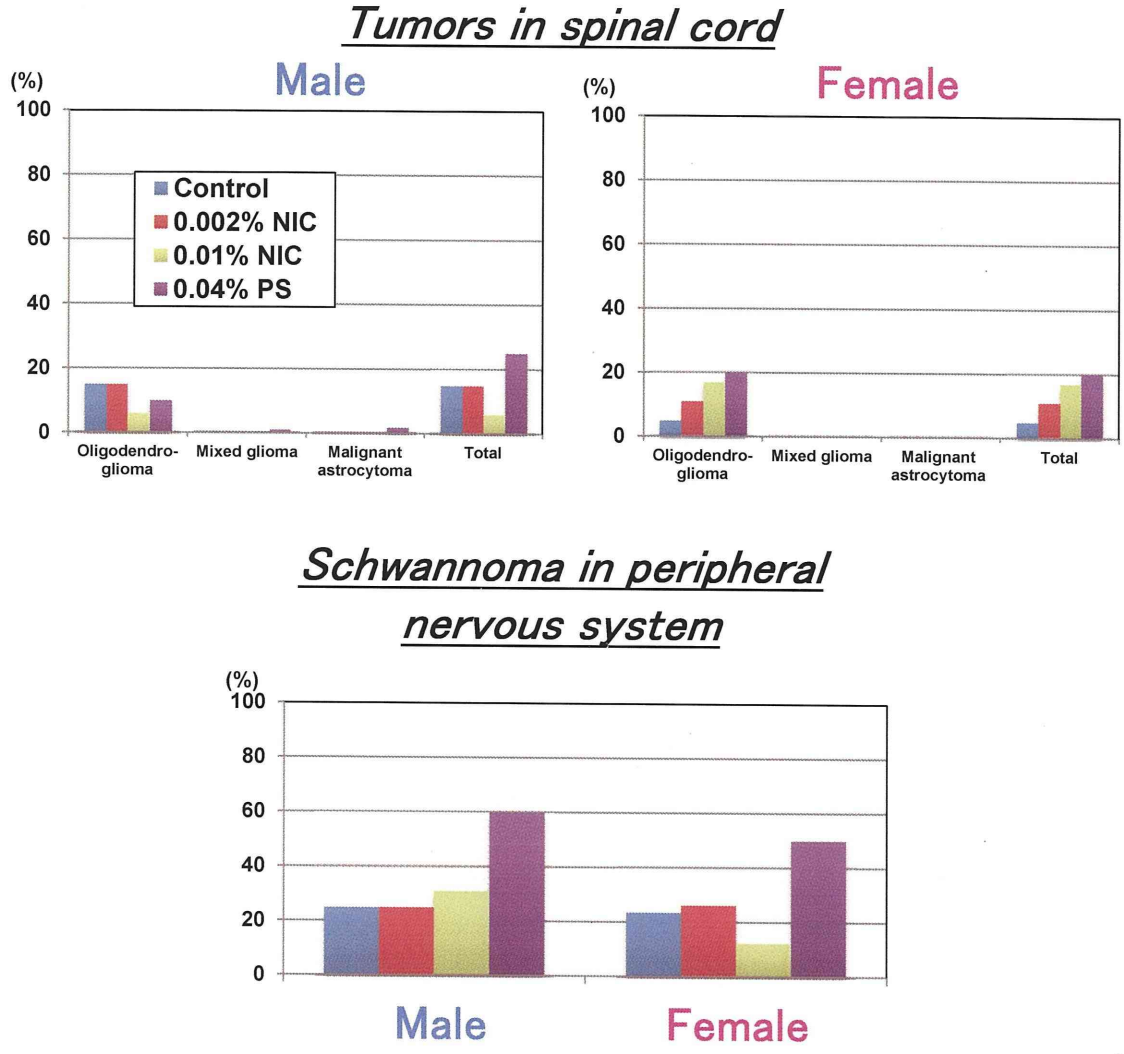

Fig. 76

Incidence of neural tumors in offspring treated with nicotine or propane sultone (Spinal cord and peripheral nervous system)

Table 62
Food consumption and manganese intake of dams

Group	No. of dams	Food consumption (g/rat/day)	Manganese intake (mg/kg b.w./day)
0% $MnCl_2$	5	29.9	0
0.002% $MnCl_2$	6	29.0	0.8
0.01% $MnCl_2$	6	29.7	4.2
0.05% $MnCl_2$	6	28.2	19.6

Table 63
Food consumption and manganese intake in offspring

Group	No. of offspring	Food consumption (g/rat/day)	Manganese intake (mg/kg b.w./day)
Male			
Control	21	14.3	0
0.002% $MnCl_2$	21	13.9	0.3
0.01% $MnCl_2$	20	13.9	1.6
0.05% $MnCl_2$	18	14.0	7.8
Female			
Control	19	9.3	0
0.002% $MnCl_2$	24	9.1	0.3
0.01% $MnCl_2$	26	9.4	1.7
0.05% $MnCl_2$	24	9.2	8.7

Each value represents the mean throughout the experiment period.

Table 64
Neuronal tumor incidences in offspring treated with manganese

Sex	Treatment	No. of animals	Brain	Spinal cord	CNS	PNS	CNS + PNS
Male	Control	21	13 (62)	4 (19)	13 (62)	6 (29)	17 (81)
	0.002% $MnCl_2$	21	12 (57)	4 (19)	14 (67)	11 (52)	18 (86)
	0.01% $MnCl_2$	20	11 (55)	2 (10)	11 (55)	8 (40)	15 (75)
	0.05% $MnCl_2$	18	12 (67)	3 (17)	12 (67)	7 (39)	15 (83)
Female	Control	19	12 (63)	4 (21)	14 (74)	8 (38)	18 (95)
	0.002% $MnCl_2$	24	18 (75)	4 (17)	19 (79)	10 (48)	20 (83)
	0.01% $MnCl_2$	26	16 (62)	3 (12)	17 (65)	8 (40)	20 (77)
	0.05% $MnCl_2$	24	13 (54)	3 (13)	15 (63)	4 (22)	18 (75)

CNS, Central nervous system

PNS, Peripheral nervous system

Table 65
Non-neuronal tumor incidences in offspring treated with manganese

Sex	Treatment	No. of animals	Lung		Kidney	
			Ad	Ad	Ad	Nb
Male	Control	21	9 (43)	4 (19)	0	0
	0.002% $MnCl_2$	21	12 (57)	4 (19)	0	1 (5)
	0.01% $MnCl_2$	20	12 (60)	7 (35)	0	0
	0.05% $MnCl_2$	18	13 (72)	10 (56)	0	0
Female	Control	19	2 (11)	0	1 (5)	0
	0.002% $MnCl_2$	24	6 (25)	1 (4)	0	0
	0.01% $MnCl_2$	26	2 (8)	0	1 (4)	0
	0.05% $MnCl_2$	24	3 (13)	0	0	1 (6)

Ad, Adenoma

Ac, Adenocarcinoma

Nb, Nephroblastoma

Table 66
Water consumption and test chemical intakes of dams treated with nicotine or propane sultone

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

Table 67 Final body and organ weights of dams

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

Each value represents the mean±S.D.

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

Table 68
Water consumption and test chemicals intake of offspring treated with nicotine or propane sultone

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

Each value represents the mean throughout the experimental period.

Table 69

Organ weights of male offspring treated with nicotine or propane sultone

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

Each value represents the mean±S.D.

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

Table 70

Organ weights of female offspring treated with nicotine or propane sultone

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

Each value represents the mean±S.D.

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

Table 71
Neural tumor incidences treated with nicotine or propane sultone

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

Table 72
Non-neural tumor incidences treated with nicotine or propane sultone

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

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

別添４

研究成果の刊行に関する一覧表

書籍

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

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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研究成果の刊行物・別刷

Reproductive Toxicology 28 (2009) 297–307

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Assessment of developmental effects of hypothyroidism in rats from in utero and lactation exposure to anti-thyroid agents

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ARTICLE INFO

Article history:
Received 18 March 2009
Received in revised form 10 April 2009
Accepted 17 April 2009
Available online 3 May 2009

Keywords:
Developmental hypothyroidism
6-Propyl-2-thiouracil
Methimazole
Growth retardation
Neuronal migration
Oligodendroglial development

ABSTRACT

To clarify the developmental effects of hypothyroidism and to establish a detection system of resultant brain retardation, pregnant rats were administered 3 or 12 ppm of 6-propyl-2-thiouracil (PTU) or 200 ppm of methimazole (MMI) in the drinking water from gestation day 10 to postnatal day 20 and maintained after weaning until 11 weeks of age (adult stage). Offspring displayed evidence of growth retardation lasting into the adult stage, which was particularly prominent in males. Except for hypothyroidism-related thyroid follicular cell hypertrophy, most histopathological changes that appeared at the end of chemical exposure were related to growth retardation and reversed by the adult stage. A delayed onset of puberty and an adult stage gonadal enlargement occurred by exposure to anti-thyroid agents, both being especially evident in males, and this effect might be related to gonadal growth suppression during exposure. At the adult stage, the distribution variability of hippocampal CA1 pyramidal neurons reflecting mismigration could be detected in animals receiving both thyrotoxins, with a dose-dependent effect by PTU. Similarly, a reduction in the area of the corpus callosum and oligodendroglial cell numbers in the cerebral deep cortex, both reflecting impaired oligodendroglial development, were detected in rats administered both chemicals. Thus, all effects, except for impaired brain development, might be linked to systemic growth retardation, and the brain morphometric methods employed in this study may be useful to evaluate the potency of chemicals to induce hypothyroidism-related brain retardation.

1. Introduction

Groups of persistent organic pollutants (POPs), such as organochlorine pesticides and polychlorinated biphenyls (PCBs), have been shown to be ubiquitous environmental pollutants because of their great chemical stability and lipid solubility [1]. POPs have been reported to cause a variety of effects including immunologic, teratogenic, reproductive, carcinogenic, and neurological effects [2]. Also, many of these compounds are known to induce hypothyroidism [3].

Developmental hypothyroidism leads to growth retardation, neurological defects and impaired performance on a variety of behavioral learning ability [4–6]. Experimentally, rat offspring exposed maternally to anti-thyroid agents such as 6-propyl-2-thiouracil (PTU) and methimazole (MMI) show brain retardation, resulting in an impairment of neuronal migration as well as white matter hypoplasia involving limited axonal myelination and oligodendrocytic accumulation [7–9]. In humans, subclinical or mild hypothyroidism is common in women and in the elderly and has been associated with an increased incidence of depression by lowering the threshold for the development of major depressive disorders [10] and other mood disorders [11,12]. In addition, mild hypothyroidism has been linked with a diminished response to standard psychiatric treatment and with cognitive dysfunction [11]. These findings indicating that even small changes in the mother's thyroid hormone status early in pregnancy may cause adverse effects on her child and may lead to an increased concern for thyroid hormone disrupting chemicals in the environment. In addition to the effects on brain development, developmental hypothyroidism affects hearing function and the immune system [13,14].

Crosstalk between the estrogen receptors (ERs) and thyroid hormone receptors (TR) by the estrogen response element (ERE) has been reported in previous studies [15,16]. Because of the similarities in the DNA binding domain of ERE and thyroid hormone response element, TR can compete with ER on the ERE and influence transcription from ER target genes [15,16]. Therefore, there

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0890-6238/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2009.04.011

is a possibility that the sexual differentiation of offspring can be affected by developmental hypothyroidism.

The present study was performed to clarify the systemic effect, including sexual differentiation, of developmental hypothyroidism as well as to establish a detection system for resultant brain retardation using rats to screen chemicals that may potentially induce developmental hypothyroidism. To distinguish chemical-specific toxicity from hypothyroidism-linked effects, two different anti-thyroid agents, PTU and MMI, were used, and dose-related responses were also examined with PTU. Both agents are known to exert inhibitory effect on thyroid hormone synthesis by interfering with thyroid peroxidase-mediated iodination of tyrosine residues in thyroglobulin [17].

2. Materials and methods

2.1. Chemicals and animals

The two chemicals, 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). Pregnant Crj:CD®(SD)IGS rats were purchased from Charles River Japan Inc. (Yokohama, Japan) at gestation day (GD) three (the day when vaginal plugs were observed was designated as GD 0). Dams were housed individually in polycarbonate cages (SK-Clean, 41.5 cm × 26 cm × 17.5 cm in size; CLEA Japan, Inc., Tokyo, Japan) with sterilized softwood chips (Sankyo Lab Service Corp., Tokyo, Japan) as bedding in a barrier-sustained animal room conditioned at 24 ± 1 °C and 55 ± 5% humidity, with a 12 h light/dark cycle. A soy-free diet (Oriental Yeast Co. Ltd., Tokyo, Japan) was chosen as the basal diet for dams to eliminate possible effects of phytoestrogens on the evaluation of this study, and water was given *ad libitum* throughout experimental period including the one-week acclimation. On the other hand, all offspring consumed a regular CRF-1 basal diet (Oriental Yeast Co. Ltd.) and water *ad libitum* from postnatal day (PND) 20 onwards (PND 0: the day of delivery). Although the formula is not open, CRF-1 contains soybean/alfalfa-derived proteins and oil including daidzin and genistin at concentrations of 87 and 102 ppm in diet according to the supplier's analysis, and coumestrol of less than 3 ppm based on the content of lucerne meal in the diet (supplier's comment). Soy-free diet was prepared based on the formulation of the NIH-07 open formula rodent diet, in which soybean meal and soy oil were replaced with ground corn, ground wheat, wheat middlings and corn oil. Values for phytoestrogens in this diet were below the detection limit (0.5 ppm), except for coumestrol with 3 ppm. Estrogen equivalents of phytoestrogens included in each CRF-1 and soy-free diet were roughly calculated as 0.91 and 0.06 ppm of β-estradiol, respectively, based on the relative binding affinities in a rat endometrial-derived experimental model [18]. Nutritional standards did not differ between SF diet and CRF-1 (supplier's analysis).

2.2. Experimental design

Dams were randomly divided into four groups including untreated controls. Eight dams per group were treated with PTU at 3 or 12 ppm or MMI at 200 ppm in the drinking water from GD 10 to PND 20. Dose finding study on PTU and MMI was preliminarily performed based on the dose range to show changes in neuronal or oligodendroglial parameters in previous reports [8,19–21]. With the dose setting at the level of 9 or 12 ppm for PTU and 200 or 250 ppm for MMI in the drinking water, dams (n = 2/dose) were treated from GD 10 to PND 20, apart from the untreated control dams (n = 2). As a result, PTU at 12 ppm and MMI at 200 ppm exhibited clear hypothyroidism-linked effects to dams, i.e., increased relative thyroid weights and thyroid follicular cell hypertrophy, but did not affect pregnancy, implantation, delivery, or nursing until PND 20 (data not shown).

In the main study, food consumption and body weight gains of dams were measured throughout the experimental period. On PND 1, the number, weights and anogenital distance (AGD) of neonates were recorded, and on PND 2, litters were culled randomly to adjust to four male and four female offspring. The offspring were weaned on PND 20. Twenty male and twenty 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) [22]. Other remaining males and females were allocated to four rats per cage and further maintained until they were 11 weeks old. The age and body weight at the onset of puberty as determined by vaginal opening for females and preputial separation for males were recorded for the offspring assigned for adult examination. Estrous cycles of females were examined by daily microscopic observation of vaginal smears from postnatal week (PNW) 8 to PNW 11. Classification was divided into proestrus, estrus, and diestrus, depending on whether specimens contained nucleated epithelial cells, cornified epithelial cells, or leukocytes, respectively. When estrus or diestrus continued for at least 3 or 4 days within cycles, 'extended estrus' or 'extended diestrus' was concluded [23,24]. 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 days 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 the necropsies of animals sacrificed on PND 20 and PNW 11, blood samples of male offspring were collected from the abdominal aorta under anesthesia. Serum was prepared and stored at −30 °C to measure thyroid-stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) concentrations at SRL, Inc. (Tokyo, Japan).

2.4. Histopathological assessment

At prepubertal necropsies of animals sacrificed on PND 20, the brain, liver, kidneys, thyroid, pituitary, adrenals, mammary glands, testes, epididymides, other male accessory sex glands (ventral prostate + seminal vesicle + coagulating gland + dorsolateral prostate), ovaries, uterus, and vagina were removed, and weights of the brain, liver, kidneys, adrenals, testes, epididymides, ovaries, and uterus were measured. Removed organs were fixed in 10% buffered formalin (pH 7.4) for three days at room temperature, except for brains and testes, which were fixed in Bouin's solution at room temperature overnight. For PNW 11 necropsies, the brain, liver, kidneys, thyroid, pituitary, adrenals, mammary glands, testes, epididymides, ventral prostate, other male accessory sex glands (seminal vesicle + coagulating gland + dorsolateral prostate), ovaries, uterus and vagina were removed and fixed in 10% buffered formalin for three days at room temperature, except for testes, which were fixed in Bouin's solution at room temperature overnight. Weights of all organs excluding the vagina and mammary glands were recorded before fixation except for those of the pituitary and ventral prostate, and other male accessory sex glands after fixation. 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 the bregma were serially sectioned at 3 μm. For detection of CNPase signals, microwave treatment was carried out with the deparaffinized brain sections for 10 min at 90 °C in 1×10^{-2} M citrate buffer (pH 6.0) using a microwave oven H2850 (EBSciences, East Granby, CT, USA). Nonspecific endogenous peroxidase activity was blocked by treatment with 0.3% H_2O_2 in absolute methanol for 30 min. After masking with 1.0% normal horse serum/0.01 M phosphate-buffered saline (PBS; pH 7.4), sections were exposed to mouse anti-human CNPase antibodies (1:300 in 0.5% casein/0.01 M PBS; Chemicon, Billerica, MA, USA) or mouse anti-mouse NeuN (1:1000 in 0.5% casein/0.01 M PBS; Chemicon) overnight at 4 °C and then subsequently to biotinylated secondary antibody for 60 min at room temperature. Immunodetection was carried out with the horseradish peroxidase–avidin–biotin complex method and a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA), with 3,3′-diaminobenzidine/H_2O_2 as the chromogen. 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 (Fig. 1A). The mean distance of the location of neurons positive for NeuN from the innermost margin of the pyramidal cell layer adjacent to the lucid layer was bilaterally measured at 0.9 mm lateral to the boundary with the subiculum under 200× magnification (Fig. 1B). Numbers of NeuN-positive nuclei within the pyramidal cell layer and outside of this layer (polymorphic layer) were also counted in the same view area (Fig. 1C).

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 (Fig. 2). In detail, the area of the corpus callosum medial to the cerebral white matter at the uppermost position of the cingulum was measured (Fig. 2A). Also, numbers of CNPase-positive oligodendrocytes were counted at layer V of the parietal isocortex dorsolateral to the cingulum under 200× magnification (Fig. 2B).

For the quantitative measurement of each tissue component, digital photomicrographs at each magnification were taken using a Vanox-S microscope (Olympus Optical Co., Ltd., Tokyo, Japan) attached to a Fujix Digital Camera System (Fujifilm, Tokyo, Japan), and quantitative measurements were carried out with the aid of the MacSCOPE image analysis software package (version 3.61, Mitani Corp., Fukui, Japan).

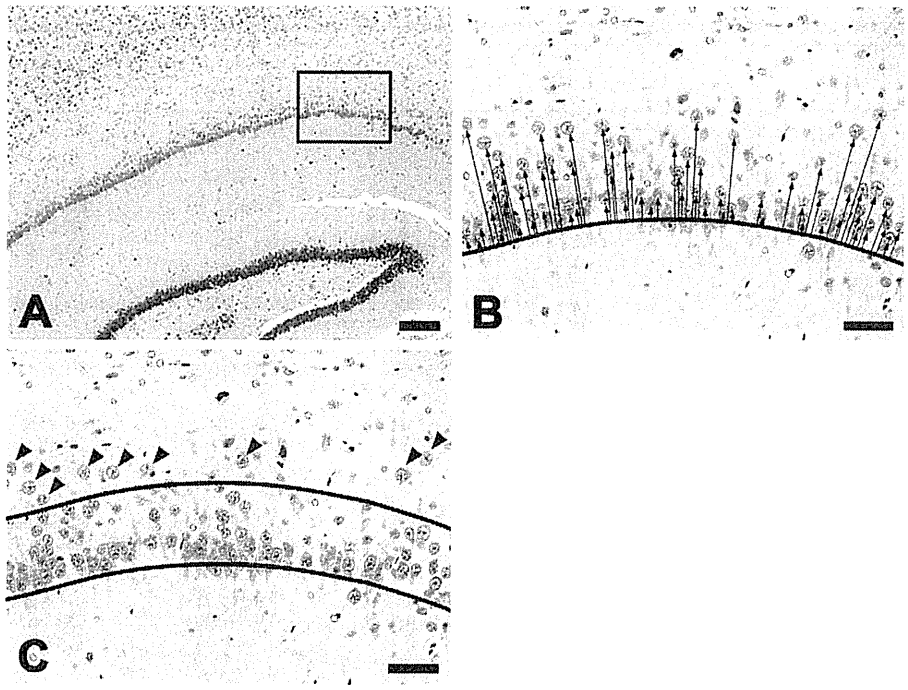

Fig. 1. Quantitative measurement of the variability in the distribution of neurons located within and lateral to the pyramidal cell layer of the hippocampal CA1 region at PNW 11. (A) Hippocampal CA1 region stained with NeuN-immunohistochemistry at 0.9 mm lateral to the boundary with the subiculum. Bar: 200 μm. (B) Measurement of distance of the location of neurons positive for NeuN from the innermost margin of the pyramidal cell layer adjacent to the lucid layer. Bar: 50 μm. (C) Number of NeuN-positive nuclei within the pyramidal cell layer and outside of this layer (polymorphic layer: arrowheads) in the same view area. Bar: 50 μm.

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.

3. Results

3.1. Effects on dams

During the gestation period, slight but statistically significant decreases of water consumption during GD 10–GD 15 and food intake during GD 15–GD 20 were observed with 200 ppm MMI compared with the untreated dams (Fig. 3). During the lactation period, both water consumption and food intake of dams decreased with 12 ppm PTU and MMI with statistical significance. However, treatment did not affect the body weight gain during the exposure period and the body weight of dams at weaning (Table 1). Thyroid weights (relative value) at this time point were statistically higher in the groups that received 12 ppm PTU and MMI.

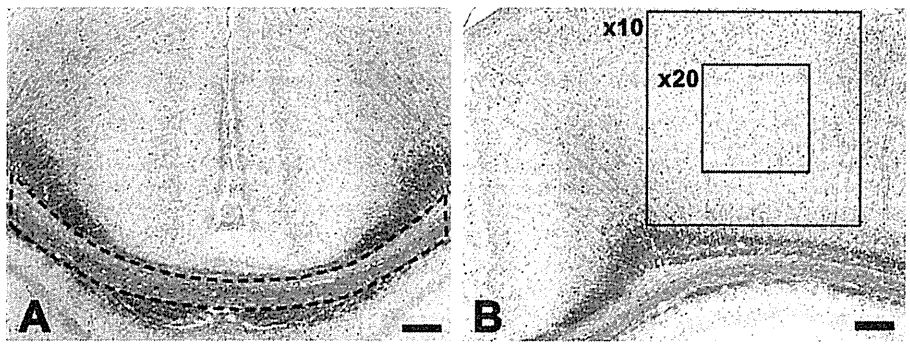

Fig. 2. Quantitative measurement of the effect on the oligodendroglial development at PNW 11. (A) Size measurement of the white matter area immunoreactive for CNPase. The area of the corpus callosum medial to the cerebral white matter at the uppermost position of the cingulum was measured. Bar: 200 μm. (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 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× Bar: 200 μm.

Table 1
Effects on dams and offspring until the prepubertal necropsy by exposure to anti-thyroid agents during the 2nd half of gestation and lactation periods.

	Control	Anti-thyroid agent in the drinking water		
		3 ppm PTU	12 ppm PTU	200 ppm MMI
No. of dams examined	8	8	8	8
Maternal parameter				
Body weight gain (g/day)				
GD 10-GD 20	11.1 ± 1.1[a]	11.6 ± 1.3	10.4 ± 1.3	10.0 ± 1.7
PND 1-PND 9	4.9 ± 1.7	5.7 ± 1.4	3.5 ± 1.6	4.3 ± 1.6
PND 9-PND 20	−0.7 ± 1.0	0.2 ± 1.4	0.8 ± 1.0	1.0 ± 0.7
PND 20				
BW (g)	304.6 ± 19.1	322.7 ± 16.8	302.2 ± 19.0	305.0 ± 26.3
Thyroid				
Relative weight (mg/100 g BW)	6.04 ± 1.17	8.85 ± 1.58	18.01 ± 4.68**	19.64 ± 4.48**
Histopathology: diffuse follicular cell hypertrophy (±/+/++/+++)[b]	1(1/0/0/0)	8#(0/0/8/0)§	8#(0/0/0/8)§	8#(0/0/0/8)§
Offspring parameter				
No. of implantation sites	13.0 ± 1.6	13.9 ± 1.7	13.0 ± 1.6	13.6 ± 1.4
No. of live offspring	12.6 ± 1.6	12.9 ± 1.4	12.3 ± 2.0	12.5 ± 2.1
Male ratio (%)	47.8 ± 9.6	49.3 ± 9.1	41.5 ± 8.5	50.8 ± 14.9
BW, PND 1 (g)				
Males	7.57 ± 0.92	7.17 ± 0.76	6.95 ± 0.83	6.56 ± 0.59
Females	7.22 ± 0.94	6.78 ± 0.74	6.63 ± 0.83	6.24 ± 0.61
AGD, PND 1 (mm)				
Males	4.02 ± 0.25	3.97 ± 0.22	4.14 ± 0.35	3.95 ± 0.15
Females	1.91 ± 0.10	1.93 ± 0.09	1.96 ± 0.13	1.88 ± 0.07
Body and organ weights, PND 20				
Males				
No. of offspring examined	10	10	10	10
BW (g)	52.2 ± 4.4	46.8 ± 8.4	35.9 ± 2.9**	35.1 ± 2.5**
Liver (g)	1.90 ± 0.25	1.62 ± 0.41	1.26 ± 0.15**	1.37 ± 0.19**
Liver (g/100 g BW)	3.62 ± 0.22	3.43 ± 0.28	3.50 ± 0.30	3.91 ± 0.39
Kidneys (g)	0.57 ± 0.07	0.50 ± 0.10	0.42 ± 0.05**	0.39 ± 0.04**
Kidneys (g/100 g BW)	1.10 ± 0.09	1.06 ± 0.07	1.17 ± 0.06	1.10 ± 0.09
Brain (g)	1.46 ± 0.06	1.47 ± 0.07	1.43 ± 0.08	1.40 ± 0.07
Brain (g/100 g BW)	2.82 ± 0.17	3.20 ± 0.42*	4.00 ± 0.33**	4.00 ± 0.28**
Adrenals (mg)	10.4 ± 4.4	7.9 ± 2.9	5.3 ± 3.5**	7.8 ± 2.1
Adrenals (mg/100 g BW)	20.0 ± 8.7	17.4 ± 7.3	14.8 ± 9.5	22.3 ± 6.5
Testes (g)	0.21 ± 0.03	0.14 ± 0.04	0.10 ± 0.02**	0.09 ± 0.01**
Testes (g/100 g BW)	0.41 ± 0.06	0.29 ± 0.04**	0.26 ± 0.04**	0.26 ± 0.02**
Epididymides (mg)	32.5 ± 7.6	35.1 ± 7.1	28.3 ± 6.6	31.4 ± 10.6
Epididymides (mg/100 g BW)	62.2 ± 13.6	76.4 ± 17.2	79.0 ± 17.8	88.9 ± 27.5*
Females				
No. of offspring examined	10	10	10	10
BW (g)	53.1 ± 2.6	45.8 ± 5.5**	34.5 ± 3.2**	34.1 ± 2.5**
Liver (g)	1.93 ± 0.16	1.59 ± 0.25**	1.25 ± 0.17**	1.32 ± 0.17**
Liver (g/100 g BW)	3.63 ± 0.15	3.47 ± 0.35	3.60 ± 0.25	3.85 ± 0.29
Kidneys (g)	0.60 ± 0.06	0.51 ± 0.05**	0.41 ± 0.05**	0.38 ± 0.04**
Kidneys (g/100 g BW)	1.12 ± 0.09	1.11 ± 0.07	1.20 ± 0.08	1.12 ± 0.09
Brain (g)	1.45 ± 0.06	1.43 ± 0.09	1.38 ± 0.06	1.37 ± 0.05*
Brain (g/100 g BW)	2.74 ± 0.14	3.14 ± 0.32*	4.03 ± 0.33**	4.03 ± 0.35**
Adrenals (mg)	7.9 ± 3.7	8.3 ± 4.8	5.9 ± 2.8	6.7 ± 2.6
Adrenals (mg/100 g BW)	14.8 ± 6.7	18.6 ± 12.0	16.6 ± 7.1	19.4 ± 7.4
Ovaries (mg)	20.4 ± 8.7	16.6 ± 6.1	8.4 ± 5.2**	11.4 ± 6.6*
Ovaries (mg/100 g BW)	38.2 ± 15.6	35.9 ± 11.8	24.2 ± 14.6	34.2 ± 20.7
Uterus (mg)	29.0 ± 7.9	27.6 ± 8.1	19.8 ± 5.4*	21.8 ± 4.8
Uterus (mg/100 g BW)	54.6 ± 14.1	56.0 ± 14.4	57.8 ± 18.0	64.4 ± 17.0

Abbreviations: AGD anogenital distance; BW body weight; GD gestational day; MMI methimazole; PND postnatal day; PTU propylthiouracil.

[a] Mean ± SD.

[b] Grade of change: (±), minimal; (+), slight; (++), moderate; (+++), marked.

* 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 Fisher's exact probability test ($P < 0.01$).

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

Though statistically non-significant, treatment with 3 ppm PTU also slightly increased the relative thyroid weight. Histopathologically, the development of a typical hypothyroidism-related thyroidal change, diffuse follicular cell hypertrophy, was evident in all animals given anti-thyroid agents. Among them, all animals in both the 12 ppm PTU and MMI groups showed a severe hypertrophy, while all dams that were administered 3 ppm PTU only showed a moderate change. Both the incidence and the severity of these lesions were significantly increased in all exposure groups.

By monitoring water consumption (data not shown), chemical intake of dams treated with 3 ppm PTU was calculated to be 0.39 mg/kg body weight/day during GD 10–GD 20 and 0.67 mg/kg body weight/day during PND 1–PND 20. In case of dams treated

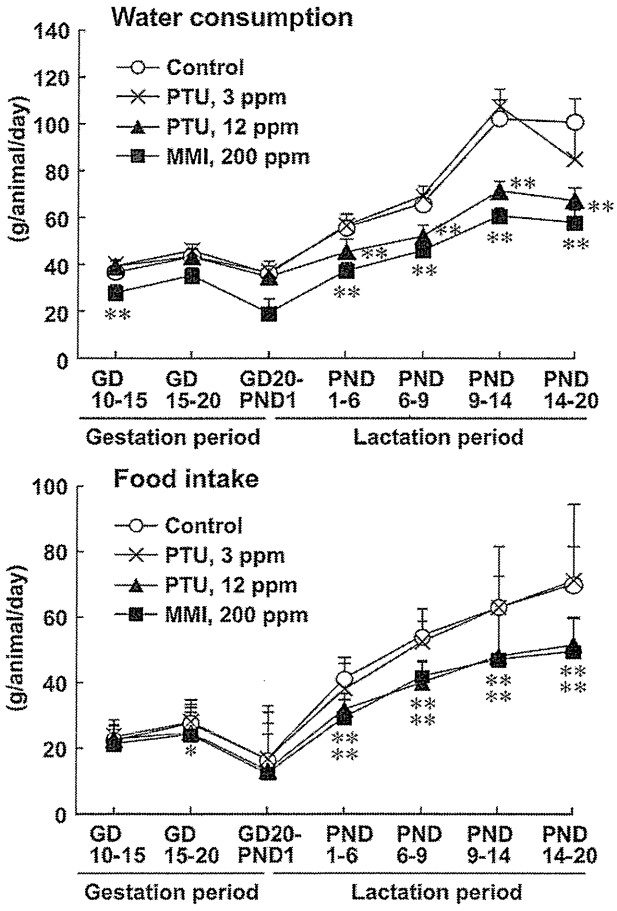

Fig. 3. Water consumption and food intake of dams during exposure to anti-thyroid agents. Significantly different from the untreated controls at $^{*}P<0.05$ and $^{**}P<0.01$, respectively.

with 12 ppm PTU, intake value was 1.54 mg/kg body weight/day during GD 10–GD 20 and 2.20 mg/kg body weight/day during PND 1–PND 20. In case of dams treated with 200 ppm MMI, intake value was 19.7 mg/kg body weight/day during GD 10–GD 20 and 31.2 mg/kg body weight/day during PND 1–PND 20.

3.2. Effects on offspring until prepubertal necropsy

With regard to the reproductive parameters, no significant alterations in the number of implantation sites, number of live offspring, and sex ratio were observed by the exposure to anti-thyroid agents (Table 1). A slight and non-significant decrease of the body weight was observed at PND 1 in all exposure groups of both sexes, while AGD at this time point was not affected by exposure to anti-thyroid agents.

At PND 20, a decrease of body weight was observed after exposure to anti-thyroid agents in both sexes, which was statistically significant in the males of the 12 ppm PTU and MMI groups and in females of all exposure groups (Table 1). These same groups displayed a statistically significant decrease in the absolute weight in the liver and kidneys. An increase of the relative brain weight was statistically significant in all exposure groups of both sexes, although the significant reduction of the absolute value was slight with females exposed to MMI. Absolute weight of the adrenals significantly decreased in males exposed to 12 ppm PTU. In males, the absolute value of testicular weight also significantly decreased after exposure to 12 ppm PTU and MMI and the relative value of testicular weight decreased significantly in all exposure groups. Absolute weight of the epididymides was not affected by exposure, whereas the relative change in the weight of the epididymides increased in the MMI group with statistical significance. In females, a significant decrease in absolute weight in the ovaries in the 12 ppm PTU and MMI groups and in the uterus of the 12 ppm PTU group was noted.

3.3. Effects on the onset of puberty and estrus cycle

After weaning, four males and six females receiving 12 ppm PTU were found dead or subjected to moribund sacrifice. During observation, many of these animals were hyperactive and aggressive in nature and sometimes raced around to bump into a cage wall. During necropsy, most of these animals showed evidence of acute hemorrhage of the brain surface.

In males, a delay in the onset of preputial separation accompanied by decreased body weight occurred in groups exposed to anti-thyroid agents, which was statistically significant in the PTU group receiving 12 ppm and the MMI group (Table 2). In females, a significantly delayed vaginal opening was evident only in the group receiving 12 ppm PTU, whereas a significantly decreased body weight was evident in both MMI-exposed rats as well as the12 ppm PTU-exposed rats.

Table 2
Onset of puberty and estrus cycles in the offspring exposed to anti-thyroid agents during the 2nd half of gestation and lactation periods.

	Control	Anti-thyroid agent in the drinking water		
		3 ppm PTU	12 ppm PTU	200 ppm MMI
Onset of puberty				
Males				
No. of animals examined	11	12	6	11
Age by day	40.5 ± 1.0[a]	43.1 ± 1.6	49.5 ± 3.0**	45.0 ± 2.2**
Body weight at the onset	204.0 ± 14.1	190.2 ± 32.4	169.0 ± 12.1*	160.1 ± 20.6**
Females				
No. of animals examined	12	12	4	12
Age by day	36.2 ± 1.9	37.3 ± 2.8	42.5 ± 3.7**	36.7 ± 3.4
Body weight at the onset	135.3 ± 17.1	129.2 ± 26.7	98.1 ± 21.4*	85.6 ± 21.0**
Estrus cycles during PNW 8–PNW 11				
No. of animals examined	10	10	4	10
Irregularity (ED/EE)	1/0	1/0	0/1	0/0

Abbreviations: ED extended diestrus; EE extended estrus; MMI methimazole; PNW postnatal week; PTU propylthiouracil.

[a] 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 ($P<0.01$).

Table 3
Serum levels of thyroid-related hormones of the offspring exposed to anti-thyroid agents during the 2nd half of gestation and lactation periods.

	Control	Anti-thyroid agent in the drinking water		
		3 ppm PTU	12 ppm PTU	200 ppm MMI
PND 20				
No. of offspring examined	10	10	9[a]	9
T_3 (ng/ml)	1.22 ± 0.1[b]	0.97 ± 0.31	0.25 ± 0.03**	0.43 ± 0.19**
T_4 (μg/ml)	4.72 ± 0.84	1.86 ± 0.41**	1.06 ± 0.32**	1.06 ± 0.44**
TSH (ng/ml)	6.80 ± 2.11	27.38 ± 13.66**	27.69 ± 5.74**	35.33 ± 12.69**
PNW 11				
No. of offspring examined	10	10	6	10
T_3 (ng/ml)	1.02 ± 0.08	0.93 ± 0.11	0.84 ± 0.10**	0.88 ± 0.09**
T_4 (μg/ml)	5.11 ± 0.70	5.12 ± 0.73	4.05 ± 0.71	4.57 ± 1.04
TSH (ng/ml)	9.81 ± 3.16	9.10 ± 3.25	7.75 ± 2.23	9.41 ± 4.40

Abbreviations: MMI methimazole; PND postnatal day; PNW postnatal week; PTU propylthiouracil; T_3 triiodothyronine; T_4 thyroxine; TSH thyroid-stimulating hormone.
[a] $N = 7$ for measurement of T_3 and T_4 levels.
[b] Mean ± SD.
** Significantly different from the controls by Dunnett's test or Dunnett-type rank-sum test ($P < 0.01$).

When estrus cycles were examined for three weeks before sacrifice at PNW 11, no apparent increase in the number of rats with irregular/abnormal cycles was observed in response to anti-thyroid agent exposure.

3.4. Serum levels of thyroid-related hormones

Serum levels of thyroid-related hormones were measured in males (Table 3). At PND 20, decreases of T_3 and T_4 were evident in group that were administered anti-thyroid agents with statistical significance in the 12 ppm PTU and MMI groups for T_3, and both PTU doses and the MMI group for T_4. Reductions of T_3 and T_4 with PTU occurred in a dose-dependent fashion. Significantly elevated TSH levels were observed with PTU at both doses and MMI. At PNW 11, a slight but statistically significant decrease of T_3 levels was observed with 12 ppm PTU and MMI groups.

3.5. Organ weight changes at the adult stage

At the necropsy of 11 week rats, only six males and four females remained in the 12 ppm PTU group, whereas 10 animals/sex remained in other groups. Offspring of dams receiving 12 ppm PTU and MMI showed a statistically significant decrease in body weight in both sexes (Table 4). In males, the absolute weight of the liver, kidneys, brain, pituitary, adrenals, dorsolateral lobe of the prostate, and seminal vesicles was significantly decreased in the 12 ppm PTU and MMI groups, while relative values for the kidneys of the 12 ppm PTU group and in the brains of the 12 ppm PTU and MMI groups were inversely increased significantly. On the other hand, absolute weight of the thyroid was significantly increased in a dose-dependent manner with PTU, with significant increase of the relative value at a dose of 12 ppm. MMI also significantly increased the relative thyroid weight. Both absolute and relative values of testicular weight were significantly increased in all exposure groups. While absolute value tended to decrease, relative epididymal weight slightly but significantly increased in the 12 ppm PTU and MMI groups. In females, a slight yet significant increase in the relative brain weight was observed with MMI administration, while absolute value did not change with the control. MMI treatment also increased the relative ovarian weight with statistical significance.

3.6. Histopathological changes

At PND 20, statistically significant increases in the incidence and severity of diffuse follicular cell hypertrophy were observed in animals of both sexes exposed maternally to anti-thyroid agents with dose-dependent increase in the severity after PTU-exposure (Table 5). In the kidneys, tubular mineralization in the cortex and/or medulla was evident in animals treated with 12 ppm PTU or MMI in both sexes with statistically significant differences in the incidence and severity. Foci of extramedullary hematopoiesis in the liver observed in the control animals were decreased by exposure to 12 ppm PTU or MMI in both sexes. In the anterior pituitary, depletion of cytoplasmic granules in the pituitary cells was evident by exposure to PTU or MMI in both sexes. In the mammary gland, cases showing proteinous secretion in the alveolar buds were increased in males, whereas in females, cases with hypoplasia of alveolar buds were increased by exposure to PTU or MMI. In the testis, delayed spermatogenesis was evident with an increase in apoptotic spermatocytes by exposure to PTU or MMI.

At PNW 11, cases with diffuse follicular cell hypertrophy were not increased by PTU or MMI in both sexes (Table 5). Renal tubular mineralization remained in the cortex and/or medulla in the anti-thyroid treatment groups with statistical significance in the incidence and severity in all groups of males and in the female groups receiving 12 ppm PTU and MMI.

3.7. Brain histopathology and morphometry

At PND 20, subcortical band heterotopia in the corpus callosum, manifested by the appearance of aberrant cortical tissue in this anatomical area, was found in 2 out of 5 MMI-exposed animals histopathologically (Table 6).

At PNW 11, hippocampal CA1 neurons showing a broad distribution at the area lateral to the pyramidal cell layer, manifested 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, and ratio of abnormally distributed neurons in total CA1 neurons, were significantly increased by both chemicals, with irregularities occurring in a dose-dependent manner after exposure to PTU (Fig. 4A–C, Table 6). The incidence of cases with subcortical band heterotopia was significantly increased by exposure to 12 ppm PTU or MMI treatment as compared with the controls (Fig. 4D, Table 6). The area of the corpus callosum was significantly and dose-dependently decreased after PTU exposure and also significantly decreased by MMI. CNPase-positive oligodendrocytes in the deep cortex of the cingulate were dose-dependently decreased by PTU and by MMI, with statistical significance at doses of 12 ppm PTU and MMI.

4. Discussion

Exposure to two different anti-thyroid agents during the period from the mid-gestation to the end of lactation resulted in typical

Table 4
Body and organ weights of offspring exposed during the period from the mid-gestation to the end of lactation to anti-thyroid agents and measured at PNW 11.

	Control	Anti-thyroid agent in the drinking water		
		3 ppm PTU	12 ppm PTU	200 ppm MMI
Males				
No. of animals examined	10	10	6	10
BW (g)	452.5 ± 32.2[a]	451.0 ± 30.7	332.4 ± 26.0**	347.4 ± 36.8**
Liver (g)	17.39 ± 1.89	17.42 ± 1.84	13.64 ± 1.22**	13.98 ± 1.48**
Liver (g/100 g BW)	3.84 ± 0.25	3.86 ± 0.26	4.11 ± 0.38	4.03 ± 0.16
Kidneys (g)	3.01 ± 0.23	2.90 ± 0.31	2.51 ± 0.25**	2.33 ± 0.22**
Kidneys (g/100 g BW)	0.67 ± 0.03	0.64 ± 0.06	0.76 ± 0.07**	0.67 ± 0.05
Brain (g)	2.10 ± 0.08	2.10 ± 0.09	1.90 ± 0.13**	1.94 ± 0.08**
Brain (g/100 g BW)	0.47 ± 0.02	0.47 ± 0.03	0.57 ± 0.04**	0.57 ± 0.07**
Pituitary (mg)	15.6 ± 1.0	15.0 ± 1.6	11.1 ± 1.1**	12.5 ± 1.1**
Pituitary (mg/100 g BW)	3.45 ± 0.22	3.34 ± 0.40	3.33 ± 0.27	3.63 ± 0.49
Thyroid (mg)	24.5 ± 3.3	29.9 ± 6.4*	30.8 ± 4.8*	26.6 ± 4.0
Thyroid (mg/100 g BW)	5.43 ± 0.82	6.66 ± 1.49	9.36 ± 1.82**	7.67 ± 1.03**
Adrenals (mg)	52.4 ± 9.8	51.2 ± 11.6	35.5 ± 4.2**	36.1 ± 6.6**
Adrenals (mg/100 g BW)	11.6 ± 2.3	11.3 ± 2.1	10.7 ± 1.1	10.4 ± 1.4
Testes (g)	3.29 ± 0.30	3.95 ± 0.33**	4.05 ± 0.45**	3.75 ± 0.29*
Testes (g/100 g BW)	0.73 ± 0.08	0.88 ± 0.09**	1.22 ± 0.08**	1.09 ± 0.01**
Epididymides (g)	0.99 ± 0.10	1.04 ± 0.10	0.87 ± 0.14	0.88 ± 0.08
Epididymides (g/100 g BW)	0.22 ± 0.01	0.23 ± 0.03	0.26 ± 0.03**	0.25 ± 0.03**
Accessory sex glands [b] (g)	0.60 ± 0.09	0.55 ± 0.11	0.45 ± 0.13*	0.49 ± 0.08*
Accessory sex glands (g/100 g BW)	0.13 ± 0.02	0.12 ± 0.02	0.13 ± 0.03	0.14 ± 0.03
Prostate, ventral (g)	0.53 ± 0.08	0.49 ± 0.13	0.38 ± 0.16	0.41 ± 0.13
Prostate, ventral (g/100 g BW)	0.12 ± 0.02	0.11 ± 0.03	0.12 ± 0.05	0.12 ± 0.03
Seminal vesicle (g)	1.16 ± 0.14	1.07 ± 0.22	0.95 ± 0.16*	0.92 ± 0.10**
Seminal vesicle (g/100 g BW)	0.26 ± 0.03	0.24 ± 0.05	0.28 ± 0.03	0.27 ± 0.04
Females				
No. of animals examined	10	10	4	10
BW (g)	281.6 ± 22.6	279.4 ± 21.3	236.5 ± 22.1*	247.4 ± 35.1*
Liver (g)	9.41 ± 1.04	9.82 ± 1.44	8.52 ± 1.09	8.89 ± 1.61
Liver (g/100 g BW)	3.34 ± 0.14	3.51 ± 0.34	3.60 ± 0.23	3.59 ± 0.29
Kidneys (g)	1.78 ± 0.19	1.78 ± 0.16	1.52 ± 0.17	1.61 ± 0.19
Kidneys (g/100 g BW)	0.63 ± 0.05	0.64 ± 0.05	0.64 ± 0.03	0.65 ± 0.05
Brain (g)	1.92 ± 0.08	1.95 ± 0.07	1.81 ± 0.09	1.86 ± 0.11
Brain (g/100 g BW)	0.68 ± 0.06	0.70 ± 0.06	0.77 ± 0.04	0.76 ± 0.08*
Pituitary (mg)	16.2 ± 1.9	13.9 ± 2.8*	10.2 ± 1.4**	11.3 ± 1.3**
Pituitary (mg/100 g BW)	5.80 ± 0.91	4.96 ± 0.90	4.19 ± 0.48*	4.64 ± 0.71*
Thyroid (mg)	20.9 ± 3.2	20.5 ± 2.4	20.2 ± 3.3	20.8 ± 4.0
Thyroid (mg/100 g BW)	7.49 ± 1.35	7.39 ± 1.09	8.50 ± 0.69	8.44 ± 1.24
Adrenals (mg)	66.6 ± 8.6	56.8 ± 16.5	50.8 ± 6.4	53.5 ± 9.5
Adrenals (mg/100 g BW)	23.8 ± 3.6	20.2 ± 4.9	21.5 ± 2.1	21.8 ± 3.4
Ovaries (mg)	78.7 ± 10.6	89.0 ± 14.5	83.3 ± 13.9	92.2 ± 18.7
Ovaries (mg/100 g BW)	28.0 ± 3.4	31.9 ± 4.8	35.4 ± 6.6	37.5 ± 6.8**
Uterus (g)	0.59 ± 0.23	0.49 ± 0.06	0.54 ± 0.22	0.44 ± 0.05
Uterus (g/100 g BW)	0.21 ± 0.08	0.18 ± 0.02	0.23 ± 0.08	0.18 ± 0.03

Abbreviations: MMI methimazole; PNW postnatal week; PTU propylthiouracil.

[a] Mean ± SD.

[b] Accessory sex glands were consisted of seminal vesicle, coagulating gland, and dorsolateral prostate.

* 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 ($P < 0.01$).

hypothyroid associated changes in serum thyroid-related hormones, weight and histopathological changes of the thyroid in the present study. In serum thyroid hormone at PND 20, both T_3 and T_4 decreased after PTU and MMI treatment, and these decreases were dose-dependent after PTU exposure. On the other hand, T_3 levels after exposure to 12 ppm PTU were lower than levels after 200 ppm MMI exposure, although T_4 levels were similar between the two groups. This effect may have been related to the differences in the biological action of the two chemicals. PTU can block the conversion of T_4 to T_3 in the thyroid and other peripheral tissues, while MMI cannot block the conversion [17]. A marked elevation of serum TSH concentration was evident in groups receiving PTU and MMI in the present study. Similar levels of TSH elevation were evident in both the 3 and 12 ppm groups, and this effect was mediated through the suppression of negative feedback through the pituitary [25]. TSH then stimulates thyroid functions to cause diffuse follicular cell hypertrophy as observed in the present study.

Exposure to PTU at 12 ppm or MMI resulted in the growth suppression in offspring of both sexes at weaning in the present study. Reductions in food intake and water consumption of dams observed during the lactation period may be related to the growth suppression of offspring. However, offspring exposed to 3 ppm PTU also exhibited reduced body weights, with a statistically significant difference in females, without a concurrent reduction of food intake and water consumption of dams, suggesting that the growth suppression was due to the development of hypothyroidism [26]. Furthermore, stunted growth and delayed maturation continued in offspring to the adult stage in groups exposed to 12 ppm PTU or MMI. Considering the high growth recovery after postnatal transient hypothyroidism [27], these results suggest a sustained growth retardation into the adult stage because the developmental hypothyroidism began during the gestation period. Compared with females, males showed lower growth recovery rate in the body weight (Reduction rate: males, 23–27%; females, 12–16%) as in postnatal transient hypothyroidism [27]. Most histopathological

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)##	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.

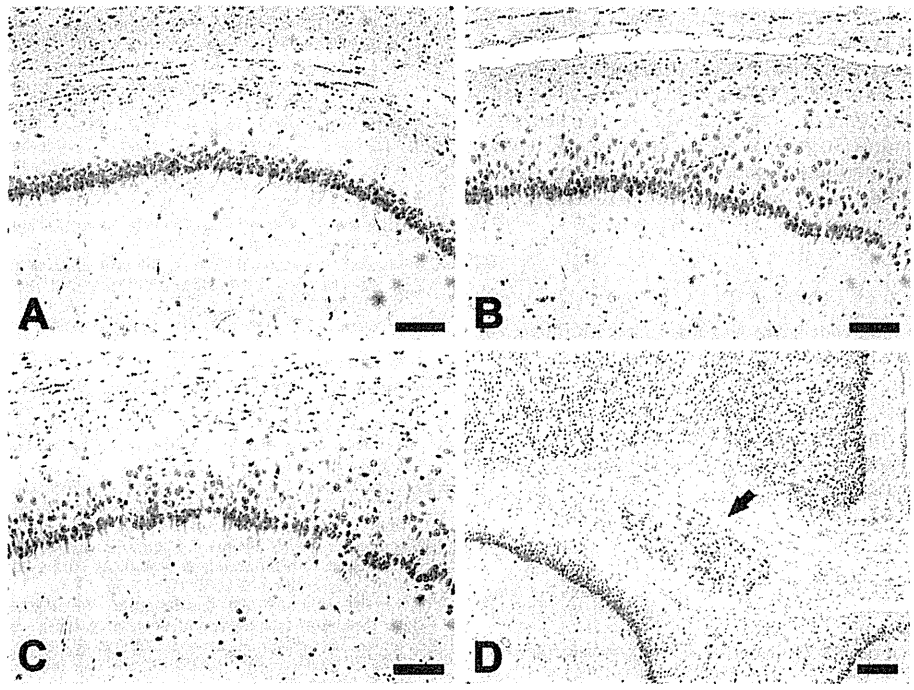

Fig. 4. Neuronal cell distribution within and lateral to the pyramidal cell layer of the hippocampal CA1 region (A–C) and subcortical band heterotopia (arrow) appeared within the corpus callosum (D) at PNW 11 stained with NeuN-immunohistochemistry. (A) Untreated control; (B) 12 ppm PTU; (C, D) 200 ppm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

Acknowledgments

We thank Miss Tomomi Morikawa and Miss Ayako Kaneko for their technical assistance in conducting the animal study. This work was supported in part by Health and Labour Sciences Research Grants (Research on Risk of Chemical Substances) from the Ministry of Health, Labour and Welfare of Japan. All authors disclose that there are no conflicts of interest that could inappropriately influence the outcome of the present study.

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