**Table 5**
Histopathologic findings for male and female offspring exposed to anti-thyroid agents during the period from the mid-gestation to the end of lactation and observed at PND 20 and PNW 11.

| | Control | PTU (ppm) | | MMI (ppm) |
|---|---|---|---|---|
| | | 3 | 12 | 200 |
| PND 20 | | | | |
| Males (No. of offspring examined) | 10 | 10 | 10 | 10 |
| *Thyroid* | | | | |
| Diffuse follicular cell hypertrophy (+/++/+++)[a] | 0 | 10**(0/6/4)## | 10**(1/3/6)## | 10**(2/4/4)## |
| *Kidneys* | | | | |
| Tubular mineralization (±/+/++/+++) | 1(0/1/0/0) | 3(2/0/1/0) | 10**(2/3/3/2)## | 9**(1/2/4/2)## |
| *Liver* | | | | |
| Extramedullary hematopoiesis (±/+) | 10(0/10) | 10(2/8) | 10(10/0)## | 10(10/0)## |
| *Pituitary, anterior lobe* | | | | |
| Depletion of cytoplasmic granules | 0 | 8** | 10** | 10** |
| *Mammary gland* | | | | |
| Secretion, alveolar buds (+/++) | 0 | 7**(6/1)#[b] | 10**(7/3)## | 8**(7/1)## |
| *Testis* | | | | |
| Delayed spermatogenesis (±/+/++) | 0 | 9**(4/4/1)## | 10**(0/2/8)## | 10**(0/6/4)## |
| Apoptotic spermatocytes (±/+) | 0 | 8**(8/0)## | 10**(9/1)## | 10**(8/2)## |
| Females (No. of offspring examined) | 10 | 10 | 10 | 10 |
| *Thyroid* | | | | |
| Diffuse follicular cell hypertrophy (+/++/+++)[a] | 0 | 10**(2/4/4)## | 9**(1/2/6)##[b] | 10**(0/3/7)## |
| *Kidneys* | | | | |
| Tubular mineralization (±/+/++/+++) | 0 | 3(3/0/0/0) | 8**(4/1/2/1)## | 6**(2/1/3/0)## |
| *Liver* | | | | |
| Extramedullary hematopoiesis (±/+) | 10(2/8) | 10(4/6) | 7(7/0)## | 10(10/0)## |
| *Pituitary, anterior lobe* | | | | |
| Depletion of cytoplasmic granules | 0 | 4* | 10* | 10** |
| *Mammary gland* | | | | |
| Hypoplasia, alveolar buds (+/++/+++) | 0 | 10**(1/4/5)## | 10**(0/8/2)## | 10**(0/9/1)## |
| PNW 11 | | | | |
| Males (No. of animals examined) | 10 | 10 | 6 | 10 |
| *Thyroid* | | | | |
| Diffuse follicular cell hypertrophy (±) | 4 | 3 | 1 | 2 |
| *Kidneys* | | | | |
| Tubular mineralization (±/+/++/+++) | 0 | 4*(3/1/0/0)# | 5**(2/2/1/0)## | 7**(2/0/4/1)## |
| Females (No. of animals examined) | 10 | 10 | 4 | 10 |
| *Thyroid* | | | | |
| Diffuse follicular cell hypertrophy (±) | 0 | 1 | 0 | 0 |
| *Kidneys* | | | | |
| Tubular mineralization (±/+/++/+++) | 1(1/0/0/0) | 2(0/2/0/0) | 4**(2/2/0/0)## | 6*(1/2/3/0)# |

*Abbreviations*: MMI methimazole; PND postnatal day; PNW postnatal week; PTU propylthiouracil.
[a] Grade of change: (±), minimal; (+), slight; (++), moderate; (+++), marked.
[b] Thyroid tissue was missing in the histological preparation in one animal in this group.
* 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 ($P < 0.01$).
\# 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 ($P < 0.01$).

changes observed at the end of chemical exposure, except for the thyroid hormone deficiency-related thyroidal changes, were considered to be a reflection of growth retardation and were reversed at the adult stage.

In males of the present study, a delayed onset of puberty was observed with 12 ppm PTU or MMI. A tendency for puberty delay was also seen at 3 ppm PTU. Postnatal hypothyroidism causes retarded spermatogenesis resulting in a reduced testicular weight [28–30]. In the present study, offspring exposed to 12 ppm PTU or MMI showed reductions in testicular weight associated with histopathologically apparent retarded spermatogenesis as well as severely retarded body growth at PND 20. This testicular retardation may be responsible for the delayed onset of puberty involving reduced testosterone levels as in the retarded cases with intrauterine malnutrition or postnatal undernutrition [31]. It is well known that a decrease in testosterone level can delay onset of puberty [32]. Suppressed body growth may also affect the puberty onset due to insufficiency of substances necessary for release of gonadotropin-releasing hormone [33]. A significant testicular enlargement observed at PNW 11 in all exposure groups is considered to be a lasting effect to enlarge ultimate testicular size after cessation of the exposure as previously reported [34,35]. Thyroid hormone receptors (TR)-α1, associated with $T_3$ binding receptors, are detected at high levels in the fetal and early neonatal stages, and then decrease significantly throughout the prepubertal period to be non-existent in the adult rat testis [36]. This pattern of TRα1 expression coincides with the proliferative stage of Sertoli cell development. Transient hypothyroidism during the lactation period decreases $T_3$ and delays the cessation of Sertoli cell proliferation [36]. Allowing for the recovery to euthyroidism at weaning, the resumption of thyroid hormone stimulates Sertoli cells to mature and increases testis size [36]. Larger testicular weight of rats receiving 12 ppm PTU compared with rats receiving MMI at PNW 11 may reflect the lower $T_3$ levels during development in the former cases.

In females, the delayed onset of puberty was observed only with 12 ppm PTU, while retarded body growth was observed equally in both 12 ppm PTU and MMI groups at PND 20. Although a delayed onset of puberty has been reported in females by intrauterine malnutrition [31], female offspring of all exposure groups did not display significantly decreased body weight at PND 1. On the other

**Table 6**
Brain histopathology and morphometry of the male offspring exposed to anti-thyroid agents during the period from the mid-gestation to the end of lactation and examined at PND 20 and PNW 11.

| | Control | Anti-thyroid agent in the drinking water | | |
|---|---|---|---|---|
| | | 3 ppm PTU | 12 ppm PTU | 200 ppm MMI |
| PND 20 | | | | |
| No. of offspring examined | 5 | 5 | 5 | 5 |
| CC | | | | |
| Subcortical band heterotopia (+) | 0 | 0 | 0 | 2 |
| PNW 11 | | | | |
| No. of offspring examined | 10 | 10 | 6 | 10 |
| Hippocampal CA1 neurons[a] | | | | |
| Mean distance of the location of neurons from the innermost margin of the pyramidal cell layer (μm) | 29.2 ± 2.6[b] | 49.7 ± 11.1* | 65.2 ± 13.0** | 61.0 ± 13.2** |
| No. of neurons located lateral to the pyramidal cell layer (/mm) | 11.0 ± 3.9 | 82.7 ± 19.5* | 110.8 ± 25.7** | 86.8 ± 32.8** |
| Ratio of abnormally distributed neurons/ CA1 neurons (%) | 4.6 ± 1.4 | 29.9 ± 7.6** | 37.0 ± 11.1** | 31.1 ± 11.1** |
| CC | | | | |
| Subcortical band heterotopia (+) | 0 | 0 | 3* | 9** |
| Area of CC ($mm^2$) | 0.14 ± 0.01 | 0.11 ± 0.02* | 0.08 ± 0.01** | 0.09 ± 0.02* |
| Cingulate deep cortex | | | | |
| CNPase (+) cell count (count/$mm^2$) | 144.0 ± 22.1 | 115.3 ± 17.5 | 92.6 ± 9.5** | 87.6 ± 6.8** |

*Abbreviations*: CC corpus callosum; CNPase 2′,3′-cyclic nucleotide 3′-phosphodiesterase; MMI methimazole; PND postnatal day; PNW postnatal week; PTU propylthiouracil.
[a] NeuN (+) neurons were subjected to analysis.
[b] Mean ± SD.
* 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 ($P < 0.01$).

hand, ovary weight was also suppressed at PND 20 in these cases similar to the prepubertal hypothyroid rat model [37]. The severity of ovary underdevelopment was higher after exposure to 12 ppm PTU compared with MMI. During ovarian development, thyroid hormone stimulates growth of ovarian follicles [38], and ovarian steroids potentiate the neuroendocrine mechanisms regulating the onset of puberty [39]. This pathway suggests that the difference in ovarian growth may be the reason for the different responses between 12 ppm PTU and MMI at the onset of puberty. Although the data for serum concentrations of thyroid-related hormones were those in male offspring, lower $T_3$ levels in offspring exposed to 12 ppm PTU compared with MMI might be responsible for the lower ovarian weight. In addition, no changes in AGD, estrus cycles or the histopathology of reproductive organs by PTU or MMI suggest there was no apparent effect on sexual differentiation involving the endocrine center, the hypothalamus. As the magnitude of the effect was milder in ovaries than in testes, a tendency for ovarian enlargement was observed in exposure groups.

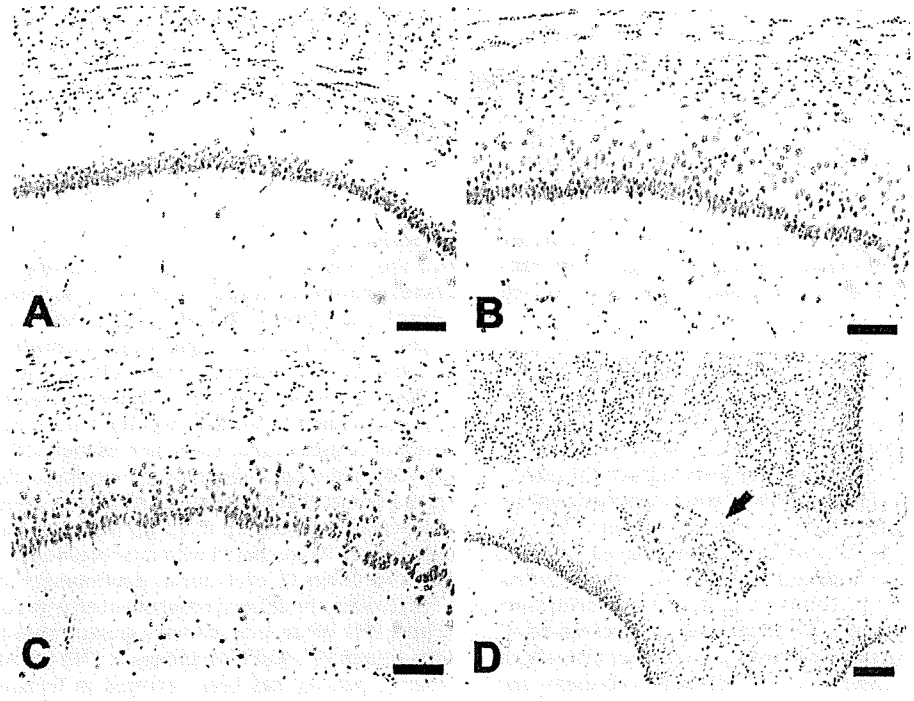


**Fig. 4.** Neuronal cell distribution within and lateral to the pyramidal cell layer of the hippocampal CA1 region (A–C) and subcortical band heterotopia (arrow) appeared within the corpus callosum (D) at PNW 11 stained with NeuN-immunohistochemistry. (A) Untreated control; (B) 12 ppm PTU; (C, D) 200 ppm MMI. (A–C) Bar: 100 μm; (D) Bar: 200 μm.

Brain regions most sensitive to perinatal hypothyroidism include the olfactory bulb, cerebral cortex, cerebellum and the hippocampal formation. In the hippocampus, perinatal hypothyroidism inhibits migration of dentate granule cells, decreases cell number, and reduces the dendritic arborization of granule and pyramidal cells [40–46]. In the present study, neuronal cell distribution as represented by hippocampal CA1 pyramidal cells was examined in terms of the mean cellular distance, and the number and ratio of abnormally distributed neurons. As a result, all exposure groups showed significant increases in all parameters, with PTU showing clear dose-dependency. Importantly, higher magnitude in the changes was observed with the latter two parameters, suggesting a high sensitivity. Subcortical band heterotopia appears in the corpus callosum by developmental hypothyroidism [9] as evident by neuronal mismigration [47]. The appearance in the present study was less sensitive to lower doses since rats receiving 3 ppm PTU lacked this aberrant structure. This was in contrast to the previous study results showing detection from a dose of 2 ppm PTU [9], although the exposure period was slightly longer than ours beginning on GD 6 and continuing until PND 30.

Thyroid hormones also regulate oligodendrocyte accumulation in developing rat brain white matter tracts, and a deficiency results in hypoplasia of the tracts [8]. In the present study, the area of the corpus callosum and the number of CNPase-positive oligodendrocytes distributed in the area of deep cortex of the cerebrum were reduced in all exposure groups. Furthermore, these changes were dose-dependent after PTU exposure, while changes in CNPase-positive cell count at 3 ppm did not attain significant difference. In another study that examined the developmental exposure effects of decabrominated diphenyl ether, a representative flame retardant, a dose-dependent reduction was observed in both oligodendroglial parameters after exposure to a dose of 100 ppm in diet (Hitoshi Fujimoto et al., manuscript in preparation).

In conclusion, a delay in the onset of puberty and adult stage gonadal enlargement appeared after maternal exposure to antithyroid agents during the period from the mid-gestation to the end of lactation were considered to result from systemic growth retardation lasting into the adult stage especially in males. The quantitative detection system of brain retardation applied here could be a useful tool to evaluate the potency of chemicals to induce hypothyroidism-related brain retardation. Furthermore, since endpoints selected for detection of brain retardation, i.e., neuronal migration and oligodendroglial growth, are general for brain development, morphometric methods applied here may also be useful for detection of basic impact of developmental neurotoxicity of other types of neurotoxicants.

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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 ($T_3$) concentration at postnatal day 20, and there was no evidence of hypothyroidism-related neuronal mismigration 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 (CNPase)-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 $T_3$ concentrations in offspring at 10,000 ppm at weaning. Increased thyroid weights and decreased serum $T_3$ concentrations were also observed in the adult stage from 1000 ppm. With regard to the effect on brain development, HBCD reduced density of CNPase-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.


## 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 mismigration 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 ± 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 (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 μm, 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 mm from

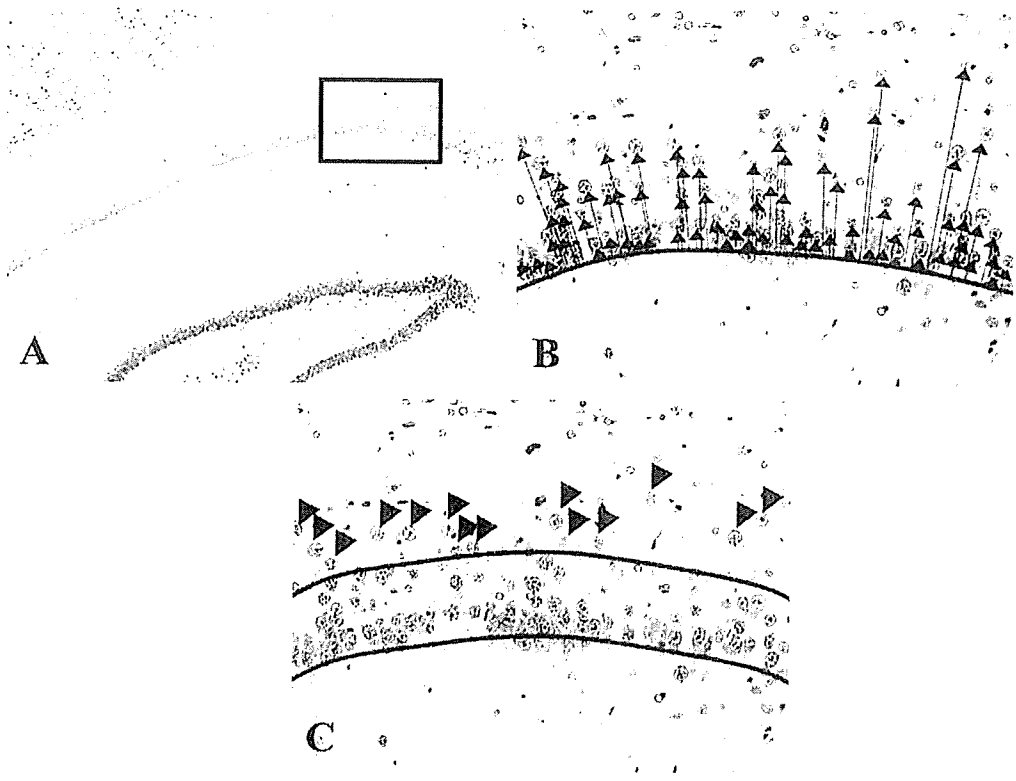


**Fig. 1.** Quantitative measurement of the variability in the distribution of neurons located within and lateral to the pyramidal cell layer of the hippocampal CA1 region at PNW 11. (A) Hippocampal CA1 region stained with NeuN-immunohistochemistry at 0.9 mm lateral to the boundary with the subiculum, 40× 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× 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× magnification.

the bregma were serially sectioned at 3 μm. Immunohistochemistry was performed according to the method described previously with 3,3′-diaminobenzidine/$H_2O_2$ 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.

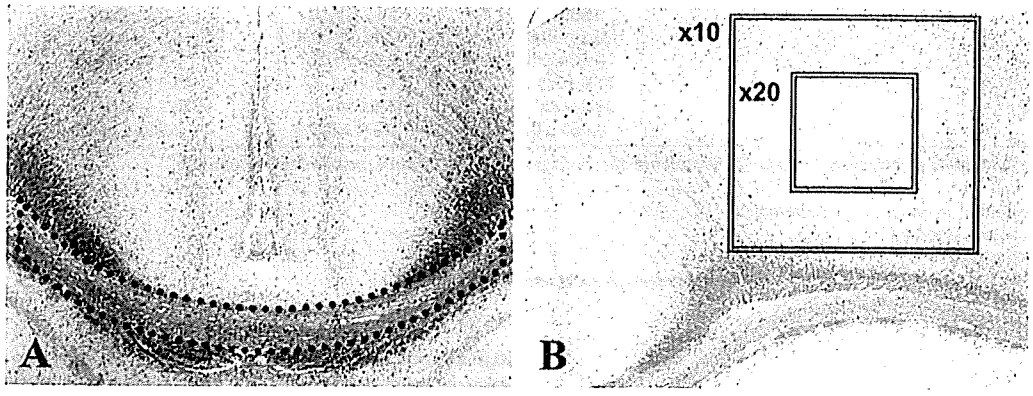


**Fig. 2.** Quantitative measurement of the effect on the oligodendroglial development at PNW 11. (A) Size measurement of the white matter area immunoreactive for CNPase. The area of the corpus callosum medial to the cerebral white matter at the uppermost position of the cingulum was measured, 40× magnification. (B) Number of CNPase-positive oligodendrocytes surrounding myelinated axons distributed at the layer V of the parietal isocortex dorsolateral to the cingulum under 200× magnification. At first, the lower and innermost ends of the view area with a 10× 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×, 40× 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) | 21.8 ± 0.5 | 21.8 ± 0.5 | 21.6 ± 0.5 | 21.5 ± 0.5 |
| Day 20 after delivery | | | | |
| BW (g) | 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* |
| 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* |
| 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* | 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* |
| 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.

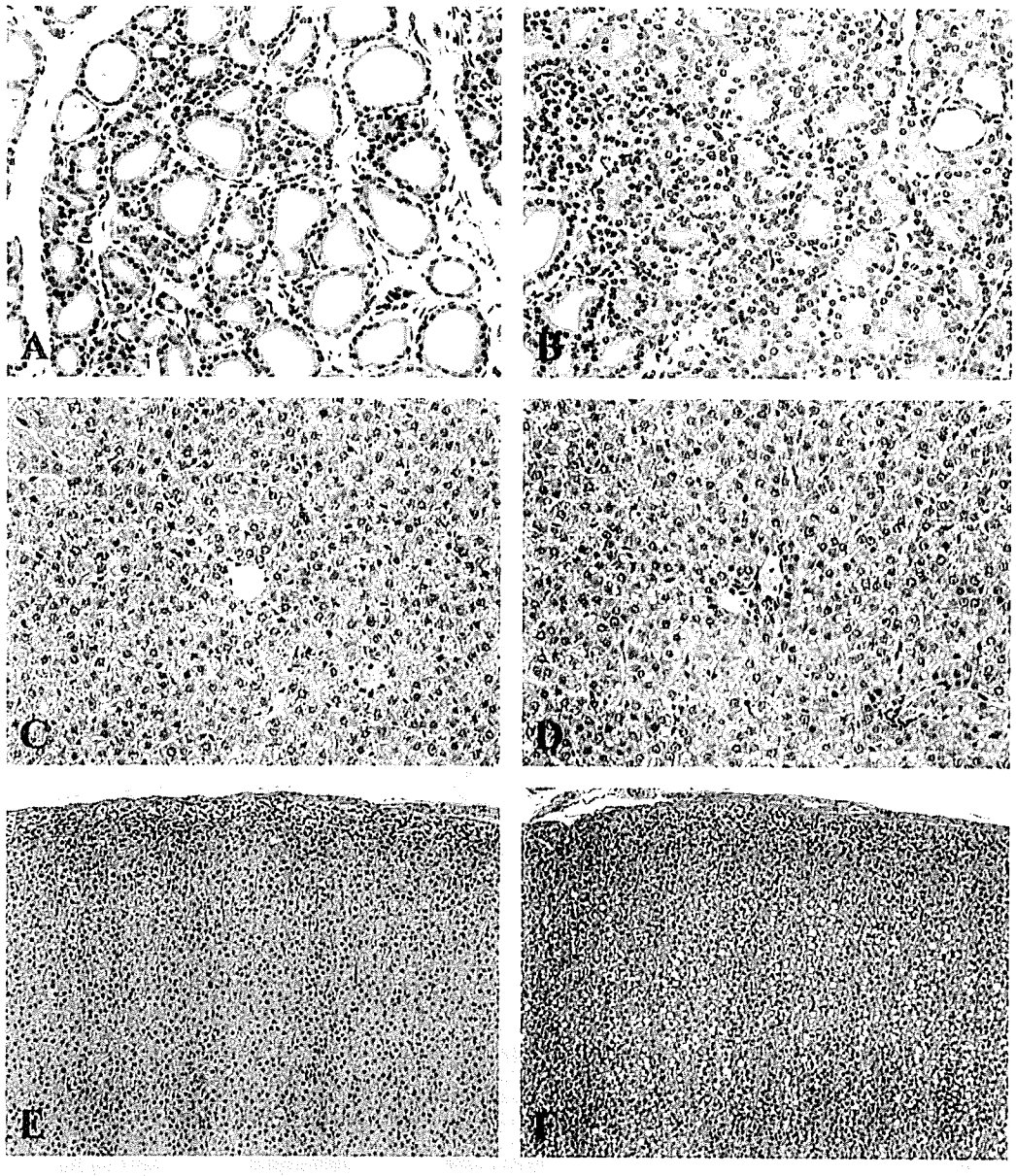


**Fig. 3.** Histopathological changes observed in dams and offspring in the HBCD study. (A and B) Thyroid gland of dams given HBCD at 0 (control) (A) or 10,000 ppm (B). As compared with the 0 ppm dose, diffuse follicular cell hypertrophy (moderate degree) is evident with 10,000 ppm HBCD, 200× magnification. (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× 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× 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)** | | | |
| | 0 | 100 | 1000 | 10,000 |
| 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; $T_3$, triiodothyronine; $T_4$, 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 $T_3$ and $T_4$ 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 $T_3$ 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 $T_4$ 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* |
| Females | | | | |
| No. of animals examined | 10 | 10 | 10 | 10 |
| Liver | | | | |
| Vacuolar degeneration, liver cells, diffuse (+/++) | 0 | 0 | 0 | 6[b,*] (0/6)[c,§§] |
| PNW 11 | | | | |
| Males | | | | |
| No. of animals examined | 10 | 10 | 10 | 10 |
| Adrenal | | | | |
| Vacuolar degeneration, diffuse, cortical cells (+/++) | 0 | 0 | 0 | 4* (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 ± 3.0[a] | 29.8 ± 2.0 | 31.5 ± 1.8 | 29.2 ± 1.6 |
| No. of neurons located lateral to the pyramidal cell layer (per mm) | 5.1 ± 1.4 | 4.7 ± 1.5 | 4.0 ± 1.6 | 4.6 ± 1.7 |
| Ratio of abnormally distributed neurons/CA1 neurons (%) | 2.3 ± 0.6 | 1.9 ± 0.5 | 1.7 ± 0.6 | 1.9 ± 0.7 |
| CC | | | | |
| Area of CC ($mm^2$) | 0.14 ± 0.01 | 0.14 ± 0.01 | 0.14 ± 0.02 | 0.14 ± 0.01 |
| Cingulate deep cortex | | | | |
| CNPase (+) cell count (count/$mm^2$) | 133.0 ± 16.7 | 120.8 ± 14.3 | 121.4 ± 15.4 | 127.1 ± 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 ± 11.8 | 49.3 ± 9.1 | 47.2 ± 11.9 | 48.2 ± 2.3 |
| No. of neurons located lateral to the pyramidal cell layer (per mm) | 4.5 ± 1.3 | 4.9 ± 0.9 | 3.3 ± 1.1 | 3.5 ± 1.1 |
| Ratio of abnormally distributed neurons/CA1 neurons (%) | 2.9 ± 1.0 | 3.1 ± 0.6 | 2.3 ± 0.9 | 2.3 ± 0.7 |
| CC | | | | |
| Area of CC ($mm^2$) | 0.15 ± 0.02 | 0.15 ± 0.02 | 0.14 ± 0.01 | 0.14 ± 0.01 |
| Cingulate deep cortex | | | | |
| CNPase (+) cell count (count/$mm^2$) | 181.6 ± 28.2 | 167.6 ± 23.2 | 160.3 ± 28.1 | 138.7 ± 23.7* |

*Abbreviations*: CC, corpus callosum; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; 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 ($P < 0.05$).

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 $\alpha 1$, 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 antithyroid 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 $\geq$100 mg/kg-d resulted in a dose-dependent increase in the liver weight. All studies showed effects on the thyroid-related parameters. Among them, a 90-d study exhibited relative liver weight increases (18–24%) by HBCD from 100 mg/kg-d. Disturbances in the thyroid hormone system ($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.

## Acknowledgments

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FULL PAPER *Pathology*

# 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 ± 1°C; relative humidity: 55 ± 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/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-$\mu$m-thick sections between ten 20 $\mu$m-thick serial sections were prepared. The 4 $\mu$m-thick sections were stained with hematoxylin and eosin for confirmation of anatomical orientation of the hippocampal substructure to aid microdissection. The 20 $\mu$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-m*l* 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

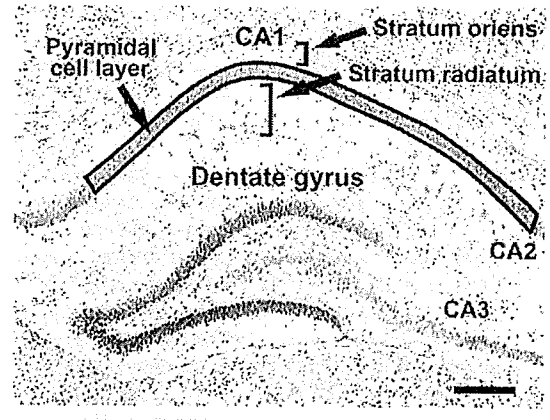


Fig. 1. Overview of the hippocampal formation of a male rat at postnatal day 20 stained with hematoxylin and eosin. Bar=200 $\mu$m. The CA1 pyramidal cell layer, enclosed by a solid line, was microdissected for the microarray and subsequent real-time RT-PCR analyses. The number of cells immunoreactive 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/$H_2O_2$ 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 |
|---|---|---|---|---|---|---|
| *Up-regulated genes (of 119 genes in total)* | | | | | | |
| 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 | Adamtsl1 | 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 | Pvrl2 | 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 | FXYD 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 |
| *Down-regulated genes (of 97 genes in total)* | | | | | | |
| 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 | Srprb 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 | Ermn | 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 |