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Brief communication

Thyroid hormone-dependent formation of a subcortical band heterotopia (SBH) in the neonatal brain is not exacerbated under conditions of low dietary iron (FeD)

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ABSTRACT

Thyroid hormones (TH) are critical for brain development and insufficiencies can lead to structural abnormalities in specific brain regions. Administration of the goitrogen propylthiouracil (PTU) reduces TH production by inhibiting thyroperoxidase (TPO), an enzyme that oxidizes iodide for the synthesis of TH. TPO activity is iron (Fe)-dependent and dietary iron deficiency (FeD) also reduces circulating levels of TH. We have previously shown that modest degrees of TH insufficiency induced in pregnant rat dams alters the expression of TH-responsive genes in the cortex and hippocampus of the neonate, and results in the formation of a subcortical band heterotopia (SBH) in the corpus callosum (Royland et al., 2008, Bastian et al., 2014, Gilbert et al., 2014). The present experiment investigated if FeD alone was sufficient to induce a SBH or if FeD would augment SBH formation at lower doses of PTU. One set of pregnant rats was administered 0, 1, 3, or 10 ppm of PTU via drinking water starting on gestational day (GD) 6. FeD was induced in a 2nd set of dams beginning on GD2. A third set of dams received the FeD diet from GD2 paired with either 1 ppm or 3 ppm PTU beginning on GD6. All treatments continued until the time of sacrifice. On PN18, one female pup from each litter was sacrificed and the brain examined for SBH. We observed lower maternal, PN2 and PN18 pup serum T4 in response to PTU. FeD reduced serum T4 in pups on PN16, but did not affect serum T4 in dams or PN2 pups. Neither did FeD in combination with PTU alter T4 levels in dams on PN18 or pups on PN2 compared to PTU treatment alone. By PN16, however more severe T4 reductions were observed in pups when FeD was combined with PTU. SBH increased with increasing dosage of PTU, but counter to our hypothesis, no SBH was detected in the offspring of FeD dams. As such, T4 levels in dams and newborn pups rather than older neonates appear to be a better predictor SBH associated with TH insufficiency. These data indirectly support previous work indicating prenatal TH insufficiency but not postnatal TH insufficiency in offspring is required for SBH formation.

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1. Introduction

Thyroid hormones (TH) are critical for early brain development. We have previously shown dietary deficiencies in iodine (I), iron (Fe), and copper (Cu) can alter circulating levels of TH in rat dams and their pups (Bastian et al., 2010, 2012; Gilbert et al., 2013; Bastian et al., 2014). These dietary deficiencies also disrupt the expression of TH-responsive genes in the cortex and hippocampus of exposed offspring (Bastian et al., 2012, 2014). Alterations in TH action as reflected in expression of TH-responsive genes are similar to those seen with the TH synthesis inhibitor propylthiouracil (PTU) (Bernal, 2002; Royland et al., 2008; Bastian et al., 2014). PTU reduces circulating levels of TH by reducing iodide organification in the thyroid gland through its interaction with thyroid peroxidase (TPO). TPO is a Fe-dependent enzyme that oxidizes iodide in the synthesis of TH (Hess et al., 2002). Thus, Fe-deficiency and PTU interact with the same enzyme system within the thyroid gland that is essential for TH production.

Developmental TH disruption induced by PTU results in a cortical dysplasia, the formation of a subcortical band heterotopia (SBH). A SBH is seen in neonates and adult offspring of hypothyroxinemic dams and its incidence and size are dose-dependently related to the degree of hypothyroidism induced (Gilbert et al., 2014). Bastian et al. (2014) have previously reported exacerbation of the detrimental effects of PTU on the expression of TH-responsive genes in the developing

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brain when low doses of PTU were combined with FeD. The shared site of action of FeD and PTU on TPO may underlie the more detrimental effects of FeD on TH action in brain when paired with PTU (Bastian et al., 2014).

The present study was conducted to extend this observation beyond serum TH concentrations and brain TH-dependent gene expression to downstream effects on brain morphogenesis. We hypothesized that the coupling of maternal FeD with modest doses of PTU would increase the incidence and/or size of SBH in exposed offspring. Consistent with previous results (Goodman and Gilbert, 2007; Shibutani et al., 2009; Powell et al., 2012; Gilbert et al., 2014), SBH volume increased with degree of TH insufficiency induced by PTU, but contrary to our predictions, no SBH was detected in the offspring of FeD dams. Neither was the presence or size of the SBH further exacerbated with combined treatments of FeD + PTU.

2. Materials and methods

2.1. Subjects

Animals used in this study were from a subset of litters previously described in Bastian et al. (2014). Briefly, time-pregnant Sprague Dawley rats (n = 35) were obtained from Charles River (Wilmington, MA) on gestational day (GD) 2 and housed individually in standard plastic hanging cages in an AAALAC-certified animal facility. Animal rooms were maintained on a 12:12 light:dark schedule and all animal treatments were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* Studies were conducted with the approval of the University of Minnesota Animal Care and Use Committee. On arrival animals were given free access to deionized water and assigned to 1 of 7 treatment conditions. Beginning on GD2, three groups of 5 rats received a semipurified diet (Harlan Laboratories) deficient in Fe (10.1 μg Fe/g of food, FeD), while the remaining four groups remained on chow with adequate amounts of Fe (83.6 μg Fe/g) (Bastian et al., 2014). This latter set of dams was administered propylthiouracil (PTU) in the drinking water at 0, 1, 3 or 10 ppm beginning on GD6. Two of the FeD groups were administered PTU at 1 ppm (n = 5) or 3 ppm (n = 5). All groups were maintained on these regimens until time of sacrifice. The day of birth was designated postnatal day (PN) 0 and litters were culled to 10 with equal numbers of males and females wherever possible. As previously reported by Bastian et al. (2014), none of these treatments had any effect on body weight gain in treated dams, or in pups exposed to the two lower doses of PTU. FeD reduced body weight in offspring when administered alone and in combination with PTU. Food and water intake is not altered by PTU, by FeD, or when FeD was combined with PTU at the dose levels used here (Gilbert, 2011; Anderson, unpublished observations).

Blood and brains were collected from littermates on PN16 for serum and brain hormone analyses as reported in Bastian et al. (2014), but were reanalyzed here to include only those litters for which neuroanatomical assessments were performed, and using litter as the unit of analysis.

2.2. Analysis of T4 by liquid-chromatography-tandem mass spectrometry

Thyroxine (T4) was measured in serum from PN2 pups using methods described in Hornung et al. (2015) with the following exception. In this study, serum were processed using 96 well solid phase extraction (SPE) plates rather than SPE cartridges and assay was performed in a 30 μl volume of serum. The method lower limit of quantification for T4 was 0.0333 ng/ml. The relative percent difference between duplicate samples was 4.7%, recovery of T4 in the matrix spiked sample was 100.7%.

2.3. Immunohistochemisry

One PN18 female from each litter was sacrificed by anesthesia followed by cardiac perfusion with 10% formalin. Tissues were post-fixed for 6 h in 10% formalin, stored in 80% ethanol and prepared for immunohistochemistry to identify SBH. Dams of these pups were sacrificed on PN18 and serum collected for hormone analysis. Brains of PN16 pups were sectioned on a vibratome (60 μm) and sections were collected beginning from the dorsal hippocampus and extending posterior to the hemispheric separation of corpus callosum. Free-floating sections (~30/brain) were processed with mouse monoclonal antisera against a neuron specific nuclear protein, NeuN (1:2500, Millipore MAB377) according to the methods of Goodman and Gilbert (2007) and Ramos et al. (2008). Briefly, after several washes in 0.1 M Tris buffer (pH 7.6), the sections were incubated in 1% hydrogen peroxide to remove endogenous peroxidase activity, washed sequentially in Tris B (0.1 M Tris, 0.1% Triton X-100, 0.05% BSA) and incubated overnight at 4 °C in 10% normal horse serum. On the second day of processing, sections were incubated in rat biotinylated secondary antisera (Vector Labs, Burlingame, CA) followed by avidin-biotin complex (ABC Elite, 1:1000, Vector Labs). Staining was visualized with the chromogen diaminobenzidine (DAB) using bright field microscopy. Each section was examined for the presence of NeuN-positive cells in white matter of the corpus callosum and imaged for area estimates. Areas were summed, multiplied by section thickness, and summed across both hemispheres to calculate volume of the SBH.

2.4. Statistics

Statistical analyses for serum T4, brain T4, and SBH volume were performed using standard general linear model analysis of variance (ANOVA) via SAS (version 9.2, SAS Institute, Cary, NC) followed by the Duncan's Multiple Range post hoc test. For SBH incidence analysis, a criterion for inclusion was nominally set at $>0.0016\ mM^3$ as this was the largest NeuN-positive cell cluster observed in control animals. The 10 ppm PTU treatment group was used as a reference positive control group for hypothyroidism and although data are presented, they were not included in statistical evaluation.

3. Results

3.1. Serum and brain hormones

Serum hormones from dams sacrificed on PN18 and pups sacrificed on PN16, were previously reported by Bastian et al. (2014) but are summarized here in Fig. 1A and 1C in the specific subset of litters assessed for neuroanatomical abnormalities (n = 5/treatment group). Serum T4 in dams was significantly reduced by 3 but not 1 ppm of PTU [$F(5,24) = 4.17$, $p < 0.0072$]. FeD did not alter dam hormones and although a slight reduction was seen when FeD was combined with 1 ppm PTU, it did not differ from FeD alone or from control. A significant reduction from control was seen in dam T4 when FeD was combined with 3 ppm PTU (FeD + 3 ppm), but dam T4 was nominally higher with combined FeD +3 ppm PTU than with 3 ppm PTU alone (Fig. 1A). of this slight rise in dam T4 in FeD animals. Although intriguing, the small sample size and lack of statistical power may have obviated statistical confirmation. Serum concentrations of T4 in pups on PN2 were much lower than in dams (compare Fig. 1A and 1B). Pup serum T4 on PN2 was reduced significantly at 1 and 3 ppm maternal dosages of PTU [$F(5,24) = 11.85$, $p < 0.0001$]. Interestingly, PTU at the low dose of 1 ppm was without effect in the dam, but reduced T4 in newborns by approximately 50%. Similar to the dams, FeD alone did not alter T4 serum levels in the PN2 pup. Finally, combined FeD and PTU treatments did not exacerbate the reduction of serum T4 in PN2 pups, compared to PTU treatment alone.