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 ($P < 0.01$).

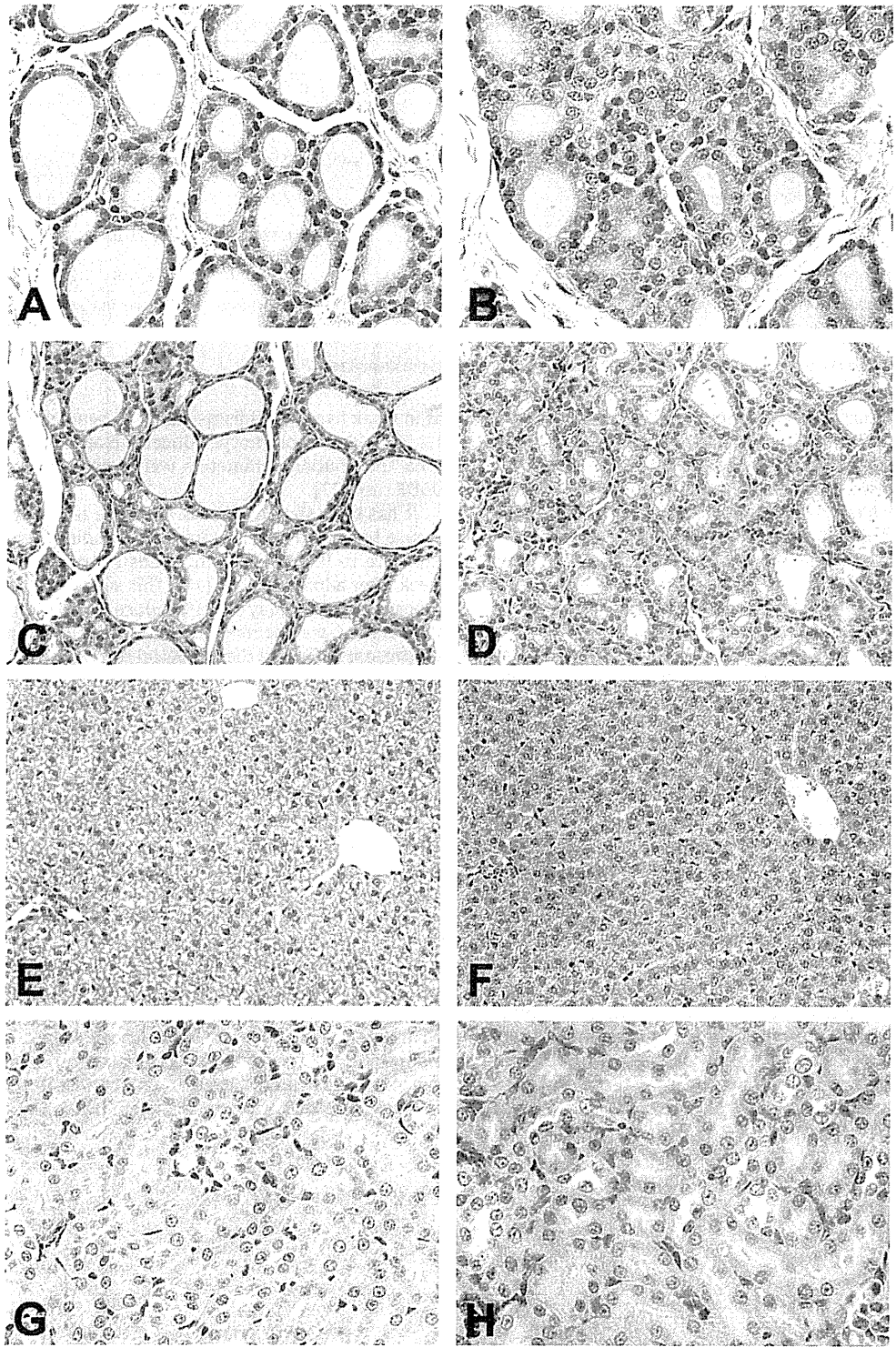

Fig. 1. Histopathological changes observed in offspring exposed to DBDE from mid-gestation to the end of lactation. (A and B) Thyroid gland of a male offspring on PND 20 given DBDE at 0 (control) (A) or 1000 ppm (B). Compared with the control, diffuse follicular cell hypertrophy (slight degree) is evident 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.

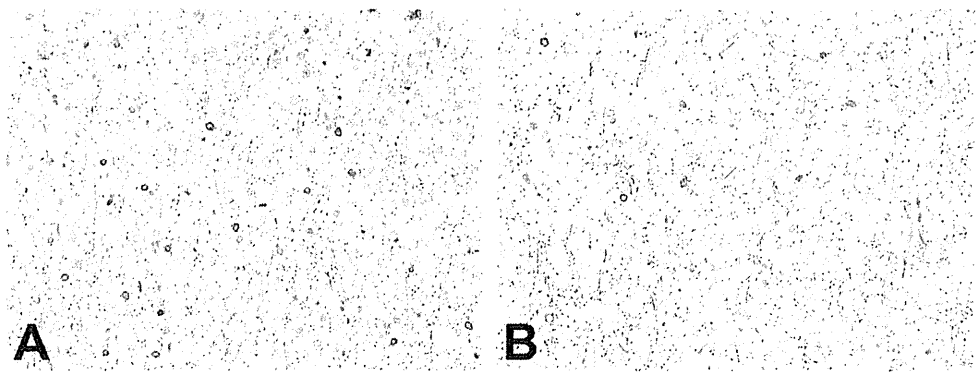

Fig. 2. Distribution of CNPase-positive oligodendrocytes in the cingulate deep cortex of the cerebrum in offspring exposed to DBDE from mid-gestation to the end of lactation. (A and B) A male offspring on PNW 11 given DBDE at 0 (control) (A) or 1000 ppm (B). Compared with the control, decrease of CNPase-positive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

Acknowledgments

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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.

References

[1] Porterfield SP. Thyroidal dysfunction and environmental chemicals—potential impact on brain development. Environ Health Perspect 2000;108(Suppl. 3):433–8.
[2] Schoonover CM, Seibel MM, Jolson DM, Stack MJ, Rahman RJ, Jones SA, et al. Thyroid hormone regulates oligodendrocyte accumulation in developing rat brain white matter tracts. Endocrinology 2004;145:5013–20.
[3] Akaike M, Kato N, Ohno H, Kobayashi T. Hyperactivity and spatial maze learning impairment of adult rats with temporary neonatal hypothyroidism. Neurotoxicol Teratol 1991;13:317–22.
[4] Rivas M, Naranjo JR. Thyroid hormones, learning and memory. Genes Brain Behav 2007;6(Suppl. 1):40–4.
[5] Goodman JH, Gilbert ME. Modest thyroid hormone insufficiency during development induces a cellular malformation in the corpus callosum: a model of cortical dysplasia. Endocrinology 2007;148:2593–7.
[6] Lavado-Autric R, Ausó E, García-Velasco JV, Arufe Mdel C, Escobar del Rey F, Berbel P, et al. Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. J Clin Invest 2003;111:1073–82.
[7] Shibutani M, Woo GH, Fujimoto H, Saegusa Y, Takahashi M, Inoue K, et al. Assessment of developmental effects of hypothyroidism in rats from in utero and lactation exposure to anti-thyroid agents. Reprod Toxicol 2009;28:297–307.
[8] Porterfield SP, Hendrich CE. Tissue iodothyronine levels in fetuses of control and hypothyroid rats at 13 and 16 days gestation. Endocrinology 1992;131:195–200.
[9] Woeber KA. Subclinical thyroid dysfunction. Arch Intern Med 1997;157:1065–8.
[10] Haggerty Jr JJ, Garbutt JC, Evans DL, Golden RN, Pedersen C, Simon JS, et al. Subclinical hypothyroidism: a review of neuropsychiatric aspects. Int J Psychiatry Med 1990;20:193–208.
[11] Birnbaum LS, Staskal DF. Brominated flame retardants: cause for concern? Environ Health Perspect 2004;112:9–17.
[12] de Wit CA. An overview of brominated flame retardants in the environment. Chemosphere 2002;46:583–624.
[13] Siddiqi MA, Laessig RH, Reed KD. Polybrominated diphenyl ethers (PBDEs): new pollutants-old diseases. Clin Med Res 2003;1:281–90.
[14] Kuriyama SN, Wanner A, Fidalgo-Neto AA, Chris E, Talsness CE, Koerner W, et al. Developmental exposure to low-dose PBDE-99: tissue distribution and thyroid hormone levels. Toxicology 2007;242:80–90.
[15] Rice DC, Reeve EA, Herlihy A, Zoeller RT, Thompson WD, Markowski VP. Developmental delays and locomotor activity in the C57BL6/J mouse following neonatal exposure to the fully-brominated PBDE, decabromodiphenyl ether. Neurotoxicol Teratol 2007;29:511–20.
[16] Meneses M, Wingfors H, Schuhmacher M, Domingo JL, Lindstrom G, Van Bavel B. Polybrominated diphenyl ethers detected in human adipose tissue from Spain. Chemosphere 1999;39:2271–8.
[17] Meironyté D, Norén K, Bergman A. Analysis of polybrominated diphenyl ethers in Swedish human milk. A time-related trend study, 1972–1997. J Toxicol Environ Health A 1999;58:329–41.
[18] Viberg H, Fredriksson A, Jakobsson E, Orn U, Eriksson P. Neurobehavioral derangements in adult mice receiving decabromodiphenyl ether (PBDE 209) during a defined period of neonatal brain development. Toxicol Sci 2003;76:112–20.
[19] Sjödin A, Patterson D, Bergman Jr GA. Brominated flame retardants in serum from U.S. blood donors. Environ Sci Technol 2001;35:3830–3.
[20] Thuresson K, Bergman A, Jakobsson K. Occupational exposure to commercial decabromodiphenyl ether in workers manufacturing or handling flame-retarded rubber. Environ Sci Technol 2005;39:1980–6.
[21] Darnerud PO, Eriksen GS, Jóhannesson T, Larsen PB, Viluksela M. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. Environ Health Perspect 2001;109(Suppl. 1):49–68.
[22] Stoker TE, Laws SC, Crofton KM, Hedge JM, Ferrell JM, Cooper RL. Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols. Toxicol Sci 2004;78:144–55.
[23] Eriksson P, Viberg H, Jakobsson E, Orn U, Fredriksson A. A brominated flame retardant, 2,2′,4,4′,5-pentabromodiphenyl ether: uptake, retention, and induction of neurobehavioral alterations in mice during a critical phase of neonatal brain development. Toxicol Sci 2002;67:98–103.
[24] Viberg H, Fredriksson A, Eriksson P. Changes in spontaneous behaviour and altered response to nicotine in the adult rat, after neonatal exposure to the brominated flame retardant, decabromodiphenyl ether (PBDE 209). Neurotoxicology 2007;28:136–42.
[25] Masutomi N, Shibutani M, Takagi H, Uneyama C, Hirose M. Dietary influence on the impact of ethinylestradiol-induced alterations in the endocrine/reproductive system with perinatal maternal exposure. Reprod Toxicol 2004;18:23–33.
[26] Teshima R, Nakamura R, Nakamura R, Hachisuka A, Sawada JI, Shibutani M. Effects of exposure to decabromodiphenyl ether on the development of the immune system in rats. J Health Sci 2008;54:382–9.
[27] Saegusa Y, Fujimoto H, Woo GH, Inoue K, Takahashi M, Mitsumori K, et al. Developmental toxicity of brominated flame retardants, tetrabromobisphenol A and 1,2,5,6,9,10-hexabromocyclododecane, in rat offspring after maternal exposure from mid-gestation through lactation. Reprod Toxicol 2009;28:456–67.
[28] IPCS (International Program on Chemical Safety). Environmental health criteria no. 162. Brominated diphenyl ethers. Geneva: WHO; 1994.

[29] Hallgren S, Sinjari T, Håkansson H, Darnerud PO. Effects of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) on thyroid hormone and vitamin A levels in rats and mice. Arch Toxicol 2001;75:200–8.
[30] Zhou T, Ross DG, DeVito MJ, Crofton KM. Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. Toxicol Sci 2001;61:76–82.
[31] Marsh G, Bergman Å, Bladh L-G, Gillner M, Jakobsson E. Synthesis of *p*-hydroxybromodiphenyl ethers and binding to the thyroid hormone receptor. Organohalogen Comp 1998;37:305–9.
[32] Brouwer A, Morse DC, Lans MC, Schuur AG, Murk AJ, Klasson-Wehler E, et al. Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. Toxicol Ind Health 1998;14:59–84.
[33] National Toxicology Program. NTP toxicology and carcinogenesis studies of decabromodiphenyl oxide (CAS No. 1163-19-5) In F344/N rats and B6C3F1 mice (feed studies). Natl Toxicol Program Tech Rep Ser 1986;309:1–242.
[34] Tseng LH, Li MH, Tsai SS, Lee CW, Pan MH, Yao WJ, et al. Developmental exposure to decabromodiphenyl ether (PBDE 209): effects on thyroid hormone and hepatic enzyme activity in male mouse offspring. Chemosphere 2008;70:640–7.
[35] Kim TH, Lee YJ, Lee E, Kim MS, Kwack SJ, Kim KB, et al. Effects of gestational exposure to decabromodiphenyl ether on reproductive parameters, thyroid hormone levels, and neuronal development in Sprague–Dawley rats offspring. J Toxicol Environ Health A 2009;72(21/22):1296–303.
[36] Eriksson P, Ankarberg E, Viberg H, Fredriksson A. The developing cholinergic system as target for environmental toxicants, nicotine and polychlorinated biphenyls (PCBs): implications for neurotoxicological processes in mice. Neurotox Res 2001;3:37–51.
[37] Eriksson P, Jakobsson E, Fredriksson A. Brominated flame retardants: a novel class of developmental neurotoxicants in our environment? Environ Health Perspect 2001;109:903–8.
[38] Eriksson P, Fischer C, Fredriksson A. Polybrominated diphenyl ethers, a group of brominated flame retardants, can interact with polychlorinated biphenyls in enhancing developmental neurobehavioral defects. Toxicol Sci 2006;94:302–9.
[39] Viberg H. Neonatal ontogeny and neurotoxic effect of decabrominated diphenyl ether (PBDE 209) on levels of synaptophysin and tau. Int J Dev Neurosci 2009;27:423–9.
[40] Xing T, Chen L, Tao Y, Wang M, Chen J, Ruan DY. Effects of decabrominated diphenyl ether (PBDE 209) exposure at different developmental periods on synaptic plasticity in the dentate gyrus of adult rats in vivo. Toxicol Sci 2009;110:401–10.
[41] Norris JM, Kociba RJ, Schwetz BA, Rose JQ, Humiston CG, Jewett GL, et al. Toxicology of octabromobiphenyl and decabromodiphenyl oxide. Environ Health Perspect 1975;11:153–61.
[42] Hardy ML, Schroeder R, Biesemeier J, Manor O. Prenatal oral (gavage) developmental toxicity study of decabromodiphenyl oxide in rats. Int J Toxicol 2002;21:83–91.
[43] Morck A, Hakk H, Orn U, Klasson WE. Decabromodiphenyl ether in the rat: absorption, distribution, metabolism, and excretion. Drug Metab Dispos 2003;31:900–7.
[44] Van der Ven LT, van de Kuil T, Leonards PE, Slob W, Cantón RF, Germer S, et al. A 28-day oral dose toxicity study in Wistar rats enhanced to detect endocrine effects of decabromodiphenyl ether (decaBDE). Toxicol Lett 2008;179:6–14.
[45] Carlson GP. Induction of xenobiotic metabolism in rats by short-term administration of brominated diphenyl ethers. Toxicol Lett 1980;5:19–25.
[46] Morck A, Hakk H, Orn U, Klasson Wehler E. Decabromodiphenyl ether in the rat: absorption, distribution, metabolism, and excretion. Drug Metab Dispos 2003;31:900–7.

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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 $IgG_{1 \text{ and } 2a}$, 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

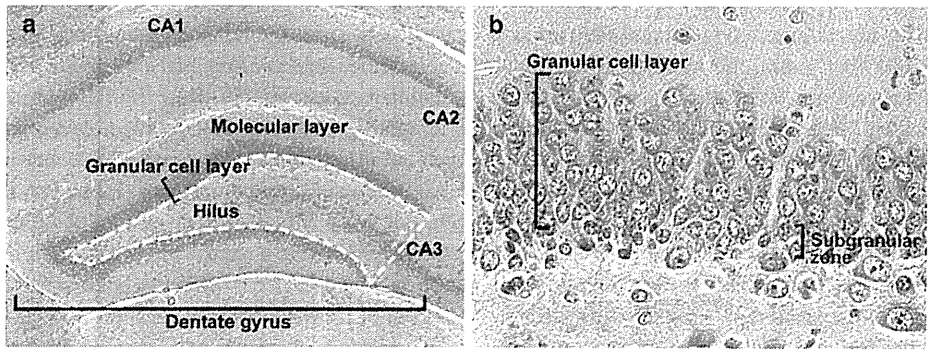

Fig. 1 Overview of the hippocampal formation of a male rat at PND 21 stained with hematoxylin and eosin. **a** The numbers of cells in the hilus of the dentate gyrus (as demarcated by the *white dotted line*) displaying immunoreactivity for Reelin, GAD67, and Calb-D-28 K were counted and normalized for 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

Table 1 Immunohistochemically positive cells and apoptotic bodies counted in AA-exposed offspring

	AA in the drinking water (ppm)				AA-injection
	0	25	50	100	50 mg/kg/day, i.p.
Reelin (/mm^2)					
Males	22.0 ± 1.1[a] (3)	35.3 ± 12.3 (4)	44.9 ± 7.3 (8)**	37.8 ± 6.3 (5)*	37.9 ± 9.5 (4)
Females	24.4 ± 8.7 (7)	31.1 ± 6.8 (6)	37.2 ± 13.6 (6)	42.6 ± 8.9 (3)*	38.4 ± 9.0 (5)*
Males + Females	23.7 ± 8.9 (10)	32.8 ± 9.0 (10)*	41.6 ± 10.7 (14)**	39.6 ± 7.2 (8)**	38.2 ± 8.7 (9)**
GAD67 (/mm^2)					
Males	17.4 ± 8.9 (3)	33.1 ± 2.8 (4)	38.8 ± 8.6 (8)**	41.6 ± 6.7 (5)**	36.2 ± 7.4 (4)**
Females	28.8 ± 4.1 (7)[†]	31.3 ± 9.1 (6)	34.4 ± 7.0 (6)	30.9 ± 10.7 (3)	33.8 ± 9.9 (5)
Males + Females	25.4 ± 7.7 (10)	32.0 ± 7.0 (10)	36.9 ± 8.0 (14)**	37.6 ± 9.4 (8)**	34.9 ± 8.5 (9)*
Calb-D-28 K (/mm)					
Males	7.96 ± 1.30 (3)	12.47 ± 1.74 (4)*	9.87 ± 3.85 (8)	11.72 ± 4.27 (5)	9.28 ± 1.50 (4)
Females	8.08 ± 2.61 (7)	7.62 ± 3.58 (6)[†]	8.05 ± 2.71 (6)	11.10 ± 2.92 (3)	10.84 ± 3.64 (5)
Males + Females	8.04 ± 2.22 (10)	9.56 ± 3.80 (10)	9.09 ± 3.42 (14)	11.49 ± 3.60 (8)*	10.14 ± 2.85 (9)
Apoptotic body (/mm)					
Males	1.35 ± 0.94 (3)	0.56 ± 0.14 (4)	0.46 ± 0.35 (8)	0.44 ± 0.28 (5)	0.70 ± 0.63 (4)
Females	1.22 ± 1.02 (7)	3.52 ± 7.38 (6)	0.72 ± 0.34 (6)	0.21 ± 0.19 (3)*	0.19 ± 0.17 (5)*
Males + Females	1.25 ± 0.94 (10)	2.34 ± 5.71 (10)	0.57 ± 0.36 (14)	0.36 ± 0.26 (8)*	0.42 ± 0.49 (9)*
PCNA (/mm)					
Males	5.46 ± 1.34 (3)	2.54 ± 0.97 (4)*	3.18 ± 1.40 (8)*	2.88 ± 1.35 (5)*	2.86 ± 1.37 (4)
Females	3.07 ± 1.65 (7)	3.56 ± 1.16 (6)	3.26 ± 0.66 (6)	2.17 ± 1.16 (3)	2.25 ± 0.63 (5)
Males + Females	3.79 ± 1.88 (10)	3.16 ± 1.16 (10)	3.21 ± 1.11 (14)	2.62 ± 1.25 (8)	2.52 ± 1.00 (9)

AA, acrylamide; GAD67, glutamic acid decarboxylase 67; Calb-D-28 K, Calbindin-D-28 K; i.p., intraperitoneal injection; PCNA, proliferating cell nuclear antigen

* $P < 0.05$, ** $P < 0.01$ versus 0 ppm group

[†] $P < 0.05$ versus males of the same group

[a] Mean ± SD

() No. of animals examined

was a trend toward decrease in females and combined values at 100 ppm. Similarly, intraperitoneal AA exposure also resulted in appreciable but non-significant decreases in the number of PCNA-positive cells, particularly in males. There were no statistically significant differences between males and females in any group.

Discussion

In our previous study, we found no apparent AA-induced neurotoxicity in the axon terminals of rat pups following exposure of the mother to toxic levels through her drinking water (Takahashi et al. 2009). In the present study, we used identical animals and detected a clear increase in the number of Reelin-positive cells in the dentate hilus of offspring from 25 ppm, comparable to pups treated with intraperitoneal AA. A concomitant increase in GAD67-positive cells as well as an increasing tendency toward expression of Calb-D-28 K after AA exposure through both maternal and intraperitoneal transfer suggests an increase in the number of Reelin-producing GABAergic interneurons (Saegusa et al. 2010). In addition, we found a sex difference in the number of GAD67-positive cells in untreated controls. Although a number of studies exist demonstrating sex differences in GAD67-expression in mammals (Searles et al. 2000; Hays et al. 2002; Catalano et al. 2010), these reports all document differences in the GABAergic neurons of the hypothalamic sexually dimorphic nucleus. There have been no previous studies reporting differences in hilar GABAergic interneurons. We did not see any sex difference in Reelin- or Calb-D-28 K-positive cells in the hilar area of untreated controls. While further studies may be necessary regarding sexual dimorphism in the hilar GABAergic interneurons, we are confident that sexual dimorphism does not exist in the numbers of Reelin-, Calb-D-28 K- or PCNA-positive cells, nor apoptotic bodies.

We have recently shown aberrant increases of Reelin-expressing interneurons in the dentate hilus following developmental exposure to anti-thyroid agents during the gestation and lactation period (Saegusa et al. 2010). Considering the role of Reelin in neuronal development,

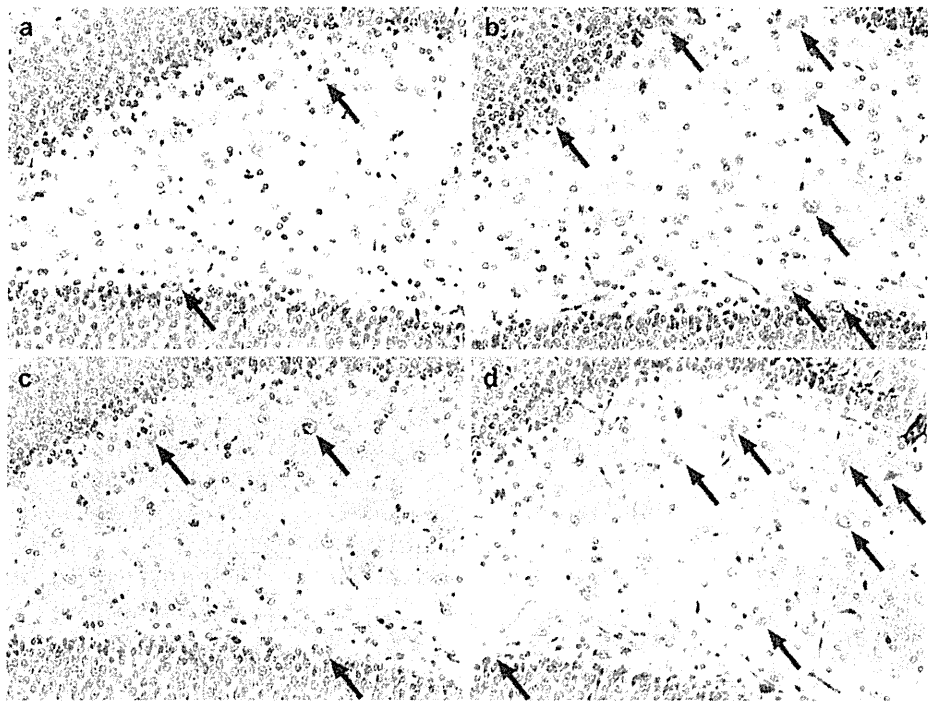

Fig. 2 Distribution of immunoreactive cells for Reelin and GAD67 in the hippocampal formation in rats at PND 21 after maternal exposure to AA. **a**, **b** Reelin-immunoreactive cells in the hilus of the dentate gyrus of offspring at 0 ppm (**a**) and 100 ppm (**b**). Reelin-positive cells with abundant cytoplasm (*indicated with arrows*) show scattered distribution within the hilar region. Note the higher number of Reelin-positive cells in (**b**) when compared with (**a**). Reelin immunohistochemistry, avidin–biotin complex method counterstained with hematoxylin. Magnification, × 200. **c**, **d** GAD67-immunoreactive cells in the hilus of the dentate gyrus of offspring at 0 ppm (**c**) and 100 ppm (**d**). Note the higher number of GAD67-positive cells indicated with arrows in (**d**) when compared with (**c**). GAD67-immunohistochemistry, avidin–biotin complex method counterstained with hematoxylin. Magnification, × 200

AA-driven increases in Reelin-expressing interneuron numbers may suggest abnormalities in neuronal migration and positioning in the dentate granular cell layer. Importantly, this aberrant increase continues until adult stages in cases of developmental hypothyroidism (Saegusa et al. 2010). Therefore, it is absolutely necessary to examine the reversibility of the interneuron changes induced by developmental AA exposure.

In contrast to the increase in apoptosis in the subgranular zone induced by developmental exposure to anti-thyroid agents (Saegusa et al. 2010), maternal transfer of AA from drinking water to offspring causes decreased apoptosis in this zone at 100 ppm, as does intraperitoneal injection of AA. Although the reason for the decrease in apoptosis is unclear, the anti-apoptotic cellular signaling machinery may be activated as a neuroprotective mechanism to compensate for an impairment of neurogenesis caused by AA. Further studies may be necessary to delineate the exact mechanisms underlying this regulation of apoptosis. Recently, impairment of adult neurogenesis, as revealed by a decrease in the number of proliferating cells in the subgranular zone, has been reported in mice treated with intraperitoneal injections of AA at 50 mg/kg with a total of 12 doses in 2 weeks (Park et al. 2010). Although the dose-relationship was not clear, we also detected a decrease in PCNA-positive (i.e. proliferating) cells in the subgranular zone in all groups of maternally exposed male offspring, as well as a similar but non-significant decrease in male pups receiving intraperitoneal injections with AA. In contrast, we did not see any apparent decrease in PCNA-positive cells in females following either maternal exposure or intraperitoneal injections. The use of a bromodeoxyuridine-based consecutive in vivo labeling method for S-phase cells such as that used by Park et al. (2010) may detect more subtle changes in proliferation activity than the method of detecting proliferating cells at the time of killing as used in the present study.

Although neurogenesis in the dentate gyrus can be affected by AA exposure during the postnatal period (Park et al. 2010), high doses of AA administered by intraperitoneal injections are necessary to induce increases in Reelin-producing interneurons during the lactation period in the present study. Importantly, we here found that much lower doses are able to stimulate changes in Reelin expression if administered maternally from the gestation period onwards. In humans, the internal level of AA in

neonates by transplacental exposure was estimated to be at least equal to that of the mother (Schettgen et al. 2004). Considering that only about a tenth of maternal AA is transferred to offspring by lactation (Takahashi et al. 2009), transplacental rather than lactational transfer of AA may be responsible for disruption of neuronal migration and neurogenesis.

Previous study has shown that short-term oral treatment with AA in rats resulted in moderate dose-dependent effects on plasma thyroxine (increased) and thyroid stimulating hormone (decreased), as well as some follicular cell hypertrophy, suggesting an acute effect of AA exposure on the thyroid (Khan et al. 1999). Conversely, an extensive recent study has shown no effect of sub-chronic AA exposure on gene expression, neurochemistry, hormone levels, or histopathology in the hypothalamus–pituitary–thyroid axis of rats (Bowyer et al. 2008). These results may suggest that AA at least does not cause developmental hypothyroidism that can trigger impaired brain development in rats.

Systemic growth retardation in offspring caused by maternal and/or offspring toxicity may cause a delay in brain development. In previous studies of developmental hypothyroidism (Shibutani et al. 2009; Saegusa et al. 2010), we observed a suppression of systemic growth in the offspring after exposure to anti-thyroid agents that caused apparent hypothyroidism in both dams and offspring. Thus, there is a possibility that the increase in the number of Reelin-expressing interneurons occurs in concert with the delayed brain growth accompanying systemic growth retardation rather than the hypothyroidism-related impairment of thyroid hormone signaling in the brain. With regard to the effects of developmental exposure to AA on offspring growth, maternal administration of AA at dose levels inducing maternal neurotoxicity has been shown to lead to reductions in offspring body size (Friedman et al. 1999; Takahashi et al. 2009). However, we have recently revealed that the systemic growth retardation in offspring resulting from maternal protein restriction affects brain growth but does not change the cellular distribution of immunoreactivity for Reelin, Calb-D-28 K, or GAD67 or of NeuN-positive post-mitotic neurons in the dentate hilus, either at weaning or in adulthood (Ohishi et al. in press). This result may suggest that the growth retardation arising from AA toxicity in offspring does not affect the population of GABAergic interneurons involved in Reelin synthesis.

In summary, using study animals known to suffer no apparent axon terminal neurotoxicity following exposure to maternally toxic levels of AA through drinking water, we here found a clear increase in the number of Reelin-producing interneurons in the dentate hilus of offspring following maternal exposure to AA at and above 25 ppm. We also observed decreased apoptosis in the neuroblast-producing subgranular zone at 100 ppm, comparable to pups given high doses of AA directly via intraperitoneal injections during the lactation period. These results suggest a compensatory mechanism that regulates and protects against the impaired neurogenesis and mismigration arising following maternal exposure to AA from low doses during neuronal development. The lowest-observed-adverse-effect level of AA was determined to be 25 ppm (3.7 mg/kg body weight/day).

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References

Bashaw GJ, Klein R (2010) Signaling from axon guidance receptors. Cold Spring Harb Perspect Biol 2:a001941

Bowyer JF, Latendresse JR, Delongchamp RR, Muskhelishvili L, Warbritton AR, Thomas M, Tareke E, McDaniel LP, Doerge DR (2008) The effects of subchronic acrylamide exposure on gene expression, neurochemistry, hormones, and histopathology in the hypothalamus-pituitary-thyroid axis of male Fischer 344 rats. Toxicol Appl Pharmacol 230:208–215

Catalano PN, Di Giorgio N, Bonaventura MM, Bettler B, Libertun C, Lux-Lantos VA (2010) Lack of functional GABA(B) receptors alters GnRH physiology and sexual dimorphic expression of GnRH and GAD-67 in the brain. Am J Physiol Endocrinol Metab 298:E683–E696

D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature 374:719–723

D'Arcangelo G, Nakajima K, Miyata T, Ogawa M, Mikoshiba K, Curran T (1997) Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. J Neurosci 17:23–31

Exon JH (2006) A review of the toxicology of acrylamide. J Toxicol Environ Health B Crit Rev 9:397–412

Friedman MA, Tyl RW, Marr MC, Myers CB, Gerling FS, Ross WP (1999) Effects of lactational administration of acrylamide on rat dams and offspring. Reprod Toxicol 13:511–520

Goodman JH, Gilbert ME (2007) Modest thyroid hormone insufficiency during development induces a cellular malformation in the corpus callosum: a model of cortical dysplasia. Endocrinology 148:2593–2597

Gould E (2007) How widespread is adult neurogenesis in mammals? Nat Rev Neurosci 8:481–488

Hays LE, Carpenter CD, Petersen SL (2002) Evidence that GABAergic neurons in the preoptic area of the rat brain are targets of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin during development. Environ Health Perspect 110(Suppl 3):369–376

Houser CR (2007) Interneurons of the dentate gyrus: an overview of cell types, terminal fields and neurochemical identity. Prog Brain Res 163:217–232

Khan MA, Davis CA, Foley GL, Friedman MA, Hansen LG (1999) Changes in thyroid gland morphology after acute acrylamide exposure. Toxicol Sci 47:151–157

Lavado-Autric R, Ausó E, García-Velasco JV, Arufe Mdel C, del Rey FE, Berbel P, de Escobar GM (2003) Early maternal hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. J Clin Invest 111:954–957

LoPachin RM (2004) The changing view of acrylamide neurotoxicity. NeuroToxicology 25:617–630

Nóbrega-Pereira S, Marín O (2009) Transcriptional control of neuronal migration in the developing mouse brain. Cereb Cortex 19(Suppl 1):i107–i113

Nuñez JL, McCarthy MM (2004) Cell death in the rat hippocampus in a model of prenatal brain injury: time course and expression of death-related proteins. Neuroscience 129:393–402

Ohishi T, Wang L, Ogawa B, Fujisawa K, Taniai E, Hayashi H, Mitsumori K, Shibutani M (2010) No effect of sustained systemic growth retardation on the distribution of Reelin-expressing interneurons in the neuron-producing hippocampal dentate gyrus in rats. Reprod Toxicol (in press)

Park HR, Kim MS, Kim SJ, Park M, Kong KH, Kim HS, Kwack SJ, Kang TS, Kim SH, Kim HS, Lee J (2010) Acrylamide induces cell death in neural progenitor cells and impairs hippocampal neurogenesis. Toxicol Lett 193:86–93

Parzefall W (2008) Minireview on the toxicity of dietary acrylamide. Food Chem Toxicol 46:1360–1364

Pesold C, Impagnatiello F, Pisu MG, Uzunov DP, Costa E, Guidotti A, Caruncho HJ (1998) Reelin is preferentially expressed in neurons synthesizing gamma-aminobutyric acid in cortex and hippocampus of adult rats. Proc Natl Acad Sci USA 95:3221–3226

Saegusa Y, Woo G-H, Fujimoto H, Kemmochi S, Shimamoto K, Hirose M, Mitsumori K, Nishikawa A, Shibutani M (2010) Sustained production of Reelin-expressing interneurons in the hippocampal dentate hilus after developmental exposure to antithyroid agents in rats. Reprod Toxicol 29:407–414

Saita K, Ohi T, Hanaoka Y, Furukawa S, Furukawa Y, Hayashi K, Matsukura S (1996) A catechol derivative (4-methylcatechol) accelerates the recovery from experimental acrylamide-induced neuropathy. J Pharmacol Exp Ther 276:231–237

Schettgen T, Kutting B, Hornig M, Beckmann MW, Weiss T, Drexler H, Angerer J (2004) Trans-placental exposure of neonates to acrylamide—a pilot study. Int Arch Occup Environ Health 77:213–216

Schoonover CM, Seibel MM, Jolson DM, Stack MJ, Rahman RJ, Jones SA, Mariash CN, Anderson GW (2004) Thyroid hormone regulates oligodendrocyte accumulation in developing rat brain white matter tracts. Endocrinology 145:5013–5020

Scotti AL, Herrmann G (2002) Reelin immunoreactivity in dissociated cultures of the postnatal hippocampus. Brain Res 924:209–218

Searles RV, Yoo MJ, He JR, Shen WB, Selmanoff M (2000) Sex differences in GABA turnover and glutamic acid decarboxylase (GAD(65) and GAD(67)) mRNA in the rat hypothalamus. Brain Res 878:11–19

Shibutani M, Lee KY, Igarashi K, Woo G-H, Inoue K, Nishimura T, Hirose M (2007) Hypothalamus region-specific global gene expression profiling in early stages of central endocrine disruption in rat neonates injected with estradiol benzoate or flutamide. Dev Neurobiol 67:253–269

Shibutani M, Woo G-H, Fujimoto H, Saegusa Y, Takahashi M, Inoue K, Hirose M, Nishikawa A (2009) Assessment of developmental effects of hypothyroidism in rats from in utero and lactation exposure to anti-thyroid agents. Reprod Toxicol 28:297–307

Takahashi M, Shibutani M, Inoue K, Fujimoto H, Hirose M, Nishikawa A (2008) Pathological assessment of the nervous and male reproductive systems of rat offspring exposed maternally to acrylamide during the gestation and lactation periods—a preliminary study. J Toxicol Sci 33:11–24

Takahashi M, Shibutani M, Nakahigashi J, Sakaguchi N, Inoue K, Morikawa T, Yoshida M, Nishikawa A (2009) Limited lactational transfer of acrylamide to rat offspring on maternal oral administration during the gestation and lactation periods. Arch Toxicol 83:785–793

WHO/IPCS (2006) Summary and conclusions of the sixty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) Rome, 8-17 February 2005. summary_report_64_final.pdf.Available from: http://www.who.int/ipcs/food/jecfa/summaries/en/i

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ORGAN TOXICITY AND MECHANISMS

Life stage-related differences in susceptibility to acrylamide-induced neural and testicular toxicity

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Abstract In order to assess age-dependence of susceptibility to acrylamide (ACR)-induced neural and testicular toxicity, 3- and 7-week-old male SD rats were given ACR at 0, 50, 100, or 200 ppm in the drinking water for 4 weeks, and the nervous and male reproductive systems were examined histopathologically. Testicular genotoxicity was evaluated with the comet assay and the micronucleus (MN) test. Glutathione *S*-transferase (GST) activity and glutathione (GSH) content in the liver and testis were also measured. In both young and adult animals, neurotoxicity was evident from 100 ppm and increased in proportion to ACR intake per body weight. In the testis, marked degeneration and exfoliation, mainly of spermatids, were observed from 100 ppm limited to young animals. The comet assay revealed ACR to significantly induce DNA damage from 100 ppm in both life stages, while MNs were found only in young rats from 100 ppm. The level of GST activity in the testis of young rats at the end of experiment was significantly lower than that of adult animals, regardless of the ACR treatment. There were no life stage-related differences in GSH contents in the liver and testis. These results suggest that susceptibility to neurotoxicity might not differ between young and adult rats when exposure levels are adjusted for body weight. Regarding testicular toxicity, young animals around puberty proved more susceptible than adult animals, possibly due to their lower level of testicular GST activity than that in adult animals.

Keywords Acrylamide · Age · Susceptibility · Neurotoxicity · Testicular toxicity · Rat

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Introduction

Acrylamide (ACR), a proven carcinogen in animals known to be a neuro- and reproductive toxicant, has found many industrial and laboratory uses and therefore is a potential hazard to man. Recently, it was found to be formed on heating from carbohydrate and amino acid components of food, so that public exposure has become a worldwide concern (Exon 2006; Parzefall 2008). Mean daily intake of ACR for adults is estimated as 1 μg/kg body weight/day, but values for infants and children are estimated to be two- to threefold higher when expressed on a body weight basis (WHO/IPCS 2006). So far, since toxicity studies of ACR have mainly been performed using adult animals, to evaluate toxicity targeting children is important for risk assessment of ACR exposure in man.

Even the limited studies on susceptibility to neurotoxicity of ACR in relation with the life stage have not generated consistent results controversial. For example, whereas a rotarod test revealed slightly higher sensitivity with adult when compared to immature rats regarding disruption of locomotor activity (Kaplan and Murphy 1972), earlier onset of neurological symptoms as well as faster induction of severe myelin disruption was found with

suckling rats given ACR at 50 mg/kg by intraperitoneal injection 3 times weekly when compared with adult rats (Suzuki and Pfaff 1973). Similarly, mice given ACR at 400 ppm in the drinking water revealed earlier onset of neurological symptoms and terminal nerve swelling by administration starting from 3 weeks of age than from 8-weeks of age (Ko et al. 1999).

As for testicular toxicity, no data on the susceptibility to ACR in relation with testicular development have been reported. In our previous study, although retardation of spermatogenesis was observed in preweaning rats exposed to ACR via maternal drinking water or direct intraperitoneal injection, no histopathological changes suggestive of testicular toxicity were noted (Takahashi et al. 2009). Therefore, testes prior to spermatogenesis could be less sensitive to ACR.

In the present study, to elucidate factors influencing susceptibility to ACR-induced neuro- or testicular toxicity, we compared histopathological changes of the nervous and male reproductive systems between rats exposed to ACR during the puberty growth period and the young adult stage. The comet assay and micronucleus (MN) test were also conducted to evaluate testicular genotoxicity induced by ACR. Additionally, to compare the capacity for ACR detoxification between the young and adult animals, glutathione *S*-transferase (GST) activity and glutathione (GSH) content were measured in the liver and testis.

Materials and methods

Experimental design

Thirteen pregnant Crj:CD (SD) rats were obtained from Charles River Japan Inc. (Yokohama, Japan) at gestational day 10. They were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature: 24 ± 1°C, relative humidity: 55 ± 5%, 12-h light/dark cycle) with basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water available ad libitum. After delivery, 40 male pups on weaning at 21 days of age were allocated to 4 groups, each consisting 10 animals from different dams, given ACR (Sigma, St. Louis, MO, USA; CAS #79-06-1) at 0, 50, 100, and 200 ppm in the drinking water for 4 weeks (**young group**). Similarly, forty male SD rats at 6 weeks of age were obtained from Charles River Japan Inc., and acclimatized with basal diet (CRF-1) and tap water ad libitum for 1 week. Then, they were randomly divided into 4 groups and given ACR at 0, 50, 100, and 200 ppm in the drinking water for 4 weeks (**adult group**). The highest dose was set as the dose that induces neurotoxicity and testicular toxicity within 4 weeks in adult male rats (Lee et al. 2005). Observations for clinical signs and mortality were conducted daily. Body weights and food consumption were recorded every week. In addition, animals were scored with respect to the appearance of gait abnormalities, as previously reported (Moser 1991; Shell et al. 1992; Lee et al. 2005), as follows: grade 1, normal gait; grade 2, slightly abnormal gait with slight degrees of ataxia, hopping gait, and foot splay; grade 3, moderately abnormal gait with moderate degrees of ataxia, foot splay, and limb abduction; grade 4, severely affected gait, including inability to support the body weight as well as foot splay.

At necropsy, all animals were killed under deep anesthesia by exsanguination, and the brain, testes, and epididymides were removed and weighed. The trigeminal nerve was also removed. The brains from 5 animals per each group were fixed in methacarn solution at 4°C overnight. The brains from another 5 rats per each group, along with the trigeminal nerves and epididymides were fixed in neutrally buffered 10% formalin. One testis was used for comet assays, and the other was fixed in Bouin's solution at room temperature overnight. All fixed tissues were routinely processed for paraffin embedding, sectioned at 3 μm, and stained with hematoxylin and eosin (HE). The sciatic nerves were exposed at autopsy and subjected to in situ fixation by immersion in ice-cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 min (Takahashi et al. 2009). The portion located at the ankle position was carefully dissected and further fixed with fresh fixative overnight, postfixed in 1% osmium tetroxide (TAAB Laboratories Equipment Ltd., Berkshire, UK) in the same buffer for 2 h at 4°C, and embedded in epoxy resin (TAAB Laboratories Equipment Ltd.). Semithin sections, 1 μm in thickness, were stained with toluidine blue for light microscopic assessment.

As a satellite study, 3- or 7-week-old male SD rats were similarly given ACR at 0 or 200 ppm in the drinking water for 4 weeks. The livers and unilateral testes were collected for measurement of GST activity and GSH content. Small portions of each testis were fixed in Bouin's solution to confirm ACR-induced lesions microscopically.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Morphometric assessment

To evaluate aberrant dot-like structures immunoreactive with synaptophysin (SYP) in the cerebellar molecular layer, methacarn-fixed cerebellum sections were subjected to immunohistochemistry for SYP, as described previously (Takahashi et al. 2008), with rabbit polyclonal antibody Ab-4 (1: 200, Lab Vision Corp., Fremont, CA, USA) as the primary antibody.

For morphometry of axonal degeneration in the sciatic nerves and SYP-immunoreactive aberrant dot-like structures in the cerebellar molecular layer, photomicrographs were taken with a digital camera attached to a microscope (DP71, Olympus Corp., Tokyo, Japan). Measurement was then performed using image analysis software (WinROOF, Version 5.7.1, Mitani Corp. Tokyo, Japan). The total number of axons/unit area and the numbers of degenerated axons and the diameters of axons were assessed in one cross-sectional area at 400× magnification of toluidine blue-stained specimens from each animal, and the density, percentage of degenerated axons, and percentage of myelinated axons less than 3 μm in diameter were calculated. For evaluation of SYP-immunoreactive aberrant dot-like structures, numbers of dots in the left cerebellar hemisphere were counted following measurement of the length of the cortex in one cross-sectional area at 12.5× magnification and the number of SYP-immunoreactive dots/unit length of the cortex was calculated.

For testicular toxicity, approximately 400–650 circularly sectioned seminiferous tubules for each rat were assessed microscopically, and then the percentages of tubules with histopathological changes were calculated.

Comet assay

The procedures for preparing and processing comet assays were performed according to the recommendation by an expert working group on the comet assay in the International Workshop on Genotoxicity Testing (IWGT) (Tice et al. 2000; Burlinson et al. 2007) and slightly modified for testes. Briefly, each testis was washed with cold mincing/homogenizing buffer containing Hanks' Balanced Salt (HBSS) Solutions (Invitrogen Corporation, Carlsbad, CA, USA), 20 mM EDTA·2Na, and 10% DMSO, minced with scissors, and placed on ice for 15–30 s to precipitate clumps of cells. The supernatant was suspended in 0.5% Nusieve GTG agarose (Lonza, Basel, Switzerland), quickly layered on a MAS-coated slide (Matsunami Glass Ind. Ltd., Osaka, Japan), immersed in lysing solution (pH10, 100 mM EDTA·2Na, 2.5 M NaCl, 10 mM Tris(hydroxymethyl)aminomethane containing 1% Triton-X and 10% DMSO) at 4°C overnight, and electrophoresed for 15 min in alkaline buffer after the unwinding treatment. Then, the cells were fixed with ethanol and stained with SYBER green (Molecular Probes, Eugene, OR, USA) according to the manufacturer's recommendation. We observed the cells under a fluorescence microscope (BX50, Olympus Co.). Round-shaped cells, considered as spermatocytes and early spermatids, were captured with a CCD camera. At least 100 cells were observed and the tail intensity of each comet image was measured using an image analysis software (Comet assay IV, Perceptive Instruments Ltd., Suffolk, UK).

Micronucleus (MN) test

The MN test for the testis was conducted according to the method of Tates et al. (1983) with a slight modification. Briefly, the testes excised from each animal were minced in HBSS Solution. The cell suspensions were incubated in 2 mg/ml collagenase solution (Wako Pure Chemicals Ind., Osaka, Japan) for 30 min at 37°C in a shaking water bath, filtered through a cell strainer, washed, and fixed in methanol. The cells were stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride), and the slide specimens were prepared with acridine orange coating (TOYOBO Co., Ltd., Tokyo, Japan). We observed 1,000 early spermatids per animal under a fluorescence microscope (BX50, Olympus Co.).

Measurement of GST activity and GSH content

The livers and testes obtained from the satellite groups were rinsed with PBS solution to remove any red blood cells. Total GSH concentrations were determined with the Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), and GST activity was measured using a Glutathione *S*-Transferase Assay Kit (Cayman Chemical). Sample preparation and measurement were conducted according to the instructions of the manufacturer.

Statistical analysis

Variance in data for body weights, food consumption, water consumption, values from morphometric assessment in the sciatic nerves, cerebellar molecular layer and testis, and data for the comet assay were 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 statistically significant differences were indicated, the Dunnett's multiple test was employed for comparisons between the 0 ppm and ACR-treated groups. Data for the MN tests, GST activity, and GSH content were analyzed by Student's or Welch's *t* test following a test for equal variance.

Results

In-life parameters and intake of ACR

Suppression of body weight gain was observed in the young groups at 100 and 200 ppm from week 2 (Fig. 1a).

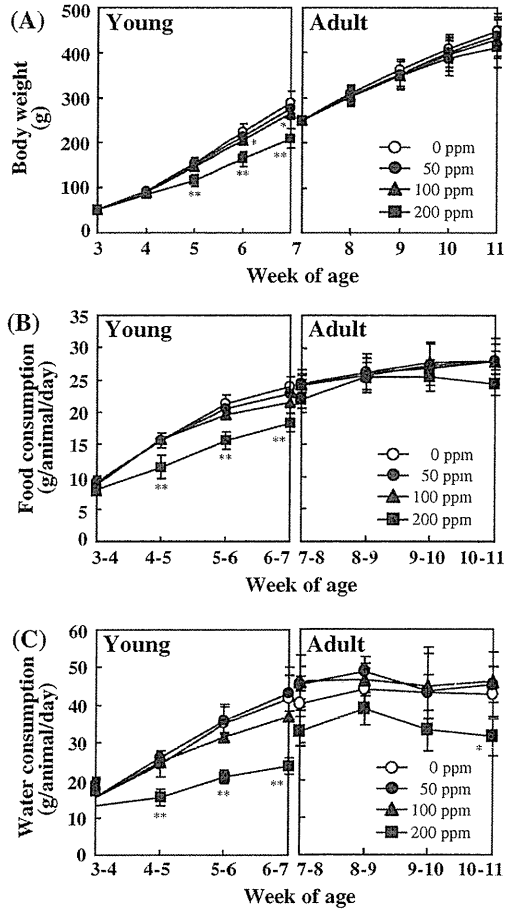

Fig. 1 Time course of change in body weights (**a**), food consumption (**b**), and water consumption (**c**) in young and adult rats given ACR in the drinking water for 4 weeks. Data are mean ± SD. *, **$P < 0.05$ and $P < 0.01$ vs. 0 ppm

In the adult groups, there were no intergroup differences in the body weight curves. Also, food consumption was suppressed only in the young group at 200 ppm (Fig. 1b). Water consumption was lowered at 200 ppm both in young and adult groups (Fig. 1c). Mean daily intakes of ACR are summarized in Table 1. Compared to adult groups, mean daily intake of ACR per kg body weight was higher in young groups at each dose.

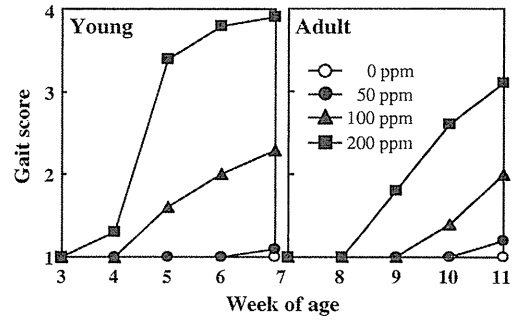

Fig. 2 Scores for gait abnormalities of young and adult rats given ACR in the drinking water for 4 weeks

Both in young and adult groups, apparent gait abnormalities were found at 100 and 200 ppm, and their severity advanced during the exposure in a dose-dependent manner (Fig. 2). Young groups showed earlier occurrence of gait abnormalities and faster progression of the symptoms than adult groups. At 200 ppm, slightly abnormal gait appeared in the young animals from week 1, and symptoms rapidly progressed so that the gait score reached 3.4 at week 2. Adult animals at 200 ppm exhibited mild gait abnormality from week 2, which progressed to score 3.1 at week 4.

Final body and organ weights are summarized in Table 2. In young groups, body weights were significantly depressed at 100 and 200 ppm. Alteration of the brain weight in young rats appeared to reflect body weight decrease. Decreases in absolute weights of the testis and epididymides observed in young and adult rats could have been linked with the histopathological changes described below.

Morphometric analysis

Data for histopathology and morphometry of lesions developing in the nervous system are shown in Table 3. Representative histopathological illustrations of the nervous systems of young and adult groups are summarized in Fig. 3 and Fig. 4, respectively. In both young and adult groups, central chromatolysis of ganglion cells in the trigeminal nerves was apparent from 100 ppm. The density of

Table 1 Mean daily intake of ACR in young and adult rats

Group	Acrylamide in the drinking water (ppm)				
		0	50	100	200
	No. of animals examined	10	10	10	10
Young	(mg/kg/day)	0 ± 0[a]	8.27 ± 0.32	15.73 ± 1.51	26.37 ± 3.51
Adult	(mg/kg/day)	0 ± 0	6.26 ± 1.10	12.63 ± 1.97	19.07 ± 3.46

[a] Mean ± SD