC	15	16.3	9.1
	0.04% PS	14	16.8	30.8
Female	Control	21	17.5	—
	0.002% NIC	18	11.4	1.7
	0.01% NIC	24	10.5	9.1
	0.04% PS	16	12.1	35.8

Each value represents the mean throughout the experimental period.

Table 4. Organ weights of male offspring

	Control	0.002% NIC	0.01% NIC	0.04% PS
No. of animals	19	20	15	14
Body weight (g)	333 ± 14	310 ± 20 **	260 ± 15 **	303 ± 24 **
Absolute				
Brain (g)	1.95 ± 0.06	1.94 ± 0.13	1.87 ± 0.04 **	1.90 ± 0.04 **
Lungs (g)	1.02 ± 0.09	1.05 ± 0.20	0.89 ± 0.10 *	1.00 ± 0.14
Spleen (g)	0.63 ± 0.04	0.61 ± 0.05	0.52 ± 0.04 **	0.62 ± 0.07
Liver (g)	9.68 ± 0.55	8.92 ± 0.69 **	7.10 ± 0.63 **	8.74 ± 0.95 **
Kidneys (g)	2.15 ± 0.16	2.06 ± 0.17	1.71 ± 0.11 **	1.93 ± 0.09 **
Relative				
Brain (%)	0.59 ± 0.03	0.63 ± 0.07	0.72 ± 0.03 **	0.63 ± 0.06
Lung (%)	0.31 ± 0.03	0.34 ± 0.07	0.34 ± 0.04 **	0.33 ± 0.06
Spleen (%)	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.02	0.21 ± 0.04
Liver (%)	2.90 ± 0.12	2.88 ± 0.12	2.73 ± 0.13 **	2.88 ± 0.18
Kidneys (%)	0.64 ± 0.04	0.67 ± 0.04	0.66 ± 0.03	0.64 ± 0.05

Each value represents the mean±S.D.

*, **: Significantly different from the control at $p<0.05$ and $p<0.01$, respectively

Table 5. Organ weights of female offspring

	Control	0.002% NIC	0.01% NIC	0.04% PS
No. of animals	21	18	24	16
Body weight (g)	182 ± 17	173 ± 5	147 ± 15 **	166 ± 13 **
Absolute				
Brain (g)	1.79 ± 0.04	1.76 ± 0.10	1.73 ± 0.05 **	1.75 ± 0.05 *
Lungs (g)	0.80 ± 0.13	0.73 ± 0.09	0.71 ± 0.10 *	0.75 ± 0.08
Spleen (g)	0.43 ± 0.05	0.41 ± 0.03	0.38 ± 0.06 **	0.39 ± 0.05 *
Liver (g)	5.02 ± 0.53	4.76 ± 0.33	3.87 ± 0.44 **	4.69 ± 0.39
Kidneys (g)	1.24 ± 0.10	1.29 ± 0.07	1.13 ± 0.09 **	1.24 ± 0.07
Relative				
Brain (%)	0.99 ± 0.10	1.02 ± 0.07	1.19 ± 0.12 **	1.06 ± 0.08
Lung (%)	0.44 ± 0.09	0.42 ± 0.05	0.48 ± 0.07	0.45 ± 0.06
Spleen (%)	0.24 ± 0.02	0.24 ± 0.02	0.26 ± 0.04	0.24 ± 0.02
Liver (%)	2.76 ± 0.18	2.75 ± 0.16	2.62 ± 0.13 **	2.83 ± 0.12
Kidneys (%)	0.68 ± 0.05	0.75 ± 0.04 **	0.77 ± 0.05 **	0.75 ± 0.05 **

Each value represents the mean±S.D.

*, **: Significantly different from the control at $p<0.05$ and $p<0.01$, respectively

Table 6. Neural tumor incidences

Sex	Treatment	No. of animals	Brain (%)	Spinal cord (%)	CNS (%)	PNS (%)	CNS + PNS (%)
Male	Control	20	9 (45)	3 (15)	10 (50)	5 (25)	14 (70)
	0.002% NIC	20	12 (60)	3 (15)	14 (70)	5 (25)	15 (75)
	0.01% NIC	16	9 (56)	1 (6)	9 (56)	5 (31)	10 (63)
	0.04% PS	20	12 (60)	5 (25)	14 (70)	12 (60)	20 (100)
Female	Control	21	11 (52)	1 (5)	12 (57)	5 (25)	14 (67)
	0.002% NIC	19	8 (42)	2 (11)	9 (47)	5 (25)	13 (68)
	0.01% NIC	24	13 (54)	4 (17)	16 (67)	3 (19)	17 (71)
	0.04% PS	20	9 (45)	4 (20)	10 (50)	10 (50)	17 (85)

CNS, Central nervous system
PNS, Peripheral nervous system

Table 7. Non-neural tumor incidences

Sex	Treatment	No. of animals	Lung	
			Adenoma (%)	Adeno-carcinoma (%)
Male	Control	20	2 (10)	0 (0)
	0.002% NIC	20	2 (10)	0 (0)
	0.01% NIC	16	2 (13)	0 (0)
	0.04% PS	20	9 (45) *	10 (50)**
Female	Control	21	2 (10)	0 (0)
	0.002% NIC	19	0 (0)	0 (0)
	0.01% NIC	24	0 (0)	0 (0)
	0.04% PS	20	2 (10)	1 (5)

*, **: Significantly different from the control at $p<0.05$ and $p<0.01$, respectively

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研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Fujimoto, H., Shibutani, M., et al.	Impaired oligodendroglial development by decabromodiphenyl ether in rat offspring after maternal exposure from mid-gestation through lactation	Reprod. Toxicol.	31(1)	86-94	2011
Ogawa, B., Shibutani, M., et al.	Disruptive neuronal development by acrylamide in the hippocampal dentate hilus after developmental exposure in rats	Arch. Toxicol.	85(8)	987-994	2011
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Fujimoto, H., Shibutani, M., et al.	Increased cellular distribution of vimentin and Ret in the cingulum induced by developmental hypothyroidism in rat offspring maternally exposed to anti-thyroid agents	Reprod. Toxicol.		(in press)	
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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).

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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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bioaccumulation. Because of these properties, several BFRs have contaminated the environment and have accumulated in wildlife, which has evoked concern for both environmental and human health [12]. Recent studies have shown that polybrominated diphenyl ethers (PBDEs), a subgroup of BFRs, can cause carcinogenic, thyrotoxic, estrogenic and neurotoxic effects in experimental animals and humans [13]. Developmental hypothyroidism in children is the major concern of exposure effect of BFRs [14,15].

Lower-brominated PBDEs (tetra-BDE, penta-BDE, and hexa-BDE) are especially persistent in the environment and have also been found in human adipose tissue and in breast milk [16,17]. Deca-BDE (DBDE), a fully brominated PBDE, is the most widely used congener of PBDEs [18]. Though DBDE is thought to have a relatively low capacity of bioaccumulation among PBDEs, it has been found in human blood [19]. Occupational exposure to DBDE has been shown to cause increased levels of DBDE in the blood of computer technicians and workers handling flame-retarded rubber [20].

Regarding the developmental toxicity of PBDEs, exposure of pregnant rats to penta-BDE from gestational day (GD) 6 to postnatal day (PND) 21 has been shown to decrease serum thyroxine (T_4) levels in offspring at PNDs 4 and 14 [21]. Pubertal exposure of male rats during PNDs 23–53 to DE-71, a commercial mixture of PBDEs also decreased serum triiodothyronine (T_3) and T_4, increased serum TSH and induced uridine diphosphate glucosyltransferase (UGT) and ethoxy- and pentoxyresorufin-*O*-deethylase [22]. These animals exhibited a delay in the preputial separation, and had reduced weights of seminal vesicles and the ventral prostate. Furthermore, neonatal exposure to penta-BDE caused disturbances in the spontaneous behavior of mice [23]. Neonatal DBDE exposure also caused behavioral abnormalities in mice and rats [18,24].

Reproduction studies and developmental neurotoxicity studies require large numbers of animals for detection of subtle dose-response changes. However, for screening purposes of many new chemicals, smaller scale studies, preferably with short-term experiments, need to be established. Based on previously reported landmarks on brain development caused by developmental hypothyroidism [2,5,6], we recently established a morphometric detection system for neuronal mismigration and aberrant oligodendroglial development using fewer animals than those required in developmental neurotoxicity studies to evaluate the potency of chemicals to induce hypothyroidism-related impaired brain development [7]. The present study was performed to assess the effects of exposure to DBDEs through the maternal diet on the development of the offspring in rats, with a particular focus on brain development parameters that are affected by hypothyroidism.

2. Materials and methods

2.1. Chemicals and animals

Decabromodiphenyl ether (DBDE; CAS No. 1163-19-5, purity: >98%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pregnant CD®(SD)IGS rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at GD 3 (the day when vaginal plugs were observed was designated as GD 0). Rats were housed individually in polycarbonate cages with wood chip bedding, maintained in an air-conditioned animal room (temperature 24 ± 1 °C, relative humidity: 55 ± 5%) with a 12 h light/dark cycle and allowed *ad libitum* access to feed and tap water. A soy-free diet (Oriental Yeast Co. Ltd., Tokyo, Japan) was chosen as the basal diet for dams to eliminate possible phytoestrogen effects on the evaluation of this study, and water was provided *ad libitum* throughout the experimental period including 1 week of acclimation. The estrogen and phytoestrogen content in the soy-free diet has been described elsewhere [25].

2.2. Experimental design

Immediately after arrival at the testing facility, dams were given a powdered soy-free diet. On GD 10, the animals were randomized into 4 groups (8 dams/group) and given a soy-free diet that contained DBDE at concentrations of 0, 10, 100, and 1000 ppm until day 20 after delivery. A preliminary dose finding study of DBDE was performed with dietary doses of 0 (control), 10, 100, or 10,000 ppm from GD 10 until the day 20 after delivery ($n = 3$ dams in each group). Although a clear dose-dependence was not found, slight increases of the absolute and relative thyroid weights (statistical analysis not applicable) and development of diffuse follicular cell hypertrophy of the thyroid (one case of minimal grade at 10 ppm; two cases of minimal grade at 100 ppm; two cases of minimal grade and one case of slight grade at 10,000 ppm) were observed in dams of all DBDE-treated groups. DBDE did not affect pregnancy, survival of offspring or delivery at any dose (data not shown). Because of the unclear dose response effect on thyroid weight from 10 ppm even with the wide dose range employed, we decided to select 10 ppm as the lowest dietary concentration for administration.

In the main study, all dams were weighed and food consumption was measured throughout the experimental period. On PND 1 (PND 0: the day of birth), the number, weights and anogenital distance (AGD) of neonates were recorded, and on PND 2 litters were randomly culled to 8 offspring per dam, comprising 4 males and 4 females. On PND 20 dosing was terminated and all dams were killed. Ten male and 10 female offspring (at least one male and one female per dam) per group were subjected to prepubertal necropsy for histopathological assessment. Another group of 10 males and 10 females were also killed to investigate the effect on the development of immune system [26]. The remaining males and females were allocated to 4 rats per cage, given regular CRF-1 basal diet (Oriental Yeast Co. Ltd.) and water *ad libitum*, and kept for adult examination at 11 weeks of age.

Prepubertal necropsies were conducted on PND 20. The organs removed and weighed, i.e., brain, liver, kidneys, adrenals, testes, epididymides, ovaries, and uterus, were subjected to histopathological assessment. Weight measurement and histopathological examination of the thyroid glands was also performed on dams. The number of implantation remnants was also recorded at this point.

All female offspring were monitored daily from PND 26 for vaginal opening and all male pups were examined for preputial separation from PND 34 until each animal reached this developmental landmark. The age and body weight at the onset of puberty was recorded for the offspring allocated for adult examination. Estrous cycles of females were examined by daily microscopic observation of vaginal smears from postnatal week (PNW) 8 to PNW 11 as described previously [25].

At PNW 11, offspring were killed and following organs were subjected to weight measurement and histopathological assessment: brain, pituitary, liver, kidneys, adrenals, testes, epididymides, ventral prostate, dorso-lateral prostate, seminal vesicle, ovaries, uterus, and thyroid. Male offspring were killed on the first day of week 11. For female offspring, killing was delayed for up to 4 days after the first day of week 11 until the animal entered the diestrous stage of the estrous cycle.

The experimental animals were weighed and killed by exsanguination from the abdominal aorta under deep anesthesia with ether. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

2.3. Thyroid-related hormone measurement

At PND 20 and PNW 11, 10 male offspring were euthanized by trunk blood withdrawal from the abdominal aorta under ether anesthesia. Serum was prepared from the collected blood and stored at −30 °C to measure thyroid stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) concentrations by electrochemiluminescence immunoassay method at SRL Inc. (Tokyo, Japan).

2.4. Histopathological assessment

Prepubertal and adult stage necropsies were performed at PND 20 and PNW 11, respectively. Organs and tissues were removed and their weights have been recorded the similar way as previously [7]. Removed organs were routinely processed for paraffin embedding, sectioned at 3 μm, and stained with hematoxylin and eosin for light microscopy.

2.5. Immunohistochemistry

Brains of male offspring obtained at PNW 11 were subjected to immunohistochemistry for 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) and neuron-specific nuclear protein (NeuN) staining to stain oligodendrocytes and postmitotic neurons, respectively. Deparaffinized coronal brain slices at the position of −3.5 mm from the bregma were serially sectioned at 3 μm. Immunohistochemistry was performed according to a method described previously with 3,3′-diaminobenzidine/H_2O_2 as the chromogen [7,27]. Sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

2.6. Morphometric assessment

For the evaluation of the irreversible effects on neuronal migration, quantitative measurement of the variability in the distribution of neurons located within and lateral to the pyramidal cell layer of the hippocampal CA1 region was performed at PNW 11 using brain sections stained with NeuN as described previously [7,27].

To evaluate the effect on oligodendroglial development, areas of the white matter tract immunoreactive for CNPase and the number of CNPase-positive oligodendrocytes surrounding myelinated axons distributed in the cerebral cortical area were measured as described previously [7,27].

2.7. Statistical analysis

Data for offspring obtained during the lactation period such as body weights on PND 1, AGD, and body weight gain, were analyzed using the litter as the experimental unit. Data after weaning and the maternal data were analyzed using the individual animal as the experimental unit. Numerical data were analyzed for homogeneity of variance using Bartlett's test. When the variance was homogeneous among the groups, a one-way analysis of variance was carried out. If significant differences were found, the mean value for each exposure group was compared with that of the control using Dunnett's test. When the variance was heterogeneous based on Bartlett's test, the Kruskal–Wallis's *H*-test was employed to check for differences among the groups. If significant differences appeared, a Dunnett-type rank-sum test was performed. The incidences of histopathological lesions and estrous cycles were statistically compared using the Fisher's exact probability test. The severity of histopathological lesions analyzed by grading the change was statistically compared using the Mann–Whitney's *U*-test.

3. Results

3.1. Maternal toxicity

During the gestation period (GD10–GD20), body weight gain and food consumption of dams were not changed by DBDE-treatment (Table 1). Also, during the lactation period from days 1 to 20 after delivery, no changes were found in either parameter as a result of DBDE-treatment. Levels of maternal daily intake of DBDE were thus concluded to be proportional to the dietary concentration. Duration of the pregnancy period and body weight at weaning were also unchanged, irrespective of the DBDE-treatment.

On sacrifice at day 20 after delivery, body weight was unaltered by DBDE-treatment. However, both absolute and relative thyroid weights were significantly increased in the groups receiving 10 and 1000 ppm DBDE. A non-significant slight increase in the absolute and relative thyroid weights was also observed with 100 ppm DBDE. On the other hand, there were no statistically significant differences in the incidence and severity of diffuse follicular cell hypertrophy of the thyroid between the untreated controls and any of the exposure groups.

3.2. Effects on offspring until prepubertal necropsy

With regard to the offspring parameters on PND 1, no external malformations were observed in any treatment group, and the number of implantation sites in the uterus, number of live offspring, male ratio, and neonatal body weights and AGD were not changed by DBDE in either sex (Table 1). Until weaning, body weights were not changed by exposure to DBDE in either sex (data not shown).

At the prepubertal necropsy, there were no statistically significant differences in body and organ weights among the control and treatment groups, except for the liver (Table 1). In males, although the dose relation was unclear, statistically significant increases were observed in absolute liver weights at 10 and 1000 ppm and in relative liver weights at doses from 10 ppm. In females, significant increases of both absolute and relative liver weights were only observed at 1000 ppm.

3.3. Effects on the onset of puberty and estrous cycle

Onset age of the preputial separation and vaginal opening and body weight at the onset time were not changed by DBDE-treatment (Table 2). In terms of the estrous cycle, there were no irregularities showing statistically significant increased incidence by DBDE-treatment as compared with the control value.

3.4. Effects on offspring until adult stage necropsy

Males of the 100 ppm group had slightly but significantly higher body weights throughout the experiment until the adult stage necropsy at PNW 11 (data not shown). Significantly higher body weights were also observed in the 10 ppm males except at PNW 4, 5, and 8. Body weight in females was not changed by DBDE (data not shown).

At the necropsy on PNW 11, a statistically significant increase in body weight was observed in 10 and 100 ppm males (data not shown). A non-significant increase in body weight was also observed in 100 ppm females. With regard to organ weights, the relative brain weight was decreased in 10 and 100 ppm males and in 100 ppm females, and the absolute weights of the kidneys and thyroid were increased in 100 ppm males (data not shown).

3.5. Serum levels of thyroid-related hormones

Serum levels of thyroid-related hormones were measured in male offspring (Table 3). In the 1000 ppm group, statistically significant decreases of T_3 and T_4 were observed on PND 20 and PNW 11, respectively.

3.6. Histopathology at the prepubertal and adult stage necropsies

Results of the histopathological findings at PND 20 and PNW 11 are summarized in Table 4.

At PND 20, diffuse hypertrophy of thyroid follicular cells was observed in males of all exposure groups, with statistically significant increases in the incidence and severity at 1000 ppm (Fig. 1A and B). Similar changes were also observed in females at 10 and 1000 ppm, but without statistically significant differences in the incidence or severity. In the liver, diffuse liver cell hypertrophy associated with increased cytoplasmic eosinophilia was observed in males of all treatment groups with statistically significant increases in both incidence and severity (Fig. 1E and F). In females, this change was observed at 100 and 1000 ppm with statistically significant increases of the incidence and severity at 1000 ppm. In the kidneys, increased cytoplasmic eosinophilia in the cortical proximal tubular epithelia was observed in all DBDE-treated groups in both sexes, with statistically significant increases in the incidence and severity from 100 ppm in males and from 10 ppm in females (Fig. 1G and H). In addition, an increase in interstitial glands of the ovaries observed at 1000 ppm was not statistically significant.

At PNW11, cases with diffuse follicular cell hypertrophy of the thyroid were observed in DBDE-treated males at 10 ppm and higher, but there were no statistically significant difference in the incidence or severity in comparison with the untreated controls (Fig. 1C and D). In females, one animal each in the 100 and 1000 ppm groups showed follicular cell hypertrophy. Incidence and/or severity of other histopathological changes at PNW11 were not statistically significant in either sex.

3.7. Brain morphometry at the adult stage

With regard to the distribution of hippocampal CA1 neurons, there were no significant differences between the untreated controls and any of the exposure groups in the mean distance of the location of NeuN-positive neurons from the pyramidal cell layer, the number of neurons located laterally to the pyramidal cell layer, or the ratio of abnormally distributed neurons in total CA1 neurons (Table 5). With regard to the oligodendroglial development-related parameters, both the CC area and the number of CNPase-positive oligodendrocytes were significantly reduced at 100 and 1000 ppm, but there was no clear dose-relation (Table 5 and Fig. 2A and B). Although statistically non-significant, slight reductions in these parameters were also observed at 10 ppm.

Table 1
Effects on dams and offspring until prepubertal necropsy by exposure to DBDE from mid-gestation to the end of lactation.

	DBDE in diet (ppm)			
	0	10	100	1000
No. of dams examined	8	8	8	8
Maternal parameter				
Body weight gain (g/day)				
GD 10–GD 20	10.4 ± 1.6[a]	10.5 ± 1.5	11.1 ± 1.7	11.0 ± 1.1
Day 1–day 9 after delivery	4.7 ± 1.2	5.6 ± 1.9	5.1 ± 1.5	5.5 ± 2.2
Day 9–day 20 after delivery	−0.2 ± 0.9	−0.4 ± 1.4	−0.7 ± 1.7	0.2 ± 1.5
Food consumption (g/day)				
GD 10–GD 20	27.8 ± 3.6	26.2 ± 3.1	26.8 ± 3.4	25.3 ± 2.7
Day 1–day 9 after delivery	46.5 ± 6.0	46.6 ± 4.8	46.1 ± 6.3	44.6 ± 3.2
Day 9–day 20 after delivery	75.6 ± 17.3	76.1 ± 12.3	74.0 ± 16.3	71.4 ± 6.3
DBDE intake (mg/kg-BW-d)				
GD 10–GD 20	0	0.7 ± 0.1	7.0 ± 0.4	66.3 ± 4.8
Day 1–day 9 after delivery	0	1.5 ± 0.2	13.9 ± 1.0	140.4 ± 5.7
Day 9–day 20 after delivery	0	2.4 ± 0.4	22.8 ± 4.2	224.3 ± 20.1
Duration of pregnancy (days)	21.6 ± 0.5	21.8 ± 0.5	21.5 ± 0.5	21.6 ± 0.5
Necropsy at weaning (day 20 after delivery)				
BW (g)	302.0 ± 25.3	302.3 ± 21.8	311.4 ± 24.0	302.2 ± 23.5
Thyroid				
Absolute weight (mg)	17.9 ± 1.8	21.8 ± 3.1*	20.1 ± 2.5	21.6 ± 3.2*
Relative weight (mg/100 g BW)	5.95 ± 0.56	7.20 ± 0.93*	6.48 ± 0.93	7.17 ± 1.08*
Histopathology: diffuse follicular cell hypertrophy (±/+)[b]	2[c] (2/0)[d]	4(3/1)	6(5/1)	5(3/2)
Offspring parameter				
No. of implantation sites	13.0 ± 2.4	13.1 ± 1.5	12.4 ± 1.9	13.4 ± 1.3
No. of live offspring	12.4 ± 2.6	12.1 ± 1.7	11.5 ± 2.4	12.5 ± 2.0
Male ratio (%)	47.5 ± 16.2	53.7 ± 14.6	46.7 ± 17.3	38.5 ± 7.0
BW, PND 1 (g)				
Males	7.46 ± 0.58	7.16 ± 1.00	7.50 ± 1.06	7.08 ± 0.73
Females	7.05 ± 0.58	6.99 ± 0.87	6.92 ± 1.10	6.69 ± 0.82
AGD, PND 1 (mm)				
Males	3.93 ± 0.15	3.90 ± 0.23	3.98 ± 0.31	3.93 ± 0.17
Females	1.70 ± 0.57	1.89 ± 0.08	1.88 ± 0.07	1.86 ± 0.07
Prepubertal necropsy on PND 20				
Males				
No. of animals examined	10	10	10	10
BW (g)	51.6 ± 6.2	55.8 ± 4.0	52.7 ± 6.0	54.0 ± 3.0
Liver (g)	1.88 ± 0.34	2.22 ± 0.24*	2.07 ± 0.35	2.37 ± 0.24**
Liver (g/100g BW)	3.62 ± 0.26	3.98 ± 0.20*	3.90 ± 0.29*	4.39 ± 0.27**
Females				
No. of animals examined	10	10	10	10
BW (g)	49.8 ± 4.2	48.4 ± 7.3	48.0 ± 4.5	51.3 ± 2.7
Liver (g)	1.85 ± 0.25	1.84 ± 0.36	1.83 ± 0.27	2.21 ± 0.20*
Liver (g/100 g BW)	3.71 ± 0.23	3.77 ± 0.26	3.80 ± 0.26	4.31 ± 0.20**

Abbreviations: AGD, anogenital distance; BW, body weight; DBDE, decabromodiphenyl ether; GD, gestational day; PND, postnatal day.
[a] Mean ± SD.
[b] Grade of change: (±) minimal; (+) slight.
[c] Total number of animals with each finding.
[d] No. of animals with each grade.
* Significantly different from the 0 ppm group (control) by Dunnett's test or Dunnett-type rank-sum test ($P < 0.05$).
** Significantly different from the 0 ppm group (control) by Dunnett's test or Dunnett-type rank-sum test ($P < 0.01$).

Table 2
Onset of puberty and estrous cycles in the offspring exposed to DBDE from mid-gestation to the end of lactation.

	DBDE in diet (ppm)			
	0	10	100	1000
Onset of puberty				
Preputial separation in males				
No. of animals examined	11	12	11	12
Age by day	41.1 ± 1.5[a]	40.1 ± 1.5	41.5 ± 1.6	41.3 ± 2.1
BW (g)	189.6 ± 14.4	192.1 ± 18.8	208.8 ± 28.6	193.2 ± 19.2
Vaginal separation in females				
No. of animals examined	11	11	12	10
Age by day	35.1 ± 2.4	34.4 ± 2.0	34.7 ± 2.4	34.8 ± 2.4
BW (g)	121.5 ± 9.0	126.0 ± 19.8	126.6 ± 15.2	121.9 ± 11.8
Estrous cycles during PNW 8–11				
No. of animals examined	10	10	10	10
Irregularity (extended diestrus)	1	1	2	1

Abbreviations: DBDE, decabromodiphenyl ether; BW, body weight; PNW, postnatal week.
[a] Mean ± SD.

Table 3
Serum levels of thyroid-related hormones of the male offspring exposed to DBDE from mid-gestation to the end of lactation.

	DBDE in diet (ppm)			
	0	10	100	1000
PND 20				
No. of animals examined	10	10	10	10
T_3 (ng/ml)	1.39 ± 0.11[a]	1.35 ± 0.15	1.33 ± 0.18	1.17 ± 0.10**
T_4 (μg/dl)	5.19 ± 0.74	4.89 ± 0.84	5.66 ± 0.71	4.89 ± 0.54
TSH (ng/ml)	5.38 ± 0.89	5.12 ± 0.71	5.85 ± 1.22	4.74 ± 0.69
PNW 11				
No. of animals examined	10	10	10	10
T_3 (ng/ml)	0.99 ± 0.09	1.01 ± 0.08	1.01 ± 0.11	1.02 ± 0.11
T_4 (μg/dl)	6.02 ± 0.70	6.00 ± 0.66	5.98 ± 0.94	5.17 ± 0.57*
TSH (ng/ml)	8.30 ± 3.40	8.81 ± 1.63	9.71 ± 3.45	10.47 ± 2.35

Abbreviations: DBDE, decabromodiphenyl ether; PND, postnatal day; PNW, postnatal week; T_3, triiodothyronine; T_4, thyroxine; TSH, thyroid-stimulating hormone.
[a] Mean ± SD.
* Significantly different from the 0 ppm group (control) by Dunnett's test or Dunnett-type rank-sum test ($P < 0.05$).
** Significantly different from the 0 ppm group (control) by Dunnett's test or Dunnett-type rank-sum test ($P < 0.01$).

4. Discussion

Lower BDEs are known to affect thyroid hormone homeostasis to affect serum T_4 levels in rats and mice [28–30]. There are two mechanisms by which lower BDE's affect thyroid hormone homeostasis. First, there is increased elimination of the thyroid hormones, especially of T_4 primarily as a result of induced activity of UGT in the liver, which leads to acceleration of hepatic clearance of T_4 and following reductions in serum levels of total and free T_4 [30]. Second, many halogenated DEs structurally resemble thyroid hormones and therefore compete for binding to thyroid hormone receptors and transporter proteins such as transthyretin [31,32]. With regard to fully brominated DBDE, a carcinogenicity study in mice resulted in increased incidences of thyroid follicular proliferative lesions, suggesting the potential for DBDE to affect thyroid function similar to lower BDEs [33]. In the present study, DBDE-exposure at 1000 ppm slightly decreased serum T_3 levels on PND 20 in male offspring. There was also a dose-related induction

Table 4
Histopathological changes for male and female offspring exposed to DBDE from mid-gestation to the end of lactation.

	DBDE in diet (ppm)			
	0	10	100	1000
PND 20				
Males				
No. of animals examined	10	10	10	10
Thyroid				
Diffuse follicular cell hypertrophy (±/+)	0	1 (1/0)	3 (2/1)	9 (3/6)**,##
Liver				
Diffuse liver cell hypertrophy with increased cytoplasmic eosinophilia (±/+/++)[a]	0	10[b] (8/2/0)[c],**,##	10 (4/6/0)**,##	10 (0/2/8)**,##
Kidney				
Increased cytoplasmic eosinophilia, cortical proximal tubules (±/+)	1 (1/0)	4 (4/0)	7 (5/2)**,#	10 (1/9)**,##
Females				
No. of animals examined	10	10	10	10
Thyroid				
Diffuse follicular cell hypertrophy (±/+)	0	2 (2/0)	0	3 (3/0)
Liver				
Diffuse liver cell hypertrophy with increased cytoplasmic eosinophilia (±/+)	0	0	3 (2/1)	7 (1/6)**,##
Kidney				
Increased cytoplasmic eosinophilia, cortical proximal tubules (±/+)	0	7 (5/2)**,##	6 (5/1)*,#	7 (4/3)**,##
Ovary				
Increase of interstitial glands (+)	0	0	0	2
PNW 11				
Males				
No. of animals examined	10	10	10	10
Thyroid				
Diffuse follicular cell hypertrophy (±/+/++)	0	3 (1/2/0)	2 (2/0/0)	4 (2/1/1)
Mammary gland				
Diffuse lobular atrophy (±/+/++)	2 (0/1/1)	3 (1/2/0)	6 (2/2/2)	3 (2/0/1)
Females				
No. of animals examined	10	11	10	11
Thyroid				
Diffuse follicular cell hypertrophy (±/+)	0	0	1 (1/0)	1 (0/1)
Uterus				
Hydrometra (+)	0	1	0	3

[a] Grade of change: (±) minimal; (+) slight; (++) moderate.
[b] Total no. of animals with each finding.
[c] No. of animals with each grade.
* Significantly different from the 0 ppm group (control) by Fisher's exact probability test ($P < 0.05$).
** Significantly different from the 0 ppm group (control) by Fisher's exact probability test ($P < 0.01$).
# Significantly different from the 0 ppm group (control) by Mann–Whitney's *U*-test ($P < 0.05$).
Significantly different from the 0 ppm group (control) by Mann–Whitney's *U*-test ($P < 0.01$).

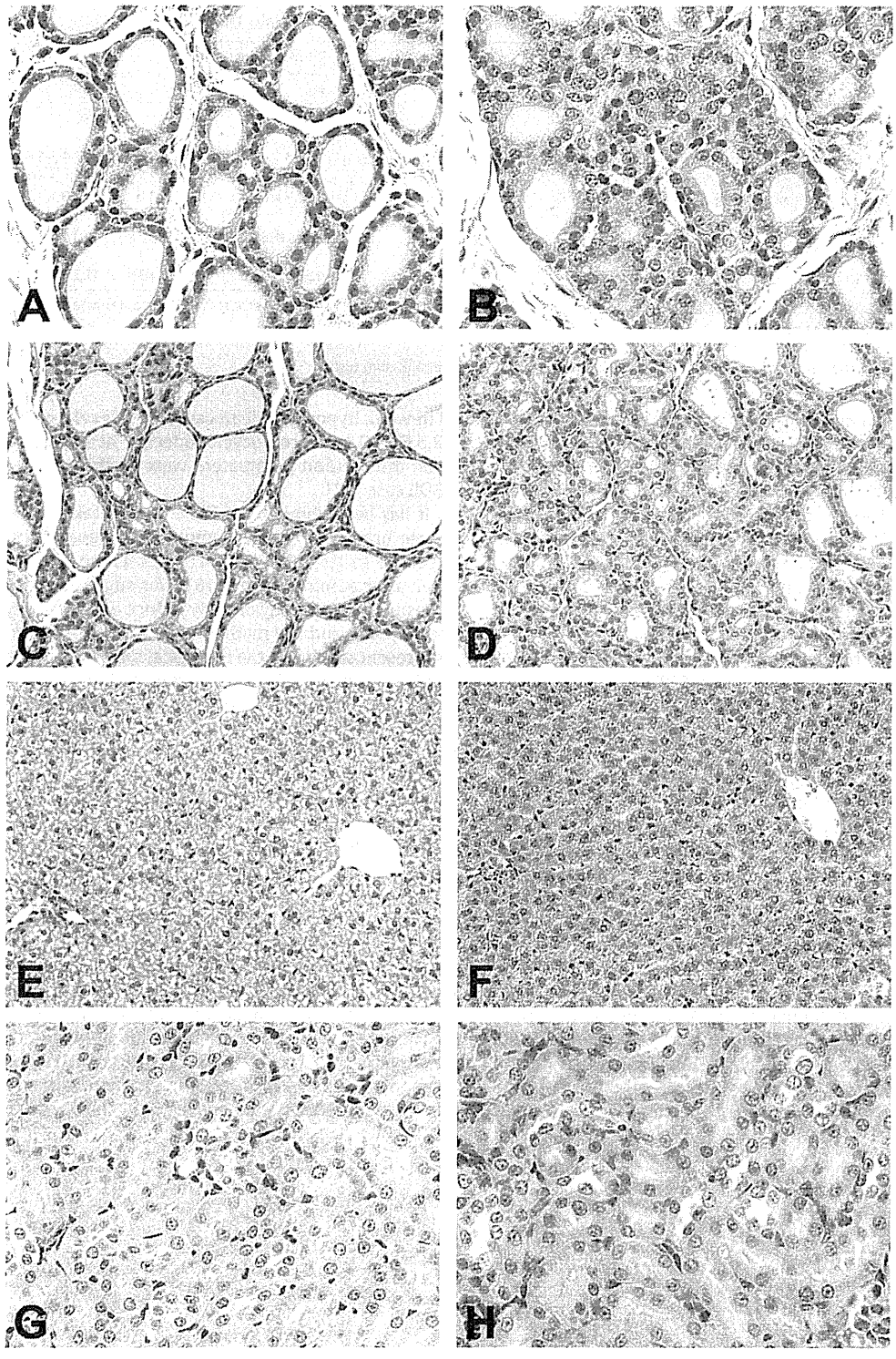

Fig. 1. Histopathological changes observed in offspring exposed to DBDE from mid-gestation to the end of lactation. (A and B) Thyroid gland of a male offspring on PND 20 given DBDE at 0 (control) (A) or 1000 ppm (B). Compared with the control, diffuse follicular cell hypertrophy (slight degree) is evident with 1000 ppm DBDE. Hematoxylin and eosin. 400× magnification. (C and D) Thyroid gland of a male offspring on PNW 11 after developmental exposure to DBDE at 0 (control) (C) or 1000 ppm (D). Compared with the control, diffuse follicular cell hypertrophy (moderate degree) is evident with 1000 ppm DBDE. Hematoxylin and eosin. 200× magnification. (E and F) Liver of a male offspring on PND 20 given DBDE at 0 (control) (E) or 1000 ppm (F). Note that liver cells show diffuse hypertrophy associated with increase of cytoplasmic eosinophilia in the 1000 ppm DBDE-exposed rat. Hematoxylin and eosin. 200× magnification. (G and H) Kidney of a female offspring on PND 20 given DBDE at 0 (control) (G) or 1000 ppm (H). Note increased eosinophilia in the cytoplasm of cortical proximal tubules in the 1000 ppm DBDE-exposed case. Hematoxylin and eosin. 400× magnification.

Table 5
Brain morphometry of the male offspring exposed to DBDE from mid-gestation to the end of lactation and examined at PNW 11.

	DBDE in diet (ppm)			
	0	10	100	1000
No. of offspring examined	10	10	10	10
Hippocampal CA1 neurons[a]				
Mean distance of the location of neurons from the innermost margin of the pyramidal cell layer (μm)	33.8 ± 4.4[b]	32.5 ± 3.4	32.3 ± 3.5	32.2 ± 5.3
No. of neurons located lateral to the pyramidal cell layer (mm^{-1})	59.5 ± 26.9	80.8 ± 35.9	65.1 ± 29.2	58.4 ± 27.0
Ratio of abnormally distributed neurons/CA1 neurons (%)	2.7 ± 0.9	3.2 ± 1.3	2.9 ± 1.1	2.6 ± 1.0
CC				
Area of CC (mm^2)	0.18 ± 0.03	0.15 ± 0.02	0.12 ± 0.02**	0.13 ± 0.03**
Cingulate deep cortex				
CNPase (+) cell count (count/mm^2)	150.9 ± 23.0	137.7 ± 10.8	122.4 ± 13.9**	124.9 ± 13.0**

Abbreviations: CC, corpus callosum; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; DBDE, decabromodiphenyl ether; PNW, postnatal week.
[a] NeuN (+) neurons were subjected to analysis.
[b] Mean ± SD.
** Significantly different from the 0 ppm group (control) by Dunnett's test or Dunnett-type rank-sum test ($P < 0.01$).

of mild thyroid follicular cell hypertrophy in these animals at this time point with a significant difference at 1000 ppm. These results suggest a weak developmental hypothyroidism caused by DBDE at least at the highest dose, but the mechanism behind the induction of hypothyroidism is likely to be different between lower BDEs and fully brominated DBDE. While prenatal exposure to DBDE resulted in decrease of serum T_3 levels at the adult stage in mice [34], we found a slight decrease of serum T_4 levels by DBDE exposure at 1000 ppm in adult rats, which has previously been shown by others [35].

Neonatal exposure to lower BDEs (tetra-BDE, penta-BDE, hexa-BDE, hepta-BDE, octa-BDE, and nona-BDE) as well as polychlorinated biphenyls causes changes in the spontaneous behavior of mice [23,36–38]. With regard to the developmental exposure effect of DBDE, there is increasing in vivo evidence of neurotoxicity involving synaptogenesis [39,40]. In a previous study we employed the same morphometric methods to study the neuroarchitecture as in the present study and confirmed hypothyroidism-related changes in the neuronal cell distribution of the hippocampal CA1 region as well as in parameters linked to oligodendroglial development [7]. Developmental hypothyroidism results in a decreased number of mature oligodendrocytes, which results in a decreased area of intrahemispheric commissures, such as the CC [2]. In the present study, reductions in the area of CC and CNPase-positive oligodendrocytes were observed after DBDE exposure of 100 ppm and higher at PNW 11, while no other changes were detected in neuronal migration parameters. These results suggest a mild effect of DBDE on oligodendroglial development, probably through a hypothyroidism-related mechanism. We recently detected a reduced density of oligodendrocytes similar as in weak hypothyroidism caused by developmental exposure to 1,2,5,6,9,10-hexabromocyclododecane at 10,000 ppm, while neuronal distribution parameters were unchanged as in the present DBDE case [27].

It has been shown that DBDE can be taken up in the neonatal mouse brain and that the amount of radioactivity after neonatal exposure to [U-^{14}C]DBDE increases in the brain during the first week after administration [18]. This suggests that developmental exposure to DBDE may directly induce neurotoxicity. Although the species used and the mode of administration were different from the present study and the biological relevance to the present study results are unclear, Viberg et al. reported that single neonatal exposure to DBDE at PND 3 (20.1 mg/kg body weight by oral gavage) caused disturbances in the spontaneous motor behavior at the adult stage in both mice and rats, and that this effect worsened with age [18,24].

In a classic developmental exposure study of the commercial product of DBDE with 77% purity (including 21.8% nona-BDE and 0.8% octa-BDE) by oral administration through gavage to pregnant rats, a dose-unrelated increase in the resorption incidence was observed at 10 and 100 mg/kg-d from GD 6 to 15, while these changes were lacking at 1000 mg/kg-d [41]. The authors concluded that the observed changes were probably due to chance rather than treatment. In the present study no effect was observed on reproductive parameters by exposure to DBDE up to 1000 ppm in the maternal diet (66.3–224.3 mg/kg-d). Likewise, no effect of treatment was observed on neonatal body weight, neonatal sex distribution, or AGD. Hardy et al. [42] reported no evidence of maternal or neonatal toxicity or developmental effect in a study using rats administered 1000 mg/kg body weight DBDE from GD 0 to GD 19.

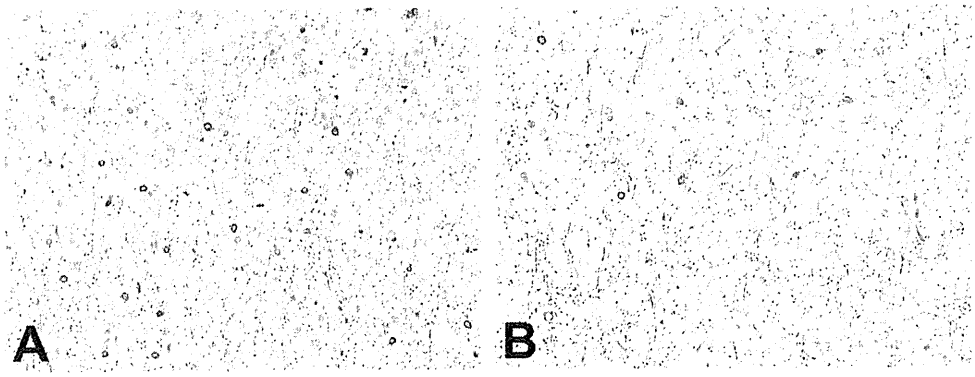

Fig. 2. Distribution of CNPase-positive oligodendrocytes in the cingulate deep cortex of the cerebrum in offspring exposed to DBDE from mid-gestation to the end of lactation. (A and B) A male offspring on PNW 11 given DBDE at 0 (control) (A) or 1000 ppm (B). Compared with the control, decrease of CNPase-positive oligodendrocytes is evident with 1000 ppm DBDE. CNPase-immunohistochemistry. 200× magnification.

In the present study, increases in relative weight and histopathological alterations in the liver, and histopathological changes in the kidneys were observed at PND 20, while these changes had entirely recovered at the adult stage. Orally administered DBDE is mainly distributed to the liver [18,21,43]. Carcinogenicity studies of orally administered DBDE using rats and mice showed an increased incidence of neoplastic nodules in the livers of rats of both sexes, and increased incidences of hepatocellular adenomas or carcinomas (combined) in male mice [33]. Recent reports have shown hepatic expressions of cytochrome P450 (CYP) 1A and CYP2B by oral administration of DBDE as well as an increase in hepatic S9 7-ethoxyresorufin *O*-deethylase activity by developmental exposure to DBDE [34,44], although there was a contradictory result regarding the induction of hepatic phase I and II enzymes including UGT in a classic study [45]. This suggests that the maternal DBDE-exposure in the present study resulted in the development of liver cell hypertrophy due to enzyme induction in the offspring. Regarding the increased eosinophilia in the proximal tubular epithelia on weaning in the present study, there have been no such reported cases by DBDE-exposure, while induction of hyaline degeneration of the renal tubules has been reported after repeated oral administration of DBDE in rats [41]. Although pathological mechanism behind the increased tubular eosinophilia on weaning was unclear, induction of similar reversible cytoplasmic eosinophilia in the liver cells at the same time may suggest a common mechanism between the liver and renal tubular cells on this change. Because administered DBDE can also be distributed to the kidney [46], cellular interaction of DBDE and/or its metabolites may be responsible increased cytoplasmic eosinophilia.

In conclusion, this study has shown that developmental exposure of DBDE at low doses causes mild hypothyroidism in male rats, which lasts into adulthood. Furthermore, we found irreversible white matter hypoplasia targeting oligodendrocytes at doses of 100 ppm and higher, which is likely to be related to developmental hypothyroidism. Although changes were reversed by adulthood, 10 ppm, translating into 0.7–2.4 mg/kg-d, was determined to be the lowest-observed-adverse-effect level of DBDE by maternal exposure judging from histopathological changes in the liver and kidneys and liver weight changes on weaning. Of note, rat pups may gradually start to consume diet from around PND 14, and therefore, mg-test-substance per kg-body weight basis of pups may actually be consuming a higher dose than adult case during their third week of the lactation period.

Conflict of interest

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2010.09.003.

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REPRODUCTIVE TOXICOLOGY

Disruptive neuronal development by acrylamide in the hippocampal dentate hilus after developmental exposure in rats

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Abstract To examine whether developmental exposure to acrylamide (AA) impairs neuronal development, pregnant Sprague–Dawley rats were treated with AA at 0, 25, 50 or 100 ppm in drinking water from gestational day 6 until weaning on postnatal day 21. Offspring were immunohistochemically examined at the end of exposure. We investigated the expression of Reelin (a molecule regulating neuronal migration and positioning) in the hilus of the hippocampal dentate gyrus. As a positive control for direct exposure, AA (50 mg/kg body weight) was administered to pups by intraperitoneal injection 3 times per week during the lactation period. As well as pups directly injected with AA, maternally exposed offspring decreased body weight at 100 ppm; increased dose-dependently the number of Reelin-immunoreactive cells (from 25 ppm AA) and glutamic acid decarboxylase 67-immunoreactive cells (from 50 ppm AA), confirming an increase in γ-aminobutyric acid-ergic interneurons. We also noted decreased apoptosis in the neuroblast-producing subgranular zone of the dentate gyrus of maternally exposed pups at 100 ppm, as well as in directly AA-injected pups. These results suggest that a compensatory regulatory mechanism exists to correct impaired neurogenesis and mismigration caused by maternal exposure to AA during neuronal development. The lowest-observed-adverse-effect level of AA was determined to be 25 ppm (3.72 mg/kg body weight/day).

Keywords Acrylamide · Developmental neurotoxicity · Neuronal migration · Neurogenesis · Dentate gyrus · GABAergic interneuron

Abbreviations

AA	Acrylamide
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
Calb-D-28 K	Calbindin-D-28 K
GABA	γ-aminobutyric acid
GAD67	Glutamic acid decarboxylase 67
GD	Gestational day
PCNA	Proliferating cell nuclear antigen
PND	Postnatal day

Introduction

Acrylamide (AA), a widely used chemical in many industries, is known to be a neuro- and reproductive toxicant and to act as a carcinogen in animals (WHO/IPCS 2006). Recently, it was found that AA is generated during heating of foods containing carbohydrate and asparagine, and risk assessment studies of AA in foodstuffs are now being conducted globally (Exon 2006; Parzefall 2008). Mean daily intake of AA for adults is estimated as 1 μg/kg body weight/day. Intake for infants and children is estimated to

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be 2–3-fold higher than for adults when expressed on a body weight basis (WHO/IPCS 2006).

It is well known that AA affects axon terminals in both the central and peripheral nervous systems (LoPachin 2004). We and others have demonstrated that developmental exposure to AA (by maternal transfer, with dose levels inducing maternal neurotoxicity) shows no obvious neurotoxicity in offspring but a reduction in body size (Friedman et al. 1999; Takahashi et al. 2009). However, direct injection into neonatal rats throughout the lactation period resulted in neurotoxicity similar to that observed in adult animals (Takahashi et al. 2009), suggesting that neonates and adult animals have comparable sensitivity to AA. The differences in the neurotoxicity of AA in maternally and directly exposed pups is likely due to limited lactational transfer and perhaps to impairment in nursing/lactation activity as a consequence of maternal neurotoxicity (Takahashi et al. 2009), which may also explain the loss of body weight in the offspring of neurotoxic dams.

Reelin is a molecule that plays an important role in neuronal migration and positioning (D'Arcangelo et al. 1997). In the hippocampal formation, neuronal subpopulations are known to produce Reelin in the embryonic period and throughout adult life. This molecule accumulates in brain areas at the timing of cortical development involving neuronal migration (D'Arcangelo et al. 1995, 1997; Pesold et al. 1998; Scotti and Herrmann 2002; Houser 2007). On the other hand, rat offspring exposed maternally to anti-thyroid agents show impaired brain development, with impaired neuronal migration and white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation (Goodman and Gilbert 2007; Lavado-Autric et al. 2003; Schoonover et al. 2004). In the hippocampus, the subgranular zone of the dentate gyrus continues to produce new neurons which are distributed in the hilus of the dentate, even during adulthood (Gould 2007). We have recently shown aberrant increases in the numbers of Reelin-expressing γ-aminobutyric acid (GABA)ergic interneurons in the dentate hilus following developmental exposure to anti-thyroid agents during gestation and lactation periods, suggestive of the reflection of disrupted neuronal migration and positioning by these agents (Saegusa et al. 2010).

Axon guidance during development and after axon injury is an important process in maintaining neuronal plasticity at the axon terminals (Bashaw and Klein 2010). It is well established that the molecular mechanisms controlling neuronal migration during development have many similarities with those described for axon guidance (Nóbrega-Pereira and Marín 2009). Therefore, both migrating neuroblasts and immature axon terminals may have sensitivity to AA.

In the present study, we used samples verified as lacking any obvious axon terminal injury either in the central or peripheral nervous system (Takahashi et al. 2009) to elucidate whether AA affects neurogenesis or neuronal migration. We investigated the distribution of interneurons expressing Reelin and/or glutamic acid decarboxylase (GAD) 67 in the dentate hilus as well as the apoptosis and cell proliferation at the subgranular zone following maternal exposure (through drinking water) during gestation and lactation periods.

Materials and methods

Chemicals and animals

Acrylamide (AA) was purchased from Sigma (St Louis, MO, USA; CAS #79-06-1) as a white powder with a purity of > 98%. A total of 18 pregnant CD® (SD) IGS rats were obtained from Charles River Japan Inc. (Yokohama, Japan) at gestational day (GD) 1 (the day when a vaginal plug was observed was designated as GD 0) and housed individually in polycarbonate cages with wood chip bedding, in an air-conditioned animal room (temperature 24 ± 1°C, relative humidity 55 ± 5%) with a 12-h light/dark cycle. They received powdered basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum during the 5-day acclimatization period.

Experimental design

The animal experiment was identical to that in our previous study (Takahashi et al. 2009). In brief, on GD 6, dams were randomly divided into four groups of four dams each and given AA at 0, 25, 50 or 100 ppm in their drinking water from GD 6 to postnatal day (PND) 21 (where PND 0 is the day of delivery). The highest dose was selected as that at which, in our previous study, dams exhibit progressive gait abnormalities due to neurotoxicity (Takahashi et al. 2008). Two dams were maintained untreated until delivery, and their offspring received AA at 50 mg/kg/day by direct intraperitoneal injections 3 times a week from PND 2 to PND 21. This dosing regimen is known to induce peripheral nerve degeneration in adult rats within 3 weeks (Saita et al. 1996). All dams were housed individually. Litters were culled randomly on PND 3 to preserve eight pups, generally four of each sex per litter. On PND 21, all dams and remaining offspring were killed by exsanguination from the abdominal aorta under deep ether anesthesia, subjected to autopsy and the brain removed. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Immunohistochemistry and Cresyl Violet staining

For immunohistochemical analysis, brains in the subgroups of offspring killed at PND 21 were fixed in methacarn solution at 4°C overnight, routinely processed for paraffin embedding and sectioned at 4 μm. Coronal slices at the positions of −3.0 and −3.5 mm from the bregma were prepared.

Immunohistochemistry was performed on the brain sections with antibodies against Reelin (clone G10, mouse $IgG_{1\kappa}$, 1:1,000; Novus Biologicals, Inc., Littleton, CO, USA), glutamic acid decarboxylase 67 (GAD67; mouse $IgG_{1\ and\ 2a}$, 1:50, Chemicon, Billerica, MA, USA), Calbindin-D-28 K (Calb-D-28 K; clone CB-955, mouse IgG_1, 1:500; Sigma Chemical Co.), and proliferating cell nuclear antigen (PCNA; clone PC10, mouse IgG_{2a}, 1:200, Dako, Glostrup, Denmark). Immunodetection was carried out using a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine/H_2O_2 as the chromogen, as previously described (Shibutani et al. 2007). Sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

For evaluation of apoptosis in the subgranular zone of the dentate gyrus, apoptotic bodies were detected by Cresyl Violet staining as described elsewhere (Nuñez and McCarthy 2004).

Morphometry of immunolocalized cells and apoptotic cells

Reelin- or GAD67-positive cells in the cytoplasm distributed in the hilus of the dentate gyrus were bilaterally counted and normalized as the number per unit area of the polymorphic layer of the hilus (as enclosed by the dotted line in Fig. 1a). Cells expressing Calb-D-28 K in the cytoplasm were largely localized beneath the subgranular zone of the dentate gyrus (Fig. 1b). Therefore, Calb-D-28 K-positive cells were bilaterally counted and their numbers normalized with the length of the granular cell layer measured. Apoptotic bodies (detected by Cresyl Violet staining) and proliferating cells (detected by nuclear immunoreactivity of PCNA) distributed in the subgranular zone were counted and normalized similarly to Calb-D-28 K-positive cells. Digital photomicrographs at 100 × magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) connected to a DP70 Digital Camera System (Olympus Optical Co.), and quantitative measurements were taken on these images using the WinROOF image analysis software package (version 5.7, Mitani Corp., Fukui, Japan).

Statistical analysis

Variance in data for body weights was checked for homogeneity by Bartlett's procedure. If the variance was homogeneous, the data were assessed by one-way analysis of variance. If not, the Kruskal–Wallis test was applied. When a statistically significant difference was indicated, the Dunnett's multiple test was employed for comparison of each AA treatment group with the control (0 ppm) group. Values for morphometric assessment of the number of immunoreactive cells or apoptotic bodies were analyzed by the Student's *t*-test when the variance was proven to be homogenous among the groups using a test for equal variance. If a significant difference in variance was observed, Welch's *t*-test was performed.

Results

In-life and reproductive parameters

Dams in the 100 ppm group exhibited gait abnormality from PND 2, which progressed to a moderate or severe

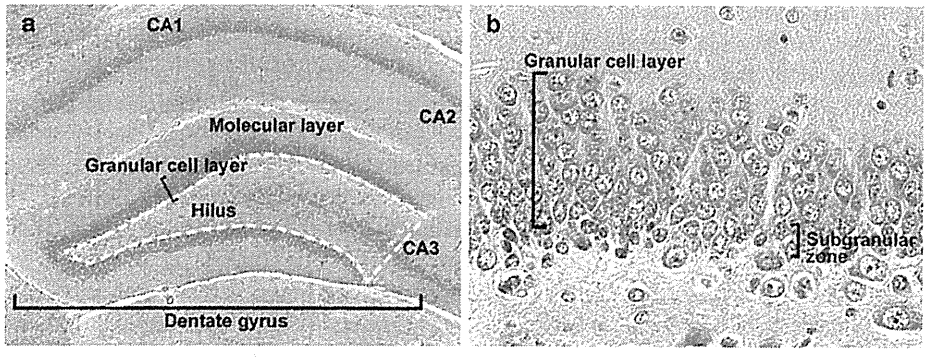

Fig. 1 Overview of the hippocampal formation of a male rat at PND 21 stained with hematoxylin and eosin. **a** The numbers of cells in the hilus of the dentate gyrus (as demarcated by the *white dotted line*) displaying immunoreactivity for Reelin, GAD67, and Calb-D-28 K were counted and normalized for the unit area. Positive immunoreactivity for these antigens was restricted to small-sized neurons in this area, as larger CA3 neurons were not immunoreactive. Magnification, × 40. **b** Higher magnification of the granular cell layer and subgranular zone. Magnification, × 400

degree at PND 21 (Takahashi et al. 2009). Body weight in this group was suppressed in parallel with the progression of neurotoxic symptoms. At 50 ppm, a slightly abnormal gait appeared from PND 18. Tendencies for decreased food and water consumption were observed at 100 ppm during the lactation period. Mean daily intake of AA by dams during the gestation and lactation periods was 3.72 ± 0.28, 7.89 ± 1.70, and 14.56 ± 2.47 mg/kg body weight/day at 25, 50, and 100 ppm, respectively. AA did not affect the gestation period, number of implantations, live birth ratio, and male pup ratio. On PND 8–12, deaths of offspring were sporadically found in all groups, including the control group. No apparent abnormalities were found on clinical observation in offspring exposed to AA maternally at any dose. In contrast, intraperitoneal injections of AA into offspring revealed gait abnormalities similar to the adult cases from PND 15.

At PND 21, the average body weight of dams treated with 100 ppm AA was reduced to 92.1% of control (0 ppm), although this difference was not statistically significant. Body weights in maternally exposed offspring (both male and female) were significantly lower at 100 ppm, when compared with those in the control group (57.8 and 54.3% of control in males and females, respectively), which is consistent with our previous study (Takahashi et al. 2008). We also noted significant reduction of body weight in offspring receiving intraperitoneal AA (to 62.3% of control).

Immunolocalization of Reelin, GAD67, and Calb-D-28 K in the hippocampal formation at PND 21

The distribution of Reelin-immunoreactive cells in the hippocampal formation, including the CA1–3 regions, was similar to that described in previous reports (Pesold et al. 1998; Saegusa et al. 2010). In the dentate gyrus, Reelin was expressed predominantly in the interneurons located in the polymorphic layer of the hilus, with only sparse distribution in the molecular layer. In offspring exposed to AA maternally through drinking water, the number of Reelin-expressing cells increased in both males and females, although with differing dose response patterns (Table 1, Fig. 2a, b). Male offspring showed a statistically significant difference in Reelin-expressing cells at 50 ppm and above, but this effect appeared not to be linked to dose, with the highest value also being attained at 50 ppm. In contrast, female offspring showed a dose-related increase from the lowest dose level, although this only became statistically significant at 100 ppm. However, there were no significant differences between males and females in each group. Combined mean values (male + female for each dose) were significantly higher in all dosed groups when compared with untreated controls. Pups treated with intraperitoneal AA during the lactation period also exhibited increased numbers of Reelin-immunoreactive cells in both sexes compared to untreated controls, with this increase being statistically significant in female offspring and combined (male + female) mean values.

With regard to GAD67-positive cells, male offspring exposed maternally to AA exhibited significant dose-related increases in the number of these cells from 50 ppm (Table 1 and Fig. 2c, d). Interestingly, females had higher numbers of positive cells under control conditions (0 ppm) than did males, and there was no significant fluctuation in these levels at any dose of maternally administered AA. However, the combined mean value (male + female at each dose) was significantly higher at 50 and 100 ppm when compared with untreated controls (Table 1). Pups treated with intraperitoneal AA showed significantly higher numbers of GAD67-positive cells both in male offspring and in the combined mean values.

We also observed a dose-independent increase in the number of Calb-D-28 K-positive cells in male offspring exposed to AA maternally, although the only statistically significant difference was at 25 ppm (Table 1). In female offspring, there was a small but non-significant increase at 100 ppm, but no apparent differences at any other dose. However, the combined mean value (male + female) at 100 ppm showed a significant increase compared to untreated controls. Males contained a higher number of positive cells in the 25 ppm group than females, although no other treatment groups showed any sex-specific difference in Calb-D-28 K expression. Intraperitoneal injections of AA into both male and female pups also resulted in non-significant increases in the number of Calb-D-28 K-positive cells.

Apoptotic and proliferating cell indices in the dentate subgranular zone

Apoptotic bodies in maternally AA-exposed males showed statistically non-significant decreases in all dose groups (Table 1). In female offspring, a decrease in the number of apoptotic bodies was observed from 50 ppm and the difference was significant at 100 ppm. Intraperitoneal AA exposure also resulted in a non-significant decrease in males and a significant decrease in females. However, there were no significant sex differences in any group. Combined mean values also showed a significant decrease in apoptotic bodies compared to control following maternal AA exposure at 100 ppm or by intraperitoneal AA exposure.

We noted a decrease in PCNA-positive cells in male offspring exposed maternally to AA at and above 25 ppm, but this pattern was apparently not related to dose (Table 1). Female offspring did not show any such decrease, nor did combined mean values, although there