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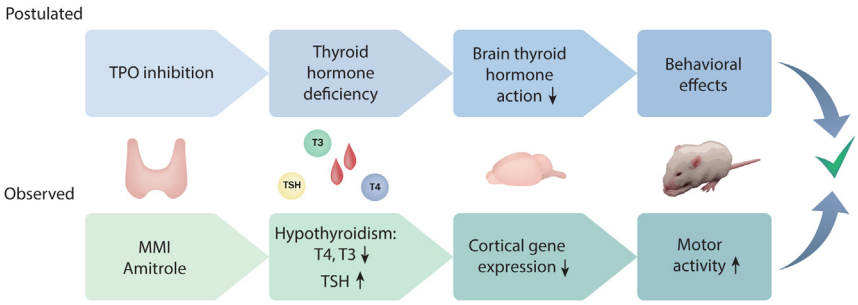
HIGHLIGHTS

- TPO inhibitors MMI and amitrole elicit similar disruptions of the thyroid hormone system.
- MMI and amitrole perturb thyroid hormone dependent rat brain development *via* TPO inhibition.
- MMI and amitrole reduce expression of thyroid hormone-sensitive genes in cerebral cortex.
- MMI and amitrole alter motor activity of perinatally exposed rat offspring.

GRAPHICAL ABSTRACT



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ABSTRACT

Disruption of the thyroid hormone system during development can impair brain development and cause irreversible damage. Some thyroid hormone system disruptors act by inhibiting the thyroperoxidase (TPO) enzyme, which is key to thyroid hormone synthesis. For the potent TPO-inhibiting drug propylthiouracil (PTU) this has been shown to result in thyroid hormone system disruption and altered brain development in animal studies. However, an outstanding question is which chemicals beside PTU can cause similar effects on brain development and to what degree thyroid hormone insufficiency must be induced to be able to measure adverse effects in rats and their offspring. To start answering these questions, we performed a perinatal exposure study in pregnant rats with two TPO-inhibitors: the drug methimazole (MMI) and the triazole herbicide amitrole. The study involved maternal exposure from gestational day 7 through to postnatal day 22, to MMI (8 and 16 mg/kg body weight/day) or amitrole (25 and 50 mg/kg body weight/day). Both MMI and amitrole reduced serum T4 concentrations in a dose-dependent manner in dams and offspring, with a strong activation of the hypothalamic-pituitary-thyroid axis. This reduction in serum T4 led to decreased thyroid hormone-mediated gene expression in the offspring's brains and caused adverse effects on brain function, seen as hyperactivity and decreased habituation in preweaning pups. These dose-dependent effects induced by MMI and amitrole are largely

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HPT-axis
Thyroxine

the same as those observed with PTU. This demonstrates that potent TPO-inhibitors can induce effects on brain development in rats and that these effects are driven by T4 deficiency. This knowledge will aid the identification of TPO-inhibiting thyroid hormone system disruptors in a regulatory context and can serve as a starting point in search of more sensitive markers of developmental thyroid hormone system disruption.

1. Introduction

Brain development during fetal and early postnatal life is critically dependent on thyroid hormone action. During these early life stages, normal brain differentiation is sensitive to a timely supply of tightly controlled concentrations of thyroid hormones in the target cells in order to regulate the expression of thyroid hormone responsive genes (Bernal, 2017; Gilbert et al., 2020). The regulation of the thyroid hormone system itself, however, involves a complex network of hormones, transporters, metabolizing enzymes and receptors, each contributing towards supplying thyroid hormones to target tissues, including the brain. Thus, to maintain a sufficient supply of thyroid hormones in the brain, all other components of the thyroid hormone system network must be functional in spatial and temporal coordination. One crucial component is the synthesis of the thyroid hormones, which exclusively takes place in the thyroid gland under the control of the enzyme thyroperoxidase (TPO) (Carvalho and Dupuy, 2017).

TPO is expressed by the thyroid follicular cells and functions to oxidize iodide to iodine, iodinate thyroglobulin and couple iodinated tyrosine residues to form the thyroid hormone iodothyronines thyroxine (T4) and 3,3′,5-tri-iodothyronine (T3) (Carvalho and Dupuy, 2017). *In vivo*, exposure to TPO inhibiting compounds leads to reduced thyroid hormone synthesis. If such exposure occurs during pregnancy, it could affect both maternal and fetal thyroid hormone synthesis. In turn, this may reduce supply of thyroid hormones to the fetal and neonatal brain and impair brain development. This cause-effect relationship has been established in rodent models, which typically have induced extreme degrees of hypothyroidism by using high doses of the anti-thyroid drugs propylthiouracil (PTU) or methimazole (MMI), destruction of the thyroid gland by radioiodine treatment, thyroidectomy or a combination thereof (Akaike et al., 1991; Berbel et al., 1994; Dussault and Ruel, 1987; Gilbert et al., 2020; Goldey et al., 1995; Iniguez et al., 1996; Mohan et al., 2012; Ruiz-Marcos et al., 1979; Uchida et al., 2021). In these models, the thyroid hormone concentrations can be suppressed to below the assay's limits of detection.

More recently, studies on moderate degrees of thyroid hormone deficiency have been performed. Although PTU is still the preferred model compound, these studies have explored the developmental effects caused by serum T4 reductions in rodent offspring to around 15–60 % of controls. This reduction in T4 is achieved by exposing rat dams to moderate PTU doses during pregnancy and lactation. This reduces dam serum thyroid hormone concentrations and reduces offspring serum and brain thyroid hormone concentrations even more (O'Shaughnessy et al., 2018). This thyroid hormone deficiency then results in compromised thyroid hormone action in the brain, as seen by disrupted expression of key thyroid hormone-regulated target genes (O'Shaughnessy et al., 2018). It also leads to impaired brain development manifesting as compromised neuronal migration, radial glial cell abnormalities, alterations to parvalbumin expressing interneurons, as well as changes to motor activity and learning and memory impairments (Ausó et al., 2004; Axelstad et al., 2008; Gilbert, 2011; Gilbert et al., 2017, 2014, 2007; Lavado-Autric et al., 2003; O'Shaughnessy et al., 2019; Shiraki et al., 2014). A potential challenge with using PTU as a model compound for TPO inhibition, however, is that PTU also inhibits the thyroid hormone metabolizing enzyme deiodinase 1 (D1), which converts the pro-hormone T4 to the active hormone T3. Thus, in addition to reduced serum T4, also reduced serum T3 and/or altered T4/T3 ratio may contribute to the adverse effects of PTU exposure. However, since D1 appears to be most active in the periphery (including the thyroid gland) and not necessarily in the brain, the D1-inhibiting properties of PTU are likely not contributing to the neurodevelopmental effects observed in rodent studies (Bárez-López et al., 2017; Calvo et al., 1990; Morreale de Escobar et al., 1988).

Many environmental chemicals have the potential to inhibit TPO, at least *in vitro* (Friedman et al., 2016; US EPA, 2020). Then, if the adverse effects seen after developmental PTU exposure are mediated through TPO-inhibition, these other potential TPO-inhibiting compounds could pose a threat to brain development. We recently showed that the TPO inhibiting pesticide amitrole can disrupt brain development of perinatally exposed rat offspring, causing a neuronal migration defect with heterotopia forming in the corpus callosum of 16-days old rat offspring (Ramhøj et al., 2021). However, it is still unclear if amitrole and other TPO inhibitors phenocopy the PTU-induced effects on the thyroid hormone system, thyroid hormone action and on the offspring's brain function. To start addressing these questions we have performed an *in vivo* study with the pesticide amitrole and the specific TPO-inhibiting drug MMI. We aimed to establish to what degree the two TPO inhibitors can disrupt the thyroid hormone system and how this disruption affects cortical thyroid hormone action and ultimately has adverse effects on behavior.

2. Materials and methods

2.1. Chemicals

Methimazole (MMI) (CAS no: 60-56-0, Sigma-Aldrich 301507-5 G, batch: WXBC9951 V, purity 99.0 %) and 3-Amino-1H-1,2,4-triazole (amitrole) (CAS no: 61-82-5, Sigma-Aldrich 8144950100, batch: S7075495 925, purity 99.7 %) were used as test compounds. MMI, also known as thiamazole, is a medical drug used to treat hyperthyroidism. Amitrole is a herbicide and biocide that, in the EU, is registered under REACH (not as a pesticide) and classified as a suspected reproductive toxicant (Repr. 2) and for its thyroid toxicity (STOT RE 2) in repeated exposure studies in adult rats (ECHA, 2021). In the ToxCast database amitrole is active in 2 out of the 451 tested assays: in the TPO inhibition assay with IC_{50} 1.76 μM and maximal inhibition of 69.1 % and in the estrogen receptor assay as agonist with IC_{50} 47.4 μM and top at 24.6 % activity (US EPA, 2020). Corn oil (Sigma-Aldrich, cat.no. C8267 – 2.5 L) was used as control compound and vehicle. Test compounds were dissolved in corn oil and stirred overnight over low heat, then homogenized with ultra turrax. Dosing solutions were stored in glass bottles and kept in the dark, at room temperature and under continuous stirring during the study.

2.2. Animals and treatment

An overview of the study is presented in Fig. 1. The study was conducted in three balanced blocks totaling 60 time-mated, nulliparous Sprague-Dawley rat dams (Crl:CD(SD) bred by Charles

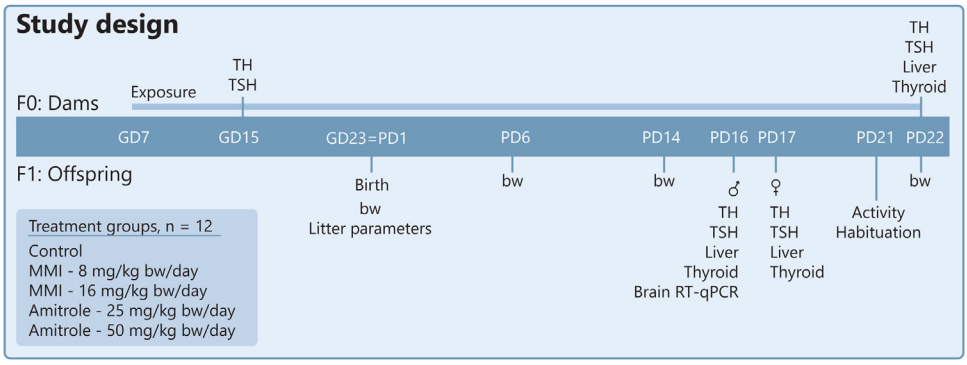

Fig. 1. Study design for the perinatal exposure study with the *in vitro* TPO-inhibitors amitrole and MMI. Dams were exposed throughout gestation and lactation; endpoints were focused on the thyroid hormone system of dams and offspring and on derived effects on the offspring brain. Amitrole: 3-Amino-1H-1,2,4-triazole, bw: body weight, GD: gestational day, MMI: methimazole, PD: pup day, TH: T4, T3, TSH: Thyroid stimulating hormone. Timeline not drawn to scale.

River Europe, distributed by SCANBUR, Denmark) with an approximate weight of 240 ± 30 g that were supplied on gestation day (GD) 3. The day of plug detection was designated as GD1 and day of expected birth was pup day 1 (PD1). Dams 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) placed in ScanTainers (ventilated cabinets) with ScanClime controlled environmental conditions (both from Scanbur, Karlslunde, Denmark): humidity 55 ± 5 %, temperature at 21 ± 1 °C and air-change 50 times per hour. Reversed light/dark cycles of 12 h with sunrise/sunset of 30 min (light from 9 PM to 9 AM, dark from 9 AM to 9 PM. The cages were equipped with aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material and Tapvei Arcade 17 aspen wood shelters (both from Brogaarden, Lynge Denmark). The animals were fed standard Altromin 1314 (soy and alfalfa- free, Altromin GmbH, Lage, Germany) diet *ad libitum*. The iodine content of the diet was 1.52 mg/kg and the selenium content was 0.26 mg/kg. Acidified tap water was provided *ad libitum* in PSU bottles (84-ACBTO702SU Tecniplast). Housing was pairwise, within treatment groups, until GD17 and individually thereafter.

On GD4 the animals were weighed and pseudo-randomly assigned into groups with similar weight distribution. Dams were exposed to test compounds by oral gavage daily from GD7 to PD22, except the day of delivery (PD1). The study included 5 treatment groups of 12 dams (n = 12): control (corn oil), 8 mg/kg body weight (bw)/day MMI, 16 mg/kg bw/day MMI, 25 mg/kg bw/day amitrole and 50 mg/kg bw/day amitrole. MMI and amitrole doses were chosen to cause marked dose-related reductions in PD16 pup serum T4 concentrations. Every morning the dams were weighed and then dosed with the corresponding amount of test compound at a constant volume of 2 mL/kg bw/day. On the morning of GD23, 56 out of 59 dams had given birth and this was assigned as PD1 for all pups (one was a control dam that had delivered by GD22 in the morning). Two dams from the 16 mg/kg MMI group gave birth in the afternoon of GD23 (GD23.5) and one dam from the 50 mg/kg amitrole group had given birth in the morning of GD24.

Two dams were lost during the study, presumably unrelated to exposure. One was incorrectly administered test compound in the lung on GD9 (8 mg/kg MMI) and another, including her litter, was euthanized on PD8 because of blocked intestine (25 mg/kg amitrole). Data from this dam and her litter was excluded from all analyses after PD1.

Dams and pups were weighed on the day of birth and the pups were sexed and counted. All pups were weighed on PD6, PD14 and on PD22 when the study ended.

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 2020−15-0201−00539. The experiments were overseen by the in-house Animal Welfare Committee for animal care and use at the National Food Institute.

2.3. Organ weights, tissue and serum sample collection

On GD15 dam blood was collected by tongue bleeding of unanaesthetized dams. All animals were weighed and killed by decapitation under CO_2/O_2 anesthesia, and trunk blood was collected and pooled from two male pups per litter on PD16, from two female pups per litter on PD17 and from dams on PD22. Serum samples were collected in Eppendorf tubes without heparin. Blood was centrifuged immediately or kept on ice (<1 h), samples were centrifuged for 10 min at 4 °C and 4000 rotations per minute (rpm), and serum was collected and stored at −80 °C until analysis.

Necropsy was performed on two male pups per litter on PD16. Thyroid gland and liver were excised and weighed from one pup. From the second pup the thyroid gland was extracted with a piece of the thyroid cartilage and fixed in neutral buffered 10 % formalin for 24 h, thereafter processed in Excelsior AS Tissue Processor (Thermo Scientific, United Kingdom), embedded in paraffin and stored until histological evaluation. The brain was extracted and an oblique slab of anterior to lateral cortex was stored in RNAlater at −80 °C until RNA extraction. From one female pup per litter thyroid gland and liver were excised and weighed. From the PD22 dams, liver and thyroid glands were excised and weighed, and uteri were excised and implantation scars counted.

2.4. Serum hormone analysis

Serum total T4 and total 3,3′,5-tri-iodothyronine (T3) were determined in GD15 dams, PD16/17 male and female offspring and PD22 dams in technical duplicates whenever possible (*e.g.* single determination used for a portion of dams with low sample volumes on GD15). Validated radioimmunoassays were used to determine serum total T4 (RIA4524, DRG Instruments, Marburg, Germany) and serum total T3 (RIA4525, DRG Instruments, Marburg, Germany). According to manufacturer's instructions a gamma counter (1277 GammaMaster; LKB Wallac, Turku, Finland) was used to determine bound radioactivity after 1 h incubation of samples and standard-curve calibrators with 125I-T4 or 125I-T3 as tracers in antibody-coated tubes. However, to increase sensitivity of the assays, the protocols were adjusted: sample and standard volumes were doubled and the standard curve was extended by diluting standard calibrators as follows. All standard calibrators were diluted by a factor two but with the lowest standard

calibrator was diluted by a factor of 4 and 8. Thus, the limit of quantification for total T4 became 4.0 nM and for total T3 0.2 nM. Intra-assay coefficients of variation were <9 % for both T4 and T3.

Serum TSH concentrations were measured by the Milliplex MAP rat pituitary magnetic bead panel (RPTMAG-86 K; EMD Millipore, Darmstadt, Germany) according to manufacturer's instructions. The limit of quantification was 3.2 pg/mL for TSH. Intra-assay coefficients of variation were <10.5 %.

2.5. Histological evaluation of thyroid glands

Sections of 3 μm (3 sections per animal) of a sub-set of male pup PD16 thyroid glands were stained with hematoxylin and eosin (H&E) and evaluated, blinded to exposure group, for signs of histological changes such as irregular follicles, follicular lumen vesicles, decreased follicular lumen area, increased cellularity, follicular epithelial cell hypertrophy and hyperplasia. The stained sections were imaged using the 40x objective in a Pannoramic Midi II digital slide scanner (3DHISTECH Ltd., Budapest, Hungary) and images evaluated with the CaseViewer software v.2.4 also from 3DHISTECH.

2.6. RNA extraction, cDNA synthesis and RT-qPCR assay

Cortex slabs from male PD16 pups (approximately 20–30 mg tissue) (n = 11–12) were homogenized in 350 μL Buffer RLT using TissueLyser II (Qiagen, Hilden, Germany) (30 osc/sec for 2 min). Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) including on-column DNase I digestion according to manufacturer's instructions. RNA quantity and purity were measured on a NanoDrop spectrophotometer (ND-1000, Fisher Scientific) (all samples had a 260/280 ratio >2.00). cDNA was synthesized from 500 ng total RNA using Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany), random primer mix (BioNordika, Herlev, Denmark), and anti-RNase (Life Technologies, USA) according to manufacturer's instructions. A total volume of 11 μl reactions were made up by 3 μl diluted cDNA (1:20), TaqMan™ Fast Universal PCR Master Mix (2X) (Life Technologies, CA, USA), and TaqMan™ Gene Expression Assay (20X) (Life Technologies, CA, USA) according to manufacturer's instructions. Samples were analyzed by RT-qPCR in technical duplicates. The used TaqMan Gene Expression Assays were: *Rps18* (Rn01428913_gH), *Ubc* (Rn01789812_g1), *Pvalb* (Rn00574541_m1), *Coll11a2* (Rn01428773_g1), *Gjb6* (Rn02042582_s1), *Itih3* (Rn00569293_m1), *Agt* (Rn00593114_m1), *Pnoc* (Rn01637101_m1), *Hopx* (Rn00592446_m1), *Hr* (Rn00577605_m1), *Mog* (Rn00575354_m1), and *Klf9* (Rn00589498_m1). RT-qPCR experiments were run on a QuantStudio 7 Flex Fast Real-Time PCR System (Thermo Fischer Scientific, MA, USA) in 384-well plates. RT-qPCR cycling conditions of initial 95 °C for 20 s followed by 45 cycles of 95 °C for 1 s and 60 °C for 20 s were used. The relative gene expression was calculated by the comparative Ct-method ($2^{-\Delta\Delta Ct}$-method) (Schmittgen and Livak, 2008) with geometric mean of stably expressed and validated reference genes *Rps18* and *Ubc* (Ramhøj et al., 2019). *Rps18* and *Ubc* had mean Ct-values of 22.0 and 21.1 cycles, respectively. In the control group mean expression was <29 Ct except *Pnoc* and *Mog* with mean thresholds of 30.8 and 30.2 Ct. All genes amplified at <35 Ct in all samples.

2.7. Motor activity and habituation

Motor activity and habituation was assessed on PD21 in one male and one female pup from each litter. Each pup was placed in clean, empty cages in activity boxes recording horizontal activity *via* photocells: interruptions of photobeams represents movement and were registered by a computer in an adjoining room. Six animals were tested simultaneously in separate sound insulated activity boxes and with both males and females tested in each run. Testing took place for 30 min. after an initial 10 s habituation and was recorded as counts in 10 periods of 3 min. Motor activity was quantified as activity counts (disruption of adjacent photobeams) that allows for determination of movement across the cage and not just of interruptions of beams in the same spot which are registered as break counts (total number of interrupted photobeams).

Total activity counts during the 30 min. were used to evaluate general activity and average activity counts during 3 periods were used to assess habituation: initial (0–9 min.), middle (10–21 min.) and last period (22–30 min.). A similar metric was achieved by averaging activity counts during the first 15 min. of the test (0–15 min.) and the last 15 min. of the test (16–30 min.).

2.8. Statistical analysis

Continuous endpoints were analyzed with all groups in the same General Linear Model (GLM), accounting for multiple testing using Dunnett's post-hoc test and the statistical significance level set at 0.05. Similarly, data was also analyzed according to treatment using GLM, but only including the control group and the two groups of either MMI or amitrole. These two approaches yielded largely similar results, supporting the robustness of the analysis. To account for litter effects only one pup from each litter was included in the analysis, or litter means were evaluated (*e.g.* pup weight gain) or litter was included in the analysis as an independent, random and nested factor (*e.g.* AGD, pup bw). When requirements for normal distribution and homogeneity of variance was not met data were log-transformed prior to analysis and covariates were included when considered appropriate (*e.g.* litter size for birth weight, body weight for motor activity). When homogeneity of variance could not be achieved for TSH concentrations, data were evaluated using the nonparametric Kruskal-Wallis with Dunn's multiple comparisons test. Organ weights were analyzed as absolute weights with body weight included as a covariate and as weights relative to body weight. Gestational length, post implantation loss and perinatal loss were analyzed using Kruskal-Wallis test. Statistical analyses were conducted in SAS Enterprise v8.3 (SAS institute, NC, US) or in GraphPad Prism 9 (GraphPad Software, CA, US).

3. Results

3.1. Toxicity and litter parameters

Exposure of pregnant rat dams to MMI or amitrole during gestation and lactation decreased dam weight gain during pregnancy but did not change gestational length, litter size, pup survival or early postnatal maternal weight gain (Table 1). Additionally, pup birth weight was reduced in both sexes when exposed to 16 mg/kg MMI and in female pups exposed to 50 mg/kg amitrole. Offspring body weights and weight gains were reduced by high dose MMI and amitrole throughout the postnatal period. Except for male pup PD6 weights and weight gain PD6–14, that were decreased by 8 mg/kg MMI, there were no effects on body weights or weight gains in the low dose groups. Apart from the reduced body weights and growth there were no other signs of overt toxicity in dams or pups. Liver weights in PD22 dams (Fig. 2B) and PD16/17 offspring (Fig. 3A and Table 2) were nominally smaller, probably as a consequence of reduced body weights as there were no effects on liver weights relative to body weight (Table 2).

Table 1
Dam and litter data after perinatal exposure to MMI or amitrole.

Dams and litters	Control (Corn oil)	MMI 8 mg/kg	MMI 16 mg/kg	Amitrole 25 mg/kg	Amitrole 50 mg/kg
No. of pregnant dams (viable litters)	12(12)	12(12)	11(11)	12(12[1])	12(12)
Dam body weight gain					
Maternal bw gain GD7-GD21 (g)	130 ± 17	113 ± 9	**109 ± 16***	134 ± 18	122 ± 22
Maternal bw gain GD7- PD1 (g)	42 ± 13	**26 ± 9***	**20 ± 13****	39 ± 17	**28 ± 13***
Maternal bw gain PD1-PD14 (g)	29 ± 8	24 ± 11	20 ± 7	33 ± 15	25 ± 13
Maternal bw gain PD14-PD22 (g)	−23 ± 10	−18 ± 11	**−9 ± 11***	−22 ± 15	−22 ± 10
Litters					
Gestational length (d)	22.9 ± 0.3	23.0 ± 0.0	23.1 ± 0.2	23.0 ± 0.0	23.1 ± 0.3
Litter size, live pups, PD1	14.4 ± 2.4	13.7 ± 1.8	13.8 ± 1.5	13.8 ± 2.8	13.6 ± 3.2
Postimplantation loss (prenatal mortality) (%)	8.0 ± 11.6	6.2 ± 6.6	6.5 ± 6.7	7.7 ± 16.7	12.3 ± 14.2
Perinatal loss (pre- and postnatal mortality) (%)	10.1 ± 11.4	8.3 ± 6.2	12.0 ± 10.1	9.6 ± 17.4	16.2 ± 12.3
Males / Females (%)	56 / 44 ± 12	56 / 44 ± 10	49 / 51 ± 10	50 / 50 ± 13	50 / 50 ± 13
Offspring					
Male birth weight (g)	7.1 ± 0.7	6.9 ± 0.7	**6.3 ± 0.6****	7.1 ± 0.5	6.7 ± 0.8
Female birth weight (g)	6.8 ± 0.6	6.5 ± 0.6	**6.0 ± 0.6****	6.7 ± 0.4	**6.2 ± 0.7***
Male bw PD6 (g)	14.8 ± 1.5	**13.0 ± 2.1***	**11.7 ± 1.5****	14.6 ± 2.2	**12.5 ± 1.5***
Female bw PD6 (g)	14.2 ± 1.3	12.7 ± 2.1	**11.3 ± 1.4****	14.1 ± 1.9	**12.1 ± 1.8***
Male bw PD14 (g)	37.9 ± 4.1	33.8 ± 4.5	**28.0 ± 4.0****	37.1 ± 6.6	**29.3 ± 4.2****
Female bw PD14 (g)	36.9 ± 4.2	32.9 ± 4.4	**28.0 ± 4.2****	36.7 ± 5.7	**28.7 ± 5.1****
Male bw PD22 (g)	68.7 ± 7.4	64.0 ± 7.3	**49.8 ± 7.9****	66.4 ± 11.8	**50.1 ± 8.9****
Female bw PD22 (g)	66.3 ± 8.0	60.8 ± 7.4	**50.5 ± 7.5****	63.4 ± 6.7	**46.5 ± 10.7****
bw gain, PD1–6, litter mean (g)	7.6 ± 1.1	**6.1 ± 1.5***	**5.4 ± 0.9****	7.3 ± 1.8	**5.8 ± 1.1****
bw gain, PD6–14, litter mean (g)	23.0 ± 3.0	20.6 ± 2.6	**16.5 ± 2.8****	22.6 ± 4.1	**16.7 ± 3.2****
bw gain, PD14–22, litter mean (g)	30.0 ± 4.2	29.2 ± 4.4	**21.6 ± 4.0****	26.2 ± 4.7	**18.9 ± 6.5****

Data represent group means based on dams or litter means ± SD. * $p < 0.05$, ** $p < 0.01$. Statistics performed on offspring body weights were based on weights of individual pups and adjusted for litter effects. Doses are given in mg/kg bw/day.
bw: body weight, GD: gestation day, PD: pup day.
[1] One of these litters lost after PD1 due to maternal intestinal block.

3.2. Dam thyroid hormone system disruption

Dam hormone data were analyzed after one week of dosing (GD15) and again at study termination after approximately five weeks of exposure (PD22). On GD15 MMI and amitrole caused similar reductions in serum T4 at both doses and similarly increased TSH concentration (Fig. 2A). However, MMI did not affect serum T3 while 25 mg/kg amitrole caused a statistically significant reduction. On PD22 the effects were more severe and high dose MMI and amitrole suppressed serum T4 to below limit of detection in the majority of the animals (Fig. 2B). In the low dose groups serum T4 was suppressed by 25 mg/kg amitrole (to 45 % of controls) while there was no effect of 8 mg/kg MMI. Consistent with hypothyroidism, high dose MMI and both doses of amitrole caused marked increases in serum TSH concentrations and thyroid gland weights (Table 2 and Fig. 2). Surprisingly, on PD22 the only effect of low dose MMI was a significant increase in serum T3 concentration (128 % of control) (Fig. 2B).

3.3. Pup thyroid hormone system disruption

Thyroid hormone concentrations were markedly reduced by MMI and amitrole exposure in the male PD16 pups (Figs. 3A and 4). Male PD16 serum samples showed similar effects by MMI and amitrole: serum T4 was reduced to 58 % and 56 % by low dose MMI and low dose amitrole. Suppression of T4 concentrations were severe at the high doses; here MMI reduced serum T4 to 10 % of controls and amitrole to 20 % of controls (Fig. 3A). Serum T3 was significantly reduced to 75 % of control in high dose MMI and in amitrole-exposed males to 65 % of control. Overall, results appeared largely similar in male and female pups but with a tendency of slightly less prominent effects in females (compare Fig. 3A with Fig. 4).

The HPT-axis was activated in both male and female pups where we observed both increased serum TSH and increased thyroid gland weights (Figs. 3A and 4 and Table 2). For both TSH and thyroid gland weights, again, effects appeared slightly more pronounced in male pups as compared to females. This was most distinct in the unaffected thyroid gland weights of the MMI-exposed female pups (Fig. 4). Also thyroid gland histological changes (Fig. 3B) in the male PD16 pups were consistent with TSH stimulation and HPT-axis activation. There were marked changes in the thyroid glands of pups exposed to high dose MMI and amitrole with very irregular follicles, colloid depletion, reduced follicular lumen area, increased cellularity and hyperplasia/hypertrophy. Effects were milder and less consistent between animals in the low dose groups where effects such as many active follicles, irregular follicles, vesicles in follicular lumen and increased cellularity were observed.

3.4. Thyroid hormone mediated gene expression in offspring cortex

We used a panel of 10 thyroid hormone sensitive genes to evaluate thyroid hormone availability and thyroid hormone action in the developing cerebral cortex of male PD16 offspring (Fig. 5). There were dose-dependent and significant reductions in the expression of 9 of the genes; *Pvalb*, *Agt*, *Itih3*, *Mog*, *Coll11a2*, *Gjb6*, *Hopx*, *Klf9* and *Hr*. The effect pattern was similar for all genes (although changes were smaller and did not reach statistical significance for *Pnoc*) and in both MMI and amitrole-exposed pups. The expression of *Pvalb*, *Agt*, *Mog*, *Gjb6* and *Hopx* was decreased also in the pups exposed to the low dose of amitrole (25 mg/kg amitrole). The largest effect size was observed for *Pvalb* in the 50 mg/kg amitrole group where the expression level was reduced with 94 % compared to control.

3.5. Offspring motor activity and habituation

Severe developmental hypothyroidism frequently leads to increased pup motor activity and a decreased habituation capacity

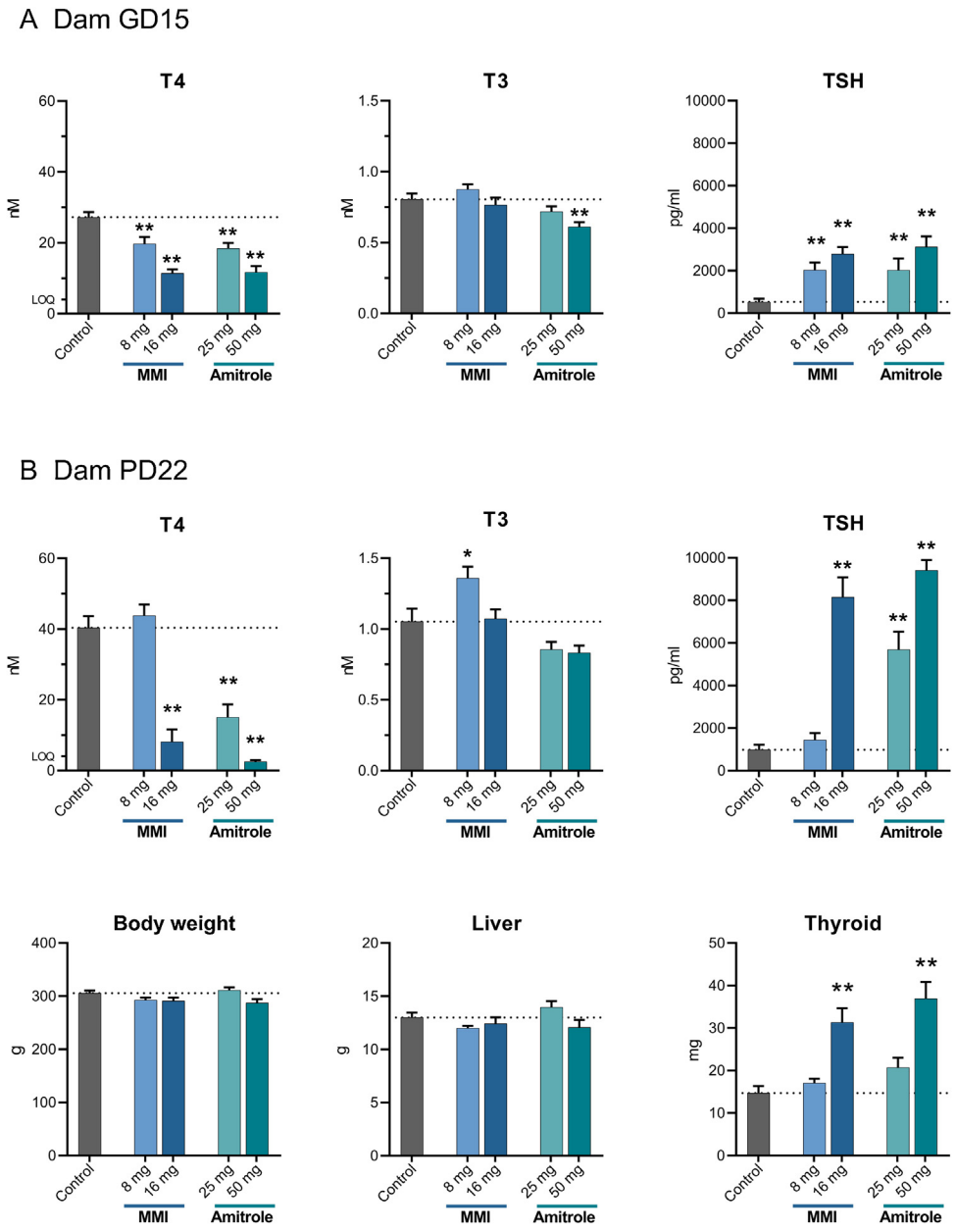

Fig. 2. Dam thyroid hormone system disruption. A) Dam GD15 serum hormone concentrations. T4: n = 11-12, T3: n = 9-12, TSH: n = 11-12 (except 25 mg/kg amitrole with n = 9). B) Dam PD22 hormone concentrations and body, liver and thyroid gland weights, n = 11-12. Mean + SEM. Dotted line represents mean of the control. * $p < 0.05$, ** $p < 0.01$.

and can thus be used as a functional output for impaired brain development resulting in altered behavior. We analyzed motor activity levels in the developing rat offspring on PD21. For total motor activity we combined results from male and female offspring and found that MMI (16 mg/kg) and amitrole (25 and 50 mg/kg) exposure increased activity in the pups (Fig. 6A). Habituation – assessed as lower activity levels towards the end of the 30 min test period – was assessed stratified by sex. Habituation was negatively affected in males that were more active than controls towards the end of the test. In the females there were also nominal increases in activity during test periods, but only in the high dose groups and this failed to reach statistical significance (Fig. 6B). Reduced body weights can be a confounder in activity test and may have influenced the results in the high dose groups, but since there were no effects in the low dose groups these results were likely not confounded by reduced body weights.

4. Discussion

Thyroid hormone deficiency during early life can impede brain development. In rodents, this can be evidenced by perinatal exposure to PTU, a potent thyroid hormone suppressing drug. Exposure to moderate doses of PTU results in suboptimal thyroid hormone concentrations and consequential disruption of neuronal migration, brain differentiation and in cognitive abnormalities (Gilbert et al., 2020). What has remained unclear, however, is if these adverse effects are caused by the TPO-inhibiting properties of PTU solely or by additional D1 inhibition, which might alter the T4/T3 ratio in circulation, or by further unidentified mechanisms. In this study, we have shown that two additional TPO-inhibitors, MMI and amitrole, which do not interfere with D1 activity, can cause the same developmental abnormalities as PTU. They do so by reducing circulating thyroid hormone concentrations, which leads

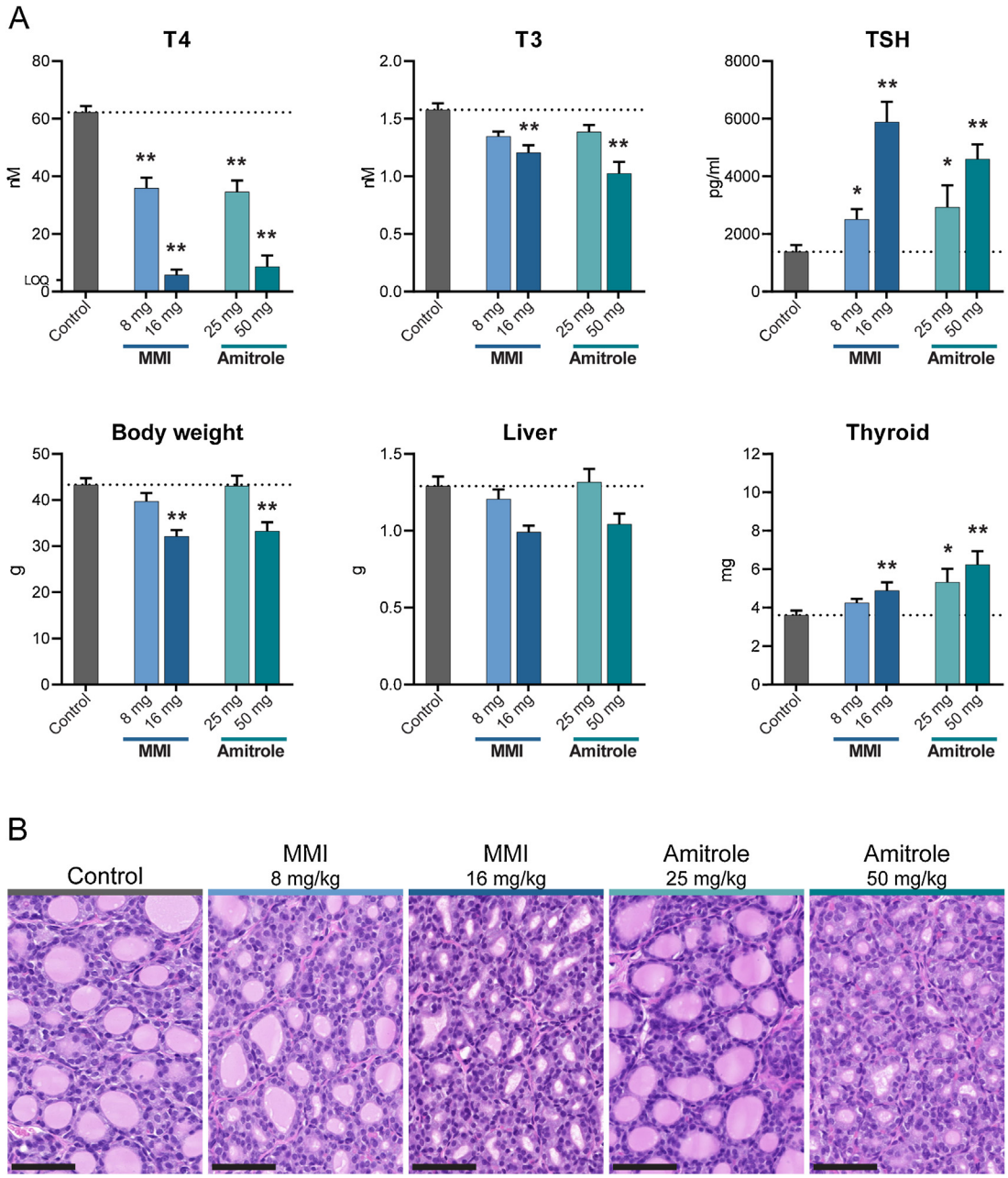

Fig. 3. Male offspring thyroid hormone system disruption. A) Male PD16 pup serum T4, T3 and TSH concentrations and body, liver and thyroid gland weights. n = 10-12. Mean + SEM. Dotted line represents mean of the control. LOQ: lower limit of quantification. * $p < 0.05$, ** $p < 0.01$. B) Male PD16 pup thyroid gland histology, representative images from each dose group. Doses were given as mg/kg body weight/day, n = 3-4. Scale bar = 50 μm.

to compromised thyroid hormone action in the brain and ultimately impaired brain function. This supports the view that TPO-inhibitors as a substance class may have the potential to adversely affect brain development in mammals through thyroid hormone system disruption.

Both MMI and amitrole were chosen as they can both be regarded model compounds for thyroid hormone system disruption through TPO inhibition. Although additional modes of action cannot be fully excluded, we are not aware of any evidence to suggest other modalities at the applied dose levels. Nevertheless, the aim of the study was to gain insight into mode of action and downstream effects of TPO-inhibitors in general, rather than toxicological information on MMI and amitrole *per se*. Being a medical drug, human exposure to MMI is intended and, at least in the European Union, amitrole is used by professional industrial workers while potential consumer exposure is unknown (ECHA, 2021). Yet, we argue that the effects by these two compounds represents potential effects of all *in vitro* TPO-inhibitors and that the combined exposure to all thyroid hormone system disrupting chemicals pose a threat to human thyroid function and brain development.

4.1. Thyroid hormone system disruption by MMI and amitrole

Overall, the TPO-inhibitors MMI and amitrole caused very similar effects on the thyroid hormone systems of both dams and offspring, albeit effects appeared quantitatively more pronounced in the pups. Both compounds induced larger reductions in T4 than in T3 serum concentrations and both activated the HPT-axis, evident by elevated serum TSH. Both compounds also increased

Table 2
Dam and offspring organ weights after perinatal exposure to MMI or amitrole.

	Control	MMI 8 mg/kg	MMI 16 mg/kg	Amitrole 25 mg/kg	Amitrole 50 mg/kg
Dams PD22 (no.)	12	12	11	11	12
Dam body weight (g)	305 ± 16	293 ± 15	291 ± 20	311 ± 18	288 ± 24
Dam relative liver weight x100	4.26 ± 0.43	4.11 ± 0.26	4.25 ± 0.53	4.48 ± 0.49	4.17 ± 0.52
Dam relative thyroid weight x100	0.0048 ± 0.0018	0.0058 ± 0.0012	**0.0107 ± 0.0034****	0.0067 ± 0.0025	**0.0130 ± 0.0051****
Male pups PD16 (no.)	12	12	11	11	12
Male body weight	43.3 ± 4.9	39.7 ± 6.2	**32.1 ± 4.5****	43.1 ± 7.3	**33.3 ± 6.7****
Male relative liver weight x100	2.96 ± 0.18	3.03 ± 0.15	3.08 ± 0.35	3.04 ± 0.27	3.13 ± 0.35
Male relative thyroid gland weight x100	0.0082 ± 0.0012	0.0109 ± 0.0019	**0.0155 ± 0.0051****	0.0119 ± 0.0045	**0.0192 ± 0.0081****
Female pups PD17 (no.)	12	12	11	11	12
Female body weight	46.8 ± 5.9	43.0 ± 6.2	**36.0 ± 5.2****	47.4 ± 7.5	**35.7 ± 8.4****
Female liver weight (g)[1]	1.59 ± 0.27	1.53 ± 0.24	1.27 ± 0.22	1.63 ± 0.30	1.19 ± 0.30
Female relative liver weight x100	3.39 ± 0.20	3.54 ± 0.16	3.53 ± 0.21	3.43 ± 0.19	3.34 ± 0.25
Female relative thyroid gland weight x100	0.0084 ± 0.0020	0.0114 ± 0.0022	**0.0135 ± 0.0044****	**0.0139 ± 0.0035****	**0.0248 ± 0.0128****

Doses were given as mg/kg body weight/day. Data shown as mean ± SD.* $p < 0.05$, ** $p < 0.01$.
[1] Statistical analysis of absolute organ weights included body weight as a covariate.

thyroid gland weights and induced hyperplasia/hypertrophy in the thyroid glands of male pups, indicating maintained bioactivity of TSH and its signaling cascade. This is consistent with findings from studies exposing perinatal rats to MMI through drinking water or feed (Comer and Norton, 1985; Fegert et al., 2012; Shibutani et al., 2009; Shiraki et al., 2012). With respect to effects of amitrole on rat pups, this has previously only been investigated in a small exploratory study (Ramhøj et al., 2021). Nevertheless, our current data, using larger group sizes, suggests a more pronounced reduction in postnatal thyroid hormone concentrations than previously reported, alongside a noticeable effect on thyroid gland histopathology. Taken together, the MMI and amitrole effect pattern is representative of classical developmental hypothyroidism, a pattern also seen with PTU.

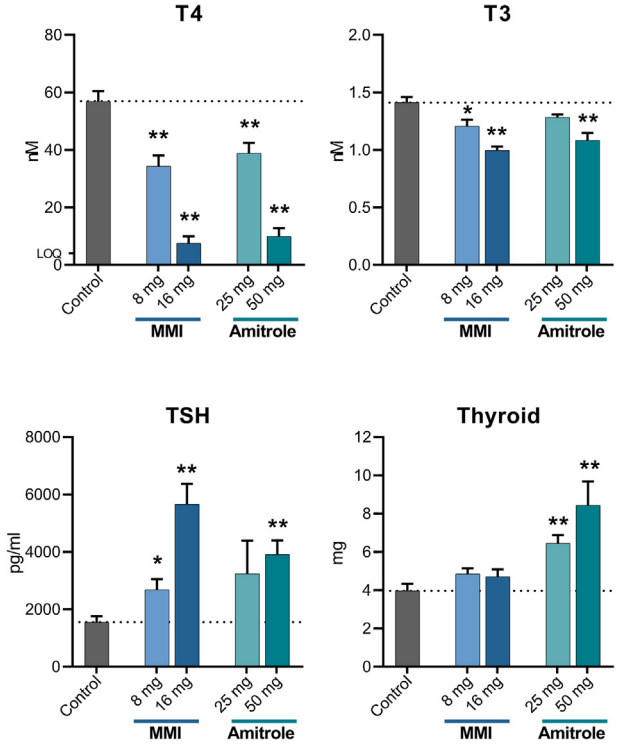

Fig. 4. Femaleoffspring thyroid hormone system disruption. Female PD17 pup serum T4, T3 and TSH concentrations and thyroid gland weights. n = 11-12. Mean + SEM. Dotted line represents mean of the control. LOQ: lower limit of quantification. * $p < 0.05$, ** $p < 0.01$.

Based on our findings, it is clear that the *in vivo* hypothyroidism effect pattern is shared between a subset of *in vitro* TPO-inhibitors. This effect pattern points to a common mechanism for thyroid hormone derived adverse effects. However, not all *in vitro* TPO-inhibitors are potent *in vivo* (Ramhøj et al., 2021) or possess the toxicokinetic properties that make them capable of disrupting both maternal, fetal and pup thyroid hormone concentrations to the same degree as PTU, MMI and amitrole. This represents a challenge for future studies, and predictive toxicology, as our current tools cannot easily predict to what degree *in vitro* TPO-inhibitors will impede on TPO activity *in vivo,* especially not in developing animals (Chang and Doerge, 2000; Handa et al., 2021; Hassan et al., 2020, 2017; Ramhøj et al., 2021).

Although our administered doses of MMI and amitrole resulted in clear dose-dependent effects on serum thyroid hormone concentrations and HPT-axis activation, animals in the higher dose regimens were highly affected. This severe hypothyroidism caused significant growth retardation, with offspring being smaller at birth and later postnatally, something that should be considered when extrapolating findings from our high dose groups. Inclusion of these high doses, however, provides a strong link between hypothyroidism and specific effect endpoints in the developing rat brain. But since many environmental chemicals causing thyroid hormone system disruption will induce more moderate degrees of hypothyroidism (Axelstad et al., 2013; Gilbert et al., 2020; Lavado-Autric et al., 2013; Paul et al., 2012; Ramhøj et al., 2021, 2020), our low dose groups would be more informative for comparisons across chemicals.

4.2. Maternal MMI and amitrole exposure decreases thyroid hormone action in the brain and alters brain function in the offspring

Previous work has identified a number of thyroid hormone sensitive genes in the cortex of young rats, including genes that show differential regulation after perinatal exposure to PTU (Bastian et al., 2014; Bernal, 2017; Chatonnet et al., 2015; Gil-Ibañez et al., 2017; O'Shaughnessy et al., 2018; Royland et al., 2008). Genes such as *Klf9* and *Hr* are directly regulated by the thyroid hormone receptor (Gil-Ibañez et al., 2017), while for instance *Pvalb,* encoding the calcium-binding protein parvalbumin in interneurons, have been implicated in thyroid hormone mediated effects on brain development (Gilbert et al., 2007; López-Espíndola et al., 2014; Shiraki et al., 2012; Venero et al., 2005). In PTU-exposed animals, these gene transcript levels correlate dose-dependently to both serum and brain thyroid hormone concentrations (O'Shaughnessy et al., 2018). Although

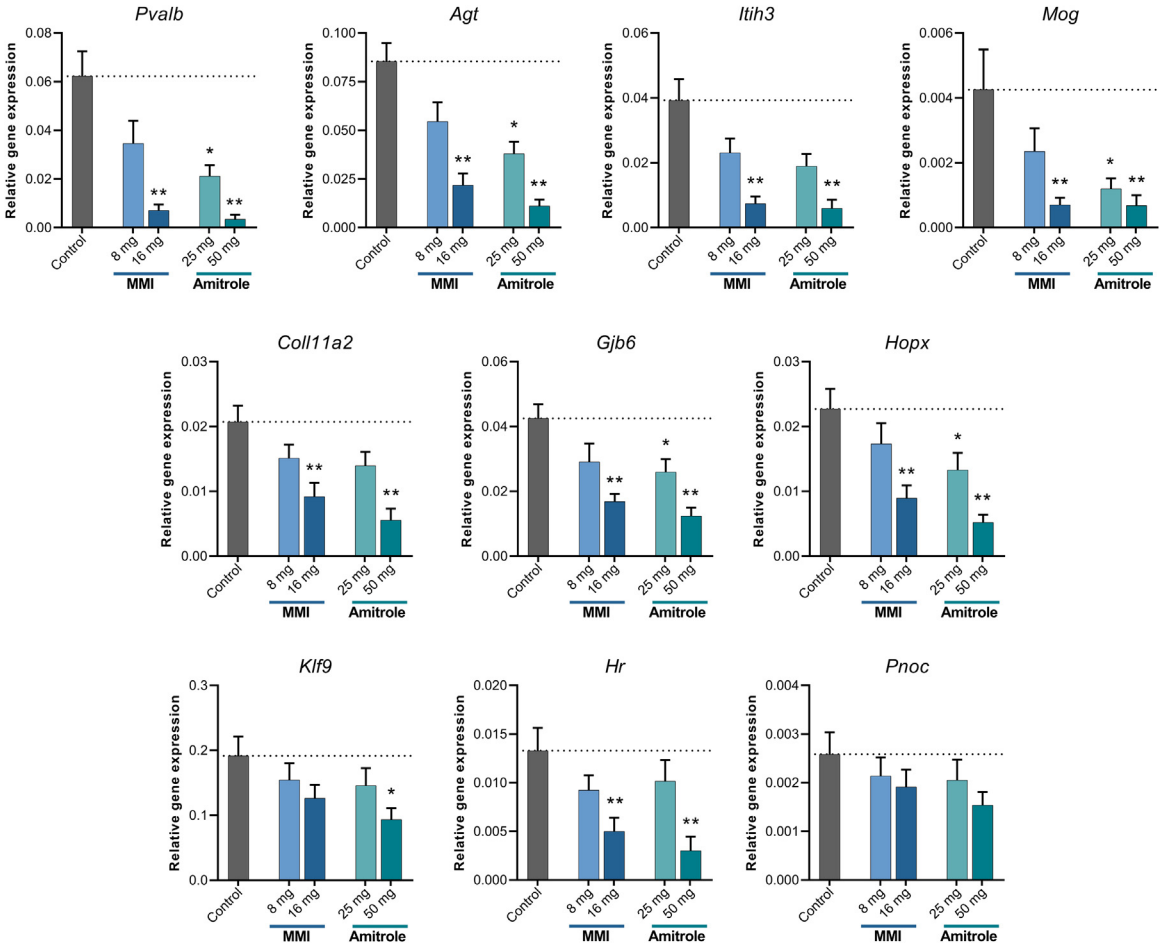

Fig. 5. Cerebral cortical gene expression in male pups PD16. Gene expression of thyroid hormone-sensitive genes were reduced in male pups exposed perinatally to MMI and amitrole. n = 10-12 (except *Mog* in amitrole 50 mg/kg with n = 7). Mean + SEM. Dotted line represents mean of the control. * $p < 0.05$, ** $p < 0.01$.

work remains to characterize to what degree many genes are directly regulated by thyroid hormone action *versus* indirect regulation, they are still useful markers for thyroid hormone deficiency in the brain (O'Shaughnessy et al., 2018). Based on previous studies, we analyzed the expression of several of these genes and found many transcript levels to be reduced. This dose-dependent downregulation of *Pvalb*, *Agt*, *Itih3*, *Mog*, *Coll11a2*, *Gjb6*, *Hopx*, *Klf9* and *Hr* was evident in both the MMI- and the amitrole-exposed pups, and the effect patterns were similar to that reported for PTU-exposed animals. Thus, *in vivo* and across three different TPO-inhibiting compounds, it appears that these gene transcripts can be used as a readout of thyroid hormone action in the developing rat brain. Furthermore, the fact that these genes are affected in the brain indicates thyroid hormone deficiency in the brain itself. This latter point is important since the morphological and functional consequences of thyroid hormone deficiency most likely arises from decreased thyroid hormone action in the brain.

We found that the reduced thyroid hormone signaling in the pups' brain led to altered motor activity early in development. This in itself is of concern, but more so since such effects are associated with permanent behavioral changes and impaired learning and memory later in life (Axelstad et al., 2008; Goldey et al., 1995; Kobayashi et al., 2005). Presumably, for MMI and amitrole, the effects on behavior are mediated through TPO-inhibition and reduced thyroid hormone synthesis with subsequent effects on thyroid hormone mediated brain development. This is supported by the reduced thyroid hormone action in the brain shown here, and the induction of the thyroid hormone specific periventricular heterotopia observed in our recent study (Goodman and Gilbert, 2007; Ramhøj et al., 2021). Thus, although all TPO-inhibitors have the potential to cause developmental neurotoxicity, the ability of a specific compound to reduce thyroid hormone concentrations *in vivo* in both dams and their developing offspring is critical. Clearly, this potential depends on the compound's potency and toxicokinetic properties. When considering the similarities between the effects caused by MMI and amitrole in this study, alongside effects known to be caused by PTU exposure (Axelstad et al., 2008; Gilbert et al., 2017, 2014, 2007; O'Shaughnessy et al., 2018, 2019; Shiraki et al., 2014), it is likely that the MMI- and amitrole-exposed pups would display a range of other adverse neurological effects. This would likely include changes to interneurons, impaired neurogenesis, neuronal migration (*e.g.* seen as heterotopia formation) or changes to neuronal glial cell populations. Some of these effect endpoints will be explored in future studies.

4.3. Sensitivity of assays for detecting thyroid hormone mediated effects on the brain

Although we observed a statistically significant effect for both gene expression and motor activity levels, our results highlight some obvious challenges when it comes to available test methods for identification of the adverse effects of disrupted thyroid hormone system on brain development. In the high dose group – both for MMI and amitrole – a marked degree of hypothyroidism was evident, yet effects on behavior remained relatively subtle. This implies that the behavioral test is quite insensitive with regard

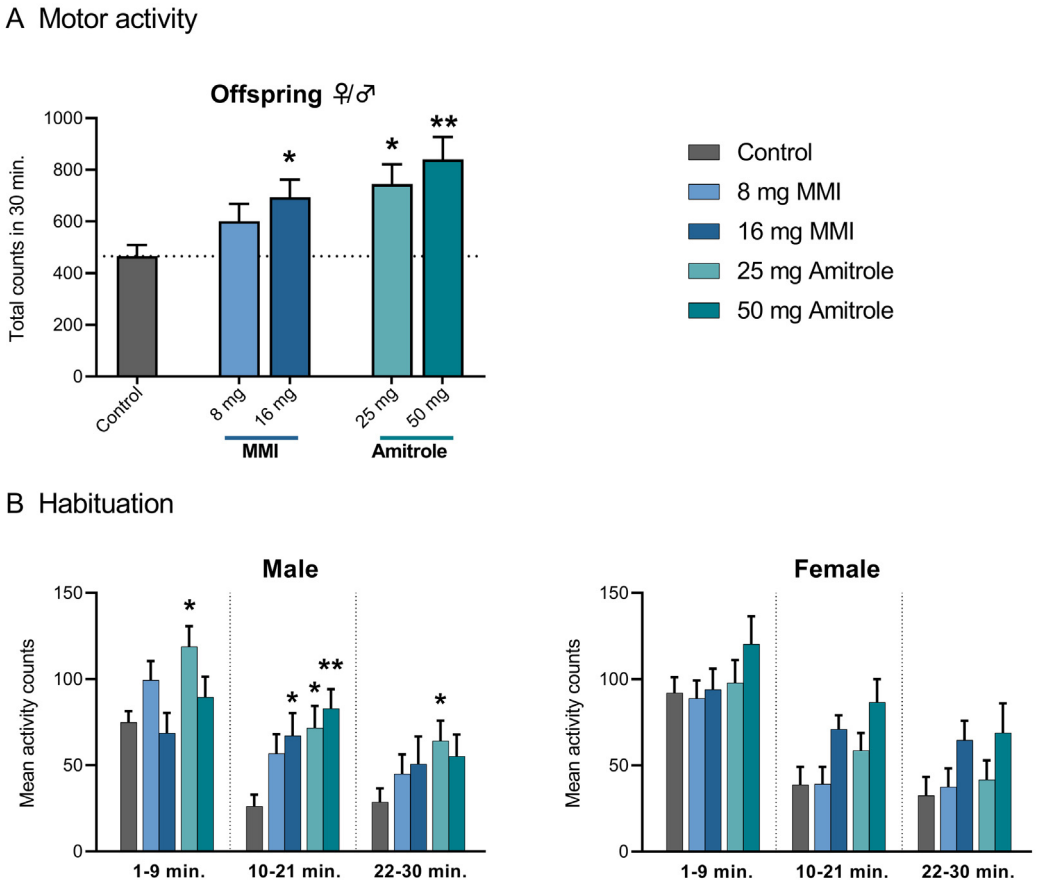

Fig. 6. Pup motor activity was increased and habituation was impaired on PD21. A) Total motor activity in PD21 pups during 30 min. n = 18-22 (9-11 litters each represented by a male and female pup. The statistical analysis included adjustment for sex and bw). B) Habituation in male and female pups. Average activity during the first, middle and last parts of the test period. Mean + SEM. Dotted line represents mean of the control. n = 9-11. * $p < 0.05$, ** $p < 0.01$.

to detecting thyroid hormone system disrupting compounds. This challenge becomes more pronounced when considering the low dose groups where a more moderate degree of hypothyroidism was induced. In these low dose groups – for both MMI and amitrole – the effects on both cortical gene expression and behavioral assay were not consistently detected, but at these exposure doses there was around a 50 % reduction in serum T4 during the postnatal period. Thus, these endpoints are not sensitive enough to detect compounds that do not decrease serum T4 beyond 50 %.

The sensitivity of our assays to detect thyroid hormone system disruption and effects on brain development becomes even more of a challenge when considering the potential degree of T4 reduction these animals experienced during fetal and neonatal life. Low dose MMI and amitrole reduced PD16/17 thyroid hormone concentrations similarly to what is seen in two-week-old offspring from low-dose PTU studies (Axelstad et al., 2008; Gilbert et al., 2014; O'Shaughnessy et al., 2018; Spring et al., 2016). Yet, for PTU we know that even greater reductions in T4 are seen in the fetal and neonatal pup serum and brain (O'Shaughnessy et al., 2018). If the kinetic patterns for MMI and amitrole are similar to PTU, it is also likely that offspring T4 concentrations in our low dose groups were reduced beyond 50 % during fetal and neonatal periods. Still, only sporadic effects on brain endpoints were observed in the low dose group offspring. Since many environmentally relevant TPO-inhibitors may be less potent than MMI and amitrole, it is obvious that current test methods will not be able to detect adverse effects of such compounds on rat brain development. This is a regulatory challenge in that humans appear very sensitive to thyroid hormone insufficiency and that far less reduction in T4 than 50 % is enough to impair brain development. In fact, epidemiological studies have correlated low, but within the reference range, maternal serum T4 concentrations to increased risk of neurobehavioral disorders, altered grey to white matter ratio and decreased IQ in the children (Ghassabian et al., 2014; Gyllenberg et al., 2016; Henrichs et al., 2010; Korevaar et al., 2016; Modesto et al., 2015; Román et al., 2013). In other words, the chemical legislative and regulatory system is inadequate with respect to testing and regulating chemicals that can perturb the thyroid hormone system in pregnant women and their fetuses/children. We would like to stress the importance of the scientific community contributing towards filling this gap and are focusing on this challenge in the ongoing ATHENA project under the EU Horizon 2020 programme (Kortenkamp et al., 2020).

5. Conclusions

The therapeutic drug MMI and the herbicide amitrole are both TPO-inhibitors that can cause developmental thyroid hormone deficiency, resulting in perturbed brain development and altered behavior in rat offspring. The adverse effect outcomes are similar to those induced by the potent thyroid hormone-suppressing drug PTU. Since PTU also inhibits TPO activity, this strongly suggests that TPO-inhibition leading to thyroid hormone deficiency is the main driver of adverse brain development for these chemical substances. Hence, our study implicate TPO-inhibiting chemicals more broadly as thyroid hormone system disruptors capable of disturbing brain development, also in humans. Future studies should focus on confirming this causal link between TPO-

inhibition and altered brain developmental processes and develop more sensitive markers for detecting consequences of developmental thyroid hormone system disruption. Such new endpoints are crucial if we are to protect human brain development from thyroid hormone system disrupting chemicals.

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Declaration of Competing Interest

The authors report no declarations of interest.

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