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Testing for heterotopia formation in rats after developmental exposure to selected *in vitro* inhibitors of thyroperoxidase☆

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ABSTRACT

The thyroperoxidase (TPO) enzyme is expressed by the thyroid follicular cells and is required for thyroid hormone synthesis. In turn, thyroid hormones are essential for brain development, thus inhibition of TPO in early life can have life-long consequences for brain function. If environmental chemicals with the capacity to inhibit TPO *in vitro* can also alter brain development *in vivo* through thyroid hormone dependent mechanisms, however, remains unknown. In this study we show that the *in vitro* TPO inhibiting pesticide amitrole alters neuronal migration and induces periventricular heterotopia; a thyroid hormone dependent brain malformation. Perinatal exposure to amitrole reduced pup serum thyroxine (T4) concentrations to less than 50% of control animals and this insufficiency led to heterotopia formation in the 16-day old pup's brain. Two other *in vitro* TPO inhibitors, 2-mercaptobenzimidazole and cyanamide, caused reproductive toxicity and had only minor sporadic effects on the thyroid hormone system; consequently, they did not cause heterotopia. This is the first demonstration of an environmental chemical causing heterotopia, a brain malformation until now only reported for rodent studies with the anti-thyroid drugs propylthiouracil and methimazole. Our results highlight that certain TPO-inhibiting environmental chemicals can alter brain development through thyroid hormone dependent mechanisms. Improved understanding of the effects on the brain as well as the conditions under which chemicals can perturb brain development will be key to protect human health.

1. Introduction

Proper brain development is critically dependent on thyroid hormones. Epidemiological studies show associations between low maternal thyroxine (T4) concentrations and neurodevelopmental effects manifesting as decreased intelligence coefficients (IQ) and cognitive disorders later in the child's life (Andersen et al., 2018; Fetene et al., 2017; Ghassabian et al., 2014; Gyllenberg et al., 2016; Haddow et al., 1999; Korevaar et al., 2016; Román et al., 2013). These associations have been confirmed in animal studies, where disruption of thyroid hormone signalling during development led to brain malformations and behavioural effects in the maturing and adult offspring (Akaike et al., 1991; Ausó et al., 2004; Axelstad et al., 2008; Gilbert et al., 2016; Gilbert and Sui, 2006; Lavado-Autric et al., 2003; Richard et al., 2020; Sharlin et al., 2008). Thus, it is reasonable to assume that exposures in early life to environmental chemicals that can disrupt the thyroid hormone system can also perturb brain development and ultimately pose a threat to human health.

The thyroid hormone system comprises a complex network of hormones, transporters, metabolizing enzymes and receptors, with tissues and organs interconnected by regulatory feedback loops. This means that environmental chemicals can disrupt the thyroid

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hormone system in a multitude of ways (Noyes et al., 2019). One established mechanism, and the focus of this study, is inhibition of the thyroperoxidase (TPO) enzyme (Crofton et al., 2019; Hassan et al., 2017). TPO is exclusively expressed in the thyroid gland where it functions to synthesise thyroid hormones. TPO oxidizes iodide to iodine, iodininates thyroglobulin and couples iodinated tyrosine residues to form the thyroid hormone iodothyronines T4 and 3,3′,5-tri-iodothyronine (T3) (Carvalho and Dupuy, 2017). Since thyroid hormones cannot be synthesized without properly functioning TPO, inhibition of this enzyme can severely diminish serum T4 concentrations. This critical role is exploited in medical treatment of hyperthyroidism, where the antithyroid drugs propylthiouracil (PTU) and methimazol (MMI) reduce serum T4 concentrations by inhibiting TPO activity. When given to pregnant and lactating rats, both PTU and MMI markedly reduce serum T4 concentrations in dams, foetuses and pups throughout development (Hasebe et al., 2008; Hassan et al., 2017; Morreale de Escobar et al., 1988; O'Shaughnessy et al., 2018b; Ruiz de Oña et al., 1988).

The suppression of thyroid hormone production during development by PTU and MMI can have severe consequences for neurological development in the offspring. One of the effects on the developing brain is disrupted neuronal migration, resulting in a type of irreversible brain damage, periventricular heterotopia, which are misplaced neurons found in the white matter of the pup's brains (Gilbert et al., 2014; Goodman and Gilbert, 2007; O'Shaughnessy et al., 2018a, 2019; Shibutani et al., 2009; Shiraki et al., 2012; Spring et al., 2016). These misplaced neurons cluster closely in the corpus callosum to form heterotopia whose size increases with increasing T4 deficiency while their formation can be prevented by co-administration of T4 (Gilbert et al., 2014; Goodman and Gilbert, 2007; O'Shaughnessy et al., 2019). Heterotopia can be detected in rodent brains even after exposure to doses of PTU that only have minor effects on maternal serum T4 concentrations, but concomitantly severely reduce foetal and neonatal thyroid hormone concentrations in serum and brain (Gilbert et al., 2014; Hassan et al., 2017; O'Shaughnessy et al., 2018b, 2019). The reduced T4 concentrations in neonatal brains after PTU administration may well explain the ability of the drug to produce heterotopia. However, PTU and MMI are highly potent drugs designed to suppress T4. Whether or not heterotopia may develop from exposure to environmental chemicals designed for other purposes, but then also found to inhibit TPO *in vitro*, remains unknown (Gilbert et al., 2020).

To test if TPO inhibitors can cause heterotopia in the brain, we leveraged information from the U.S. Environmental Protection Agency's (EPA's) Toxicity Forecaster (ToxCast) which has identified a host of chemicals that can inhibit TPO in *in vitro* assays (Friedman et al., 2016; US EPA, 2020). Our aim was to establish whether environmental chemicals that are *in vitro* TPO inhibitors can disrupt the thyroid hormone system to such an extent that brain development is severely disrupted and heterotopia are formed. Such studies are urgently needed to identify hazards for brain development that may be associated with *in vitro* TPO inhibition.

2. Materials and methods

2.1. Test chemicals

We applied a systematic selection process using Toxcast, published literature and the REACH registration database to identify three TPO inhibitors for developmental toxicity screening *in vivo* (see Fig. 1 and 3.1). We chose compounds that were potent TPO inhibitors *in vitro*, showed clear evidence of thyrotoxic potential *in vivo*, and had a presumed lack of carcinogenicity and neurotoxicity through modes of action other than TPO inhibition. Furthermore, some published toxicity studies with pregnant animals needed to be available to allow for dose selection to avoid doses causing overt maternal toxicity. Lastly, we excluded well established goitrogens that were considered positive controls for TPO inhibition. Based on this procedure, we selected the following compounds for *in vivo* testing: 2-Mercaptobenzimidazole (MBI) (CAS no: 583-39-1, Sigma-Aldrich M3205-5G, lot: STBH8752, purity 98%), 3-Amino-1H-1,2,4-triazole (amitrole) (CAS no: 61-82-5, Sigma-Aldrich 8144950100, batch: S7075495 847, purity >97.0%) and cyanamide (CAS no: 420-04-2, Sigma-Aldrich, 187,364-5G, lot: STBH8303, purity 99%).

2.2. Animals and treatments

Time-mated nulliparous Sprague-Dawley rats (Crl:CD(SD) bred by Charles River Europe, distributed by SCANBUR, Denmark) with a body weight of approximately 240 ± 30 g were received at gestation day (GD) 3 of pregnancy (the day of plug detection designated as GD1). On GD4 the 28 dams were weighed, randomized and assigned to 7 dose groups with similar weight distributions (n = 4 per group). The exposure groups were: a vehicle control group, and two doses each of MBI (10 and 20 mg/kg bw/day), amitrole (25 and 50 mg/kg bw/day) and cyanamide (7 and 15/11.25 mg/kg bw/day).

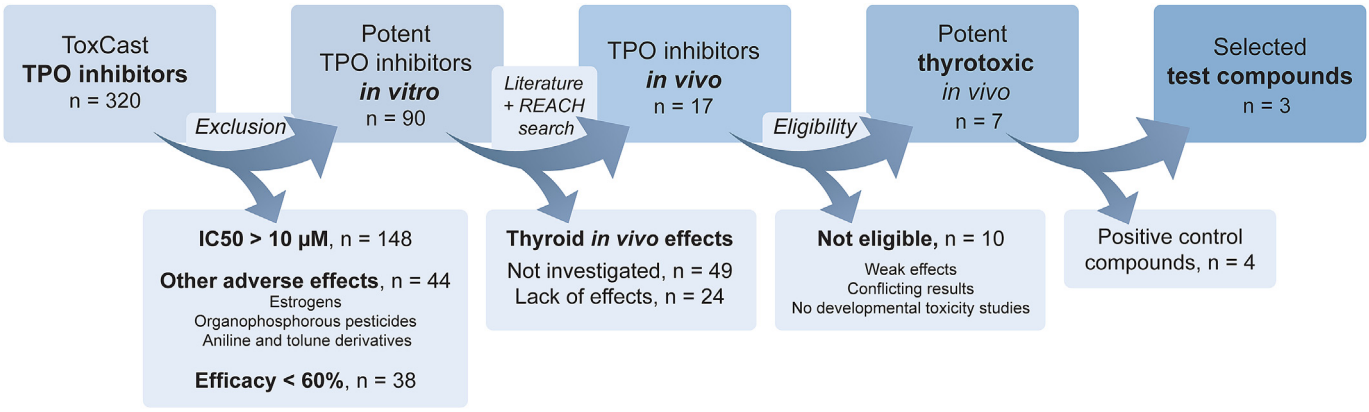

Fig. 1. Work flow for systematic prioritization strategy of thyroperoxidase (TPO) inhibitors for *in vivo* testing for heterotopia linked to thyroid hormone insufficiency. Out of 93 potent inhibitors of TPO that were not toluene or aniline derivatives there were no available *in vivo* data on thyroid effects for more than 50% of the compounds. For another ~25% there were data showing investigated thyroid gland endpoints unaffected. The remaining 20 compounds were investigated for their suitability for developmental studies. Finally, 3 compounds were chosen for this study. REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals, TPO: Thyroperoxidase, IC: Inhibitory concentration.

Due to reduced weight gains in the pregnant dams, the initial dose of 15 mg/kg cyanamide was lowered to 11.25 mg/kg from GD16 onwards. All dams were dosed daily, in the morning, by oral gavage and with a constant volume of 2 ml/kg bw. Dosing began at GD7 and lasted to postnatal day (PD) 22, except the day of birth. Corn oil (Sigma-Aldrich) was used as a vehicle and in the control group.

The dams were housed pairwise within treatment groups until GD17, and individually thereafter. The animals were housed in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast S.p.A, Buguggiate, Italy) (15 × 27 × 43 cm). Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark), and Tapvei Arcade 17 (aspen wood) shelters (Brogaarden) were provided. Cages were placed in ScanTainers (ventilated cabinets, Scanbur) with ScanClime (Scanbur) controlled environmental conditions: humidity 55 ± 5%, temperature at 21 ± 1 °C and airchange 50 times per hour. Reversed light/dark cycles of 12 h with sunrise/sunset of 30 min (light from 9 p.m. to 9 a.m., dark from 9 a.m. to 9 p.m.). All animals were provided *ad libitum* acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast) and were fed *ad libitum* on a standard diet with Altromin 1314 (soy and alfalfa-free, Altromin GmbH, Lage, Germany). The iodine content of this diet was 1.52 mg/kg and the selenium content 0.26 mg/kg.

Dam body weights were recorded daily before dosing and all animals were inspected twice daily for signs of toxicity. Dams from the control, amitrole and cyanamide groups all gave birth on GD23 in the morning (assigned as PD1 for all pups). Dams from the 10 mg/kg MBI group all gave birth in the afternoon of GD23 and all dams in the high dose MBI (20 mg/kg) had to be killed due to parturition problems (one dam on GD22, the remaining three in the afternoon on GD23). Thus, the 20 mg/kg group did not deliver any viable litters and was excluded from most analyses.

On the day of birth (PD1), pups were weighed, sexed and counted. Pup weights were recorded on PD6, 14 and 22 when the study was terminated. One male and one female pup per litter were killed by decapitation on PD6. On PD16, three males and two females were killed by decapitation under CO_2/O_2 anesthesia, and on PD22 the same procedure was used to terminate dams and the remaining pups.

The animal experiments were carried out in the BioFacility of the Technical University of Denmark. Ethical approval was given by the Danish Animal Experiments Inspectorate, authorization number 2015-15-0201-00553 C3. The experiments were overseen by the in-house Animal Welfare Committee for animal care and use at the National Food Institute.

2.3. Serum hormone concentrations

Serum samples were collected in Eppendorf tubes without heparin, on GD15 by tongue bleeding of unanaesthetized dams. Trunk blood was collected on PD6 (one male and one female pup per litter) and PD16 (three males pooled and 2 females pooled for each litter) and dams on PD22. Blood was kept on ice (<1 h) until centrifugation for 10 min at 4 °C and 4000 rotations per minute (rpm), serum was collected and stored at −80 °C until analysis.

Serum total T3 and T4 were determined in duplicates in dams at GD15 and PD22 and in offspring at PD6 (only T4 due to small volume) and PD16 using validated radioimmunoassays (RIA4524 resp. RIA4525; DRG Instruments (Marburg, Germany)). To improve sensitivity, two additional concentrations were added to the standard curves by diluting the lowest standard calibrator by a factor of 4 and 8 with the zero standard provided with the assay kit. All other standard calibrators were diluted by a factor 2 with the zero standard. Also to improve sensitivity the sample and standard volumes were doubled when compared to the manufacturer's protocol. According to the manufacturer's protocol samples and calibrators for a standard curve were incubated with 125I-T4 or 125I-T3 as a tracer in antibody-coated tubes for 1 h. Bound radioactivity was determined in a gamma counter (1277 GammaMaster; LKB Wallac, Turku, Finland). The limit of quantification was 4.0 nM and 0.20 nM for total T4 and T3, respectively. Intra-assay coefficients of variation were <10% and <8%, respectively.

Serum TSH was measured in GD15 dams and PD16 offspring by the Milliplex MAP rat pituitary magnetic bead panel (RPTMAG-86 K; EMD Millipore, Darmstadt, Germany). The TSH Milliplex assay was performed following the manufacturer's instructions. The limit of quantification was 3.2 pg/ml for TSH. Intra-assay coefficients of variation were <10.5%.

2.4. Organ weights and tissue samples

Necropsy was performed on two male pups per litter on PD16 and on PD22 dams. The pups were weighed, decapitated and thyroid gland and liver were excised and weighed. Brain with cerebellum (one pup per litter from high dose only) was excised and immersion fixed in 10% formalin for 5 days. After fixation, the brains were blocked with coronal cuts anterior and posterior to the hippocampus. The blocks (containing the hippocampus) were processed and paraffin embedded. The brains were sectioned at 10 μm and every 3rd section collected. Collection took place from the anterior hippocampus to the division of the corpus callosum. On PD22, at termination of the study, the dams were killed, and thyroid glands and livers removed and weighed. The uteri were excised and implantation scars counted.

2.5. Immunohistochemistry and detection of heterotopia

Immunohistochemical staining was performed on brain sections with NeuN antibody in Shandon Sequenza Immunostaining racks (Thermo Scientific, Roskilde, Denmark). Sections were boiled in a microwave for 15 min in Tris/EDTA buffer (pH 8.95–9.05) and blocked in 1% bovine serum albumin in PBS buffer for 30 min. Sections were incubated overnight at 4 °C with 1:15,000 NeuN (MAB377 Neuronal Nuclei, EMB Millipore Corp, now Merck, Darmstadt, Germany) in 1% bovine serum albumin. Endogenous peroxidase was blocked with 3% H_2O_2 in PBS for 10 min and then incubated for 30 min with EnVision + System-HRP Labelled Polymer Anti-mouse (K4001, Dako, Glostrup, Denmark). Sections were stained in DAB+ (Dako, Glostrup, Denmark) for 10 min, and counterstained in Meyer's hematoxylin.

Sections were evaluated for heterotopia, as first described by Goodman and Gilbert (2007), by light microscopy in the corpus callosum from the midline and more lateral until inferior to the frontal cortex area 2. A heterotopia was defined as a minimum of 5 large NeuN + cells in the near vicinity of each other and present on at least 2 adjacent sections over the subiculum and present on sections towards the posterior part of the hippocampus. Hemispheres on which a heterotopia was identified were counted and summed for left and right hemisphere per animal, then averaged across groups.

2.6. Statistical analysis

Data from continuous endpoints (e.g. hormones, organ weights) were analyzed for dose-related effect differences to the controls by General Linear Models (GLM), with data from all treatment groups analyzed in the same model. If deemed necessary, data were log-transformed prior to data analysis, and covariates were included in data analysis when considered appropriate (e.g., litter size for

birth weight, body weight for all organ weights, pup sex for the hormones). Litter effects were accounted for by analysing data from one pup per litter, by using litter means or for male pup organ weights (where two pups per litter were included) by using litter as an independent, random and nested factor in the statistical analysis. Gestation length, post-implantation loss and perinatal loss were investigated for differences to the controls by the non-parametric Kruskal-Wallis test. Statistical testing between control and treatment groups was Dunnett-adjusted to account for multiple testing (α = 5%, two-sided). Heterotopia formation was assumed to follow a Poisson distribution (according to unpublished data), and significant differences between the control and treatment means were investigated by contrast testing in a Poisson regression model, with p-values adjusted for multiple testing according to the Holm-Sidak (Abdi, 2007). All statistical analyses were conducted in SAS Enterprise v8.3.

3. Results

3.1. Systematic selection of test compounds

We used the ToxCast database as a starting point for selecting TPO inhibitors for our studies and applied a systematic selection process (Fig. 1) (US EPA, 2020). A list of the most potent *in vitro* TPO inhibitors was extracted from the database (n = 320). Compounds with IC50 values > 10 μM were excluded, as were estrogens, aniline derivatives suspected of carcinogenicity, potentially directly neurotoxic compounds (organic toluene solvents and organophosphorus pesticides) (n = 44) and compounds with maximal TPO inhibition (efficacy) < 60% in the Toxcast assay (n = 38). For the remaining 90 compounds, we searched for *in vivo* studies of thyroid hormone system disrupting effects. According to our searches in Pubmed (https://pubmed.ncbi.nlm.nih.gov/) and in the REACH registration database (https://echa.europa.eu/home) more than 50% of the compounds had not been studied for thyroid related effects *in vivo,* and these were excluded (n = 49). Also excluded were compounds that did not show effects on thyroid-related endpoints (n = 24). Notably, most of these compounds had been inadequately investigated; "no effect"-data on thyroid histopathology and cancer development were available for most compounds, but effects on thyroid hormone concentrations had only been investigated for a few of them. Eligibility was determined for the remaining 17 compounds: for 10 of them, *in vivo* effects appeared weak or inconsistent, or there were no developmental studies available. At the end of this process, we were left with 7 compounds that fitted our selection criteria (Table 1). After excluding compounds that could be regarded as positive controls (6-propyl-2-thiouracil, 6-methyl-2-thiouracil, methimazole and ethylene thiourea) (n = 4), we arrived at 3 suitable test compounds all in current use in the European Union: 2-Mercaptobenzimidazole (MBI), amitrole and cyanamide. MBI is used as a rubber antioxidant and anti-corrosive agent, amitrole is a herbicide and biocide, and cyanamide is applied in agriculture as a rest-breaking agent to stimulate the uniform opening of buds.

Table 1
TPO inhibitory properties, selected test compounds and positive control compounds.

Compound	IC_{50} (μM)	% E_{max} (inhibition)[a]	Test compound
2-Mercaptobenzimidazole (MBI)	0.06	97%	yes
Methimazole (MMI)	0.06	83%	–
6-Propyl-2-thiouracil (PTU)	0.23	93%	–
6-Methyl-2-thiouracil (MTU)	0.46	95%	–
Amitrole	1.76	69%	yes
Cyanamide	2.42	86%	yes
Ethylene Thiourea (ETU)	7.75	102%	–

Data from ToxCast (US EPA, 2020).
[a] Percent maximal inhibition of thyroperoxidase (TPO).

3.2. Systemic, reproductive and developmental toxicity

The three compounds chosen for *in vivo* testing – MBI, amitrole and cyanamide – produced surprisingly different toxic effect patterns in dams and offspring. Most striking were the effects by MBI, where dams experienced problems giving birth (dystocia) at the highest dose (20 mg/kg). As a result, one dam had to be killed on GD22 and the remaining three on GD23 (Table 2). All the dams that received the lower dose of 10 mg/kg MBI delivered viable, although smaller, litters after prolonged gestation (i.e. in the afternoon on GD23, while animals exposed to control, amitrole and cyanamide all had delivered by the morning of GD23) (p = 0.001). In the 10 mg/kg group there was reduced pup survival with a perinatal loss of 18%. Offspring body weight was lower in MBI treated litters compared with controls. This effect was statistically significant in the male offspring on PD16.

Amitrole caused diminished pup growth in the later postnatal period (Table 2), an effect that potentially can be ascribed to thyroid hormone insufficiency (see below). Amitrole did not produce effects on dam body weights or other signs of maternal toxicity.

Lastly, cyanamide reduced dam gestational body weight gains. After 10 days of treatment it became evident that the highest dose (15 mg/kg bw/day) caused a marked decrease in body weight gain in the pregnant dams (33 ± 10 g) compared to control dams (52 ± 7 g). Consequently, we lowered this high dose to 11.25 mg/kg bw/day from GD16 onwards. This dose reduction restored body weight gains from GD16-GD21 to levels similar to those seen with the lower cyanamide dose (7.5 mg/kg) and with untreated controls (~63 g in all three groups). However, both doses of cyanamide produced markedly decreased body weights in the offspring. In male pups, these decreases reached statistical significance from PD14 onwards; in female pups this was the case on PD22 (Table 2). Thus, testing cyanamide at doses exceeding 11.25 mg/kg bw/day would have produced excessive systemic toxicity in the dams and offspring.

None of the compounds significantly affected absolute liver weights (adjusted for body weight in the statistical analysis), neither in the dams nor the offspring (Table 2).

In summary, MBI induced clear reproductive toxicity, rendering dams unable to give birth at the high dose (20 mg/kg) and, at the lower dose of 10 mg/kg, prolonging gestation and causing pup loss. Cyanamide (15 mg/kg) decreased maternal gestational weight gains and amitrole, along with the other two compounds, decreased pup body weights postnatally.

3.3. Maternal thyroid hormone system disruption

After one week of exposure to MBI, amitrole and cyanamide (GD15), and at study termination (PD22), we measured maternal thyroid hormone serum concentrations. On GD15 there were no statistically significant changes to T4, T3 or TSH after exposure to amitrole and cyanamide. In contrast, MBI reduced serum T3 (p = 0.037) and caused increased TSH concentrations (p = 0.017) in the high dose group (20 mg/kg), indicating activation of the HPT-axis to maintain T4 concentrations (Fig. 2, A). At the end of the study on PD22, amitrole had caused a pronounced reduction in T4 concentrations at both 25 and 50 mg/kg bw/day, whereas exposure to the lower dose of MBI (10 mg/kg) and both doses of cyanamide (Fig. 2, B) left T4 and T3 concentrations unchanged. Dam thyroid gland mean weights were increased after dosing with 10 mg/kg

Table 2
Pregnancy, litter data and organ weights.

	Control (corn oil)	**MBI-10**, mg/kg bw/day	**MBI-20**, mg/kg bw/day	**Amitrole-25**, mg/kg bw/day	**Amitrole-50**, mg/kg bw/day	**Cyanamide-7.5**, mg/kg bw/day	**Cyanamide-15/11.25**, mg/kg bw/day
Pregnancy and litters							
Time-mated females (no.) (viable litters)	4(4)	4(4)	4(0)	4(4)	4(4)	4(4)	4(4)
Pregnant, but could not give birth and were killed	0	0	4	0	0	0	0
Maternal bw GD7 (g)	245.5 ± 28.2	253.4 ± 36.3	247.9 ± 19.2	242.6 ± 12.3	250.7 ± 29.0	245.6 ± 12.8	245.3 ± 19.8
Maternal bw gain GD7-GD21 (g)	125.5 ± 14.8	123.4 ± 20.0	94.8 ± 14.3	130.7 ± 14.0	127.6 ± 18.8	120.1 ± 14.6	104.5 ± 20.9
Maternal bw gain GD7- PD1 (g)	38.6 ± 3.1	42.4 ± 8.2	–	42.4 ± 10.3	34.6 ± 6.8	37.7 ± 10.9	36.5 ± 5.7
Maternal bw gain PD1-PD14 (g)	41.3 ± 11.3	45.5 ± 3.7	–	36.3 ± 13.6	49.8 ± 12.4	32.0 ± 12.3	42.5 ± 8.5
Maternal bw gain PD14-PD22	−29.3 ± 7.5	−19.5 ± 1.3	–	−9.3 ± 14.2	−19.3 ± 8.2	−24.5 ± 11.7	−19.0 ± 14.5
Gestational length (d)	23 ± 0	**23.5 ± 0****	–	23 ± 0	23 ± 0	23 ± 0	23 ± 0
Litter size (no.)	14.0 ± 1.4	12.0 ± 2.8	–	14.0 ± 2.2	14.5 ± 2.9	14.8 ± 1.3	13.8 ± 4.0
Litter size. live pups. PD 1 (no.)	14.0 ± 1.4	11.0 ± 2.4	–	14.0 ± 2.2	14.3 ± 2.5	14.5 ± 1.3	13.8 ± 4.0
Postimplantation loss (prenatal mortality) (%)	5.2 ± 6.8	11.7 ± 10.0	**100.0 ± 0.0***	0.0 ± 0.0	1.6 ± 3.6	5.9 ± 6.8	11.3 ± 18.4
Perinatal loss (pre- and postnatal mortality) (%)	5.2 ± 6.8	18.3 ± 12.6	**100.0 ± 0.0***	4.5 ± 9.1	4.3 ± 5.4	11.8 ± 14.4	11.3 ± 18.4
Dam organs							
Dam bw PD22 (g)	296.0 ± 20.9	321.8 ± 36.5	–	312.0 ± 23.1	315.8 ± 38.6	290.8 ± 11.8	295.3 ± 34.7
Dam liver weight PD22 (g)	13.7 ± 0.8	14.5 ± 2.3	–	13.9 ± 2.7	15.8 ± 3.7	13.5 ± 0.4	14.1 ± 2.5
Offspring body weight							
Birth weight. Male pups (g)	7.2 ± 0.3	7.2 ± 0.3	–	7.5 ± 0.4	7.2 ± 0.4	7.3 ± 0.5	7.0 ± 0.7
Birth weight. Female pups (g)	6.9 ± 0.1	6.8 ± 0.4	–	6.9 ± 0.3	6.9 ± 0.4	6.8 ± 0.5	6.5 ± 0.5
Body weight. PD 6. Male pups (g)	15.0 ± 0.6	14.0 ± 1.7	–	13.3 ± 1.4	13.8 ± 1.3	13.7 ± 1.5	14.0 ± 2.0
Body weight. PD 6. Female pups (g)	14.1 ± 1.1	13.5 ± 1.9	–	12.5 ± 0.7	13.0 ± 1.1	13.1 ± 1.7	13.4 ± 1.8
Body weight. PD 14. Male pups (g)	39.9 ± 1.2	34.7 ± 4.0	–	**29.3 ± 2.8****	**30.7 ± 1.7***	**31.6 ± 5.3***	**31.3 ± 6.0***
Body weight. PD 14. Female pups (g)	37.7 ± 4.1	33.6 ± 3.6	–	29.0 ± 1.8	30.4 ± 1.8	30.2 ± 5.8	30.8 ± 5.8
Body weight. PD 22. Male pups (g)	73.9 ± 2.1	65.0 ± 0.0[a]	–	**54.6 ± 5.4****	**56.0 ± 3.4****	**57.0 ± 7.1***	**52.4 ± 4.0****
Body weight. PD 22. Female pups (g)	72.9 ± 3.6	65.0 ± 6.7	–	**54.0 ± 2.9****	**55.6 ± 4.5***	**57.6 ± 9.4***	**56.3 ± 8.9***
Offspring organ weights							
Male pup weight PD16 (g)[b]	45.7 ± 2.4	**38.6 ± 4.6***	-	**32.7 ± 2.7****	**34.9 ± 1.5****	**35.2 ± 6.0****	**35.2 ± 6.8****
Male pup liver weight PD16 (g)[c]	1.38 ± 0.11	1.14 ± 0.18	–	0.88 ± 0.06	0.94 ± 0.06	0.98 ± 0.22	0.99 ± 0.20
Male pup thyroid weight PD16 (mg)[c]	3.6 ± 0.4	4.2 ± 1.6	–	**5.0 ± 1.7****	**5.8 ± 1.7****	2.8 ± 1.0	2.4 ± 1.2

Bw: body weight. Data shown as litter means ± standard deviation.
*p < 0.05, **p < 0.01 compared to the control. For organ weights body weight was included as covariate in the statistical analysis.
[a] On PD22 a single litter had remaining male pups.
[b] Weight of the pups from which the liver weights were obtained.
[c] Data from two pups per litter and accounting for litter effects in the statistical analysis.

MBI and in both amitrole-exposed groups. However, there were considerable variations between dams and the weight increases did not reach statistical significance. Cyanamide did not cause significant changes to thyroid hormone concentrations, TSH or thyroid gland weights in rat dams.

In summary, our study demonstrated clear thyroid hormone system disruption at both doses of amitrole. There were indications of thyroid hormone system-disrupting effects in MBI-exposed dams, most pronounced on GD15 in dams that received the high dose, but these dams did not survive giving birth. In contrast, there were no signs of thyroid system disrupting effects in pregnant rats at highest tested dose of 15/11.25 mg/kg cyanamide, and higher doses could not be used due to maternal toxicity.

3.4. Thyroid hormone system disruption in offspring

On PD6 and PD16 we assayed T4 serum concentrations in the pups (Fig. 3) and observed effect patterns like those seen in the dams. On PD6 (the end of the critical window for heterotopia formation (O'Shaughnessy et al., 2019)) and on PD16, amitrole reduced serum T4 concentrations ($p = 0.0312$). On PD16, T3 serum concentrations were also suppressed, albeit without a clear dose-response relationship. These changes were accompanied by a pronounced activation of the HPT-axis with increased TSH concentrations and increased thyroid gland weights in male pups (females were not tested). Thyroid weights were significantly increased both in terms of absolute weights with body weight as a covariate and weights relative to body weight. MBI and cyanamide did not affect any of these parameters.

3.5. Developmental neurotoxicity in the form of periventricular heterotopia

We investigated whether maternal exposure to MBI, amitrole and cyanamide can lead to the formation of periventricular heterotopia in offspring. We found large heterotopia in both hemispheres of all PD16 male pups exposed to 50 mg/kg amitrole ($p < 0.0001$, Fig. 4). In contrast, heterotopia formation in MBI and cyanamide exposed animals were similar to the control, consistent with the lack of effect on T4 concentrations. Curiously, the

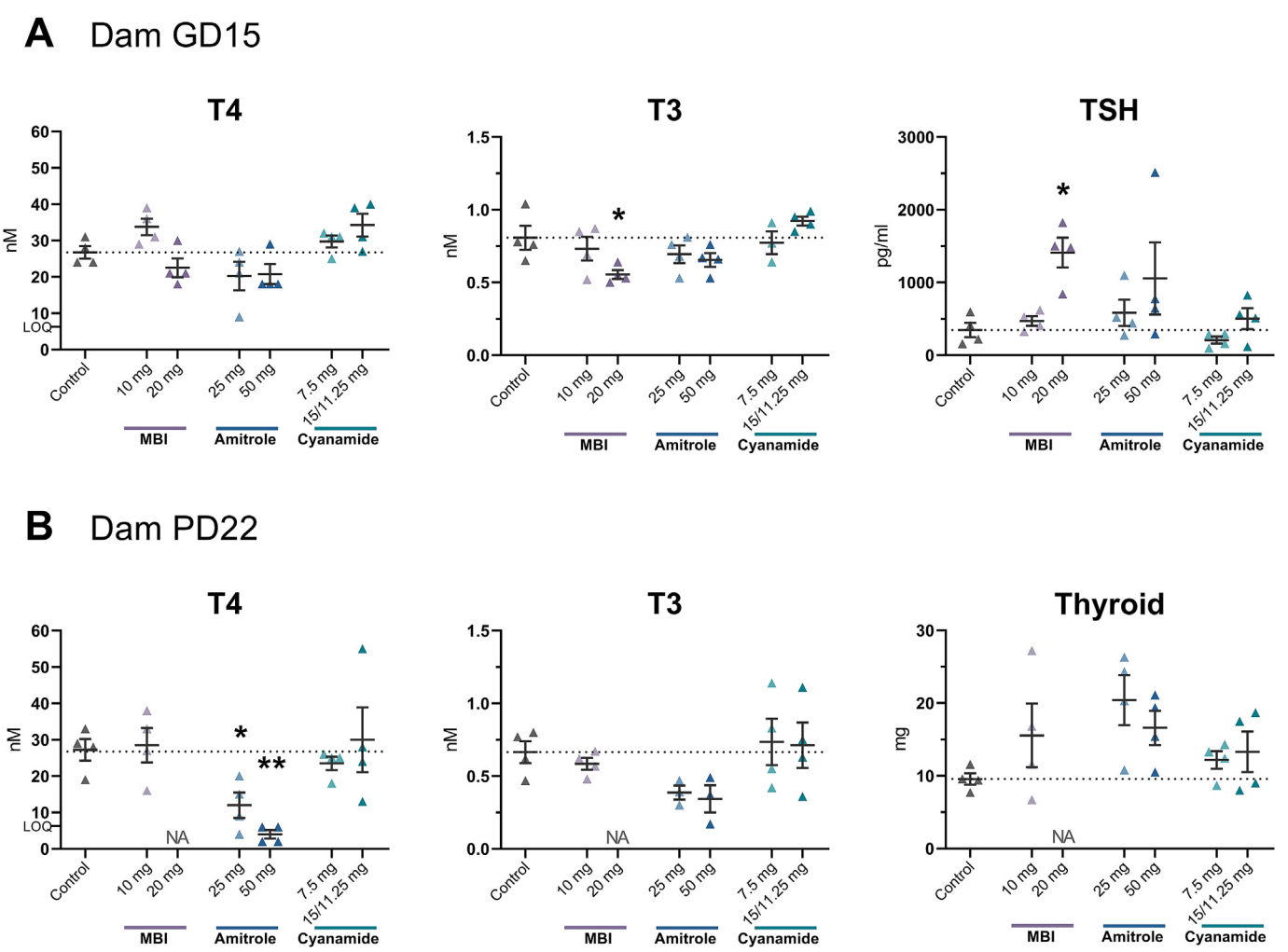

Fig. 2. Serum hormone concentrations and thyroid gland weights in dams exposed to MBI, amitrole or cyanamide from GD7-PD22. A) T4, T3 and TSH serum concentrations in dams on GD15. B) Dam T4 and T3 serum concentrations and thyroid gland weights on PD22. GD: gestational day, NA: not available, PD: postnatal day, T4: thyroxine, T3: 3,3′,5-triiodothyronine, TSH: thyroid stimulating hormone, LOQ: limit of quantification. n = 4. Data shown as scatter with mean ± SEM. Dotted line shows the mean of the controls. *$p < 0.05$, **$p < 0.01$.

heterotopia in animals treated with 15/11.25 mg/kg cyanamide were statistically significantly ($p < 0.01$) smaller in male pups, an effect we consider a chance finding.

4. Discussion

We have shown that exposure to amitrole during pregnancy and lactation can induce large heterotopia in the brains of 16 day old male offspring. The periventricular heterotopia consists of erroneously migrated neurons and likely arises from fetal and neonatal thyroid hormone insufficiency. In contrast, MBI and cyanamide did not induce heterotopia formation in the rat offspring, likely because pup thyroid hormone concentrations were not reduced by these two compounds. To our knowledge, this is the first demonstration of thyroid hormone dependent heterotopia formation by an environmental chemical with *in vitro* TPO-inhibiting properties.

While amitrole did not significantly lower maternal T4 and T3 serum concentrations at GD15, the PD6 pups did show pronounced effects on serum T4 with reductions of 50% relative to controls (below the quantification limit of our assay). It is reasonable to assume that the pups must have experienced a significant drop in serum T4 during the entire critical brain developmental window spanning late foetal to early postnatal life (GD19 to PD6) (O'Shaughnessy et al., 2018a, 2019), including low T4 in the brains themselves (O'Shaughnessy et al., 2018b, 2019). Consequently, this drop must have been sufficient to disrupt neuronal migration, as evidenced by the formation of periventricular heterotopia; an irreversible malformation of the brain. For MBI (10 mg/kg) and cyanamide (15/11.25 mg/kg) we observed no reduction in dam and pup serum T4 concentrations. The absence of heterotopia formation in 16-days old pups was therefore not surprising. If anything, we saw a minor reduction in heterotopia after exposure to cyanamide as compared to sporadically present small heterotopia in control animals. The fact that neither MBI nor cyanamide suppressed serum T4 concentrations in pregnant dams or their offspring was somewhat unexpected based on their *in vitro* TPO inhibitory properties and capacity to disrupt the thyroid hormone system *in vivo*. The most likely explanation to these discrepancies are doses and time to effect. For MBI we only had pups in the lower dose group (10 mg/kg). Here, MBI increased serum T4 in PD6 pups but did not cause any other effects on thyroid hormone concentrations. At higher exposure doses, MBI appears to affect the thyroid hormone system *in vivo*. For instance, at 20 mg/kg MBI we found signs of activation of the hypothalamic-pituitary-thyroid axis, with increased TSH concentrations in the dams at GD15. Notably, MBI obstructed parturition (dystocia) in pregnant rats, with not a single

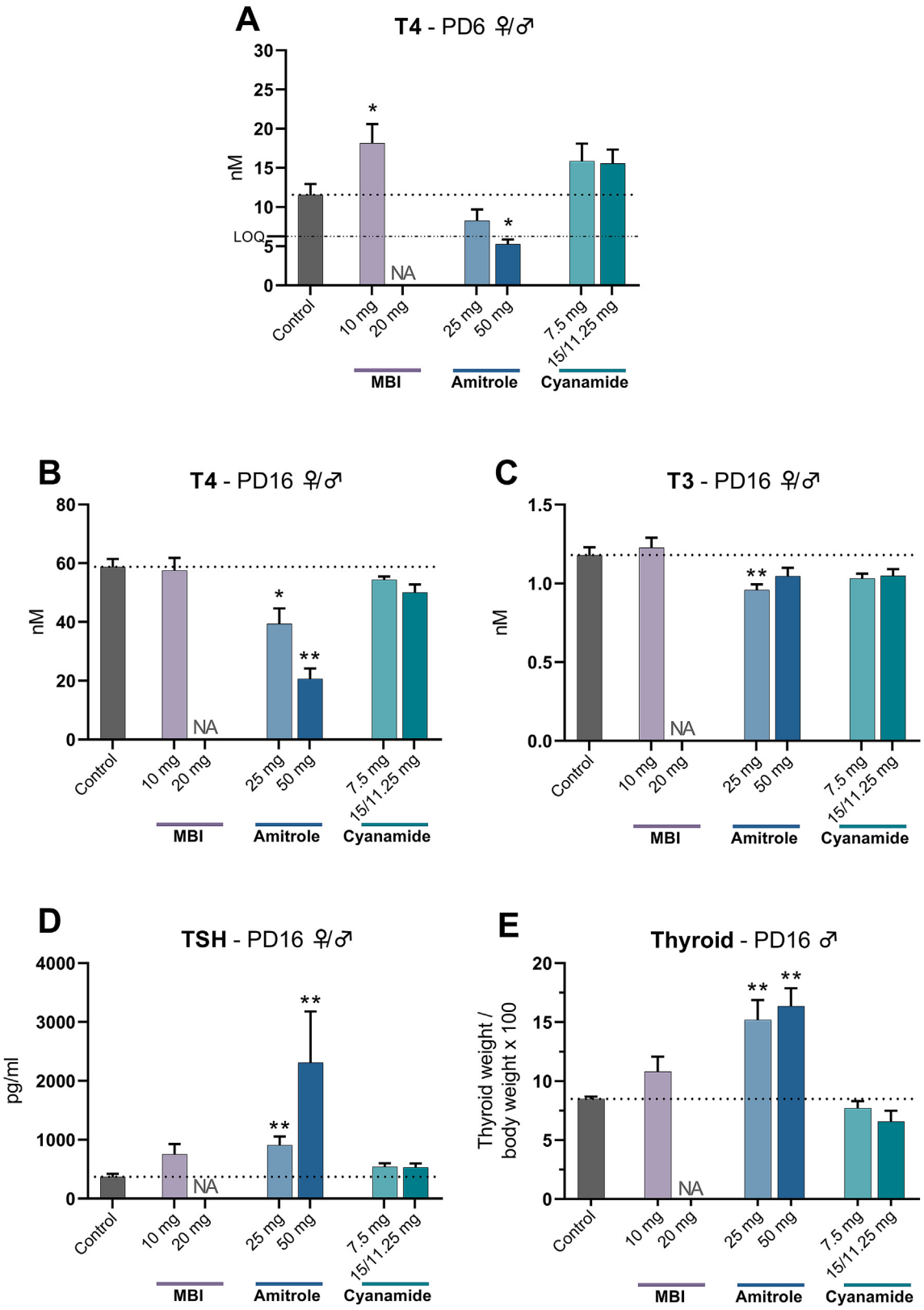

Fig. 3. Serum hormone concentrations and thyroid gland weights in offspring developmentally exposed to MBI, amitrole or cyanamide from GD7-PD22. A) T4 serum concentrations in pups on PD6. n = 8: 4 litters each represented by a male and female pup. LOQ with dotted line shows limit of quantification. B-D) T4, T3 and TSH serum concentration in pups on PD16. n = 8, 4 litters represented by a male sample (serum poled from three male pups) and a female sample (sample pooled from two female pups). E) Male pup thyroid gland weight on PD16. n = 8: 4 litters each represented by 2 male pups, and litter effects accounted for in the statistical analysis. NA: not available, PD: postnatal day, T4: thyroxine, T3: 3,3',5-tri-iodothyronine, TSH: thyroid stimulating hormone, LOQ: limit of quantification (dashed line in A). Data shown as mean + SEM. Dotted line shows the mean of the controls. *p < 0.05, **p < 0.01.

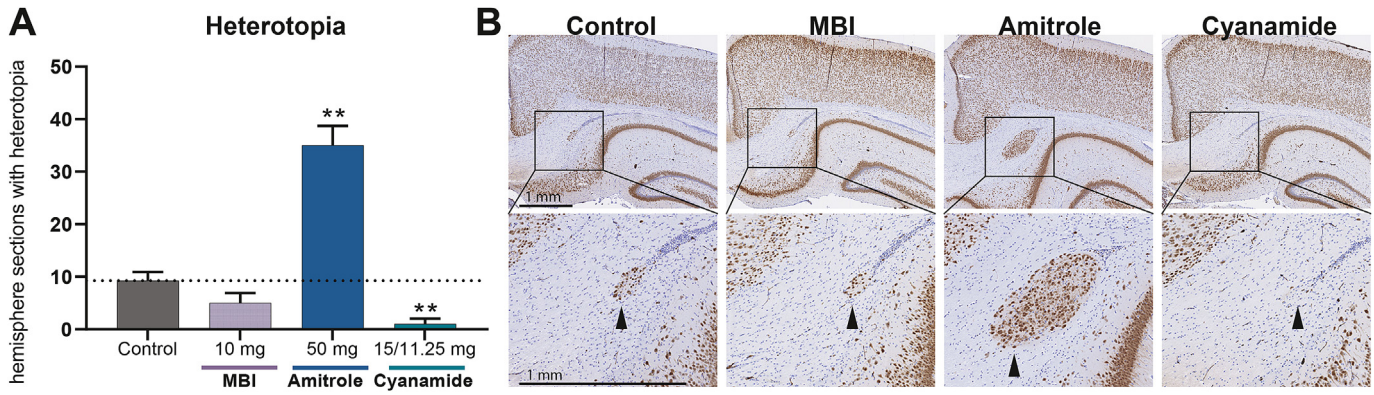

Fig. 4. Heterotopia formation in PD16 male offspring exposed to MBI, amitrole or cyanamide during perinatal development. A) Amitrole caused marked heterotopia formation with a heterotopia in each brain hemisphere of all 4 pups exposed to amitrole. Heterotopia in MBI and cyanamide-exposed animals were similar to the controls. The rostral-caudal length of one heterotopia in amitrole exposed animals was on average 525 μm. n = 4. Data shown are the mean number of sections with heterotopia in the left or right hemisphere +SEM. Dotted line shows the mean of the controls.**p < 0.01 compared to control. B) Representative images of heterotopia from each exposure group at two magnifications. Arrowheads indicate heterotopia or its expected periventricular location. Scale bars = 1 mm, applies to all images, the lower row showing insets of the upper row.

dam able to give birth in the 20 mg/kg dose group, resulting in loss of all the litters. This adverse toxicological effect precluded us from drawing any conclusions on potential effects on thyroid hormones and brain development in the offspring at the high dose. In addition to our report of increased TSH in pregnant rat dams at 20 mg/kg MBI, thyroid gland weights were increased in pregnant rat dams at 10 and 30 mg/kg (Yamano et al., 1995) but, to our knowledge, no other studies have examined thyroid hormone concentrations in pregnant rats exposed to MBI. Still, in adult male rats exposed for 15 days to 25 and 50 mg/kg MBI there were severe thyroid hormone reductions, with T4 concentrations below 20% of controls and TSH increased by approximately 10-fold (Kawasaki et al., 1998). Two further studies reported hyperplasia/hypertrophy of thyroid follicular cells at 1.2 mg/kg and enlarged thyroid glands from 4 mg/kg after repeated exposure (ECHA, 2021a). Taken together, the TPO inhibiting properties of MBI appear strong enough to cause thyroid hormone system disruption *in vivo* (and to a degree that could affect brain development) but only at doses that cause toxicity in pregnant rat dams. For cyanamide we did not see any effects on thyroid hormone concentrations in pregnant dams and their offspring up to the dose of 15/11.25 mg/kg. As was the case for MBI, the cyanamide dose of 15/11.25 mg/kg represented the highest possible exposure dose for pregnant rats, as higher doses will induce severe toxic responses. However, a few previous studies show that cyanamide can cause some thyroid hormone system disruption *in vivo*. A single developmental study has examined thyroid-related effects of cyanamide; a two-generation reproductive toxicity study administered cyanamide in the drinking water at doses of 0, 20, 60 and 180 ppm to Wistar rats (ECHA, 2021b). At 180 ppm, there was increased thyroid gland weight and thyroid histopathological changes in the parental generation suggesting the HPT-axis had been activated. Thyroid histopathological effects have also been reported in repeated dose toxicity studies in adult animals exposed to cyanamide (ECHA, 2021b; National Cancer Institute, 1979), but only two studies measured thyroid hormone concentrations. These two studies were of very long duration, a 1-year study in dogs that found T4 concentrations decreased at 5 mg/kg bw/day, and a chronic toxicity study in rats that found decreased T3 in females from 2.5 mg/kg bw/day and above and decreased male T4 at 7.5 mg/kg bw/day (ECHA, 2021b). Thus, as with MBI, there are *in vivo* data supporting the view that cyanamide is a thyroid hormone system-disrupting chemical. In fact, cyanamide received a STOT-RE Category 2 classification (presumed toxic to humans following repeated exposure on the basis of evidence from studies in experimental animals) for thyroid effects (ECHA, 2021c). However, available data also indicate that thyroid hormone system disrupting effects probably occur mainly at higher doses and at longer exposure times. Based on our data, we surmise that the toxicological properties of cyanamide make it nearly impossible to administer doses to pregnant rats that are sufficiently high to cause thyroid hormone system disruption.

As discussed, MBI, amitrole and cyanamide all appear to reduce thyroid hormone concentrations *in vivo*. This is likely through their TPO-inhibitory properties. Notably, amitrole caused *in vivo* thyroid hormone system disruption and adverse effects on brain development through relatively weak TPO−inhibitory properties as shown in Table 1. The only other compounds so far proven to cause heterotopia are PTU and MMI, both clinically used drugs, with lower IC_{50} and higher efficacy in their TPO inhibition (Table 1). However, the environmental chemical amitrole with coincidental TPO-inhibiting capacity turned out to also reduce serum T4 concentrations in rat pups to a degree producing heterotopia in the brain. At the same time, MBI and cyanamide have more potent *in vitro* TPO-inhibitory properties than amitrole, but do not reduce T4 concentrations in dams and pups in our experimental setup. This appears paradoxical but can likely be explained by dosing and chemical specific toxicokinetics (i.e. absorption, distribution, metabolism and excretion parameters. This includes the placental and lactational transfer of compound to foetuses and pups which is all important for effects in the thyroid hormone system). However a full elucidation of the relationship between administered dose of compound, actual internal exposure, and *in vivo* reductions in T4 over the course of development, would require in-depth toxicokinetic investigations and T4 measurements in foetuses and in pups at several time points; investigations that were out of scope for this study, but which will be pursued in future investigations of amitrole.

It is already established that the pesticide amitrole can affect thyroid hormone concentrations and induce effects on the thyroid gland (Alexander, 1959; ECHA, 2021d; Stringer et al., 1981). The fact that amitrole can affect foetal and neonatal thyroid hormone concentrations and impact brain development is new knowledge that is of serious concern. In fact, our study is the first to report any developmental neurotoxic effects by amitrole, a pesticide that since 2016 is no longer approved for use in the EU (European Commission, 2021), but is still registered under REACH and used at 10−1000 tons per year. Of concern, it is found in groundwater samples (Ministry of Environment and Food of Denmark, 2020).

Our findings thus highlight the need to conduct further studies on potential effects on brain development caused by amitrole and other thyroid hormone system disrupting chemicals. It also highlights inadequacy of regulatory testing requirements and underscores the need for new and more sensitive methods that can detect effects on brain development after developmental thyroid hormone system disruption.

Our study was specifically designed to test if environmental chemicals could cause heterotopia in rats after developmental exposure to TPO inhibitors. However, our results also revealed that both MBI and cyanamide can cause reproductive toxicity effects. MBI had only been investigated in a single teratogenicity study where pregnant dam and foetuses were examined after MBI-exposure from GD7-17. MBI reduced foetal weights, foetal visceral variations and delayed ossification, resulting in a classification for developmental effects (rep cat 1) (ECHA, 2021a; Yamano et al., 1995). We now show that adverse reproductive toxicity effects of MBI also include increased gestation length at 10 mg/kg bw/day and dystocia with total litter loss at a dose of 20 mg/kg bw/day administered GD7–22. This effect pattern of prolonged gestation and disrupted parturition is one MBI shares with many other azole fungicides. The fungicides interfere with cytochrome P450 enzymes of the steroidogenesis pathway (Draskau et al., 2019; Kjærstad et al., 2010; Osawa et al., 1987; Taxvig et al., 2008; Zhang et al., 2002) and cause sustained increases in serum progesterone concentrations, ultimately preventing the pronounced drop in serum progesterone that is necessary for parturition (Taxvig et al., 2007; Zakar and Hertelendy, 2007). Parturition was not affected in the cyanamide-exposed animals, but dam weight gain during gestation was reduced and indicates that 11.25 mg/kg cyanamide can be considered a threshold for maternal toxicity in SD rats in reproductive toxicology studies. Although the REACH registration dossier for cyanamide (ECHA, 2021b) contains several reproductive and developmental toxicity studies that investigated doses up to 45 mg/kg and reported severe maternal toxicity it was unexpected that we found excessive toxicity also at 15 mg/kg.

5. Conclusions

We have shown that amitrole is a developmental thyroid hormone system disruptor that can adversely affect brain development, manifesting as heterotopia in rat offspring. Amitrole has coincidental TPO-inhibiting properties that are much weaker than drugs such as PTU and MMI. Yet, it reduces T4 serum concentrations in both dams and their offspring, likely causing the periventricular heterotopia found in PD16 male pups. Thus, we show that current testing regimens are not sensitive enough to identify developmental neurotoxicity through thyroid hormone system disruption and that heterotopia formation could be used as an endpoint in regulatory toxicity testing, as demonstrated here for the TPO-inhibiting pesticide amitrole. Future studies need to further quantify the relationship between serum and brain T4 concentrations during the critical window, heterotopia formation and the underlying mechanism.

Author statements

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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.

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