Is periventricular heterotopia a useful endpoint for developmental thyroid hormone system disruption in mouse toxicity studies?

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Is periventricular heterotopia a useful endpoint for developmental thyroid hormone system disruption in mouse toxicity studies?

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ABSTRACT

In rats, hypothyroidism during fetal and neonatal development can disrupt neuronal migration and induce the formation of periventricular heterotopia in the brain. However, it remains uncertain if heterotopia also manifest in mice after developmental hypothyroidism and whether they could be used as a toxicological endpoint to detect TH-mediated effects caused by TH system disrupting chemicals. Here, we performed a mouse study where we induced severe hypothyroidism by exposing pregnant mice (n = 3) to a very high dose of propylthiouracil (PTU) (1500 ppm) in the diet. This, to obtain best chances of detecting heterotopia. We found what appears to be very small heterotopia in 4 out of the 8 PTU-exposed pups. Although the incidence rate could suggest some utility for this endpoint, the small size of the ectopic neuronal clusters at maximum hypothyroidism excludes the utility of heterotopia in mouse toxicity studies aimed to detect TH system disrupting chemicals. On the other hand, parvalbumin expression was manifestly lower in the cortex of hypothyroid mouse offspring demonstrating that offspring TH-deficiency caused an effect on the developing brain. Based on overall results, we conclude that heterotopia formation in mice is not a useful toxicological endpoint for examining TH-mediated developmental neurotoxicity.

1. Introduction

Proper brain development depends on appropriate thyroid hormone (TH) action during fetal and perinatal life. Disruption of the thyroid hormone (TH) system during these early life stages can thus lead to severe developmental neurotoxicity in animals and humans (Bernal, 2022; Gilbert and Zoeller, 2011). Many chemicals have been shown to interfere with the thyroid hormone system (Miller et al., 2009; Mughal et al., 2018); however, robust and sensitive toxicological markers of TH system disruption in animal studies, that are indicative of disrupted brain function, are currently missing (ECHA/EFSA, 2018; Gilbert et al., 2020). One promising effect biomarker in rat toxicity studies is the formation of periventricular heterotopia, an irreversible malformation of the brain (Kortenkamp et al., 2020). These malformations are observed in rats that were severely hypothyroid during development, but it is an outstanding question whether heterotopia could also be used as effect biomarker in mouse toxicity studies.

Periventricular heterotopia are a neuronal migration defect seen as clusters of misplaced neurons in the corpus callosum. It is considered a malformation and regarded as an adverse effect outcome in rat toxicity studies. In severely hypothyroid rat offspring, heterotopia associate with several other effects on the brain, including learning and memory deficits, altered motor activity, cell population changes and reduced myelination and dendritic arborization (Bernal, 2022; Gilbert and Zoeller, 2011). Heterotopia form consistently, irreversibly and dose-dependently, in rat studies where pregnant and lactating dams are exposed to thyroperoxidase (TPO)-inhibiting compounds such as propylthiouracil (PTU); in these studies marked reductions in serum and brain T4 and T3 (>50–60%) in both fetus and neonatal rat pups are achieved (Gilbert et al., 2014; O'Shaughnessy et al., 2019; O'Shaughnessy et al., 2018;O'Shaughnessy, Wood, et al., 2018; Ramhøj et al., 2021; Shibutani et al., 2009). From a toxicological viewpoint, it is particularly useful that heterotopia are TH-mediated, a permanent malformation and that they arise in a dose-dependent manner with large

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volume increases after more pronounced hypothyroidism (Gilbert et al., 2014; Goodman and Gilbert, 2007). This is the case in rats, but the question still remains if heterotopia formation also occurs in hypothyroid mouse offspring. This question is important to answer, as heterotopia formation in mice would open up for toxicity testing, translational studies as well as giving access to sophisticated knock-out models to decipher the mechanisms behind TH-mediated heterotopia formation.

Reduced expression of parvalbumin (PVALB) in the brain of rats (Berbel et al., 1996; Gilbert et al., 2007; Ramhøj et al., 2022; Shiraki et al., 2012) and mice (Harder et al., 2018; Mayerl et al., 2022; Richard et al., 2020; Uchida et al., 2021; Venero et al., 2005; Wallis et al., 2008) is another observable effect of developmental hypothyroidism. PVALB is a calcium-binding protein expressed in a subset of the inhibitory gamma-aminobutyric acid (GABAergic) neurons, the PV+-interneurons, and is important in the inhibitory control of neuronal activity. In developmentally hypothyroid offspring there are dose-dependent effects on the number of PV+-cells, on general PV-immunoreactivity, including in neuronal processes, and on protein quantity (Gilbert et al., 2007). It is thus possible that changes to PV+-cell populations can serve as a marker of developmental thyroid hormone system disruption (Gilbert et al., 2020; Kortenkamp et al., 2020).

In this study, we aimed to answer two questions. Firstly, do heterotopia form in the mouse in response to developmental hypothyroidism as in rats? Secondly, if yes, does heterotopia hold any utility as an adverse effect measurement in toxicological studies for the identification of TH system disrupting chemicals? To answer these questions, we performed a pilot study in mice where we induced severe hypothyroidism by exposing pregnant mice to iodine deficiency and a very high dose of PTU via the diet. This exposure leaves pups hypothyroid during development (Vancamp et al., 2022, 2023). The exposure regimen was deliberately chosen as the highest dose possible causing severe hypothyroidism and offspring growth retardation. The rationale being that if heterotopia manifests in hypothyroid mouse offspring, they should be detected by this study design. On the other hand, if heterotopia are not detected, it is likely that this effect does not occur in mice, as it does in rats, and that it is not a potential morphological biomarker of TH-mediated developmental neurotoxicity in mice. To monitor disrupted TH-mediated brain development by separate means, we also investigated the expression of PVALB in the mouse offspring.

2. Materials and methods

2.1. Animals, exposure and tissue collection

Pregnant C57bl/6 mice were received on gestational day (GD) 4 (Janvier-Labs) and housed at 23 °C, under a 7:30 a.m. to 8:30 p.m. light/dark cycle. From GD7 to postnatal day (PD) 14, dams were fed ad libitum with either standard chow (LASQC Rod16 R, Altromin, Germany) (control), or iodine deficient pellets containing 1500 ppm (0.15%) propylthiouracil (PTU) (Teklad TD.95125 Envigo, Madison, USA) (n = 3). All experiments were carried out in accordance with the European Community Council Directive of September 22, 2010 (2010/63/EU) regarding the protection of animals used for experimental and other scientific purposes. The research project was approved by a local animal care and use committee (C2EA015) and authorized by the French Ministry of Research (project #14063–2018022215397455).

On PD14, dams were killed by intraperitoneal injection of a combination of xylazine (25 mg/kg) and ketamine (130 mg/kg). Pups were killed by decapitation and whole brains were extracted from 2 to 3 pups per litter and fixed in 10% formalin for 5 days at room temperature. Blocked brains were processed in an Excelsior AS Tissue Processor from Thermo Scientific (United Kingdom), thereafter embedded in paraffin and stored until sectioning.

2.2. Immunohistochemistry and evaluations

Paraffin-embedded brains were coronally sectioned at 10 μm. To identify heterotopia, every 3rd section from the anterior to posterior hippocampus was stained with NeuN antibody. To assess parvalbumin expression, one section containing the anterior hippocampus was stained with PV antibody. Immunohistochemical staining of all sections was performed as described below, note different specifics for the two antibodies. Sections were deparaffinized and boiled in Tris/EDTA buffer (pH 8.95–9.05) for 15 min in a microwave, then blocked with 1% bovine serum albumin (in PBS buffer) for 30 min. Antibody incubation was overnight at 4 °C with 1:20,000 NeuN (MAB377 anti-neuronal nuclei antibody, EMB Millipore Corp, now Merck, Darmstadt, Germany) and 60 min at room temperature with 1:8000 PV (ab11427, anti-parvalbumin antibody, Abcam plc, Wales, England). Endogenous peroxidase was blocked by 10 min treatment with 3% H_2O_2, then sections were incubated for 30 min with EnVision + System-HRP Labelled Polymer Anti-mouse (K4001, Dako, Glostrup, Denmark). Staining was performed with 10 min DAB+ (Dako, Glostrup, Denmark) incubation, then counterstaining with Mayer's hematoxylin.

Stained and cover slipped mouse sections were imaged with a 40x objective in a Pannoramic Midi II digital slide scanner (3DHISTECH Ltd., Budapest, Hungary) and evaluated with the CaseViewer software (v.2.4 from 3DHISTECH) and ImageJ 1.53t (Wayne Rasband and contributors, National Institutes of Health, USA).

Heterotopia were assessed (as first described by (Goodman and Gilbert, 2007)) by examining the corpus callosum, from the midline and out laterally superior to the hippocampus, on every 3rd section from anterior to posterior hippocampus. Cell clusters were assessed as heterotopia when a minimum of 5 large NeuN+-cells were clustered close together and present on at least two adjacent sections. The area of each heterotopia on each section was measured, then multiplied by 30 μm to obtain the heterotopia volume for each animal (up to two heterotopia per animal were identified). Heterotopia were assessed in at least one male and one female pup per litter, and for 5 of 6 litters an additional male or female pup was also analyzed: Thus, total group sizes were 9 control pups and 8 exposed to PTU.

Parvalbumin immunoreactivity was assessed by counting PV+-cells within a fixed square (760 μm^2) overlayed on the cortex (~90 cells counted per control animal). Cell density was calculated as the number of positive cells/area. Furthermore, each section was scored for diffuse PV+ staining in the cortex (yes/no scoring of presence of light brown staining). PV+ was assessed in 9 controls and 8 PTU-exposed but in unequal numbers across litters. For the control litters, one male and one female pup was included from one litter, two males and one female from another litter and two males and two females from the third litter. For the PTU-exposed litters there was one male from one litter, two males and one female from the second litter and 3 males and one female from the third litter. Total group sizes for parvalbumin analysis were 9 control and 8 PTU-exposed animals.

3. Results

3.1. Periventricular heterotopia

We investigated the corpus callosum for heterotopia by examining coronal brain sections from the anterior to posterior hippocampus and calculated their volumes (Fig. 1A). There were heterotopia in 4 of the 8 PTU-exposed animals (one animal with unilateral, 3 with bilateral), in a slightly more posterior location than normally found in rats (Fig. 1B) (Gilbert et al., 2014; Ramhøj et al., 2021). The heterotopia were present in just a few sections per animal (Fig. 1A) and were found in two males and in one female from one litter and in one female from another litter (no heterotopia in the male sibling). In the last PTU-exposed litter there were no heterotopia in the two males and one female. Visual evaluation and a comparison of the heterotopia volumes with those found in

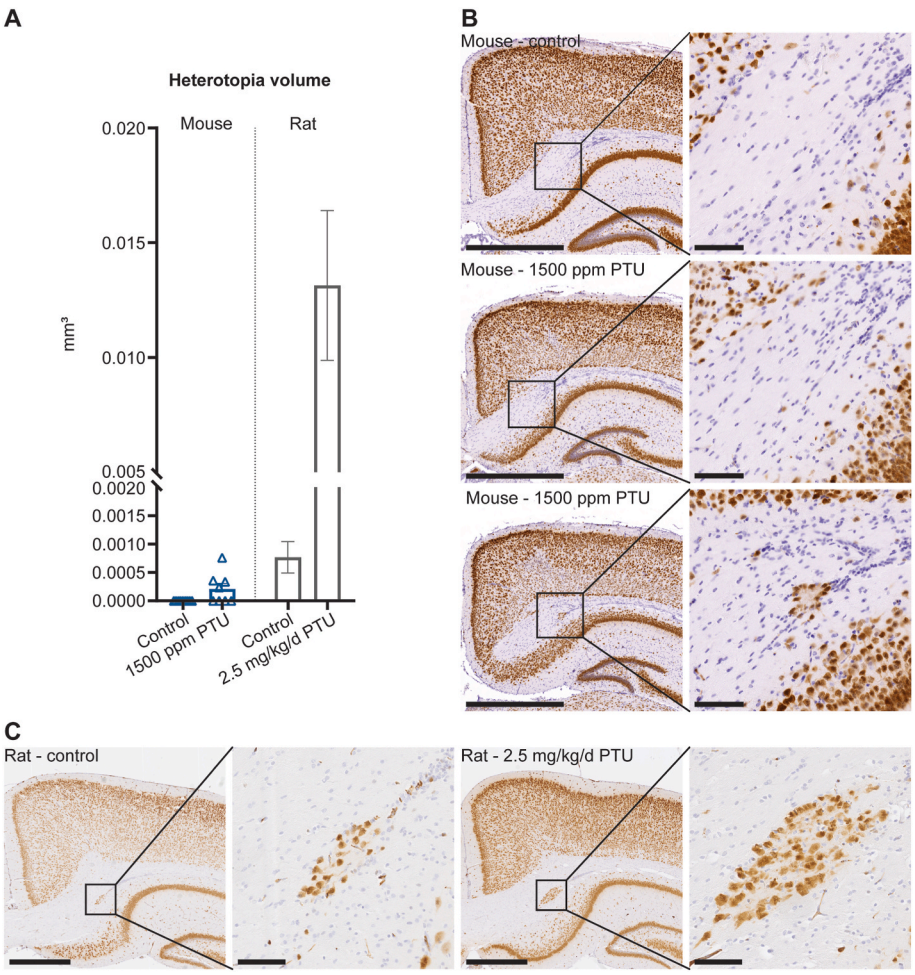

Fig. 1. Minor heterotopia formation in mouse offspring after perinatal exposure to 1500 ppm PTU via feed. Heterotopia formation in rats after a 2.5 mg/kg/d oral gavage exposure to PTU is shown for comparison (unpublished data). n = 8–9 mice per group, n = 15 control rats and 10 exposed to 2.5 mg/kg/d PTU (oral gavage) (all rats were from different litters). A) Periventricular heterotopia volumes in mouse (left) and rat (right) offspring exposed to PTU during development. Few and minimal heterotopia were identified in mice whereas they are larger in control rats and much larger in developmentally hypothyroid rats. Data shown as mean+SEM and with individual data-points for the mice. B) No heterotopia in mice were found in control (top row) or PTU-exposed pups (middle row) in the region where they usually form in rat pups (see C). Few small heterotopia were found in PTU-exposed pups (bottom row) at a location more posterior than the rat heterotopia. Displayed are overview images (left, scalebar = 1000 μm) and magnifications (right, scalebar = 100 μm) of indicated area. C) heterotopia in control and PTU-exposed rat offspring. Displayed is a large control rat heterotopia (left set of images) and a heterotopia in a PTU-exposed rat pup. Scalebars on overview images are 1000 μm and on magnifications 100 μm.

PTU-exposed rats (unpublished data) showed that the mouse heterotopia were considerably smaller, within the size range seen in control rats. In contrast to control (unexposed) rats there were no heterotopia in control mice (Gilbert et al., 2014; Ramhøj et al., 2021).

3.2. Parvalbumin immunoreactivity

We investigated parvalbumin protein expression to determine whether there was altered TH-mediated brain development in the PTU-exposed mice. We observed profound changes in PV immunoreactivity in the cortex of PTU-exposed offspring compared to controls (Fig. 2). PV+-cell density in the cortex was approximately 115 cells/mm^2 in controls but there were no PV+-cells in PTU-exposed offspring (Fig. 2A and B). We also scored diffuse PV+ staining in the cortex and found diffuse staining in all control animals, but it was absent in PTU-exposed pups (Fig. 2B and C). Qualitatively, in the hippocampus, there were PV immunoreactivity in both control and exposed animals, but in the PTU-treated animals there were fewer PV+-cells and less staining of neuronal processes (Fig. 2B).

4. Discussion

In our mouse model of severe hypothyroidism we observed heterotopia in 4 out of 8 animals, or in 2 out of 3 PTU-exposed litters; however, the heterotopia were very small as compared to those forming in hypothyroid rats. This raises the question if the small heterotopia in severely hypothyroid mice can be considered *bona fide* heterotopia and therefore also a malformation of the brain, or if they simply represent small clusters of misplaced cells similarly to clusters of neurons sometimes observed in control rats. It has previously been argued that 'small heterotopia' in control rats represents a normal background condition that is distinct from the larger, dose-dependent heterotopia induced by hypothyroidism in rats (Gilbert et al., 2014, 2021).

Although we observed heterotopia in 50% of the PTU-exposed offspring, and absence of heterotopia in all controls, it should be stressed that our sample size was very small. Accordingly, it is possible that a much larger sample size would result in a smaller, or larger, prevalence of heterotopia. Likewise, a thorough examination should include other mouse strains than the C57bl/6 used herein. However, it was not the intention of this pilot study to establish a prevalence rate of heterotopia in hypothyroid mice, but rather to determine if they manifest as robustly as they do in rats. A number of additional factors further substantiate our notion that heterotopia does not seem to be a useful toxicological effect endpoint for assessing TH-mediated developmental neurotoxicity in mice:

- First, we used severe hypothyroidism to cause the largest possible effect; i.e. the lowest possible TH levels during development leading to largest effects on brain development. Under these conditions, parvalbumin expression was manifestly reduced, demonstrating that the mice were indeed hypothyroid, yet only small neuronal clusters formed.
- Second, 3 of the 4 heterotopia were from the same litter, so we cannot exclude a genetic factor, or susceptibility, contributing to the formation of the small clusters of neurons; i.e. the overall prevalence in larger populations may be different from the 50% observed in this small pilot study.

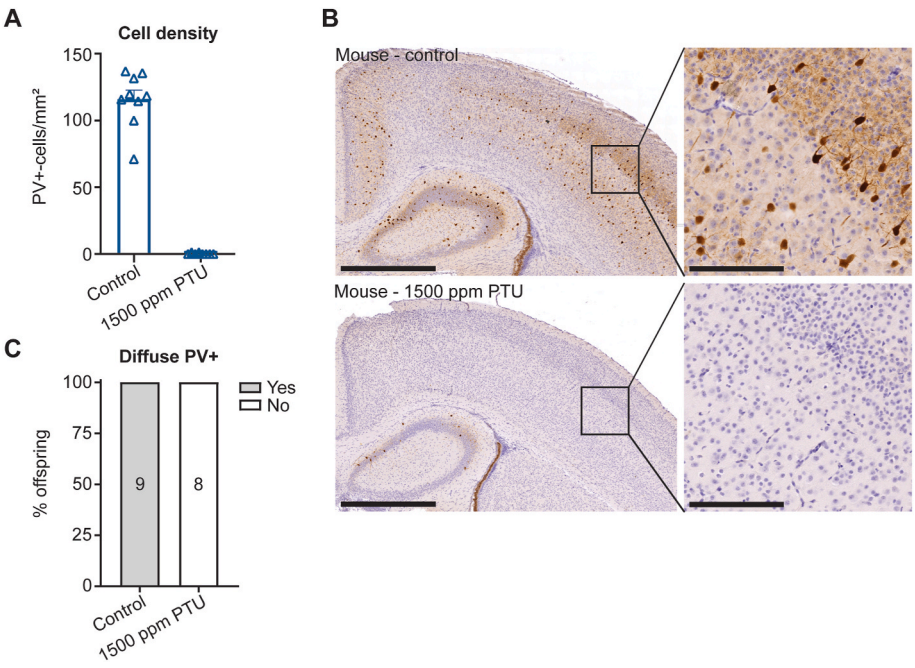

Fig. 2. Parvalbumin protein expression in cortex of mouse offspring perinatally exposed to PTU. A) Density of PV+-cells in cortex. There were no PV+-cells in PTU-exposed animals. Data shown as individual points and mean+SEM. B) Representative images of parvalbumin expression (brown, DAB and Mayer's hematoxylin as blue counterstain) in the hippocampus and cortex of control (upper row) and PTU-exposed animals (lower row). There are positive cells and diffuse PV-staining in the cortex and hippocampus of control animals. In PTU-exposed animals there was no parvalbumin expression in the cortex but slight diffuse staining and a few positive cells in the hippocampus. Scalebar is 1000 μm on left column images and 200 μm on right column magnifications of indicated area. C) Diffuse cortical PV+ staining in offspring scored as yes/no and shown as percentage of offspring receiving each score. No PTU-exposed animals displayed diffuse staining while all control animals did. Numbers in bars indicate sample size. ppm: parts per million, PTU: propylthiouracil, PV: parvalbumin. n = 8–9 offspring from 3 litters.

- Third, to hold value as a toxicological readout of TH disruption, the effect endpoint should be sensitive enough to detect effects at lower doses (i.e. milder TH-insufficiency) and ideally also identify TH disruptors that are not so potent as PTU. Clearly, barely detectable small clusters of neurons in a severely hypothyroid animal model cannot be considered sensitive enough to hold any real utility as a morphometric biomarker.
- Fourth, a toxicological endpoint useful for hazard identification should have a large dynamic range with large differences between controls and exposed, and a demonstrable dose-response relationship. Neither of these parameters seem feasible based on the results in our pilot mouse study. In other words, although there were no heterotopia observed in control animals, our data suggest that, even if heterotopia form in mice, it would be an endpoint with limited sensitivity, dose-dependency and dynamic range and thus not suitable as a toxicological endpoint.

The absence of large heterotopia in the mouse offspring could be due to a general absence of effects on the developing brain in response to developmental hypothyroidism. To make sure this was not the case, and to further substantiate the usefulness of our model, we measured parvalbumin immunoreactivity in the offspring's cortex. There was a clear and nearly complete suppression of parvalbumin in this tissue, which demonstrates that TH-deficiency in the developing mice indeed caused an effect on the brain. Thus, even if small heterotopia can manifest in mice, it appears to be a much less sensitive endpoint than parvalbumin expression. This is different to the situation in rats where heterotopia formation seem more sensitive and with a larger dynamic range than changes in parvalbumin expression (Berbel et al., 1996; Gilbert et al., 2007, 2014). This suggests that it may be useful to pursue parvalbumin immunoreactivity as a TH-mediated adverse effect endpoint in mouse toxicity studies.

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CRediT authorship contribution statement

Louise Ramhøj: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Romain Guyot:** Investigation, Writing – review & editing. **Terje Svingen:** Conceptualization, Resources, Writing – original draft, Writing – review & editing. **Andreas Kortenkamp:** Conceptualization, Writing – review & editing. **Frédéric Flamant:** Conceptualization, Resources, Writing – review & editing. **Marta Axelstad:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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