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Original Article

Optimal testing time for cerebral heterotopia formation in the rat comparative thyroid assay, a downstream indicator for perinatal thyroid hormone insufficiency

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Abstract: In a past study, we proposed a modified Comparative Thyroid Assay (CTA) with additional examinations of brain thyroid hormone (TH) concentrations and brain histopathology but with smaller group sizes. The results showed that the modified CTA in Sprague Dawley rats detected 10 ppm 6-propylthiouracil (6-PTU)-induced significant suppressions of serum/brain TH concentrations in offspring. To confirm the reliability of qualitative brain histopathology and identify the optimal testing time for heterotopia (a cluster of ectopic neurons) in the modified CTA, brain histopathology together with serum/brain TH concentrations were assessed in GD20 fetuses and PND2, 4, 21, and 28 pups using a similar study protocol but with a smaller number of animals (N=3-6/group/time). Significant hypothyroidism was observed and brain histopathology revealed cerebral heterotopia formation in PND21 and PND28 pups, with likely precursor findings in PND2 and PND4 pups but not in GD20 fetuses. This study confirmed that the optimal testing time for cerebral heterotopia in rat CTA was PND21 and thereafter. These findings suggest that cerebral heterotopia assessment at appropriate times may be a useful alternative to the original CTA design. (DOI: 10.1293/tox.2024-0004; J Toxicol Pathol 2024; 37: 173–187)

Key words: comparative thyroid assay, developmental neurotoxicity, endocrine disruption, heterotopia, hypothyroidism, thyroid hormone

Introduction

Thyroid hormones (THs) are essential for normal physiological functions, including neurodevelopment, growth, and metabolism. Adequate TH concentrations are required during the perinatal period for normal brain development in humans and animals[1–4]. Over the past two decades, increasing evidence has demonstrated that environmental chemicals disrupt aspects of thyroid signaling and function; thus, regulatory concerns have been raised that TH disruptors identified in adult rats may potentially interfere with the developing brain[5–11]. Therefore, there is a great need to better assess the TH disruption potential of the concerned chemicals in prenatal and postnatal offspring, which are sensitive to the periods of brain development[12, 13].

Comparative thyroid assay (CTA) is a screening method designed to detect peripheral TH disruption in dams and offspring[13]. CTA was designed to determine whether the potentially sensitive life stages for TH disruption are adequately protected by the points of departure used for hazard assessments and not for further elucidation of endocrine and/or neurodevelopmental hazard[13, 14]. However, this does not allow the determination of whether the effects detected in the peripheral serum adequately reflect hormone concentrations in the target tissue (i.e., developing brain)[15], leading to potential uncertainties in the adverse points of departure in hazard assessments. Recently, we verified the feasibility, sensitivity, and/or reliability of a modified CTA by adding parameters such as examinations of brain THs concentrations and brain histopathology but by reducing the number

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of rats (10 maternal rats per dose group per cohort)[16, 17]. The results showed that the modified CTA could detect 10 ppm 6-propylthiouracil (6-PTU) dietary treatment-induced severe (>70%) suppression of serum THs in dams, with significant (>50%) suppression of serum/brain TH concentrations in fetuses on gestation day (GD) 20 and pups on postnatal days (PND) 4 and 21, and significantly increased heterotopia in the cerebrum and external granular layer of the cerebellum in PND 21 pups[16].

Heterotopia is a cluster of ectopic neurons in the brain indicative of altered neuronal migration (Fig. 1), which has been observed in rats and humans and is associated with neurodevelopmental disorders, such as epilepsy and learning disabilities[18, 19]. Cerebral heterotopia is comprised of glutamatergic and GABAergic neurons and is positioned bilaterally in the corpus callosum[19] (Fig. 1). Cells with this abnormality are structurally and functionally connected to the cortical neurons[18]. Rat heterotopia is ameliorated by maternal T4 infusion, demonstrating that prenatal TH insufficiency is required for its formation[18, 20]. Interestingly, the severity of the phenotype is dependent on the degree of hypothyroidism and is observable even at low or moderate levels of maternal TH disruption induced by 6-PTU[18, 19, 21–23], methimazole[18, 20], amitrole[24] and perchlorate[25]. The same observation is identifiable when maternal TH disruption is induced by iodine deficiency[26], demonstrating that heterotopia formation is not limited to exposure to specific substances, such as 6-PTU or methimazole. Furthermore, heterotopia persists in adult offspring despite a return to euthyroid status upon termination of exposure[19]. Given these properties, heterotopia is a useful downstream indicator of perinatal TH insufficiency during rat brain development[16, 19, 25, 27]. Abnormal radial migration is one of the most reproducible and sensitive markers of TH activity *in vivo*[15, 25].

O'Shaughnessy *et al*. reported that GD19-LD2 is a critical time window for heterotopia formation upon exposure to 10 ppm 6-PTU in drinking water in maternal rats[28]. The postnatal test cohort of CTA (treatment period from GD6 to PND21)[13] covers this critical time window for heterotopia formation. Since brain histopathology is not required in the original CTA[13], we hypothesized that adding brain histopathology (especially examining cerebral heterotopia formation) and brain TH measurement may add value to the original CTA in rats[16]. In addition, such augmentation may allow for a reduction in the number of test animals (originally 20 rats per dose group per cohort[13]; a total of 160 rats for 4 dose study).

To test our hypothesis, we verified the feasibility, sensitivity, and reliability of the modified CTA in rats. Previously, we reported that modified CTA could detect significant cerebral heterotopia formation in PND21 pups by classical standard histopathology (but step sectioning with grading is necessary) upon exposure to 10 ppm 6-PTU by feeding it in the diet of maternal rats[16]. Several previous studies have confirmed that heterotopia examinations on PND14[2, 25, 27, 28], PND16[24], PND18[22, 27], and later[19, 27] are usable periods for examination. Therefore, PND21, which is an examination point in the postnatal test cohort of the original CTA protocol[13], may be acceptable for assessing heterotopia on CTA. However, it is important to investigate the optimal timing

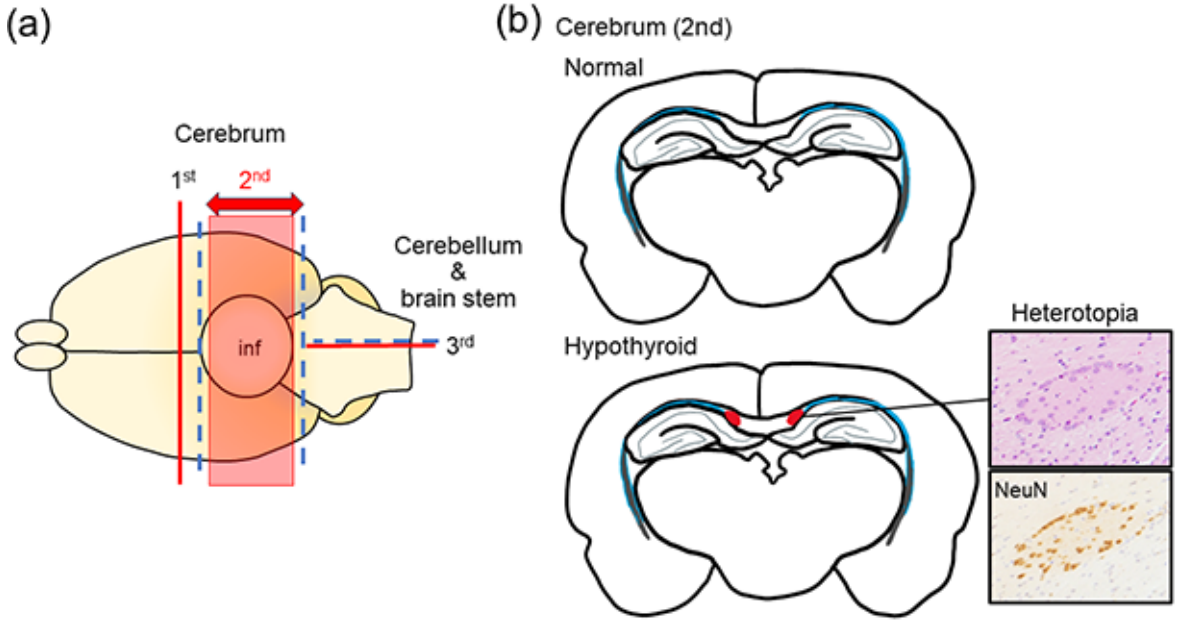

Fig. 1. (a) Diagram (from the base of the brain) of the cutting planes and examination planes in the pups. Blue dashed lines; macroscopic cutting planes. Red lines; microscopic examination planes. Inf; the infundibulum. Heterotopia was evaluated from step sections of the 2nd cerebrum region (shown in red squares) including the hippocampus. (b) Location of heterotopia. Heterotopia can be detected in the 2nd cerebrum area (red-filled area), located adjacent to median side of the ventricular epithelium (blue line) and above the hippocampus in the corpus callosum in the hypothyroid rats with increase of incidence and size.

for the assessment of heterotopia formation using modified CTA.

In the present study, heterotopia formation was assessed on GD20, PND4, and PND21 (the examination days for serum TH concentrations designated by the original CTA[13]), PND2 (the terminal of the critical time window for heterotopia formation[28]), and PND28 (7 days after the end of administration) to confirm the flexibility of test timing. At each time point, heterotopia formation was concomitantly assessed using serum/brain TH concentrations. In addition to cerebral heterotopia, the external granular layer in the cerebellum may also be a useful downstream indicator of TH insufficiency in rats because the migration of neurons from the external to the internal granular layer in the rat cerebellum occurs postnatally and is dependent on the presence of TH[29]. However, CTA is considered less reliable because it can be affected by a variety of internal (e.g., neurotransmitters [glutamate] and neuropeptides [somatostatin, PACAP]) and external factors (light stimuli, methylmercury, and ethanol)[30, 31]. Qualitative histopathology of the cerebellum was performed to confirm whether this consideration was correct.

Materials and Methods

Test chemicals

6-PTU (CAS# 51-52-5; Lot No.WDN6541; purity ≥98%; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used. 6-PTU is a potent thyroperoxidase (TPO)-inhibiting drug (and also acts by inhibiting the enzyme deiodinase type 1 [D1], which converts T4 to its active form, T3), resulting in TH disruption and altered brain development in rat studies[16, 18, 19, 21–23, 27, 28, 32]. 6-PTU is suggested as a positive control in the USEPA CTA guidance[13].

Animals, husbandry, and mating

All non-histopathology experiments were conducted at the Institute of Environmental Toxicology (IET), which was the same facility used in previous studies[16, 17] and were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) in accordance with the Animal Care and Use Program of IET (IET IACUC Approval No. AC21019). Briefly, specific pathogen-free (SPF) Crl:CD(SD) rats of both sexes were purchased from the Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (currently Jackson Laboratory Japan, Kanagawa, Japan) at 12 weeks of age. After quarantine and acclimatization for 10–14 days, males and females were paired at 13–14 weeks of age. Animals were housed in a barrier-sustained animal room with targeted controlled temperature (22 ± 2°C), humidity (50 ± 20%), ventilation (at least 10 times per hour; all fresh air system), and illumination (12 hours per day, lights on at 7:00 a.m. and off at 7:00 p.m.) throughout the study period. A commercially available solid or pulverized diet (MF or MF Mash diet; Oriental Yeast Co., Ltd., Tokyo, Japan) and local tap (chlorinated) water were provided *ad libitum* throughout the study. Overnight fasting of the animals before sacrifice, which is often conducted in the tox studies, was not done in this experiment. Copulated females were prepared using standard reproductive study methods, and the day on which vaginal plugs and/or sperm were observed was designated as GD0.

Study design

This study was conducted with some modifications to the CTA protocol designated by the USEPA guidance[13] (Fig. 2). The treatment regimen was consistent with that described in a previous study[16]. 6-PTU was exposed to pregnant rats at 0 and 10 ppm (10 and 6 females, respectively) in the diet from GD6 to GD20 in the prenatal test cohort. In the postnatal test cohort, 6-PTU-containing diets at 0 and 10 ppm were fed to another set of pregnant rats (10 and 6 females, respectively) from GD6 to LD21. The concentration of 6-PTU in the test diet was reduced to 5 ppm from LD13 to 21 to avoid excessive toxicity due to excessive substance intake during later lactation because of the expected increased food consumption (more than 2-fold of that during early lactation)[16]. In addition, 6-PTU-containing diets at 0 and 10 ppm were fed to another set of pregnant rats (three females/dose group) from GD6 through LD2. LD2 is the end of the critical time window for heterotopia formation[28]. Pups culled on PND4[13] were also assessed for TH concentration and histopathology. Furthermore, to determine the persistence/transience of the brain abnormalities on PND28, which was one week after the termination of the treatment on PND21, some selected weanlings in the postnatal test cohort were housed in groups with the same conditions described above without 6-PTU treatment, and brain histopathology and serum/brain TH concentrations of these weanlings were assessed on PND28. Data from control animals were shared with a sodium phenobarbital study conducted concurrently (published elsewhere).

During treatment, cage-side observations of female mothers were made once daily for clinical signs and mortality. Each female was also observed in more detail for the presence of abnormalities, such as excitement, convulsion, sedation, and abnormal gait, when weighed, instead of cage-side observation. During breeding, females were examined for pregnancy and parturition. All the observed abnormalities were recorded for individual females. Body weights and food consumption of the dams and litter were monitored periodically throughout the study.

Blood and tissue collection

To minimize the impact of circadian changes in hormone concentrations, sampling was performed from the animals in the two groups at similar times of the day and performed alternately between the control group and the 6-PTU group to avoid bias. In the prenatal test cohort, pregnant females in each group were laparotomized under isoflurane anesthesia to collect blood samples from the posterior vena cava using plain syringes (19G needle) on GD20 (Fig. 2). Sera were separated from the blood for hormone analysis.

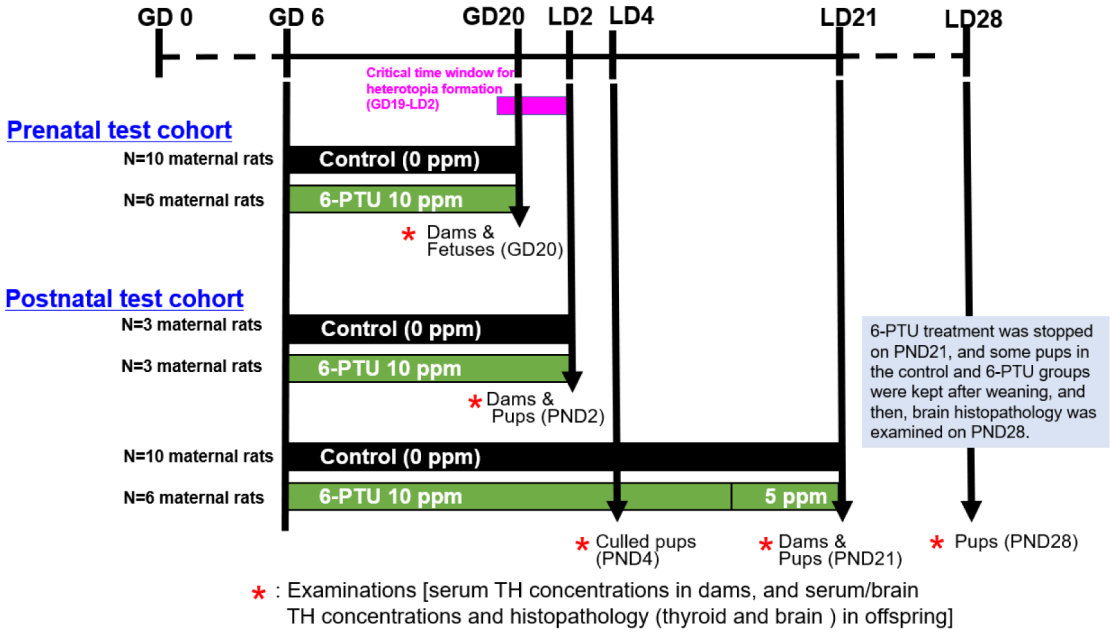

Fig. 2. Schematic representation of study design. The study design of the present study was mostly derived from the Comparative Thyroid Assay (CTA) of USEPA guidance (2005) and the modified CTA previously reported (Minami *et al.*[16]). To confirm that cerebrum heterotopia is an optimal brain histopathological indicator for perinatal TH insufficiency and its appropriate testing time point in the modified CTA, brain histopathology and hormone concentrations (THs and TSH) were assessed in offspring on GD20 (latest gestation), PND2 (terminal of the critical time window for heterotopia formation; O'Shaughnessy *et al.*[28]), PND4 (culled pups), PND21 (dosing terminal in the CTA), and additionally PND28 (to confirm persistence or transience of the brain findings 1 week after treatment cessation). Maternal serum TH and TSH concentrations were also determined on GD20, LD2 and LD21. GD, gestation day; LD, lactation day; PND, postnatal day; TH, thyroid hormone (T3 and T4); TSH, thyroid stimulating hormone.

Blood sampling was generally performed in the afternoon (13:00–16:00) to ensure practical flexibility in conducting the studies, as previously reported[16, 17]. The rats were held in a curtained-off holding area adjacent to the necropsy room to avoid the effects of stress on hormone concentrations. The sera were stored in a freezer (−70°C or below) until use. The pregnant females underwent cesarean section and gross necropsy after blood collection. The thyroids of the dams were isolated, weighed, and fixed in 10% neutral-buffered formalin for histopathology.

After external examination, blood was collected from all live fetuses by making a small cut on the neck without any form of anesthesia[17]. The collected blood was pooled by sex in each litter as suggested by USEPA[13], processed for sera, and stored in a freezer (−70°C or below). After blood sampling, the whole body of the fetuses (2 per sex per litter) was fixed in 10% neutral-buffered formalin with the cranium, thoracic cavity, and abdominal cavity being incised and preserved in 0.01M phosphate-buffered saline at 4°C following 2-day formalin fixation for histopathology. In addition, the brain was removed from another set of fetuses (up to 2 per sex per litter, if available), snap-frozen, and stored in a freezer (−70°C or below) for brain hormone analyses.

In the postnatal test cohort, all dams were allowed to deliver pups. The day of completion of parturition was designated as LD0 for dams (PND0 for offspring). On LD2, three litters per group from the control and 6-PTU groups were euthanized for blood and tissue sampling and then examined (Fig. 2). The methods of euthanization and sera/tissue collection from dams were the same as those described for the prenatal test cohort. After LD2 sampling, 10 litters in the control group and 6 litters in the 6-PTU group were left for subsequent examination. On PND4, the size of each litter was standardized by random pup selection to eight pups, four males and four females, if possible, to avoid eliminating runts only (Fig. 2). Maternal females were laparotomized under isoflurane anesthesia to obtain blood samples from the posterior vena cava on LD2 and LD21 using plain syringes. Serum samples were prepared and stored in the same manner as those in the prenatal test cohort for both groups. The thyroid glands of the dams were weighed and stored for the same analyses as those of the prenatal test cohort.

Blood samples were collected from the pups on PND2, PND4, PND21, and PND28 (pooled by sex by litter on PND2 and PND4, as suggested by the USEPA[13]). PND4 pups mean the culled pups on PND4. For PND2 and PND4 pups, the methods of euthanization and serum collection were the same as those described for the fetuses, with the following exception: the pups were sedated while collecting blood with an intraperitoneal injection of a thiamylal sodium solution (ISOZOL, Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan). For PND21 and PND28 pups (Fig. 2), blood was collected from the posterior vena cava under isoflurane anesthesia using plain syringes and 21G needles. The brains

were also collected from the blood-sampled animals, at least 1 per sex per litter per time point (if available), snap-frozen, and stored for brain hormone analyses. Another set of pups, at least 1 per sex per litter per time point (if available), was sacrificed in the same manner for the collection of histopathology samples: brain and thyroid (fixed in the same manner as the fetuses, except that at necropsy, the thyroid and brain were removed at PND21 and later pups). Tissue/serum collection from pups at each time point was performed in the afternoon as previously[16].

Thyroid hormone analyses

Frozen brains of fetuses and pups (1 per sex per litter) were individually weighed and homogenized in the same volume of water for injection. Serum samples collected from maternal rats and fetuses and homogenized brain samples were analyzed for T3 and T4 concentrations using liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS). After the study we reported[16], we have slightly modified the analytical method using a purification column and higher-grade MS/MS, as previously described[17]. An aliquot (50 µL) of serum or brain homogenate was placed into a test tube, added with 50 µL of water for injection and 300 µL of internal standard solution (methanol solution). After mixing, the mixture solution was centrifuged (16,000 × g, 5 minutes, 10°C). Then, 200 µL of 0.1% formic acid was added to the solution and the mixture solution was centrifuged (16,000 × g, 5 minutes, 10°C). An aliquot of the supernatant was purified using MonoSpin Phospholipid (GL Sciences Inc., Tokyo, Japan). The eluate was then injected into the Online SPE LC-MS/MS (LC: 1290 HPLC, Agilent Technologies, Santa Clara, CA, USA; MS/MS: 6470 Triple Quad LC/MS, Agilent Technologies; SPE column: Shim-pack MAYI-ODS (G), 2.0 mm × 10 mm, Shimadzu Corporation, Kyoto, Japan; LC column: ZORBAX Eclipse C18, 1.8 µm × 2.1 mm × 50 mm, Agilent Technologies). The limits of quantification (LOQ) for serum T3 and T4 were 0.010 and 0.2 ng/ml, respectively, and the limits of detection (LOD) for serum T3 and T4 were 0.005 and 0.1 ng/mL respectively. The LOQ for brain T3 and T4 were 0.02 and 0.20 ng/g brain weight, respectively, and the LOD for brain T3 and T4 were 0.01 and 0.10 ng/g brain weight respectively. When the result was below the LOQ, the mean value was calculated using the LOQ as the measured value for each animal.

Serum thyroid-stimulating hormone (TSH) analyses

Serum TSH concentration was measured using an immuno-bead assay using the Milliplex Map Rat Thyroid Hormone TSH Panel (EMD Millipore, Burlington, MA, USA)[16, 17]. Each serum sample was incubated with beads and detection antibodies, according to the manufacturer's instructions. The fluorescence intensity of each sample was analyzed after gating the immune bead population on the forward and side scatter areas using a flow cytometer (FACSVerse, BD, Tokyo, Japan) with the FACSuite program. The LOQ for TSH was 62.5 pg/mL and none of the cases were below the LOQ value in this study. Regarding the intra-assay precision of the TSH immunobead assay in the analytical validation, coefficients of variation (CVs) ranged from 3.0–13.6% and 4.3–9.1% for eight samples each for males and females, respectively.

Histopathology

The preserved brain and thyroid (with the trachea) of fetuses (after removal from the preserved whole body), pups (1 per sex per litter), and dams were processed and subjected to histopathological examination. Both lobes of the thyroid gland were processed and examined under a light microscope. For fetal and pup brains (Fig. 1), the cerebrum was cut coronally at levels anterior and caudal to the infundibulum, as vertically as possible, to make two paraffin blocks. The cerebellum and brainstem were cut in the midsagittal plane as vertically as possible to create another paraffin block. After trimming, the brains, liver, and thyroids were embedded in paraffin, sectioned at 3 µm, stained with hematoxylin and eosin (H&E), and examined by light microscopy.

The three blocks of the brain prepared as described above were carefully thinned to obtain three levels of planes (the first level included the cerebral cortex and caudate/putamen; the second level included the cerebral cortex, hippocampus, thalamus, and hypothalamus; and the third level included the cerebellum and brain stem along the mid-sagittal plane) homogeneously throughout all animals (Fig. 1). Histopathological examination of the offspring brain was conducted at the three levels described above to evaluate qualitative changes[16]. In addition to these three levels of examination, for PND2, PND4, PND21, and PND28 pups, "step sections" of 3 µm thickness were made every third section in the mid-region of the brain including the hippocampus using the posterior block of the cerebrum[16].

Neuronal clusters of five or more cells were diagnosed as heterotopia[16]. Heterotopia is distinguished from other cells, such as glial cells, based on its larger nuclear size, small but visible cytoplasm, and expression pattern as clusters[16, 18, 19]. The number of neural cells in the maximal plane of heterotopia was counted and graded as ± for 5–9 cells, + for 10–19 cells, and 2+ for 20 or more cells to assess incidence and size[16]. Brain heterotopia on PND21 pups has been previously published[16]. To confirm the heterotopia observed by H&E staining, immunohistochemical staining using anti-NeuN antibody was conducted for representative affected PND21 pups, with step sections taken for immunostaining of other sets of every three sections prepared at the same time as the sections for H&E staining. Immunohistochemistry was performed using a HISTOSTAINER 48A (Nichirei Bioscience Inc., Tokyo, Japan) in accordance with the manufacturer's instructions[16]. After deparaffinization and heat-induced epitope retrieval in HEAT PROCESSOR Solution pH9 (Nichirei Bioscience Inc.) using HEAT PRO II (Nichirei Bioscience Inc.), endogenous peroxidase was inhibited using 3% hydrogen peroxide (H_2O_2) solution and the sections were incubated with rabbit monoclonal anti-NeuN antibody [EPR12763] (catalog no. ab177487, Abcam, Cam-

bridge, UK) with 100 times dilution for one hour following the incubation with Histofine Simple Stain™ Rat MAX PO (MULTI) (catalog no. 414191F, Nichirei Bioscience Inc.) for 30 minutes. Immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB)/H_2O_2 and counterstained using hematoxylin. Regarding thyroid histopathology, follicular cell hypertrophy was diagnosed when thyroid follicles were lined by taller cuboidal to columnar epithelium compared to the control animals and scored according to the degree of increase in cell height ratio to the nucleus as ± for <1.5, + for 1.5–<2.0, and 2+ for ≥2.0. Follicular cell hyperplasia was diagnosed only with ± grade for a slightly diffuse change with partial multiple layers of follicular epithelium protruding into the follicular lumen.

Statistical analyses

The following statistical tests were used to evaluate the significance of the differences between the control and treated groups. Litter was the statistical unit for all analyses except for the multi-way analysis of variance (ANOVA). The data sets of body weights, body weight gain, food consumption, and organ weights were first evaluated by the F test (α=0.05) for equality of variances. When group variances were homogenous, Student's t-test (α=0.05 or 0.01) was performed to detect any statistically significant difference between the control group and the treated group. When group variances were not homogenous, the Aspin–Welch test (α=0.05 or 0.01) was used. Serum and brain hormone measurements were also analyzed using Student's t-test (α=0.05 or 0.01) or Aspin–Welch test (α=0.05 or 0.01) when all data sets were above the LOQ values; otherwise, these data sets were analyzed using the Wilcoxon–Mann–Whitney test (α=0.05 or 0.01) with the <LOQ data replaced by the LOQ value. Fisher's exact probability test or Wilcoxon-Mann-Whitney test (α=0.05 or 0.01) was also used for the incidences and/or the grades of pathological findings. Multiway ANOVA was used to assess the influencing effects and interactions among the factors of treatment, age, and sex.

Results

The group mean values and standard deviations for each examination item are shown in Supplementary Tables 1–7.

6-PTU intake in dams

Averages of group mean 6-PTU intake of dams in the 6-PTU group during prenatal and postnatal periods were 0.58 and 1.00 mg/kg/day, respectively. The overall 6-PTU intake during the study period was approximately 0.80 mg/kg/day. Temporal changes in 6-PTU intake by the dams during the dosing period are shown in Supplementary Fig. 1a.

General condition of dams

No 6-PTU-related clinical signs were noted on palpation or cage-side observations during gestation or lactation in either cohort. The 6-PTU treatment significantly decreased maternal food consumption at the end of the gestation period and throughout the lactation period (except for LD4 and LD17) (Supplementary Fig. 1b). 6-PTU treatment slightly but significantly decreased maternal body weights on GD18 and GD20 but the body weights of the treated animals slightly exceeded the control level on LD21 (Supplementary Fig. 1c). The body weight gain of the 6-PTU group was slightly decreased (−11%) during GD6-GD20, but slightly increased (+11%) during LD0-LD13, and then significantly increased (+180%) during LD0-LD21 (Supplementary Table 1).

Thyroid effects in dams

Gross pathological examination showed that 6-PTU induced enlargement of the thyroid on GD20 (2/5 rats), LD2 (1/3 rats), and LD21(2/6 rats); however, these increases were not statistically significant. Histopathologically, 6-PTU significantly induced mild-to-moderate follicular cell hypertrophy and slight follicular cell hyperplasia on GD20 and LD21 (Table 1). 6-PTU treatment revealed non-significant or significant increases in absolute and relative thyroid weights; the relative values compared to the control were +73% and +78% in GD20 dams, +108% and +108% in LD2 dams, and +92% and +84% in LD21 dams, respectively (Supplementary Table 1, Supplementary Fig. 2).

Control serum T3 and T4 (but not TSH) increased as a function of age in samples collected from dams on GD20, LD2, and LD21 (Fig. 3), with significant effects of age on serum T3 and T4, as evaluated using two-way ANOVA test (Supplementary Table 8).

6-PTU treatment significantly decreased serum T3 concentrations at all time points evaluated in dams (Fig. 3a), with a significant effect of treatment analyzed by the ANOVA test; however, there was no significant treatment × age interaction on serum T3 (Supplementary Table 8), reflecting that serum T3 suppression by 6-PTU was similarly observed on GD20, LD2, and LD21 (Fig. 3a, Supplementary Fig. 3a).

Significant suppression of serum T4 concentrations was also observed on GD20, LD2, and LD21, and the absolute suppression values from the concurrent control group increased with age (Fig. 3b, Supplementary Fig. 3b). These conclusions were supported by the significant effects of treatment and treatment × age interaction, as analyzed using ANOVA (Supplementary Table 8). Consequently, serum T4 suppression by 6-PTU was greater on LD21 than on GD20 and LD2, even at higher T4 concentrations in the control LD21 dams (Fig. 3b, Supplementary Fig. 3b).

Concomitant significant increases in serum TSH concentrations were observed in the GD20 and LD21 dams (Fig. 3c). There was also a non-significant increase in TSH concentration on LD2 (8-fold control) (non-significance may be partly due to the small number of animals tested [N=3/group]). The significant effect on serum TSH level was due to treatment, as analyzed using ANOVA, but no significant effects were observed on age or treatment × age interaction (Supplementary Table 8). Therefore, 6-PTU treatment similarly increased serum TSH concentrations at all the time

Table 1. Summary of Thyroid Gland Histopathology in Dams and Offspring

Test animals		Examination day	Findings in thyroid gland	Control	6-PTU 10 ppm
Dams		GD20	Follicular cell hypertrophy	0/10	5/5 (+, 3; 2+, 2) ##
			Follicular cell hyperplasia	0/10	5/5 (±, 5) ##
		LD21	Follicular cell hypertrophy	0/10	6/6 (+, 4; 2+, 2) ##
			Follicular cell hyperplasia	0/10	6/6 (±, 6) ##
Male offspring	Fetuses	GD20	Decreased follicle lumen	4/10 (±, 4)	5/5 (±, 5) #
	Pups	PND2	Follicular cell hypertrophy	0/3	3/3 (±, 2; +, 1)
			Single cell necrosis, follicular epithelium	0/3	1/3 (±, 1)
		PND4	Follicular cell hypertrophy	0/5	3/3 (±, 1; +, 2)
			Single cell necrosis, follicular epithelium	0/5	1/3 (±, 1)
		PND21	Follicular cell hypertrophy	0/10	6/6 (±, 3; +, 3) ##
			Follicular cell hyperplasia	0/10	4/6 (±, 4) ##
		PND28	Follicular cell hypertrophy	0/10	6/6 (±, 3; +, 3) ##
			Follicular cell hyperplasia	0/10	4/6 (±, 4) ##
Female offspring	Fetuses	GD20	Decreased follicle lumen	4/10 (±, 4)	5/5 (±, 5) #
	Pups	PND2	Follicular cell hypertrophy	0/3	3/3 (±, 2; +, 1)
			Single cell necrosis, follicular epithelium	0/3	1/3 (±, 1)
		PND4	Follicular cell hypertrophy	0/6	2/2 (±, 1; +, 1)
		PND21	Follicular cell hypertrophy	0/10	4/4 (±, 2; +, 2) ##
			Follicular cell hyperplasia	0/10	3/4 (±, 3) ##
		PND28	Follicular cell hypertrophy	0/8	4/4 (±, 2; +, 2) ##
			Follicular cell hyperplasia	0/8	3/4 (±, 3) ##

Data present incidence/number of animals examined. The thyroid gland from PND28 pups were examined to determine transition in the incidences of findings after the termination of treatment at PND21. Severity grades for graded histopathological findings: slight ±, mild +, moderate 2+, severe 3+. Significantly different from control (Fisher test or Wilcoxon–Mann–Whitney test): $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$. GD: gestation day; LD: lactation day; PND: postnatal day.

points tested (Fig. 3c, Supplementary Fig. 3c).

Serum T3, T4, and TSH concentrations in offspring

Generally, similar responses to the treatment were observed in both sexes (Supplementary Figs. 4 and 6), supported by the lack of significant effects on sex and no interactions of treatment × sex and age × sex (Supplementary Table 8). Therefore, only the data for males are presented in Fig. 4a–4c (data for females are shown in Supplementary Fig. 5a–5c). Serum T3 concentrations (Fig. 4a) in offspring were two orders of magnitude lower than serum T4 concentrations (Fig. 4b), as in the maternal animals (Fig. 3a and 3b). In both sexes of GD20 fetuses, 6-PTU-treatment to dams dramatically decreased serum T3 (more than −50%) and T4 concentrations (more than −95%), with concomitant significant increases in serum TSH concentrations (4–5-fold control). However, the magnitude of the decrease in serum T3 was imprecise because all animals (10/10 rats) in the 6-PTU group were below the LOQ (0.010 ng/mL) and the control concentration (0.018 ± 0.004 ng/mL, Supplementary Table 3) was close to the LOQ. There were significant effects of treatment on serum T3, T4, and TSH concentrations (Supplementary Table 8), consistent with the above conclusions on 6-PTU effects.

There were significant effects of age on serum T3, T4, and TSH concentrations (Supplementary Table 8) reflecting age-dependent increases in serum T3 and T4 concentrations and a slight decrease in serum TSH concentrations (Fig. 4). Furthermore, significant treatment × age interactions by the ANOVA test were observed in serum T4 and TSH (but not T3) (Supplementary Table 8), which resulted from 6-PTU-induced decreases in serum T4 concentrations with an age-dependent increase (Fig. 4b) and 6-PTU-induced increases in serum TSH concentrations with an age-dependent reduction (Fig. 4c).

After one week of withdrawal treatment, the effects observed in PND28 pups were generally similar to those observed in PND21 pups (Fig. 4a–4c).

Brain T3 and T4 concentrations in offspring

Generally, similar responses to the treatment were observed in both sexes (Supplementary Figs. 7 and 8), supported by no significant effects on sex and no interactions of treatment × sex and age × sex (Supplementary Table 8). Therefore, only data for males are presented in Fig. 4d and 4e (data for females are shown in Supplementary Fig. 5d and 5e). Unlike serum, brain T3 and T4 concentrations in fetuses and pups were found to be of the same order of magnitude (Fig. 4d and 4e). Brain T3 concentrations were decreased by 6-PTU in GD20 fetuses (approximately −80%), PND2 pups (−35 to −60%), and PND4 pups (approximately −70%) but not in PND21 and PND28 pups (Fig. 4d). There were significant effects of treatment and age (but not sex) on brain T3 concentrations (Supplementary Table 8). A significant interaction of doing × age was observed in brain T3 (Supplementary Table 8) that resulted from a treatment effect from

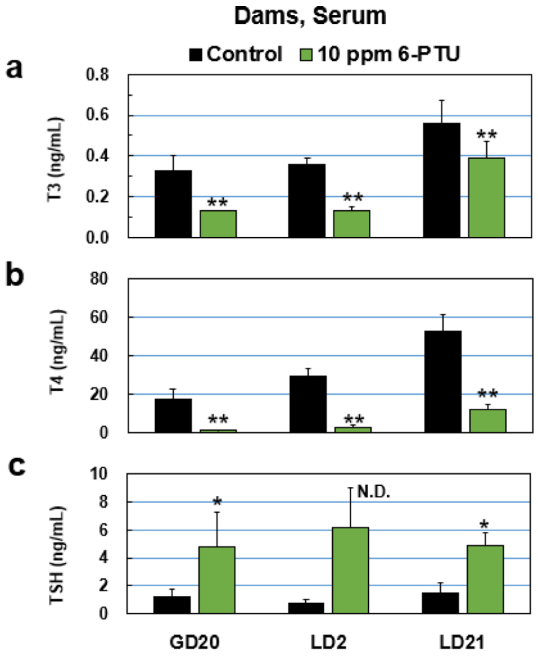

Fig. 3. Effects of 10 ppm 6-PTU on serum (a) T3, (b) T4, and (c) TSH concentrations in dams on gestation day (GD) 20 and lactation day (LD) 2 and 21. Values represent mean ± standard deviation of absolute values. Original data are shown in Supplementary Tables 1 and 2. The absolute suppression values from the concurrent control are shown in Supplementary Fig. 3. The control and 6-PTU groups comprised 10 and 5 animals on GD20, 3 each on LD2, 10 and 6 on PND21, respectively. Significantly different from control; *p≤0.05, **p≤0.01 by Student's t-test or Aspin–Welch test. N.D. means statistically no significant (p>0.05).

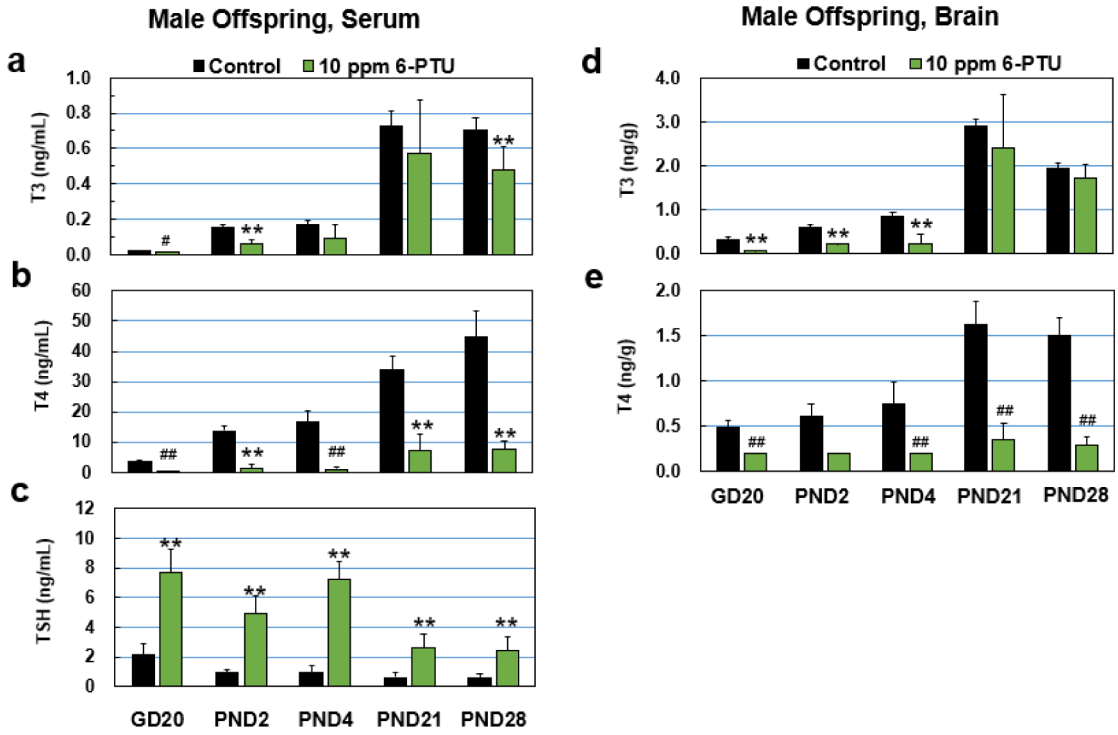

Fig. 4. Effects of 10 ppm 6-PTU on (a–c) serum and (d and e) brain hormone concentrations in male offspring on gestation day (GD) 20 and postnatal days (PND) 2, 4, 21 and 28. Values represent mean ± standard deviation. No significant sex differences were observed in the effects of 6-PTU treatment on hormone concentrations, therefore, only the data for males are plotted here. The data for females are shown in Supplementary Fig. 5. Original data are shown in Supplementary Tables 3–7. The control and 6-PTU groups comprised 10 and 5 animals on GD20, 3 each on LD2, 10 and 4 on PND4, and 10 and 6 on PND21 and PND28, respectively. Data on PND28 were collected to confirm persistence of the brain findings observed on PND21 after 1 week of withdrawal of treatment. Significantly different from control by Student's t-test or Aspin–Welch test (*p≤0.05, **p≤0.01) when all data sets were above the LOQ values; otherwise, these data sets were analyzed by the Wilcoxon–Mann–Whitney test ($^{\#}$p≤0.05, $^{\#\#}$p≤0.01) with the <LOQ data replaced by the LOQ value. Brain T4 concentrations in the 6-PTU groups were below the LOQ (0.20 μg/g) across all animals on GD20, LD2, and LD4, and in some animals on PND21 (3/12) and PND28 (2/12). Statistically non-significant reductions were observed in brain T3 of female PND2 pups and brain T4 of male and female PND2 pups, lack of significance possibly because of small number of animals examined (n=3 per each control and 6-PTU group).

GD20 to PND4 but not on PND21 and 28 (Fig. 4d).

In contrast to brain T3, brain T4 concentrations were significantly and consistently decreased during the study (−60% to −80%), even one week after cessation of 6-PTU treatment (i.e., in PND28 pups) (Fig. 4e). However, the magnitude of these decreases especially in GD20 fetuses and PND2 and PND4 pups is underestimated because brain T4 concentrations in all animals of the 6-PTU group were below the LOQ (0.20 ng/g brain weight; 10/10 rats on GD20, 6/6 rats on PND2, 8/8 rats on PND4), and control concentrations (0.49–0.89 ng/g brain weight, Supplementary Tables 3–5) were close to the LOQ. Brain T4 concentrations in 3/12 rats on PND21 and 2/12 rats on PND28 were also lower than the LOQ but control concentrations were higher than 1.50 ng/g brain weight (Supplementary Tables 6 and 7). There were significant effects of treatment and age (but not sex) on brain T4 concentrations (Supplementary Table 8). A significant treatment × age interaction was also observed in brain T4 concentrations (Supplementary Table 8) that resulted from a slightly larger treatment effect with age from GD20 to PND21 (GD20<PND2<PND4<PND21; Fig. 4e, Supplementary Figs. 7 and 8). One week after withdrawal, the effects on brain T4 concentrations observed in PND21 pups were also noted in PND28 pups (Fig. 4e), similar to those of serum T4 (Fig. 4b).

Histopathology in offspring

Gross pathological examination did not reveal any abnormalities in the thyroid or brain of the pups. In qualitative histopathological examinations, decreased follicle lumens were observed in all fetuses in the 6-PTU group tested on GD20 but some control fetuses also showed the same findings (Table 1). Hypertrophy and/or hyperplasia of follicular cells in the thyroid increased in both sexes at PND2, PND4, PND21, and PND28 in the 6-PTU group, with statistical significance in PND21 and PND28 pups. In this group, some pups showed single cell necrosis of the follicular epithelium on PND2 and PND4 but these findings were not observed in the control pups (Table 1).

No treatment-related changes were observed in the brains of GD20 fetuses (Table 2). Cell aggregation in the heterotopia forming region of the cerebrum (Fig. 5a and 5b) was observed in the control and treated pups on PND2 and PND4 but the severity of the finding increased in the 6-PTU group (Table 2). In the PND21 pups, cerebral heterotopia (Fig. 5c) was observed in five of six males and four of four females in the step sections, with statistically significantly increased severity in the 6-PTU group compared to control animals (Table 2). Cerebral heterotopia (Fig. 5d) was also observed in five of the five males and three of the three females on PND28 in the 6-PTU group (Table 2). Dislocation of the cerebrum was also noted on PND21 in the 6-PTU group (Table 2). We use the term "Dislocation" for the imbalanced figures of the cerebrum which included posterior components even though trimmed and examined at the same level, as previously reported[16]. This likely indicates a brain size difference between the control and 6-PTU groups (shorter longitudinal axis length in the 6-PTU group).

In addition, the severity and incidence of the external granular layer in the cerebellum (Fig. 6a) were significantly increased in both sexes of PND21 pups in the 6-PTU group (Table 2). However, this was not observed in PND28 pups (Fig. 6b, Table 2).

Discussion

Hypothyroidism induced by 10 ppm 6-PTU in the feeding study of modified CTA

According to the USEPA CTA guidance[13], oral administration (gavage) is typical for test chemicals; however, another route (method) of administration is used if justification and reasoning for its selection are provided. In the present study, dietary administration was selected because most pesticide toxicity studies have been conducted using feeding studies. The overall 6-PTU intake in the present study during the experimental period was approximately 0.80 mg/kg/day. Since Spring *et al.* [22] reported that pronounced heterotopia in terms of incidence and volume was induced by treatment with 3 ppm 6-PTU via drinking water (corresponding to approximately 0.3–0.4 mg/kg/day, which was reported by Marty *et al.*)[33], it appears that sufficient intake of 6-PTU was achieved in our experiment. In the present study, significant hypothyroidism was induced in both dams and offspring of the 6-PTU group.

Serum and brain TH concentrations in the control offspring gradually increased with age, which is consistent with previous findings[34]. Even under such conditions, a continuous suppression rate relative to concurrent control (more than approximately 80%) was observed in offspring T4 concentrations, consistent with a previous report[27]; and thus, absolute T4 reduction by 6-PTU was increased with aging in both serum and brain. This resulted from lower TH transfer from dams and the direct TH inhibition effect of 6-PTU, which was transferred through the placenta and milk[27, 35]. The critical period for heterotopia formation has been reported as GD19-PND2[28]. In the 6-PTU group, since serum T4 concentrations in PND4 and PND21 pups (approximately 1 and 7 ng/mL, respectively) were lower than control concentrations in PND2 pups (approximately 14–16 ng/mL), the T4 concentrations during GD19-PND2 in the 10 ppm 6-PTU group appeared to be low enough for heterotopia formation to occur and thus, the current study conditions were appropriate for seeking testing time points for heterotopia formation.

Although serum T3 was suppressed in dams at all time points, there were some age-specific suppressions in serum/brain T3 concentrations offspring: the suppression effects of 6-PTU were reduced with age, consistent with a previous report[27]. The suppression of T3 concentrations in serum and brain (more than 50% relative to concurrent control) was greater than thresholds of statistically significant neurodevelopmental effects (>20% and statistically significant offspring serum T3 reduction[33]). Furthermore, age-specific T3 reduction appears to be induced during the critical period

Table 2. Summary of Brain Histopathology in Offspring

Test animals		Examination day	Findings in brain	Control	6-PTU 10 ppm
Male offspring	Fetuses	GD20	Within normal limits	10/10	5/5
	Pups	PND2	Cell aggregation, HFR, cerebrum (step section)	3/3 (±, 3)	3/3 (+, 3) [#]
		PND4	Cell aggregation, HFR, cerebrum (step section)	5/5 (±, 5)	3/3 (±, 1; +, 2)
		PND21	Heterotopia, cerebrum (step section)	4/10	5/6 [##]
			± (Max = 5–9 cells)	4	0
			+ (Max = 10–19 cells)	0	0
			2+ (Max ≥20 cells)	0	5
			Dislocation, cerebrum	0/10	2/6
			External granular layer, cerebellum	1/10 (±, 1)	5/6 (+, 5) [##]
		PND28	Heterotopia, cerebrum (step section)	2/10	5/5[##]
			± (Max = 5–9 cells)	1	0
			+ (Max = 10–19 cells)	1	1
			2+ (Max ≥20 cells)	0	4
			Dislocation, cerebrum	0/10	0/5
			External granular layer, cerebellum	0/10	0/5
Female offspring	Fetuses	GD20	Within normal limits	10/10	5/5
	Pups	PND2	Cell aggregation, HFR, cerebrum (step section)	2/3 (±, 2)	3/3 (+, 3) (p=0.059)
		PND4	Cell aggregation, HFR, cerebrum (step section)	5/6 (±, 5)	2/2 (±, 1; +, 1)
		PND21	Heterotopia, cerebrum (step section)	0/10	4/4 [##]
			± (Max = 5–9 cells)	0	2
			+ (Max = 10–19 cells)	0	1
			2+ (Max ≥20 cells)	0	1
			Dislocation, cerebrum	0/10	2/4
			External granular layer, cerebellum	1/10 (±, 1)	4/4 (+, 4) [##]
		PND28	Heterotopia, cerebrum (step section)	3/8	3/3[#]
			± (Max = 5–9 cells)	3	0
			+ (Max = 10–19 cells)	0	1
			2+ (Max ≥20 cells)	0	2
			Dislocation, cerebrum	0/10	0/5
			External granular layer, cerebellum	0/10	0/5

Data present incidence/number of animals examined. Data of the brain on PND21 pups in the control and 6-PTU groups were previously published (Minami *et al.*[16]). The brain from PND28 pups were examined to determine transition in the incidences of heterotopia in the brain after the termination of treatment at PND21. Severity grades for graded histopathological findings: slight ± , mild +, moderate 2+. Significantly different from control (Fisher test or Wilcoxon–Mann–Whitney test): [#]p≤0.05, [##]p≤0.01. GD: gestation day; PND: postnatal day; HFR: heterotopia forming region.

for heterotopia formation (GD19-PND2)[28]. To identify the appropriate time point to test for heterotopia formation, attenuated TH suppression during the late lactation period in the present study had little impact on the primary research purpose of the present study (i.e., heterotopia assessment).

The reasons for the lack of significant reduction in serum and brain T3 concentrations in PND21 pups remain unknown. Because maternal milk does not contain substantial amounts of TH[36], the contribution of changes in maternal T3 transfer is unlikely. T3 production in PND21 pups may be enhanced by increased serum TSH concentrations induced by 6-PTU. Thus, this is thought to be the result of a balance between the direct suppression of T3 synthesis by 6-PTU and the stimulation of T3 synthesis by increased TSH, which is a result of the functional growth of the hypothalamus-pituitary-thyroid axis in pups.

Interestingly, the reduction of offspring T4 concentrations in the serum (−77 to −84%) and brain (−80%) was observed one week after secession of the 6-PTU treatment (i.e., PND28). In a previous study[27], serum 6-PTU concentrations decreased to undetectable levels during a 4-day recovery period; however, a reduction in serum T3 and T4 by 6-PTU in pups on PND14 was still observed in pups on PND18 but to a lesser extent. These findings suggest that T4 insufficiency was retained in the pups for at least one week without internal 6-PTU exposure.

Reliable testing time point for cerebral heterotopia formation in the modified CTA

Under the experimental hypothyroid conditions described above, heterotopia formation with increased incidence and severity was observed in PND21 pups in the 6-PTU group. Similar findings were observed in PND28 pups, suggesting that examination of heterotopia formation is acceptable even after PND21 in CTA. This is also supported by previous reports showing that fetal TH perturbations lead to irreversible malformations of the cortex, such as heterotopia[19, 28].

Increased severity of cell aggregation was also observed in the heterotopia forming region of the cerebrum

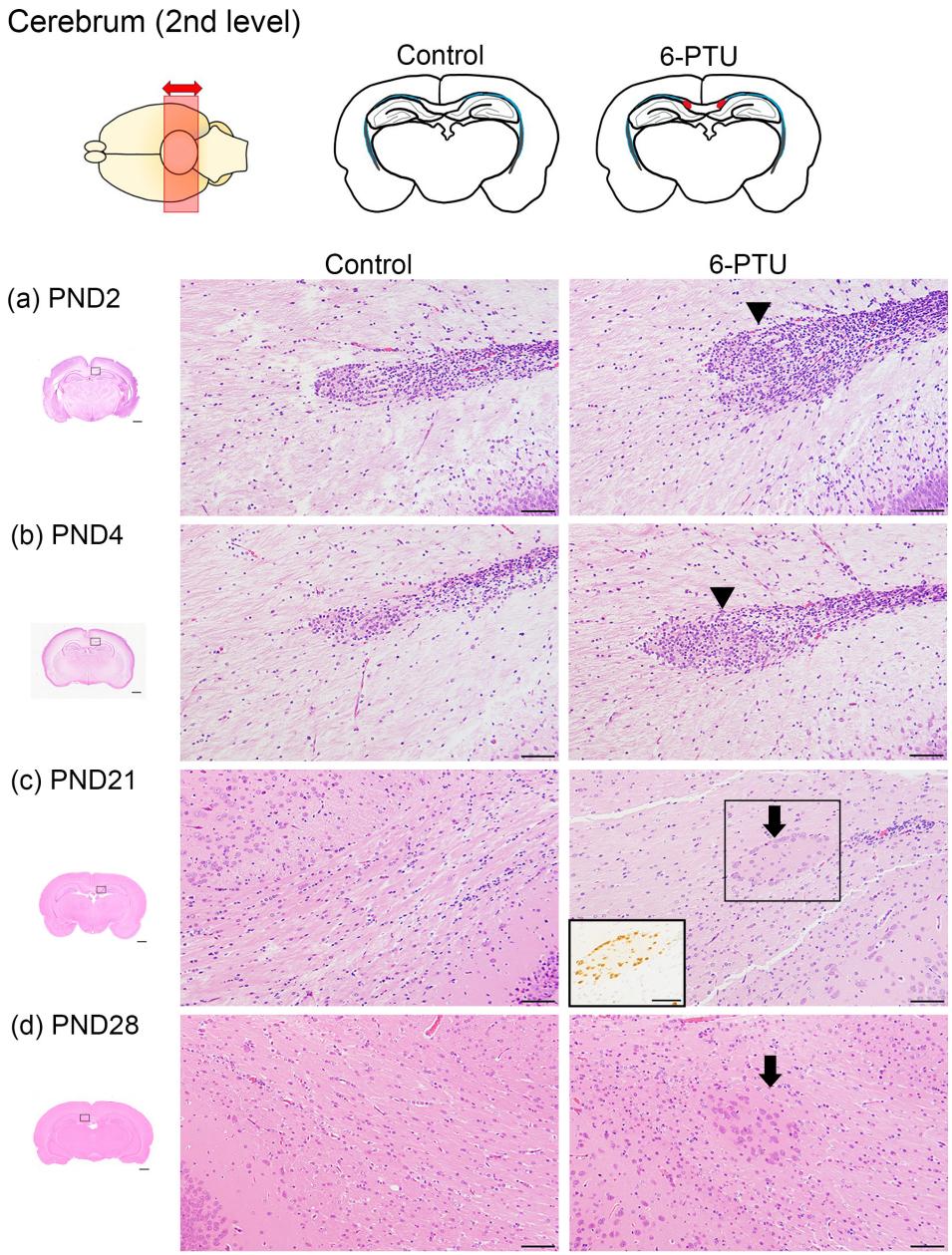

Fig. 5. Microphotographs in the cerebrum (2nd level area) in pups. On (a) PND2 and (b) PND4, cell aggregation in the heterotopia forming region adjacent to several layers of ventricular epithelium was increased in size and number in pups of the 6-PTU group (arrow heads). On (c) PND21 and (d) PND28, ventricular epithelium appeared to be more indistinct. Heterotopia was observed in the same area as with on PND2 and PND4 in the corpus callosum in pups of the 6-PTU group (arrows). Heterotopia was confirmed by anti-NeuN immunohistochemical staining (black frame). Hematoxylin and Eosin (H&E) staining unless otherwise specified. Scale bars=1 mm (low magnification H&E), 50 µm (high magnification H&E and anti-NeuN immunohistochemistry). These photos were taken from males as representative.

in PND4 pups in the 6-PTU group, consistent with a previous study[16]. This finding was also observed, but with less severity, in most control animals on PND4. This was also observed in animals younger than PND4 (i.e., PND2 pups). PND2 represents the end of a critical time window for heterotopia formation[28]. When the heterotopia assessment was conducted in this age range, a similar finding was observed, even in control animals, consistent with a previous report that tested on PND6[28]. Thus, heterotopia assessment on PND2 or PND4 may complicate the interpretation of test substance-induced results. In contrast to PND2 and PND4, the incidence and severity of heterotopia formation were much lower in the control animals on PND21 (and PND28) because normal cell migration was complete in most animals, making it relatively easy to detect the effect of the test substance. Indeed, even in the small number of rats tested,

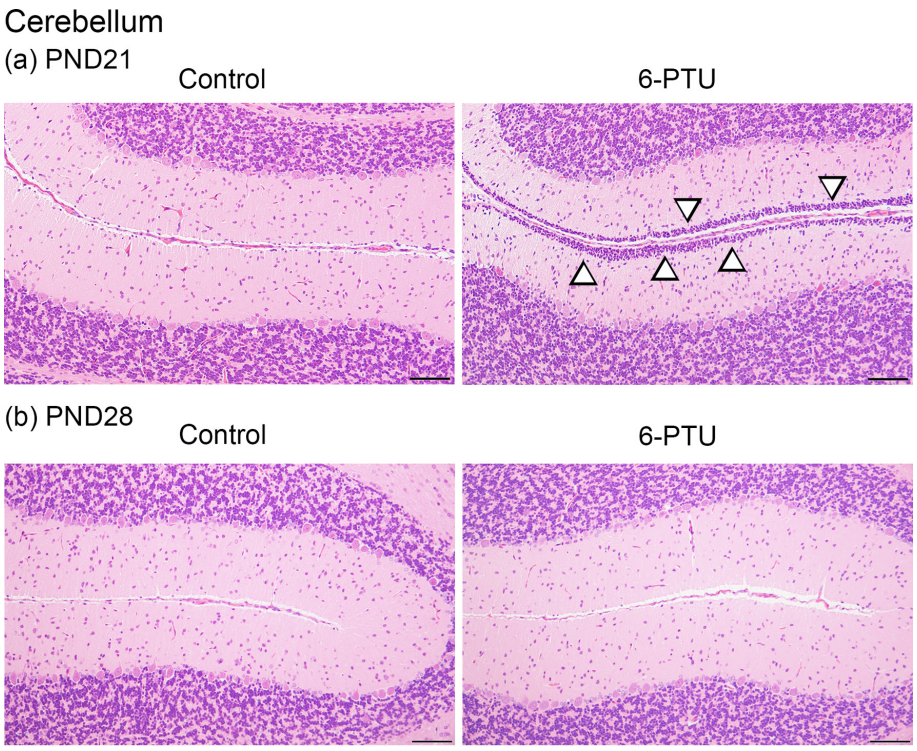

Fig. 6. Microphotographs of cerebellum in (a) PND21 and (b) PND28 pups. On PND21, the external granule layer (consisting of several layers) was clearly observed in the 6-PTU group, whereas it was almost or completely disappeared in the control group. On PND28, the external granule layer was no longer observed in both the control and 6-PTU groups. Hematoxylin and Eosin (H&E) staining. Scale bars=50 μm. These photos were taken from females as representative.

the effect of a relatively high dose of 6-PTU (10 ppm in the diet) on heterotopia formation was significantly detectable in our study. Gilbert *et al.* reported the specific characterization of heterotopia as a subcortical band as opposed to being periventricular in their investigations of heterotopia studies primarily performed in animals from PND14 to adulthood[2, 25, 27, 28]. Thus, in modified CTA, it is reasonable to conduct a heterotopia assessment on PND21 (and thereafter).

Allowing heterotopia assessment after PND21 is of practical value in CTA. The basal setting for heterotopia assessment would be primally on PND21 (one of the original test points in the CTA; USEPA[13]); however, if tissue sampling for histopathology is not available on PND21 due to practical reasons (e.g., manpower shortage) in the laboratory, later sampling (e.g., PND28) is also acceptable because heterotopia is a permanent finding[19, 27]. In this paper, we present experimental data using 6-PTU; however, as indicated in the Introduction, heterotopia formation is not exclusively associated with 6-PTU. Specifically, heterotopia has been reported in offspring examined after PND14, following perinatal TH deficiency induced not only by 6-PTU, but also by methimazole[18, 20] and amitrole[24], both of which are TPO inhibitors, as well as by perchlorate[25], an inhibitor acting on a different site, namely the sodium/iodide symporter (NIS), and in cases of an iodine-deficient diet[26]. Moreover, the presence of small heterotopias (approximately 0.005 mm^3) slightly above background levels in postnatal pups beyond PND28 has been associated with brain T4 concentrations modeled from fetal serum T4 changes of approximately 35% on GD20[37]. Therefore, assessing periventricular heterotopia in the postnatal rat brain from PND14 onwards is informative for evaluating TH insufficiency on CTA. However, further studies using other compounds or follow-up studies in other laboratories are required to confirm the sensitivity of the qualitative assessment of heterotopia formation in the proposed CTA.

Lack of sex differences in heterotopia formation in the modified CTA

In the present study, 10 ppm 6-PTU significantly increased the incidence and severity of heterotopia formation in both sexes of PND21 pups; thus, our study confirmed that a significant effect of 6-PTU treatment was observed without sex differences in TH disruptions (all p-values >0.05, for treatment × sex interaction) and heterotopia formation. To directly assess the potential sex-dependent effects on heterotopia formation, O'Shaughnessy *et al.* conducted an experiment in which dams were exposed to 3 ppm 6-PTU in drinking water from GD6 through GD14 and male and female littermates were directly compared for heterotopia volume[27]. This study demonstrated that heterotopia formation and severity were not sex dependent[27]. Therefore, the lack of sex differences in heterotopia formation observed in

the present study is reasonable.

The USEPA CTA guidance[13] states in Section (ii)C, *"Fetal blood should be collected and pooled by sex within litters for biochemical analyses"* and Section (iii)C, *"On PND 4 and PND 21, pup blood should be collected from one randomly chosen male and female offspring per litter. If necessary to increase sample volume, blood from all culled pups may be pooled by sex within litters".* Therefore, separate blood collection from male and female offspring is recommended. However, the present study demonstrated that no sex differences were detected in basal TH concentrations and chemical-induced suppression of serum and brain TH concentrations, which was consistent with previous findings[27, 33, 34, 38]. Considering that heterotopia formation also does not appear to have sex effects[27], we propose that the modified CTA can be conducted using examinations of one sex of offspring per litter.

Reliability of cerebellum findings as a useful downstream indicator for TH insufficiency in the modified CTA

In addition to heterotopia in the cerebrum, the external granular layer in the cerebellum was also significantly increased in PND21 pups in the 6-PTU group in a previous study[16]. However, the present study revealed that while significant serum/brain T4 reductions were observed in both PND21 and 28 pups of the 6-PTU group, this cerebellar finding was observed in PND21 pups but not in PND28 pups, suggesting that granular cell migration was completed on PND28 in both the control and 6-PTU groups. Granular cell migration in the cerebellum can be influenced by TH insufficiency but can also be affected by various internal and external factors[30, 31]. Thus, unlike cerebral heterotopia, the external granular layer in the cerebellum may not be a valid endpoint for modified CTA because it is less specific for TH insufficiency and is transient.

Conclusions

The present study confirms that cerebral heterotopia could be a useful downstream indicator of perinatal TH insufficiency in offspring if assessed at an appropriate time point. We suggest PND21 (and thereafter, if needed) and do not recommend PND4. This examination, together with measurement of brain TH concentrations in offspring, can augment the CTA performance as a reliable screening test for offspring TH disruptors, while further studies need to determine more a precise quantitative relationship among reductions of serum TH concentrations in dams, reduction of serum/brain TH concentrations in fetuses, and brain heterotopia formation in pups in the CTA protocol. A previous study using drinking water administration of 6-PTU demonstrated that the severity of cerebral heterotopia is dependent on the degree of hypothyroidism and is observable under low or moderate concentrations of maternal TH disruption in rats[18, 19, 21, 22, 25], we are currently examining the dose response of 6-PTU for cerebral heterotopia formation in a rat perinatal study via dietary treatment.

Heterotopia formation can be assessed by standard pathological examination but requires step sections with grading[16], which is practically available in many contract laboratories. However, because this pathological examination (step section with grading) requires considerable manpower, the development of a more efficient system, such as adaptation of diagnosis using artificial intelligence, will be necessary.

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