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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

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ABSTRACT

To evaluate developmental exposure effects of two brominated flame retardants, tetrabromobisphenol A (TBBPA) and 1,2,5,6,9,10-hexabromocyclododecane (HBCD), pregnant Sprague–Dawley rats were administered either chemical at doses of 100, 1000 or 10,000 ppm in a soy-free diet from gestation day 10 until the day 20 after delivery. Offspring exposed to TBBPA showed dose-unrelated slight decreases of serum triiodothyronine (T₃) concentration at postnatal day 20, and there was no evidence of hypothyroidism-related neuronal migration and impaired oligodendroglial development as judged by morphometric analyses of NeuN-immunoreactive neuronal distribution in the hippocampal CA1, and area of corpus callosum as well as density of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNase)-immunoreactive oligodendrocytes in the cingulate deep cortex at the adult stage. On the other hand, HBCD exerted a weak hypothyroidism evident with increases in thyroid weight, thyroid follicular cell hypertrophy and serum concentrations of thyroid-stimulating hormone as well as decreases of serum T₃ concentrations in offspring at 10,000 ppm at weaning. Increased thyroid weights and decreased serum T₃ concentrations were also observed in the adult stage from 1000 ppm. With regard to the effect on brain development, HBCD reduced density of CNase-positive oligodendrocytes at 10,000 ppm, suggesting an impaired oligodendroglial development. Results thus suggest that TBBPA did not exert developmental brain effects, while HBCD did, and 100 ppm was determined to be the no-observed-adverse-effect level of HBCD from changes in thyroid parameters at the adult stage by maternal exposure, translating into 8.1–21.3 mg/kg-d.

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1. Introduction

Thyroid hormones are required for normal brain development during the fetal and neonatal periods [1–4], and developmental hypothyroidism during this period leads to growth retardation, neurological defects and impaired performance on a variety of behavioral learning ability [5–7]. Experimentally, rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) show brain retardation, resulting in impaired neuronal migration as well as white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation

[4,8–10]. Maternal serum thyroid hormone (TH) levels directly affect TH levels in fetuses [11]. Recent studies, indicating that even small changes in the mother's TH status early in pregnancy may cause adverse effects on her child, have led to an increase in concern for TH disrupting chemicals in the environment [12].

Brominated flame retardants (BFRs) have been used as the most efficient flame retardants and are commonly used to protect a variety of commercial products such as computers, televisions, mobile phones, furniture, carpet, insulation boards and mattresses [13]. Many of these BFR compounds have highly lipophilic and persistent characteristics and are believed to have the highest potential for bioaccumulation. Because of these properties, several BFRs have contaminated the environment to bioaccumulate in wildlife, which has evoked concern for both environmental and human health [14,15]. Recent studies have shown that polybrominated diphenyl ethers (PBDEs), a subgroup of BFRs, can cause carcinogenic, thyrotoxic, estrogenic and neurotoxic effects in experimental animals

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and/or humans [16]. As a children's health risk, developmental hypothyroidism is the major concern of exposure effect of BFRs [17,18].

Among the variety of BFRs, PBDEs, tetrabromobisphenol A (TBBPA) and 1,2,5,6,9,10-hexabromocyclododecane (HBCD) are the most widely used BFRs throughout the world. Among these, TBBPA has the largest production volume in the world, and accounted for 58.7% (about 120,000 metric tons per year) of total market demand for BFRs in 2001 [19]. HBCD is the third most widely used BFRs in the world, accounting for 8.2% (16,700 metric tons per year) of total market demand for BFRs in 2001. Both TBBPA and HBCD are detected in human blood and breast milk [13,20–22].

Toxicity of TBBPA in experimental animals has been suggested to be low. In a two-generation reproductive toxicity study, TBBPA did not induce effects on fertility or reproductive performance at doses up to 1000 mg/kg body weight and had no convincing effects on neurodevelopmental end points [23], while anti-estrogenic activity was detected in uterotrophic activity [24]. On the other hand, *in vitro* studies of TBBPA have shown immunological effects [25], antagonistic activity on TH receptors [24,26], and inhibition of synaptic neurotransmitter uptake [27]. Recent studies have shown neurobehavioral effects in offspring in a one-generation reproduction study [28,29].

Regarding toxicity of HBCD, several repeated oral dose toxicity studies have been performed in rats, and hepatic enlargement associated with liver cell vacuolation and hypothyroidism-related effects were reported [30]. With regard to the effects on reproductive system parameters, high doses of HBCD inhibited oogenesis in pregnant rats and increased the prostate weight in rats [30]. In the two previously performed developmental toxicity studies in rats, HBCD did not exert any fetotoxicity or teratogenic potential on offspring [30]. In the recently published study on two-generation reproductive toxicity in rats, on the other hand, a variety of changes occurred including a reduction in the number of ovarian primordial follicles, a lower viability index and body weight, a low incidence of the completion of eye opening and completed mid-air righting in offspring generations, as well as the hypothyroidism-related changes and liver enlargement in both parents and offspring [31]. Similar to TBBPA, HBCD inhibited neurotransmitter uptake into synaptosomes and dopamine uptake into synaptic vesicles [27]. HBCD exposure during brain development in mice affected spontaneous behavior, learning and memory later in life [32].

Regular reproduction studies and developmental neurotoxicity studies require large number of animals for detection of subtle changes with dose-response. On the other hand, for screening purposes to confront many new chemicals, smaller scale studies with preferably short-term experiment are necessary to be established. Recently, we established a morphometric detection system of neuronal migration and aberrant oligodendroglial development using smaller numbers of animals than those required in developmental neurotoxicity study to evaluate the potency of chemicals to induce hypothyroidism-related brain retardation [10], based on previously reported landmarks on brain retardation due to developmental hypothyroidism [4,8,9]. The present study was performed to assess the developmental exposure effects of TBBPA and HBCD on rat offspring through maternal diet in small scale animal studies, with a particular focus on brain development parameters to be affected by hypothyroidism.

2. Materials and methods

2.1. Chemicals and animals

The two chemicals, tetrabromobisphenol A (TBBPA; CAS No.79-94-7, Catalog No. T0032, Lot No. GH01, purity: >98%) and 1,2,5,6,9,10-hexabromocyclododecane (HBCD; CAS No. 3194-55-6, Catalog No. H0544, Lot No. GN01, purity: >95%) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Pregnant Crj:CD®(SD)IGS

rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at gestational day (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 $24 \pm 1^\circ\text{C}$, relative humidity: $55 \pm 5\%$) with a 12 h light/dark cycle and allowed *ad libitum* access to feed and tap water. A soy-free (SF) 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 given *ad libitum* throughout experimental period including the 1 week acclimation. Content of estrogens and phytoestrogens in the SF diet was described elsewhere [33].

2.2. Experimental design

The TBBPA and HBCD studies were performed individually. In each study, dams were randomized into 4 groups on GD 10 (8 dams per group in TBBPA study, 10 dams per group in HBCD study) and provided with a SF diet that contained 0 (control), 100, 1000, or 10,000 ppm of TBBPA or HBCD from GD 10 until the day 20 after delivery (the day of weaning). A preliminary dose finding study was performed for TBBPA and HBCD with the same dose settings at dietary levels of 0 (control), 1000, or 10,000 ppm from GD 10 until the day 20 after delivery, and as a result, the highest dose of TBBPA and HBCD exhibited a weakly positive response from dams, i.e., slightly increased relative thyroid weights and thyroid follicular cell hypertrophy, but did not affect pregnancy, implantation or delivery (data not shown).

In the main study, all dams were weighed and food consumption was measured throughout the experimental period. On postnatal day (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 culled randomly to adjust to eight offspring per dam comprised of four males and four females. On the day 20 after delivery, dosing was terminated, and all dams were killed. Twenty male and 20 female offspring (at least one male and one female per dam) per group were subjected to prepubertal necropsy for histopathological assessment (10 males and 10 females per group), and for other experimental purposes (10 males and 10 females per group). The remaining males and females were allocated to four rats per cage, given regular CRF-1 basal diet (Oriental Yeast Co. Ltd.) and water *ad libitum*, and maintained further for adult examination when they were 11 weeks old.

Prepubertal necropsies were conducted on PND 20. The organs/tissues were removed and weighed, and histopathological assessment was performed. Dams were subjected to organ weight measurement and histopathological examination of the thyroid glands, and the numbers of implantations were also recorded at this time point.

All female offspring were monitored daily for vaginal opening from PND 26 and all male pups were examined for preputial separation from PND 34 until each animal acquired this developmental landmark. The age 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 [33,34].

At PNW 11, offspring were sacrificed and tissues were subjected to histopathological assessment and thyroid-related hormone measurement. Male offspring were killed on the first day of week 11. For female offspring, killing was delayed for up to 4 d after the first day of week 11 until the animal entered the diestrus stage of the estrus cycle.

The experimental animals were weighed and sacrificed 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, ten 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/tissues removed and those subjected to weight measurement were described previously [10]. Removed organs were routinely processed for paraffin embedding, sectioned at $3\ \mu\text{m}$, and stained with hematoxylin and eosin (HE) 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) to stain oligodendrocytes and neurons, respectively. Deparaffinized coronal brain slices at the position of $-3.5\ \text{mm}$ from

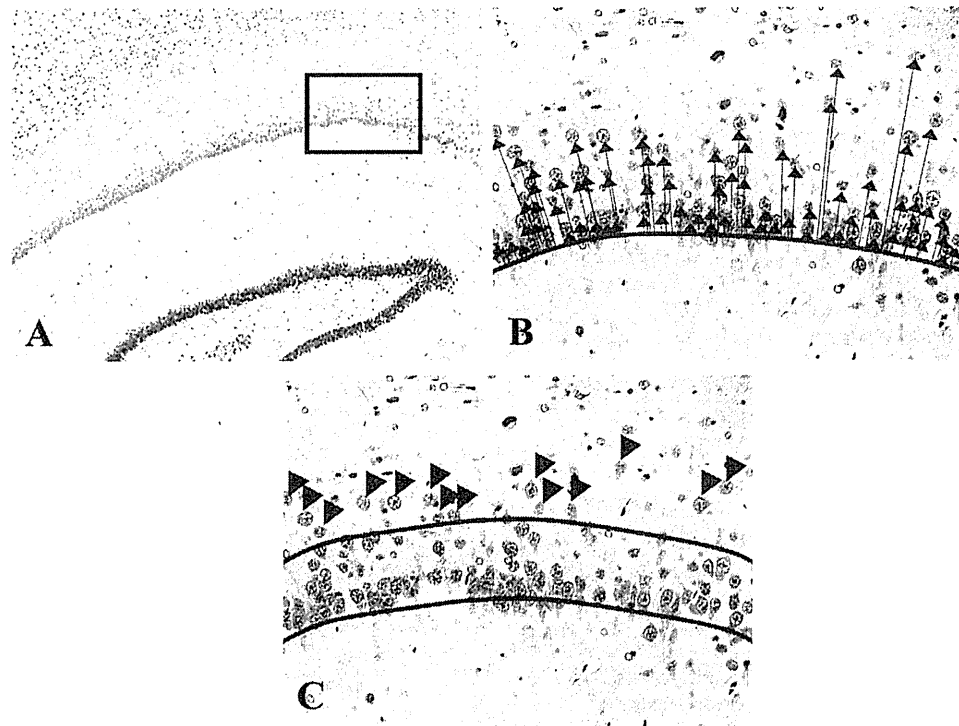


Fig. 1. Quantitative measurement of the variability in the distribution of neurons located within and lateral to the pyramidal cell layer of the hippocampal CA1 region at PNW 11. (A) Hippocampal CA1 region stained with NeuN-immunohistochemistry at 0.9 mm lateral to the boundary with the subiculum, 40 \times magnification. (B) Measurement of the distance of the location of neurons positive for NeuN from the innermost margin of the pyramidal cell layer adjacent to the lucid layer, 200 \times magnification. (C) Number of NeuN-positive nuclei within the pyramidal cell layer and outside of this layer (polymorphic layer: arrowheads) in the same view area, 200 \times magnification.

the bregma were serially sectioned at 3 μ m. Immunohistochemistry was performed according to the method described previously with 3,3'-diaminobenzidine/H₂O₂ as the chromogen [10]. 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 [10] (Fig. 1A–C).

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 [10] (Fig. 2A and B).

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 as well as 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 (ANOVA) 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 estrus 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.

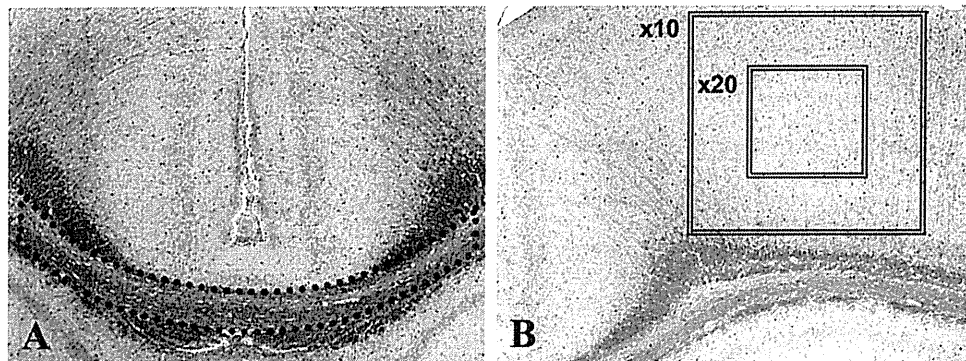


Fig. 2. Quantitative measurement of the effect on the oligodendroglial development at PNW 11. (A) Size measurement of the white matter area immunoreactive for CNPase. The area of the corpus callosum medial to the cerebral white matter at the uppermost position of the cingulum was measured, 40 \times magnification. (B) Number of CNPase-positive oligodendrocytes surrounding myelinated axons distributed at the layer V of the parietal isocortex dorsolateral to the cingulum under 200 \times magnification. At first, the lower and innermost ends of the view area with a 10 \times objective lens were fixed at the uppermost position of the cingulum, then magnification of the view area for cellular counting was increased by changing the lens to 20 \times , 40 \times magnification.

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

	TBBPA in diet (ppm)			
	0	100	1000	10,000
No. of dams examined	8	8	8	8
Maternal parameter				
Body weight gain (g/d)				
GD 10–GD 20	11.33 ± 1.40 ^a	11.66 ± 1.50	11.39 ± 1.30	11.55 ± 0.90
Day 1–day 9 after delivery	6.58 ± 2.80	7.16 ± 1.96	6.30 ± 1.95	6.80 ± 1.68
Day 9–day 20 after delivery	0.31 ± 0.59	0.53 ± 0.73	0.46 ± 0.65	1.36 ± 0.70 [*]
Food consumption (g/d)				
GD 10–GD 20	31 ± 5.2	32.4 ± 7.5	29.7 ± 3.5	28.0 ± 3.2
Day 1–day 9 after delivery	57.7 ± 13.1	56.5 ± 12.6	51.5 ± 10.7	47.6 ± 4.8
Day 9–day 20 after delivery	73.7 ± 18.3	77.7 ± 30.3	70.3 ± 15.6	75.3 ± 14.6
TBBPA intake (mg/kg BW/d)				
GD 10–GD 20	0	9.5 ± 2.2	86.8 ± 10.3	818.9 ± 93.5
Day 1–day 9 after delivery	0	18.3 ± 4.0	159.0 ± 33.1	1465.9 ± 148.6
Day 9–day 20 after delivery	0	22.9 ± 8.9	202.1 ± 44.8	2129.2 ± 413.3
Duration of pregnancy (day)				
Day 20 after delivery	21.8 ± 0.5	21.8 ± 0.5	21.6 ± 0.5	21.5 ± 0.5
BW (g)				
Day 20 after delivery	335.4 ± 25.0	334.0 ± 23.5	328.6 ± 26.3	338.4 ± 24.7
Thyroid				
Relative weight (mg/100 g BW)	5.81 ± 1.24	6.41 ± 0.89	6.28 ± 0.67	6.67 ± 0.97
Histopathology: diffuse follicular cell hypertrophy (±/+/++) ^b	3 ^c (1/1/1) ^d	3 (1/2/0)	7 (3/4/0)	7 (5/2/0)
Offspring parameter				
No. of implantation sites				
	13.9 ± 1.9	13.5 ± 2.4	13.3 ± 3.2	14.0 ± 1.1
No. of live offspring				
	12.8 ± 2.6	12.8 ± 3.0	12.4 ± 3.1	13.1 ± 0.6
Male ratio (%)				
	51.9 ± 13.8	47.3 ± 14.2	47.8 ± 9.5	51.7 ± 15.6
BW, PND 1 (g)				
Males	7.59 ± 0.71	7.51 ± 1.04	7.49 ± 1.04	6.90 ± 0.35
Females	7.17 ± 0.80	7.06 ± 0.92	6.96 ± 0.94	6.60 ± 0.32
AGD, PND 1 (mm)				
Males	4.15 ± 0.22	4.01 ± 0.20	4.05 ± 0.24	4.00 ± 0.17
Females	2.03 ± 0.13	1.95 ± 0.12	2.03 ± 0.09	2.00 ± 0.08
Relative organ weights, PND 20				
No. of offspring examined				
	10	10	10	10
Males				
BW (g)	54.3 ± 4.4	53.0 ± 5.9	59.0 ± 7.0	52.6 ± 3.0
Liver (g/100 g BW)	3.88 ± 0.25	3.60 ± 0.41	3.91 ± 0.25	3.76 ± 0.20
Kidneys (g/100 g BW)	1.14 ± 0.06	1.12 ± 0.07	1.09 ± 0.08	1.11 ± 0.06
Brain (g/100 g BW)	2.77 ± 0.18	2.88 ± 0.26	2.64 ± 0.32	2.82 ± 0.19
Spleen (g/100 g BW)	0.39 ± 0.06	0.38 ± 0.09	0.37 ± 0.05	0.35 ± 0.06
Thymus (g/100 g BW)	0.39 ± 0.06	0.37 ± 0.08	0.40 ± 0.03	0.37 ± 0.06
Adrenals (mg/100 g BW)	26.3 ± 6.5	24.9 ± 4.2	29.0 ± 2.4	29.0 ± 2.8
Testes (g/100 g BW)	0.42 ± 0.04	0.40 ± 0.04	0.41 ± 0.03	0.40 ± 0.05
Epididymides (g/100 g BW)	0.065 ± 0.013	0.061 ± 0.007	0.065 ± 0.010	0.062 ± 0.007
Females				
BW (g)	50.4 ± 4.3	51.1 ± 5.1	53.3 ± 5.6	52.4 ± 2.7
Liver (g/100 g BW)	3.86 ± 0.19	3.86 ± 0.14	3.94 ± 0.32	3.84 ± 0.20
Kidneys (g/100 g BW)	1.17 ± 0.05	1.18 ± 0.06	1.13 ± 0.09	1.16 ± 0.10
Brain (g/100 g BW)	2.90 ± 0.17	2.83 ± 0.20	2.75 ± 0.28	2.77 ± 0.15
Spleen (g/100 g BW)	0.39 ± 0.06	0.42 ± 0.08	0.37 ± 0.02	0.38 ± 0.06
Thymus (g/100 g BW)	0.40 ± 0.07	0.39 ± 0.06	0.44 ± 0.05	0.42 ± 0.08
Adrenals (mg/100 g BW)	21.6 ± 5.3	24.3 ± 4.5	23.3 ± 4.9	22.0 ± 5.8
Ovaries (mg/100 g BW)	24.9 ± 7.8	30.0 ± 5.6	25.3 ± 10.5	27.1 ± 8.5
Uterus (g/100 g BW)	0.069 ± 0.008	0.074 ± 0.013	0.068 ± 0.011	0.074 ± 0.012

Abbreviations: AGD, anogenital distance; BW, body weight; GD, gestational day; PND, postnatal day; TBBPA, tetrabromobisphenol A.

^a Mean ± S.D.

^b Grade of change: (±) minimal; (+) slight; (++) moderate.

^c Total no. of animals with each finding.

^d No. of animals with each grade.

^{*} Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.05$).

3. Results

3.1. Maternal toxicity

In the TBBPA study, an increased body weight gain of dams was observed from the day 9 to 20 after delivery at

10,000 ppm, while the body weight at the day 20 after delivery was unchanged between the control and TBBPA-treated dams (Table 1). Maternal food consumption was not affected during the gestation and lactation periods, and thus levels of maternal daily intake of TBBPA were considered to be proportional to the dose. Duration of pregnancy was not affected at any

Table 2

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

	HBCD in diet (ppm)			
	0	100	1000	10,000
No. of dams examined	10	10	10	10
Maternal parameter				
Body weight gain (g/d)				
GD 10–GD 20	11.22 ± 1.85 ^a	11.08 ± 1.34	10.72 ± 1.18	11.34 ± 1.12
Day 1–day 9 after delivery	4.70 ± 1.82	4.20 ± 2.70	4.00 ± 1.49	5.02 ± 1.74
Day 9–day 20 after delivery	1.58 ± 1.11	0.64 ± 0.96	1.16 ± 1.15	1.13 ± 1.17
Food consumption (g/d)				
GD 10–GD 20	30.6 ± 5.4	28.1 ± 3.7	27.7 ± 5.4	27.8 ± 3.5
Day 1–day 9 after delivery	42.7 ± 4.9	45.0 ± 7.2	45.0 ± 9.5	45.6 ± 6.0
Day 9–day 20 after delivery	70.5 ± 10.0	72.4 ± 19.4	74.1 ± 26.9	78.9 ± 15.7
HBCD intake (mg/kg BW/d)				
GD 10–GD 20	0	8.1 ± 1.1	80.7 ± 15.9	803.2 ± 101.9
Day 1–day 9 after delivery	0	14.3 ± 2.3	138.7 ± 29.4	1404.8 ± 184.5
Day 9–day 20 after delivery	0	21.3 ± 5.7	212.9 ± 77.3	2231.3 ± 445.0
Duration of pregnancy (day)	21.4 ± 0.5	21.6 ± 0.5	21.6 ± 0.5	21.7 ± 0.5
Day 20 after delivery				
BW (g)	339.3 ± 27.3	323.3 ± 26.6	334.0 ± 25.7	336.5 ± 26.4
Thyroid				
Relative weight (mg/100 g BW)	5.73 ± 0.90	6.75 ± 0.99	6.30 ± 0.80	7.47 ± 1.05 ^c
Histopathology: diffuse follicular cell hypertrophy (±/+/+/+/+) ^b	3 ^c (0/3/0/0) ^d	5 (2/3/0/0)	6 (1/3/2/0)	9 [#] (0/3/4/2) ^{§§}
Offspring parameter				
No. of implantation sites	13.7 ± 1.9	14.2 ± 1.6	12.4 ± 1.4	14.0 ± 1.6
No. of live offspring	13.0 ± 1.8	13.0 ± 1.6	11.6 ± 1.6	12.9 ± 1.4
Male ratio (%)	48.5 ± 16.2	59.3 ± 14.2	48.7 ± 20.2	45.7 ± 9.5
BW, PND 1 (g)				
Males	7.11 ± 0.66	7.22 ± 0.56	7.65 ± 0.95	7.15 ± 0.80
Females	6.53 ± 0.59	6.84 ± 0.50	7.28 ± 0.75	6.84 ± 0.81
AGD, PND 1 (mm)				
Males	3.88 ± 0.23	3.96 ± 0.20	4.08 ± 0.30	4.01 ± 0.23
Females	2.13 ± 0.60	1.94 ± 0.08	2.00 ± 0.17	2.03 ± 0.12
Relative organ weights, PND 20				
No. of offspring examined	10	10	10	10
Males				
BW (g)	54.3 ± 3.5	51.2 ± 7.3	56.7 ± 4.1	54.0 ± 3.3
Liver (g/100 g BW)	3.68 ± 0.11	3.82 ± 0.31	3.98 ± 0.15	4.66 ± 0.35 ^c
Kidneys (g/100 g BW)	1.12 ± 0.05	1.12 ± 0.05	1.12 ± 0.05	1.09 ± 0.05
Brain (g/100 g BW)	2.76 ± 0.20	3.00 ± 0.46	2.67 ± 0.18	2.78 ± 0.34
Spleen (g/100 g BW)	0.39 ± 0.05	0.35 ± 0.09	0.41 ± 0.06	0.37 ± 0.06
Thymus (g/100 g BW)	0.36 ± 0.06	0.40 ± 0.06	0.42 ± 0.04	0.39 ± 0.05
Adrenals (mg/100 g BW)	26.6 ± 2.5	29.7 ± 2.6	31.9 ± 5.2 ^c	31.0 ± 5.5
Testes (g/100 g BW)	0.43 ± 0.04	0.43 ± 0.03	0.43 ± 0.05	0.40 ± 0.03
Epididymides (g/100 g BW)	0.063 ± 0.016	0.068 ± 0.008	0.071 ± 0.012	0.068 ± 0.013
Females				
BW (g)	50.3 ± 3.4	50.0 ± 6.0	53.7 ± 5.5	51.3 ± 2.9
Liver (g/100 g BW)	3.77 ± 0.17	3.83 ± 0.23	4.01 ± 0.25	4.83 ± 0.26 ^c
Kidneys (g/100 g BW)	1.21 ± 0.07	1.12 ± 0.08	1.17 ± 0.08	1.17 ± 0.05
Brain (g/100 g BW)	2.88 ± 0.23	2.89 ± 0.29	2.72 ± 0.23	2.73 ± 0.12
Spleen (g/100 g BW)	0.38 ± 0.05	0.36 ± 0.06	0.41 ± 0.05	0.37 ± 0.04
Thymus (g/100 g BW)	0.39 ± 0.08	0.41 ± 0.09	0.46 ± 0.07	0.42 ± 0.07
Adrenals (mg/100 g BW)	31.4 ± 6.3	30.3 ± 3.6	30.3 ± 2.1	27.8 ± 4.9
Ovaries (mg/100 g BW)	32.3 ± 3.9	30.9 ± 4.9	28.1 ± 6.3	28.7 ± 3.4
Uterus (g/100 g BW)	0.078 ± 0.013	0.078 ± 0.010	0.075 ± 0.010	0.071 ± 0.011

Abbreviations: AGD, anogenital distance; BW, body weight; GD, gestational day; HBCD, hexabromocyclododecane; PND, postnatal day.

^a Mean ± S.D.^b Grade of change: (±) minimal; (+) slight; (++) moderate; (+++) severe.^c Total no. of animals with each finding.^d No. of animals with each grade.^{*} Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.05$).[#] Significantly different from the controls by Fisher's exact probability test ($P < 0.05$).^{§§} Significantly different from the controls by Mann–Whitney's *U*-test ($P < 0.01$).

dose level. Dams receiving TBBPA did not show statistically significant change in the relative thyroid weights as compared with those in the control group at the day 20 after delivery, while a dose-unrelated increasing tendency was noted in the

relative thyroid weight of all treatment groups. Histopathologically, though not significant, the incidence of diffuse thyroid follicular cell hypertrophy showed a marginal increase from 1000 ppm.

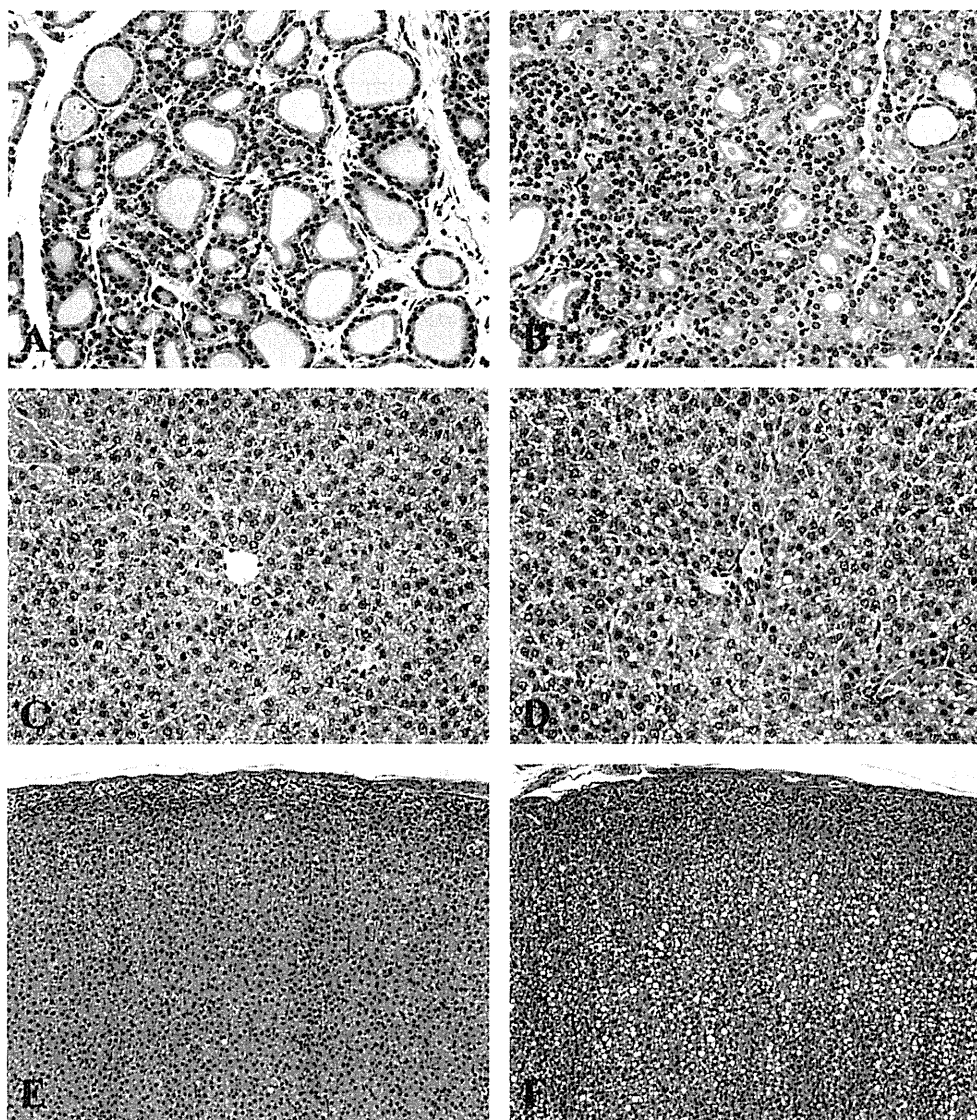


Fig. 3. Histopathological changes observed in dams and offspring in the HBCD study. (A and B) Thyroid gland of dams given HBCD at 0 (control) (A) or 10,000 ppm (B). As compared with the 0 ppm dose, diffuse follicular cell hypertrophy (moderate degree) is evident with 10,000 ppm HBCD, 200 \times magnification. (C and D) Liver of female offspring on PND 20 given HBCD at 0 (control) (C) or 10,000 ppm (D). Note that liver cells show vacuolar degeneration in the 10,000 ppm HBCD-exposed rat, 200 \times magnification. (E and F) Adrenal gland of male offspring on PNW 11 given HBCD at 0 (control) (E) or 10,000 ppm (F). Note diffuse vacuolar degeneration in the cortex in the 10,000 ppm HBCD-exposed case, 100 \times magnification.

In the HBCD study, maternal body weight and food consumption did not change throughout the gestation and lactation periods (Table 2). Duration of pregnancy was not influenced at any dose level. The body weight at day 20 after delivery was unchanged between the control and HBCD-treated dams, and thus levels of maternal daily intake of HBCD were concluded to be proportional to the dose. On the other hand, the relative weight of the thyroid was significantly increased at 10,000 ppm. A tendency for an increase was also noted in other treatment groups. Histopathologically, an increased incidence of diffuse thyroid follicular cell hypertrophy was observed at 10,000 ppm associated with an increase in the severity (Fig. 3A and B). At 100 and 1000 ppm, the incidence of thyroid follicular cell hypertrophy showed a tendency to increase, although this change was not significant.

3.2. Effects on offspring until prepubertal necropsy

In the TBBPA study, no offspring parameters showed any abnormalities in the clinical observation, number of implantation sites, number of live offspring, male ratio, and body weight and AGD at PND 1 in all groups (Table 1). The body weight in both sexes from PND 1 through weaning was not affected (data not shown). At the prepubertal necropsy, there were no obvious body and organ weight changes at any dose of TBBPA in either gender.

In the HBCD study, no offspring parameters revealed any abnormalities in the clinical observation, number of implantation sites, number of live offspring, male ratio, and body weight and AGD at PND 1 in all groups (Table 2). The body weight in both sexes from PND 1 through weaning was not affected (data not shown). At the prepubertal necropsy, an increased relative weight of the liver

Table 3

Onset of puberty and estrus cycles in the offspring exposed to tetrabromobisphenol A or hexabromocyclododecane from mid-gestation to the end of lactation.

TBBPA study	TBBPA in diet (ppm)			
	0	100	1000	10,000
Onset of puberty				
Males				
No. of animals examined	12	12	12	12
Age by day	41.0 ± 1.0 ^a	41.8 ± 1.5	41.2 ± 1.1	42.7 ± 2.1
BW (g)	204.9 ± 12.0	218.2 ± 12.3	209.0 ± 14.7	221.2 ± 17.6 [*]
Females				
No. of animals examined	12	12	12	12
Age by day	34.9 ± 1.6	33.3 ± 1.4	33.5 ± 1.9	34.1 ± 2.4
BW (g)	129.2 ± 11.2	123.9 ± 11.8	122.1 ± 8.5	128.7 ± 16.0
Estrus cycles during PNW 8–11				
No. of animals examined	10	10	10	10
Irregularity (extended diestrus)	0	0	0	1
HBCD study				
HBCD in diet (ppm)				
Onset of puberty				
Males				
No. of animals examined	13	14	12	14
Age by day	40.7 ± 1.9	40.6 ± 1.3	40.8 ± 2.0	41.1 ± 1.6
BW (g)	204.3 ± 15.7	198.3 ± 20.4	203.2 ± 15.0	195.8 ± 10.1
Females				
No. of animals examined	14	12	14	13
Age by day	35.4 ± 1.9	35.6 ± 1.8	34.9 ± 1.7	34.4 ± 2.1
BW (g)	130.8 ± 11.7	133.8 ± 10.8	129.2 ± 13.5	118.6 ± 11.7 [*]
Estrus cycles during PNW 8–11				
No. of animals examined	10	10	10	10
Irregularity (extended diestrus)	1	2	0	0

Abbreviations: BW, body weight; HBCD, hexabromocyclododecane; PNW, postnatal week; TBBPA, tetrabromobisphenol A.

^a Mean ± S.D.

^{*} Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.05$).

was observed at 10,000 ppm in both sexes. Also, although dose-unrelated, an increase in the relative adrenal weight at 1000 ppm in males and a decrease in the relative kidney weight at 100 ppm in females were observed.

3.3. Effects on the onset of puberty and estrus cycle

In the TBBPA study, there were no effects on the onset of puberty in either sex after weaning, while a higher body weight was observed in males at 10,000 ppm as compared with the untreated controls at the onset of puberty (Table 3). In females, no significant irregularity in estrus cycle was observed after exposure to TBBPA.

In the HBCD study, there were no effects on the onset of puberty in both sexes after weaning, while a lower body weight was observed in females at 10,000 ppm as compared with the untreated controls at the onset of puberty (Table 3). Male offspring also showed a tendency for a decrease in the body weight at the onset. In females, no significant irregularity in estrus cycle was observed by exposure to HBCD.

3.4. Serum levels of thyroid-related hormones

Changes in serum concentrations of thyroid-related hormones were examined at PND 20 and PNW 11 in male offspring (Table 4).

In the TBBPA study, a dose-unrelated, but statistically significant decrease of serum T_3 concentration was observed at 100 and 1000 ppm on PND 20, although a significant alteration was lacking at 10,000 ppm. As for serum T_4 and TSH concentrations, there

was no significant change at any dose. On PNW 11, there were no exposure-related changes in the serum concentration of any thyroid-related hormones in any group.

In the HBCD study, a statistically significant decrease of serum T_3 concentration was observed at 10,000 ppm on PND 20, while serum T_4 concentration was unaltered at any dose. On the other hand, a statistically significant increase of serum TSH concentration was observed at 10,000 ppm on PND 20. On PNW 11, a statistically significant decrease of serum T_3 concentration was observed at both 1000 and 10,000 ppm, although a dose relationship was unclear. Serum T_4 and TSH concentrations were unaltered at any dose at this time point.

3.5. Organ weight changes at the adult stage

In the TBBPA study, body and organ weights did not change in males compared with the untreated controls at necropsy on PNW 11 (Table 5). In females, decreases in relative kidney and uterus weights were observed at 1000 and 10,000 ppm, respectively.

In the HBCD study, a tendency to decrease was noted in the body weights of both sexes at 10,000 ppm at the necropsy on PNW 11, although this measurement was not statistically significant as compared with the untreated controls (Table 5). No other groups showed fluctuations in body weight. With regard to the relative organ weights, thyroid weight increased in all groups and was statistically significant from 1000 ppm. Dose-unrelated increases in relative liver weights and decreases of relative epididymal weights were observed at 100 ppm. In females, no obvious changes were noted in organ weights.

Table 4

Serum levels of thyroid-related hormones of the male offspring exposed to tetrabromobisphenol A or hexabromocyclododecane from mid-gestation to the end of lactation.

TBBPA study	TBBPA in diet (ppm)			
	0	100	1000	10,000
PND 20				
No. of offspring examined	10	10	10	10
T ₃ (ng/ml)	1.31 ± 0.12 ^a	1.13 ± 0.12 [*]	1.15 ± 0.08 [*]	1.20 ± 0.13
T ₄ (μg/dl)	4.86 ± 0.50	4.66 ± 0.64	4.85 ± 0.43	5.12 ± 0.52
TSH (ng/ml)	7.09 ± 1.32	6.68 ± 2.51	6.17 ± 1.78	5.45 ± 0.56
PNW 11				
No. of offspring examined	10	10	10	10
T ₃ (ng/ml)	0.89 ± 0.08	0.89 ± 0.05	0.92 ± 0.08	0.87 ± 0.04
T ₄ (μg/dl)	4.77 ± 0.53	5.11 ± 0.93	5.03 ± 0.40	4.49 ± 0.80
TSH (ng/ml)	7.12 ± 2.06	7.19 ± 2.23	6.72 ± 1.90	6.23 ± 1.62
HBCD study	HBCD in diet (ppm)			
	0	100	1000	10,000
PND 20				
No. of offspring examined	10	10	10	10
T ₃ (ng/ml)	1.09 ± 0.11	1.13 ± 0.12	1.06 ± 0.08	0.93 ± 0.10 ^{**}
T ₄ (μg/dl)	4.39 ± 0.93	4.20 ± 0.77	4.78 ± 0.49	4.20 ± 0.52
TSH (ng/ml)	5.40 ± 0.62	6.66 ± 1.24	6.07 ± 1.41	7.00 ± 1.31 [*]
PNW 11				
No. of offspring examined	10	10	10	10
T ₃ (ng/ml)	0.96 ± 0.06	0.93 ± 0.07	0.88 ± 0.05 [*]	0.89 ± 0.06 ^{**}
T ₄ (μg/dl)	4.77 ± 0.70	4.84 ± 0.59	5.21 ± 0.65	5.20 ± 0.98
TSH (ng/ml)	4.74 ± 0.62	5.81 ± 1.72	5.36 ± 1.11	4.96 ± 0.80

Abbreviations: HBCD, hexabromocyclododecane; PND, postnatal day; PNW, postnatal week; TBBPA, tetrabromobisphenol A; T₃, triiodothyronine; T₄, thyroxine; TSH, thyroid-stimulating hormone.

^a Mean ± S.D.

^{*} Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.05$).

^{**} Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.01$).

3.6. Histopathology at the prepubertal and adult stage necropsies

In the TBBPA study, no treatment-related histopathological changes were observed in any other organs/tissues in either gender at PND 20 or PNW 11 (data not shown).

In the HBCD study, a statistically significant increased incidence of diffuse vacuolar degeneration of liver cells was observed at 10,000 ppm in both sexes on PND 20 (Fig. 3C and D, and Table 6). On PNW 11, a significantly increased incidence of adrenocortical vacuolar degeneration was observed in males at 10,000 ppm (Fig. 3E and F).

3.7. Brain morphometry at the adult stage

Brain morphometric changes in terms of neuronal migration and oligodendroglial development were examined in male offspring at the adult stage (Table 7).

In the TBBPA study, there were no significant differences between the untreated controls and any exposure groups in the distribution of hippocampal CA1 neurons measured by the mean distance of the location of NeuN-positive neurons from the pyramidal cell layer, the number of neurons located lateral to the pyramidal cell layer, or ratio of abnormally distributed neurons in total CA1 neurons. The area of the CC and the number of CNPase-positive oligodendrocytes in the cingulate deep cortex were also unaltered by TBBPA.

In the HBCD study, there were also no significant differences between the untreated controls and any exposure groups in the parameters described above to measure the distribution of hippocampal CA1 neurons. With regard to the oligodendroglial development-related parameters, a significant reduction in the number of CNPase-positive oligodendrocytes in the cingulate deep cortex was observed at 10,000 ppm as compared with the value in the untreated controls, while CC area was unaltered at any dose.

4. Discussion

Disruption of thyroid homeostasis has been proposed to be the primary toxic effect of TBBPA, as well as other BFRs [13]. Exposure to TBBPA during gestation increases fetal plasma TSH concentration in rats, but circulating concentrations of T₃ and T₄ are unaltered in dams or offspring [35]. In contrast, in the present study, TBBPA-treated dams did not show any toxic changes except for a tendency to increase the incidence of hypothyroidism-related thyroid follicular cell hypertrophy from 1000 ppm, and there was a dose-unrelated increasing tendency for relative thyroid weight in all treatment groups. No obvious toxic changes were also noted in offspring except for a dose-unrelated reduction of serum T₃ concentration at 100 and 1000 ppm. However, these animals did not show any changes in the histopathology or weight of the thyroid or the serum concentrations of T₄ and TSH. These results may suggest a limited evidence of the effects of developmental hypothyroidism by TBBPA in the experimental conditions of the present study. Retarded body growth, a typical alteration during the transient developmental hypothyroidism [36], was also lacking in offspring exposed to TBBPA during gestation through lactation. Unchanged values for both neuronal migration and oligodendroglial development at the adult stage may support a negligible effect on the thyroid function of TBBPA. However, in a recently published one-generation reproductive toxicity study of TBBPA by feeding, auditory response effects, typically related to developmental hypothyroidism, were observed in offspring at the adult stage in association with serum TH changes [28,29]. Although the reason for the discrepancy of hypothyroidism-related effects between the reproduction study and ours was unclear, a difference in the duration of exposure might have contributed. At the very least, changes in thyroid hormone levels detected in the reproduction study might have been because of the continued dietary administration of TBBPA to offspring until sacrifice after cessation of maternal exposure.

Table 5

Body and organ weights of the offspring exposed to tetrabromobisphenol A or hexabromocyclododecane from mid-gestation to the end of lactation and examined at PNW 11.

TBBPA study	TBBPA in diet (ppm)			
	0	100	1000	10,000
Males				
No. of animals examined	10	10	10	10
BW (g)	456.1 ± 15.9 ^a	465.4 ± 29.1	452.6 ± 23.9	455.7 ± 33.3
Brain (g/100 g BW)	0.48 ± 0.02	0.47 ± 0.02	0.47 ± 0.02	0.48 ± 0.04
Pituitary (mg/100 g BW)	3.41 ± 0.38	3.32 ± 0.17	3.19 ± 0.21	3.37 ± 0.45
Spleen (g/100 g BW)	0.19 ± 0.02	0.19 ± 0.03	0.19 ± 0.02	0.21 ± 0.04
Thymus (g/100 g BW)	0.11 ± 0.03	0.13 ± 0.03	0.11 ± 0.02	0.14 ± 0.03
Liver (g/100 g BW)	3.76 ± 0.18	3.68 ± 0.21	3.64 ± 0.28	3.53 ± 0.36
Kidneys (g/100 g BW)	0.68 ± 0.04	0.69 ± 0.04	0.72 ± 0.05	0.69 ± 0.04
Adrenals (mg/100 g BW)	12.4 ± 1.3	13.1 ± 1.7	12.6 ± 1.9	14.0 ± 2.3
Testes (g/100 g BW)	0.75 ± 0.08	0.72 ± 0.10	0.74 ± 0.11	0.76 ± 0.06
Epididymides (g/100 g BW)	0.22 ± 0.02	0.21 ± 0.03	0.22 ± 0.03	0.23 ± 0.02
Prostate, ventral (mg/100 g BW)	0.11 ± 0.02	0.12 ± 0.02	0.11 ± 0.02	0.12 ± 0.02
Prostate, dorso-lateral (mg/100 g BW)	0.12 ± 0.01	0.12 ± 0.03	0.12 ± 0.02	0.12 ± 0.01
Seminal vesicles (mg/100 g BW)	0.25 ± 0.03	0.24 ± 0.04	0.25 ± 0.02	0.25 ± 0.04
Thyroid (mg/100 g BW)	4.72 ± 0.95	5.40 ± 0.96	5.09 ± 0.92	5.58 ± 0.86
Females				
No. of animals examined	10	10	10	10
BW (g)	285.4 ± 12.4	285.1 ± 20.7	289.7 ± 26.2	296.1 ± 25.0
Brain (g/100 g BW)	0.70 ± 0.03	0.71 ± 0.6	0.68 ± 0.05	0.67 ± 0.06
Pituitary (mg/100 g BW)	6.04 ± 0.58	6.16 ± 0.63	5.62 ± 0.93	5.82 ± 1.00
Spleen (g/100 g BW)	0.22 ± 0.03	0.23 ± 0.05	0.19 ± 0.02	0.20 ± 0.02
Thymus (g/100 g BW)	0.19 ± 0.04	0.16 ± 0.02	0.16 ± 0.03	0.20 ± 0.05
Liver (g/100 g BW)	3.49 ± 0.25	3.47 ± 0.22	3.32 ± 0.20	3.51 ± 0.27
Kidneys (g/100 g BW)	0.71 ± 0.04	0.70 ± 0.03	0.66 ± 0.05 [†]	0.71 ± 0.04
Adrenals (mg/100 g BW)	24.2 ± 3.5	25.4 ± 2.4	22.8 ± 2.5	24.3 ± 2.8
Ovaries (mg/100 g BW)	31.3 ± 3.8	34.9 ± 3.9	33.3 ± 3.2	34.6 ± 4.7
Uterus (g/100 g BW)	0.18 ± 0.03	0.19 ± 0.03	0.18 ± 0.07	0.14 ± 0.02 [†]
Thyroid (mg/100 g BW)	7.19 ± 1.00	7.46 ± 1.78	6.60 ± 1.01	7.25 ± 1.09
HBCD study				
	HBCD in diet (ppm)			
	0	100	1000	10,000
Males				
No. of animals examined	10	10	10	10
BW (g)	454.3 ± 25.4	456.9 ± 24.8	450.8 ± 33.4	435.1 ± 24.6
Brain (g/100 g BW)	0.46 ± 0.03	0.46 ± 0.02	0.47 ± 0.04	0.47 ± 0.02
Pituitary (mg/100 g BW)	3.35 ± 0.19	3.43 ± 0.35	3.30 ± 0.21	3.24 ± 0.30
Spleen (g/100 g BW)	0.18 ± 0.02	0.21 ± 0.03	0.19 ± 0.02	0.19 ± 0.02
Thymus (g/100 g BW)	0.13 ± 0.03	0.14 ± 0.03	0.12 ± 0.04	0.12 ± 0.03
Liver (g/100 g BW)	3.45 ± 0.27	3.81 ± 0.23 ^{**}	3.58 ± 0.24	3.53 ± 0.22
Kidneys (g/100 g BW)	0.66 ± 0.05	0.67 ± 0.05	0.67 ± 0.04	0.66 ± 0.05
Adrenals (mg/100 g BW)	13.0 ± 1.5	12.4 ± 1.2	11.6 ± 1.8	12.3 ± 2.5
Testes (g/100 g BW)	0.77 ± 0.07	0.73 ± 0.04	0.78 ± 0.09	0.74 ± 0.05
Epididymides (g/100 g BW)	0.23 ± 0.02	0.21 ± 0.01 [†]	0.22 ± 0.02	0.21 ± 0.01
Prostate, ventral (mg/100 g BW)	0.13 ± 0.02	0.13 ± 0.04	0.12 ± 0.03	0.12 ± 0.01
Prostate, dorsolateral (mg/100 g BW)	0.13 ± 0.03	0.13 ± 0.01	0.14 ± 0.03	0.13 ± 0.02
Seminal vesicles (mg/100 g BW)	0.27 ± 0.05	0.26 ± 0.03	0.26 ± 0.05	0.26 ± 0.05
Thyroid (mg/100 g BW)	4.85 ± 0.69	5.66 ± 0.67	5.78 ± 0.82 [†]	6.20 ± 1.03 ^{**}
Females				
No. of animals examined	10	10	10	10
BW (g)	286.2 ± 25.2	293.4 ± 21.5	289.2 ± 24.4	270.7 ± 19.6
Brain (g/100 g BW)	0.68 ± 0.07	0.65 ± 0.05	0.68 ± 0.06	0.71 ± 0.04
Pituitary (mg/100 g BW)	5.94 ± 1.00	5.63 ± 0.64	5.72 ± 1.31	5.71 ± 0.63
Spleen (g/100 g BW)	0.19 ± 0.02	0.20 ± 0.03	0.21 ± 0.02	0.20 ± 0.03
Thymus (g/100 g BW)	0.18 ± 0.03	0.19 ± 0.06	0.17 ± 0.04	0.16 ± 0.05
Liver (g/100 g BW)	3.35 ± 0.20	3.59 ± 0.19	3.44 ± 0.25	3.30 ± 0.22
Kidneys (g/100 g BW)	0.69 ± 0.03	0.65 ± 0.03	0.69 ± 0.06	0.65 ± 0.05
Adrenals (mg/100 g BW)	21.1 ± 3.4	22.6 ± 2.0	23.7 ± 2.3	24.2 ± 4.7
Ovaries (mg/100 g BW)	31.8 ± 6.1	32.8 ± 2.6	32.2 ± 5.7	34.0 ± 4.8
Uterus (g/100 g BW)	0.16 ± 0.04	0.15 ± 0.02	0.16 ± 0.02	0.17 ± 0.03
Thyroid (mg/100 g BW)	8.20 ± 2.94	6.84 ± 0.81	7.35 ± 0.87	7.72 ± 0.83

Abbreviations: BW, body weight; HBCD, hexabromocyclododecane; PNW, postnatal week; TBBPA, tetrabromobisphenol A.

^a Mean ± S.D.[†] Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.05$).^{**} Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.01$).

In the HBCD study, we found increases in the relative thyroid weight and an increased incidence of thyroid follicular cell hypertrophy in dams at weaning when given 10,000 ppm. Tendencies to increase for these parameters were also noted in dams in other

treatment groups. Offspring at 10,000 ppm at this time point also showed a decrease of T_3 and an increase of TSH in the serum, while the magnitude of these alterations were small and histopathological thyroidal changes were not found at this time point. At the

Table 6
Histopathologic changes for male and female offspring exposed to hexabromocyclododecane from mid-gestation to the end of lactation.

	HBCD in diet (ppm)			
	0	100	1000	10,000
PND 20				
Males				
No. of animals examined	10	10	10	10
Liver				
Vacuolar degeneration, liver cells, diffuse (+) ^a	0	0	0	6 ^c
Females				
No. of animals examined	10	10	10	10
Liver				
Vacuolar degeneration, liver cells, diffuse (+/++)	0	0	0	6 ^{b,c} (0/6) ^{c,§§}
PNW 11				
Males				
No. of animals examined	10	10	10	10
Adrenal				
Vacuolar degeneration, diffuse, cortical cells (+/++)	0	0	0	4 ^c (2/2) [§]

Abbreviations: HBCD, hexabromocyclododecane; PND, postnatal day; PNW, postnatal week.

^a Grade of change: (+) slight; (++) moderate.

^b Total no. of animals with each finding.

^c No. of animals with each grade.

^{*} Significantly different from the controls by Fisher's exact probability test ($P < 0.05$).

[§] Significantly different from the controls by Mann–Whitney's *U*-test ($P < 0.05$).

^{§§} Significantly different from the controls by Mann–Whitney's *U*-test ($P < 0.01$).

Table 7

Brain morphometry of the male offspring exposed to tetrabromobisphenol A or hexabromocyclododecane from mid-gestation to the end of lactation and examined at PNW 11.

TBBPA study	TBBPA in diet (ppm)			
	0	100	1000	10,000
No. of offspring examined	10	10	10	10
Hippocampal CA1 neurons				
Mean distance of the location of neurons from the innermost margin of the pyramidal cell layer (μm)	30.9 \pm 3.0 ^a	29.8 \pm 2.0	31.5 \pm 1.8	29.2 \pm 1.6
No. of neurons located lateral to the pyramidal cell layer (per mm)	5.1 \pm 1.4	4.7 \pm 1.5	4.0 \pm 1.6	4.6 \pm 1.7
Ratio of abnormally distributed neurons/CA1 neurons (%)	2.3 \pm 0.6	1.9 \pm 0.5	1.7 \pm 0.6	1.9 \pm 0.7
CC				
Area of CC (mm^2)	0.14 \pm 0.01	0.14 \pm 0.01	0.14 \pm 0.02	0.14 \pm 0.01
Cingulate deep cortex				
CNPase (+) cell count (count/ mm^2)	133.0 \pm 16.7	120.8 \pm 14.3	121.4 \pm 15.4	127.1 \pm 18.8
HBCD study	HBCD in diet (ppm)			
	0	100	1000	10,000
No. of offspring examined	10	10	10	10
Hippocampal CA1 neurons				
Mean distance of the location of neurons from the innermost margin of the pyramidal cell layer (μm)	50.9 \pm 11.8	49.3 \pm 9.1	47.2 \pm 11.9	48.2 \pm 2.3
No. of neurons located lateral to the pyramidal cell layer (per mm)	4.5 \pm 1.3	4.9 \pm 0.9	3.3 \pm 1.1	3.5 \pm 1.1
Ratio of abnormally distributed neurons/CA1 neurons (%)	2.9 \pm 1.0	3.1 \pm 0.6	2.3 \pm 0.9	2.3 \pm 0.7
CC				
Area of CC (mm^2)	0.15 \pm 0.02	0.15 \pm 0.02	0.14 \pm 0.01	0.14 \pm 0.01
Cingulate deep cortex				
CNPase (+) cell count (count/ mm^2)	181.6 \pm 28.2	167.6 \pm 23.2	160.3 \pm 28.1	138.7 \pm 23.7

Abbreviations: CC, corpus callosum; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; HBCD, hexabromocyclododecane; PNW, postnatal week; TBBPA, tetrabromobisphenol A.

^a Mean \pm S.D.

^{*} Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.05$).

adult stage, a slight reduction of serum T_3 concentration, as well as slight increase of relative thyroid weight, was found from a dose

of 1000 ppm. These results suggest a slight but sustained hypothyroidism until adult stage beginning at a dose of 1000 ppm. Slight

but nonsignificant reductions in body weight from the onset of puberty in groups receiving 10,000 ppm in both sexes may support the effect of hypothyroidism [36]. With regard to the brain morphometric parameters of offspring, the oligodendroglial cell number decreased in the 10,000 ppm groups, while no other changes were detected in neuronal migration parameters and in the size of the CC area. Developmental hypothyroidism results in a decreased number of mature oligodendrocytes, which results in a decreased area of intrahemispheric commissures, such as the CC as measured in the present study [4]. These results may suggest a mild effect of HBCD on oligodendroglial development at 803.2–2231.3 mg/kg body weight/d by maternal exposure level, probably through a hypothyroidism-related mechanism.

In rats, HBCD is known to act as an inducer of cytochrome P450 (CYP) [37] and uridine diphosphate-glucuronosyltransferase [38] in rats. This biological potential may be linked to the facilitation of TH metabolism to cause an elevation of serum TSH concentration through the suppression of negative feedback by HBCD at 10,000 ppm on PND 20 in the present study. Alternatively, Yamada-Okabe et al. [3] have shown that TH receptor-mediated gene expression is affected by HBCD in a reporter gene assay in HeLaTR cells expressing human TH receptor α 1, suggesting a direct action of HBCD on TH receptors. In the present study, a decrease in serum T_3 concentration and increase of the thyroid weight continued until the adult stage by HBCD at doses from 1000 ppm, probably due to strong propensity for bioaccumulation [13,39].

Sui and Gilbert [40] demonstrated that experimentally induced developmental hypothyroidism via maternal administration of PTU impairs the functional integrity of synaptic communication in the hippocampal CA1 region. Furthermore, an experimentally induced iodine deficiency causing hypothyroidism during gestation resulted in aberrant migration of neurons, such as those in somatosensory cortex and hippocampal CA1 [9]. In our preceding study we employed the same morphometrical methods to study the neuroarchitecture as in the present study and confirmed hypothyroidism-related changes in the neuronal cell distribution in the CA1 region as well as those in parameters linked to oligodendroglial development [10]. After exposure to two graded doses of PTU and a single dose of methimazole (MMI), another anti-thyroid agent, during the period from GD 10 to PND 20, the PTU group showed changes that were clearly dose-dependent [10]. However, we could not detect aberrant neuronal distribution in the CA1 region by either TBBPA or HBCD in the present study. Recently, there are two studies reporting genes identified to show altered expression by microarray analysis in the cerebral cortex and hippocampus of rats under developmental hypothyroidism [41,42]. Molecules detected by such analysis may provide a beneficial tool as a marker for rapid screening of chemicals to cause disruption of brain differentiation in terms of neuronal migration and plasticity in a small scale of animals study.

In the HBCD study, an increase of relative liver weight and vacuolar degeneration of liver cells were observed in both sexes at PND 20, similar to the previously performed 28 d and 90 d toxicity studies of HBCD in rats [30]. Hepatic enlargement may be partly related to liver enzyme induction by HBCD as mentioned above [37]. In 4 out of 10 males, diffuse vacuolar degeneration in the adrenal cortex was observed by exposure to 10,000 ppm HBCD at the adult stage, but the change was not reflected in the adrenal weight. Any other repeated toxicity studies of HBCD did not show similar changes, and the effect was lacking in females, suggesting a low toxicological relevance.

With regard to repeated-oral-dose-toxicity of HBCD, there are three data available studies in rats; two 28-d and one 90-d studies [38,39,43]. In these studies, doses of ≥ 100 mg/kg-d resulted in a dose-dependent increase in the liver weight. All studies

showed effects on the thyroid-related parameters. Among them, a 90-d study exhibited relative liver weight increases (18–24%) by HBCD from 100 mg/kg-d. Disturbances in the thyroid hormone system (T_4 and TSH) also occurred at this dose [39]. Similarly, a recently reported two-generation reproductive toxicity study has also shown the liver and thyroid system to be target organs [31]. In this study, the middle dose (<101–141 mg/kg-d) was considered to be a level showing apparent effects on the liver. For effects on the thyroid system, the middle dose (<101–141 mg/kg-d) was also a clear effect level, with decreased thyroid follicle size and increased serum TSH. In the present study, the most sensitive effect to offspring was detected on thyroid parameters at the adult stage at the maternal exposure levels from 1000 ppm (80.7–212.9 mg/kg-d), that is similar to the effect levels similar to other studies above mentioned. Although the species and the mode of administration were different from the present study, Eriksson et al. [32] reported neurobehavioral abnormalities in male mice after a single direct administration of HBCD by gavage at 0.9 and 13.5 mg/kg body weight on PND 10. The discrepancy in the dose–effect relationship between the present and previous study could be explained by the difference in the actual intake of HBCD in pups between the direct exposure of pups and maternal exposure, indirectly to pups via maternal milk, and by differences in the animal species used in these studies. Further studies are needed to clarify the difference in the mechanism of action of HBCD to affect the nervous system between the present and previous study.

In conclusion, maternal exposure to TBBPA through diet exhibited limited evidence of developmental hypothyroidism on rat offspring that could be negligible for thyroid function, and there was no evidence of impaired brain development such as hypothyroidism-related neuronal migration or oligodendroglial development. With regard to the maternal exposure effect of HBCD, a weak hypothyroidism effect was evident with weight and histopathological changes of the thyroid and serum T_3 and TSH concentrations on offspring receiving 10,000 ppm at weaning. An increase of thyroidal weight and decrease of serum T_3 concentration continued until the adult stage in groups receiving at least 1000 ppm. With regard to the effect on brain development, HBCD showed evidence that oligodendroglial development was affected at a dose of 10,000 ppm, probably as a result of developmental hypothyroidism. Thus, based on the developmental brain effect, 100 ppm was determined to be the no-observed-adverse-effect level (NOAEL) of HBCD from changes in thyroid parameters, translating into 8.1–21.3 mg/kg-d by maternal exposure level. NRC estimated that the average oral dose rate was 0.026 mg/kg body weight/d [44]. The estimated human intake of HBCD is well below the NOAEL in the present study.

Conflict of interest

None declared.

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Gene Expression Profiling and Cellular Distribution of Molecules with Altered Expression in the Hippocampal CA1 Region after Developmental Exposure to Anti-Thyroid Agents in Rats

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ABSTRACT. To determine whether developmental hypothyroidism causes permanent disruption of neuronal development, we first performed a global gene expression profiling study targeting hippocampal CA1 neurons in male rats at the end of maternal exposure to anti-thyroid agents on weaning (postnatal day 20). As a result, genes associated with nervous system development, zinc ion binding, apoptosis and cell adhesion were commonly up- or down-regulated. Genes related to calcium ion binding were up-regulated and those for myelination were often down-regulated. We, then, examined immunohistochemical cellular distribution of Ephrin type A receptor 5 (EphA5) and Tachykinin receptor (Tacr)-3, those selected based on the gene expression profiles, in the hippocampal formation at the adult stage (11-week-old) as well as at the end of exposure. At weaning, both EphA5- and Tacr3-immunoreactive cells with strong intensities appeared in the pyramidal cell layer or stratum oriens of the hippocampal CA1 region. Although the magnitude of the change was decreased at the adult stage, Tacr3 in the CA1 region showed a sustained increase in expressing cells until the adult stage after developmental hypothyroidism. On the other hand, EphA5-expressing cells did not show sustained increase at the adult stage. The results suggest that developmental hypothyroidism caused sustained neuronal expression of Tacr3 in the hippocampal CA1 region, probably reflecting a neuroprotective mechanism for mismigration.

KEY WORDS: developmental hypothyroidism, EphA5, hippocampal CA1 region, Tacr3.

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Thyroid hormones are essential for normal fetal and neonatal brain development. They control neuronal and glial proliferation in definitive brain regions and regulate neural migration and differentiation [12, 18, 21]. In humans, maternal hypothyroxinemia, early in pregnancy, may have adverse effects on fetal brain development and importantly, even mild-moderate hypothyroxinemia may result in suboptimal neurodevelopment [4]. These results may increase the concern of thyroid hormone-disrupting chemicals in the environment.

Experimentally, developmental hypothyroidism leads to growth retardation, neurological defects and impaired performance on a variety of behavioral learning actions [1, 2]. Rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) show brain retardation, with impaired neuronal migration and white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation [6, 8, 21]. The outcome of this type of brain retardation is permanent and is accompanied by apparent structural and functional abnormalities. However, it is still unclear whether the molecular aberrations remain

in the retarded brain after maturation.

Histological lesion-specific gene expression profiling provides valuable information on the mechanisms underlying lesion development. We have established molecular analysis methods for DNA, RNA and proteins in paraffin-embedded small tissue specimens utilizing an organic solvent-based fixative, methacarn, with high performance close to that achieved with unfixed frozen tissue specimens [22, 26, 27]. We have previously applied these techniques to analyze global gene expression changes in microdissected lesions [23, 28].

Hippocampal CA1 region is a well-known target of developmental hypothyroidism [8], and we, in our recent study, detected a distribution variability of hippocampal CA1 pyramidal neurons reflecting mismigration in rat offspring at the adult stage after developmental exposure to anti-thyroid agents [24]. The present study was performed to determine whether developmental hypothyroidism triggers sustained aberrations in neuronal development associated with neuronal mismigration until the adult stage. For this purpose, we first performed a global gene expression profiling of the CA1-pyramidal cell layer in rat offspring at the end of developmental exposure to anti-thyroid agents. To distinguish chemical-specific expression changes from hypothyroidism-linked ones, two different anti-thyroid

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agents, PTU and 2-mercapto-1-methylimidazole (MMI), were used, and dose-related responses were also examined with PTU. To extract the neuronal cell layer-specific gene expression profile, microdissection technique was applied for microarray analysis. Based on the expression profiles obtained, cellular localization of the molecules showing altered expression were then immunohistochemically examined in the hippocampus at the adult stage as well as at the end of the developmental exposure.

MATERIALS AND METHODS

Chemicals and animals: 6-propyl-2-thiouracil (PTU; CAS No. 51-52-5) and methimazole (2-mercapto-1-methylimidazole; MMI; CAS No. 60-56-0) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pregnant Crj:CD®(SD)IGS rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at gestation day (GD) 3 (appearance of vaginal plugs was designated as GD 0). Animals were housed individually in polycarbonate cages with wood chip bedding, maintained in an air-conditioned animal room (temperature: $24 \pm 1^\circ\text{C}$; relative humidity: $55 \pm 5\%$) with a 12-hr light/dark cycle and allowed *ad libitum* access to food and tap water. A soy-free diet (Oriental Yeast Co., Ltd., Tokyo, Japan) was chosen as the basal diet for the maternal animals to eliminate possible phytoestrogen effects [10], and water was given *ad libitum* throughout the experimental period including the 1-week acclimation period.

Animal experiments: The animal experiments were identical to those in a previous study [24]. In brief, maternal animals were randomly divided into four groups including untreated controls. Eight dams per group were treated with 3 or 12 ppm of PTU or 200 ppm of MMI in the drinking water from GD 10 to postnatal day (PND) 20 (PND 0: the day of delivery). On PND 2, the litters were culled randomly, leaving four male and four female offspring. On PND 20, 20 male and 20 female offspring (at least one male and one female per dam) per group were subjected to prepubertal necropsy [13, 24].

The remaining animals were maintained until postnatal week (PNW) 11. All offspring consumed the CRF-1 basal diet and tap water *ad libitum* from PND 21 onwards. At PNW 11, all pups were subjected to adult stage necropsy [13, 24].

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

Preparation of tissue specimens and microdissection: For microarray and subsequent real-time RT-PCR analyses, the whole brain of male offspring was removed at prepubertal necropsy on PND 20 ($n=4/\text{group}$) and was fixed with methacarn solution for 2 hr at 4°C [22]. Coronal brain slices taken at the position of -3.5 mm from the bregma were

dehydrated and embedded in paraffin. The embedded tissue blocks were stored at 4°C until tissue sectioning for microdissection [9].

For microdissection, $4\text{-}\mu\text{m}$ -thick sections between ten $20\text{-}\mu\text{m}$ -thick serial sections were prepared. The $4\text{-}\mu\text{m}$ -thick sections were stained with hematoxylin and eosin for confirmation of anatomical orientation of the hippocampal substructure to aid microdissection. The $20\text{-}\mu\text{m}$ -thick sections were mounted onto PEN-foil film (Leica Microsystems GmbH, Welzlar, Germany) overlaid on glass slides, dried in an incubator overnight at 37°C , and then stained using an LCM staining kit (Ambion, Inc., Austin, TX, U.S.A.). Bilateral sides of the hippocampal CA1 pyramidal cell layer in the sections were subjected to laser microbeam microdissection (Leica Microsystems GmbH) (Fig. 1). Twenty sections from each animal were used for microdissection, and the bilateral microdissected samples were collected and stored in separate 1.5-ml sample tubes at -80°C until the extraction of total RNA.

RNA preparation, amplification and microarray analysis: Total RNA extraction from hippocampal CA1 samples, quantitation of the RNA yield, and amplification of RNA samples were performed using previously described methods [9, 28].

For microarray analysis, second-round-amplified biotin-labeled antisense RNAs were subjected to hybridization with a GeneChip® Rat Genome 230 2.0 Array (Affymetrix, Inc., Santa Clara, CA, U.S.A.), as previously described [28].

The selection of genes and normalization of the expression data were performed using GeneSpring® software (ver7.2, Silicon Genetics, Redwood City, CA, U.S.A.). Per chip normalization was performed according to a previously described method [28]. Genes showing signals judged to be "absent" in all eight samples of untreated controls and in the anti-thyroid agent-exposed group were excluded. Genes

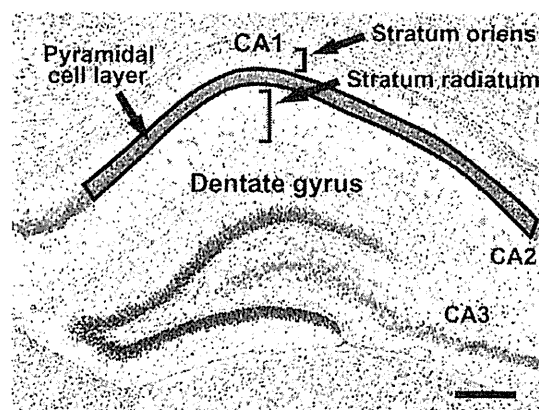


Fig. 1. Overview of the hippocampal formation of a male rat at postnatal day 20 stained with hematoxylin and eosin. Bar=200 μm . The CA1 pyramidal cell layer, enclosed by a solid line, was microdissected for the microarray and subsequent real-time RT-PCR analyses. The number of cells immunoreactive for the candidate molecules in this area was normalized for the length of CA1 used.

showing expression changes with differences of at least twofold in magnitude from the untreated controls were selected, and the "presence" signal in more than 3/4 of samples in each group showing higher expression values were selected. Genes showing altered expression in common in the anti-thyroid agent-exposed groups were also selected.

Real-time RT-PCR: Quantitative real-time RT-PCR was performed to confirm the expression values obtained with microarrays using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan). Genes those showing altered expression (≥ 2 -fold, ≤ 0.5 -fold) in common in the anti-thyroid agent-exposed groups as compared with untreated control offspring were randomly selected, irrespective of the presence or absence of statistically significant difference. As a result, the following seven genes (four up-regulated and three down-regulated) with known function were selected as targets: Tachykinin receptor 3 (*Tacr3*), Calbindin 1, Slit homolog 2 (*Drosophila*) and Pleomorphic adenoma gene-like 1 (*Plagl1*) as up-regulated examples, and Myelin-associated oligodendrocytic basic protein (*Mobp*), Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8 and CCAAT/enhancer binding protein as down-regulated. RT was performed using first-round antisense RNAs prepared for microarray analysis. For real-time PCR analysis of the genes selected, ABI Assays-on-Demand™ TaqMan® probe and primer sets from Applied Biosystems (available at <https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601267>)(n=4/group) were used. For quantification of the expression data, a standard curve method was applied. The expression values were normalized to two housekeeping genes, Glyceraldehyde 3-phosphate dehydrogenase and Hypoxanthine-guanine phosphoribosyltransferase.

Immunohistochemistry: To evaluate the immunohistochemical distribution of the molecules selected by microarray analysis, the brains of male pups obtained at PND 20 or PNW 11 were fixed in Bouin's solution at room temperature overnight. Six animals were used as untreated controls, six for 200 ppm MMI, eight for 3 ppm PTU, and nine for 12 ppm PTU on PND 20. On PNW 11, 10 animals were used as untreated controls and 10 for 200 ppm MMI, nine for 3 ppm PTU, and six for 12 ppm PTU.

Immunohistochemistry was performed on the brain tissue sections of PND 20 and PNW 11 animals with antibodies against Ephrin type A receptor 5 (EphA5; rabbit IgG, 1:50; Abcam, Cambridge, U.K.) and Tacr3 (rabbit polyclonal antibody, 1:3,000, Novus Biologicals, Inc., Littleton, CO, U.S.A.), which were incubated with the tissue sections overnight at 4°C. Antigen retrieval treatment was not performed for these antigens. Immunodetection was carried out using a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.) with 3,3'-diaminobenzidine/H₂O₂ as the chromogen, as previously described [23]. The sections were then counterstained with hematoxylin and coverslipped for microscopic examination.

With regard to EphA5, *Efna5*, a gene encoding the representative ligand for this receptor molecule [5], was found to

be up-regulated (≥ 2 -fold) by microarray analysis in all of the groups exposed to anti-thyroid agents in the present study (Table 1). Because distribution of EphA5 has been confirmed in the pyramidal cells of the hippocampal CA1 region at both developmental and adult stages in mice and at adult stage in humans [3, 17], we selected this molecule to examine distribution changes in the present study. Tacr3 was also up-regulated in all of the MMI and PTU groups by microarray analysis and real-time RT-PCR in the present study (Table 1). Expression of Tacr3 in the hippocampal CA1 pyramidal neurons has also been confirmed in rats [11], and therefore, we also selected this molecule for examination in the expression changes in the present study.

Morphometry of immunolocalized cells and apoptotic cells: EphA5- or Tacr3-immunoreactive cells distributed in the pyramidal cell layer or stratum oriens of the hippocampal CA1 region were bilaterally counted and normalized to the number in the length of the CA1 region measured (Fig. 1). Tacr3-immunoreactive cells in the subgranular zone of the dentate gyrus were also bilaterally counted and normalized for the number in the length of the granular zone measured. For quantitative measurement of each immunoreactive cellular component, digital photomicrographs at 100-fold magnification were taken using a BX51 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a DP70 Digital Camera System (Olympus Optical Co., Ltd.), and quantitative measurements were performed using the WinROOF image analysis software package (version 5.7, Mitani Corp., Fukui, Japan).

Statistical analysis: Numerical data of the number of immunoreactive cells were assessed using Student's *t*-test to compare the untreated controls with each of the anti-thyroid agent-exposed groups when the variance was homogenous among the groups using a test for equal variance. If a significant difference in variance was observed, Aspin-Welch's *t*-test was used instead. The data for gene expression levels from real-time RT-PCR analysis were analyzed by the Kruskal-Wallis test, followed by Bartlett's test. When statistically significant differences were indicated, Dunnett's multiple test was used for comparisons with the untreated controls. For the microarray data, statistical analysis was performed with GeneSpring® software, and the significance of gene expression changes was analyzed by Student's *t*-test or ANOVA between the untreated controls and each of the anti-thyroid agent-exposed groups.

RESULTS

Microarray analysis: Figure 2 shows the Venn diagram of genes showing altered expression in the microdissected CA1 pyramidal neurons in the exposure groups in combination or individually in each exposure group. Many genes were found to be up- or down-regulated in common in two of the three groups. The numbers of genes classified into common categories between the groups or individually in each group were similar in terms of up- and down-regulated genes. The number of genes showing up- or down-regula-

Table 1. List of representative genes showing up- or down-regulation common to 2-mercapto-1-methylimidazole (MMI), 3 and 12 ppm 6-propyl-2-thiouracil (PTU) (≥ 2 -fold, ≤ 0.5 -fold)

Gene function	Accession No.	Gene title	Symbol	MMI	3 ppm PTU	12 ppm PTU
<i>Up-regulated genes (of 119 genes in total)</i>						
Nervous system development	AI101660	Slit homolog 2 (Drosophila)	Slit2	3.04	2.62	7.08
Nervous system development	NM_024358.1	Notch gene homolog 2 (Drosophila)	Notch2	2.52	2.01	2.02
Nervous system development	AW527295	Ephrin A5	Efna5	3.12	3.46	4.31
Nervous system development	NM_053465.1	Fucosyltransferase 9	Fut9	2.13	6.75	2.11
Nervous system development	BE106256	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1	Spock1	3.22	3.13	2.15
Calcium ion binding	X04280.1	Calbindin 1	Calb1	4.48	4.85	9.00
Calcium ion binding	BM386119	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	Galnt3	2.43	2.30	2.63
Calcium ion binding	BI279663	Desmocollin 2	Dsc2	2.82	2.04	5.62
Calcium ion binding	AI105369	Calmodulin-like 4	Calml4	3.40	2.25	5.59
Zinc ion binding	BE098686	Similar to Tnf receptor-associated factor 1	LOC687813	3.10	2.04	2.78
Zinc ion binding	BF562032	RAN binding protein 2	Ranbp2	3.49	2.67	2.78
Zinc ion binding	BF397925	ADAMTS-like 1	Adamts11	6.22	2.55	7.63
Zinc ion binding	BF395606	Splicing factor, arginine/serine-rich 7	Sfrs7	4.93	2.06	2.90
Apoptosis	NM_012760.1	Pleomorphic adenoma gene-like 1	Plagl1	3.10	4.28	6.86
Apoptosis	NM_057130.1	Harakiri, BCL2 interacting protein (contains only BH3 domain)	Hrk	2.63	2.73	3.18
Cell Adhesion	AA850909	Poliovirus receptor-related 2	Pvr12	4.74	2.46	2.61
Cell Adhesion	AA819731	Hyaluronan and proteoglycan link protein 4	Hapln4	4.13	6.67	3.46
Cell Adhesion	BI287851	Collagen, type VI, alpha 2	Col6a2	3.45	2.19	5.12
Ion channel activity	AA851939	FXVD domain-containing ion transport regulator 6	Fxyd6	4.73	2.61	7.85
Other	NM_017053.1	Tachykinin receptor 3	Tacr3	7.32	6.19	12.49
<i>Down-regulated genes (of 97 genes in total)</i>						
Nervous system development	NM_031018.1	Activating transcription factor 2	Atf2	0.41	0.36	0.36
Neuron migration	BF390065	Roundabout homolog 3 (Drosophila)	Robo3	0.06	0.31	0.04
Neuron differentiation	AF115249.1	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8	Edg8	0.40	0.06	0.08
Neuron differentiation	NM_024125.1	CCAAT/enhancer binding protein (C/EBP), beta	Cebpb	0.31	0.43	0.26
Myelination	X89638.1	Myelin-associated oligodendrocytic basic protein	Mobp	0.35	0.18	0.12
Myelination	NM_017190.1	Myelin-associated glycoprotein	Mag	0.47	0.36	0.29
Myelination	NM_022668.1	Myelin oligodendrocyte glycoprotein	Mog	0.44	0.32	0.19
Myelination	NM_012798.1	Mal, T-cell differentiation protein	Mal	0.37	0.28	0.28
Myelination	AA945178	Signal recognition particle receptor, B subunit transferrin	Strprb Tf	0.33	0.27	0.15
Zinc ion binding	NM_012566.1	Growth factor independent 1 transcription repressor	Gfi1	0.20	0.44	0.41
Zinc ion binding	AW529624	Zinc finger protein 91	Zfp91	0.33	0.32	0.38
Actin binding	AW522439	Ermin, ERM-like protein	Ermin	0.43	0.42	0.28
Apoptosis	BG377720	Solute carrier family 5 (sodium/glucose cotransporter), member 11	Slc5a11	0.25	0.19	0.19
Apoptosis	U21955.1	Eph receptor A	Epha7	0.34	0.48	0.18
Cell Adhesion	BM391100	Mucin 4, cell surface associated	Muc4	0.43	0.36	0.27
Other	AW435010	Protein tyrosine phosphatase, non-receptor type 3	Ptpn3	0.38	0.46	0.36
Other	AF312319.1	gamma-aminobutyric acid (GABA) B receptor 1	Gabbr1	0.33	0.41	0.39
Other	NM_053936.1	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	Edg2	0.47	0.31	0.31

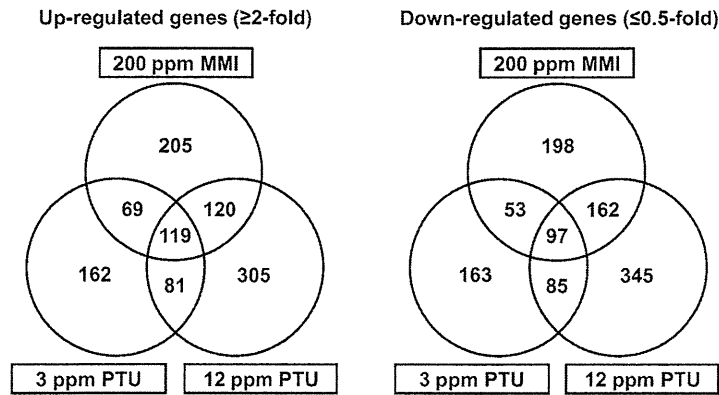


Fig. 2. Venn diagram of gene populations showing altered expression in the hippocampal CA1 pyramidal cell layer at postnatal day 20 in response to maternal exposure to propylthiouracil and/or 2-mercapto-1-methylimidazole compared with the untreated controls. (Left) Up-regulated genes (≥ 2 -fold). (Right) Down-regulated genes (≤ 0.5 -fold). Abbreviations: MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil.

Table 2. Validation of microarray data by real-time RT-PCR

Gene	200 ppm MMI			3 ppm PTU			12 ppm PTU		
	Microarray	Real-time RT-PCR normalized to		Microarray	Real-time RT-PCR normalized to		Microarray	Real-time RT-PCR normalized to	
		Hprt ^{a)}	Gapdh ^{b)}		Hprt	Gapdh		Hprt	Gapdh
Tacr3 ^{c)}	7.32 ± 2.21**	4.29 ± 1.27	4.08 ± 1.15*	6.19 ± 2.19**	3.46 ± 1.42	3.76 ± 1.51*	12.49 ± 1.56**	9.23 ± 3.00**	8.81 ± 1.60**
Calb1 ^{d)}	4.48 ± 0.66*	3.96 ± 0.74	3.67 ± 0.16	4.85 ± 2.53*	4.74 ± 2.48	4.93 ± 3.79	9.00 ± 1.85**	11.13 ± 2.13**	10.53 ± 3.26**
Slit2 ^{e)}	3.04 ± 0.79	2.83 ± 0.90	4.08 ± 1.15*	2.62 ± 1.16	1.33 ± 0.67	3.67 ± 1.51*	7.08 ± 2.15**	4.72 ± 2.57**	8.81 ± 1.60**
Plg11 ^{f)}	3.10 ± 1.57	12.67 ± 5.00	11.5 ± 7.50	4.28 ± 2.88	18.33 ± 6.00	19.00 ± 9.00*	6.86 ± 2.85**	30.67 ± 5.33**	27.00 ± 8.00**
Mobp ^{g)}	0.35 ± 0.15**	0.6 ± 0.22*	0.52 ± 0.16**	0.18 ± 0.07**	0.24 ± 0.07**	0.24 ± 0.05**	0.12 ± 0.02**	0.18 ± 0.04**	0.16 ± 0.04**
Edg8 ^{h)}	0.40 ± 0.11*	0.49 ± 0.16*	0.43 ± 0.13*	0.06 ± 0.05**	0.29 ± 0.10**	0.28 ± 0.08**	0.08 ± 0.07**	0.21 ± 0.07**	0.18 ± 0.03**
Cebpb ⁱ⁾	0.31 ± 0.06**	0.43 ± 0.04**	0.38 ± 0.06**	0.43 ± 0.18**	0.77 ± 0.07	0.76 ± 0.10	0.26 ± 0.04**	0.39 ± 0.16**	0.35 ± 0.22**

a) Hprt, Hypoxanthine-guanine phosphoribosyltransferase; b) Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; c) Tacr3, Tachykinin receptor 3; d) Calb1, Calbindin 1; e) Slit2, Slit homolog 2 (Drosophila); f) Plg11, Pleomorphic adenoma gene-like 1; g) Mobp, Myelin-associated oligodendrocytic basic protein; h) Edg8, Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8; i) Cebpb, CCAAT/enhancer binding protein (C/EBP), beta.

Values are mean ± SD (n=4) relative to the expression level in the untreated controls. Real-time RT-PCR analysis of Hprt and Gapdh was performed in the analysis of each target gene.

*, **. Significantly different from the untreated controls at $P < 0.05$ and $P < 0.01$, respectively (Dunnett's multiple comparison test).

tion in response to 12 ppm PTU was approximately 2-fold higher than that with 3 ppm PTU. The number of genes showing up- or down-regulation in response to 200 ppm MMI was in between that elicited by 3 or 12 ppm PTU. One-hundred nineteen genes were up-regulated in common by MMI and PTU, with PTU showing up-regulation from 3 ppm. On the other hand, 97 genes showed down-regulation in all MMI and PTU groups. Representative genes showing up- or down-regulation in all three groups are shown in the Table 1. Among the genes listed, genes associated with nervous system development, zinc ion binding, apoptosis and cell adhesion were commonly up- or down-regulated. Genes related to calcium ion binding were found to be up-regulated and those for myelination were often down-regulated.

Real-time RT-PCR analysis: For confirmation of the microarray data, four genes that were up-regulated and three that were down-regulated in response to anti-thyroid agents were selected for mRNA expression analysis by real-time RT-PCR and the results are summarized in Table 2.

In all exposure groups, many of the expression changes were similar in the two analysis systems, except for much higher expression of *Plag11* in all exposure groups by real-time RT-PCR as compared with findings from the microarray system.

Although we performed expression analysis of *EfnA5* by real-time RT-PCR, expression values were rather low with great variability between samples, and therefore, reliable quantitative data could not be obtained (data not shown).

Immunolocalization of EphA5 and Tacr3 in the hippoc-

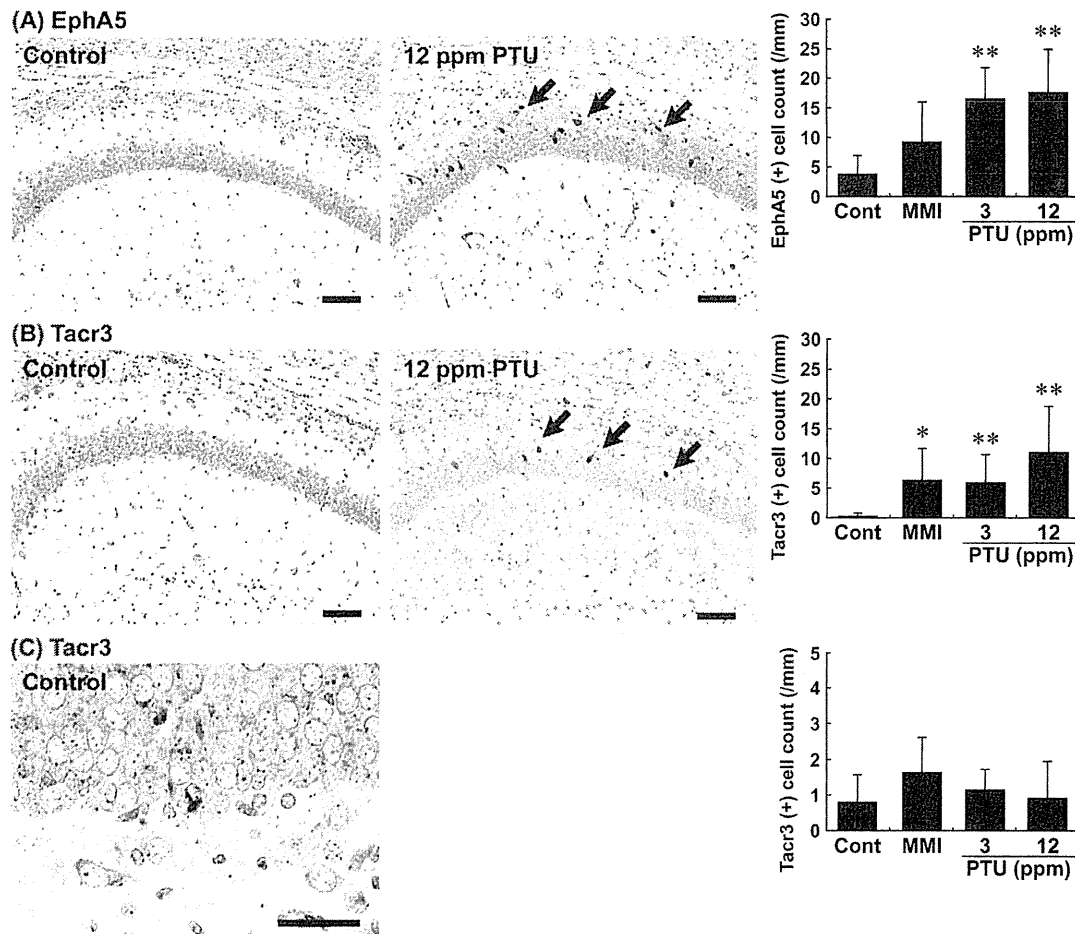


Fig. 3. Distribution of immunoreactive cells for EphA5 and Tacr3 in the hippocampal formation in rats at PND 20 after maternal exposure to anti-thyroid agents. (A) EphA5-immunoreactive cells with strong intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Note the higher number of EphA5-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Bar = 100 μ m. The graph shows the number of EphA5-positive cells/unit length (mm) of the CA1 region of the bilateral hemispheres. ** $P < 0.01$ versus untreated controls (Student's *t*-test). (B) Tacr3-immunoreactive cells with strong intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Note the higher number of Tacr3-positive cells in a case exposed to 12 ppm PTU (Right) as compared with the control animal (Left). Bar = 100 μ m. The graph shows the number of Tacr3-positive cells/unit length (mm) of the CA1 region of bilateral hemispheres. * $P < 0.05$, ** $P < 0.01$ versus untreated controls (Student's *t*-test). (C) Tacr3-immunoreactive cells located in the subgranular zone of the dentate gyrus. Bar = 50 μ m. The graph shows the number of Tacr3-positive cells/unit length (mm) of the subgranular zone of bilateral hemispheres. Abbreviations: EphA5, Ephrin type A receptor 5; MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil; Tacr3, Tachykinin receptor 3.

ampal formation: Immunohistochemical localization of EphA5 and Tacr3 in the hippocampal formation was examined at PND 20 and PNW 11.

On PND 20, EphA5 showed weak immunoreactivity in the pyramidal neurons throughout the hippocampal formation in the untreated controls. This immunoreactivity was unchanged by exposure to anti-thyroid agents. On the other hand, very sparse distribution of strongly immunoreactive cells for EphA5 was observed in the region of the CA1 pyramidal cell layer and stratum oriens in the untreated control

animals, but immunoreactive cells were significantly increased showing scattered distribution by PTU at both 3 and 12 ppm (Fig. 3A). MMI-exposed animals also showed a small increase in the number of strongly positive cells with EphA5. Increased intensity in immunoreactivity of EphA5 was also observed in the gray matter consisting of neuropil at the stratum oriens of the CA1 region (Fig. 3A), and also in the molecular layer of the dentate gyrus at PND 20 after exposure to anti-thyroid agents, especially in PTU-exposed groups (data not shown).

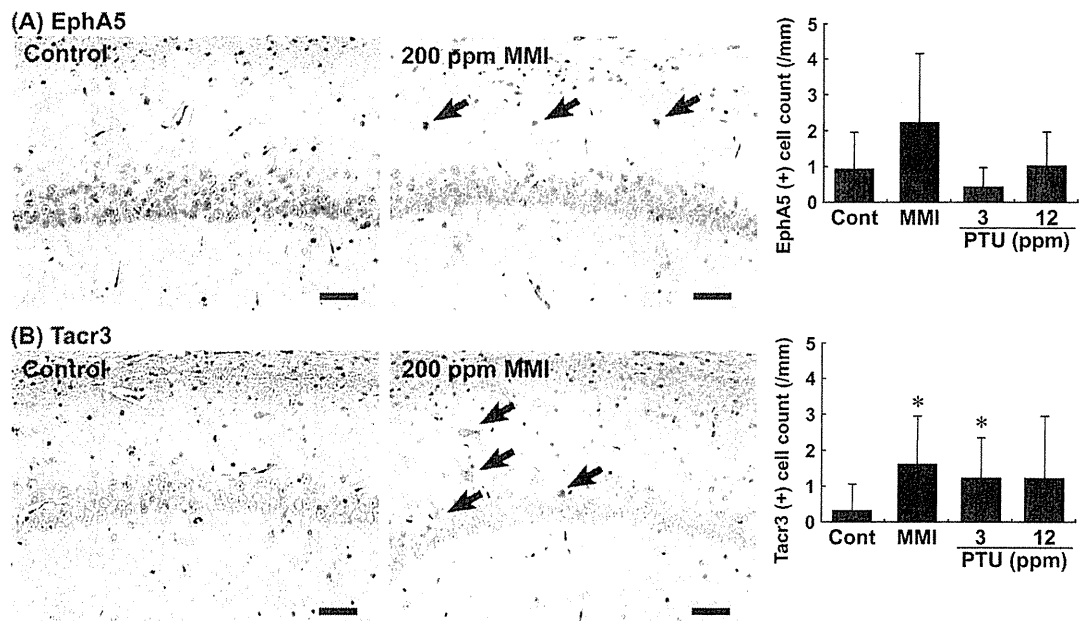


Fig. 4. Distribution of immunoreactive cells for EphA5 and Tacr3 in the hippocampal formation at PNW 11 of rats exposed maternally to anti-thyroid agents. (A) EphA5-immunoreactive cells with moderate staining intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region. EphA5-positive cells in a case exposed to 200 ppm MMI (Right) as compared with the control animal (Left). The arrows show positive cells. Bar = 50 μ m. The graph shows the number of EphA5-positive cells/unit length (mm) of the CA1 region of the bilateral hemispheres. (B) Tacr3-immunoreactive cells with weak to moderate staining intensity located within the pyramidal cell layer and stratum oriens of the hippocampal CA1 region (arrows). Immunoreactivity is rather faint as compared with that observed at PND 20. Note the higher number of Tacr3-positive cells in a case exposed to 200 ppm MMI (Right) as compared with the control animal (Left). Bar = 50 μ m. The graph shows the number of Tacr3-positive cells/unit length (mm) of the CA1 region of bilateral hemispheres. * $P < 0.05$ versus untreated controls (Student's *t*-test). Abbreviations: EphA5, Ephrin type A receptor 5; MMI, 2-mercapto-1-methylimidazole; PTU, 6-propyl-2-thiouracil; Tacr3, Tachykinin receptor 3.

With regards to Tacr3, the number of positive cells was increased with a scattered distribution showing strong intensity in the CA1 region similarly to that of EphA5 in the animals exposed to MMI or PTU on PND 20, but they were mostly absent in the untreated controls (Fig. 3B). Similarly, Tacr3-immunoreactive cells were sparse in the subgranular zone of the dentate gyrus in the MMI and PTU-exposed animals and in the untreated controls, but there were no differences in the number of positive cells as compared with the untreated controls (Fig. 3C). In addition, increased intensity in neuropil-immunoreactivity of Tacr3 was also observed in the strata oriens and radiatum of the CA1 region in all exposure groups of anti-thyroid agents (Fig. 3B).

On PNW 11, EphA5 showed weak immunoreactivity in the pyramidal neurons throughout the hippocampal formation in the untreated controls. This immunoreactivity was unchanged by exposure to anti-thyroid agents. EphA5-immunoreactive cells with moderate staining intensity were very sparsely observed in the region of the CA1 pyramidal cell layer and stratum oriens in the untreated control animals. There was no statistically significant increase in the

number of these immunoreactive cells after exposure to PTU, while animals exposed to MMI showed a tendency for an increased number of immunoreactive cells (Fig. 4A). Increased neuropil-immunoreactivity of EphA5 as observed at PND 20 in exposure groups of anti-thyroid agents was mostly disappeared at PNW 11 (data not shown).

As well as at PND 20, Tacr3-immunoreactive cells were mostly absent in the untreated controls at PNW 11; however, a few immunoreactive cells with weak to moderate intensity were observed in the stratum oriens of the CA1 region in the animals exposed to anti-thyroid agents. There was a statistically significant difference in the animals treated with MMI or 3 ppm PTU compared with the untreated controls (Fig. 4B). Although the change was non-significant and lacked dose-dependence, 12 ppm PTU also showed an increasing tendency in the number of Tacr3-immunoreactive cells. In addition, increased neuropil-immunoreactivity of Tacr3 as observed at PND 20 in exposure groups of anti-thyroid agents was mostly disappeared at PNW 11 (data not shown).