Gestational-neonatal iron deficiency suppresses and iron treatment reactivates IGF signaling in developing rat hippocampus

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Tran PV, Fretham SJ, Wobken J, Miller BS, Georgieff MK. Gestational-neonatal iron deficiency suppresses and iron treatment reactivates IGF signaling in developing rat hippocampus. Am J Physiol Endocrinol Metab 302: E316–E324, 2012. First published November 8, 2011; doi:10.1152/ajpendo.00369.2011.—Gestational-neonatal iron deficiency, a common micronutrient deficiency affecting the offspring of more than 30% of pregnancies worldwide, leads to long-term cognitive and behavioral abnormalities. Preclinical models of gestational-neonatal iron deficiency result in reduced energy metabolism and expression of genes critical for neuronal plasticity and cognitive function, which are associated with a smaller hippocampal volume and abnormal neuronal dendrite growth. Because insulin-like growth factor (IGF) modulates early postnatal cellular growth, differentiation, and survival, we used a dietary-induced rat model to assess the effects of gestational iron deficiency on activity of the IGF system. We hypothesized that gestational iron deficiency attenuates postnatal hippocampal IGF signaling and results in downstream effects that contribute to hippocampal anatomic and functional deficits. At postnatal day (P) 15 untreated gestational-neonatal iron deficiency markedly suppressed hippocampal IGF activation and protein kinase B (PKB) signaling, and reduced neurogenesis, while elevating extracellular signal-regulated kinase 1/2 (ERK1/2) expression and hypoxia-inducible factor-1α (HIF1α). Iron treatment beginning at P7 restored IGF signaling, increased neurogenesis, and normalized all parameters by the end of rapid hippocampal differentiation (P30). Expression of the neuron-specific synaptogenesis marker, disc-large homolog 4 (PSDH4), increased more rapidly than the glia-specific myelination marker, myelin basic protein, following iron treatment, suggesting a more robust response to iron therapy in IGF-I-dependent neurons than IGF-II-dependent glia. Collectively, our findings suggest that IGF dysfunction is in part responsible for hippocampal abnormalities in untreated iron deficiency. Early postnatal iron treatment of gestational iron deficiency reactivates the IGF system and promotes neurogenesis and differentiation in the hippocampus during a critical developmental period.

hippocampal development; micronutrient deficiency; insulin receptor; insulin-like growth factor I receptor; neurogenesis; extracellular signal-regulated kinase; protein kinase B

IRON DEFICIENCY IS THE MOST COMMON NUTRIENT DEFICIENCY, affecting approximately two billion people and 30–50% of pregnancies and their fetuses worldwide (49). Common clinical conditions during pregnancy such as severe maternal iron deficiency anemia, intrauterine insufficiency due to maternal hypertension (i.e., intrauterine growth restriction), and maternal diabetes mellitus as well as nonclinical conditions such as cigarette smoking result in fetal iron deficiency (15, 28, 46, 57). In humans, early life iron deficiency leads to long-term cognitive deficits and behavioral abnormalities (39), which have been phenotypically reproduced in preclinical rodent models (13, 23, 39). Moreover, iron-deficient (ID) rat pups have decreased energy metabolism, abnormal neuronal morphology and neurotransmission, and reduced expression of genes critical for neural plasticity in the hippocampus, particularly during the period of peak hippocampal dendritogenesis, synaptogenesis, and myelination (10, 16, 21, 34, 35, 48, 51, 60). These abnormalities in gene expression and neuronal morphology can persist beyond the period of early iron deficiency into adulthood (10, 61).

The insulin-like growth factors (IGFs) regulate cellular metabolism and growth via endocrine, autocrine, and paracrine mechanisms by activating multiple critical intracellular pathways during postnatal development. While circulating IGF-I is primarily synthesized in the liver and functions in an endocrine manner, local organ production (e.g., in brain) of IGF is important for its autocrine and paracrine activity. In postnatal hippocampus, local production of IGF facilitates cellular proliferation and differentiation (7, 18, 44). IGF-I and IGF-II are two closely related family members that have a critical role in neurodevelopment (18). Both IGF-I and -II bind IGFIIR and insulin receptor (IR), activating protein kinase B (Akt) and extracellular signal-regulated kinase (Erk) 1/2 pathways (36, 56). IGF-induced Akt signaling promotes cell survival in part by suppressing activity of forkhead transcription factors (FoxO1, FoxO4) through phosphorylation of FoxO1 at serine 319 (26, 30). This particular FoxO1 phosphorylation shuttles it from nucleus to cytoplasm and thereby represses FoxO1 transcriptional activity on its target genes, including the cell-death factor Bim (26, 41, 62). IGF-induced Akt also facilitates cellular growth by activating mammalian target of rapamycin (mTOR)/p70S6K signaling and proliferation by repressing activity of cell-cycle inhibitors p21 and p27 (41, 66). Likewise, IGF-induced Erk1/2 signaling mediates cell growth by regulating activity of transcription factors such as early growth response gene 1 and c-fos (56, 58).

IGF transport and activity are modulated by a family of IGF-binding proteins (e.g., IGFBP1–6), whose expression is regulated both temporally and locally (18). IGFBP3, a main carrier protein for circulating IGF-I, is highly expressed in the developing rat hippocampus and may colocalize with IGF-I (37, 47). Of relevance to iron biology, IGFBP3 has been shown to interact with transferrin, the major iron-binding protein, leading to suppression of in vitro IGFBP3-mediated cellular proliferation and apoptosis (65). While perinatal iron deficiency upregulates transferrin expression in the hippocampus...
dams were given ID diet similar to the ID group until P7, after which they were fed a purified IS diet (200 ppm Fe, TD 01583; Harlan Teklad). The timing of this iron treatment regimen was designed to model the provision of iron at the brain developmental equivalent of term birth in humans (2). In this model, iron treatment beginning at P7 reduces the severity of brain iron deficiency from a maximum of 40–45%, although the hippocampus remains ID (25% lower than control) at P30 (34). Complete iron repletion occurs in this model before P56 (10). Pups from dams given IS diet throughout the experiment served as IS controls. All litters were culled to eight pups (6 males, 2 females) per litter at P0.

Tissue dissection and collection. To assess IGF signaling at time points relevant to hippocampal development, samples were collected at P7, P15, and P30. These time points were chosen because P7 represents a stage of late proliferation/neurogenesis (5), P15 represents early differentiation with a marked increase in dendritogenesis, synaptogenesis, and myelination, and P30 represents late differentiation (48). Rats were killed by an intraperitoneal injection of pentobarbital (100 mg/kg). Brains were removed and bisected along the midline. Hippocampi were dissected, flash-frozen in liquid nitrogen, and stored at −80°C.

Quantitative RT-PCR. Assays were carried out as described previously (60) with modifications. Total RNA was isolated from dissected

Table 1. Identification of mRNA transcript assessed

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Bank Accession No.</th>
<th>ABI Assay ID</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>NM_178866</td>
<td>Re00710306_ml</td>
<td>Signaling molecule</td>
</tr>
<tr>
<td>IGF-II</td>
<td>NM_031511</td>
<td>Re01454518_ml</td>
<td>Signaling molecule</td>
</tr>
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<td>IGF1R</td>
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<td>Re00583837_ml</td>
<td>Membrane receptor</td>
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<td>Re00567070_ml</td>
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<tr>
<td>InsR/IR(total)</td>
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<td>Re01403522_ml</td>
<td>Membrane receptor</td>
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<tr>
<td>IGFBP3</td>
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<td>Re01401821_ml</td>
<td>Carrier protein</td>
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<tr>
<td>Hif1α</td>
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<td>c-fos</td>
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<td>Dlg4b</td>
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<td>Re00575179_ml</td>
<td>Synaptic density protein</td>
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<tr>
<td>Rps18</td>
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<td>Re01428915</td>
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IGF, insulin-like growth factor; IGF1R, insulin-like growth factor-I receptor; InsR/IR(FL), insulin receptor full-length variant; InsR/IR(total), total insulin receptor; IGFBP, insulin-like growth factor-binding protein; Hif1α, hypoxia-inducible factor 1α; Dlg4b, disc-large homolog 4; Mbp, myelin basic protein.

MATERIALS AND METHODS

Animals. The University of Minnesota Institutional Animal Care and Use Committee approved the experiments in this study. Timed-pregnant Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). Rats were kept in a 12:12-h light-dark cycle with ad libitum food and water. Pups were weaned at P21. Three groups were generated for the experiment: always iron deficient (ID), iron deficient treated with iron at P7 (IDT), and always iron sufficient control (IS).

Diet manipulations. Gestational-neonatal iron deficiency was induced by diet manipulation as previously described with the dietary modifications achieving a 40% loss of total brain iron at P10 (34, 52), a degree of brain iron deficiency equivalent to that seen in term newborn humans (46). For the ID group, pregnant dams were maintained on a purified ID diet (4 ppm Fe, TD 80396; Harlan Teklad, Madison, WI) from gestational day 2 to P15. The ID group was not maintained beyond P15 because mortality associated with untreated ID increases beyond that time point. For the IDT group, the nursing
hippocampus using the RNAqueous kit (Ambion, Austin, TX), and concentrations were determined by absorbance at 260 nm using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Total RNA (2 μg) was used to generate cDNA using the High-Capacity cDNA Kit (ABI, Foster City, CA). The resulting cDNA was diluted 10-fold to give a final volume of 200 μl. All quantitative PCR experiments were performed with the use of Taqman Gene Expression Assay probes (see Table 1) and one-half of the manufacturer’s recommended volume (ABI). Thermocycling was carried out according to the manufacturer’s protocol (ABI) using a MX3000P instrument (Stratagene, La Jolla, CA).

**Western blot analysis.** Analysis was carried out as described previously (12). In brief, flash-frozen hippocampal tissues were lysed by sonication. Protein lysate (30 μg) was separated using a 4–12% gradient SDS-PAGE (Invitrogen, Carlsbad, CA). Proteins were transferred onto nitrocellulose membranes (Pierce, Rockford, IL) using wet transfer (Invitrogen X-cell II Blot Module). Membranes were blocked in Blocking Buffer with 0.1% Tween 20 and 0.01% SDS at room temperature for 45 min. Following PBT washes (4X), blots were incubated in antibody diluted in Blocking Buffer overnight at 4°C with rocking. Membrane blots were rinsed in PBS with 0.1% Tween 20 (PBT, 4X) to remove unbound antibody and incubated in secondary antibody diluted in secondary antibody diluted in Blocking Buffer with 0.1% Tween 20 and 0.01% SDS at room temperature for 45 min. Following PBT washes (4X), blots were imaged with an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) and the integrated intensity of the protein of interest was standardized to total protein or β-actin. Specific primary antibodies including phosphorylated (P)-IGF1Rβ [binds both IGF1Rβ<sub>Y1135, 1136</sub> and IGF1Rβ<sub>Y1151, 1152</sub>; 1:1,000], IGF1Rβ (1:1,000), P-Akt<sub>S473</sub> (1:500), Akt (1:500), P-FoxO1<sub>S319</sub> (1:1,000), P-p70S6K<sub>Thr389</sub> (1:1,000), P-Erk1/2<sub>Thr202/Y204</sub> (1:1,000), Erk1/2 (1:1,000), P-FOXO1<sub>S319</sub> (1:1,000), and P-Erk1<sub>S383</sub> (1:1,000), were purchased from Cell Signaling (Danvers, MA) and used according to the manufacturer’s recommendation. β-Actin (mouse monoclonal antibody, 1:5,000; Sigma, St. Louis, MO) was used as an internal control. Secondary antibodies included Alexa Fluor 700-conjugated anti-mouse (1:12,500 vol/vol; Invitrogen) and Infrared Dye 800-conjugated anti-rabbit (1:12,500 vol/vol; Rockland) antibody. Images were captured in color for quantification and converted to gray scale for presentation.

**Brain sectioning.** Rats were deeply anesthetized with pentobarbital (100 mg/kg) and perfused transcardially with PBS and 4% paraformaldehyde fixative. Brains were removed and further fixed in 4% paraformaldehyde overnight at 4°C. Fixed brains were cryoprotected by immersion in 15% then 30% sucrose/PBS solution and embedded in frozen section medium (Neg-50; Richard-Allan Scientific, Kalamazoo, MI). Coronal sections (20 μm) were cut using a cryostat (CM1900; Leica, Bannockburn, IL), mounted on a glass slide (Super-frosted; Fisher Scientific, Pittsburgh, PA), and stored at −20°C.

**Measurement of apoptotic cell death (TdT-dUTP nick end-labeling).** Apoptotic cell death was examined in P15 brain sections of three treatment groups using a TdT-dUTP nick end-labeling (TUNEL) method described previously (59). In brief, fragmented DNA was labeled with Biotin-dUTP using terminal transferase. Labeled DNA was then detected with Cy3-streptavidin (1:100; Invitrogen). TUNEL<sup>+</sup> cells were counted from six hippocampal sections per rat, three rats for the ID group and four rats for IS and IDT groups.

**Measurement of neurogenesis.** Brain slices were equilibrated to room temperature and rehydrated in PBS. Sections were immersed in 85°C 20 mM sodium citrate (pH 8.0) and cooled to room temperature to unmask antigens, permeabilized for 1 h in 0.2% (vol/vol) Triton X-100 diluted in PBS, and rinsed three times in PBS. Sections were then blocked in BSA (crystalline BSA fraction V, 10 mg/ml in PBS; Sigma) for 30 min and incubated in mouse monoclonal anti-histone H3 (PhosphoS10) antibody (1:5,000 vol/vol, ab14955; Abcam, Cambridge, MA) diluted in antibody-diluent (1 mg/ml BSA, PBS, 0.1%, 0.01% SDS; 200 μl) overnight at 4°C. Following rinses (3×) in wash buffer (PBS, 0.1% Tween 20), sections were incubated overnight with Alexa-488-conjugated secondary antibody (1:200 vol/vol; Invitrogen, Eugene, OR) in antibody diluent. Excess antibody was removed with wash buffer rinses (3×). Slides were mounted and covered with a glass cover slip using Vectashield mounting medium containing propidium iodide (Vector Laboratories, Burlingame, CA). Sections were selected to represent dorsal and ventral hippocampus, and three to four serial sections were examined per hippocampal region per rat.

**Statistical analyses.** P7 and P30 data were analyzed by two-tailed unpaired t-test. P15 data were analyzed by ANOVA and post hoc Bonferroni-corrected t-test, since there were three treatment groups. Significance was set at an α of 0.05. Graphs and statistical calculations were performed with GraphPad Prism (GraphPad Software, San Diego, CA).

**RESULTS**

Decreased mRNA level of IGF-I, IGF-II, IGFBP3, and IGF-I receptors in ID hippocampus. Transcript levels of IGFs, receptors, and IGFBP-3 were assessed during early postnatal development in the rat hippocampus to determine whether...
gestational iron deficiency and its neonatal treatment alter expression of the IGF system. In IS control hippocampus, expression of all the measured IGF system components demonstrated developmental activation with peak expression occurring at P15 (Fig. 1, IS). Untreated iron deficiency resulted in lower levels of all measured transcripts at P15 in ID compared with IS control hippocampus (Fig. 1, ID). Iron treatment beginning at P7 restored hippocampal IGF-I to IS levels by

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**Fig. 3.** Decreased hippocampal protein kinase B (Akt) signaling in P15 ID rats. A and B: Western blots showing P-Akt and total Akt (A) with quantified ratio of P-Akt over total Akt (B). C and D: levels of P-FoxO1S319 (C) and P-p70S6K (D), downstream signaling targets of P-Akt. Values are means ± SE; n = 4–9/group. *P < 0.05, **P < 0.01, and ***P < 0.001.

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**Fig. 4.** Increased phosphoinositide 3-kinase (PI3K)-independent extracellular signal-regulated kinase (Erk) 1/2 signaling in P15 ID hippocampus. A and B: Western blot images showing P-Erk1/2 with respective total Erk1/2 (A) and P-Erk1/2 with respective β-actin (B). Corresponding quantified data are shown on right. C and D: increased phosphorylated levels of Elk1 (C) and P-p90Rsk (D), downstream effectors of P-Erk1/2 signaling. Values are means ± SE; n = 4–9/group. *P < 0.05, **P < 0.01, and ***P < 0.001.
P15 (Fig. 1A, IDT). Iron treatment also increased IGF-II, IGF1R, and InsR expression compared with untreated ID pups at P15, although the levels remained lower than IS (Fig. 1, B–E). Levels of IGFBP3 were lower in the ID and IDT groups at P15 (Fig. 1F). Continuation of iron treatment resulted in all analyzed transcripts in the IDT group returning to IS levels by P30.

Reduced activation of IGF receptors and Akt signaling accompanied by increased Erk signaling in P15 ID hippocampus. We assessed levels of IGF receptor activation and its downstream intracellular signaling pathways (i.e., Akt and Erk) to evaluate the molecular consequences of reduced IGF expression in ID hippocampus, since these are prominent pathways regulating cellular proliferation, survival, and differentiation. Compared with age-matched IS controls, P15 ID showed lower levels of phosphorylated IGF receptors (IGR1Rβ and IRβ) (Fig. 2, A–C). Iron treatment restored the phosphorylated IR level, but the phosphorylated IGF1R level remained lower, in the P15 hippocampus (Fig. 2, B and C, IDT). Notably, IGR1Rβ concentrations were approximately threefold less than IRβ at this time point, and the ratios of IGR1Rβ-IRβ were not different among groups (Fig. 2D).

The ratio of P-Akt/total Akt in P15 hippocampus was lower in the ID group compared with age-matched IS or IDT groups (Fig. 3, A and B) as were levels of P-FoxO1S119 and P-p70S6K (Fig. 3, C and D). Iron treatment increased levels of P-FoxO1 and P-p70S6K compared with untreated ID at P15, although the P-p70S6K level remained lower than IS (Fig. 3, C and D, IDT). In contrast, levels of P-Erk1/2 standardized by total Erk or β-actin were higher in the P15 ID compared with age-matched IS (Fig. 4, A and B). Iron treatment decreased the elevated P-Erk1/2 (Fig. 4, A and B, IDT). Consistent with increased Erk activation, levels of P-Elk-1 and P-p90RSK were elevated in P15 ID hippocampus (Fig. 4, C and D) and were normalized by iron treatment in the IDT group (Fig. 4, C and D). There were no differences among groups in these signaling molecules at P30 (Table 2).

Reduced expression of IGF-dependent genes in the developing ID hippocampus. To further analyze the effects of lowered IGF activity, we measured mRNA levels of IGF-signaling target genes, including the neuronal marker disc-large homolog 4 (Dlk4b [PSD95]) and the glial marker myelin basic protein (Mbp). Levels of both transcripts showed a marked downregulation in P15 ID hippocampus and were partially restored by iron treatment (Table 3). The recovery was more pronounced for Dlk4b than for Mbp. Both transcripts were normalized with iron treatment by P30.

Increased expression of hypoxia-inducible factor 1α and c-fos in developing ID hippocampus. To evaluate whether anemia-induced tissue hypoxia, which accompanies this dietary-induced iron deficiency model (12), might contribute to increased Erk signaling and to confirm the effect of increased Erk signaling in P15 ID hippocampus, we examined mRNA levels of hypoxia-inducible factor 1α (Hif1α) and Erk signaling-dependent target gene c-fos. Hypoxia-ischemia induces similarly divergent effects as iron deficiency (i.e., reduced Akt and increased Erk signaling) on the developing rat brain (63). Hif1α expression was elevated in the P15 ID hippocampus and was readily normalized with iron treatment to IS levels by P15 (Table 3). Hippocampal c-fos levels were upregulated in the ID group and normalized with iron treatment by P15 but remained greater than IS control levels at P30 (Table 3).

Iron treatment increases suppressed hippocampal neurogenesis of ID rats. We analyzed neuronal programmed cell death and found no difference in the number of TUNEL+ cells in the hippocampus among treatment groups (Table 4). Next, we assessed the effect of iron treatment on neurogenesis in the developing ID hippocampus. Cells immunoreactive for phosphorylated histone H3 (pH3-ir), a marker of mitotically active cells, were counted in postnatal rat hippocampus (Fig. 5, A–C). Perinatal iron deficiency reduced neurogenesis in the hippocampus (Fig. 5D, ID). The number of pH3-ir cells was lowered in dentate gyrus (DG) at P7 ID; however, all three areas (CA1, CA3, and DG) showed substantially fewer pH3-ir cells in P15 ID hippocampus (Fig. 5D).

Table 2. Ratio of phosphorylated proteins between IDT and IS group in P30 rat hippocampus

<table>
<thead>
<tr>
<th>Protein</th>
<th>P30 IDT/IS</th>
<th>Unpaired t-Test P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-Akt</td>
<td>1.06</td>
<td>0.17</td>
</tr>
<tr>
<td>P-FoxO1</td>
<td>0.75</td>
<td>0.06*</td>
</tr>
<tr>
<td>P-p70S6K</td>
<td>1.03</td>
<td>0.85</td>
</tr>
<tr>
<td>P-Erk1/2</td>
<td>0.88</td>
<td>0.58</td>
</tr>
<tr>
<td>P-p90RSK</td>
<td>0.88</td>
<td>0.10</td>
</tr>
<tr>
<td>P-Elk1</td>
<td>0.97</td>
<td>0.74</td>
</tr>
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</table>

Phosphorylated proteins were normalized by total protein or β-actin. Sample size (n) = 5–6 rats/group. IDT, iron deficient treated with iron; IS, iron sufficient control; P30, postnatal day 30; P, phosphorylated; Akt, protein kinase B; FoxO1, forkhead box O1; Erk, extracellular signal-regulated kinase. #Trend toward significant difference between groups.

Table 3. Quantitative measurement of transcript levels in rat hippocampus

<table>
<thead>
<tr>
<th>Gene</th>
<th>P7 IS</th>
<th>ID</th>
<th>P15 IS</th>
<th>ID</th>
<th>P30 IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hif1α</td>
<td>1.00 ± 0.12 (5)</td>
<td>1.02 ± 0.13 (5)</td>
<td>0.60 ± 0.08 (11)</td>
<td>0.84 ± 0.05** (7)</td>
<td>0.56 ± 0.16 (10)</td>
</tr>
<tr>
<td>c-fos</td>
<td>1.00 ± 0.10 (5)</td>
<td>2.03 ± 0.09** (4)</td>
<td>5.40 ± 1.43 (7)</td>
<td>9.77 ± 1.23** (4)</td>
<td>4.63 ± 1.05 (7)</td>
</tr>
<tr>
<td>Dlk4b (PSD95)</td>
<td>1.00 ± 0.16 (6)</td>
<td>0.47 ± 0.09*** (6)</td>
<td>2.85 ± 0.47 (11)</td>
<td>1.68 ± 0.25** (8)</td>
<td>2.59 ± 0.28 (11)</td>
</tr>
<tr>
<td>Mbp</td>
<td>1.00 ± 0.20 (3)</td>
<td>0.91 ± 0.06 (6)</td>
<td>1.56 ± 117 (5)</td>
<td>784 ± 61** (6)</td>
<td>986 ± 35** (5)</td>
</tr>
</tbody>
</table>

Values are means ± SD; no. of rats in parentheses. NS, not significant. Letters indicate significant intragroup difference with a < b < c by ANOVA. Intergroup difference as follows: *P < 0.05, **P < 0.01, and ***P < 0.001 by unpaired t-test.

Table 4. TUNEL+ cells in P15 hippocampus

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>TUNEL+ Cell/Section</th>
<th>ANOVA P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS</td>
<td>0.25 ± 0.09 (4)</td>
<td></td>
</tr>
<tr>
<td>ID</td>
<td>0.26 ± 0.17 (3)</td>
<td>0.14 (NS)</td>
</tr>
<tr>
<td>IDT</td>
<td>0.09 ± 0.10 (4)</td>
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Values are means ± SD; no. of rats in parentheses. NS, not significant.
of pH3-ir cells outside of the pyramidal and granular layers of P7 and P15 ID hippocampi. The suppression was reversed following iron treatment by P15 in the IDT group; iron treatment increased the number of proliferating cells in all three areas with complete normalization by P30 (Fig. 5D, IDT).

**DISCUSSION**

Previous studies of the hippocampus in preclinical models of gestational-neonatal iron deficiency demonstrate striking neurodevelopmental deficits that include abnormal neuronal energy metabolism and reduced expression of growth factors relevant for dendritic arborization, synaptogenesis, and myelination (6, 10, 16, 40, 60). These findings establish potential molecular and cellular bases underlying the persistently abnormal cognitive function observed in these rodent models and in human populations. The present study demonstrates that untreated early life iron deficiency attenuates the IGF signaling system and is accompanied by reduced neurogenesis in the rat hippocampus. Iron treatment restores these deficits with an apparent prioritization favoring neurons over glia. Our data support the hypothesis that changing the iron status of the developing rat hippocampus leads to local alterations in the production of IGFs and subsequently to changes in neuronal proliferation, myelination, and survival. However, we could not exclude the possibility that iron therapy alters the permeability of the blood-brain barrier to IGFs, leading to IGF-dependent changes in the developing rat hippocampus (1, 27, 69). A precise mechanism of IGF actions following iron therapy of early life iron deficiency will require additional investigation.

The occurrence of peak expression level of the IGF system at the beginning of rapid hippocampal dendritic arborization, synaptogenesis, and myelination (16, 48) underscores that IGF signaling is critical for normal hippocampal growth and differentiation (14, 18). Although its role in the hippocampus is less well-established, IGF-II may also have an important role in facilitating aspects of hippocampal glial development since it is expressed primarily in glia of postnatal brain (53) and is capable of compensating for myelination deficits observed in mice with loss of IGF-I function (18, 19, 64). Our finding of a more than fourfold greater expression of $\text{IGF-II}$ than $\text{IGF-I}$ (Fig. 6) in postnatal rat hippocampus is therefore consistent with IGF-II’s key role in myelination. Unlike IGF-I, which acts through IR only at high concentrations, IGF-II can act through IGF1R or IR with equal affinity (22, 24); thus, the high levels of IGF-II and IR suggest a prominent role for IGF-II during this period of rapid hippocampal growth.

Iron deficiency downregulates hippocampal IGF expression and diminishes IGF signaling during this critical time period without evidence of compensatory upregulation of its receptors. This lack of a compensatory response is similar to what we have previously reported with hippocampal brain-derived neurotrophic factor signaling during iron deficiency (60).
Iron deficiency impairs hippocampal IGF signaling

Growth factors are key determinants of neuronal cellular outputs such as cell size and survival, protein translation rate, and actin polymerization with their actions mediated through the phosphatidylinositol 3-kinase (PI3K)/mTOR signaling pathway (66). Specifically, protein translation rates are regulated by p70S6K phosphorylation, which was suppressed by early iron deficiency in the current study. The suppression of IGF levels without a compensatory increase in receptor expression seen in early iron deficiency is consistent with a hypometabolic state (20, 52) and results in a reduction in these critical cell functions during a time of rapid regional brain development. The repression of IGF signaling by iron deficiency occurring during the period of neuronal proliferation in the hippocampus led to reduced hippocampal neurogenesis, but not increased apoptosis (Table 3), in the present study. The absence of increased hippocampal cell death was somewhat unexpected, since the IGF system inhibits neuronal apoptosis in the developing hippocampus (38), but was in line with a recent report suggesting that reduced levels of IGF1R and IR could confer apoptotic resistance (9). Thus, the lack of compensatory upregulation of the receptors in the current study may have conferred neural protection by maintaining a lower, but sustainable, amount of cellular mTOR activity as an adaptive response. It is also consistent with increased PI3K-independent activation of the Erk1/2 pathway, which promotes cell survival (45), in ID hippocampus (Fig. 4). The present study suggests differential cellular effects in response to levels of IGF signaling. Whereas increased levels of IGF promote neurogenesis and synaptogenesis (44), reduced expression levels of IGF and P-p70S6K in the ID hippocampus impair these specific developmental processes without inducing apoptosis. The findings are consistent with our previous studies demonstrating reduced synaptogenesis and dendritic complexity in this model (10). In spite of these cellular effects, it is also possible that iron deficiency also causes deficits in neuronal growth (soma and axon) and that iron therapy ameliorates these deficits in the developing hippocampus.

There were some unexpected findings regarding the effects of untreated iron deficiency in the current study. First, lower levels of P-FoxO1S319, which would increase FoxO1 nuclear localization and transcriptional activity (26, 62), should have resulted in increased apoptosis in P15 ID hippocampus. Instead, we found an absence of increased cell death in ID relative to IS hippocampus. Second, we expected that lower IGF signaling would result in lower Erk signaling in P15 ID hippocampus; increased Erk signaling was found instead. We speculate that increased Erk activation was likely driven by anemia-induced hypoxia in ID rats (11, 29, 33, 63). This possibility is further corroborated by increased hippocampal Hif1α expression in P15 ID rat as well as a 40% lower P-Erk level observed in a hippocampal-specific DMT-1 knockout mouse model (unpublished observations), where iron deficiency occurs without anemia and thus tissue hypoxia (13). Provision of the deficient substrate (i.e., iron) rapidly normalizes IGF-I, but not IGF-II, levels by P15, suggesting differential responses in gene regulation immediately following iron treatment even though the hippocampus remains 30–40% ID (34). Coupled with a marked upregulation of the exclusively neuronal marker Dlgh4 (PSD95) relative to the exclusively glial marker Mbp and the role of IGF-I in multiple neural populations (18, 53), the rapid response of IGF-I suggests that it receives high priority compared with other genes, including IGF-II, and suggests that neurons have a more robust response to iron treatment compared with glia during these early postnatal ages. Ultimately, these specific effects may lead to resumption of normal activity earlier in development and thus may protect the hippocampus from long-term damage that would be induced by having the suppression last beyond the critical period of hippocampal development. Interestingly, mRNA levels of IGFBP3, a main IGF carrier protein that can regulate availability of IGF to IGF1R and IR at a local level (18), were downregulated in P15 ID animals and did not...
respond to iron treatment until P30. IGFBP3 has its own IGF-independent effects that inhibit cell proliferation or promote apoptosis (65). Lower levels of IGFBP3 in the P15 ID hippocampus in the present study may reflect a role of IGFBP3 in hippocampal development through IGF-dependent or -independent mechanisms. Lack of immediate recovery of IGFBP3 levels with iron therapy (IDT) may act to heighten availability of locally synthesized IGF and transferrin. For example, up-regulation of transferrin expression observed in previous studies (12) and IGF-II in the present study following iron treatment may facilitate the recovery from the hypomyelination seen in the ID hippocampus (3, 12, 67). The mechanism underpinning the differential responses between neuron and glia to the effects of iron therapy in the developing brain will need a detailed examination in future studies.

Iron mediates cell proliferation by regulating the activity of ribonucleotide reductase as well as cell cycle regulators (68). Iron deficiency reduces cell proliferation by limiting the available pool of deoxyribonucleotides critical for DNA replication and inducing cell cycle arrest. The present study is one of the first to demonstrate reduced neurogenesis in postnatal ID hippocampus. It was surprising, however, that iron therapy can normalize neurogenesis in the P15 even though there is still a 40% reduction of hippocampal iron (34). This observation suggests that, in the developing hippocampus, neurogenesis is a high-priority event that utilizes iron substrate as soon as the substrate becomes available. Thus, by extension, repletion of the pool of functional iron compounds has priority over ferritin iron storage following iron therapy of early life iron deficiency. Based on the partial recovery of IGF signaling following iron treatment, we propose that IGF is partly responsible for neurogenesis in ID hippocampus, consistent with its role in postnatal brain development (42). Whether increased neurogenesis following iron treatment is mediated by upregulation and/or posttranslational modification of relevant cell cycle regulators in ID hippocampus, whether iron deficiency reduces the number of neural stem cells, and whether these newly born cells exhibit characteristics of pyramidal or granular neurons will be the focus of future studies.

**Perspectives and Significance**

The dysregulation of hippocampal IGF signaling in the present study, summarized in a working model (Fig. 7), may have implications for other early nutrient deficiencies, including copper deficiency, iodine/thyroid hormone deficiency, and the protein-energy malnutrition found in intrauterine growth restriction. All are characterized by hypomyelination and abnormal cognitive function and are interrelated; copper deficiency induces iron deficiency, whereas both induce a hypothyroid state in the brain (4, 25, 54, 55). Although iron deficiency is common to these perinatal nutrient deficiencies (4, 50), the similarity of brain developmental defects suggests that hippocampal IGF signaling is likely impaired in all of these preclinical rodent models. In this regard, reduced IGF signaling may be a common mechanism underlying hypomyelination in thyroid, copper, and ID brains (4, 6, 17, 31), abnormal neural differentiation in ID hippocampus (34, 60), and lower granular cell number observed in the DG of the rodent model of perinatal nutrition insufficiency (8). Thus, targeting the IGF signaling pathway may lead to novel therapeutic strategies to prevent long-term neurological abnormalities associated with perinatal nutrient deficiencies.

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**DISCLOSURES**

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