Fetal and Neonatal Iron Deficiency Exacerbates Mild Thyroid Hormone Insufficiency Effects on Male Thyroid Hormone Levels and Brain Thyroid Hormone-Responsive Gene Expression

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Fetal/neonatal iron (Fe) and iodine/TH deficiencies lead to similar brain developmental abnormalities and often coexist in developing countries. We recently demonstrated that fetal/neonatal Fe deficiency results in a mild neonatal thyroidal impairment, suggesting that TH insufficiency contributes to the neurodevelopmental abnormalities associated with Fe deficiency. We hypothesized that combining Fe deficiency with an additional mild thyroidal perturbation (6-propyl-2-thiouracil [PTU]) during development would more severely impair neonatal thyroidal status and brain TH-responsive gene expression than either deficiency alone. Early gestation pregnant rats were assigned to 7 different treatment groups: control, Fe deficient (FeD), mild TH deficient (1 ppm PTU), moderate TH deficient (3 ppm PTU), severe TH deficient (10 ppm PTU), FeD/1 ppm PTU, or FeD/3 ppm PTU. FeD or 1 ppm PTU treatment alone reduced postnatal day 15 serum total T_4 concentrations by 64% and 74%, respectively, without significantly altering serum total T_3 concentrations. Neither treatment alone significantly altered postnatal day 16 cortical or hippocampal T_3 concentrations. FeD combined with 1 ppm PTU treatment produced a more severe effect, reducing serum total T_4 by 95%, and lowering hippocampal and cortical T_3 concentrations by 24% and 31%, respectively. Combined FeD/PTU had a more severe effect on brain TH-responsive gene expression than either treatment alone, significantly altering *Pvalb*, *Dio2*, *Mbp*, and *Hairless* hippocampal and/or cortical mRNA levels. FeD/PTU treatment more severely impacted cortical and hippocampal parvalbumin protein expression compared with either individual treatment. These data suggest that combining 2 mild thyroidal insults during development significantly disrupts thyroid function and impairs TH-regulated brain gene expression. **(*Endocrinology* 155: 1157–1167, 2014)**

Thyroid hormone is critical for normal mammalian brain development (1, 2). Much of what we know about the role of TH in mammalian brain development comes from studies on severe iodine/TH deficiency. However, it is estimated that mild thyroidal impairments could be 150–200 times more prevalent during pregnancy than congenital hypothyroidism (3). Importantly, recent evidence suggests that maternal hypothyroxinemia and subclinical hypothyroidism contribute to impaired offspring cognitive outcome (4–7). Rodent studies demonstrate that even mild TH insufficiencies impair neonatal brain gene and protein expression (8–12), cellular differentiation, proliferation, and migration (11–16), short- and long-term memory (16), and seizure susceptibility (14) during mammalian brain development, leading to permanent abnormalities in offspring (13, 14, 17–19).

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Abbreviations: CA1, Cornu Ammonis 1; E, embryonic day; Fe, iron; FeD, Fe deficient; GABA, γ-aminobutyric acid; MDC, minimum detectable concentration; P, postnatal day; PTU, 6-propyl-2-thiouracil; PVALB, parvalbumin; qPCR, quantitative real-time PCR; TBST, Tris-buffered saline containing 0.1% Tween 20; TT_4, total T_4.

There are several environmental factors that disrupt thyroidal status, including environmental endocrine disrupting chemicals, dietary goitrogens, and micronutrient deficiencies. Individually, dietary goitrogens, environmental chemicals, and micronutrient deficiencies may exert relatively minor effects on the thyroid axis. However, in combination, these effects may exacerbate each other, thus potentiating a more severe insult to the thyroid axis and more severe developmental impairments. Given their profound impact on child development and prosperity, it is important to understand how micronutrient deficiencies and other thyroid disruptors interact to affect the thyroid axis during fetal and infant development.

Insufficient iodine intake is the main cause of thyroidal dysfunction and affects an estimated 1.88 billion people worldwide (20). Iron (Fe) deficiency anemia, affecting approximately 50% of the estimated 1.62 billion anemic people worldwide (21), also impairs thyroid function (22). Fe deficiency anemia coexists with iodine deficiency in goitrous North and West African school-age children, blunting the efficacy of iodine supplementation (23–25). Combined Fe/iodine supplementation improves thyroid volume, goiter prevalence, and serum total T_4 (TT_4) in these children (23, 25). Importantly, Zimmermann et al (26) recently showed that low body Fe stores are predictive of higher serum TSH and lower serum TT_4 in mildly iodine-insufficient pregnant women. Thus, Fe deficiency could compound the already deleterious effects of iodine deficiency on TH-dependent brain development.

In developing rodents, fetal/neonatal Fe and TH deficiencies produce similar brain developmental abnormalities, including impaired gene expression, myelination, neuronal maturation, synapse formation and function, and neurotransmitter signaling (1, 27). We recently demonstrated that fetal/neonatal Fe deficiency results in a mild impairment to neonatal thyroidal status, leading to altered brain TH-responsive gene expression (9, 28). These data suggest that TH insufficiency may contribute to or exacerbate some of the deficits in brain development associated with Fe deficiency. This led us to hypothesize that combining Fe deficiency with an additional mild thyroidal disruptor would lead to a more severe effect on neonatal thyroidal status and TH-responsive brain gene expression. In this study, we show that concurrent Fe deficiency and mild or moderate 6-propyl-2-thiouracil (PTU) treatment results in a more severe effect on neonatal circulating and brain TH levels compared with Fe deficiency or PTU treatment alone. This combined effect on thyroidal status translates to a more severe impairment of *Pvalb*, *Dio2*, *Hr*, *Mbp*, and *Agt* mRNA levels and parvalbumin (PVALB) protein levels in the neonatal brain.

Materials and Methods

Animals and diets

Sperm-positive Sprague Dawley female rats were purchased from Charles River Laboratories. At gestational day 2 (embryonic day [E]2), 42 pregnant rats were randomly assigned to 1 of 7 groups: control, Fe deficient (FeD), 1 ppm (1 mg/L) PTU (Sigma-Aldrich) treatment, 3 ppm (3 mg/L) PTU treatment, 10 ppm (10 mg/L) PTU treatment, concurrent FeD/1 ppm PTU, and concurrent FeD/3 ppm PTU (n = 6 dams per group). Beginning at E2, FeD dams were fed a semipurified diet (Harlan Laboratories) deficient in Fe. The Fe content of the FeD diet was increased from 5–7 μg/g in our previous studies (9, 28) to 10.1 $\pm$ 0.5 μg/g in order to produce a less severe Fe deficiency. Control and PTU dams were fed an Fe-adequate diet containing 83.6 $\pm$ 6.9 μg/g Fe, which was similar to previous studies (9, 28). Dams in the control and FeD groups drank deionized water. Beginning at E6, dams in the 1, 3, and 10 ppm PTU groups were offered deionized water containing 1, 3, or 10 ppm PTU. Dams in the combined FeD/PTU groups were fed the FeD and were offered water containing either 1 or 3 ppm PTU. Day of birth was designated as postnatal day (P)0 and at P2; all litters were culled to 10 pups. At P15 and P16, male pups were killed to evaluate Fe biomarkers, serum and brain regional TH concentrations, and brain regional mRNA expression. Dams were killed at P18 to evaluate metal and TH status.

Animals were given free access to diet and drinking water throughout the study and were housed at constant temperature and humidity on a 12-hour light, 12-hour dark cycle. All animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health guide for the Care and Use of Laboratory Animals. The local Institutional Animal Care and Use Committee approved these procedures.

Sample collection

At P15, 1 or 2 male pups per litter were killed (11–12 total per group). From each pup, trunk blood was collected after decapitation and kept on ice until centrifuged to collect serum. Serum was stored at −80°C until analyzed. The brain was removed, and the hippocampus and cerebral cortex were dissected and placed immediately into RNAlater RNA stabilization reagent (QIAGEN). After 24 hours at 4°C, tissues in RNAlater were stored at −20°C. At P16, 1 or 2 male pups per litter were killed (9–12 total per group). Serum was collected as described above. The brain was removed, and the hippocampus and cerebral cortex were dissected, weighed, and placed immediately into liquid nitrogen. Hippocampi and cerebral cortices were stored at −80°C until analyzed for tissue T_3 concentrations. At P16, a second cohort of 1 male pup per litter was killed (5–6 total per group). Serum was collected as described above, and a 5-μL blood sample was removed to determine hemoglobin concentrations. The brain was removed, bisected at the midline, and the hippocampus and cerebral cortex were dissected from one half-brain, weighed, and placed immediately into liquid nitrogen. Hippocampi and cerebral cortices were stored at −80°C until later use for Western blotting experiments.

Metal, biochemical, and hormone analyses

Fe, Cu, Zn, and hemoglobin measurements were performed as described (28). Serum TT_4 and TT_3 concentrations were mea-

sured using RIA kits (Siemens Medical Solutions Diagnostics) modified for rodent use as described (28). The minimum detectable concentration (MDC) for T_4 (1.09 ng/mL) and T_3 (6.67 ng/dL) RIAs was calculated statistically as 3 SDs above the 0 calibrator (n = 10). Samples with T_4 or T_3 concentrations below the MDC were set to the MDC for statistical purposes. Serum TSH concentrations were measured using a rodent TSH ELISA kit (Endocrine Technologies). Brain regional T_3 concentrations were measured in extracts from the hippocampus and cerebral cortex as described (9, 28).

Brain mRNA analysis

RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR) for P15 hippocampus and cerebral cortex was carried out as described (9). Primer pairs for the assayed genes are outlined in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. Relative mRNA levels were calculated relative to an internal whole-brain cDNA sample from a P12 control rat pup to allow comparisons between independent experiments and PCR reactions.

Western blotting

Cerebral cortices and hippocampi were homogenized in 9 volumes of homogenization buffer (50mM Tris [pH 7.5], 0.25M sucrose, 1mM EDTA, 10mM sodium fluoride, 5mM sodium pyrophosphate, and 1× complete protease inhibitor cocktail [Roche Applied Science]). The homogenate was treated with 0.5% Triton X-100 and then centrifuged at 13 000*g* for 15 minutes at 4°C. Total protein levels of homogenates were determined using a bicinchoninic acid protein assay kit (Pierce). Homogenates were diluted to 4 μg/mL in homogenization buffer containing 0.5% Triton X-100 and subsequently diluted to a final concentration of 2 μg/mL in an equal volume of 2× sample buffer (4% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125M Tris-Cl [pH 6.8]). Twenty micrograms of protein were boiled for 5 minutes and then subjected to sodium dodecyl sulfate-PAGE on a 15% gel. Protein was transferred to a 0.2-μm nitrocellulose membrane (Whatman) at 100 V for 1.5 hours and blocked for 3 hours in 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature. To assess PVALB protein expression, membranes were probed overnight at 4°C with a rabbit polyclonal antibody (Genscript) at a concentration of 0.5 μg/mL in TBST. After 5 5-minute washes in TBST, membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody (Jackson ImmunoResearch) at a concentration of 0.16 μg/mL in TBST. After 5 5-minute washes in TBST, immunoreactive proteins were detected using an enhanced chemiluminescence system (GE Healthcare). Chemiluminescence imaging and densitometry was performed using the ChemiDoc XRS Molecular Imager and Quantity One software (Bio-Rad Laboratories). Membranes were stripped for 5 minutes at room temperature using Restore Plus Western Blot Stripping buffer (Thermo Scientific) and then blocked for 1 hour at room temperature. Lactate dehydrogenase protein expression was assessed as a loading control by incubating the stripped membranes overnight at 4°C with a goat polyclonal antibody at a concentration of 2 μg/mL (Chemicon International), followed by incubation for 1 hour at room temperature with a horseradish peroxidase-conjugated bovine antigoat IgG secondary antibody (Santa Cruz Biotechnology, Inc) at a concentration of 0.04 μg/mL. On each gel, along with 1 sample from each of the 7 treatments, duplicates of a P14 hippocampus internal control sample were loaded to enable quantitative comparison across gels. For PVALB densitometry calculations, each band intensity value was normalized to the average of the 2 internal control band intensity values for that gel.

Statistical analysis

One-way ANOVA was used for making statistical comparisons between treatment groups for datasets where 1 pup per litter was analyzed. When more than 1 pup per litter was included, a nested ANOVA was used to account for possible litter effects. Bartlett's test was used to assess homogeneity of variances. When variances were equal across groups, Tukey's post hoc test was used. When variances were unequal, data were ln transformed and Tukey's test was used. When transformation did not normalize the variances, Scheffe's post hoc test was used on the untransformed data. Statistical outliers were determined by Grubbs' test (graphpad.com/quickcalcs/Grubbs1.cfm). All data are presented as mean ± SEM. Statistical analyses and data graphing were carried out using JMP (SAS Institute, Inc), Prism (GraphPad Software), or Kaleidagraph (Synergy Software) software. An $\alpha = 0.05$ was chosen to define significant differences. The 10 ppm PTU group served as a positive hypothyroid control and was excluded from ANOVA analyses but was included in figures for comparison.

Results

Evaluating dam and pup Fe status

Table 1 shows Fe and TH characteristics of the rat dams on postpartum day 18. Serum Fe levels were lower in FeD,

Table 1. Fe and Thyroid Hormone Status of Rat Dams

Characteristic	Control	FeD	1 ppm PTU	3 ppm PTU	FeD/1 ppm PTU	FeD/3 ppm PTU	10 ppm PTU
Body weight, g	281 ± 14.7	295 ± 8.7	275 ± 2.9	317 ± 12.2	310 ± 13.5	319 ± 5.8	285 ± 9.1
Hemoglobin, g/L	143 ± 4.1^{ab}	115 ± 8.0^{b}	155 ± 3.7^{a}	142 ± 4.0^{ab}	126 ± 10.4^{ab}	111 ± 9.4^{b}	146 ± 11.2^{ab}
Serum Fe, μg/mL	3.6 ± 0.48^{a}	0.93 ± 0.03^{b}	4.1 ± 0.27^{a}	4.1 ± 0.50^{a}	1.1 ± 0.18^{b}	0.95 ± 0.12^{b}	4.0 ± 0.51^{a}
Serum TT_4, ng/mL	35.9 ± 3.2^{a}	42.9 ± 5.1^{a}	29.2 ± 6.0^{ab}	15.2 ± 3.9^{bc}	30.4 ± 3.6^{a}	25.2 ± 5.2^{a}	5.5 ± 1.7^{c}
Serum TT_3, ng/dL	77.6 ± 7.1	87.3 ± 5.5	91.6 ± 9.2	80.2 ± 15.5	105 ± 7.0	100 ± 10.3	125 ± 24.8

Data are presented as the mean ± SEM (n = 5). Within a specific row, means not sharing a common letter superscript are significantly different by one-way ANOVA and Tukey's multiple comparison test ($P < .05$). If the lettered superscripts are the same, no significant differences were observed between groups.

FeD/1 ppm PTU, and FeD/3 ppm PTU dams compared with controls. Serum Fe levels were not significantly altered in the 1, 3, or 10 ppm PTU dams. Blood hemoglobin concentrations were not significantly altered in dams of any treatment group. Serum TT_4 concentrations were 58% and 85% lower in 3 and 10 ppm PTU dams, respectively, but were not altered in the other treatment groups. Serum TT_3 concentrations were not altered in dams of any treatment group. Litter sizes from each treatment group were similar (data not shown).

To evaluate Fe status of P16 pups, serum Fe and blood hemoglobin levels were measured (Table 2). Serum Fe levels were lower in FeD, FeD/1 ppm PTU, and FeD/3 ppm PTU P16 pups compared with controls. Blood hemoglobin concentrations were also lower in FeD, FeD/1 ppm PTU, and FeD/3 ppm PTU P16 pups compared with controls. As seen previously, serum Fe and blood hemoglobin concentrations were 113% and 31% higher in 10 ppm PTU P16 pups compared with controls (28). P15 pup body weights were lower in FeD, FeD/1 ppm PTU, FeD/3 ppm PTU, and 10 ppm PTU groups.

Effects of combined PTU treatment and Fe deficiency on thyroidal status

To determine whether combining Fe deficiency with mild or moderate PTU treatment during fetal and neonatal development more severely impacts neonatal thyroidal status, serum TT_4, TT_3, and TSH concentrations were measured in P15 pups (Figure 1). Individually, Fe deficiency and 1 ppm PTU treatment lowered P15 serum TT_4 concentrations by 64% and 74%, respectively, compared with controls (Figure 1A). Supporting our hypothesis, the combined FeD/1 ppm PTU treatment led to a more severe effect, lowering P15 serum TT_4 concentrations by 95% compared with controls. Serum TT_4 concentrations were 91%, 96%, and 98% lower in 3 ppm PTU, FeD/3 ppm PTU, and 10 ppm PTU P15 pups, respectively, compared with controls. Serum TT_3 concentrations were 80% lower in 10 ppm PTU P15 pups but were unaltered in the other treatment groups (Figure 1B). Serum TT_4 concentrations for 2 out of 12 FeD/1 ppm PTU, 6 out of 12 FeD/3 ppm PTU, and 12 out of 12 10 ppm PTU samples fell below the MDC.

Serum TSH concentrations were dose dependently increased in 1, 3, and 10 ppm PTU P15 pups compared with controls (Figure 1C). As reported previously, Fe deficiency did not alter neonatal serum TSH concentrations (9). Serum TSH concentrations were higher in the combined FeD/1 ppm PTU and FeD/3 ppm PTU P15 pups compared with controls.

Effects of combined PTU treatment and Fe deficiency on brain thyroidal status

To assess whether concurrent Fe deficiency and PTU treatment produces a more severe effect on neonatal brain thyroidal status, T_3 levels were measured in the P16 cerebral cortex and hippocampus (Figure 2). Individually, Fe deficiency and 1 ppm PTU treatment did not significantly alter cerebral cortical (Figure 2A) or hippocampal (Figure 2B) T_3 concentrations. However, the combined FeD/1 ppm PTU treatment lowered cerebral cortical and hippocampal T_3 concentrations by 31% and 24%, respectively, compared with controls. T_3 concentrations were similarly affected in the 3 ppm PTU and FeD/3 ppm PTU cerebral cortex (48% lower compared with controls) and hippocampus (44% and 33% lower, respectively, compared with controls); 10 ppm PTU treatment lowered cerebral cortical and hippocampal T_3 concentrations by 84% and 82%, respectively, compared with controls.

Effect of Fe deficiency and PTU treatment on hippocampal and cerebral cortical mRNA expression

We previously demonstrated that fetal/neonatal Fe deficiency alters cerebral cortical or hippocampal mRNA levels for several TH-responsive genes to a similar extent as mild PTU treatment (9). qPCR was performed to test whether combining Fe deficiency with a mild or moderate PTU treatment would lead to a more severe effect on cerebral cortex and hippocampus TH-responsive mRNA expression. The 10 ppm PTU hypothyroid positive control treatment significantly altered *Pvalb*, *Dio2*, *Hr*, *Agt*, *Mbp*, and *Enpp6* mRNA levels in both the cerebral cortex and hippocampus (Figure 3). *Gapdh* mRNA levels were not significantly altered by any of the treatments in either

Table 2. Characteristics of P16 Rat Pups After Fetal and Neonatal Fe Deficiency or PTU Treatment

Characteristic	Control	FeD	1 ppm PTU	3 ppm PTU	FeD/1 ppm PTU	FeD/3 ppm PTU	10 ppm PTU
Body weight, g	44.3 ± 0.93^{a}	29.8 ± 2.0^{c}	41.7 ± 0.62^{a}	40.4 ± 0.36^{ab}	27.6 ± 1.3^{c}	31.5 ± 1.1^{bc}	35.2 ± 1.2^{b}
Hemoglobin, g/L	105 ± 3.3^{a}	41.3 ± 5.9^{b}	103 ± 2.3^{a}	113 ± 3.2^{a}	43.4 ± 7.2^{b}	42.2 ± 4.5^{b}	138 ± 2.8^{c}
Serum Fe, μg/mL	0.94 ± 0.05^{a}	0.29 ± 0.08^{b}	1.1 ± 0.09^{a}	1.2 ± 0.09^{a}	0.33 ± 0.05^{b}	0.29 ± 0.07^{b}	2.0 ± 0.22^{c}

Data are presented as the mean ± SEM (n = 5–6). Within a specific row, means not sharing a common letter superscript are significantly different by one-way ANOVA and Tukey's or Scheffe's multiple comparison test ($P < .05$).

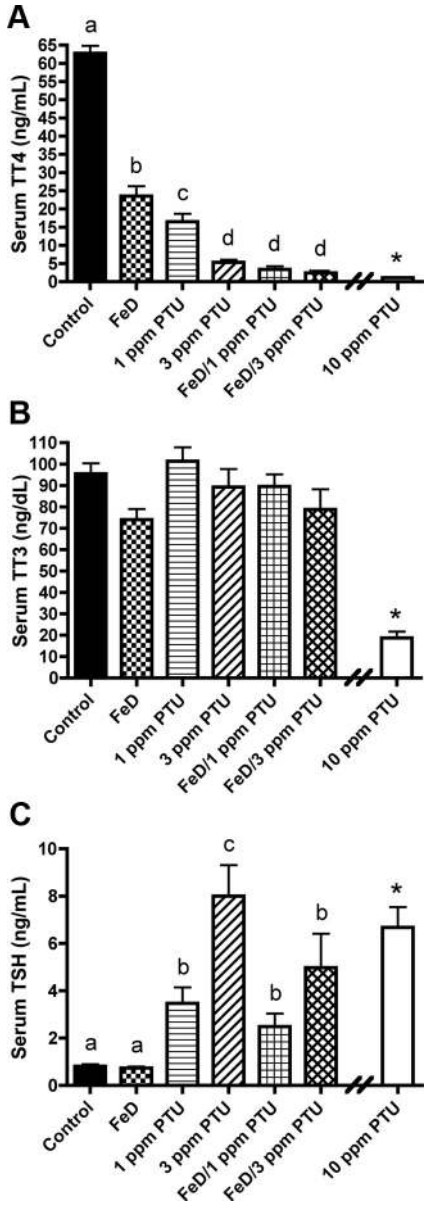

Figure 1. Fetal and neonatal Fe deficiency augments the effect of 1 ppm PTU treatment on serum TT_4 concentrations. Serum was collected from 1 or 2 P15 male pups per litter (n = 11–12), and TT_4 (A), TT_3 (B), and TSH (C) concentrations were measured. Data are presented as the mean ± SEM. Groups not sharing a common letter superscript are significantly different by nested one-way ANOVA and Tukey's multiple comparison test ($P < .05$). If the lettered superscripts are the same, no significant differences were observed between groups. The 10 ppm PTU group served as a positive hypothyroid control. Asterisks indicate statistical difference between 10 ppm PTU and control.

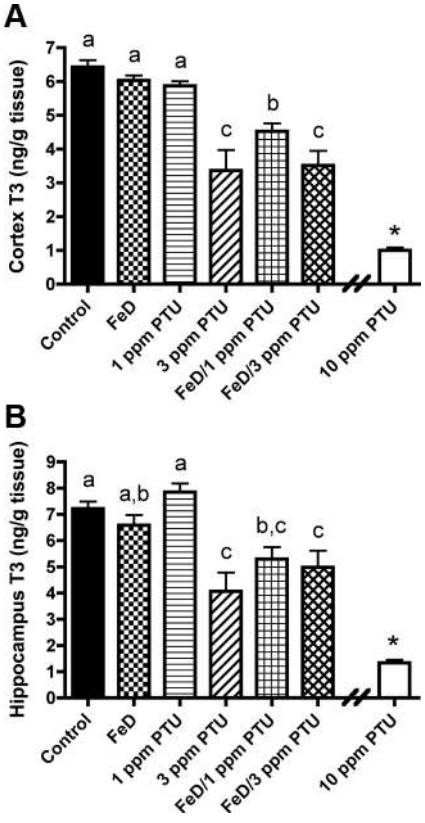

Figure 2. Fetal and neonatal Fe deficiency exacerbates the effect of 1 ppm PTU treatment on hippocampus and cerebral cortex T_3 concentrations. Hippocampi (A) or cerebral cortices (B) were collected from 1 or 2 P16 male pups per litter (n = 9–12), and T_3 concentrations were measured. Data are presented as the mean ± SEM. Groups not sharing a common letter superscript are significantly different by nested one-way ANOVA and Tukey's multiple comparison test ($P < .05$). The 10 ppm PTU group served as a positive hypothyroid control. Asterisks indicate statistical difference between 10 ppm PTU and control.

the cerebral cortex or the hippocampus (Supplemental Figure 1).

Individually, Fe deficiency and 1 ppm PTU treatment lowered P15 cerebral cortex *Pvalb* mRNA levels by 25% and 40%, respectively, compared with controls (Figure 3A). Concurrent Fe deficiency and 1 ppm PTU treatment had a more severe effect on P15 cerebral cortex *Pvalb* expression, lowering mRNA levels by 63%. *Pvalb* mRNA levels were significantly reduced in the 3 ppm PTU and FeD/3 ppm PTU P15 cerebral cortex. In the P15 hippocampus, Fe deficiency, 1 ppm PTU, and 3 ppm PTU did not significantly alter *Pvalb* mRNA levels, compared with controls (Figure 3A). In contrast, the FeD/1 ppm PTU and FeD/3 ppm PTU treatments significantly lowered P15 hippocampal *Pvalb* mRNA levels by 29% and 38%, respectively.

Fe deficiency, 1 ppm PTU, and 3 ppm PTU did not significantly alter P15 cerebral cortical or hippocampal *Dio2* mRNA levels, compared with controls (Figure 3B). However, combining Fe deficiency with 1 ppm PTU produced a more severe impact on *Dio2* expression, significantly increasing cerebral cortical and hippocampal *Dio2* mRNA levels by 48% and 32%, respectively. Likewise, *Dio2* mRNA levels were elevated in the FeD/3 ppm PTU P15 cerebral cortex and hippocampus.

Fe deficiency and 1 ppm PTU did not significantly alter *Hr* mRNA levels in the P15 hippocampus, compared with

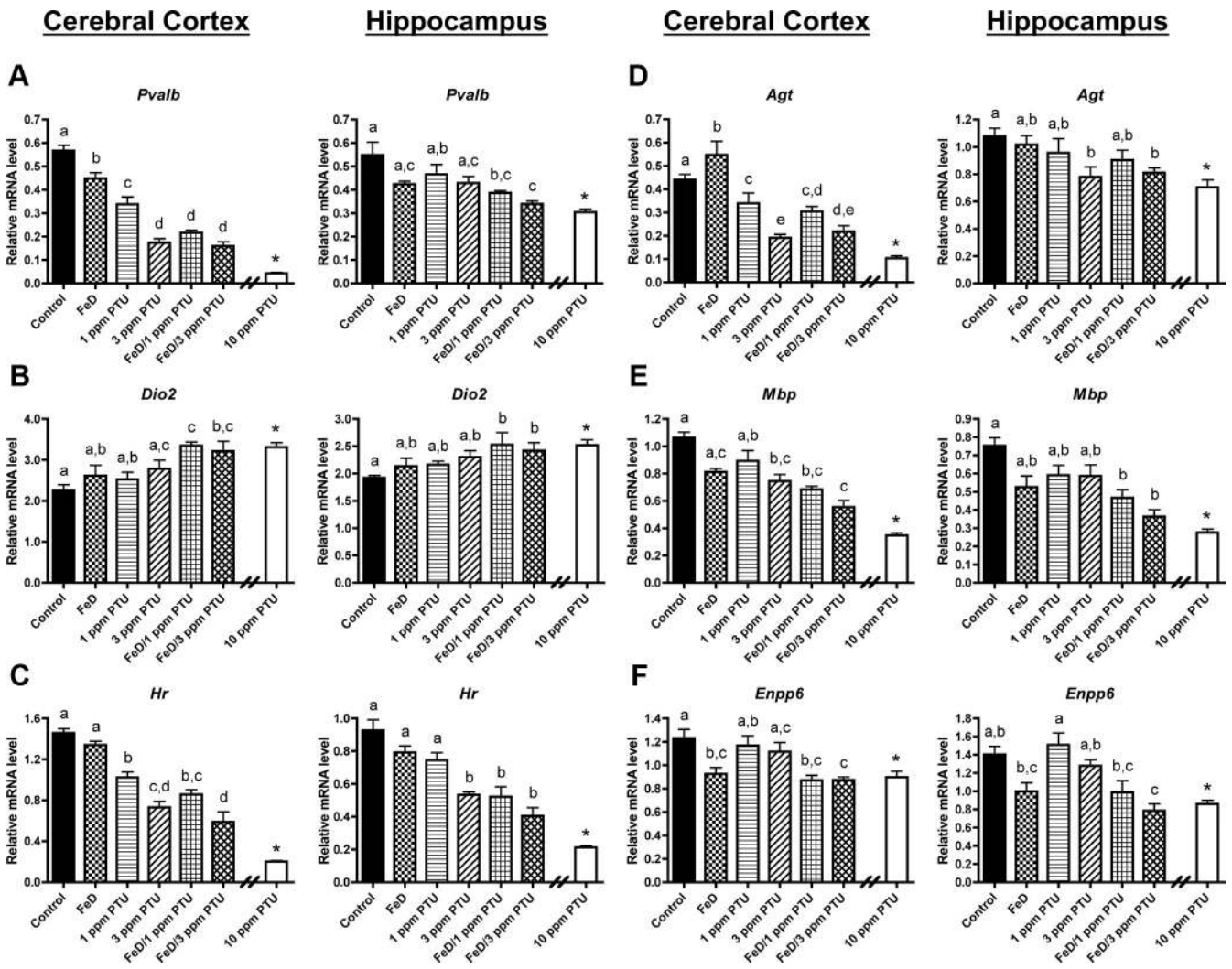

Figure 3. Fetal and neonatal Fe deficiency exacerbates the effect of 1 or 3 ppm PTU treatment on hippocampus and cerebral cortex TH-responsive gene expression. Hippocampi or cerebral cortices were harvested from 1 or 2 P15 male pups per litter (n = 11–12), total RNA was extracted, and cDNA was synthesized. qPCR was performed for several TH-responsive genes. PVALB, *Pvalb* (A), type 2 deiodinase, *Dio2* (B), hairless, *Hr* (C), myelin basic protein, *Mbp* (D), and ectonucleotide pyrophosphatase/phosphodiesterase 6, *Enpp6* (E). Relative mRNA levels are calculated relative to an internal control P12 whole-brain cDNA sample. Data are presented as the mean ± SEM. Groups not sharing a common letter superscript are significantly different by nested one-way ANOVA and Tukey's multiple comparison test ($P < .05$). The 10 ppm PTU group served as a positive hypothyroid control. Asterisks indicate statistical difference between 10 ppm PTU and control.

controls (Figure 3C). Combined FeD/1 ppm PTU again had a more severe effect than independent treatments, significantly lowering hippocampal *Hr* mRNA levels by 44%. Hippocampal *Hr* mRNA levels were also significantly reduced in P15 3 ppm PTU and FeD/3 ppm PTU pups. In the cerebral cortex, Fe deficiency did not alter *Hr* mRNA levels, compared with controls. *Hr* mRNA levels were 31%, 50%, 41%, and 60% lower in the 1 ppm PTU, 3 ppm PTU, FeD/1 ppm PTU, and FeD/3 ppm PTU P15 cerebral cortex, respectively. Thus, the FeD/PTU combined effect was present but attenuated for *Hr* mRNA levels in the P15 cerebral cortex.

Fe deficiency increased *Agt* mRNA levels in the P15 cerebral cortex, compared with controls (Figure 3D). *Agt* cerebral cortical mRNA levels were reduced in 1 ppm PTU, 3 ppm PTU, FeD/1 ppm PTU, and FeD/3 ppm PTU pups. Hippocampal *Agt* mRNA levels were less TH-responsive, significantly altered in only the 3 ppm PTU and FeD/3 ppm PTU pups (28% and 25% lower, respectively, compared with controls) (Figure 3D). No significant differences in *Agt* mRNA levels were observed between the 1 ppm PTU or combined FeD/1 ppm PTU groups in either the cortex or hippocampus.

FeD or 1 ppm PTU treatment alone did not significantly alter *Mbp* mRNA levels in the cerebral cortex or hippocampus (Figure 3E). Fe deficiency independently lowered *Enpp6* mRNA levels by 26% and 31% in the P15 cerebral cortex and hippocampus (Figure 3F). Three parts per million PTU treatment lowered cerebral cortical *Mbp* mRNA levels by 30% but did not alter hippocampal *Mbp*

mRNA levels or *Enpp6* mRNA levels in either brain region. Although combining Fe deficiency with 1 or 3 ppm PTU appeared to have a significant additive effect for *Mbp* in cerebral cortex and hippocampus, there was no combinatorial effect for *Enpp6*. Altogether, the mRNA data demonstrate that concurrent presence of 2 mild thyroidal perturbations leads to a more severe disruption of the *Pvalb*, *Dio2*, *Mbp*, and *Hr* genes in the developing brain.

One possibility is that combining Fe deficiency with 1 or 3 ppm PTU lowers brain Fe status, leading to the more severe effects on mRNA levels observed in these combined treatment groups. We assessed mRNA levels for *TfR1*, an Fe-responsive gene that codes for the transferrin receptor protein involved in cellular Fe uptake (9, 27), in both the cerebral cortex and hippocampus to address this issue. The 1, 3, and 10 ppm PTU treatments did not alter *TfR1* mRNA levels in the P15 cerebral cortex or hippocampus (Figure 4). *TfR1* mRNA levels were significantly elevated in the FeD, FeD/1 ppm PTU, and FeD/3 ppm PTU P15 cerebral cortex compared with controls (Figure 4A). Cerebral cortex *TfR1* mRNA levels were significantly higher in the FeD group compared with both FeD/PTU groups. Fe deficiency, FeD/1 ppm PTU, and FeD/3 ppm PTU also increased P15 hippocampal *TfR1* mRNA levels (Figure 4B). The ratio of hippocampal to cerebral cortical *TfR1* mRNA levels indicates a similar degree of Fe deficiency in these brain regions for the FeD groups (Figure 4C). These data demonstrate that, if anything, the FeD/PTU brains were slightly less FeD than FeD brains. Thus, brain Fe status is unlikely to contribute to the more severe effect of combined FeD/PTU on brain mRNA levels.

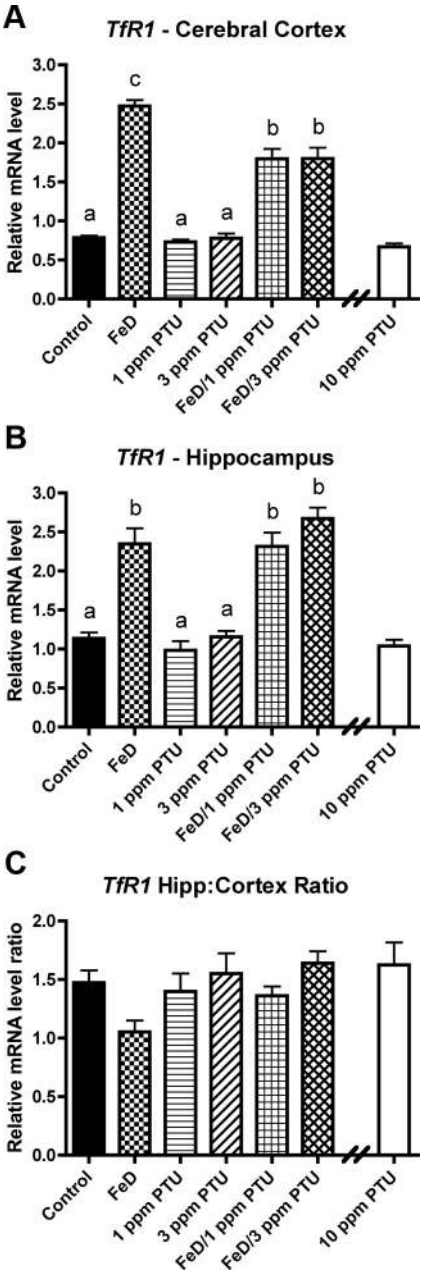

Figure 4. Fetal and neonatal Fe deficiency produces a similar degree of Fe deficiency in the neonatal hippocampus and cerebral cortex. Hippocampi or cerebral cortices were harvested from 1 or 2 P15 male pups per litter (n = 11–12), total RNA was extracted, and cDNA was synthesized. qPCR was performed for transferrin receptor 1, *TfR1*, in the hippocampus (A) and cerebral cortex (B). Relative mRNA levels are calculated relative to an internal control P12 whole-brain cDNA sample. The ratio of hippocampus *TfR1* relative mRNA level to cerebral cortex *TfR1* relative mRNA level was calculated to assess the relative degree of Fe deficiency in these brain regions (C). Data are presented as the mean ± SEM. Groups not sharing a common letter superscript are significantly different by nested one-way ANOVA and Tukey's multiple comparison test ($P < .05$). The 10 ppm PTU group served as a positive hypothyroid control. Asterisks indicate statistical difference between 10 ppm PTU and control.

Effect of Fe deficiency and PTU treatment on hippocampal and cerebral cortical PVALB protein expression

Pvalb mRNA expression has been established as one of the most sensitive biomarkers of mildly impaired brain thyroidal status (9, 10). Whether mild thyroidal disruptions, alone or in combination, also impact brain PVALB protein levels had not been assessed. One part per million PTU treatment lowered P16 cerebral cortical PVALB protein levels by 32% compared with controls (Figure 5A). When combined, Fe deficiency and 1 ppm PTU had an additive effect, lowering P16 cerebral cortical PVALB protein levels by 52%. PVALB protein levels were 76% and 79% lower in the 3 ppm PTU and FeD/3 ppm PTU cerebral cortex, respectively. In the P16 hippocampus, Fe deficiency, 1 ppm PTU, 3 ppm PTU, and FeD/1 ppm PTU did not significantly alter PVALB protein levels (Figure 5B). However, Fe deficiency combined with 3 ppm PTU produced a significant effect on hippocampal PVALB, lowering protein levels by 42%. The 10 ppm PTU treatment significantly lowered PVALB protein levels in both the cerebral cortex and hippocampus.

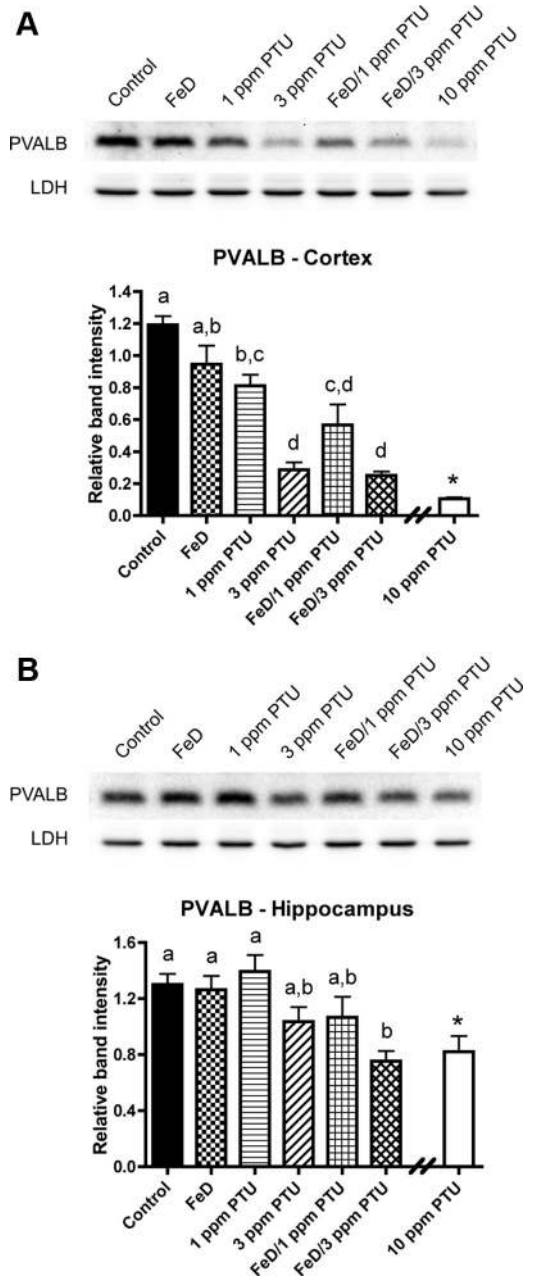

Figure 5. Fetal and neonatal Fe deficiency exacerbates the effect of 1 or 3 ppm PTU treatment on hippocampus and cerebral cortex PVALB protein levels. Western blottings for PVALB and lactate dehydrogenase (LDH) were performed on P16 hippocampus (A) and cerebral cortex (B) homogenates from 1 P16 male pup per litter (n = 5–6). For each brain region, a representative immunoblot is shown. Graphs depict quantification of PVALB band intensity from 6 different blots (n = 5–6 per treatment group). Densitometry data are presented as the mean ± SEM. Groups not sharing a common letter superscript are significantly different by one-way ANOVA and Tukey's multiple comparison test ($P < .05$). The 10 ppm PTU group served as a positive hypothyroid control. Asterisks indicate statistical difference between 10 ppm PTU and control.

Discussion

In this study, concurrent fetal/neonatal Fe deficiency and 1 ppm PTU treatment lowered circulating TT_4 and brain T_3 concentrations in neonatal rats to a greater extent than either FeD or 1 ppm PTU alone, indicating a compounding deleterious effect on neonatal thyroidal status. TH predominately acts at the level of regulating gene transcription and thus, we predicted that concurrent FeD/PTU treatment would also have a more severe effect on TH-responsive gene expression. Indeed, Fe deficiency also exacerbated the effect of PTU on TH-responsive mRNA (*Pvalb*, *Dio2*, *Hr*, and *Mbp*) and protein (*Pvalb*) levels in the neonatal cerebral cortex and hippocampus. Interestingly, Fe deficiency and 1 ppm PTU alone did not significantly reduce cerebral cortical and hippocampal T_3 concentrations, cerebral cortical *Dio2* mRNA levels, and hippocampal *Pvalb*, *Dio2*, and *Hr* mRNA levels. This finding suggests that defining nutritional micronutrient requirements and acceptable environmental exposure limits must take into consideration the potential for combinatorial effects on the thyroid, especially during development. This is especially important in light of recent studies demonstrating that maternal hypothyroxinemia, low normal free T_4 concentrations with normal TSH concentrations, is associated with impaired offspring neurobehavioral development (4, 6, 7, 29).

Two recent studies identified *Pvalb* expression as an excellent biomarker of brain sensitivity to mild thyroidal disruption, because fetal/neonatal 1 ppm PTU treatment significantly reduces *Pvalb* mRNA levels in the P10 and P14 cerebral cortex (9, 10). Here, we demonstrate that concurrent Fe deficiency and 1 ppm PTU treatment lowers *Pvalb* mRNA levels in both the hippocampus and cerebral cortex to a greater extent than either individual treatment, indicating that the combined FeD/PTU effect on PVALB expression may functionally impact the developing brain.

In moderate to severe TH deficiencies, hippocampal PVALB protein expression and the number of PVALB-expressing γ-aminobutyric acid (GABA)-ergic interneurons in the developing hippocampus and cerebral cortex are reduced (30). Another nutritional model of Fe deficiency anemia demonstrates reductions in P15, P30, and P65 PVALB mRNA and P30 and P65 PVALB protein in the hippocampus without altering the number of PVALB-positive interneurons (31). Hippocampal Cornu Ammonis subregion 1 (CA1)-specific Fe deficiency, without anemia, reduces the number of PVALB-positive cells in the CA1 hippocampal region (32). The effects of TH and Fe deficiencies on PVALB are permanent, persisting in adult offspring despite repletion (30, 31). PVALB is predominately expressed in a subset of GABA-ergic inhibitory interneurons and plays an important role in synaptic calcium signaling, synaptic plasticity, excitatory/inhibitory balance, seizure susceptibility, and memory formation in the developing rodent brain (33–36). Both Fe and TH de-

ficiencies alter measures of short- and long-term synaptic plasticity, including paired-pulse facilitation and long-term potentiation, in CA1 of the developing hippocampus, and these impairments persist into adulthood (37–39). Thus, reduced *Pvalb* expression may explain some of the synaptic plasticity impairments observed in Fe and TH deficiencies (30, 31). The more severe effect of FeD/PTU on *Pvalb*/PVALB expression suggests exacerbation of synaptic function deficits observed in Fe and TH deficiencies alone. However, differences in study design and analysis parameters of hippocampal electrophysiology experiments (37–39) preclude comparison of the specific effects of Fe and TH deficiencies on synaptic function and plasticity in this study.

The appearance of PVALB-expressing GABA-ergic interneurons also marks the beginning of a critical period of experience-dependent neuronal plasticity (40). Thus, reduced *Pvalb*/PVALB expression in Fe- and TH-deficient neonatal brains may indicate a poorly defined or delayed critical period window. Critical period alterations may be damaging in the Fe- or TH-deficient brain or provide an opportunity to restore normal Fe and TH levels before closure of the window. Thus, clearly defining the critical period window is important for both deficiencies.

Increased *Dio2* expression is a typical response of the brain to TH insufficiency (41), indicating an attempt to maintain brain T_3 levels by increasing conversion of T_4 to T_3. Similarly, lower *Hr* expression suggests an attempt to maintain TH-responsive gene expression levels in the absence of T_3, as the hairless protein is a corepressor for TH-responsive genes (42). Therefore, the *Dio2* and *Hr* mRNA results provide further evidence that the FeD/1 ppm PTU brain is sensing a more severe TH insufficiency than either FeD or 1 ppm PTU alone.

Both FeD and PTU treatment clearly impair the neonatal cortical and hippocampal mRNA expression of the myelin-associated genes, *Mbp* and *Enpp6* (Figure 3, E and F). Despite producing a thyroidal effect similar to 1 ppm PTU, Fe deficiency lowers cortical and hippocampal *Enpp6* mRNA levels to a greater extent. These data suggest that the aberrant myelination associated with Fe deficiency may be partially or fully driven through a mechanism independent of altered thyroidal status, such as impaired function of Fe-dependent enzymes involved in fatty acid and cholesterol metabolism or altered energy status of oligodendrocytes (43).

In addition to the maladaptive effects of TH insufficiency associated with Fe deficiency, altered TH economy may also provide an adaptive response to Fe deficiency. Both TH and Fe play important stimulatory roles in carbohydrate, fat, and protein metabolic pathways and in mitochondrial ATP production (44, 45). Therefore, in terms of both cellular and whole-body energy economy, it may actually be beneficial and adaptive to lower TH levels when cellular energy production is already reduced due to Fe deficiency. It will be important to determine both the positive and negative contributions of TH insufficiency during developmental Fe deficiency.

The lack of stimulatory effect of fetal/neonatal Fe deficiency on neonatal serum TSH concentrations was previously described and possible explanations discussed in detail (9). Fetal/neonatal FeD/1 ppm PTU and FeD/3 ppm PTU treatments produced a dose-dependent increase in serum TSH (Figure 1C). These data demonstrate that the hypothalamic and pituitary arms of the hypothalamic-pituitary-thyroid axis are functional in the neonatal FeD rat. Whether fetal/neonatal Fe deficiency attenuates TRH and/or TSH response to reduced serum TH concentrations is unknown.

Consistent with our previous study (9), the combined effect of FeD and 1 ppm PTU treatment on brain TH-responsive gene expression was both gene- and region specific. The effect of FeD/1 ppm PTU, compared with FeD or 1 ppm PTU alone, on *Pvalb* mRNA expression was much more pronounced in the P15 cerebral cortex than the hippocampus. This was also true for PVALB protein levels. In general, the cerebral cortex at this age appears to be more sensitive than the hippocampus to mild TH insufficiencies, because both *Pvalb* and *Hr* cerebral cortical mRNA levels were significantly reduced in 1 ppm PTU pups despite a nonsignificant reduction in cerebral cortical T_3 levels. In contrast to *Pvalb*, however, the impact of combined FeD/1 ppm PTU on *Hr* expression was more pronounced in the hippocampus compared with the cerebral cortex. This appears to be due to the more severe effect of 1 ppm PTU alone in the cerebral cortex compared with the hippocampus.

The findings of this study may have serious implications for human health, because there are numerous environmental chemicals, dietary goitrogens, and micronutrient deficiencies that impair thyroid function. Individually, these thyroidal disruptors may exert a mild thyroidal perturbation and in turn have subtle effect on TH-dependent brain development. However, our data suggest that in combination, mild thyroidal disruptors can have an additive deleterious effect on thyroidal status and TH-dependent brain development.

Iodine deficiency often coexists with other potential thyroid disrupting micronutrient deficiencies, such as Fe, selenium, vitamin A, and zinc deficiencies in developing countries, due to diets lacking nutrient diversity, disease, and other factors (22, 46). The Micronutrient Initiative estimates that approximately 13%–27% of preschool children (~100 million children) are experiencing concur-

rent Fe, iodine, and/or vitamin A deficiencies (47). A high prevalence of multiple concurrent micronutrient deficiencies has also been reported in pregnant women and infants (48–50). In addition to thyroid disrupting micronutrient deficiencies, chronic consumption of staple foods containing dietary goitrogens is common in developing countries and can contribute to impaired thyroid function and micronutrient status (51, 52). Thus, it is important to consider multiple interacting insults to the thyroid axis as source of developmental cognitive impairments in these populations.

In the United States, approximately 18% of pregnant women have insufficient Fe levels (53), and 57% of pregnant women have insufficient iodine levels (54). In addition, potential thyroid disrupting environmental chemicals are detectable in American pregnant women (55). Therefore, even in developed countries, there is significant risk of multiple concurrent mild thyroidal perturbations during pregnancy, which may lead to reduced cognitive potential of offspring. Our results highlight the importance of eliminating all possible insults to the thyroid axis, especially in pregnant women and children and in populations where multiple thyroidal disruptors are present.

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