Fetal and Neonatal Iron Deficiency Reduces Thyroid Hormone-Responsive Gene mRNA Levels in the Neonatal Rat Hippocampus and Cerebral Cortex

Thomas W. Bastian, Jeremy A. Anderson, Stephanie J. Fretham, Joseph R. Prohaska, Michael K. Georgieff, and Grant W. Anderson

Department of Pharmacy Practice and Pharmaceutical Sciences (T.W.B., J.A.A., G.W.A.), University of Minnesota Duluth, College of Pharmacy, Duluth, Minnesota 55812; Department of Pediatrics and Center for Neurobehavioral Development (S.J.F., M.K.G.), University of Minnesota Medical School, Minneapolis, Minnesota 55455; and Department of Biomedical Sciences (J.R.P.), University of Minnesota Medical School Duluth, Duluth, Minnesota, 55812

Copper (Cu), iron (Fe), and thyroid hormone (TH) deficiencies produce similar defects in late brain development including hypomyelination of axons and impaired synapse formation and function, suggesting that these micronutrient deficiencies share a common mechanism contributing to these derangements. We previously demonstrated that fetal/neonatal Cu and Fe deficiencies lower circulating TH concentrations in neonatal rats. Fe deficiency also reduces whole-brain T_3 content, suggesting impaired TH action in the developing Fe-deficient brain. We hypothesized that fetal/neonatal Cu and Fe deficiencies will produce mild or moderate TH deficiencies and will impair TH-responsive gene expression in the neonatal cerebral cortex and hippocampus. To test this hypothesis, we rendered pregnant Sprague Dawley rats Cu-, Fe-, or TH-deficient from early gestation through postnatal d 10 (P10). Mild and moderate TH deficiencies were induced by 1 and 3 ppm propylthiouracil treatment, respectively. Cu deficiency did not significantly alter serum or tissue TH concentrations or TH-responsive brain mRNA expression. Fe deficiency significantly lowered P10 serum total T_3 (45%), serum total T_4 (52%), whole brain T_3 (14%), and hippocampal T_3 (18%) concentrations, producing a mild TH deficiency similar to 1 ppm propylthiouracil treatment. Fe deficiency lowered *Pvalb*, *Enpp6*, and *Mbp* mRNA levels in the P10 hippocampus. Fe deficiency also altered *Hairless*, *Dbm*, and *Dio2* mRNA levels in the P10 cerebral cortex. These results suggest that some of the brain defects associated with Fe deficiency may be mediated through altered thyroidal status and the concomitant alterations in TH-responsive gene transcription. **(*Endocrinology* 153: 5668–5680, 2012)**

Globally, billions of people suffer from insufficient dietary intake of micronutrients such as zinc, vitamin A, selenium, copper (Cu), iron (Fe), and iodine (1). Micronutrient deficiencies are most prevalent in developing countries and often coexist due to consumption of diets lacking nutrient diversity (1). Given their profound impact on child development and prosperity, it is critical to understand how micronutrients interact during fetal and infant neurodevelopment.

Interactions between Fe and iodine/thyroid hormones (THs) have serious implications for human health. Approximately 1.9 billion people have insufficient iodine intake and 1.6 billion people suffer from anemia or Fe deficiency (2, 3). Data from Zimmermann *et al.* (4–6) indicate that 20–44% of West and North African children suffer from both deficiencies. Treatment of Fe deficiency in these children improves the efficacy of iodine supplementation, reducing thyroid volume and goiter prevalence (4, 6) and increasing serum total T_4 concentrations, indicating an Fe-specific effect on thyroidal status (6). Additional cross-sectional studies demonstrate correlations between Fe and thyroidal parameters (reviewed in Ref. 7).

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Abbreviations: Bdnf, Brain-derived neurotrophic factor; CuD, copper-deficient; E, gestational day; Enpp6, ectonucleotide pyrophosphatase/phosphodiesterase 6; FeD, iron-deficient; Mbp, myelin basic protein; MDC, minimum detectable concentration; P, postnatal day; PTU, 6-propyl-2-thiouracil; qPCR, quantitative real-time PCR; TH, thyroid hormone; TSH, thyroid stimulating hormone; TT4, total T_4; TT3, total T_3.

Interestingly, a recent study in pregnant Swiss women shows that low maternal body Fe stores are correlated with increased serum thyroid stimulating hormone (TSH) and reduced total T_4 (TT4) concentrations, suggesting Fe deficiency may compound the deleterious effects of developmental iodine deficiency (8).

Studies in FeD rodents have provided additional data to support the hypothesis that Fe is important for TH synthesis and/or metabolism. Fe deficiency in adolescent or adult rats decreases circulating TT4 and total T_3 concentrations (9–12), and free T_4 and T_3 concentrations (11), plasma and pituitary TSH concentrations (9, 10), hepatic T_4 deiodination (10, 11), and thyroid peroxidase activity (12). Cu deficiency lowers plasma Fe levels, circulating TT4 and total T_3 (TT3) concentrations, and peripheral conversion of T_4 to T_3 (13, 14).

Cu, Fe, and iodine/TH deficiencies have their most profound effects on early life brain development and ultimately on adult intellectual outcome (15, 16). Interestingly, Cu, Fe, and iodine/TH deficiencies result in similar defects in rodent hippocampal and cerebral cortical development including aberrant myelination, blunted neuronal maturation, impaired synapse formation and function, altered neurotransmission, and changes in tissue energy metabolism (15–17). The hippocampal and cerebral cortical developmental defects associated with these deficiencies persist into adulthood and result in permanent behavioral abnormalities including impaired learning, memory, and sensorimotor function (15–20).

We recently demonstrated that fetal/neonatal Cu and Fe deficiencies decrease neonatal serum TT4 and TT3 concentrations (18, 21). Neonatal FeD rats also have reduced whole-brain T_3 content with concomitant alterations in whole brain mRNA expression for some TH-responsive genes (21). However, the expression of several additional Fe- or TH-responsive genes was not significantly altered in Fe- or TH-deficient whole brains. A potential explanation for this observation is that, when analyzing whole brains, Fe- or TH-dependent changes in mRNA expression in discrete brain regions are diluted by unaffected brain regions. Therefore, we hypothesized that Cu and Fe deficiencies will impair TH-responsive gene expression in the neonatal cerebral cortex and/or hippocampus, two brain regions that are sensitive to Cu, Fe, and TH insufficiencies during development. In addition, we hypothesized that Cu and Fe deficiencies will disrupt the thyroid axis to a similar extent as a mild or moderate TH deficiency. In this study we show that fetal/neonatal Fe deficiency decreases circulating and brain TH concentrations, producing a mild TH-deficient state in neonatal rats. We also demonstrate that fetal/neonatal Fe deficiency impairs hippocampal or cerebral cortical mRNA expression for several TH-responsive genes.

Materials and Methods

Animals and diets

Sperm-positive Sprague Dawley female rats were purchased from Charles River Laboratories (Wilmington, MA). At gestational day (E) 2, 25 sperm-positive rats were randomly assigned to one of five groups: control, copper-deficient (CuD), iron-deficient (FeD), 1 ppm (1 mg/liter) 6-propyl-2-thiouracil (PTU; Sigma-Aldrich; St. Louis, MO) treatment, and 3 ppm (3 mg/liter) PTU treatment (n = 5 dams per group). The PTU groups are denoted as 1 ppm PTU and 3 ppm PTU. Beginning at E2, CuD and FeD dams were fed a semipurified diet (Harlan Laboratories, Madison, WI) deficient in Cu or Fe, respectively (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The Fe content of the FeD diet was increased compared with our previous study (21) to produce a less severe Fe deficiency. Control and PTU dams were fed a Cu- and Fe-adequate diet (Supplemental Table 1). Dams in the control, CuD, and FeD groups drank deionized water. Beginning at E6, 1 and 3 ppm PTU dams were offered deionized 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 P10, male pups were killed to evaluate Cu and Fe biomarkers, serum and brain TH concentrations, and brain mRNA expression. Dams were killed at P11 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-h light, 12-h 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 P10, one or two male pups per litter were killed (seven to 10 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. A 5-μl blood sample was removed to determine hemoglobin concentrations. A blood sample was also drawn from the trunk into heparanized microhematocrit tubes. A portion of liver was removed, rinsed with deionized water, weighed, and processed for metal analyses. Brains were removed and bisected at the midline. Five half-brains for metal analyses were rinsed with deionized water and weighed. Seven to 10 half-brains for TH analysis were weighed and flash frozen in liquid nitrogen. A second cohort of one or two P10 male pups per litter was killed (eight to10 total per group). Serum was collected as described above. Hippocampi and cerebral cortices were dissected, removed, and placed immediately into an RNAlater RNA stabilization reagent (QIAGEN, Valencia, CA). Finally, a third cohort of two P10 male pups per litter (10 total per group) was killed and intracardially perfused with PBS to remove blood from the brains. Hippocampi and cerebral cortices were dissected, removed, and flash frozen in liquid nitrogen for tissue TH measurement.

Metal and biochemical analyses

Cu, Fe, Zn, hemoglobin, and ceruloplasmin measurements were performed as described (21). Microhematocrit tubes were

TABLE 1. Cu, Fe, and TH status of rat dams

Characteristic	Control	CuD	FeD	1 ppm PTU	3 ppm PTU
Body weight (g)	342 ± 5.4[a]	329 ± 9.5[a]	337 ± 13.4[a]	325 ± 7.2[a]	341 ± 7.9[a]
Hemoglobin (g/liter)	172 ± 1.5[a]	158 ± 4.3[a]	136 ± 5.8[b]	167 ± 4.5[a]	161 ± 0.9[a]
Ceruloplasmin (U/liter)	203 ± 18.3[a]	0.54 ± 0.14[b]	190 ± 11.3[a]	208 ± 27.0[a]	168 ± 27.1[a]
Serum Fe (μg/ml)	5.37 ± 0.47[a]	2.68 ± 0.66[b]	0.97 ± 0.20[c]	4.81 ± 0.09[a]	5.02 ± 0.39[a]
Serum TT4 (ng/ml)	37.0 ± 2.37[a,b]	43.3 ± 3.05[a]	38.5 ± 5.74[a,b]	27.6 ± 3.62[b]	8.51 ± 1.40[c]
Serum TT3 (ng/dl)	37.3 ± 2.76[a]	41.2 ± 3.01[a]	40.7 ± 4.33[a]	40.2 ± 5.19[a]	36.5 ± 5.83[a]

Data are presented as the mean ± SEM (n = 5). Within a specific row, groups not sharing a *common superscript* are significantly different by one-way ANOVA and Tukey's or Scheffé's multiple comparison test ($P < 0.05$).

centrifuged and hematocrit was measured using a standard microcapillary reader.

Serum and brain hormone measurements

Serum TT4 and TT3 concentrations were measured using RIA kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA) modified for rodent use as described (21). The minimum detectable concentration (MDC) for T_4 (1.7 ng/ml) and T_3 (7.6 ng/dl) RIAs was calculated statistically as 3 SDs above the zero calibrator (n = 10). Samples with T_4 or T_3 concentrations below the MDC were set to the MDC for statistical purposes. The serum TSH concentrations were measured using a rodent TSH ELISA kit (Endocrine Technologies, Newark, CA). The serum was diluted 1:2 in the provided sample diluent for the TSH measurements.

THs were extracted from half brains, hippocampi, and cerebral cortices as described (21). For hippocampi, the following modifications were used due to the small size of this brain region. Hippocampi were homogenized for 30 sec at approximately 20,000 rpm with a PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA). The volume of ^{125}I-T4 tracer was reduced to 50 μl. All steps of hippocampal TH extractions were carried out in 1.5-ml polypropylene screw-cap tubes. Each lyophilized hippocampal sample was resuspended in 250 μl hormone-stripped rat serum.

Brain mRNA analysis

Total RNA was extracted from brain subregions using QIAGEN RNeasy midi (hippocampi) or maxi (cerebral cortices) kits. The optional on-column deoxyribonuclease digestion was performed to remove genomic DNA. RNA integrity and purity was established spectrophotometrically and by RNA gels. cDNA was synthesized from 2 μg (hippocampi) or 3 μg (cerebral cortices) total RNA using SuperScript III first-strand synthesis supermix and random hexamers (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qPCR) was performed using Rotor-Gene SYBR Green PCR kits (QIAGEN) and a Corbett RotorGene RG-3000 (QIAGEN). Primer pairs for the assayed genes are outlined in Supplemental Table 2. PCR reactions were performed on cDNA equivalent to 80 ng total RNA according to the manufacturer's protocol except that a final volume of 12.5 μl was used. Quantification cycle values were determined in the log-linear amplification phase. Relative mRNA levels were calculated relative to internal hippocampal or cerebral cortical cDNA samples from a P10 control rat pup.

Statistical analysis

One-way ANOVA was used for making statistical comparisons between treatment groups. Bartlett's test was used to assess homogeneity of variances. When variances were equal across groups, a Tukey's *post hoc* test was used. When variances were unequal, data were ln transformed and a Tukey's test was used. When transformation did not normalize the variances, Scheffé's *post hoc* test was used on the untransformed data. Statistical outliers were determined by Grubbs' test (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). All data are presented as mean ± SEM. Linear regressions were performed to compare serum TT4 concentrations with hippocampal or cerebral cortical mRNA levels for individual pups. Statistical analyses and data graphing were carried out using Prism (GraphPad Software, La Jolla, CA) or Kaleidagraph (Synergy Software, Reading, PA) software. An $\alpha = 0.05$ was chosen to define significant differences. Cu deficiency did not impact serum or brain TH concentrations or TH-responsive gene expression and thus was excluded from statistical analyses. The 3-ppm PTU group served as a positive hypothyroid control and was excluded from ANOVA analyses but was included in linear regressions and figures for comparison.

Results

Evaluating dam and pup Cu and Fe status

Cu deficiency was confirmed in CuD dams as measured by a 99% reduction in serum ceruloplasmin activity, compared with controls (Table 1). Fe deficiency anemia was confirmed in FeD dams as measured by significant reductions in serum Fe (98% lower than controls) and hemoglobin (21% lower than controls) levels (Table 1). TH deficiency was confirmed in 3-ppm PTU dams as determined by a 77% reduction in serum TT4 (Table 1). Serum TT3 concentrations were not altered in dams of any treatment (Table 1). Litter sizes from each treatment group were similar (data not shown).

In P10 pups (Table 2), blood hemoglobin, hematocrit, serum Fe concentrations, and serum ceruloplasmin activity were lower in both CuD and FeD pups compared with controls. Cu deficiency lowered liver and brain Cu levels and Fe deficiency lowered liver and brain Fe levels. In keeping with our previous studies using this CuD diet,

TABLE 2. Characteristics of P10 rat pups after fetal and neonatal Cu deficiency, Fe deficiency, or PTU treatment

Characteristic	Control	CuD	FeD	1 ppm PTU	3 ppm PTU
Body weight (g)	26.5 ± 0.88[a]	25.0 ± 0.81[a]	19.8 ± 1.06[b]	24.3 ± 1.15[a]	25.7 ± 0.74[a]
Hemoglobin (g/liter)	118 ± 2.16[a]	96.4 ± 2.16[b]	58.2 ± 4.02[c]	116 ± 2.99[a]	123 ± 4.88[a]
Hematocrit (%)	40.4 ± 1.01[a]	30.7 ± 1.94[b]	17.8 ± 1.02[c]	37.9 ± 1.19[a]	40.0 ± 1.62[a]
Ceruloplasmin (U/liter)	46.2 ± 3.01[a]	4.91 ± 0.55[b]	27.5 ± 3.55[c]	36.0 ± 1.87[a,c]	51.7 ± 10.5[a]
Serum Fe (μg/ml)	1.52 ± 0.15[a]	0.82 ± 0.08[b]	0.31 ± 0.04[b]	1.90 ± 0.20[a]	1.65 ± 0.08[a]
Liver Cu (μg/g)	49.9 ± 2.02[a]	1.93 ± 0.10[b]	103 ± 9.68[c]	46.4 ± 5.67[a]	42.6 ± 2.82[a]
Liver Fe (μg/g)	45.0 ± 6.40[a]	45.0 ± 8.35[a]	21.7 ± 2.85[a]	48.8 ± 8.16[a]	35.4 ± 2.43[a]
Liver Zn (μg/g)	76.8 ± 4.06[a]	65.5 ± 5.75[a]	54.7 ± 4.63[b]	72.5 ± 3.42[a]	72.2 ± 5.73[a]
Brain Cu (μg/g)	0.93 ± 0.02[a]	0.51 ± 0.02[b]	1.18 ± 0.03[c]	0.96 ± 0.02[a]	0.86 ± 0.02[a]
Brain Fe (μg/g)	7.65 ± 1.63[a]	5.40 ± 0.39[a]	3.37 ± 0.08[b]	6.02 ± 0.41[a]	5.85 ± 0.16[a]
Brain Zn (μg/g)	9.02 ± 0.27[a]	8.86 ± 0.33[a]	8.88 ± 0.08[a]	8.57 ± 0.28[a]	8.45 ± 0.27[a]

Data are presented as the mean ± SEM (n = 4–10). Within a specific row, groups not sharing a *common superscript* are significantly different by one-way ANOVA and Tukey's or Scheffé's multiple comparison test ($P < 0.05$).

liver and brain Fe levels were not significantly altered compared with controls (18, 21). Brain Cu content was higher in FeD pups, as reported previously (21). The 1- and 3-ppm PTU treatment did not significantly alter Fe or Cu status. Together these data demonstrate that Fe and Cu deficiencies were generated in P10 pups nursing on dams fed nutritionally restricted diets.

Effects of Cu and Fe deficiency on thyroidal status

We previously reported that Cu and Fe deficiencies during fetal/neonatal development reduce neonatal circulating TH concentrations (21). In this study, Cu deficiency did not significantly alter P10 circulating hormone concentrations (data not shown). In keeping with our hypothesis, Fe deficiency reduced P10 serum TT4 by 52% and serum TT3 by 45% (Fig. 1, A and B). Fe deficiency did not alter serum TSH concentrations (Fig. 1C). Serum TT4 was 66% lower and serum TT3 was 34% higher in 1-ppm PTU P10 pups (Fig. 1, A and B). Serum TSH concentrations were 2.3 times higher in 1-ppm PTU pups, compared with controls (Fig. 1C). Both serum TT4 (93%) and TT3 (57%) were lower in 3-ppm PTU pups, compared with controls (Fig. 1, A and B). Serum TSH concentrations were 4.1 times higher in 3-ppm PTU pups (Fig. 1C), compared with controls, indicating these pups are TH-deficient and are an appropriate hypothyroid control group. Serum TT4 con-

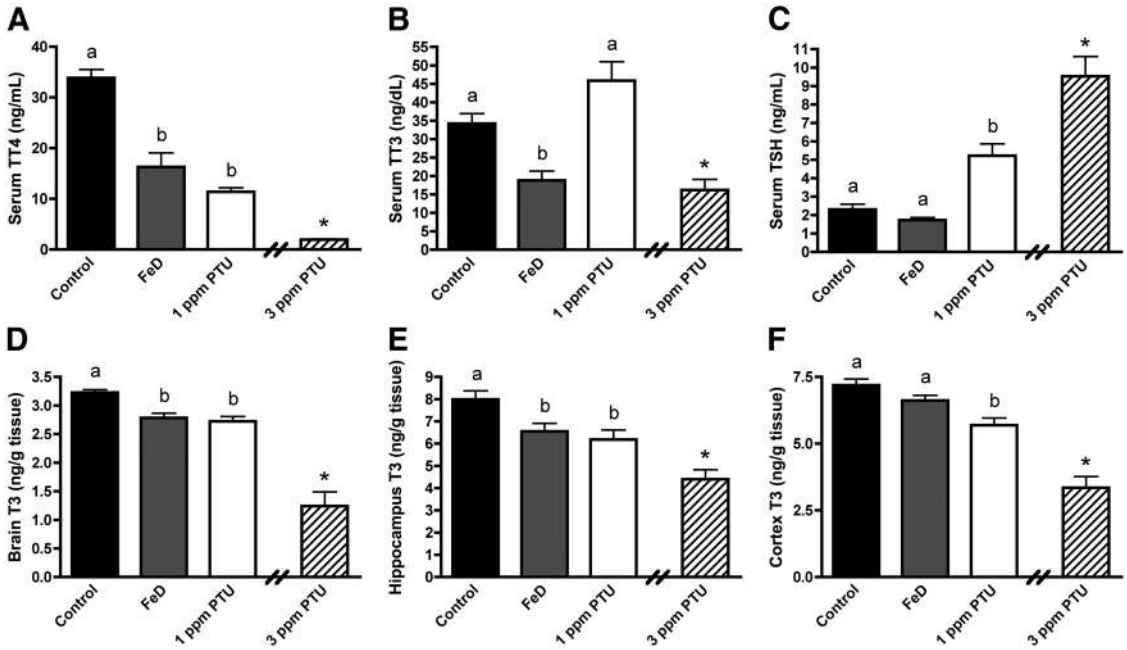

FIG. 1. Fetal and neonatal Fe deficiency reduces serum and brain TH levels. A, Serum TT4. B, Serum TT3. C, Serum TSH. Serum was harvested from P10 male pups (n = 8–10). D, Whole-brain T_3. E, Hippocampal T_3. F, Cerebral cortical T_3. Half brains (n = 6–9), hippocampi (n = 9–10), and cerebral cortices (n = 9) were harvested from P10 male pups. Data are presented as the mean ± SEM. Groups not sharing a *common superscript* are significantly different by one-way ANOVA and Tukey's or Scheffé's multiple comparison test ($P < 0.05$). The 3-ppm PTU group served as a positive hypothyroid control. *Asterisks* indicate a statistical difference between 3 ppm PTU and control.

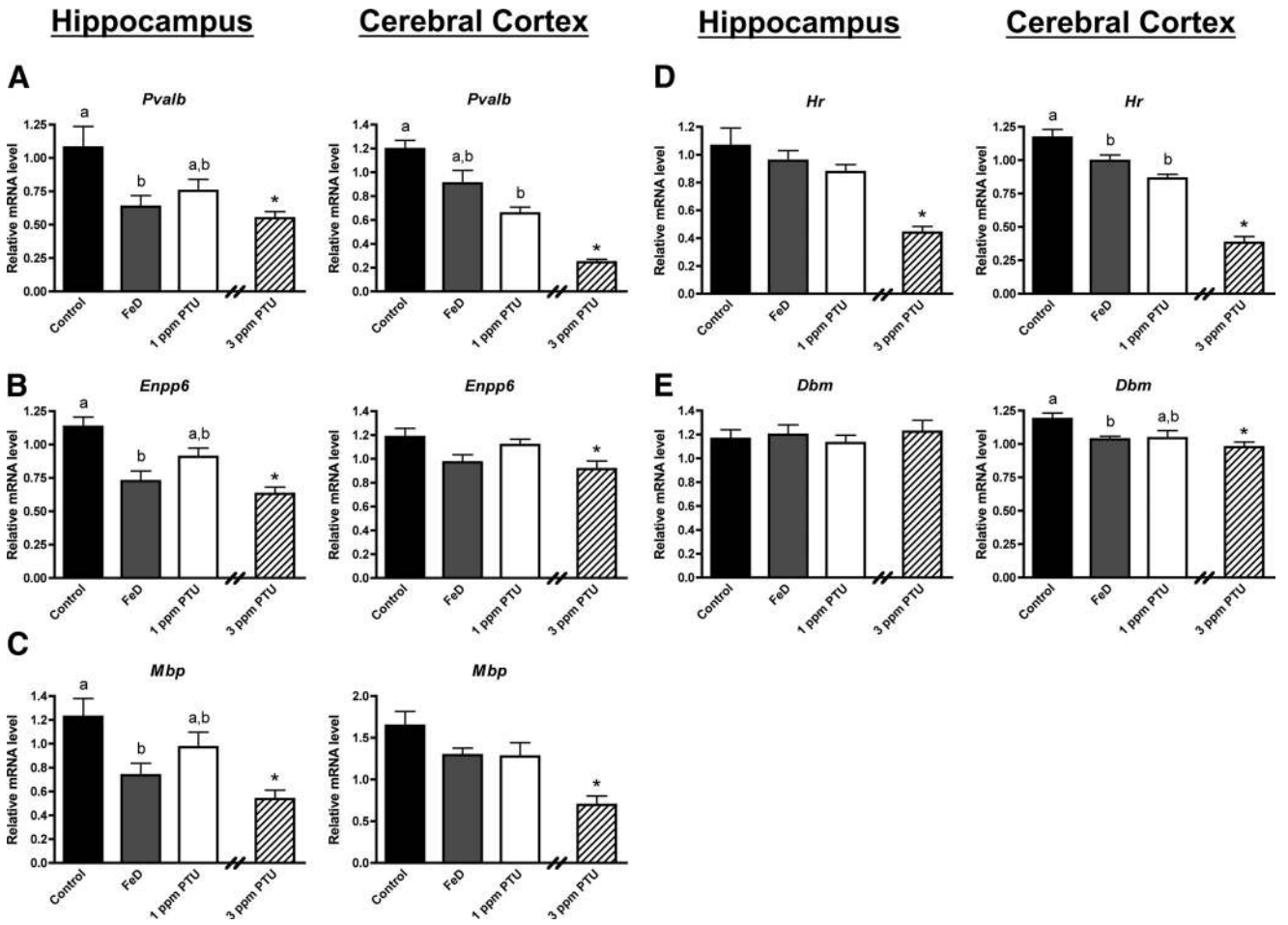

FIG. 2. Fe deficiency impairs TH-dependent gene expression for some genes in the neonatal hippocampus or cerebral cortex. Hippocampi or cerebral cortices were harvested from P10 male pups (n = 8–10), total RNA was extracted, and cDNA was synthesized. Quantitative real-time PCR was performed for several TH-responsive genes. A, Parvalbumin (*Pvalb*). B, Ectonucleotide pyrophosphatase/phosphodiesterase 6 (*Enpp6*). C, Myelin basic protein (*Mbp*). D, Hairless (*Hr*). E, Dopamine β-monooxygenase (*Dbm*). Relative mRNA levels are calculated relative to an internal control cDNA sample. Data are presented as mean ± SEM. Groups not sharing a *common superscript* are significantly different by one-way ANOVA and Tukey's or Scheffé's multiple comparison test ($P < 0.05$). The 3-ppm PTU group served as a positive hypothyroid control. *Asterisks* indicate statistical difference between 3 ppm PTU and control.

centrations for six of nine 3-ppm PTU samples fell below the MDC. Serum TT3 concentrations for three of nine 3-ppm PTU samples and one out of 12 FeD samples fell below the MDC.

Given the reduction in circulating TH concentrations in FeD pups, brain subregions that are dependent on TH for development and function may be susceptible to altered tissue TH concentrations. T_3 content was measured in P10 half-brains, hippocampi, and cerebral cortices. Brain T_3 content was not significantly lower in CuD pups (data not shown). P10 whole-brain T_3 content was 14% lower in FeD pups, 16% lower in 1-ppm PTU pups, and 62% lower in 3-ppm PTU pups (Fig. 1D). P10 hippocampus T_3 content was 18% lower in FeD pups, 23% lower in 1-ppm PTU pups, and 45% lower in 3-ppm PTU pups (Fig. 1E). P10 cerebral cortex T_3 content was not statistically lower in FeD pups but was 21% lower in 1-ppm PTU pups, and 53% lower in 3-ppm PTU pups (Fig. 1F).

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

In our previous study, several known TH-responsive genes were not altered in Fe- or TH-deficient whole brains (21). This led us to hypothesize that the effect of Fe deficiency on TH-responsive gene expression will be more pronounced in brain subregions such as the cerebral cortex or hippocampus. To test this hypothesis, qPCR was performed for several genes, including some that are known Fe- or TH-responsive genes in the developing brain.

Figure 2 shows qPCR data for genes with mRNA expression that was altered by both FeD and 1 or 3 ppm PTU in the P10 hippocampus or cerebral cortex. *Pvalb*, *Enpp6*, and *Mbp* mRNA expression levels were significantly lower in the P10 FeD hippocampus compared with controls (Fig. 2, A–C). In contrast the mRNA expression of

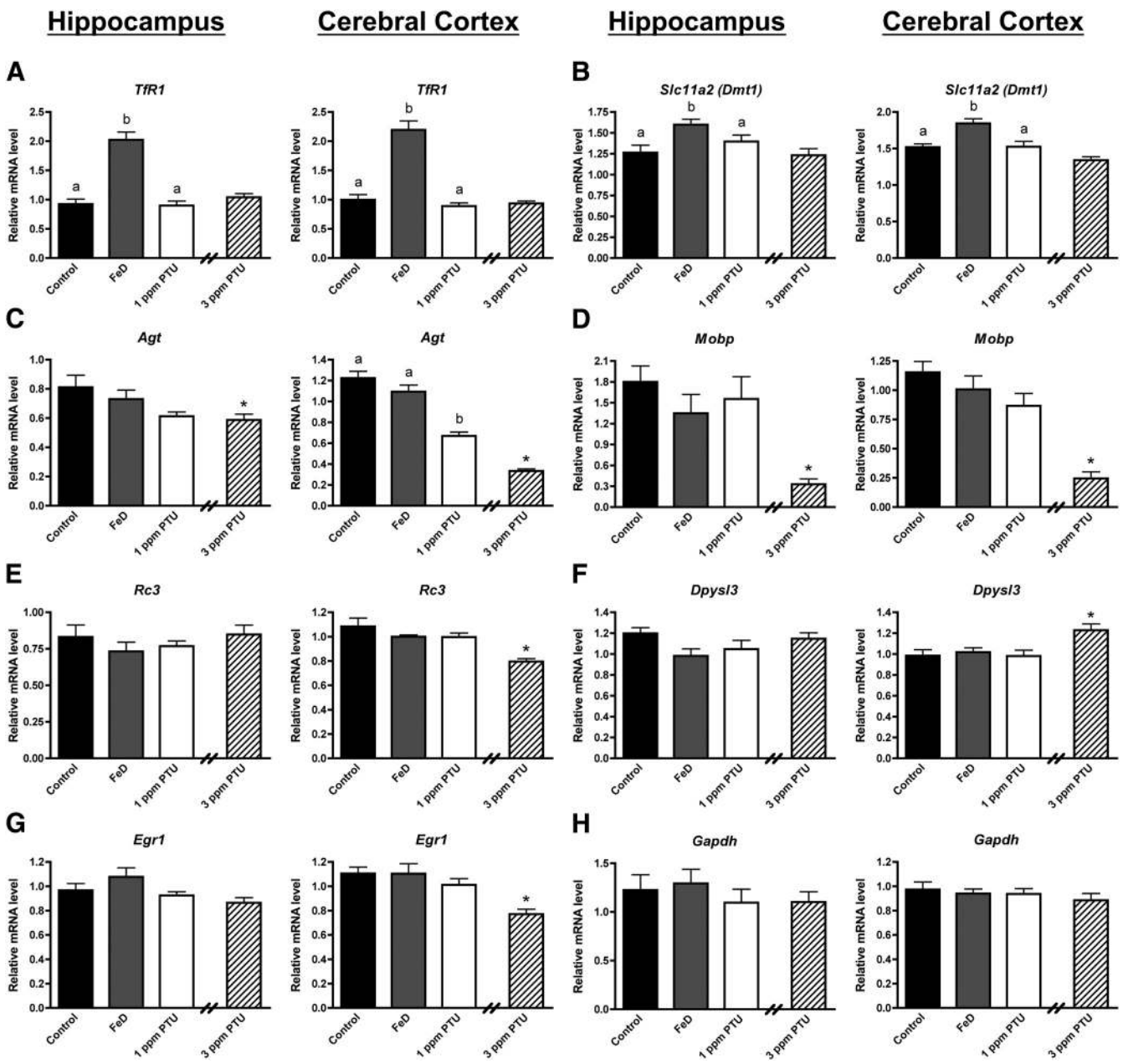

FIG. 3. The expression of some genes is Fe-responsive, TH-responsive, or neither in the neonatal hippocampus and cerebral cortex. Hippocampi or cerebral cortices were harvested from P10 male pups (n = 8–10), total RNA was extracted, and cDNA was synthesized. Quantitative real-time PCR was performed for several Fe-responsive, TH-responsive, or negative control genes. A, Transferrin receptor 1 (*TfR1*). B, Divalent metal transporter 1 (*Dmt1*). C, Angiotensinogen (*Agt*). D, Myelin-associated oligodendrocyte basic protein (*Mobp*). E, Neurogranin (*Rc3*). F, Dihydropyrimidinase-like 3 (*Dpysl3*). G, Early growth response factor 1 (*Egr1*). H, Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Relative mRNA levels are calculated relative to an internal control cDNA sample. Groups not sharing a *common superscript* are significantly different by one-way ANOVA and Tukey's or Scheffé's multiple comparison test ($P < 0.05$). The 3-ppm PTU group served as a positive hypothyroid control. *Asterisks* indicate statistical difference between 3 ppm PTU and control.

Pvalb, *Enpp6*, and *Mbp* was not significantly altered in the FeD cerebral cortex. These data are similar to changes in the T_3 contents observed in the hippocampus and cerebral cortex (Fig. 1). Interestingly, *Hr* and *Dbm* mRNA expression levels were significantly reduced in the P10 FeD cerebral cortex but not the hippocampus (Fig. 2, D and E). The hippocampal and cerebral cortical mRNA levels of *Pvalb*, *Enpp6*, *Mbp*, *Hr*, and *Dbm* were reduced to a similar extent in the FeD and 1-ppm PTU pups. One part per million of PTU significantly lowered cerebral cortical *Pvalb* and *Hr* mRNA levels compared with controls. The mRNA expression of *Pvalb*, *Enpp6*, *Mbp*, and *Hr* demonstrated a dose response to 1 and 3 ppm PTU treatment in the hippocampus and cerebral cortex, indicating TH-dependent regulation. Together these data suggest that altered thyroidal status may con-

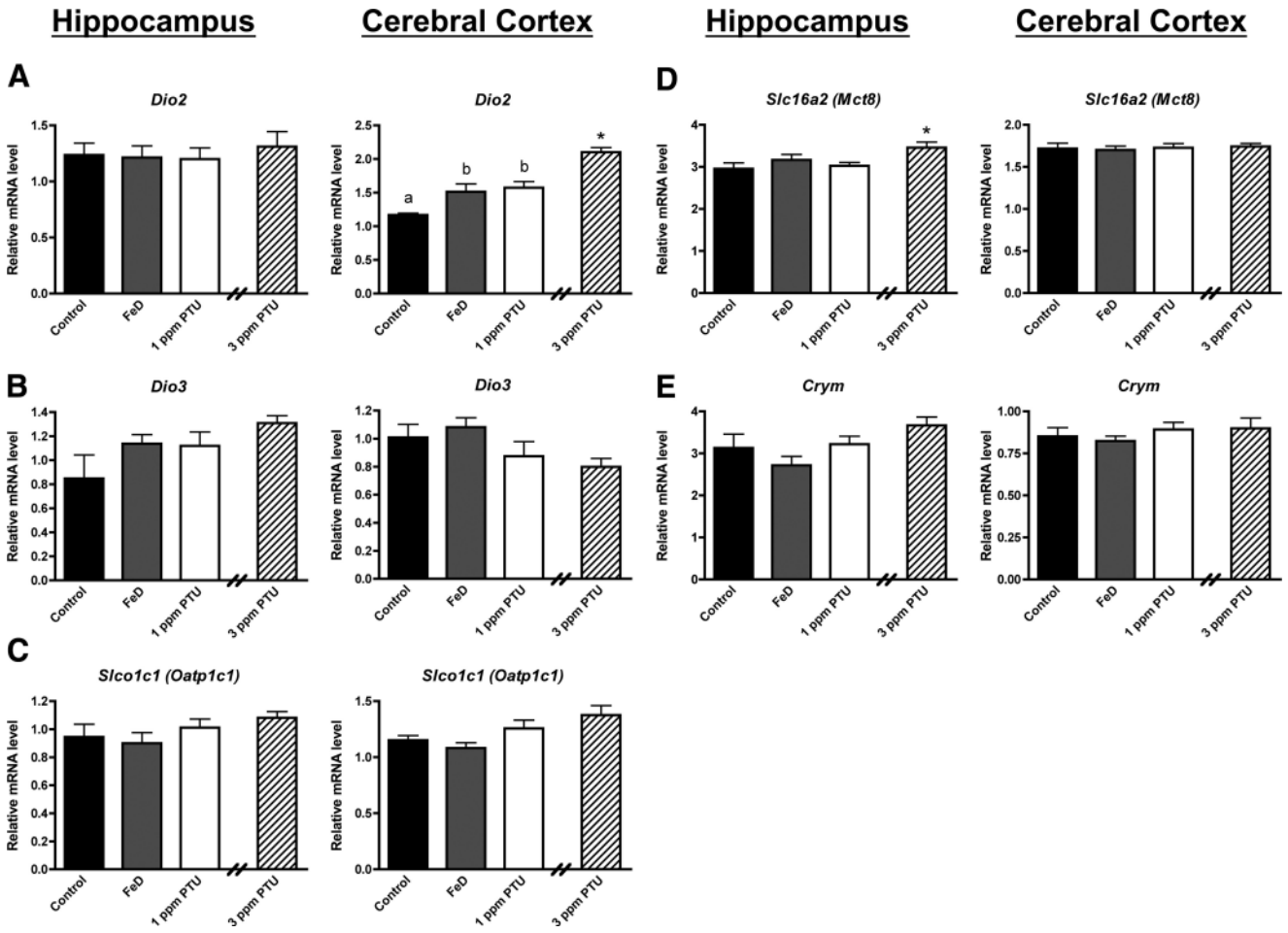

FIG. 4. The neonatal cerebral cortex, but not hippocampus senses mild TH deficiencies and up-regulates compensatory mechanisms. Hippocampi or cerebral cortices were harvested from P10 male pups (n = 8–10), total RNA was extracted, and cDNA was synthesized. Quantitative real-time PCR was performed for several genes involved in maintaining tissue TH homeostasis. A, Type 2 deiodinase (*Dio2*). B, Type 3 deiodinase (*Dio3*). C, Organic anion transporting polypeptide 1c1 (*Oatp1c1*). D, Monocarboxylate transporter 8 (*Mct8*). E, μ-Crystallin (*Crym*). Relative mRNA levels are calculated relative to an internal control cDNA sample. Data are presented as the mean ± SEM. Groups not sharing a *common superscript* are significantly different by one-way ANOVA and Tukey's or Scheffé's multiple comparison test ($P < 0.05$). The 3-ppm PTU group served as a positive hypothyroid control. *Asterisks* indicate statistical difference between 3 ppm PTU and control.

tribute to aberrant gene expression in the neonatal FeD brain.

This study also revealed several genes that are selectively responsive to Fe or TH. The *TfR1* and *Dmt1* genes code for proteins involved in tissue Fe uptake (16). Fe response element regulated *TfR1* and *Dmt1* expression is increased in neonatal FeD brains (22, 23) and therefore served as Fe-responsive controls. Fe deficiency, but not 1 or 3 ppm PTU treatment, increased *TfR1* and *Dmt1* mRNA levels in the P10 hippocampus and cerebral cortex (Fig. 3, A and B). *Agt* and *Mobp* mRNA levels were significantly reduced in the 1- and/or 3-ppm PTU hippocampus and cerebral cortex but not in the FeD hippocampus and cerebral cortex (Fig. 3, C and D). Interestingly, the TH-responsive genes *Rc3*, *Dpysl3*, and *Egr1* were significantly altered only in the 3-ppm PTU cerebral cortex (Fig. 3, E–G), suggesting differential responsivity of these genes to altered T_3 levels in the cerebral cortex compared with the hippocampus. Fe and TH deficiencies did not significantly alter *Bdnf IV*, *Bdnf VI*, and total *Bdnf* mRNA levels in the P10 hippocampus or cerebral cortex (Supplemental Fig. 1). *Gapdh* mRNA expression was measured as a negative control and was not altered in FeD or PTU brain regions (Fig. 3H).

Finally, we examined mRNA levels for several genes involved in maintaining brain TH homeostasis (Fig. 4). Interestingly, *Dio2* mRNA expression was significantly increased in P10 FeD, 1-ppm PTU, and 3-ppm PTU cerebral cortex but not the hippocampus (Fig. 4A). These data may explain the larger reduction in hippocampal T_3 compared with cerebral cortical T_3 for FeD pups (Fig. 1, D and E). *Mct8* mRNA expression was significantly increased only in the 3-ppm PTU hippocampus (Fig. 4D). We did not observe significant changes in *Dio3*, *Oatp1c1*, or *Crym* mRNA expression in either brain region for any treatment groups (Fig. 4, B, C, and E).

To further assess brain region differences in gene TH responsivity, we performed linear regression between se-

rum TT4 and cerebral cortex or hippocampus mRNA levels (Fig. 5 and Supplemental Fig. 2). The relationships between *Pvalb*, *Enpp6*, *Hr*, *Mbp*, *Mobp*, and *Agt* mRNA levels and serum TT4 concentrations had slopes that were significantly different from zero in both the cerebral cortex and the hippocampus. Correlation coefficients (R^2 values) demonstrate regional differences in relationship strengths, with *Pvalb*, *Mobp*, and *Agt* having stronger relationships

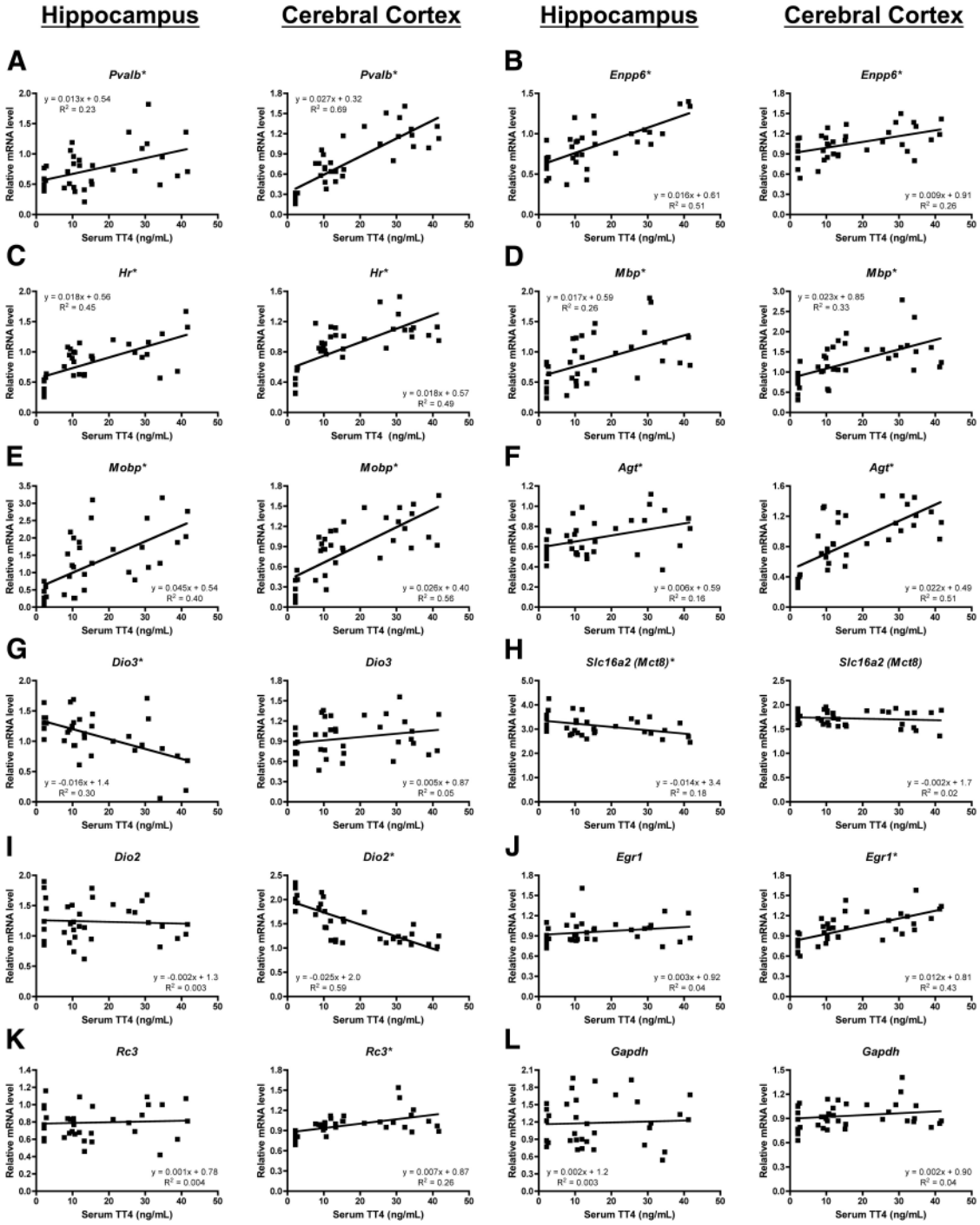

FIG. 5. Serum TT4 and brain mRNA correlations demonstrate regional differences in TH responsivity. Linear regression scatter plots demonstrating the relationship between serum total T_4 concentrations and either hippocampus or cerebral cortex mRNA levels in individual rat pups are shown for several genes. A, Parvalbumin (*Pvalb*). B, Ectonucleotide pyrophosphatase/phosphodiesterase 6 (*Enpp6*). C, Hairless (*Hr*). D, Myelin basic protein (*Mbp*). E, Myelin-associated oligodendrocyte basic protein (*Mobp*). F, Angiotensinogen (*Agt*). G, Type 3 deiodinase (*Dio3*). H, Monocarboxylate transporter 8 (*Mct8*). I, Type 2 deiodinase (*Dio2*). J, Early growth response factor 1 (*Egr1*). K, Neurogranin (*Rc3*). L, Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). The slope, y-intercept, and Pearson correlation R^2 values are shown for each linear regression analysis. *Asterisks* indicate relationships with slopes that significantly deviate from zero ($P < 0.05$).

in the cerebral cortex than the hippocampus and *Enpp6* having a stronger relationship in the hippocampus than the cerebral cortex. *Hr* and *Mbp* had similar R^2 values in the cerebral cortex and hippocampus. Only two genes, *Dio3* and *Mct8*, had correlations with slopes that significantly deviated from zero in the hippocampus but not the cerebral cortex. In contrast, *Dio2*, *Egr1*, *Rc3*, *Oatp1c1*, *Dpysl3*, and *Dbm* had relationships with slopes that were significantly different from zero in the cerebral cortex but not the hippocampus. Together these data reveal gene- and brain region-specific TH sensitivities in the developing rat brain.

Discussion

Fe deficiency affects greater than 1 billion people worldwide, having its most profound impact on fetal/infant neurodevelopment and the long-term cognitive and behavioral outcome of affected children (3, 24). In rodents, fetal/neonatal Fe and TH deficiencies lead to similar brain developmental defects including aberrant myelination, blunted neuronal maturation and neurotransmission, and impaired synapse formation and function (16, 17). Brain developmental impairments associated with these deficiencies often persist into adulthood and result in permanent behavioral abnormalities including impaired learning and memory (16, 20). Whether impaired thyroid function contributes to the derangements in brain development associated with Fe deficiency is unknown.

We recently demonstrated that Fe deficiency reduces both circulating and whole-brain TH concentrations in the neonatal rodent (21). In the current study, we confirm these findings and also demonstrate that Fe deficiency lowers neonatal brain T_3 concentrations in a brain region-specific manner, more severely impacting the hippocampus compared with the cerebral cortex. We show that both Fe and mild or moderate TH deficiencies alter the mRNA expression of several TH-responsive genes, including *Pvalb*, *Enpp6*, *Mbp*, *Hr*, *Dbm*, and *Dio2* in the neonatal cerebral cortex or hippocampus. These data suggest that some aspects of mammalian brain development may be partially impaired by reduced TH content in the FeD brain.

Fe and TH deficiencies were previously shown to reduce *Pvalb* mRNA expression in the neonatal whole brain (21, 23) or cerebral cortex (25). Here we also show that moderate TH insufficiency impairs *Pvalb* mRNA expression in the neonatal hippocampus. Fe deficiency significantly reduced *Pvalb* mRNA expression in the P10 hippocampus but not the cerebral cortex, correlating with the observed brain region T_3 contents. These data suggest that *Pvalb* mRNA expression may be altered in the FeD neonatal hippocampus due to altered tissue T_3 content. Parvalbumin is expressed in inhibitory GABAergic (γ-aminobutyric acid) interneurons and has been implicated in synaptic calcium signaling, synaptic plasticity, and memory formation in the developing rodent brain (26–28). Therefore, TH deficiency may contribute to some of the defects in hippocampal neuronal signaling and the associated deficits in learning and memory observed in FeD rats.

Mild and moderate TH deficiency was previously shown to reduce *Enpp6* mRNA expression in the neonatal hippocampus (25). Our data confirm these findings in the hippocampus and also demonstrate that *Enpp6* is TH-responsive in the neonatal cerebral cortex. In accordance with our tissue T_3 data, Fe deficiency significantly reduced *Enpp6* mRNA levels in the hippocampus but not the cerebral cortex. *Mbp* mRNA expression is down-regulated throughout the brain including the cerebral cortex and hippocampus of developing hypothyroid rats (25, 29) and in P21 FeD rat whole brains (23). Fe deficiency reduced *Mbp* mRNA expression in the hippocampus and cerebral cortex. The magnitude of this reduction was similar to the 1-ppm PTU group and was statistically significant only in the hippocampus. Myelin basic protein (Mbp) is one of the most highly enriched proteins in the myelin sheath surrounding central nervous system neurons, and thus, Mbp expression is a good indicator of myelination status (30). Ectonucleotide pyrophosphatase/phosphodiesterase 6 (Enpp6) may also be important for myelination of neuronal axons because it is involved in choline metabolism and is highly expressed in differentiating rat oligodendrocytes and the myelin fraction of mouse brain homogenates (30–32). Our *Mbp* and *Enpp6* expression data suggest that secondary TH deficiency may contribute to the well-established hypomyelination in developing FeD brains.

Fe deficiency altered the mRNA expression of three genes, *Hr*, *Dbm*, and *Dio2*, in the cerebral cortex but not the hippocampus despite a nonsignificant decrease in cerebral cortex T_3 content in FeD pups. Interestingly, the 1-ppm PTU treatment also altered the expression of these three genes in the cerebral cortex but not the hippocampus, suggesting that TH sensitivity and responsiveness of *Hr*, *Dbm*, and *Dio2* mRNA expression is brain region specific in the P10 rat brain. Noradrenergic synaptic efficacy (33) and ligand binding to the norepinephrine transporter (34) are impaired in the FeD hippocampus and locus ceruleus, respectively. Our data suggest that conversion of dopamine to norepinephrine may be impaired in the FeD cerebral cortex due to reduced *Dbm* mRNA expression and that reduced brain T_3 levels may contribute to this phenomenon.

Hairless is a well-characterized TH-responsive gene in the developing rodent brain, coding for a corepressor involved in transcriptional repression of TH-responsive genes (35). Type II deiodinase catalyzes the conversion of T_4 to T_3 and thus has an important role in maintaining brain TH homeostasis. The elevated *Dio2* mRNA expression in the FeD cerebral cortex suggests that this brain region is sensing the altered thyroidal status and is attempting to maintain adequate T_3 concentrations. This result may explain the differential effect of Fe deficiency on hippocampal and cerebral cortical T_3 concentrations. Together the altered cerebral cortical *Hr* and *Dio2* mRNA expression provides support that TH action is impaired in the developing FeD rodent brain.

Bdnf expression initiates from one of several different promoters resulting in 11 different gene products (36). In accordance with previous work in our laboratory with a different model of nutritional Fe deficiency (37), *Bdnf IV* and *Bdnf VI* [formerly *Bdnf III* and *IV*, respectively (36)] expression was reduced in the FeD hippocampus. However, in this study, the reduction in *Bdnf* expression was not statistically significant. In severe models of TH deficiency, *Bdnf* TH responsivity is regulated in a promoter-, age-, and brain region-specific fashion (reviewed in Ref. 17). In agreement with our mRNA data, Lasley *et al.* (38) recently demonstrated that fetal/neonatal 1, 2, and 3 ppm PTU treatment does not alter brain-derived neurotrophic factor (Bdnf) protein expression in the neonatal hippocampus, cerebral cortex, or cerebellum.

Cu deficiency lowered P12 and P24 serum TH concentrations in our previous studies (18, 21) but did not significantly alter P10 serum TH concentrations in this study. Although the same CuD diet was used, differences in the severity of Cu deficiency, anemia, and secondary Fe deficiency may explain the lack of significant impact on serum TH concentrations. All P10 CuD pups in this study, compared with no P12 CuD pups in the previous study (21), had measurable levels of ceruloplasmin activity, suggesting a less severe Cu deficiency. Cu deficiency lowered P10 hemoglobin and serum Fe levels by 18 and 46% (Table 2), compared with 23 and 56% in P12 pups from the previous study, suggesting less severe anemia and secondary Fe deficiency. Additionally, rat age (P10 *vs.* P12) may contribute to the severity of Cu deficiency, secondary Fe deficiency, and the accompanying effects on serum TH concentrations.

Unlike FeD neonatal pups, serum TT3 concentrations were not reduced in 1-ppm PTU pups. It is likely that 1-ppm PTU pups maintain normal serum TT3 concentrations through peripheral T_4-to-T_3 conversion, suggesting that peripheral T_4-to-T_3 conversion may be impaired in neonatal FeD rats. Supporting this hypothesis, hepatic T_4-to-T_3 conversion is impaired in adolescent or adult FeD rodents (10, 11). In addition, Fe deficiency may alter circulating TH concentrations through impaired thyroidal T_4 and T_3 synthesis as activity of thyroid peroxidase, an Fe-containing enzyme, is reduced in adolescent FeD rats (12).

Interestingly, Fe deficiency did not alter circulating TSH concentrations, despite decreased serum and brain TH concentrations. These data could indicate that fetal/neonatal Fe deficiency is associated with nonthyroidal illness, which is characterized by decreased circulating T_3 concentrations and normal or low circulating TSH concentrations. However, our TSH data could also indicate that Fe deficiency directly impairs TRH and/or TSH synthesis or secretion, leading to a secondary or tertiary hypothyroid state. Previous studies show that Fe deficiency in weanling rats reduces plasma TSH concentrations (9, 10) and pituitary TSH content (9). Exogenous TRH administration did not completely normalize plasma TSH concentrations in FeD rats but did induce an increase in plasma TSH concentrations similar to Fe-sufficient controls. These data suggest Fe deficiency decreases hypothalamic TRH production and/or release. Further research is warranted to determine the mechanism(s) by which Fe deficiency reduces circulating and tissue TH concentrations.

In our previous study, the effects of Fe and Cu deficiencies on neonatal thyroidal status were compared with severe TH deficiency (10 ppm PTU), making it difficult to determine the degree of thyroidal impairment. We now have directly compared circulating and brain TH concentrations in FeD neonatal rats to mild (1 ppm PTU) and moderate (3 ppm PTU) thyroidal insults. Fetal/neonatal Fe deficiency reduced P10 serum TT4 concentrations and whole brain and hippocampal T_3 content to a similar extent as 1 ppm PTU. Interestingly, Fe deficiency did not significantly alter cerebral cortical T_3 content, suggesting a brain region-specific difference in TH metabolism. As discussed above, *Dio2* mRNA expression was significantly increased in the FeD cerebral cortex, which may explain the difference in T_3 content of these brain regions. Together the TH data indicate that fetal/neonatal Fe deficiency produces a mild TH deficiency in the neonatal circulation and brain.

Demonstrating that the developing FeD brain experiences a TH insufficiency comparable with 1 ppm PTU treatment provides a basis for developing hypotheses about the contribution of reduced TH levels to impairments in the developing FeD brain. The fetal/neonatal 1 ppm PTU treatment model is associated with reduced cerebral cortex and hippocampus Bdnf protein expression in adult offspring (38), aberrant neonatal cerebral cortex and hippocampus gene expression [(25, 39) and current study], reduced neonatal corpus callosum cellular density (40), and impaired long-term potentiation in the dentate gyrus of adult offspring (41). Fe deficiency is also

associated with altered neonatal brain gene expression [(21–23) and current study], reduced hippocampus BDNF mRNA and protein expression in adult offspring (42), and impaired hippocampal long-term potentiation in adult offspring (43). Thus, it will be important to directly evaluate the contribution of altered thyroidal status to the brain developmental defects associated with Fe deficiency through TH repletion studies.

Previous work demonstrated that TH regulates brain gene expression in a gene-, age-, and brain region-specific fashion (reviewed in Ref.17). Our data provide further support for a model of TH-regulated brain gene expression in which individual genes have unique, brain region-specific sensitivities to changes in TH levels. *Dbm*, *Rc3*, *Dpysl3*, *Egr1*, and *Dio2* mRNA expression was significantly altered in the cerebral cortex but not the hippocampus of 3-ppm PTU pups, indicating a brain region-specific TH-responsivity for these genes at this developmental stage. In addition, *Pvalb*, *Hr*, and *Agt* mRNA expression was significantly altered in both the 1- and 3-ppm PTU cerebral cortex but only the 3-ppm PTU hippocampus, indicating a brain region-specific difference in TH sensitivity for these TH-responsive genes. Our data correlating mRNA expression levels to serum TT4 concentrations also provide support for brain region-specific differences in TH responsivity. *Dio2*, *Oatp1c1*, *Rc3*, *Egr1*, *Dpysl3*, and *Dbm* mRNA levels were significantly correlated with serum TT4 concentrations in the cerebral cortex but not the hippocampus. Alternatively, the only genes with mRNA levels significantly correlated with serum TT4 concentrations in the hippocampus but not the cerebral cortex were *Dio3* and *Mct8*. These data indicate an increased TH responsivity in the P10 cerebral cortex compared with the hippocampus, in keeping with previous findings (25).

Brain T_3 can be acquired from two different sources: transport into the brain from the circulation or local conversion from T_4. Morte *et al.* (44) recently demonstrated that some TH-responsive genes are differentially dependent on the source of T_3, requiring locally generated T_3 for regulation in the developing mouse cerebral cortex. Interestingly, *Dio2* mRNA was increased in the FeD, 1-ppm PTU, and 3-ppm PTU cerebral cortex but not the hippocampus, and mRNA for the T_3 transporter, *Mct8*, was increased in the 3-ppm PTU hippocampus but not cerebral cortex. These data suggest that a difference in the source of available T_3 may explain the apparent increase in cerebral cortical TH responsivity compared with the hippocampus. Although the molecular mechanisms controlling the brain region-specific differences in TH responsivity are complex and not fully understood, one possible explanation is differential TH receptor and cofactor expression and/or recruitment to gene promoters. In addition, mRNA levels for the intracellular T_3 binding protein, Crym, are strikingly high in the hippocampus compared with the rest of the rodent brain (Supplemental Fig. 3). Differential cytosolic T_3 binding could contribute to differences in T_3 responsivity of brain subregions by regulating T_3 entry into the nucleus and/or altering T_3 interaction with TH receptors. In addition, brain region-specific differences in developmental timing including cellular differentiation and formation of cell-cell connections could also contribute to differences in TH responsivity.

A major implication of these data is that even mild perturbations to thyroidal status can aberrantly impact the developing mammalian brain. In humans, thyroidal perturbations could be due to naturally occurring goitrogens (*e.g.* thiocyanates, isoflavanoids), anthropogenic thyroid disrupting chemicals (*e.g.* bisphenol A, polychlorinated biphenyl, perfluorinated compounds), or micronutrient deficiencies (*e.g.* iodine, Fe, selenium, vitamin A, zinc). In developing countries millions of people are deficient in not one, but multiple micronutrients due to eating a diet lacking nutrient diversity (1). In addition, people living in developing countries are often exposed to thyroid disrupting chemicals, such as thiocyanate precursors in cassava, on a daily basis. Individually, dietary goitrogens, environmental chemicals, and micronutrient deficiencies may exert only minor effects on the thyroid axis. However, in combination, these effects may be exacerbated, resulting in 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.

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Address all correspondence and requests for reprints to: Grant W. Anderson, 232 Life Science, 1110 Kirby Drive, Duluth, Minnesota 55812. E-mail: ander163@umn.edu.

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