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Combining transcriptomics and metabolomics to assess neurodevelopmental alteration caused by in utero exposure of mice to three putative thyroid hormone system disruptors.

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Highlights:

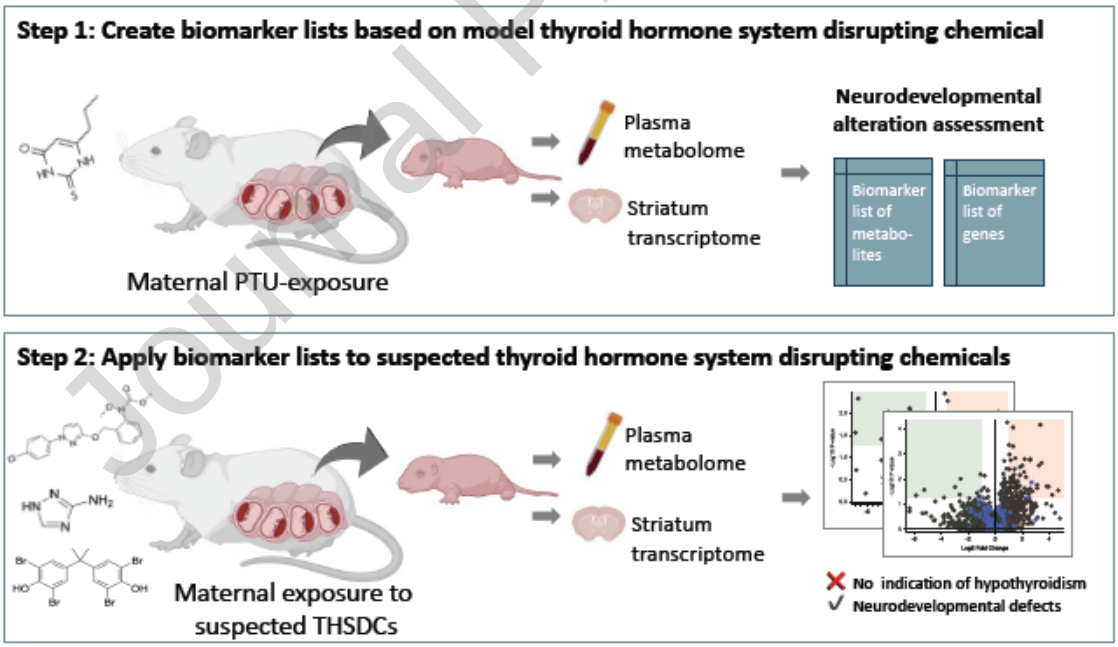
- Transcriptomics and metabolomics biomarkers are available to test for thyroid hormone system disruption in mice.
- None of the chemicals studied produced a brain transcriptomic signature indicative of hypothyroidism
- Omics reveal changes suggestive of neurodevelopmental defects in exposed pups.

Keyword: thyroid hormone/ endocrine disruptors / transcriptomics/metabolomics

Abstract

Gestating mice were exposed to three chemicals, tetrabromo-bisphenol A (TBBPA; 2 mg/kg/day)), amitrole (25 and 50 mg/kg/day) and pyraclostrobin (0.4 and 2 mg/kg/day) to assess their capacity to act as thyroid hormone disruptors and compromise neurodevelopment. Propyl-thio-uracyl, a known pharmacological inhibitor of thyroid gland secretion, was used at both high and low dose as a reference thyroid hormone disruptor (1 ppm, 1500 ppm). A combination of plasma metabolomics and striatum transcriptomics revealed the induced change in pups at the postnatal stages. Although the underlying mechanism is unlikely to involve thyroid hormone disruption, these chemicals had a detectable effect on pups' neurodevelopment.

Graphical abstract



1. Introduction

Thyroid hormones (TH, including 3,3',5-triiodo-L-thyronine, T3, and its less active precursor thyroxine, T4) are essential regulators of vertebrate development (Wojcicka et al., 2013; Bernal, 2017). The mammalian fetus relies on the transfer of maternal TH across the placenta, until the fetal thyroid gland becomes functional, at a late stage of pregnancy (Richard and Flamant, 2018). The main consequences of early TH deficiency, or congenital hypothyroidism, are a blunted skeletal growth and an irreversible mental retardation. Mild forms of hypothyroidism are also associated with neurodevelopmental defects that cause low IQ, an increased incidence of attention deficit, hyperactivity, and autism spectrum disorders (Andersen et al., 2014). A transient decrease in TH level in the fetal brain has also irreversible long-term consequences on neurodevelopment, which is very difficult to measure directly (Royland et al., 2008; O'Shaughnessy et al., 2019). Even hypothyroxinemia, i.e. low maternal T4 with normal plasma T3 and TSH levels, is detrimental to brain development (Berbel et al., 2009).

For these reasons, early exposure to environmental chemicals called thyroid hormone system disrupting chemicals (THSDCs) is a growing concern (Cediel-Ulloa et al., 2022). These chemicals can interfere with the production of THs by the thyroid gland or with the response of tissues to THs. Therefore, measuring circulating levels of T4 and T3 is not sufficient to identify their adverse effects. Although efforts have been dedicated to the identification of putative THSDCs in cell cultures (Paul-Friedman et al., 2019; Zekri et al., 2021), their *in vivo* biodistribution and metabolism are hardly predictable with current *in vitro* assays. Therefore, animal exposure remains indispensable to assess the actual developmental toxicity of THSDCs, and more specifically their neurodevelopmental toxicity (Vandenberg, 2021).

In this context, we previously showed (Poulsen et al., 2023) that unbiased 'omics' methods possess outstanding statistical power and the capacity to detect minor changes in small animal cohorts. As a proof of principle, we recently exposed mice during gestation and lactation to a very low dose of the antithyroid drug propyl-thio uracyl (PTU). Although the plasma level of T4 and T3 were not altered in the progeny, clear adverse effects could be evidenced at post-natal day 15 both in the plasma metabolome and in the striatum (Poulsen et al., 2023) a brain area that is highly sensitive to hypothyroidism (Diez et al., 2008). Importantly, although they were performed at a postnatal stage, these analyses allowed us to identify the consequences of a period of mild hypothyroidism that only occurred during the fetal life, but had long lasting consequences on neurodevelopment (Poulsen et al., 2023). Therefore, the signature of low PTU exposure is different from the signature of high dose exposure, which mainly reflects overt hypothyroidism at the time of analysis (Boumaza et al., 2019; Richard et al., 2020). Overall, these previous experiments produced lists of biomarkers from the plasma metabolome and striatum transcriptome that are now suitable to detect minor signs of present or previous hypothyroidism in exposed mouse pups. The aim of the present study is to assess the ability of three environmental xenobiotics to act as THSDCs during gestation and lactation, using the same procedure. The three tested chemicals were suspected to act as THSDCs for different reasons:

TBBPA is a common brominated flame retardant. It shows some structural similarity with T4, binds to the transthyretin serum transporter and acts as a low-affinity antagonist of the TRα1 nuclear receptor (Oka et al., 2013; Guyot et al., 2014; Ren and Flamant, 2023). While it is often considered as a THSDC, different analyses of its influence on the T3-dependent amphibian metamorphosis led to conflicting results (Thambirajah et al., 2019) Exposed reporter mice

show signs of hypothyroidism in only some organs (Sinko et al., 2022). Amitrole (3-amino-1H-1,2,4-triazole) is a widely used pesticide that inhibits thyroperoxidase activity at high concentrations (Alexander, 1959). It thus prevents the oxidation of iodide on tyrosine residues in thyroglobulin and affects the synthesis of thyroid hormones by thyrocytes. Rats exposed during gestation develop hypothyroidism. This is associated with periventricular heterotopia, a histological alteration of the brain, which is typical of congenital hypothyroidism in this species (Ramhoj et al., 2021; Ramhoj et al., 2022). Pyraclostrobin is a pesticide of the strobilurin family, which has been recently found to inhibit the *in vitro* cellular response to T3 (Zekri et al., 2021). Molecular docking suggests that it could act as an antagonist ligand of the T3 receptors (Yang et al., 2021). It also alters the inflation of the swim bladder in zebrafish embryos, which would be consistent with a defect in TH signaling (Yang et al., 2021). To our knowledge, its capacity to act as a THSDC has not yet been fully assessed *in vivo*.

The 3 chemicals were tested for their capacity to acts as THSDC, by using the procedure of transcriptome and metabolome analysis previously validated with the PTU reference compound.

2. Material and methods

2.1 Study setup and sampling

All animal experiments are complied with the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines and were approved by a local committee (C2EA015) and subsequently authorized by the French Ministry of Research (Project APAFIS#14063-2018022215397455) in compliance with the EU Directive 2010/63/EU for animal experiments. We followed the protocol described in Figure 1, which is identical to the previously described

protocol. High dose PTU exposure was achieved by feeding mice with food supplemented with 0.15% of PTU (Envigo ref TD.95125), while the control group received non-spiked food. Other chemicals (3,3',5,5'-Tetrabromobisphenol A; CAS: 79-94-7 Purity > 96.5 %; Amitrole CAS: 61-82-5 Purity ≥ 98.0 %; pyraclostrobin CAS: 175013-18-0 ≥ 98.0 % all purchased from Sigma Aldrich) were dissolved in corn oil (ref. C8267 Sigma Aldrich) and delivered by gavage (3/week). All doses were calculated to be inferior to 1% of the known LD50, and proved to have no visible adverse effect on mouse health. Controls were given pure corn oil. Pregnant C57BL/6 mice (Charles Rivers Laboratories, France) were received at gestational day four. Gavage started at gestational day 7 (GD7) and ended at postnatal day 13 (PND13). On postnatal day 14 (PND14), mice were deeply anesthetized and decapitated. Blood samples were collected *post-mortem* from dams and pups, and striatum were frozen in liquid nitrogen (Suppl. Table S1).

2.2 RNA analysis

Striatum RNA was extracted using the Macherey-Nagel NucleoSpin RNA II kit. Quality control and quantification (>10 μg/sample) were performed by UV spectrometry (Nanodrop; (Thermo Fisher scientist) and electrophoresis (Tapestation 4150 Agilent). cDNA libraries were prepared for deep sequencing (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) (>10^7 read/library; >85% mapped reads). For TBBPA treated mice, Ion AmpliSeq Transcriptome Mouse Gene Expression Assay and a Proton sequencer (Thermo Fisher

scientist; >5.10^6 mapped reads/library). Count tables are in the supplementary data. Differential gene expression analysis was performed using Deseq2 (Galaxy Version 2.11.40.7) (Love et al., 2014). Deseq2 analysis was also used to verify the absence of batch effect among the group of mice. It also showed that the sex of the pups only alters the expression of 3 genes located on the Y chromosome (*Eif2s3y, Kdm5d* and *Ddx3y*) and does not interact with the response to chemical exposure.

2.3 GeneSet Enrichment Analysis

The GSEA software (https://www.gsea-msigdb.org/gsea/index.jsp) (Subramanian et al., 2005) was used to evaluate the distribution of gene sets in the lists of genes ranked by their fold-changes over control condition, as produced by Deseq2, averaged for each group of mice.

2.4 Sample preparation for thyroid hormone quantification and plasma metabolomics

Plasma samples were prepared as described (Poulsen et al., 2023) based on a validated method (Hansen et al., 2016), with the exception that the samples from each litter were combined. In brief, 50 µL plasma was spiked with isotopic-labelled ($^{13}C_6$)-thyroid hormone standards (internal standards (IS), cT2, cT3 and cT4) and mixed. After antioxidant treatment (100 µL, 25 mg/mL ascorbic acid, R,R-dithiothreitol and citric acid solution) and protein denaturation by urea (8 M in 1% ammonium hydroxide), the samples were enriched using solid-phase micro-extraction (SOLAµ HRP 10 mg/1 mL 96 well plate, ThermoFisher Scientific, Denmark) and reconstituted in 100 µL 5% methanol containing an instrument control standard (ICS, crT3). Procedural blanks containing water instead of plasma were included from the beginning. The samples were extracted and analyzed in 3 batches each with their own control group (Suppl. table S1).

2.5 Thyroid hormone quantification

The thyroid hormones were quantified on an Agilent 6495c triple-quadrupole system with a hyphenated Agilent 1290 Infinity II UHPLC system (binary pump, degasser, and autosampler; Agilent Technologies) as described (Davidsen et al., 2022). Targeted analytes were thyroxine (T4), 3,3',5-triiodo- thyronine (T3), 3,3',5'-triiodothyronine (rT3), 3,5-diiodothyronine (3,5-T2), 3,3'-diiodothyronine (T2), 3-iodothyronine (T1), thyronine (T0), 3-iodothyroacetic acid (T1Ac), 3,5-Diiodothyroacetic acid (Diac), triiodothyroacetic acid (Triac) and tetraiodothyroacetic acid (Tetrac). Limits of detection and quantification are in Suppl. table S2. Quantifiable hormones in our samples were T4, T3, rT3, 3,5-T2, T1 and T1Ac (Table S2). Neat standard ten-point equimolar calibration curves (0.04–20.0 pmol/mL TH, n = 2) were prepared in 5% methanol and all vials contained a fixed amount IS and ICS (15.2 pmol/mL. Data analysis was conducted in MassHunter version 10.1 (Agilent Technologies). The relative difference to control animals remains the focus of the study, hence the absolute values were not corrected for recovery. This however means that the absolute values of T1 and T1Ac, for which no compound specific C13-labelled standard exist, should be taken with caution. Statistical analyses were performed in R (ver. 4.3.0; https://www.r-project.org/) using non-parametric Kruskal-Wallis tests followed by pairwise comparison by Dunn's test (Dinno, 2015) at a significance level of 0.05. Each batch was compared to their own control group. To allow for statistical comparisons, TH levels below the limit of detection (LoD) were replaced by ½ LoD. Data between the LoD and limit of quantification (LoQ) remained as they were. The data was visualized using ggplot2 (Wickham, 2009).

2.6 Metabolomics

The metabolomics analysis was performed as described (Poulsen et al., 2023) using a nanoflow UHPLC Orbitrap mass spectrometer system (ThermoFisher Scientific) with a preconcentration trap-column setup. The instrument was operated in data-dependent mode by automatically switching between MS and MS/MS fragmentation and positive electrospray ionization mode. Samples were analyzed in randomized order and a pooled sample was injected in between every seven samples to correct for any instrumentational fluctuations and monitor systematic errors. Quality and validity of the analysis was confirmed by principal component analysis (PCA) showing that the composite quality control (QC) samples were centrally located in the plot (Suppl. figure S1) and boxplots showing homogenous data variance (Suppl. figure S2). Sample acquisition for the low dose of pyraclostrobin exposure in litters unfortunately failed for two replicates leaving too few replicates for statistical analysis, so this treatment was not considered in the metabolome analysis.

Compound Discoverer (CD) software version 3.3 (Thermo Scientific) was used for data processing and analysis as previously described (Poulsen et al., 2023). Univariate statistical analysis (ANOVA with Tukey as post-hoc test and p-values adjusted by Benjamin-Hochberg algorithm) was also performed in CD. Annotation of the compounds was performed with SIRIUS version 5.8.5 (Duhrkop et al., 2019). This software offers *de novo* molecular formula annotation for database-independent annotation. Molecular fingerprints were predicted by the CSI:FingerID (Duhrkop et al., 2015) (Hoffmann et al., 2021) and the tool CANOPUS (Djoumbou Feunang et al., 2016; Duhrkop et al., 2021; Kim et al., 2021) predicts compound classes from the molecular fingerprint without database searches. Hence, this software provides unbiased structural information for compounds without the need for spectral or structural reference data.

2.7 Biomarker lists for metabolomics

For List 3 we first exposed dams to a high dose of PTU (1500 ppm) contained is food pellets (ENVIGO ref. TD95125) and analyzed the plasma content in the pups to identify biomarkers of overt hypothyroidism as previously described (Poulsen et al., 2023). Sparse partial-least-squares discriminant analysis was performed with the package mixOmics (ver 6.24.0, Using R ver 4.3.0) (Rohart et al., 2017). For details see supplementary methods and for classification performance and visualization of the models see Table S11 and Figure S4.

3. Results

3.1 TH levels measurement

T4 and T3 levels were not significantly altered in dams after chemical exposure (Figure 2A-C). We thought however that measuring the level of other iodinated compounds would help to reveal minor changes in thyroid economy. Interestingly, the low dose of pyraclostrobin, and the high dose of amitrole, had a visible effect of 3,3',5'-triiodothyronine (reverse-T3 or rT3), a known inactive product of T4 catabolism. The two chemicals, but not TBBPA, also affected the concentration of 3-monoiodothyronine (3-T1). In comparison, the high dose of PTU decreased T4, T3 and rT3, but not T1 (Suppl. figure S5A). Although the metabolism of these compounds is not fully understood, these are suggestive for a change in the maternal metabolism of TH, possibly an increased turnover of TH, which might potentially affect the fetuses' development.

The analysis of the pups' plasma revealed a significant increase of T4 and rT3 in pups exposed to pyraclostrobin (Figure 2D-F). Amitrole only modified the level of monoiodothyronine acetate (T1ac) if used at high dose and TBBPA did not affect TH levels. In comparison the high dose of PTU decreased T4, T3 and rT3, and did not affect T1Ac significantly (Suppl. figure S5B).

Although this targeted analysis was performed on a small number of samples, it suggests that none of the 3 chemicals act as genuine THSDCs, and that pyraclostrobin has the opposite effect.

3.2 Selection of genes and metabolites used as biomarkers of previous or current hypothyroidism

Omics analyses previously allowed us to identify the consequences of a period of mild hypothyroidism that only occurred during the fetal life, but had long lasting consequences on neurodevelopment. Based on this, genes and metabolites can be selected as more sensitive biomarkers of present or previous hypothyroidism. The targeted analysis of the striatum transcriptome and plasma metabolome requires to use reference data listing biomarkers that have a coordinated behavior when the level of TH varies in the offspring of dams exposed during gestation and lactation. Three of these lists are available from our previous work:

- List 1 (Suppl. Table S3) gathers genes whose transcription is directly activated by the liganded T3 receptors in striatum neurons and are the most reliable biomarkers of T3 signaling for transcriptome analyses. We previously defined this list as follows: a) exposure to a high dose of PTU, which causes overt hypothyroidism, decreases the level of the corresponding mRNA level in the striatum of hypothyroid pups on postnatal day 15 (P15) b) a genetic mutation of the *Thra* gene that prevents the T3 response of striatum neurons has the same effect. c) The mRNA level is quickly restored when hypothyroid mice are treated with TH (Richard et al., 2020).

- List2 (Suppl. table S4) contains genes whose expression is up- or down-regulated in the brain striatum of pups after exposure to a very low dose of PTU (1 ppm) (Poulsen et al., 2023). Our

previous analysis revealed that the associated changes in gene expression do not correspond to brain hypothyroidism. Instead, they are the postnatal consequences of transient fetal hypothyroidism, which are primarily interpreted as a defect in the differentiation of oligodendrocytes. This second list can thus trace the long-term effect of a mild alternation of TH signaling in the fetal brain.

- List 3 (Suppl. table S5): We completed the reference dataset by analyzing the maternal and pups plasma metabolome exposed to a high dose of PTU (Suppl. figure S3). This list of biomarkers consists of compounds that are either up- or down concentrated in the plasma of the pups after exposure to a high dose of PTU. Sparse partial-least-squares discriminant analysis was applied to identify the metabolites that best discriminate the PTU-exposed pups from the control pups. Multivariate statistics indicated that a list of 16 biomarkers is sufficient to define overt hypothyroidism (Suppl. figure S4A).

- List 4 (Suppl. table S6): The same discriminant analysis was performed with our previous data, obtained after exposing dams to a very low dose of PTU (1 ppm)(Poulsen et al., 2023). The list gathers the 55 biomarkers for transient and mild fetal hypothyroidism caused by this exposure. Only one compound was present in both list 3 and 4. These 4 lists of biomarkers provide a complete overview for the disruption of TH signaling in the striatum and plasma of PTU exposed pups (Suppl. figure S4B).

3.3 Study design

We followed the exact same procedure of *in vivo* exposure of gestating mice that was previously used to analyze the effects of the model THSDC, PTU (Figure 1). Two doses of each chemical were tested, based on known limit doses (Table I). An exception was made for TBBPA

for which a preliminary RNA-seq analysis failed to detect any effect of the high dose (200 mg/kg/day) and for which we only tested a low dose by combined transcriptome and metabolome analysis. The tested chemicals were administered to gestating mice by gavage from gestation day 7 to postnatal day 14 (PND14). The pups were euthanized at this stage, to collect blood and striatum. Maternal blood was also collected. Mass-spectrometry was then used to analyze the metabolome of the plasma. RNA was extracted from striatum for transcriptome analysis. For both metabolome and transcriptome analysis, we considered only a single individual per litter or pooled samples of littermates, to take into account a possible litter effect. Each experimental group contains pups from at least 4 litters (Table I).

3.4 Metabolomics

We first performed an unsupervised analysis to annotate metabolites that characterized the exposure to each chemical. Suppl. table S7 lists the metrics for the metabolomics analysis including number of features and compounds detected. In the maternal metabolomes (Suppl. Figure S6) TBBPA (2 mg/kg/d) significantly affected 12 metabolites (adj. p-value < 0.05, the 8 annotated metabolite are in Suppl. table S8). One of these compounds, a steroid ester, was also affected by the high dose (2 mg/kg/d) of pyraclostrobin (adj. p-value <0.05). Both low- and high dosage of amitrole and the low dose of pyraclostrobin showed no significant effects on the maternal metabolome (adj. p-value>0.05).

We also did not find significant changes in pups plasma with p-values adjusted for multiple tests. However, with non-adjusted p-values, tendencies for up- and down-concentrated metabolites were identified (Suppl. figure S7). This was the case for amitrole exposure, for which 59 compounds were affected (p-value < 0.01). This included 20 lipids and lipid-like

molecules, of which 11 were found to be fatty acyls and belong to fatty acid metabolism (Suppl. table S9). For most of these compounds, they were affected at both the high and low concentration and exhibited a pattern of dose-dependency, which suggests that the variation is exposure dependent (Suppl. figure S8). For both pyraclostrobin and TBBPA, we only obtained metabolomics data for one group and therefore we could not analyze the dose-response patterns. 29 and 36 metabolites were affected (p-value < 0.01) by pyraclostrobin and TBBPA, respectively, and 5 of these overlapped (Suppl. table S9). Among the commonly affected compounds were two steroids; a hydroxysteroid and a pregnane steroid (Suppl. figure S9). Furthermore, the concentrations of a lysophosphatidylcholine and a fatty acyl were increased by TBBPA and pyraclostrobin, respectively, which are compound classes that we also found affected by PTU. Therefore, the signatures were very different for the 3 compounds tested. Overall, these data suggest that chemical exposure during development had a minor influence on the plasma metabolome after birth. Considering the necessity to correct for multiple tests, these differences cannot reach statistical significance if the analysis is performed on a small number of litters.

3.5 Biomarker analysis

The metabolome dataset was then used for a suspect screening analysis of metabolites of which the concentration is changed by either low-dose or high-dose exposure to PTU. A coordinated change in the concentration of these previously defined lists of biomarkers might reveal a weak THSDC activity of the tested chemicals. Four of the biomarkers present in list 3 and 4 were significantly affected by one of the chemicals (p-value<0.05) (Suppl. figure S10) and many were modified to some degree. Despite this, the corresponding volcano plot did not reveal an obvious general trend (Suppl. figure S6 and S7). An exception was the lowest dose

of amitrole, which tended to displace these markers in a coordinated manner in dams plasma, compatible with a weak THSDC activity (Suppl. figure S6D). This suggests that some of the changes caused by these chemicals are related to TH signalling, but the effects are chemical specific and also different from the PTU induced fingerprint.

3.6 Transcriptome analysis

Overall, we identified only a few changes in gene expression in pups striatum after chemical exposure (Figure 3). Compared to PTU (Richard et al., 2020; Poulsen et al., 2023) the 3 tested chemicals had a very limited effect on gene expression, and the response did not increase with the dose (Table II). The genes whose expression is sensitive to PTU did not show an obvious trend which would indicate that the chemicals induced a minor brain hypothyroidism. We then used the supervised GeneSet Enrichment Analysis (GSEA) to more precisely evaluate the influence of the chemicals on TH signaling (Table II). None of the chemical exposure had a significant effect on the T3-responsive genes present in list1, arguing against possible overt brain hypothyroidism. However, list 2 genes, which are biomarkers to a transient fetal hypothyroidism were up regulated in animals exposed to 2 mg/kg/d of pyraclostrobin. This suggests that this chemical influences neurodevelopment, acting in the opposite direction of PTU, i.e., accelerating oligodendrocytes differentiation, which would be consistent with a minor increase in the intracerebral content in TH. Although amitrole had an influence on these biomarkers, and a number of other genes, the orientation of the observed changes and the comparisons between the two doses do not support a mode of action typical of a THSDC.

4. Discussion

4.1 Results of in vivo assessments

We assessed the capacity of three chemicals to act as THSDCs, by combining analyses of plasma metabolome and striatum transcriptome. Both approaches provided a global view of the consequences of chemical exposure during gestation and lactation. The new lists of biomarkers established with the reference compound PTU complete our previous analysis (Poulsen et al., 2023) and are now available to address the capacity of other chemicals to act as THSDCs. It is important to outline that the signature of low dose exposure of PTU, which only causes maternal hypothyroxinemia, and high dose of PTU, which results in overt hypothyroidism are different. We previously concluded that the postnatal analysis of litters exposed to 1 ppm of PTU probably detects a mild and transient fetal hypothyroidism (Poulsen et al., 2023), as reported in rats (Royland et al., 2008), while high dose causes overt hypothyroidism. Although it is probably less amenable to mid-throughput analyses the RNA-seq approach proved to be less variable and thus more suitable than plasma metabolomics to detect modest effects on small groups of animals.

The three tested chemicals were active at doses, which are an order of magnitude lower than LD50. The effects on metabolites such as rT3 and T1, showed that these chemicals do affect the biochemistry related to the TH system. However, our experiments failed to confirm that they act as genuine THSDCs at the tested doses, which were chosen to match previous studies in rats. Only pyraclostrobin had a visible influence on TH signaling, although in the opposite direction than anticipated: while the T4 and T3 levels remain within normal range in the exposed dams, they tend to be increased in their litter. Accordingly, the changes in gene expression observed in the striatum were consistent with a moderate and transient excess of TH in the developing brain. This might be sufficient to have an adverse effect on cognitive functions (Virgili et al., 1991). The fact that only at the lowest dose increased TH signaling in

striatum might suggest that the response to this chemical is not monotonous within the tested range. These complications highlight that many molecular events might lead to the same adverse outcomes as described in the network based adverse outcome pathway framework (Knapen et al., 2018).

In summary, amitrole and TBBPA do not act as THDSCs under the tested conditions. This conclusion differs from the ones of previous studies on rats. This discrepancy is unlikely to be the result of a lower sensitivity of mice to THDSCs, compared to rats, because we previously demonstrated that our procedure is effective to detect the modest effect of exposing mice to 1 ppm of PTU. It seems therefore that mice and rats differ in their response to the tested chemicals. Meanwhile, our data clearly show that the chemicals tested have an effect on both plasma content and gene expression in the brain, not related to a disruption of TH signaling.

The non-target metabolomics analysis revealed some interesting trends in the data that help uncover the molecular fingerprint of the individual chemicals. Steroids were affected by both TBBPA and pyraclostrobin in mothers and in pups, suggesting a cross talk between hormone systems. Interestingly, amitrole had very minor effects on the maternal metabolome, but was observed to influence the lipidome in the pups with mainly fatty acyls and glycerophospholipids being affected. Amitrole is known to be a catalase inhibitor and inhibit the alpha-oxidation of fatty acids (Casteels et al., 1994; Nitta et al., 2020). Alpha oxidation occurs in peroxisomes and involves the degradation of branched chain fatty acids. Two metabolites of this compound class were found up-concentrated by both doses of amitrole. Generally, almost all the affected metabolites were up-concentrated, illustrating an imbalance in the lipidome. At a much higher dose than investigated here (500 mg/kg/day), amitrole has been found to induce fat loss in mice with induced metabolic syndrome (Nunes-Souza et al.,

2020). In conclusion, even if the perturbation in the plasma and brain of exposed pups cannot be called endocrine disruptions, they are sufficient to suggest an adverse effect on neurodevelopment.

4.2 Study limitations

Our aim was to comply to the 3R rules by reducing the number of animals used to assess the capacity of the compounds to act as THSDCs. It should be emphasized that, due to the well-known litter effects, litters, not pups, must be considered as statistical units (Jimenez and Zylka, 2021). While omics compensates for the small numbers of animals per group, by providing large datasets, we may have missed some moderate effects. Notably, some changes in the metabolome would have almost certainly reached statistical significance with larger animal groups. Additionally, most of the putatively affected metabolites were lipids. Hence, the search for biomarkers may be more effective if the analysis platform is directed towards lipidomics. Despite the low-powered study setup we detected clear tendencies for effects on the metabolome. The analysis of a single time point precludes to address the reversibility of the observed changes. The use of omics is not sufficient to define biological effects as adverse effects. It is however likely that the observed effects will have long term consequences, and generally reasonable to consider changes in gene expression in the brain as adverse effects.

4.3. Conclusions

The exposure during gestation and lactation to either pyraclostrobin, amitrole or TBBPA does not induce clear signs of thyroid hormone signaling alteration in pups plasma and brain. However, plasma metabolomics and brain transcriptomics indicate that these chemicals

induce other perturbations, which suggest an adverse effect on neurodevelopment and should prompt further investigation.

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Competing interest statement

The authors have nothing to declare.

Data availability

The metabolomics datasets generated for this study can be found in the MassIVE repository under acces-sion: MSV000090272 and **MSV000095502**. The full count tables for RNA-seq are in supplementary data.

Author contributions

Yanis Zekri : Investigation, formal analysis, reviewing and editing. Rikke Poulsen: Investigation, formal analysis, writing, reviewing, and editing. Martin Hansen: Funding acquisition, supervision, reviewing and editing. Frédéric Flamant: Conceptualization, funding acquisition,

supervision, writing. Romain Guyot: Conceptualization, supervision, formal analysis, reviewing and editing.

Figures legend

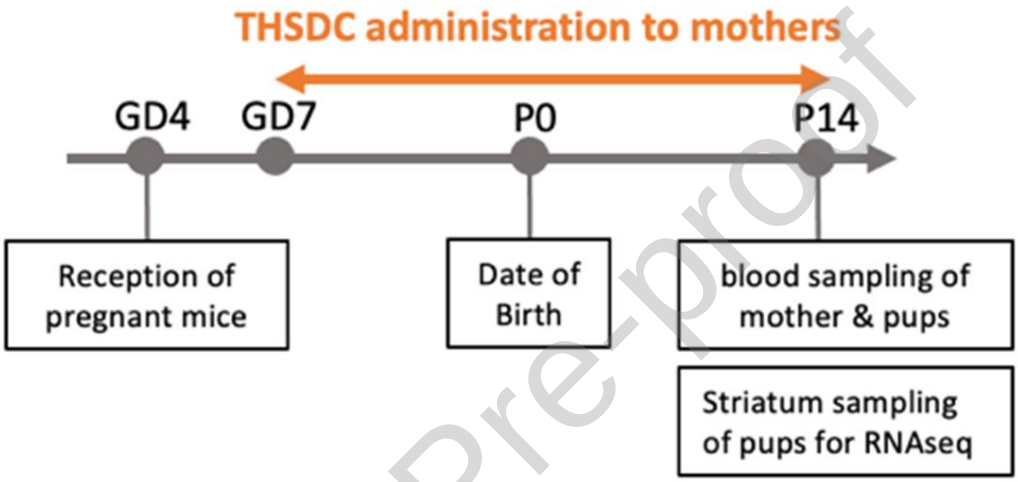

Figure 1: Gestating mice were exposed between gestation day 4 and PND13. Plasma from the dams and pups was collected at PND14. Striatum was extracted from the pup's brain for RNA analysis.

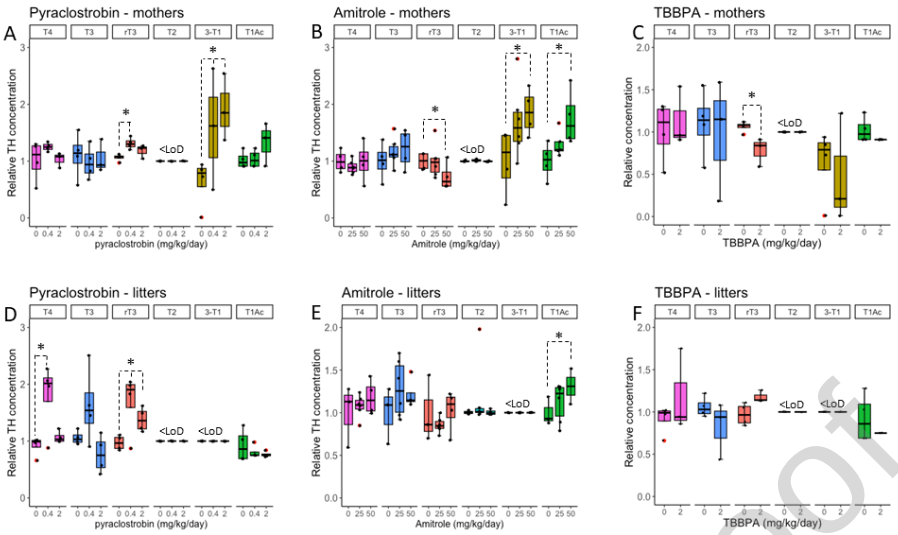

Figure2: measurement of blood content in iodinated metabolites. The concentration is shown relative to the control average (Table S2). Plasma was collected from the mothers (A-C) and litters (E-F) and analyzed by LC-MS/MS. Data were extracted for the two active hormones thyroxine (T4) and 3,3’,5-triiodo-L-thyronine (T3) and the other quantifiable iodinated metabolites: 3,3',5'-Triiodo-L-thyronine or reverse T3 (rT3) 3,3’ di-iodo-thyronine (T2) 3-monoiodothyronine (3-T1) and its acetylated derivative (T1ac). *: p-value <0.05 (Kruskal-

Wallis tests followed by pairwise comparison by Dunn's test). For group size (n) see Table S1

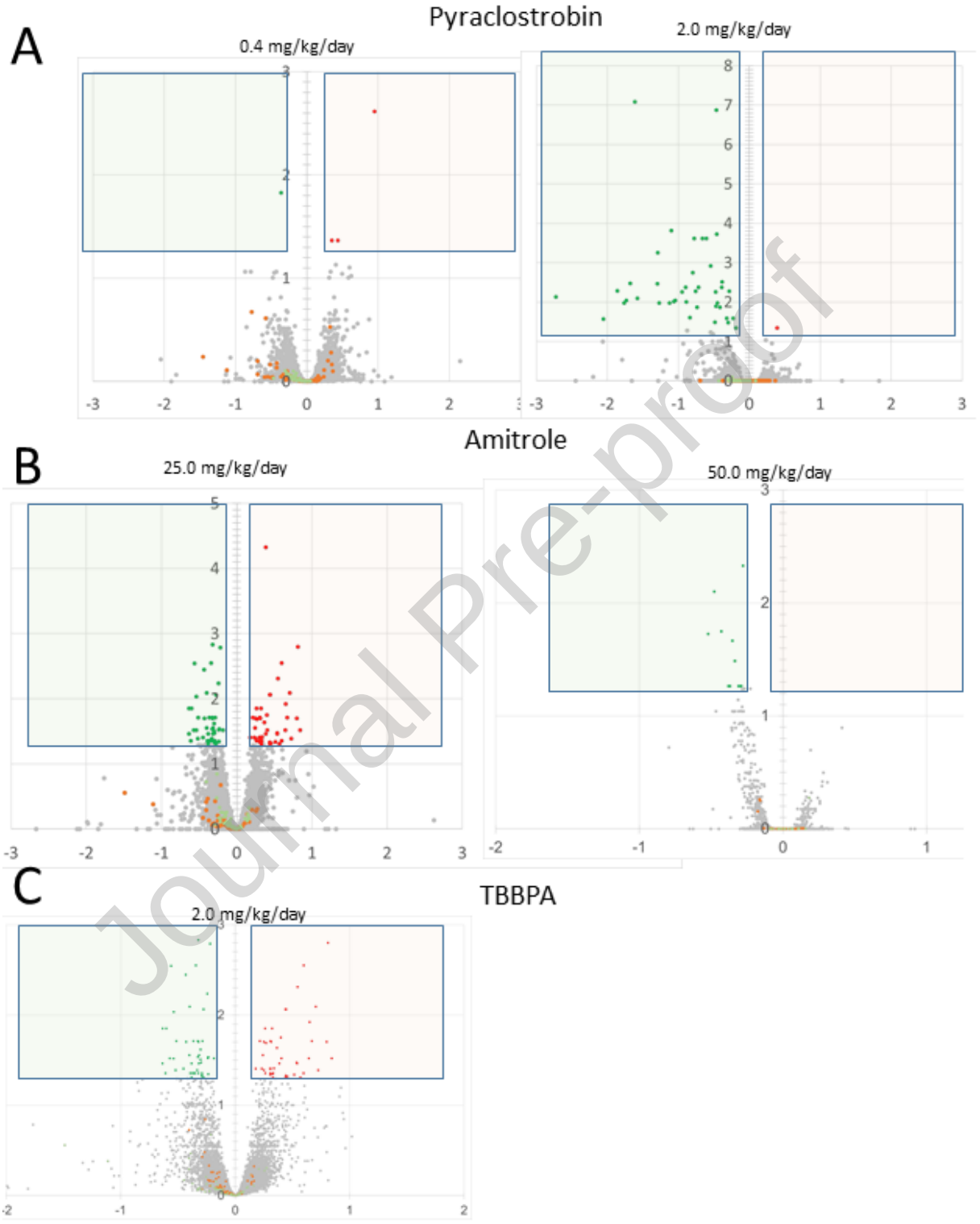

Figure 3: Gene expression analysis in pups striatum. Volcano plots depict the changes caused by exposure to pyraclostrobin (A), amitrole (B) and TBBPA (C) during gestation and lactation.

Green areas and green dots correspond to genes, which are downregulated compared to unexposed controls. Red areas and red dots correspond to up-regulated genes. x-axis: log2 fold-change. Y-axis: - log10 (adjusted p-value). Orange and dots are genes known to be up regulated in pups exposed to a high dose of PTU. Light green dots are genes known to be down regulated in pups exposed to a high dose of PTU. Note the tendency of pyraclostrobin (2 mg/kg/day) and PTU to act in opposite direction.

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Table 1:

Overview of chemicals tested. The tested dose (mg/kg/day) and the number of litters in the treated and control (Ctrl) group is given as well as the oral LD50 (g/kg) reported in literature.

Chemical	LD50 Oral (g/kg)	Dose (mg/kg/day)	Nb of litters
Pyraclostrobin	1.0	0.4	n= 4 (2 ♂ + 2♀)
		2	n= 4 (2 ♂ + 2♀)
Amitrole	14.7	25	n= 3* (3♂)
		50	n= 4 (4♂)
TBBPA	4.5	2	n= 4 (2 ♂ + 2♀)
Control		0	n=19 (8 ♂ + 3♀)

* 1 outlier was excluded from the analysis.

Table 2:

Results of gene expression analysis. For each administered dose; first the number of differentially expressed genes (DE) and the overlap between the genes affected by the high and low doses of the chemical. Then the outcome of the GeneSet Enrichment Analysis (GSEA) showing the p-value and Normalized Enrichment Score (NES) and the status regarding the directionality of the effect compared to the PTU treatments.

		Differential gene expression			GeneSet Enrichment Analysis (GSEA)			
Chemical	Dose (mg/kg/day)	Number of DE Genes	Overlap		Up low dose PTU	Down low dose PTU	Up high dose PTU*	Down high dose PTU*
Pyraclostrobin	0.4	4	0	Status				
				NES				
				p-val.				
	2	43		Status	↓	↑	=	=
				NES	-2.29	1.88	1.06	-1.14
				p-val.	0.00	0.00	0.27	0.28
Amitrole	25	67	10	Status	↑	↑	↑	↓
				NES	1.61	1.67	2.72	-1.95
				p-val.	0.002	0.002	0.0	0.0
	50	15		Status	=	=	↑	↓
				NES	1.03	-0.62	1.65	-1.69
				p-val.	0.38	0.99	0.0	0.01
TBBPA	2	16	0	Status	↓	=	=	=
				NES	-1.58	-1.09	-0.82	1.44
				p-val.	0.006	0.29	0.94	0.08

* and responsive to a 24h treatment with TH. NES: Normalized Enrichment Score

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights:

- Transcriptomics and metabolomics biomarkers are available to test for thyroid hormone system disruption in mice.
- None of the chemicals studied produced a brain transcriptomic signature indicative of hypothyroidism
- Omics reveal changes suggestive of neurodevelopmental defects in exposed pups.