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

Louise Ramhøj a, Caroline Frädrich b, Terje Svingen a, Martin Scholze c, Eva K. Wirth d, e, Eddy Rijnjtes b, Josef Köhle b, Andreas Kortenkamp c, Marta Axelstad b, e

a Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kgs. Lyngby, DK-2800, Denmark
b Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Institut für Experimentelle Endokrinologie, 10115, Berlin, Germany
c Division of Environmental Studies, College of Health, Medicine and Life Sciences, Brunel University London, Kingston Lane, Uxbridge, UB8 3PH, UK
d Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Department of Endocrinology and Metabolism, 10115, Berlin, Germany
e DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, Germany

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 chemicals can perturb 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 (Akaik et al., 1991; Ause 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
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 synthesize thyroid hormones. TPO oxidizes iodide to iodine, iodinates thyroglobulin and couples iodinated tyrosine residues to form the thyroid hormone iodothyronines T4 and 3,3',5-triiodothyronine (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 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: STB8752, 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, 187364-5G, lot: STB8303, purity 99%).

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: STB8752, 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, 187364-5G, lot: STB8303, 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 91 potent inhibitors of TPO that were not tolune 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 x 27 x 43 cm). Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lyngby, Denmark), and Tapvei Arcade 17 (aspen wood) shelves (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-ACBTO072S 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 and all dams in the high dose MBI (20 mg/kg) all gave birth on GD23 and all the remaining pups.

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 CO2/O2 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 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 Danish 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 –20 °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 dissected 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% H2O2 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 Mayer’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 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.

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 1 | TPO inhibitory properties, selected test compounds and positive control compounds. |
|---------|---------------------------------------------------------------------------------|
| **Compound** | **IC50 (μM)** | % E_max (inhibition)* | **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).

* Percent maximal inhibition of thyroperoxidase (TPO).
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
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 pooled 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.
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 IC50 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; Kjaerstad 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

Louise Ramhøj: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing — original draft. Caroline Frädrich: Investigation, Writing — review & editing. Terje Svingen: Conceptualization, Funding acquisition, Methodology, Writing — original draft. Martin Scholze: Formal analysis, Writing — review & editing. Eva K. Wirth: Investigation, Writing — review & editing. Eddy Rijntjes: Investigation, Writing — review & editing. Josef Köhrle: Funding acquisition, Resources, Writing — review & editing. Andreas Kortenkamp: Funding acquisition, Writing — review & editing. Marta Axelstad: Conceptualization, Funding acquisition, Methodology, Writing — original draft.

Funding

This work is part of the ATHENA project (Assays for the identification of Thyroid Hormone axis-disrupting chemicals: Elaborating Novel assessment strategies) (Kortenkamp et al., 2020) funded by the EU Horizon 2020 programme, grant number 825161, which is gratefully acknowledged.

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.

Acknowledgment

We thank Camilla Taxvig for contributions to the ToxCast selection process and review of in vitro data. We would also like to thank Mette Voigt Jessen, Heidi Letting, Lillian Sztuk, Dorte Lykkegaard Korsbech, Stine Styssiek and Anja Fischbach for invaluable technical assistance. Last, we thank the Biofacility at the Technical University of Denmark for animal care and husbandry.

References

Abdi, H., 2007. The bonferroni and sidák corrections for multiple comparisons. In: Steyvers, N.J. (Ed.), Encyclopedia of Measurement and Statistics. Sage Publications, Thousand Oaks, (CA), pp. 103–107.
Akaike, M., Kato, N., Ohno, H., Kobayashi, T., 1991. Hyperactivity and spatial maze learning impairment of adult rats with temporary neonatal hypothryoidism. Neurotoxicol. Teratol. 13, 317–322.
Alexander, N.M., 1959. Antithyroid action of 3-amino-1,2,4-triazole. J. Biol. Chem. 234, 148–150. https://doi.org/10.1016/s0021-9258(18)30353-0.
Andersen, S.L., Andersen, S., Vestergaard, P., Olsen, J., 2018. Maternal thyroid function in early pregnancy and child neurodevelopmental disorders: a Danish nationwide case-cohort study. Thyroid 28, 537–546. https://doi.org/10.1089/thy.2017.0425.
Ausz, E., Lavado-autric, R., Cuevas, E., Escobar del Rey, F., Morreale de Escobar, G., Berbel, P., 2004. A moderate and transient deficiency of maternal thyroid function at the beginning of fetal neurocorticogenesis alters neuronal migration. Endocrinology 145, 4037–4047.
Axelstad, M., Hansen, P.R., Boberg, J., Bonnichsen, M., Niellemann, C., Lund, S.P., Hougaard, K.S., Hass, U., 2008. Developmental neurotoxicity of Propylthiouracil (PTU) in rats: relationship between transient hypothyroxinaemia during development and long-lasting behavioural and functional changes. Toxicol. Appl. Pharmacol. 232, 1–13. https://doi.org/10.1016/j.taap.2008.05.020.
Carvalho, D.P., Dupuy, C., 2017. Thyroid hormone biosynthesis and release. Mol. Cell. Endocrinol. 458, 6–15. https://doi.org/10.1016/j.mce.2017.01.038.
Crofton, K.M., Gilbert, M.E., Friedman, K.P., Demeneix, B., Marty, M.S., Zoeller, R.T., 2019. Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals [WWW Document]. AOPWiki. URL: accessed 2.1.21.https://aopwiki.org/aops/42.
Draskau, M.K., Boberg, J., Taxvig, C., Pedersen, M., Frandsen, H.L., Christiansen, S., Svingen, T., 2019. In vitro and in vivo endocrine disrupting effects of the azole fungicides triticonazole and flusilazole. Environ. Pollut. 255 https://doi.org/10.1016/j.envpol.2019.113309.
ECHA, 2021a. Benzimidazol-2-thiol [WWW Document]. Regist. Doss. URL: https://echa.europa.eu/da/registration-dossier/-/registered-dossier/6188/1.
ECHA, 2021b. Cyanamide [WWW Document]. Regist. Doss. URL: https://echa.europa.eu/da/registration-dossier/-/registered-dossier/158237/9/1.
ECHA, 2021c. Cyanamide [WWW Document]. Subst. Inf. URL: accessed 2.1.21.https://echa.europa.eu/da/substance-information/-/substanecieinfo/100006.358.
ECHA, 2021d. Amitrole [WWW Document]. Regist. Doss. URL: https://echa.europa.eu/da/registration-dossier/-/registered-dossier/21685/1.
European Commission, 2021. Amitrole [Aminotriazole] [WWW Document]. EU Pesticide Database. URL: accessed 2.1.21.https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/active-substances?event¼as.details&as_id¼337.
Fetene, D.M., Betts, K.S., Alati, R., 2017. Mechanisms in endocrinology maternal thyroid dysfunction during pregnancy and behavioural and psychiatric
disorders of children: a systematic review. Eur. J. Endocrinol. 177, R261–R273. https://doi.org/10.1530/EJE-16-0860.

Friedman, K.P., Wootton, H.M., Hodge, J.M., Judson, R.S., Crofton, K.M., Houck, K.A., Simons, S.O., 2016. Tiered high-throughput screening approach to identify thyroperoxidase inhibitors within the toxtcast phase I and II chemical libraries. Toxicol. Sci. 151, 160–180. https://doi.org/10.1093/toxsci/kfw034.

Ghassemi, N., El Madani, F., Wiers, R.P., Jørgensen, V., Hofman, A., Verheugen, F.C., Tiemeier, H., White, T., 2014. Downstream effects of maternal hypothryoxinemia in early pregnancy: nonverbal IQ and brain morphology in school-age children. J. Clin. Endocrinol. Metab. 99, 2383–2390. https://doi.org/10.1210/jc.2013-2481.

Gilbert, M.E., O’Shaughnessy, K.L., Axell, M., 2020. Regulation of thyroid-disrupting chemicals to protect the developing brain. Endocrinol. (United States) 161, 1–17. https://doi.org/10.1210/endocr/bqa0106.

Gilbert, M.E., Ramos, R.L., McCloskey, D.P., Goodman, J.H., 2014. Subcortical band heterotopia in rat offspring following maternal hypothryoxinemia: structural and functional characteristics. J. Neuroendocrinol. 26, 528–541. https://doi.org/10.1111/jene.12175.

Gilbert, M.E., Sanchez-Huerta, K., Wood, C., 2016. Mild thyroid hormone insufficiency during development compromises activity-dependent neuroplasticity in the hippocampus of adult male rats. Endocrinology 157, 774–787. https://doi.org/10.1210/en.2015-1643.

Gilbert, M.E., Sui, L., 2006. Dose-dependent reductions in spatial learning and synaptic function in the dentate gyrus of adult rats following developmental thyroid hormone insufficiency. Brain Res. 1069, 10–22.

Goodman, J.H., Gilbert, M.E., 2007. Modest thyroid hormone insufficiency during development induces a cellular malformation in the cortex callosum: a model of cortical dysplasia. Endocrinology 148, 2593–2597. https://doi.org/10.1210/en.2006-1276.

Gyllenborg, D., Squaroun, A., Surcel, H.M., Hinkka-Yli-Salmokivi, S., McKeague, I.W., Brown, A.S., 2016. Hypothyroxinemia during gestation and offspring schizophrenia in a national birth cohort. Biol. Psychiat. 79, 962–970. https://doi.org/10.1016/j.biopsych.2015.06.014.

Haddow, J.E., Palomaki, G.E., Allan, W.C., Williams, J.R., Knight, G.J., Gagnon, J., O’Heir, C.E., Mitchell, M.L., Hermos, R.J., Waisben, S.E., Faix, J.D., Klein, R., 2016. Maternal Thyroid hormone deficiency during pregnancy and subsequent neuropsychological development of the child. N. Engl. J. Med. 374, 549–555. https://doi.org/10.1056/NEJMoa1602015.

Hasebe, M., Matsumoto, I., Imagawa, T., Uehara, M., 2008. Effects of an anti-thyroid drug, methimazole, administration to rat dams on the cerebellar cortex development in their pups. Int. J. Dev. Neurosci. 26, 409–414. https://doi.org/10.1016/j.ijdevneu.2008.05.011.

Hassan, L., El-Masri, H., Kosian, P.A., Ford, J., Degitz, S.J., Gilbert, M.E., 2017. Neurodevelopmental disorders of children: a systematic review. Eur. J. Endocrinol. 177, R261–R273. https://doi.org/10.1530/EJE-16-0860.

Heir, C.E., Mitchell, M.L., Hermos, R.J., Waisbren, S.E., Faix, J.D., Klein, R., 1999. Thyroid hormone action in the fetal and neonatal rat brain. Toxicol. Sci. 166, 163–170. https://doi.org/10.1093/toxsci/kfy016/4827576.

Houck, K.A., Simmons, S.O., 2016. Tiered high-throughput screening approach to identify thyroperoxidase inhibitors within the toxcast phase I and II chemical libraries. Toxicol. Sci. 151, 160–180. https://doi.org/10.1093/toxsci/kfw034.

Huang, Y., Yamano, T., Noda, T., Shimizu, M., Morita, S., 1995. The adverse effects of oral 2-mercaptothiazole on neurobehavioural development in the rat. J. Neuroendocrinol. 25, 218. https://doi.org/10.1111/j.1365-2833.1993.tb01848.x.

Hypothyroxinemia alters histogenesis and cerebral cortex cytoarchitecture of the progeny. J. Clin. Invest. 111, 1073–1082. https://doi.org/10.1172/JCI3016262. Ministry of Environment and Food of Denmark, 2020. Fagligt Notat Om Resultater AF Masscreening 2019. Aalborg Øst.

Morreale de Escobar, G., Jesus Obregon, M., De Oia, C.R., Del Rey, F.E., 1988. Transfer of thyroxine from the mother to the rat fetus near term: effects on brain 3,5,3’-triiodothyronine deficiency. Endocrinology 122, 1521–1531. https://doi.org/10.1210/endo-122-4-1521.

National Cancer Institute, 1979. Bioassay of Calcium Cyanamide CAS No. 156-62-2. Noyes, P.D., Friedman, K.P., Browne, P., Haselman, J.T., Gilbert, M.E., Hornung, M.W., Barone, S., Crofton, K.M., Laws, S.C., Stoker, T.E., Simons, S.O., Tietje, J.E., Degitz, S.J., 2019. Evaluating chemicals for thyroid disruption: opportunities and challenges. In: Ghassemi, N., El Madani, F., Wiers, R.P., Jørgensen, V. (Eds.), Minstry of Environment and Food of Denmark, 2020. Fagligt Notat Om Resultater AF Masscreening 2019. Aalborg Øst.

Mergenthaler, P., Nikolov, N., Pasquier, D., Du, Peeters, R.P., Jorde, Y.B., Vinggaard, A.M., Hass, U., Axelstad, M., Metzdorff, S., Nellemann, C., 2008. Endocrine-disrupting properties in vivo of widely used azole fungicides. Int. J. Environ. Res. Public Health 5, 318. https://doi.org/10.3390/ijerph5040318.

Merkel, S., Eberts, T.E.,沃思, J.L., Hertelendy, F., 2007. Progesterone withdrawal: key to parturition. Am. J. Obstet. Gynecol. 196, 289–296. https://doi.org/10.1016/j.ajog.2006.09.005.

Morris, G., Kramer, J.B., Nolte, H., Tyndale, R.F., Sellers, E.M., 2002. Inhibition of cytochromes P450 by antifungalimidazole derivatives. Drug Metab. Dispos. 30, 314–318. https://doi.org/10.1212/01.dmd.000034.314.