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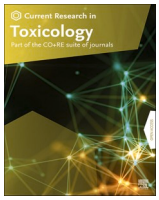

Prenatal test cohort of a modified rat comparative thyroid assay adding brain thyroid hormone measurements and histology but lowering group size appears able to detect disruption by sodium phenobarbital

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ABSTRACT

The Comparative Thyroid Assay (CTA, USEPA) is a screening test for thyroid hormone (TH) disruption in peripheral blood of dams and offspring. Recently, we began investigating feasible improvements to the CTA by adding examination of offspring brain TH concentrations and brain histopathology. In addition, we hypothesize that the number of animals required could be reduced by 50 % while still maintaining sensitivity to characterize treatment related changes in THs. Previously, we showed that the prenatal test cohort of the modified CTA could detect 1000 ppm sodium phenobarbital (NaPB)-induced suppression of brain T3 (by 9 %) and T4 (by 33 %) with no significant changes in serum T3 and T4 (less than 8 %). In the current study we expanded the dose response in a prenatal test cohort. Pregnant SD rats (N = 10/group) were exposed to 0, 1000 or 1500 ppm NaPB in the diet from gestational days (GD) 6 to GD20. Serum THs concentrations in GD20 dams together with serum/brain THs concentrations and brain histopathology in the GD20 fetuses were examined. NaPB dose-dependently suppressed serum T3 (up to −26 %) and T4 (up to −44 %) in dams, with suppression of T3 in serum (up to −26 %) and brain (up to −18 %) and T4 in serum (up to −26 %) and brain (up to −29 %) of fetuses but without clear dose dependency. There were no remarkable findings that deviated significantly from controls in GD20 fetal brain by qualitative histopathology. Overall, the present study suggests that the prenatal test cohort of this modified CTA is able to detect the expected fetal TH disruptions by prenatal exposure to NaPB, while also reducing the number of animals used by 50 %, consistent with the results of our previous study. These findings add to the suggestion that lowering group sizes and adding endpoints may be a useful alternative to the original CTA design.

Introduction

Adequate fetal and newborn thyroid hormone (TH) concentrations are essential for normal brain development in humans and animals (Gore et al., 2015; Landers and Richard, 2017; Gilbert et al., 2020, 2021; Sauer et al., 2020). Since some xenobiotic substances disrupt thyroid homeostasis, including effects on TH synthesis, metabolism, excretion and signaling, concern has been raised that TH disruptors may potentially interfere with the developing brain (Capen, 1997; Brucker-Davis, 1998; Hill et al., 1998; Crofton, 2008; Miller et al., 2009; Murk et al., 2013; Noyes et al., 2019).

The comparative thyroid assay (CTA) is a screening method designed to detect peripheral TH disruptions in dams and offspring (USEPA, 2005). More specifically, the 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 (Marty et al., 2021). However, it does not allow determination of whether effects detected in peripheral serum adequately characterize target tissue (i.e., developing brain) hormone concentrations (O'Shaughnessy and Gilbert, 2020), and thus may under- or over-estimate adverse points of departure in hazard assessments.

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Historically, serum THs and thyroid weight/histopathology have been common endpoints used in the regulatory setting (DeVito et al., 1999; USEPA, 2005). Although serum T4 is the primary source for brain THs (Landers and Richard, 2017), circulating concentrations may not be representative for the local TH changes (Reyns et al., 2003). Therefore, reliance on peripheral THs alone has been suggested to be insufficient predictive markers of adverse neurodevelopment (Gilbert et al., 2020, 2021; O'Shaughnessy and Gilbert, 2020). In addition, the original CTA requires a considerable number of animals (20 pregnant rats per dose group per cohort, total 160 pregnant rats for 4-dose group study) (USEPA, 2005), making adding other parameters practically difficult. Recently, to resolve such concerns, we proposed a modified CTA with additional parameters, such as examination of brain THs concentrations and brain histology as suggested previously (Gilbert et al., 2020, 2021, 2023, 2024; O'Shaughnessy and Gilbert, 2020) but using reduced numbers of rats (10 maternal rats per dose group per cohort) (Minami et al., 2023).

We began verifying the feasibility, sensitivity and reliability of the modified CTA, and we have already shown that the modified CTA could detect 10 ppm 6-propylthiouracil (6-PTU)-induced severe (>70 %) suppression of serum THs in dams, with >50 % suppressed serum/brain TH concentrations in fetuses/pups and brain heterotopia in postnatal day (PND) 21 pups using step sections with grading (Minami et al., 2023). 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 (Zoeller and Crofton, 2005; O'Shaughnessy et al., 2018). 6-PTU is a suggested positive control in the USEPA CTA guidance (USEPA, 2005).

When looking at the real world of the chemical hazard assessment, most chemicals are not likely to be as effective TH disruptors as the potent TH synthesis inhibitor 6-PTU, but may only result in mild TH disruption even at maximum tolerated dose levels. Thus, the utility of the CTA depends on its specificity (the response pathognomonic for alterations in thyroid function), sensitivity (the response of the method to low doses or to weak-acting chemicals), test duration, simplicity, and technical limitations of testing laboratories. Phenobarbital (PB or its salt, NaPB) produces a dose-dependent increase in hepatic UDPGT activity toward T4 via CAR activation and increases TH glucuronidation, that then produces a dose-dependent reduction in serum TH concentrations, increases in serum TSH, and ultimately increased TH synthesis in the thyroid follicular cells in rats (McClain et al., 1989; Liu et al., 1995; Hood et al., 1999a; O'Connor et al., 1999). Thus, PB is also recognized as a prototypical inducer of hepatic UDPGTs and also a well-known mild peripheral TH disrupter in adult rats (McClain et al., 1989; Liu et al., 1995; Hood et al., 1999a; O'Connor et al., 1999). PB appears to be transferable to the fetuses through the placenta and is rapidly distributed to fetal tissue (Ejiri et al., 2005). The concentration of RNAs for P-450 isoforms and UDPGTr2 remained nearly stable from late gestation to adult age, and PB stimulated the accumulation of UDPGTr2 RNA exclusively in hepatocytes even in fetuses of late gestation (Marie and Cresteil, 1989), suggesting that PB may induce TH disruption in fetuses via direct effects together with low transfer of TH from maternal rats. Many chemicals, like PB, produce mild TH disruption in adult rats through upregulation of liver enzymes, although the relevance of this to humans appears limited based on differences in specific kinetics between species as reviewed in Dellarco et al., 2006; Strupp et al., 2020; Foster et al., 2021. Nevertheless, for assessment of sensitivity of the modified CTA, since PB appears to exhibit the potential for TH disruption in pregnant animals and their offspring, its use as a reference chemical is valuable in strategic planning for testing many similar chemicals with TH disruption via liver enzyme induction. Therefore, in our previous verification study for the modified CTA, NaPB was selected as a second reference chemical together with 6-PTU (Minami et al., 2023). However, as PB or NaPB is a highly neuroactive drug (Dingemanse et al., 1989; Lumley et al., 2021), it is likely to affect brain development (Diaz and Schain, 1978; Middaugh et al., 1981). Nevertheless, considering that the CTA is not a test for DNT and the purpose of the CTA is to examine the extent to which a mild reduction of maternal TH can affect the TH concentration in offspring, the use of NaPB as a reference chemical with maternal mild TH disruption in the present study would provide useful information.

Significant hepatic enzyme up-regulation and mild but significant TH reductions were reported at 1200 ppm PB (T3 and T4 concentrations decreased 16–38 % and 18–28 % respectively (Hood et al., 1999b). Based on these findings, the dietary concentration of 1000 ppm NaPB was selected as a reference chemical for mild TH disruption in our previous study (Minami et al., 2023). Consequently, our previous study confirmed that 1000 ppm NaPB induced mild suppression of serum THs in GD20 dams (T3 and T4 concentrations decreased by 24 % and 16 % respectively), no significant changes in serum T3, T4, or TSH in GD20 fetuses, and suppression of brain TH concentrations in male (33 %) and female (15 %) fetuses but not in male and female pups (Minami et al., 2023). Since the magnitude of the effect in GD20 dams treated with 1000 ppm NaPB were less than our expectation, such <35 % suppression of brain TH concentrations in fetuses may be due to lower NaPB dosing in maternal rats.

In the present study, to better assess the sensitivity of the modified CTA to mild or moderate maternal TH disruption via liver enzyme induction, the effects of a somewhat higher dose of NaPB (e.g., 1500 ppm) were investigated in a prenatal test cohort of the modified CTA. To properly put in perspective the effects of 1500 ppm NaPB, 1000 ppm NaPB was also concurrently examined in the present study to assess the dose response, and to determine if the modified CTA maintains or improves the assay sensitivity. The modified CTA included the following changes: 1) a 50 % reduction in group size (i.e., from N = 20 in the CTA to N = 10); and 2) the additional endpoints including, fetal brain hormone concentrations and a qualitative assessment of fetal brain histopathology. We also measured hormones separately for male and female fetuses to support, or refute, the need for sex dependent measurements.

Materials and methods

Test chemicals

Phenobarbital sodium salt (NaPB; CAS# 57-30-7, Lot No.KCP6847; purity >98 %; FUJIFILM Wako Pure Chemical Co, Ltd., Japan) was used.

Animals, husbandry and mating

This study was conducted in the Institute of Environmental Toxicology (IET), which was the same facility as the previous study (Minami et al., 2023), 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 Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (currently Jackson Laboratory Japan, Kanagawa) at 12 weeks of age. After quarantine and acclimatization for 10 to 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 ℃), humidity (50 ± 20 %), ventilation (at least 10 times per hour; all fresh air system), and illumination (12 h 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) and local tap (chlorinated) water were provided *ad libitum* throughout the study. The animals were not fasted overnight prior to sacrifice. Copulated females were prepared by standard reproductive study methods (Aoyama et al., 2002, 2005, 2012); the day on which vaginal plugs and/or sperm were observed was designated as GD0. Pregnancy was confirmed by gross observation of the uterine

contents on GD20. The fertility index (number of pregnancies/number of females copulated × 100) was 100 % in each group.

Study design

The study was conducted with modifications of the CTA protocol designated by the USEPA Guidance (USEPA, 2005). In the original CTA, both prenatal test cohort and postnatal test cohort are designated. The present work utilized only the prenatal test cohort. NaPB was administered to pregnant rats, 10 females/dose group, at 0, 1000 or 1500 ppm based on the salt in diet, from GD6 through 20. Data from common control animals were shared with a 6-propylthiouracil study conducted concurrently to identify appropriate testing time of heterotopia in the CTA (to be published elsewhere). Mortality, clinical signs, body weights and food consumption of dams were periodically monitored throughout the study.

Clinical signs and mortality in maternal rats

Cage-side observations were made of maternal females 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 it was weighed instead of cage-side observation. During breeding, females were examined for the status of pregnancy. All abnormalities observed were recorded for individual females.

Tissue collection

To minimize the impact of circadian changes in hormone, samples were collected from the animals in all three groups at similar times of day and in an order of a control dam, a 1000 ppm dam, a 1500 ppm dam, followed by a control dam, a 1000 ppm dam, and so on. Maternal blood was collected from the posterior vena cava under isoflurane anesthesia using plain syringes on GD20. The blood was allowed to clot and centrifuged at 2000*g* for 10 min. at 4 ℃ to separate sera for hormone analyses. Blood sampling was generally performed in the afternoon (13:00–16:00) to ensure practical flexibility in conducting studies as previously reported (Minami et al., 2023). Rats were held in a curtained-off holding area adjacent to the necropsy room to avoid effects of any stress on hormone levels. In the NaPB-groups, plasma for measurement of blood PB concentrations was additionally prepared from approximately 2 mL of collected blood from the posterior vena cava. The sera and plasma were stored in a freezer (−70 ℃ or below) until use. Pregnant females were subjected to cesarean sectioning and gross necropsy after blood collection. The thyroid and liver of the dams were weighed and fixed in 10 % neutral-buffered formalin for histopathology. Portions of the left lateral lobe of the liver were also collected for UDPGT activity measurements and mRNA analyses prior to fixation.

Live fetuses were removed from the uteri and weighed individually. After external examination, blood was collected from all live fetuses into a serum separator tube by making a small cut on the neck without anesthesia. The collected blood was pooled by sex in each litter, processed for sera as noted above, and stored in a freezer (−70 ℃ or below). The fetal brain, thyroid and liver from a set of individuals (up to 2/sex/litter, if available) were fixed in 10 % neutral-buffered formalin for 2 days with the cranial, thoracic, and abdominal cavities exposed through incisions and preserved in 0.01 M phosphate-buffered saline at 4 ℃ until processed. In addition, the brain was removed from another set of fetuses (up to 2/sex/litter, if available), snap-frozen and stored in a freezer (−70 ℃ or below) for brain hormone analyses. Liver samples (left lateral lobe) were also collected from one fetus per sex per litter for mRNA analyses.

Thyroid hormone analyses

The frozen brains of fetuses (1/sex/litter) were individually weighed and homogenized with the same weight of water for injection as the brain sample. Serum samples collected from maternal rats and fetuses and homogenized brain samples were analyzed for the concentrations of T3 and T4 by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) analysis in singlicate. From the previous study (Minami et al., 2023), we slightly augmented the method of analysis by using a purification column and higher-grade MS/MS described below. 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 containing [$^{13}C_6$]T3 and [$^{13}C_6$]T4). After mixing, the mixture solution was centrifuged (16,000*g*, 5 min, 10 ℃). Then, 200 μL of 0.1 % formic acid was added to the solution, and the mixture solution was centrifuged (16,000*g*, 5 min, 10 ℃). An aliquot of the obtained supernatant was purified by a MonoSpin (MonoSpin Phospholipid, GL Sciences Inc., Tokyo). The eluate was then injected into the On-Line SPE LC-MS/MS (LC: 1290 HPLC, Agilent Technologies, Santa Clara, California; MS/MS: 6470 Triple Quad LC/MS, Agilent Technologies; SPE column: Shim-pack MAYI-ODS (G), 2.0 mm × 10 mm, Shimadzu Corporation, 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. Serum T3 concentrations in one male and female fetus in the NaPB 1500 ppm group were below the LOQ value (0.010 ng/ml), and thus group mean values for the NaPB 1500 ppm group were calculated as 0.01 ng/ml as individual data for these animals.

Serum thyroid stimulating hormone (TSH) analyses

Serum TSH concentration was measured by Immuno-beads assay using Milliplex Map Rat Thyroid Hormone TSH Panel (EMD Millipore, Burlington, Massachusetts) in singlicate. Each serum sample was incubated with beads and detection antibody according to the kit manual. Then, the fluorescence intensity of each sample was analyzed using a flow cytometer (FACSVerse, BD, Tokyo) with the FACSuite program. The LOQ for TSH was 62.5 pg/ml. No samples from dams or fetuses were below the LOQ value in this study. For the TSH Millipore assay, intra-assay precision was assessed by measuring 6 times for each sample using serum samples of 8 males and 8 females in SD rats. Coefficients of variation (CVs) ranged from 3.0 to 13.6 % in male and 4.3–9.1 % in female samples, which were within 15 %.

Liver UDPGT activity measurement

The frozen samples of the liver were homogenized with 1.15 % KCl using a homogenizer (Ultra Turrax®, Janke and Kunkel GmbH, Germany). The microsomal fraction was prepared by stepwise centrifugation at 9000*g* for 10 min and 105,000*g* for 60 min. For each microsomal suspension derived from the frozen liver samples of the maternal rats, microsomal protein content was determined by DC Protein Assay Kit (Nippon Bio-Rad Laboratories, Tokyo) according to the augmented method of Lowry. To determine the enzyme activity, as presented previously (Minami et al., 2023), a diluted microsomal suspension (at the constant concentration of 0.2 mg protein/mL) was incubated with 5 mM 4-hydroxybiphenyl and 30 mM UDP-glucuronic acid at 37 ℃ for 30 min in the presence of 50 mM $MgC1_2$ in 0.14 M Tris-HC1 buffer (pH 7.4). An assay was stopped by 0.5 M perchloric acid. Then, fluorescence intensity of the 4-hydroxybiphenyl glucuronide was determined at 325 nm with excitation at 290 nm using a fluorescence spectrophotometer. The enzyme activity is expressed as a percentage of the glucuronide

fluorescence intensity per mg protein over the group mean value of the controls.

Measurement of plasma phenobarbital concentrations

Concentrations of phenobarbital (PB) were determined in maternal plasma by LC-MS/MS analysis. The sample solution of plasma/methanol/water was prepared by adding the solution of methanol/water to the plasma and then subjecting it to a solid-phase extraction using PLS-2 cartridge (InertSep PLS-2, 270 mg/6 mL; GL Sciences Inc., Tokyo), and PB was determined by LC-MS/MS (LC: 1290 HPLC, Agilent Technologies; MS/MS: 6460 Triple Quad LC/MS, Agilent Technologies; LC column: ZORBAX Eclipse Plus C18, 1.8 μm × 2.1 mm × 50 mm, Agilent Technologies).

Measurements of organ weights and mRNA expression levels by quantitative real-time PCR

These measurements were conducted by standardized methods. Details of the measurements are presented in Supplemental information.

Histopathology

Liver and thyroid glands from the dams, and fetal brain were processed and subjected to histopathological examination. For fetal brain, the cerebrum was cut coronally at the levels anterior and caudal to the infundibulum, as vertically as possible, to make two paraffin blocks. Cerebellum and brainstem were cut in the mid-sagittal plane as vertically as possible to make 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.

Since there is theoretical concern that NaPB may have direct effects on brain tissue as a barbiturate antiepileptic drug (Dingemanse et al., 1989; Lumley et al., 2021), histopathological examination of the brain for fetuses was conducted at the three levels to evaluate for any qualitative changes. The three blocks of the brain prepared as described above were used and carefully thinned to obtain the three levels of planes (the first level [cerebrum 1] included cerebral cortex and caudate/putamen, the second level [cerebrum 2] included cerebral cortex, hippocampus, thalamus, and hypothalamus, and the third level included cerebellum and brain stem along the mid-sagittal plane) homogeneously throughout all animals, which was modified for GD20 from Garman et al. (2016) (Supplemental Fig. 1). As shown in the *Results Section,* the fetal brain TH reductions were similarly observed at 1000 and 1500 ppm (approximately 20 % for T3 and 30 % for T4). Thus, histopathological examination of the fetal brain was conducted in the 1000 ppm group to eliminate the possible NaPB' pharmacological effects as much as possible. Left lateral lobe and right medial lobes of the liver and both thyroids were processed and subjected to light microscopic examination for dams.

Statistical analyses

The following statistical tests were used to evaluate significance of differences between the control and each NaPB group. The data sets, including body weight, food consumption, organ weight, UDPGT activity and mRNA expression in maternal females; body weight and UDPGT mRNA expression in fetuses, were first evaluated for homogeneity of variance by Bartlett's test ($\alpha = 0.05$). When group variances were homogenous, a parametric one-way ANOVA in one-way classifications ($\alpha = 0.05$) was used to determine if there was a main effect of treatment. When the ANOVA was significant, Dunnett's multiple comparison test ($\alpha = 0.05$ or 0.01) was performed to detect statistically significant difference between each treated group and their corresponding controls. When Bartlett's test indicated that the variances were not homogeneous, the Kruskal-Wallis test ($\alpha = 0.05$ or 0.01) was used for test for a main effect of treatment, and when significant, Dunnett-type nonparametric multiple comparison ($\alpha = 0.05$ or 0.01) was performed to detect statistical differences between each treated group and their corresponding controls. The litter was the statistical unit for above analyses. Serum hormone data in dams were also analyzed in the same manner as the other parametric data (e.g., body weight) with Dunnett's multiple comparison test. Regarding with serum and brain hormone data in fetuses, a two-way ANOVA was used to analyze for main effects of treatment and sex, and for a treatment-by-sex interaction. When there were significant effect of treatment and no treatment-by-sex interaction, Dunnett's multiple comparison test ($\alpha = 0.05$ or 0.01) was performed to detect statistically significant difference between each treated group and their corresponding controls in the combined data of males and females. Fisher's exact probability test or Wilcoxon-Mann-Whitney test ($\alpha = 0.05$ or 0.01) was also used for the incidences of pathological findings.

Statistical power and required group sizes were determined using EZR, version 1.61 (Kanda, 2013). Since not enough historical background data were available at the time, the statistical power was estimated using mean ± standard deviation (SD) in the control group. To detect a 25 % decrease in TH or a 40 % increase in TSH (Li et al., 2019; Marty et al., 2021), the number of animals required per group, as well as the statistical power in the case of 10 animals per group, were calculated. These changes were detectable in the dose group compared to the control group using Student's *t*-test ($\alpha = 0.05$). Based on data from the control group and each NaPB group in the present study, post hoc statistical power was also calculated to detect a hormone concentration which can be detected in dose group compared to controls by Student's *t*-test ($\alpha = 0.05$) and shown in Supplemental Tables 2 and 3.

Results

Group mean values or incidence of each examination in dams and GD20 fetuses are summarized in Supplemental Tables 2 and 3, respectively.

NaPB intake and plasma PB concentrations in dams

Group mean NaPB intake as estimated from food intake data is shown in Fig. 1A. During the treatment period, NaPB intake was generally stable. Average NaPB intakes of the 1000 and 1500 ppm groups during GD6-20 were 60 and 84 mg/kg/day, respectively. As the NaPB intakes in the 1500 ppm group were 1.4-fold of that of the 1000 ppm group, planned target dose ratio was generally achieved. Plasma PB concentrations of the 1000 and 1500 ppm groups after designated treatment periods were dose-dependently detected in GD20 dams; 86.4 ± 19.5 mg/L and 139 ± 29.9 mg/L, respectively. PB concentration in the 1500 ppm group was 1.6-fold of the 1000 ppm group, which was consistent with the NaPB intakes.

General condition and cesarean section parameters of dams

NaPB treatment did not lead to increased maternal deaths. There was also no effect of NaPB on maternal body weights (Fig. 1B) or food consumption (Fig. 1C). NaPB treatment did produce staggered gait in 10 of 10 animals in the 1500 ppm. No treatment effect was detected for number of implantations, number of live fetuses, % resorption and fetal death, and sex ratio (Supplemental Table 2).

Serum concentrations of thyroid hormones and thyroid stimulating hormone in GD20 dams

In GD20 dams, NaPB-treatment decreased both serum T3 and T4 concentrations with dose-dependency, but statistical significance was only observed in the 1500 ppm group (Fig. 2A, B). Serum TSH concentrations were not significantly increased but rather decreased by 46 % in

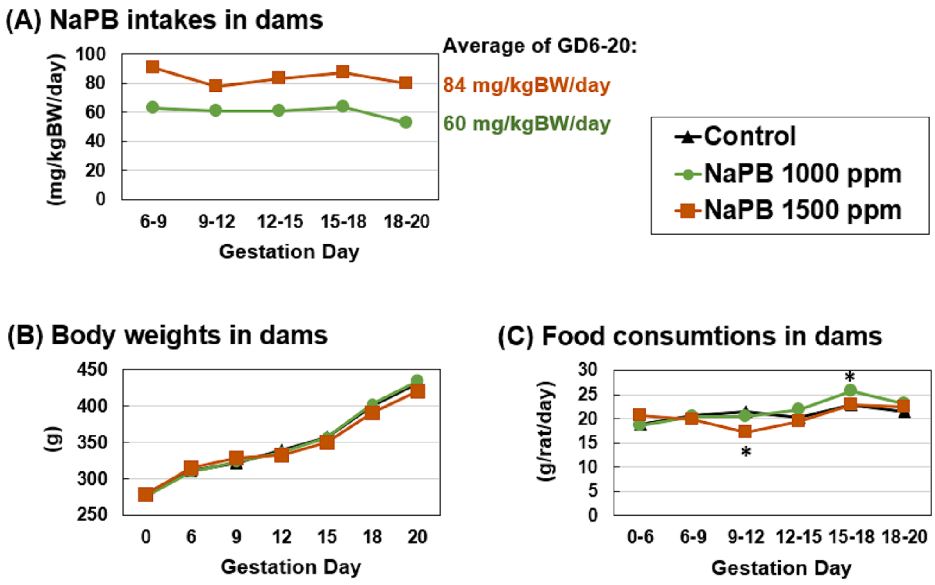

Fig. 1. NaPB intakes (A), group mean body weights (B) and food consumptions (C) in dams. N = 10 animals per group. Significantly different from control; * $p \leq 0.05$ by Dunnett's test following one-way ANOVA or Dunnett's-type test following Kruskal-Wallis test.

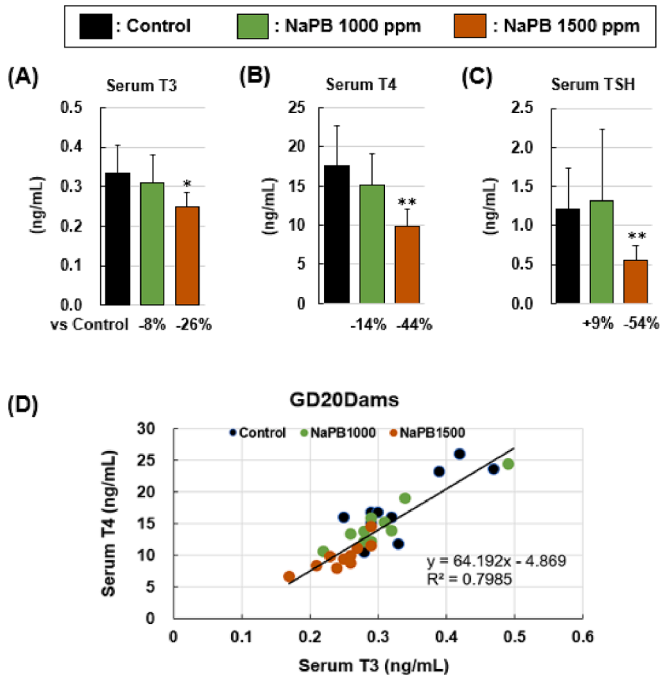

Fig. 2. Effects of NaPB on serum T3 (A), T4 (B) and TSH (C) concentrations in dams on gestation day 20. Scatter plots of individual values of serum T3 and T4 concentrations in dams (D). Values represent mean ± standard deviation, N = 10 animals per group. Significantly different from control; ** $p \leq 0.01$ by Dunnett's test following one-way ANOVA or Dunnett's-type test following Kruskal-Wallis test. The absolute values are presented in Supplemental Table 2.

the 1500 ppm group (Fig. 2C).

Scatter plots of individual values revealed that there was a high correlation between serum T3 and serum T4 concentrations ($R^2 = 0.80$, Fig. 2D). However, clear correlation ($R^2 > 0.5$) was not observed between serum TSH and T3 ($R^2 = 0.29$) or T4 ($R^2 = 0.20$) concentrations (Supplemental Fig. 2A, B). When focused on the control and 1500 ppm groups ($R^2 = 0.34$ for T3, $R^2 = 0.22$ for T4) compared to when focused on the control and 1000 ppm groups ($R^2 = 0.18$ for T3, $R^2 = 0.05$ for T4), the R^2 remained small but increased (Supplemental Fig. 2C-F). Serum T3 and T4 concentrations were reduced dose-dependently (Fig. 2A, B), while the increases in hepatic UDPGT activities were similarly observed in GD20 dams in the 1000 and 1500 ppm groups as described below (Fig. 3D). Consequently, no correlation was observed ($R^2 < 0.21$) between serum TH concentrations and liver UDPGT activity in GD20 dams (Supplemental Fig. 4B). Overall, these findings suggest that, in the 1500 ppm group, significantly lower serum T3 and T4 concentrations may partially contribute to the decreased serum TSH concentrations in addition to the increased TH catabolism via increased UDPGT activity by NaPB.

Liver and thyroid alterations in GD20 dams

NaPB-treatment at both 1000 and 1500 ppm significantly induced liver responses in GD20 dams; including the increased liver weights but without dose dependency (Fig. 3A), the increased *Cyp2b1/2* [a marker for constitutive androstane receptor (CAR) activation] (Fig. 3B) and *Ugt2b1* mRNA (Fig. 3C) levels with dose dependency, and the increased UDPGT activities from all animals (n = 10 per group) without dose dependency (Fig. 3D). However, when focusing on animals that mRNA was examined (n = 6 per group), the increased UDPGT showed some dose dependency (Supplemental Fig. 3A), and thus, scatter plots of individual values revealed that there was a correlation between UDPGT activity and *Ugt2b1* mRNA ($R^2 = 0.56$, Supplemental Fig. 3B). Correlations were not observed ($R^2 < 0.21$) between serum TH concentrations and liver *Ugt2b1* mRNA expression or UDPGT activity in GD20 dams (Supplemental Fig. 4A, B). Slight centrilobular hypertrophy of hepatocytes was observed in all GD20 dams of the 1000 ppm NaPB group tested (Table 1).

In the thyroids, slight follicular cell hypertrophy was observed in one GD20 dam of the NaPB groups but not observed in the other animals (Table 1), and no thyroid weight changes were observed (Fig. 3E).

Serum concentrations of thyroid hormones and thyroid stimulating hormone in GD20 fetuses

For serum T3 in GD20 fetuses, there was no significant interaction between treatment and sex, and there was a significant main effect of treatment, but not main effect of sex (Table 2). Serum T3 concentrations in the control GD20 fetuses were quite low (0.019 ± 0.004 ng/ml, n = 20). In the 1500 ppm NaPB group, 1 of 10 fetuses per sex had less than

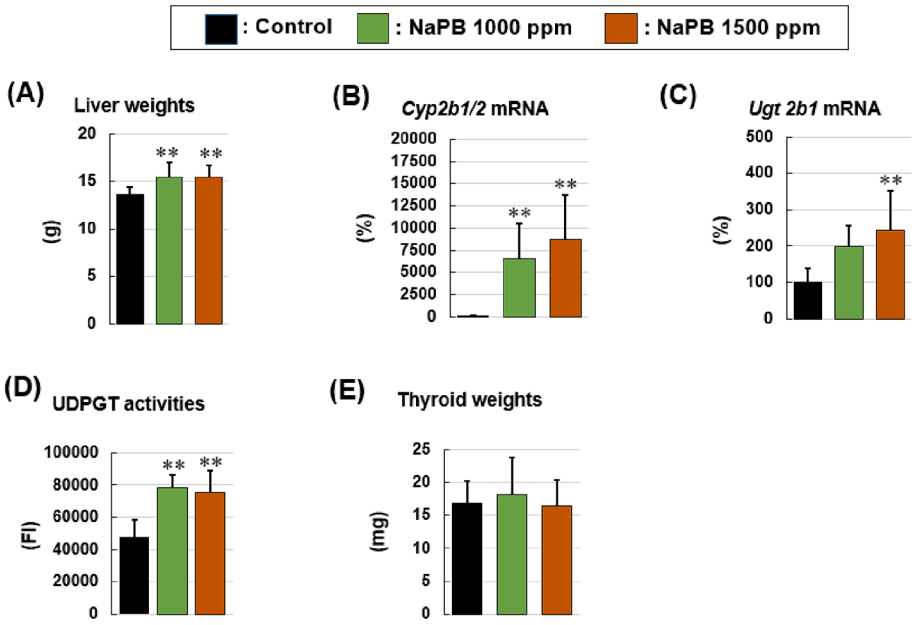

Fig. 3. Effects of NaPB on liver and thyroid in dams on gestation day 20. Values represent mean ± standard deviation, N = 10 animals per group. Expression levels of mRNA (B and C) were examined in 6 animals per group, and mRNA levels were normalized to those of *Actb* mRNA. FI in (D) means glucuronide fluorescence intensity (per 0.2 mg protein). Significantly different from control; * $p \leq 0.05$, ** $p \leq 0.01$ by Dunnett's test following one-way ANOVA or Dunnett's-type test following Kruskal-Wallis test. The absolute values are presented in Supplemental Table 2.

Table 1
Summary of histopathology.

Dose levels	Control	NaPB 1000 ppm
GD20 Dams		
Liver: Centrilobular hepatocyte hypertrophy	0/10	10/10[±, 10] ##
Liver: Necrosis, hepatocyte, focal	1/10	1/10
Thyroid: Follicular cell hypertrophy	0/10	1/10[±, 1]
GD20 male fetuses		
Brain: Within normal limits	10/10	10/10
GD20 female fetuses		
Brain: Within normal limits	10/10	9/9

GD, gestation day.
Data present incidence/number of animals examined. Since apparent abnormality of the brain was not observed in the 1000 ppm group, histopathology was not conducted in the 1500 ppm group. The thyroid glands were examined only for dams.
Severity grades for graded histopathological findings: slight ±, mild +, moderate ++, severe +++.
Significantly different from control (Fisher test or Wilcoxon-Mann-Whitney test):## $p \leq 0.01$.

Table 2
Interaction analysis of treatment and sex in serum THs and TSH and brain THs in GD20 fetuses.

	Interaction of treatment and sex	Main effects of treatment	Main effects of sex
Serum T3	Not significant (P = 0.73)	**	Not significant (P = 0.49)
Serum T4	Not significant (P = 0.60)	***	**
Serum TSH	Not significant (P = 0.96)	**	Not significant (P = 0.47)
Brain T3	Not significant (P = 0.99)	***	Not significant (P = 0.91)
Brain T4	Not significant (P = 0.77)	***	Not significant (P = 0.06)

Interaction of treatment and sex in serum TH and TSH and brain TH in fetuses was analyzed by two-way ANOVA.
Significantly different from control (two-way ANOVA): * ≤0.05, **≤0.01, ***≤0.001.

LOQ value (0.010 ng/ml). Significant suppressions of serum T3 were observed by Dunnett's test in the 1000 and 1500 ppm NaPB groups (Fig. 4A). Little dose-dependence of these changes was observed.

As for serum T4, a two-way ANOVA revealed no significant interaction between treatment and sex (Table 2). There were main effects of both treatment and sex. Serum T4 concentrations were also significantly decreased by both dose levels of NaPB by Dunnett's test, and the reductions were also with little dose-dependence (Fig. 4B).

For serum TSH in GD20 fetuses, there was no interaction between treatment and sex while there was a significant main effect of treatment, but not for sex (Table 2). Serum TSH concentrations appeared to be slightly but significantly increased by 1000 and 1500 ppm NaPB by Dunnett's test but the increase was not dose dependent (Fig. 4C). Scatter plots analyses revealed no correlations between serum TSH and T3 concentrations ($R^2 = 0.09$) or serum TSH and T4 concentrations ($R^2 = 0.18$) (Supplemental Fig. 5A, B). No correlation between serum T3 and serum T4 concentrations ($R^2 = 0.13$) was also observed (Supplemental Fig. 5C).

Comparison of effects of NaPB on serum hormone concentrations between GD20 dams and GD20 fetuses

Alteration rates by NaPB in serum T3, T4 and TSH concentrations of GD20 dams and GD20 fetuses are presented in Fig. 5A. Compared to dams, predominant responses were observed in serum T3, T4 and TSH of the 1000 ppm fetuses. In the 1500 ppm group, the fetal response in serum T3 was similar to dams, but the fetal response in serum T4 was lower than that in dams. In terms of serum TSH concentrations, significant increases were observed in fetuses, but significantly decreased in dams (Fig. 5A).

For serum T4, potentiation with increasing doses was smaller in fetuses [x1.3 (26 %/20 %) when using average of males and females] than

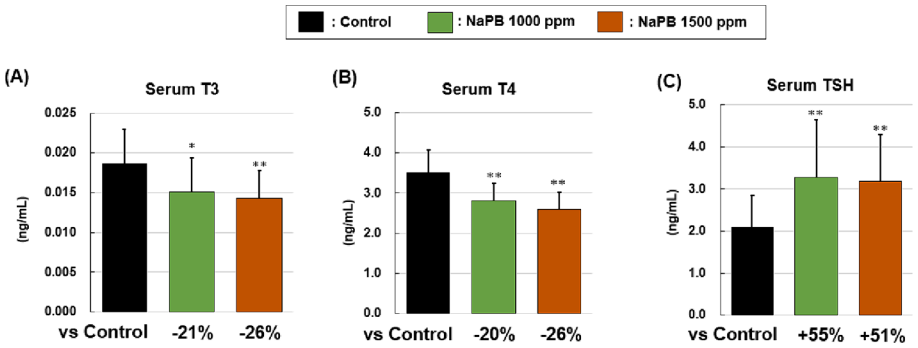

Fig. 4. Effects of NaPB on serum T3 (A) and T4 (B) and TSH (C) concentrations in fetuses on gestation day 20. Since a two-way ANOVA revealed no significant interaction between treatment and sex in serum T3, T4 and TSH, combined data from both sexes were presented. Values represent mean ± standard deviation, N = 20 animals per group. Significantly different from control; * $p \leq 0.05$, ** $p \leq 0.01$ by Dunnett's test. The absolute values of group mean in each sex are presented in Supplemental Table 3.

(A)

	Serum hormone	T3	T4	TSH
	[Expected alterations]	[Decrease]	[Decrease]	[Increase]
1000 ppm NaPB	GD20 dams	NE (-8%)	↓ (-14%)	↑ (+9%)
	GD20 fetuses	**↓ (-21%*)** [a]	**↓ (-20%**)** [a]	**↑ (+55%**)** [a]
1500 ppm NaPB	GD20 dams	↓ (-24%*)	↓ (-44%**)	↓ (-54%**) [c]
	GD20 fetuses	↓ (-26%**)	**↓ (-26%**)** [b]	**↑ (+51%**)** [a]

↓ and ↑ means "Decrease" and "Increase", respectively.

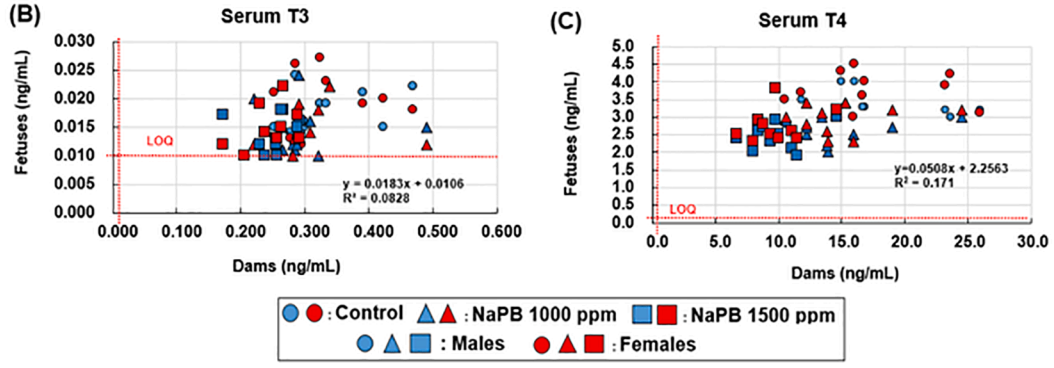

Fig. 5. Comparison of effects of NaPB-treatment on serum TH concentrations between dams and their fetuses. (A) Percent change from control mean values induced by NaPB. Original data are presented in Supplemental Tables 2 and 3. In fetuses, since a two-way ANOVA revealed no significant interaction between treatment and sex in serum T3, T4 and TSH, combined data from both sexes were used for analysis of statistically significant difference between each treated group and their corresponding controls. Values in parenthesis represent % of control mean value. NE means no effect because of minor difference (less than 10 %) from control values. [a] Predominant response in fetuses compared to dams, which are shown with bold red highlight. [b] Lesser responses in fetuses compared to dams, which are shown with bold blue highlight. [c] Unexpected alterations in dams but considered biologically of little significance. Significantly different from control in dams (Dunnett's test following one-way ANOVA or Dunnett's-type test following Kruskal-Wallis test: * $p \leq 0.05$, ** $p \leq 0.01$.). Significantly different from control in fetuses (Dunnett's test following two-way ANOVA: * $p \leq 0.05$, ** $p \leq 0.01$.). (B and C) Scatter plots of individual values of serum T3 and T4 concentrations in dams and fetuses. For serum T3 in fetuses, one fetus in both sexes of the NaPB 1500 ppm group showed less than limits of quantification (LOQ) but plotted as LOQ. Total 30 fetuses per sex including all animals from the control, and NaPB 1000 and 1500 ppm groups were used for scatter plot analysis. The LOQ for serum T3 and T4 were 0.010 and 0.2 ng/ml, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in dams [x3.1 (44 %/14 %)] (Fig. 5A). A similar difference was also observed for serum T3. Scatter plot analysis of individual values of serum T3 and T4 revealed little co-relationship between dams and fetuses for either hormone (R^2 for T3 and T4 were 0.08 and 0.17, respectively) (Fig. 5B, C). For serum TSH concentrations, the NaPB-induced effects were in the opposite direction in fetuses and dams (Fig. 5A). Although the decreased THs and the increased TSH were observed as expected in fetuses, no correlations between serum TSH and T3 or T4 were observed in fetuses (R^2 for T3 and T4 were 0.09 and 0.18, respectively, as mentioned above) (Supplemental Fig. 5A, B). There was no co-relationship between serum T3 and T4 concentrations ($R^2 = 0.13$) (Supplemental Fig. 5C).

Brain concentrations of thyroid hormones in GD20 fetuses

For brain T3 and T4 in GD20 fetuses, there was no significant interaction of treatment and sex (Table 2). There was a significant main effect of treatment, but not for sex (Table 2). Brain T3 and T4 concentrations were significantly decreased at 1000 and 1500 ppm of NaPB by Dunnett's test, but no dose-dependency (Fig. 6A, B). Scatter plot analysis

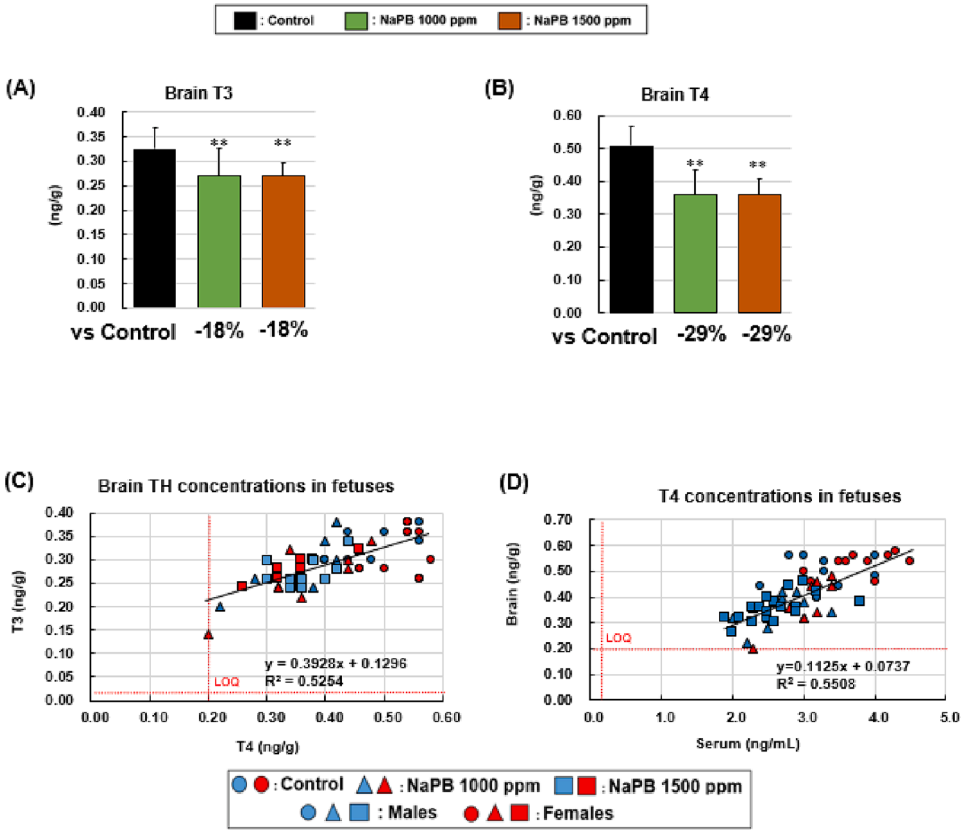

Fig. 6. Effects of NaPB on brain T3 (A) and T4 (B) concentrations in fetuses on gestation day 20. Since a two-way ANOVA revealed no significant interaction between treatment and sex, combined data from both sexes are presented. Values represent mean ± standard deviation, N = 20 animals per group. Significantly different from control; ** $p \leq 0.01$ by Dunnett's test. The absolute values of group mean in each sex are presented in Supplemental Table 3. Scatter plots of individual values of brain T3 and T4 concentrations (C) and serum and brain T4 concentrations (D) in GD20 fetuses.

Table 3
Statistical power analysis.

		Mean ± SD in the control group	Number of animals examined per group	Coefficient of variation (%)	25 % decrease in TH, 40 % increase in TSH supported by Li et al (2019) and Marty et al (2021)	
					Statistical power (%) for detecting:	Required group size for detecting:
GD20 Dams	Serum T3 (ng/mL)	0.336 ± 0.070	10	21	77	13
	Serum T4 (ng/mL)	17.6 ± 5.1	10	29	49	22
	Serum TSH (ng/mL)	1.2 ± 0.54	10	45	51	20
GD20 male fetuses	Serum T3 (ng/mL)	0.018 ± 0.004	10	22	80	11
	Serum T4 (ng/mL)	3.3 ± 0.5	10	15	96	6
	Serum TSH (ng/mL)	2.17 ± 0.76	10	35	61	12
	Brain T3 (ng/g)	0.33 ± 0.04	10	12	99	4
	Brain T4 (ng/g)	0.49 ± 0.07	10	14	97	6
GD20 female fetuses	Serum T3 (ng/mL)	0.020 ± 0.005	10	25	61	16
	Serum T4 (ng/mL)	3.8 ± 0.5	10	13	99	5
	Serum TSH (ng/mL)	2.04 ± 0.74	10	36	70	14
	Brain T3 (ng/g)	0.33 ± 0.05	10	15	95	7
	Brain T4 (ng/g)	0.53 ± 0.04	10	8	100	2

GD = gestational day.

for individual values of T3 and T4 concentrations in brain revealed co-relationship ($R^2 = 0.53$) (Fig. 6C). T4 concentrations also revealed co-relationship between serum and brain ($R^2 = 0.55$) (Fig. 6D). There was no co-relationship between serum and brain T3 concentrations in ($R^2 = 0.03$) (Supplemental Fig. 6).

Power calculation for thyroid hormone concentrations in serum and brain of GD20 fetuses

In the GD20 dams, post hoc power was 80 ~ 89 % for serum T3, T4 and TSH in the NaPB 1500 ppm group, but much less (5 ~ 22 %) in the NaPB 1000 ppm group (Supplemental Table 2). In GD20 fetuses, post hoc powers of serum T3 and brain T3 were 32 ~ 63 % and 46 ~ 76 %, respectively, and post hoc powers of serum T4 and brain T4 were 64 ~ 92 % and 92 ~ 97 %, respectively (Supplemental Table 3). Post hoc powers of serum TSH in GD20 fetuses were 49 ~ 64 % (Supplemental Table 3).

When statistical power in case of 10 individuals as group size was calculated to detect a 25 % decrease in T3 or T4 changes and a 40 % increase in TSH changes which can be detected in dose group compared to controls (Li et al., 2019; Marty et al., 2021), the powers were more than 49 % (based on serum T4) in GD20 dams but more than 61 % (based on serum T3 and TSH) in GD20 fetuses (Table 3). However, when focusing on T4, the powers were more than 96 % for serum T4 and more than 97 % for brain T4 (Table 3). When focusing on brain T3 and T4, the powers were more than 95 % for brain T3 and more than 97 % for brain T4 (Table 3).

Required group size for detecting 25 % decrease in serum T4 was less than 10 animals per group in GD20 fetuses, while GD20 dams need 22 animals per group (Table 3), approximately consistent with request by the original CTA guidance (20 maternal rats per group). Required group size for detecting 25 % decrease in serum T3 was more than 10 animals per group in both GD20 dams and fetuses. In contrast to serum, for brain T3 or T4, required group size for detecting 25 % decrease was less than 10 animals per group in GD20 fetuses (Table 3). For serum TSH, required group size for detecting 40 % increase was 20 animals per group in GD20 dams, while 12 ~ 14 animals per group in GD20 fetuses (Table 3).

Brain histopathology in GD20 fetuses

In the qualitative histopathological examination, no treatment-related remarkable findings were observed in the cerebrum and cerebellum of the brain in both sexes of GD20 fetuses in the NaPB groups (Table 1, Fig. 7).

Liver enzyme mRNA alterations in GD20 fetuses

In both sexes of GD20 fetuses, liver *Cyp2b1/2* and *Ugt2b1* mRNA levels were significantly increased up to approximately 890 and 540 % in the NaPB groups, with dose-dependency (Fig. 8A, B). There were some correlations between *Ugt2b1* mRNA in GD20 dams and GD20 fetuses ($R^2 > 0.47$, Supplemental Fig. 7). No correlations ($R^2 < 0.27$) were observed between hepatic *Ugt2b1* mRNA expression and serum/brain T3 and T4 concentrations (Supplemental Fig. 8A–D).

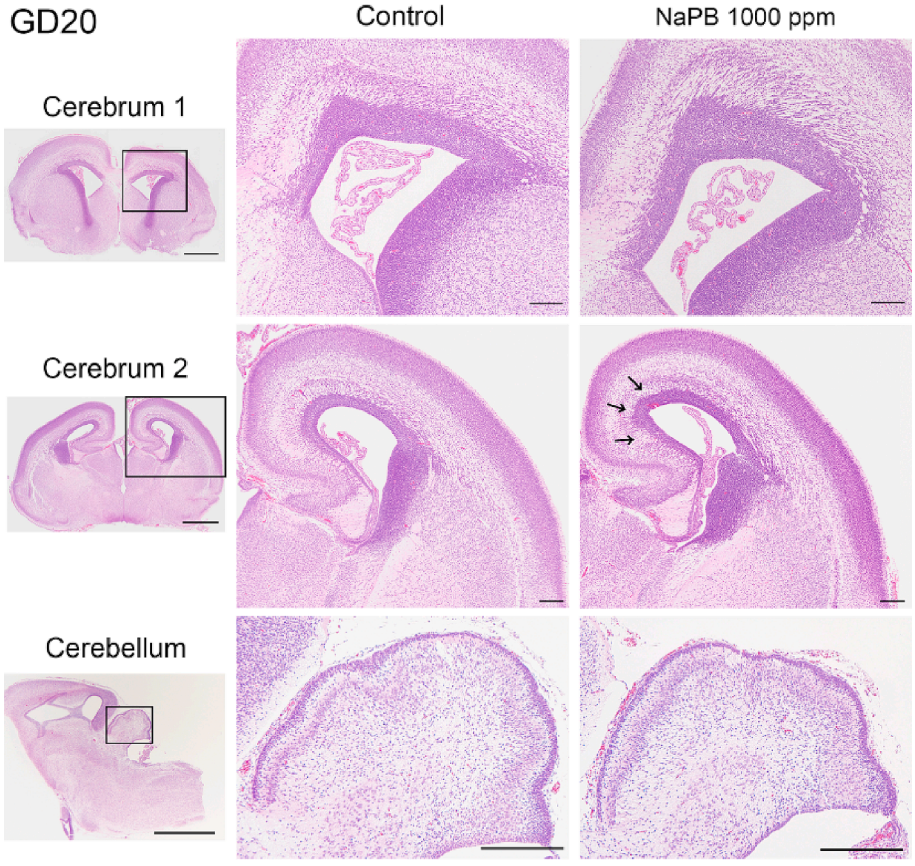

Fig. 7. Microphotographs of cerebrum and cerebellum in GD20 fetuses. In GD20 fetuses of the NaPB 1000 ppm group, no abnormalities were noted in the cerebrum at two levels and cerebellum of the brain compared to the control fetuses. No remarkable changes were observed in and around the ventricular epithelium (arrows) of the posterior forebrain (as shown in the photo in Cerebrum 2). There were also no remarkable abnormalities in the cerebellum. Hematoxylin and eosin (H&E). Scale bars = 1 mm (low magnification images), 200 μm (high magnification images). GD, gestational day.

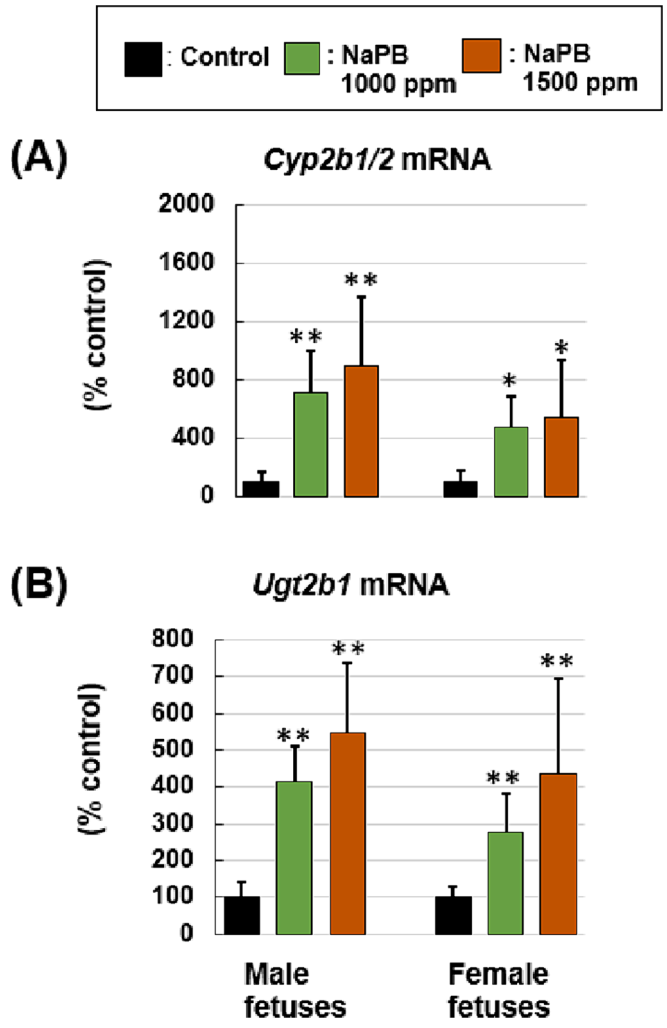

Fig. 8. Effects of NaPB on relative liver enzyme mRNA expression levels in fetuses on gestation day 20. Target mRNA levels were normalized to those of *Actb*. Values represent mean ± standard deviation. N = 6 animals per group. Significantly different from control; * $p \leq 0.05$, ** $p \leq 0.01$ by Dunnett's test following one-way ANOVA or Dunnett's-type test following Kruskal-Wallis test. The absolute values are presented in Supplemental Table 3.

Discussion

Recently, not only for US but also European registration, the CTA may be required if existing data for the pesticide provides evidence of a treatment-related effect on thyroid structure or function in other studies (ECHA/EFSA, 2018; Kucheryavenko et al., 2019). However, acceptable ranges of serum THs alterations in rats (i.e., ranges not considered causally related to adverse effects) are not currently described by either USEPA or OECD. Regardless, changes in peripheral concentrations may not reflect target tissue concentrations, which are more likely to have higher correlation with adverse outcomes (Hassan et al., 2017; Noyes et al., 2019). Thus, we believe that it is important to understand quantitative relationships between reductions of serum TH concentrations in dams and fetal brain concentrations. To more accurately characterize this relationship, appropriate test models must be both sensitive and reliable. Furthermore, we believe that reducing the number of animals examined is an important challenge from the perspective of animal welfare. The data presented herein, coupled with our previous publication (Minami et al., 2023), show that the modified CTA, with brain TH concentration measurements and reductions in the number of maternal rats per group is able to detect <30 % TH suppression.

Evaluation of the prenatal test cohort of the modified CTA protocol

Number of test animals and reliable endpoints

The USEPA CTA guidance recommends 20 maternal rats per group per cohort (USEPA, 2005). Our hypothesis was that the CTA can be conducted with 50 % less animals without losing the power to detect TH disruption. Statistical power is a critical point for defining the limits of the selected study type to detect changes in hormone concentrations under the chosen experimental conditions with the necessary sensitivity (Kucheryavenko et al., 2019; Li et al., 2019; Marty et al., 2021).

Overall, the present study suggests that lowering group size to 10 from 20 animals per group per cohort in the CTA appears able to detect TH disruption (approximately 20–30 % reduction) if fetal serum and brain TH concentrations are assessed. Such power should be acceptable based on the previous reports by Crofton in which a 50–60 % decrease in circulating T4 was needed to significantly impact hearing function in rats (Crofton, 2004), and also a recent review (Marty et al., 2022) that reported that thresholds of >60 %/>50 % (at top-/lower-dose groups) offspring serum T4 reduction indicate an increased likelihood for statistically significant neurodevelopmental effects based on analyses reported in many publications, including with amitrole (Ramhøj et al., 2021), perchlorate (Gilbert and Sui, 2008), perfluorohexane sulfonate (Ramhøj et al., 2020; Gilbert et al., 2021), Aroclor 1254 (Crofton et al., 2000), and others.

In the present study with NaPB, no additional value of brain measurements over serum alone was observed for T4 assessment in GD20 fetuses (i.e., high correlation between serum T4 and brain T4 concentrations, consistent with the findings of control fetuses in a previous study (Hassan et al., 2017)), however, T3 assessment by brain measurement was improved (Supplemental Table 4), as the group size required to detect a 25 % reduction should be greater than 10 animals for serum and less than 10 animals for brain (Table 3). Again, a recent review (Marty et al., 2022) reported that thresholds of >20 % and statistically significant offspring serum T3 reduction indicate an increased likelihood for statistically significant neurodevelopmental effects based on analysis of many publications described above. On the other hand, there was a correlation between fetal brain T3 and T4 concentrations in the NaPB study, so it may not be necessary to measure fetal brain T3 concentrations when measuring brain T4. To the best of our knowledge, there is no known inhibitory effect of NaPB on deiodinase enzymes. Therefore, when considering the necessity of measuring brain T3 concentrations in the modified CTA, investigating the effects of deiodinase inhibitors in the modified CTA is expected to provide useful information. Overall, this issue is still not fully resolved.

Based on this study alone, it cannot be concluded that fetal brain TH measurements do not provide added value to serum TH measurements in the CTA with small group size. Recent research studies using environmental contaminants on TH disruption were conducted using varying numbers of animals, e.g., less than or about 10 animals per dose (Ramhøj et al., 2018, 2022; Gilbert et al., 2021, 2022, 2023), 15–16 animals per dose (Ramhøj et al., 2020), or two balanced blocks and comprised three treatment groups of 22 dams each (Ramhøj et al., 2022). Although these are informative, they are not sufficient to draw conclusions because brain TH concentrations were not measured in all cases. Once sufficient data on brain TH have accumulated, regulatory use of the data should be comprehensively discussed by the scientific community and regulatory authorities. Further data from the modified CTA using other chemicals and by other laboratories are needed.

Lack of sex-effects on TH disrupting in GD20 fetuses

The USEPA CTA guidance (USEPA, 2005) 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, it apparently

recommends separate blood collection from male and female offspring. However, previous reports demonstrated that no sex differences were detected at basal TH concentrations and chemical-induced suppressions in the serum and brain TH concentrations (Gilbert and Sui, 2008; O'Shaughnessy et al., 2018; Marty et al., 2022; Ford et al., 2023). Especially, this is likely in fetuses at an early gestation period because the majority of fetal TH comes from the dams via the placenta (Bárez-López and Guadaño-Ferraz, 2017). Consistent with these previous reports, we confirmed no significant interaction of NaPB treatment and sex effects in serum and brain fetal TH concentrations. Control serum T4 concentration in GD20 fetuses was slightly higher in females than in males in the present study, whereas the previous study found the opposite (Minami et al., 2023), suggesting that this sex difference represents a false positive (normal variation) and not biologically relevant. Therefore, we consider that separate blood collection of males and females is not necessary, and thus pooling of fetal samples by litter, regardless of sex, would be acceptable. It is useful for additional availability such as assessment of the toxicokinetics of the test substance in the fetus.

Effects of NaPB in dams

In the present study, average NaPB intakes of the 1000 and 1500 ppm groups during GD6-GD20 were 60 and 84 mg/kg/day, respectively. Compared to the 1000 ppm group, the 1500 ppm group achieved 1.4-fold intake of NaPB and resulted in 1.6-fold PB concentration in peripheral blood. Although no maternal deaths were observed during the study, all maternal animals tested revealed staggered gait and/or some maternal animals revealed eyelid sebum in the 1500 ppm group. However, neither NaPB groups had significant alterations of maternal body weight and food consumption during the treatment period. Overall, the present findings suggest that the tested concentrations of NaPB in the diet (i.e., 1000 and 1500 ppm) did not cause excessive toxicities (e.g., maternal death, severely reduced food consumption due to drowsiness), and thus did not confound findings concerning possible TH disruption. Therefore, in this study, the dose–response of NaPB's effect on maternal serum TH concentrations was adequately investigated.

The present findings confirmed that NaPB-treatment also induced a significant CAR-mediated effects in the offspring liver consistent with that previously observed in adult rats (Liu et al., 1995; Hood et al., 1999a,b; Yamada et al., 2014; Haines et al., 2019). This is supported by changes in both UDPGT activity and *Ugt2b1* mRNA. The increase in UDPGT activity was maximal at 1000 ppm in the present study conditions. On the other hand, in the NaPB groups, mild to moderate reductions of serum TH concentrations were concomitantly observed in GD20 dams in a dose-dependent manner, but statistical significance was only observed at 1500 ppm (serum T3, −26 %; serum T4, −44 %). Since a strong correlation between serum T3 and T4 concentration was observed, this concomitant change was considered as a biological response to NaPB treatment. However, this mild to moderate TH reduction in serum was not accompanied by an increase in serum TSH concentrations, rather, serum TSH concentration was decreased at 1500 ppm contrary to our initial predictions, and negative correlations between serum TSH and serum TH were only seen when comparing the control and 1500 ppm groups. The decrease in TSH is not consistent with other studies (e.g., Hood et al., 1999a,b), and the reason for the decrease is currently unknown. Overall, the present study showed that the expected mild to moderate serum TH suppression by NaPB was induced in dams in the modified CTA protocol.

Effects of NaPB in GD20 fetuses

In GD20 fetuses of the NaPB groups, liver *Ugt2b1* mRNA expression levels were significantly increased, consistent with the previous fetal rat study (reported as UDPGTr2 RNA) (Marie and Cresteil, 1989). Regarding *Ugt2b1* mRNA expression levels, a moderate correlation was observed between in GD20 dams and GD20 fetuses, suggesting that NaPB could transfer from dams to fetuses through the placenta. Therefore, TH suppression in fetuses is likely due to both reduced TH transfer from the dams to the fetuses, and a direct increase in fetal TH excretion via hepatic enzyme induction as postulated for triclosan and perfluorohexane sulfonate (Gilbert et al., 2021).

The maternal origin of fetal T4 and T3 at term of gestation was reported as around 17.5 % and 47 %, respectively (Bárez-López and Guadaño-Ferraz, 2017). Hassan et al (2017) reported that the relative proportion of T4 emanating from the dam and contributing to the fetal circulation at late stage of gestation appears to increase with increasing concentrations of 6-PTU from 17 % at 0 ppm to 34 %-46 % at 3 ppm. Despite this increase, fetal T4 still falls precipitously with increasing dose of 6-PTU suggesting a direct action of 6-PTU to inhibit TPO in the fetal thyroid gland (Hassan et al., 2017). These findings support that fetal thyroid gland at term of gestation is functional for TH production (Bárez-López and Guadaño-Ferraz, 2017). Therefore, the direct increase in fetal TH excretion via liver enzyme activation by NaPB is suggested to be predominant during late gestation.

The increase in liver *Ugt2b1* mRNA expression levels in fetuses was in a dose-related manner; the increase of the 1500 ppm group was 1.3 ~ 1.6 fold of that in the 1000 ppm group. UDPGT activity in GD 20 fetuses was not examined in the present study. Considering the 1.2 fold increase in *Ugt2b1* mRNA with increasing dose level from 1000 to 1500 ppm but lack of a dose response in UDPGT activity in GD 20 dams, no increase in UDPGT activity was considered in GD 20 fetuses of the 1500 ppm group compared to the 1000 ppm group. Therefore, TH reductions in serum via liver UDPGT induction by NaPB appeared to be nearly maximal at 1000 ppm in GD20 fetuses. When looking at serum TH concentrations in the present study with NaPB, there were no correlations between serum TH concentrations in maternal and fetal rats, suggesting that TH suppression in fetal serum (and then brain) by NaPB is partially due to the reduced TH transfer from the dams to the fetuses. Consequently, it is again suggested that serum TH reduction in GD20 fetuses is primally due to a direct increase in fetal TH excretion via hepatic enzyme induction. However, fetal serum TH concentrations have little correlation with fetal *Ugt2b1 mRNA* expression levels. Taken together with the functional thyroid gland (Bárez-López and Guadaño-Ferraz, 2017) and the increased serum TSH in fetuses of the NaPB groups in the present study, the decrease in serum THs by NaPB appeared to be partially compensated by the functional hypothalamic-pituitary-thyroid (HTP) axis in GD20 fetuses.

Under such conditions, predominant responses were observed in serum T3, T4 and TSH of the 1000 ppm fetuses compared to dams in the same group. This may be contributed by the fact that in dams with high homeostatic activity of the HPT axis, the TH-disrupting effect is apparently smaller than in fetuses with relatively immature activity of the HTP-axis (Howdeshell, 2002; Fisher et al., 2012). These data suggest that TH testing limited to the mother may underestimate the effects on the fetus, especially at low dose of test chemicals with mild maternal TH disrupting activity.

To further evaluate biological relevance of serum T4 decreases, brain T4 concentrations were also assessed. Serum and brain TH concentrations, which are prior and subsequent key events in the adverse outcome pathways for DNT (Hassan et al., 2017; Noyes et al., 2019), were significantly decreased by NaPB at both 1000 and 1500 ppm without dose-dependency, and brain T4 concentrations were correlated to serum T4 concentrations. In addition to this, individual values of brain T3 and T4 concentrations were co-related in the present study. T4 is the primary source of T3 in the brain (Bárez-López and Guadaño-Ferraz, 2017). As well as in fetal serum TH concentrations, the decrease in the fetal brain TH concentrations by NaPB were approximately −20 % for T3 and −30 % for T4. Consequently, the present study confirmed that our modified CTA protocol could detect <30 % but significant TH disruption, which was induced by a prototypical liver enzyme inducer NaPB, in serum and brain of GD20 fetuses.

Toxicological impact of NaPB-induced disruption of TH (<30 %) in fetal brain

Our present qualitative histopathology on GD20 fetuses did not observe any treatment-related remarkable finding, consistent with the previous findings in GD20 fetuses in the 1000 ppm NaPB group (Minami et al., 2023), suggesting that there was no obvious direct effects on fetal brain tissue as a barbiturate antiepileptic drug (Dingemanse et al., 1989; Lumley et al., 2021). To understand the impact of the observed alterations (approximately 20–30 %) in brain TH concentrations on brain development in fetuses of the NaPB groups, we should evaluate postnatal brain morphology and function, as the brain undergoes significant development after birth (Howdeshell, 2002; O'Shaughnessy et al., 2019). Thyroid hormone disruption can result in heterotopias during development, with deficits in brain T4 concentrations during gestation being highly correlated ($R^2 = 0.99$) with increases in the heterotopia size or volume (Hassan et al., 2017). The presence of small heterotopias just above background levels in postnatal pups after PND28 were associated with brain T4 concentrations, modeled from fetal serum T4 changes, of approximately 35 % at GD20 (Hassan et al., 2017). Results from the present study revealed reduced brain T4 concentrations in GD20 fetuses of approximately 30 % for the 1000 and 1500 ppm exposures with NaPB. As the persistence of TH reduction after birth, in addition to the extent of TH decrease during the fetal period, is considered a significant factor in heterotopia formation (O'Shaughnessy et al., 2018, 2019; Gilbert et al., 2023), the assessment of offspring brain TH concentrations and heterotopia formation in the postnatal test cohort of the CTA with NaPB becomes important. We will discuss this issue in a separate paper on the postnatal test cohort with NaPB.

Conclusions

We are currently evaluating the feasibility, sensitivity, and reliability of the modified CTA by adding examination of brain THs and brain histopathology (especially, heterotopia assessment), while also reducing the number of test animals, even for chemicals with mild maternal TH disruption. As a part of this project, the present study focused on prenatal exposure, and confirmed that the modified CTA protocol could detect mild but statistically significant TH disruption induced by NaPB (a prototypical liver enzyme inducer in adult rats) in pregnant dams, and also could detect <30 % but statistically significant serum and brain TH disruption in their GD20 fetuses. These findings suggest that lowering group sizes (N = 10/group/cohort) in the CTA may be acceptable by adding additional endpoints (especially brain TH concentrations). However, due to the difficulty in measuring brain TH concentrations, it is necessary to confirm that other laboratories can achieve the same level of competency in this measurement. Since clear morphological changes occur in rats after birth, toxicological impact of the perinatal brain TH disruption could be assessed by heterotopia formation in PND21 pups of the postnatal test cohort in the CTA. To confirm sensitivity of brain TH concentrations and qualitative assessment of heterotopia formation in the modified CTA, further studies using other compounds or follow-up studies in other laboratories will be required.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References

Aoyama, H., Hojo, H., Takahashi, K.L., Shimizu, N., Araki, M., Harigae, M., Tanaka, N., Shirasaka, N., Kuwahara, M., Nakashima, N., Yamamoto, E., Saka, M., Teramoto, S., 2005. A two-generation reproductive toxicity study of 2,4-dichlorophenol in rats. J Toxicol Sci. 30 Special Issue, 59-78. doi: 10.2131/jts.30.s59.

Aoyama, H., Kikuta, M., Shirasaka, N., Hojo, H., Takahashi, K.L., Shimizu, N., Harigae, M., Taguchi, F., Teramoto, S., 2002. Historical control data on reproductive abilities and incidences of spontaneous fetal malformations in Wistar Hannover galas rats. Congenit. Anom. (Kyoto) 42, 194–201. https://doi.org/10.1111/j.1741-4520.2002.tb00893.x.

Aoyama, H., Hojo, H., Takahashi, K.L., Shimizu-Endo, N., Araki, M., Takeuchi-Kashimoto, Y., Saka, M., Teramoto, S., 2012. Two-generation reproduction toxicity study in rats with methoxychlor. Congenit. Anom. (Kyoto) 52, 28–41. https://doi.org/10.1111/j.1741-4520.2011.00344.x.

Bárez-López, S., Guadaño-Ferraz, A., 2017. Thyroid hormone availability and action during brain development in rodents. Front. Cell. Neurosci. 11, 240. https://doi.org/10.3389/fncel.2017.00240.

Brucker-Davis, F., 1998. Effects of environmental synthetic chemicals on thyroid function. Thyroid 8, 827–856. https://doi.org/10.1089/thy.1998.8.827.

Capen, C.C., 1997. Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol. Pathol. 25, 39–48. https://doi.org/10.1177/019262339702500109.

Crofton, K.M., 2004. Developmental disruption of thyroid hormone: Correlations with hearing dysfunction in rats. Risk Anal. 24, 1665–1671. https://doi.org/10.1111/j.0272-4332.2004.00557.x.

Crofton, K.M., 2008. Thyroid disrupting chemicals: Mechanisms and mixtures. Int. J. Androl. 31, 209–223. https://doi.org/10.1111/j.1365-2605.2007.00857.x.

Crofton, K.M., Kodavanti, P.R., Derr-Yellin, E.C., Casey, A.C., Kehn, L.S., 2000. PCBs, thyroid hormones, and ototoxicity in rats: Cross-fostering experiments demonstrate the impact of postnatal lactation exposure. Toxicol. Sci. 57, 131–140. https://doi.org/10.1093/toxsci/57.1.131.

Dellarco, V.L., McGregor, D., Berry, S.C., Cohen, S.M., Boobis, A.R., 2006. Thiazopyr and thyroid disruption: Case study within the context of the 2006 IPCS human relevance framework for analysis of a cancer mode of action. Crit. Rev. Toxicol. 36, 793–801. https://doi.org/10.1080/10408440600975242.

DeVito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A.O., Christensen, R., Colborn, T., Cooke, P., Crissman, J., Crofton, K., Doerge, D., Gray, E., Hauser, P., Hurley, P., Kohn, M., Lazar, J., McMaster, S., McClain, M., McConnell, E., Meier, C., Miller, R., Tietge, J., Tyl, R., 1999. Screening methods for thyroid hormone disruptors. Environ. Health Perspect. 107, 407–415. https://doi.org/10.1289/ehp.99107407.

Diaz, J., Schain, R.J., 1978. Phenobarbital: Effects of long-term administration on behavior and brain of artificially reared rats. Science 199, 90–91. https://doi.org/10.1126/science.199.4324.90.

Dingemanse, J., van Bree, J.B., Danhof, M., 1989. Pharmacokinetic modeling of the anticonvulsant action of phenobarbital in rats. J. Pharmacol. Exp. Ther. 249, 601–608 https://jpet.aspetjournals.org/content/249/2/601.long.

ECHA/EFSA, 2018. ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC), 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. EFSA Journal 16(6):5311, 135 pp. https://doi.org/10.2903/j.efsa.2018.5311. Accessed April 02, 2024.

Ejiri, N., Katayama, K., Doi, K., 2005. Induction of cytochrome p450 isozymes by phenobarbital in pregnant rat and fetal livers and placenta. Exp. Mol. Pathol. 78, 150–155. https://doi.org/10.1016/j.yexmp.2004.07.002.

Fisher, J., Lumen, A., Latendresse, J., Mattie, D., 2012. Extrapolation of hypothalamic-pituitary-thyroid axis perturbations and associated toxicity in rodents to humans: Case study with perchlorate. J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev. 30, 81–105. https://doi.org/10.1080/10590501.2012.653889.

Ford, J., Riutta, C., Kosian, P.A., O'Shaughessy, K., Gilbert, M., 2023. Reducing uncertainties in quantitative adverse outcome pathways by analysis of thyroid hormone in the neonatal rat brain. Toxicol. Sci. 193, 192–203. https://doi.org/10.1093/toxsci/kfad040.

Foster, J.R., Tinwell, H., Melching-Kollmuss, S., 2021. A review of species differences in the control of, and response to, chemical-induced thyroid hormone perturbations leading to thyroid cancer. Arch. Toxicol. 95, 807–836. https://doi.org/10.1007/s00204-020-02961-6.

Garman, R.H., Li, A.A., Kaufmann, W., Auer, R.N., Bolon, B., 2016. Recommended methods for brain processing and quantitative analysis in rodent developmental neurotoxicity studies. Toxicol. Pathol. 44, 14–42. https://doi.org/10.1177/0192623315596858.

Gilbert, M.E., O'Shaughnessy, K.L., Axelstad, M., 2020. Regulation of thyroid disrupting chemicals to protect the developing brain. Endocrinology 161, 1–17. https://doi.org/10.1210/endocr/bqaa106.

Gilbert, M.E., O'Shaughnessy, K.L., Thomas, S.E., Riutta, C., Wood, C.R., Smith, A., Oshiro, W.O., Ford, R.L., Hotchkiss, M., Hassan, I., Ford, J.L., 2021. Thyroid disruptors: Extrathyroidal sites of chemical action and neurodevelopmental outcome - an examination using triclosan and perfluorohexane sulfonate. Toxicol. Sci. 183, 195–213. https://doi.org/10.1093/toxsci/kfab080.

Gilbert, M.E., Hassan, I., Wood, C., O'Shaughnessy, K.L., Spring, S., Thomas, S., Ford, J., 2022. Gestational exposure to perchlorate in the rat: Thyroid hormones in fetal thyroid gland, serum, and brain. Toxicol. Sci. 188, 117–130. https://doi.org/10.1093/toxsci/kfac038.

Gilbert, M.E., O'Shaughnessy, K.L., Bell, K.S., Ford, J.L., 2023. Structural malformations in the neonatal rat brain accompany developmental exposure to ammonium perchlorate. Toxics 11, 1027. https://doi.org/10.3390/toxics11121027.

Gilbert, M.E., Hassan, I., O'Shaughnessy, K.L., Wood, C., Stoker, T.E., Riutta, C., Ford, J. L., 2024. Ammonium perchlorate: Serum dosimetry, neurotoxicity, and resilience of the neonatal rat thyroid system. Toxicol. Sci. 198, 113–127. https://doi.org/10.1093/toxsci/kfad133.

Gilbert, M.E., Sui, L., 2008. Developmental exposure to perchlorate alters synaptic transmission in hippocampus of the adult rat. Environ. Health Perspect. 116, 752–760. https://doi.org/10.1289/ehp.11089.

Gore, A.C., Chappell, V.A., Fenton, S.E., Flaws, J.A., Nadal, A., Prins, G.S., Toppari, J., Zoeller, R.T., 2015. EDC-2: The endocrine society's second scientific statement on endocrine-disrupting chemicals. Endocr. Rev. 36, E1–E150. https://doi.org/10.1210/er.2015-1010.

Haines, C., Chatham, L.R., Vardy, A., Elcombe, C.R., Foster, J.R., Lake, B.G., 2019. Comparison of the hepatic and thyroid gland effects of sodium phenobarbital and pregnenolone-16alpha-carbonitrile in wild type and constitutive androstane receptor (CAR)/pregnane X receptor (PXR) knockout rats. Xenobiotica 49, 227–238. https://doi.org/10.1080/00498254.2018.1437300.

Hassan, I., El-Masri, H., Kosian, P.A., Ford, J., Degitz, S.J., Gilbert, M.E., 2017. Neurodevelopment and thyroid hormone synthesis inhibition in the rat: Quantitative understanding within the adverse outcome pathway framework. Toxicol. Sci. 160, 57–73. https://doi.org/10.1093/toxsci/kfx163.

Hill, R.N., Crisp, T.M., Hurley, P.M., Rosenthal, S.L., Singh, D.V., 1998. Risk assessment of thyroid follicular cell tumors. Environ. Health Perspect. 106, 447–457. https://doi.org/10.1289/ehp.98106447.

Hood, A., Hashmi, R., Klaassen, C.D., 1999a. Effects of microsomal enzyme inducers on thyroid-follicular cell proliferation, hyperplasia, and hypertrophy. Toxicol. Appl. Pharmacol. 160, 163–170. https://doi.org/10.1006/taap.1999.8752.

Hood, A., Liu, J., Klaassen, C.D., 1999b. Effects of phenobarbital, pregnenolone-16alpha-carbonitrile, and propylthiouracil on thyroid follicular cell proliferation. Toxicol. Sci. 50, 45–53. https://doi.org/10.1093/toxsci/50.1.45.

Howdeshell, K.L., 2002. A model of the development of the brain as a construct of the thyroid system. Environ. Health Perspect. 110, 337–348. https://doi.org/10.1289/ehp.02110s3337.

Kanda, Y., 2013. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 48, 452–458. https://doi.org/10.1038/bmt.2012.244.

Kucheryavenko, O., Lurman, G., Lehmann, A., Braz, J., Niemann, L., Chahoud, I., Mantovani, A., Hakansson, H., Schneider, S., Strauss, V., Coder, P.S., Freyberger, A., O'Connor, J.C., Rauch, M., Renko, K., Solano, M.L.M., Andersson, N., Blanck, O., Ritz, V., Solecki, R., 2019. Report from the BfR expert hearing on practicability of hormonal measurements: Recommendations for experimental design of toxicological studies with integrated hormonal end points. Arch. Toxicol. 93, 1157–1167. https://doi.org/10.1007/s00204-019-02436-3.

Landers, K., Richard, K., 2017. Traversing barriers – how thyroid hormones pass placental, blood-brain and blood-cerebrospinal fluid barriers. Mol. Cell. Endocrinol. 458, 22–28. https://doi.org/10.1016/j.mce.2017.01.041.

Li, A.A., Makris, S.L., Marty, M.S., Strauss, V., Gilbert, M.E., Blacker, A., Zorrilla, L.M., Coder, P.S., Hannas, B., Lordi, S., Schneider, S., 2019. Practical considerations for developmental thyroid toxicity assessments: What's working, what's not, and how can we do better? Regul. Toxicol. Pharm. 106, 111–136. https://doi.org/10.1016/j.yrtph.2019.04.010.

Liu, J., Liu, Y., Barter, R.A., Klaassen, C.D., 1995. Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: A dose-response study. J. Pharmacol. Exp. Ther. 273, 977–985, 977.full.pdf (aspetjournals.org).

Lumley, L.A., Marrero-Rosado, B., Rossetti, F., Schultz, C.R., Stone, M.F., Niquet, J., Wasterlain, C.G., 2021. Combination of antiseizure medications phenobarbital, ketamine, and midazolam reduces soman-induced epileptogenesis and brain pathology in rats. Epilepsia Open. 6, 757–769. https://doi.org/10.1002/epi4.12552.

Marie, S., Cresteil, T., 1989. Phenobarbital-inducible gene expression in developing rat liver: Relationship to hepatocyte function. BBA 1009, 221–228. https://doi.org/10.1016/0167-4781(89)90106-1.

Marty, S., Beekhuijzen, M., Charlton, A., Hallmark, N., Hannas, B.R., Jacobi, S., Melching-Kollmuss, S., Sauer, U.G., Sheets, L.P., Strauss, V., Urbisch, D., Botham, P. A., van Ravenzwaay, B., 2021. Towards a science-based testing strategy to identify maternal thyroid hormone imbalance and neurodevelopmental effects in the progeny - part II: How can key events of relevant adverse outcome pathways be addressed in toxicological assessments? Crit. Rev. Toxicol. 51, 328–358. https://doi.org/10.1080/10408444.2021.1910625.

Marty, M.S., Sauer, U.G., Charlton, A., Ghaffari, R., Guignard, D., Hallmark, N., Hannas, B.R., Jacobi, S., Marxfeld, H.A., Melching-Kollmuss, S., Sheets, L.P., Urbisch, D., Botham, P.A., van Ravenzwaay, B., 2022. Towards a science-based testing strategy to identify maternal thyroid hormone imbalance and neurodevelopmental effects in the progeny - part III: How is substance-mediated thyroid hormone imbalance in pregnant/lactating rats or their progeny related to neurodevelopmental effects? Crit. Rev. Toxicol. 52, 546–617. https://doi.org/10.1080/10408444.2022.2130166.

McClain, R.M., Levin, A.A., Posch, R., Downing, J.C., 1989. The effect of phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicol. Appl. Pharmacol. 99, 216–228. https://doi.org/10.1016/0041-008x(89)90004-5.

Middaugh, L.D., Thomas, T.N., Simpson, L.W., Zemp, J.W., 1981. Effects of prenatal maternal injections of phenobarbital on brain neurotransmitters and behavior of young C57 mice. Neurobehav. Toxicol. Teratol. 3, 271–275.

Miller, M.D., Crofton, K.M., Rice, D.C., Zoeller, R.T., 2009. Thyroid-disrupting chemicals: Interpreting upstream biomarkers of adverse outcomes. Environ. Health Perspect. 117, 1033–1041. https://doi.org/10.1289/ehp.0800247.

Minami, K., Suto, H., Sato, A., Ogata, K., Kosaka, T., Hojo, H., Takahashi, N., Tomiyama, N., Fukuda, T., Iwashita, K., Aoyama, H., Yamada, T., 2023. Feasibility study for a downsized comparative thyroid assay with measurement of brain thyroid hormones and histopathology in rats: Case study with 6-propylthiouracil and sodium phenobarbital at high dose. Regul. Toxicol. Pharm. 105283. https://doi.org/10.1016/j.yrtph.2022.105283.

Murk, A.J., Rijntjes, E., Blaauboer, B.J., Clewell, R., Crofton, K.M., Dingemans, M.M., Furlow, J.D., Kavlock, R., Kohrle, J., Opitz, R., Traas, T., Visser, T.J., Xia, M., Gutleb, A.C., 2013. Mechanism-based testing strategy using in vitro approaches for identification of thyroid hormone disrupting chemicals. Toxicol. In Vitro 27, 1320–1346. https://doi.org/10.1016/j.tiv.2013.02.012.

Noyes, P.D., Friedman, K.P., Browne, P., Haselman, J.T., Gilbert, M.E., Hornung, M.W., Barone Jr, S., Crofton, K.M., Laws, S.C., Stoker, T.E., Simmons, S.O., Tietge, J.E., Degitz, S.J., 2019. Evaluating chemicals for thyroid disruption: Opportunities and challenges with in vitro testing and adverse outcome pathway approaches. Environ. Health Perspect. 127, 095001 https://doi.org/10.1289/ehp5297.

O'Shaughnessy, K.L., Kosian, P.A., Ford, J.L., Oshiro, W.M., Degitz, S.J., Gilbert, M.E., 2018. Developmental thyroid hormone insufficiency induces a cortical brain malformation and learning impairments: A cross-fostering study. Toxicol. Sci. 163, 101–115. https://doi.org/10.1093/toxsci/kfy016.

O'Connor, J.C., Frame, S.R., Davis, L.G., Cook, J.C., 1999. Detection of thyroid toxicants in a tier I screening battery and alterations in thyroid endpoints over 28 days of exposure. Toxicol. Sci. 51, 54–70. https://doi.org/10.1093/toxsci/51.1.54.

O'Shaughnessy, K.L., Gilbert, M.E., 2020. Thyroid disrupting chemicals and developmental neurotoxicity - new tools and approaches to evaluate hormone action. Mol. Cell. Endocrinol. 518, 110663 https://doi.org/10.1016/j.mce.2019.110663.

O'Shaughnessy, K.L., Thomas, S.E., Spring, S.R., Ford, J.L., Ford, R.L., Gilbert, M.E., 2019. A transient window of hypothyroidism alters neural progenitor cells and results in abnormal brain development. Sci. Rep. 9, 4662. https://doi.org/10.1038/s41598-019-40249-7.

Ramhøj, L., Hass, U., Boberg, J., Scholze, M., Christiansen, S., Nielsen, F., Axelstad, M., 2018. Perfluorohexane sulfonate (PFHXS) and a mixture of endocrine disrupters reduce thyroxine levels and cause antiandrogenic effects in rats. Toxicol. Sci. 163, 579–591. https://doi.org/10.1093/toxsci/kfy055.

Ramhøj, L., Hass, U., Gilbert, M.E., Wood, C., Svingen, T., Usai, D., Vinggaard, A.M., Mandrup, K., Axelstad, M., 2020. Evaluating thyroid hormone disruption: Investigations of long-term neurodevelopmental effects in rats after perinatal exposure to perfluorohexane sulfonate (PFHXS). Sci. Rep. 10, 2672. https://doi.org/10.1038/s41598-020-59354-z.

Ramhøj, L., Fradrich, C., Svingen, T., Scholze, M., Wirth, E.K., Rijntjes, E., Kohrle, J., Kortenkamp, A., Axelstad, M., 2021. Testing for heterotopia formation in rats after developmental exposure to selected in vitro inhibitors of thyroperoxidase. Environ. Pollut. 283, 117135 https://doi.org/10.1016/j.envpol.2021.117135.

Ramhøj, L., Svingen, T., Mandrup, K., Hass, U., Lund, S.P., Vinggaard, A.M., Hougaard, K. S., Axelstad, M., 2022. Developmental exposure to the brominated flame retardant DE-71 reduces serum thyroid hormones in rats without hypothalamic-pituitary-thyroid axis activation or neurobehavioral changes in offspring. PLoS One 17, e0271614. https://doi.org/10.1371/journal.pone.0271614.

Reyns, G.E., Venken, K., Morreale de Escobar, G., Kühn, E.R., Darras, V.M., 2003. Dynamics and regulation of intracellular thyroid hormone concentrations in embryonic chicken liver, kidney, brain, and blood. Gen. Comp. Endocrinol. 134, 80–87. https://doi.org/10.1016/s0016-6480(03)00220-x.

Sauer, U.G., Asiimwe, A., Botham, P.A., Charlton, A., Hallmark, N., Jacobi, S., Marty, S., Melching-Kollmuss, S., Palha, J.A., Strauss, V., van Ravenzwaay, B., Swaen, G., 2020. Toward a science-based testing strategy to identify maternal thyroid hormone imbalance and neurodevelopmental effects in the progeny - part I: Which parameters from human studies are most relevant for toxicological assessments? Crit. Rev. Toxicol. 50, 740–763. https://doi.org/10.1080/10408444.2020.1839380.

Strupp, C., Quesnot, N., Weber-Parmentier, C., Richert, L., Bomann, W.H., Singh, P., 2020. Weight of evidence and human relevance evaluation of the benfluralin mode of action in rats (part II): Thyroid carcinogenesis. Regul. Toxicol. Pharm. 117, 104736 https://doi.org/10.1016/j.yrtph.2020.104736.

USEPA, 2005. Guidance for thyroid assays in pregnant animals, fetuses and postnatal animals, and adult animals. Office of Pesticide Programs Health Effects Division Washington DC. Https://www.Epa.Gov/pesticide-registration/guidance-thyroid-assays-pregnant-animals-fetuses-and-postnatal-animals-and. (accessed March 29, 2024).

Yamada, T., Okuda, Y., Kushida, M., Sumida, K., Takeuchi, H., Nagahori, H., Fukuda, T., Lake, B.G., Cohen, S.M., Kawamura, S., 2014. Human hepatocytes support the hypertrophic but not the hyperplastic response to the murine nongenotoxic hepatocarcinogen sodium phenobarbital in an *in vivo* study using a chimeric mouse with humanized liver. Toxicol. Sci. 142, 137–157. https://doi.org/10.1093/toxsci/kfu173.

Zoeller, R.T., Crofton, K.M., 2005. Mode of action: Developmental thyroid hormone insufficiency–neurological abnormalities resulting from exposure to propylthiouracil. Crit. Rev. Toxicol. 35, 771–781. https://doi.org/10.1080/10408440591007313.