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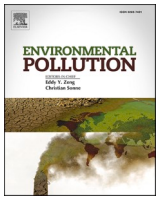

Effect of in utero and lactational exposure to a thyroid hormone system disrupting chemical on mouse metabolome and brain transcriptome☆

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ABSTRACT

Mice were exposed to a low dose of the model thyroid hormone disruptor, propylthiouracil. Although this had only a modest effect on maternal thyroid hormones production, postnatal analysis of the pups' plasma by mass spectrometry and the brain striatum by RNA sequencing gave evidence of low lasting changes that could reflect an adverse effect on neurodevelopment. Overall, these methods proved to be sensitive enough to detect minor disruptions of thyroid hormone signalling *in vivo*.

1. Introduction

Thyroid hormones (TH) are essential regulators of vertebrate development (Mullur et al., 2014; Warner and Mittag, 2012). The most bioactive TH is T3 (3,3′,5-triiodo-L-thyronine), which is mainly synthesised from T4 (thyroxine). It exerts a pleiotropic effect on cell proliferation and differentiation by binding to nuclear receptors that are present in most, if not all, cell types (Flamant et al., 2006). The development of the foetus depends on maternal TH, which crosses the placenta, until its own thyroid gland becomes functional at a late stage of pregnancy (Richard and Flamant, 2018). The main consequences of early TH deficiency are a blunted skeletal growth and an irreversible mental retardation. Mild forms are associated with low IQ and increased incidence of attention deficit hyperactivity and autism spectrum disorders ((Andersen et al., 2014)). Even hypothyroxinemia, i.e. low maternal T4 with normal T3 and TSH levels in serum is detrimental to brain development (Berbel et al., 2009).

For these reasons, early exposure to environmental chemicals called thyroid hormone disruptors, or thyroid hormone system disrupting chemicals (THSDCs), which interfere either with TH production or tissues response to TH, is a matter of growing concern (Cediel-Ulloa et al., 2022). Although intensive efforts are dedicated to *in vitro* screening for putative environmental THSDCs (Paul-Friedman et al., 2019), animal exposure remains indispensable to assess their developmental toxicity, and more specifically neurodevelopmental toxicity, of putative THSDCs (Vandenberg, 2021). Here, we combined transcriptomic and metabolomic analyses to address the consequence of *in vivo* exposure to a low dose of propylthiouracil (PTU 1 mg/L). This pharmacological model compound impairs the production of TH by thyroid follicular cells, selectively inhibiting thyroid peroxidase (Motonaga et al., 2016) and TH deiodinases (Nogimori et al., 1986). This exposure of gestating mice only provoked a selective and moderate reduction in maternal T4 level. It is relevant to the modest changes expected to result from a realistic environmental exposure to THSDCs (Gilbert et al., 2020; Grossklaus et al., 2023). We detected, by mass-spectrometry, changes in the metabolite concentrations of the pups' plasma. We also observed via RNA deep sequencing (RNA-seq) changes in gene expression in the pups' striatum. This area of the brain was chosen because of its relatively limited cellular diversity, which consists mainly of GABAergic neurons and glial cells. This brain area is highly sensitive to hypothyroidism (Gil-Ibañez et al., 2013; Rodriguez-Peña et al., 1993; Vargiu et al., 2001) and its T3-response has been fully characterized (Richard et al., 2020).

These novel data confirm that neurodevelopment is sensitive to minor alterations of TH signalling and that omics are suitable to capture the adverse consequences of these alterations.

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2. Materials and methods

2.1. Study setup and sampling

Pregnant C57bl/6 mice were purchased from Charles River and received at gestational day 4 (GD4). From GD 7 to postnatal day 14 (P14), mice were exposed to low dose PTU in drinking water (1 mg/L) exemplifying perinatal exposure in utero and lactational. The others acted as non-exposed controls. Water consumption was measured and did not differ between the two groups of mice. It raised from an average of 6 mL during gestation to 12 mL during the post-natal period. As the body weight of dams also changed during exposure, due to the progression of gestation, the daily intake of PTU varies between 0.15 or 0.5 mg/kg/day, depending on the stages. The offspring is thus continuously exposed, from midgestation, to the consequences of the maternal exposure to a low dose of PTU. At P14, dams (n = 5 treated; n = 4 controls) and pups (n = 39) were sacrificed to collect blood and striatum samples, which were immediately frozen in liquid nitrogen.

2.2. Plasma metabolome

Plasma samples of pups (n = 19 controls, n = 19 PTU-treated) were extracted as described previously(Hansen et al., 2016) with slight modifications as described in supplementary data. The analyses were performed on a nanoflow ultra-high performance liquid chromatography system (ThermoFisher Scientific) with a pre-concentration trap--column setup previously described (Ma et al., 2020). For the untargeted workflow the instrument was operated in data-dependent mode by automatically switching between MS and MS/MS fragmentation (further details provided in supp. info). Compound Discoverer software version 3.2.0.421 (Thermo Scientific) was used for data processing and analysis (see suppl. info. for setting and parameters). Putatively annotated compounds were based on comparisons of the experimental MS2-spectrum with in-house spectral libraries (ID level 1) the online spectral library, mzCloud, (ID level 2). Putatively characterized compound classes (ID level 3) were based on assigned predicted composition, the plausible candidates and *in silico* fragmentation. Compounds that did not meet these criteria were assigned to level 4 and not incorporated in the functional analysis. Quality and validity of the chemical analysis was confirmed by principal component analysis (PCA) showing that the composite quality control samples were centrally located in the plot (Fig. S1). In order to identify statistically significant differences in relative metabolite concentrations between PTU treated mice pups and controls, an univariate statistical analysis was performed with MetaboAnalystR (Pang et al., 2020) using R version 3.6.3 incorporated in RStudio ver. 2022.02. Significant differences in the data were decided with T-test analysis and significance threshold based on a false discovery rate below 0.05 (FDR<0.05).

2.3. Thyroid hormone quantification

Concentration (pg/mL ± SD) of free T3 and T4 in maternal plasma quantified on a Cobas 6000 automat with the Cobas e601module (Roche, ECL analysers) using electrochemiluminescence immunoassay (ECLIA) following manufacturer's instructions. For plasma from the pups, the quantification of TH and associated metabolites were obtained by orbitrap high-resolution tandem mass spectrometry (nanoLC-HRMS/MS, Q Exactive HF, ThermoFisher Scientific) operated in parallel-reaction monitoring acquisition mode using an ion inclusion list including thyroxine (T4), 3,3′,5-triiodothyronine (T3), 3,3′,5′-triiodothyronine (rT3), 3,5-diiodothyronine (3,5-T2), 3,3-diiodothyronine (3,3-T2), 3-iodothyronine (T1), thyronine (T0), 3-iodothyroacetic acid (T1Ac), 3,5-Diiodothyroacetic acid (Diac), triiodothyroacetic acid (Triac) and tetraiodothyroacetic acid (Tetrac). Data analysis was conducted in TraceFinder v4.1 (Thermo Scientific inc.). For quantification, a six-point calibration curve (0.24–7.8 pmol/mL) run in duplicates was applied. The statistical analysis was performed using a mixed model in the package lme4 (Bates et al., 2015) with litter included as a co-variate. R version 3.6.3 incorporated in RStudio ver. 2022.02.3 was applied.

2.4. RNA analysis

Striatum RNA was extracted using RNeasy Mini Kit (Qiagen) and concentrations were measured with a Nanodrop

spectrophotometer (Thermo Fisher Scientific). One μg of each RNA sample was reverse transcribed using murine leukemia virus reverse transcriptase (Promega) and random DNA hexamer primers. Quantitative PCR was performed according to a Biorad recommandations, using the Biorad iQ SYBRGreen kit, CFX96 thermocycler (Biorad) and the *Hprt* housekeeping gene as an internal control. For each pair of primers a standard curve was established and PCR efficiency was controlled to be within useable range (90%–110%) before analysis using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). cDNA libraries were prepared for RNA-seq using the total RNA SENSE kit (Lexogen, Vienna Austria) and single-end deep sequencing was performed on a NextSeq500 sequencer (Illumina) as described (Richard et al., 2020).

Raw reads were mapped on the mouse genome (Grcm38/mm10 version) using Bowtie2 (Galaxy Version 2.2.6.2) (Langmead et al., 2009). Count tables were prepared using htseq-count (Galaxy Version 0.6.1galaxy3) (Anders et al., 2015). Differential gene expression analysis was performed using Deseq2 (Galaxy Version 2.11.40.7) (Love et al., 2014). The GSEA software (https://www.gsea-msigdb.org/gsea/index.jsp) (Subramanian et al., 2005) was used to evaluate the distribution of specific genesets in the lists of genes ranked by expression level (adjusted base counts produced by Deseq2) in each group of RNA sample.

3. Results

3.1. Setting a protocol for exposure to low-dose PTU

We analysed the status of mice exposed during gestation and lactation at P14, a stage at which the striatum only contains postmitotic cells (Fentress et al., 1981). We chose to expose mice to 1 mg/L of PTU in the drinking water (Fig. 1) because this low dose is the lowest for which an adverse effect has been reported in rats (Gilbert et al., 2020).

3.2. Targeted analysis of thyroid hormones

PTU significantly increased T4 in PTU-exposed dams but T3 was not significantly affected. An increase of T4 deiodination can maintain the T3 level when the secretion of the thyroid gland is moderately decreased. As a result, the low-dose PTU induces a moderate and selective decrease in maternal T4 level. The T4 and T3 levels in pups, which only drink maternal milk, also remained unaltered (Table 1). TH quantification in pups' plasma were obtained by a targeted mass

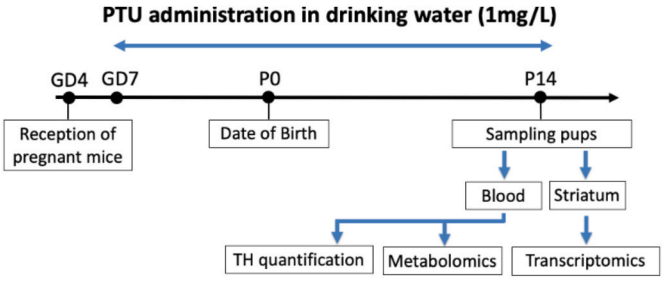

Fig. 1. Experimental design. PTU was introduced in the drinking water of gestating dams at gestation day 4 (GD4). Plasma from the dams and pups was collected at post-natal day 14 (P14). Striatum was extracted from the pup's brain for RNA analysis.

Table 1
Thyroid hormone concentrations (pg/mL) in maternal- and pup plasma. Free T3 and T4 in maternal plasma was quantified using a electrochemiluminescence immunoassay (ECLIA) assay while total T3 and T4 was quantified in pup plasma by LC-MS/MS. Asterisks indicate a significant difference between PTU treated and controls (Student t-test. p-value <0.01).

	T3 (pg/mL)	T4 (pg/mL)
Control dams (n = 7)[a]	3.2 ± 0.6	25.5 ± 2.3
PTU treated dams (n = 9) [a]	3.6 ± 0.8	**20.5 ± 2.5***
Control pups (n = 19) [b]	3.9 ± 0.2	118.2 ± 4.9
PTU treated pups (n = 19) [b]	3.7 ± 0.2	119.1 ± 6.0

* Student t-test. p-value <0.01.
[a] ECLIA quantification.
[b] LC-MS/MS quantification.

spectrometry analysis presented below, which also provided access to 9 other thyroid hormone metabolites. However, the concentrations of all these TH related compounds were below the limit of quantification (data not shown, limits of detection provided in supplementary material).

3.3. Unbiased analysis of pup plasma metabolome

Looking for novel biomarkers of PTU exposure, we detected a total of 76,631 features in the pups' plasma. All features were grouped into 9081 compounds of which 4830 were filtered out as background, leaving 4574 metabolites for analysis (Table S1). A total of 137 metabolites were found to differ significantly (FDR <0.05) in their relative concentration when comparing PTU-treated animals to controls (Fig. 2A). Annotation showed that PTU treatment affected the metabolites belonging to lipid- and fatty acid metabolism. This included several acyl carnitines, fatty acyls, two compounds involved in linoleic acid metabolism and a glycerophospholipid (Table S2). Two medium-chained acyl carnitines, decanoyl-L-carnitine and dodecenoylcarnitine, which were identified with matching standards, showed particularly high fold changes (x1000 and x60, respectively). Furthermore, two compounds tentatively annotated (ID level 3) as sphingolipids were found to be significantly

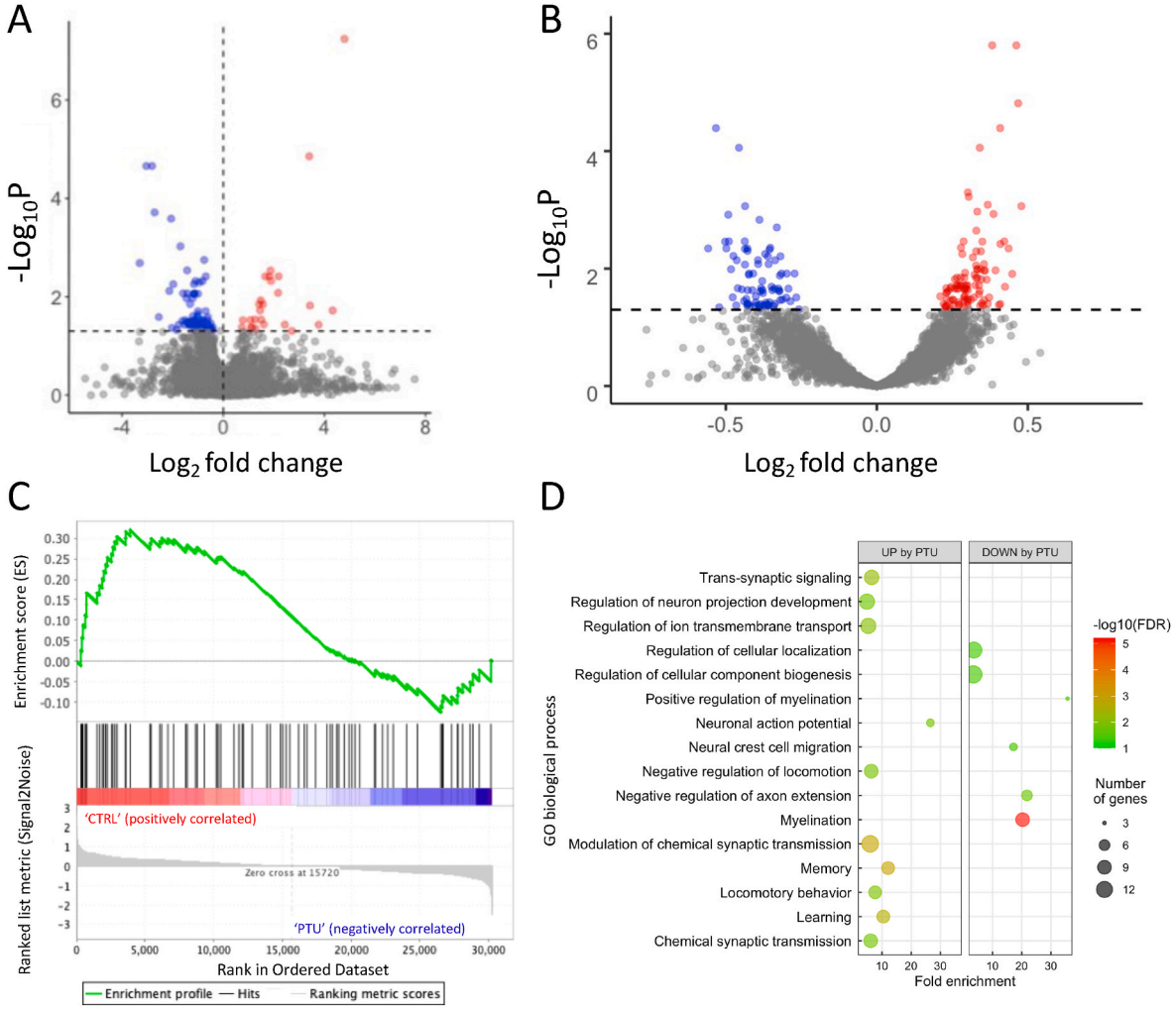

Fig. 2. Plasma metabolome and striatum transcriptome. A) Volcano plot of plasma metabolites detected in the non-target analysis (n = 4574). Negative log10 of the p-value is plotted against the Log2 fold change in relative concentration between the PTU exposed pups versus controls. The horizontal line marks a false discovery rate of 0.05, hence blue dots represent metabolites that are significantly down-concentrated and red dots those significantly up-concentrated. B) RNA-seq analysis of pup striatum at P14. Volcano plot showing the observed changes in gene expression in exposed pups (red: up-regulated genes, blue: down-regulated). C) GSEA analysis of T3-responsive genes in response to PTU exposure. A subset of T3 responsive genes is significantly (p-value <0.05) downregulated in PTU-exposed mice. D) Heatmap of gene ontology of enriched biological processes. Genes, which are up-regulated in exposed pups, tend to relate to neuronal function. The most significant change is for down-regulated genes, which mark a defect in the genetic programme responsible for axon myelination by oligodendrocytes. Color code corresponds to adjusted p-value. Circle sizes reflect the number of differentially expressed genes falling into a Gene Ontology category. Note the identification of the "myelination" term, corresponding to the presence of many genes involved in oligodendrocytes terminal differentiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

decreased (FDR<0.05 fold-changes x0.06 and x0.01; Table S2).

3.4. Unbiased analysis of the transcriptome of pup striatum

Differential analysis showed that 76 genes were up-regulated and 66 were down-regulated (padj<0.05) after PTU exposure (Fig. 2B and Table S3). Among these the usual markers of brain hypothyroidism were absent (*Hr, Kfl9, Nrgn*, etc.) which might lead to the conclusion that PTU does not cause brain hypothyroidism. To more precisely address the presence of a minor TH disruption, we used GeneSet Enrichment analysis (GSEA) which is a more sensitive method to detect minor coordinated changes in gene expression within a well-defined group of genes. We ran GSEA for the set of T3/TRα1 target genes in striatum neurons defined previously [13] as follows: 1) the expression of these genes decreases with high-dose exposure to PTU. 2) It is also low in animals that express a TRα1 receptor mutation only in neurons. 3) It increases quickly (24 h) when animals exposed to PTU are treated with TH. 4) The TRα1 receptor is associated with chromatin in a region located within 30 kb of the transcription start site of these genes. This defined a small group of 35 genes, which are highly reliable endogenous biomarkers of TH signalling level in striatum. GSEA indicates that when genes are ranked according to expression level, the average rank of the 35 genes tends to increase, indicating a trend toward lower expression (Fig. 2C p-value<0.05). Therefore, PTU-exposed juvenile mice display minor signs of brain hypothyroidism, at the postnatal stage where the analysis was performed.

We then used all the available predefined gene sets for an unbiased and global GSEA analysis to better capture the meaning of the PTU-induced changes in gene expression. This highlights a major enrichment in genes of which the expression is restricted to the oligodendrocyte lineage (Fig. 2D). This is a strong indication that PTU-induced changes in the pups' striatum reflect a delay, or an impairment, in oligodendrocytes differentiation. The set of genes that were found to be upregulated in pups were also enriched in genes expressed in neurons, which might also indicate an alteration in neuronal maturation.

We finally used qRT-PCR to confirm the deregulation of few genes on a larger number of mice. These genes were selected among the ones, which have the highest fold-changes (Fig. 3, Table S3).

4. Discussion

PTU is an antithyroid drug used to treat hyperthyroidism, which has been extensively used as a model THSDC. It is a selective inhibitor of thyroid peroxidase, which impairs TH production by thyrocytes, and acts in a dose dependent manner with very limited side effects. Overall, exposure of mice to PTU at 1 mg/L appears to provide a model relevant to human mild hypothyroxinemia during pregnancy, which is currently considered to have an adverse effect on cognitive functions (Berbel et al., 2009).

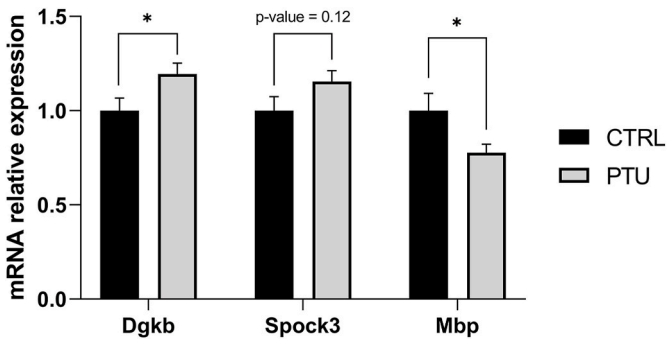

Fig. 3. RT-qPCR confirmation of dysregulation of gene expression in the striatum of PND14 PTU treated mouse pups (n = 8) compared to control littermates (n = 8). * p-value <0.05 (unpaired Student's *t*-test).

We show here that both transcriptome and metabolome analysis can detect the effect of this exposure to PTU in mice. The very low dose of PTU only causes a modest reduction in maternal T4 levels, without altering the T4 and T3 levels in pups. However, due to the statistical power of these methods that we used, analysing as little as four litters proved to be sufficient to detect minor changes induced by exposure to PTU. Meanwhile, repeating the statistical analysis, leaving one sample out failed to detect any transcriptional changes, indicating that four is a strict minimum for the differential analysis to detect these changes. Our study presents two main limitations: first, we only considered a single time point and do not know whether the observed alterations are permanent. Secondly, as studies showing behavioural alterations have only been performed at doses above 3 mg/L in rats (Axelstad et al., 2008; Gilbert et al., 2016; O'Shaughnessy et al., 2018) and 5 mg/L in mice (Amano et al., 2018) we cannot ascertain that the observed effects are adverse effects.

We found that the plasma metabolome contains biomarkers of low-dose PTU exposure. This is emphasized by the 137 differentially concentrated metabolites and especially the annotated ones presented in Table S2. This appears to be a promising result for the future risk assessment of THSDCs. The data interpretation is however more difficult, as few studies have analysed the broader influence of TH deficiency on other plasma metabolites levels leaving the mechanistic links undetermined. Metabolomics analysis of hypothyroid pregnant women has, however, previously showed that lipids content of their plasma differs significantly from those of healthy pregnant women (Cai et al., 2022; Fotakis et al., 2022; Li et al., 2021). The differences include up-concentration of glycerophospholipids and down-concentration of sphingolipids which is parallel to what we detected in the current study. Our metabolomics data are also consistent with data obtained with plasma of hypothyroid rats, in which sphingolipids, glycerophospholipids, and fatty acid transportation were the most affected metabolic pathways (Wu et al., 2013). The accumulation of medium chain acyl carnitines in plasma that we observed has been previously recognised as a biomarker for disorders of fatty acid metabolism (McCann et al., 2021). As T3 activates energy metabolism in most cell types, PTU-induced changes might reflect an alteration of mitochondrial functions. Other metabolomics platforms, that target different molecule, have also found effects of TH in amino acids, ubiquinone and other intermediates of terpenoid-quinone biosynthesis (Boumaza et al., 2019; Fotakis et al., 2022; Wu et al., 2013). However, these pathways were not measurable on the platform applied in the current study. Further investigations into the possible mechanistic links between these metabolic changes and maternal TH deficiency is warranted.

The analysis of the striatum transcriptome outlines a very likely impairment in oligodendrocyte differentiation, which is a typical effect of TH deficiency (Barres et al., 1994; Picou et al., 2012; Rodriguez-Peña et al., 1993). However, the TH receptor target genes were only marginally deregulated in striatum neurons, which argue against an overt brain hypothyroidism at P14. The most likely explanation, which is consistent with previous experiments performed in rats for different areas of the brain(Gilbert et al., 2020, 2016; O'Shaughnessy et al., 2018; Royland et al., 2008; Sharlin et al., 2010; Spring et al., 2016) is that PTU induces transient hypothyroidism in the foetal brain. While the T3 levels in brain is quickly recovered after birth, the process of oligodendrocytes differentiation and myelination is delayed, and the RNA-seq analysis captures the long-lasting consequences of this prenatal defect. Therefore, our post-natal analysis captures a decreased expression for genes expressed in mature oligodendrocytes, but no change for markers of TH signalling. The cell cycle exit of the progenitors of oligodendrocytes and their terminal differentiation is well known to be dependent on T3-signaling (Billon et al., 2001; Durand and Raff, 2000; Picou et al., 2012). A long-lasting defect could alter myelin formation and compromise neuronal survival, however as mentioned, such long term effects were not included in the current study.

In conclusion, the omics methods we developed proved to be suitable

for detecting TH disruption on a small group of animals and, thus, comply to the 3R rules (Burgdorf et al., 2019). Compared to thyroid hormones, other, more sensitive biomarkers were uncovered with omics tools, i.e. 142 differentially expressed genes and 137 differentially concentrated metabolites, which can be applied in future risk assessment of THSDCs.

Author contribution statement

Rikke Poulsen: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization; Yanis Zekri: Methodology, Investigation, Writing - Review & Editing, Visualization; Romain Guyot: Methodology, Investigation, Writing - Review & Editing, Visualization; Frédéric Flamant: Conceptualization, Methodology, Validation, Writing - Original Draft, Supervision, Funding acquisition; Martin Hansen: Conceptualization, Methodology, Validation, Resources, Writing - Review & Editing, Funding acquisition.

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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

The metabolomics datasets generated for this study can be found in the MassIVE repository under acces-sion: MSV000090272

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122783.

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