Early-Life Iron Deficiency Anemia Alters Neurotrophic Factor Expression and Hippocampal Neuron Differentiation in Male Rats¹–³

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Abstract

Fetal-neonatal iron deficiency alters hippocampal neuronal morphology, reduces its volume, and is associated with acute and long-term learning impairments. However, neither the effects of early-life iron deficiency anemia on growth, differentiation, and survival of hippocampal neurons nor regulation of the neurotrophic factors that mediate these processes has been investigated. We compared hippocampal expression of neurotrophic factors in male rats made iron deficient (ID) from gestational d 2 to postnatal d (P) 7 to iron-sufficient controls at P7, 15, and 30 with quantitative RT-PCR, Western analysis, and immunohistology. Iron deficiency downregulated brain-derived neurotrophic factor (BDNF) expression in the hippocampus without compensatory upregulation of its specific receptor, tyrosine-receptor kinase B. Consistent with low overall BDNF activity, we found lower expression of early-growth response gene-1 and -2, transcriptional targets of BDNF signaling. Doublecortin expression, a marker of differentiating neurons, was reduced during peak iron deficiency, suggesting impaired neuronal differentiation in the ID hippocampus. In contrast, iron deficiency upregulated hippocampal nerve growth factor, epidermal growth factor, and glial-derived neurotrophic factor accompanied by an increase in neurotrophic receptor p75 expression. Our findings suggest that fetal-neonatal iron deficiency lowers BDNF function and impairs neuronal differentiation in the hippocampus. J. Nutr. 138: 2495–2501, 2008.

Introduction

Iron deficiency is one of the foremost early-life nutrient deficiencies, affecting ~30–50% pregnancies worldwide, including an estimated 80% of pregnancies in developing countries (1). Late gestational and neonatal (perinatal) iron deficiency arises from 3 common maternal gestational conditions: severe iron deficiency anemia, placental vascular insufficiency resulting from maternal hypertension, and diabetes mellitus (2–4). In humans, neonatal iron deficiency causes deficits in cognitive function during the period of iron deficiency and poor school performance well after the period of iron deficiency (5,6). With early postnatal iron deficiency, although certain developmental deficits can be corrected with iron treatment, other neurological and cognitive developmental deficits persist up to 10 y after iron treatment (7–9). The neural basis of these developmental deficits continues to be investigated, with evidence from animal models suggesting that multiple developing brain processes, such as myelination, monoamine metabolism, energy metabolism, and dendritic arborization, might be affected (10–14). Based on the ontogeny of human brain development, perinatal iron deficiency may have large effects on rapidly differentiating regions such as the hippocampus (15). Consistent with this notion, peak iron import into the rat hippocampus occurs between postnatal d (P) 5 and P15, just prior to maximal cellular differentiation, synaptogenesis, and dendritic growth and arborization (16).

Fetal-neonatal iron deficiency particularly affects the hippocampus as evidenced by decreased energy metabolism, impaired neuronal morphology and transmission, and increased susceptibility to infarction (13,14,17,18). A recent study profiling altered gene expression induced by perinatal iron deficiency identified alterations in salient molecular pathways involved in neuronal

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differs (19), most notably the mammalian target of rapamycin (mTOR) pathway, which integrates external stimuli such as nutrients and growth factors to regulate gene expression necessary for synaptic maturation and plasticity in the hippocampus (20–22). However, the specific effect of perinatal iron deficiency on the expression of neurotrophic growth factors critical for inducing and maintaining hippocampal neurogenesis, differentiation, and plasticity has not been investigated.

Among the known neurotrophic growth factors, brain-derived neurotrophic factor (BDNF) influences multiple aspects of hippocampal development and synaptic plasticity. BDNF regulates neurogenesis, survival, dendritic growth and branching, and plasticity across the life span (23–25). Induction of long-term potentiation (LTP), a cellular phenomenon associated with memory formation, in the rodent hippocampus rapidly increases BDNF transcript levels (26–28), whereas suppression of BDNF expression and genetic deletion of BDNF leads to impairment of learning, LTP formation, and affective behavior (29,30). BDNF signaling is mediated preferentially by tyrosine-receptor kinase B (TrkB) and neurotrophic receptor p75 (p75NTR) (31). Whereas BDNF/TrkB facilitates long-term depression and reduces neurite outgrowth (32–34). Chronic stress, chronic antidepressant administration, learning, and LTP induction regulate both BDNF and TrkB expression in the hippocampus (35,36).

Based on our prior findings that perinatal iron deficiency induces defects in hippocampal dendritic morphology and neurotransmission, we hypothesized that iron deficiency would result in dysregulation of neurotrophic factors involved in neuronal differentiation and synaptic plasticity. Here, we present evidence that perinatal iron deficiency reduces BDNF activity and alters neuronal development in rat hippocampus.

Materials and Methods

Animals. All animal experiments were conducted with the approval of the University of Minnesota Institutional Animal Care and Use Committee. Timed-pregnant Sprague-Dawley rats were purchased from Harlan. Fetal-neonatal iron deficiency was induced as previously described to achieve a 40% loss of total brain iron at P10 (12), a degree of brain iron deficiency that is critical for inducing and maintaining hippocampal neurogenesis, survival, dendritic growth and branching, and plasticity across the life span (23–25). Induction of long-term potentiation (LTP), a cellular phenomenon associated with memory formation, in the rodent hippocampus rapidly increases BDNF transcript levels (26–28), whereas suppression of BDNF expression and genetic deletion of BDNF leads to impairment of learning, LTP formation, and affective behavior (29,30). BDNF signaling is mediated preferentially by tyrosine-receptor kinase B (TrkB) and neurotrophic receptor p75 (p75NTR) (31). Whereas BDNF/TrkB promotes neurogenesis, neurite outgrowth, and synaptic plasticity, BDNF/p75NTR facilitates long-term depression and reduces neurite outgrowth (32–34). Chronic stress, chronic antidepressant administration, learning, and LTP induction regulate both BDNF and TrkB expression in the hippocampus (35,36).

Diet. IS control (198 mg/kg iron, Rx 241632) and ID (3 mg/kg iron, Rx 247497) nonpurified diets were purchased from Harlan Teklad. The composition of both the IS and ID diets has been described previously (12).

Tissue dissection and collection. Male rats at P7, 15, and 30 were killed by an intraperitoneal injection of Beuthanasia (100 mg/kg). Specific postnatal ages were selected on the basis of the ontogeny of the hippocampal formation; P7 marks the end of the proliferative stage and P15 and P30 represent early and late differentiation, respectively (37,38). Brains were removed and bisectioned along the midline. Hippocampus was dissected, flash-frozen in liquid nitrogen, and stored at −80°C.

Quantitative RT-PCR. Total RNA was isolated from dissected hippocampus using a RNA isolation kit (Stratagene) and concentrations were measured by absorbance at 260 nm (A260/280) using a NanoDrop ND-1000 (NanoDrop Technologies). Approximately 4 µg of total RNA was used to generate cDNA by reverse transcription using SuperScript III (Invitrogen) and random hexamer primers per manufacturer recommendation. The resulting cDNA was diluted 7-fold to give a final volume of 140 µL. All quantitative RT-PCR experiments were performed with one-half the manufacturer’s recommended volume (Applied Biosystems) consisting of 4 µL of diluted cDNA, 5 µL 2× Taqman quantitative RT-PCR Universal mix (No AmpErase), and 0.5 µL 20× Taqman Gene Expression Assay primer/probe mix. Thermocycling was conducted according to the manufacturer’s protocol (ABI) using a MX3000P instrument (Stratagene). The transcripts that were analyzed are listed in Supplemental Table 1.

Western blot analysis. Protein isolation was conducted as described previously (19). In brief, flash-frozen hippocampal tissues were lysed by sonication. Approximately 31 µg of total protein was loaded and separated in 10% SDS-PAGE gels. Protein was transferred onto Nitrocellulose membranes (Pierce) using semidyed transfer (Bio-Rad). Membranes were blocked in 3% nonfat powder milk diluted in TBST (Tris buffer pH 7.4 + 0.1% Tween-20) for 1 h at room temperature. Membranes were incubated in primary antibody diluted in TBST containing 0.1% nonfat powder milk overnight at 4°C with rocking and rinsed in TBST (4×) to remove excess antibody. Membranes were then incubated in horseradish peroxidase-conjugated secondary antisera at room temperature for 2 h and excess antisera were removed with TBST washes (5×). The protein-antiser complex was detected using an ECL kit (GE Healthcare) and a darkroom equipped with a super-cored charge coupled device camera (Bio-Rad). For quantification, integrated intensity of the protein of interest was standardized to actin, whose expression is not affected by iron deficiency and thus acts as an internal control. The primary antibodies included anti-TrKB (1:1000) rabbit polyclonal (Cell Signaling), anti-actin (1:500) mouse monoclonal (Sigma), and anti-p75NTR (1:1000) rabbit polyclonal (a generous gift from Dr. William Engeland, University of Minnesota).

Immunohistology. Rats were deeply anesthetized with pentobarbital (100 mg/kg) and perfused transcardially with PBS and 10% formalin fixative. Brains were removed and further fixed in 10% formalin for 4–6 h at 4°C. Fixed brains were cryoprotected by immersion in 30% sucrose/PBS solution and embedded in frozen section medium (Neg-50, Richard-Allan Scientific). Twenty-µm coronal sections were obtained using a cryostat (Leica CM1900) and stored at −20°C. For fluorescence immunohistology, sections were equilibrated to room temperature and rehydrated in TBS. Antigen unmasking was performed by immersing sections in hot (95°C) 10 mmol/L Na citrate, pH 8.6, and then allowing them to cool to room temperature in a water bath. Sections were permeabilized in TBS + 0.2% Triton X-100 and incubated for 1 h in blocking solution (10 g/L bovine serum albumin diluted in TBS + 0.1% Tween-20) and then incubated in primary antibody overnight at 4°C. Excess antibody was removed with TBST washes. Sections were retreated with blocking solution, incubated in fluorescence-labeled secondary antibody overnight at 4°C, and washed with TBST. Finally, sections were mounted in aqueous mounting media with 4',6-diamidino-2-phenylindole (Vector Laboratories). Antibodies included biotin-conjugated anti-BDNF (5 mg/L) chicken polyclonal (A&D System), anti-Neuronal Nuclei (1:200) raised in mouse (MAB377, Chemicon International), anti-doublecortin (Dcx) (1:50) goat polyclonal (Santa Cruz Biotechnology), and anti-p75NTR (1:1000) rabbit polyclonal. Fluorescence-labeled secondary antibodies were purchased from Molecular Probes (Invitrogen) and used according to the manufacturer’s recommendation. Confocal images were captured with a Nikon Digital-Eclipse C1 microscope system.

Terminal deoxynucleotidyl transferase-mediated biotin-DUTP nick end labeling staining. Detection of enzyme-mediated DNA fragmentation was carried out by terminal deoxynucleotidyl transferase (TdT)-mediated biotin-DUTP nick end labeling (TUNEL) staining (39) with modifications. In brief, following rehydration in TBS, 20-µm brain sections were permeabilized with TBS + 0.2% Triton X-100, rinsed in TdT buffer (30 mmol/L Tris, pH7.2, 0.02% CoCl₂, 3% Na cacodylate), and incubated for 1 h at 37°C in terminal transferase solution (400 U
Acute upregulation of p75NTR receptor in ID hippocampus. ID rats had 90% greater p75NTR mRNA expression at P15 compared with IS rats (Table 1) with a corresponding increase in protein level (Supplemental Fig. 2B). Levels of p75NTR mRNA normalized at P30 in the ID group (Table 1). The p75NTR protein was localized primarily in the neurites of stratum oriens and stratum lucidum (Supplemental Fig. 3). In the pyramidal cell layer, p75NTR was localized to the cellular membrane and was enriched in cells with lower NeuN expression (Supplemental Fig. 3). Moreover, p75NTR and BDNF were not extensively colocalized in the developing hippocampus of either the IS or ID group (Supplemental Fig. 3).

Reduced early growth response gene-1 and -2 expression and decreased neuronal death in ID hippocampus. To assess whether lower BDNF expression in the developing ID hippocampus would lead to decreased BDNF activity, mRNA transcript levels of early growth response gene-1 (Egr-1) and Egr-2, 2 known transcriptional targets of BDNF signaling (40,41), were examined in the IS and ID hippocampus. Egr-1 and -2 were
Fetal-neonatal iron deficiency affects structure and function of many of which last beyond the period of iron deficiency (1,8). Deficits associated with fetal-neonatal iron deficiency anemia, as 50% that of the IS group (42,43). Apoptotic cell death was diminished in the dorsal ID hippocampus (Fig. 3).

**Decreased Dcx expression associated with delayed NeuN-nuclear accumulation in ID hippocampus.** We assessed the expression of Dcx, a microtubule-associated protein expressed in differentiating neurons (44), to further define how iron deficiency affects neural differentiation. At P30, the IS and ID groups did not differ, but at P15, Dcx protein in the ID group was 0.6 that of the IS group (P < 0.05). Dcx was histologically localized to CA3 pyramidal cells and to dentate granular and hilus cells of P15 and P30 hippocampi (Supplemental Fig. 4). NeuN accumulation in pyramidal cell nuclei occurred by P30 in IS rats but was absent in ID rats (Supplemental Fig. 4).

**Discussion**

We and others have established neurological and behavioral deficits associated with fetal-neonatal iron deficiency anemia, many of which last beyond the period of iron deficiency (1,8). Fetal-neonatal iron deficiency affects structure and function of the hippocampus among other brain regions responsible for learning and memory formation (15), yet little is known about the cellular mechanism that may be responsible for this altered development. This study demonstrates that early-life iron deficiency decreases BDNF levels and increases GDNF, EGF, and NGF levels in the developing rat hippocampus. Compensatory TrkB expression does not occur despite reduced BDNF expression and is accompanied by downregulation of BDNF target genes and altered neuronal differentiation. The upregulation of GDNF, EGF, and NGF as well as p75NTR suggests utilization of alternate signaling pathways to compensate for the abnormalities in BDNF-driven pathways. These findings suggest that iron homeostasis is critical for proper neurotrophic factor expression during early life and provide a possible molecular basis for the neuro-morphologic and behavioral deficits in perinatal iron deficiency (8).

Our previous study showed that iron deficiency downregulates mRNA and protein levels of factors important for synaptic structure and plasticity, including calmodulin-regulated kinase-Ila and postsynaptic density 95 (19). The translation of these factors at the synapse is in turn regulated by mTOR, a basic intracellular signaling pathway that integrates stimuli, including growth factors, nutrient and energy availability, and oxidative stress, to regulate protein translation and cellular growth (22,45,46). Iron deficiency significantly alters components of the mTOR signaling pathway (19). In turn, BDNF modulates the activity of critical components of the mTOR signaling pathway, including Akt (22,45). We speculate that decreased BDNF, coupled with altered components of the mTOR signaling cascade, serve as the basis for the reduced

**TABLE 1** Comparison of neurotrophic factor and receptor expression in the developing IS and ID rat hippocampus

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Fold of P7 IS</th>
<th>P7</th>
<th>IS</th>
<th>ID</th>
<th>Fold of P15 IS</th>
<th>IS</th>
<th>ID</th>
<th>Fold of P30 IS</th>
<th>IS</th>
<th>ID</th>
<th>2-Way ANOVA P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNTF</td>
<td>1.0 ± 0.0a</td>
<td>1.3 ± 0.1</td>
<td>1.9 ± 0.1b</td>
<td>2.1 ± 0.2</td>
<td>2.2 ± 0.2b</td>
<td>2.2 ± 0.2</td>
<td>0.11</td>
<td>&lt;0.01</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDNF</td>
<td>1.0 ± 0.0a</td>
<td>1.8 ± 0.1*</td>
<td>1.7 ± 0.1b</td>
<td>2.4 ± 0.2*</td>
<td>1.5 ± 0.2b</td>
<td>1.6 ± 0.2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>1.0 ± 0.0b</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.2b</td>
<td>2.2 ± 0.2*</td>
<td>2.8 ± 0.3b</td>
<td>2.9 ± 0.4</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGF</td>
<td>1.0 ± 0.0c</td>
<td>1.0 ± 0.1</td>
<td>3.8 ± 0.4c</td>
<td>5.2 ± 0.5*</td>
<td>5.8 ± 0.3c</td>
<td>6.5 ± 0.3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.07</td>
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<tr>
<td>TrkB</td>
<td>1.0 ± 0.0a</td>
<td>1.0 ± 0.0</td>
<td>2.3 ± 0.3b</td>
<td>2.2 ± 0.3</td>
<td>3.0 ± 0.2c</td>
<td>2.8 ± 0.3</td>
<td>0.38</td>
<td>&lt;0.01</td>
<td>0.91</td>
<td></td>
<td></td>
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<tr>
<td>TrkB</td>
<td>1.0 ± 0.0b</td>
<td>1.2 ± 0.1</td>
<td>4.0 ± 0.8b</td>
<td>3.7 ± 0.2</td>
<td>2.4 ± 0.2b</td>
<td>2.9 ± 0.2</td>
<td>0.22</td>
<td>&lt;0.01</td>
<td>0.14</td>
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<tr>
<td>p75</td>
<td>1.0 ± 0.0c</td>
<td>1.6 ± 0.2*</td>
<td>1.1 ± 0.1b</td>
<td>1.9 ± 0.11</td>
<td>0.8 ± 0.0a</td>
<td>0.9 ± 0.1</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>Egr-1</td>
<td>1.0 ± 0.0c</td>
<td>1.0 ± 0.1</td>
<td>9.2 ± 0.4b</td>
<td>4.9 ± 0.5*</td>
<td>5.8 ± 0.4b</td>
<td>7.1 ± 1.0</td>
<td>0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>Egr-2</td>
<td>1.0 ± 0.0b</td>
<td>1.4 ± 0.1</td>
<td>7.0 ± 1.3b</td>
<td>3.8 ± 0.5*</td>
<td>2.6 ± 0.2b</td>
<td>4.1 ± 0.3</td>
<td>0.30</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
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</table>

1 Values are means ± SEM, n = 4–6. Means in a row with superscripts without a common letter differ, P < 0.05. *Different from IS at that age, *P < 0.05. IS bars without a common letter differ, P < 0.05.
expression of calmodulin-regulated kinase-IIα and postsynaptic density 95, ultimately leading to the documented impairment of synaptic plasticity in the ID rat (47,48).

The reduced BDNF levels in the current study during periods of neural proliferation and early differentiation, P7 and P15, respectively (37,38), may underlie compromised cell number and dendritic complexity in the ID hippocampus. Either or both might account for the smaller hippocampal size (R. Rao and M. K. Georgieff, unpublished observation) and abnormal Cornu Ammonis area 1 dendritic structure in ID rats (13). The effect of iron deficiency on proliferation and cell number remains unresolved. Determining the consequences of perinatal iron deficiency on cell proliferation will be valuable in understanding how BDNF participates in modulating early hippocampal development. Downregulation of BDNF target genes (Egr-1 and Egr-2), which also promote neuronal differentiation (49), together with decreased Dcx expression and delayed nuclear accumulation of NeuN provide evidence for impaired neuronal differentiation in the ID hippocampus during a period of normally rapid dendritogenesis and synaptogenesis. These data suggest that iron deficiency places a brake on the developing hippocampus. It would be of interest to determine whether promotion of BDNF expression by alternative means such as exposure to an enriched environment or antidepressant drugs (50,51) would reverse or prevent the neural differentiation defects of perinatal iron deficiency.

In contrast to a model of chronic placental insufficiency, which had a decreased BDNF transcript level accompanied by increased TrkB expression (52,53), we found no compensatory expression of total TrkB mRNA levels in ID rats. However, the finding of a greater TrkB<sub>1</sub>-BDNF ratio suggests an increased availability of this TrkB isoform for BDNF binding in ID hippocampus. We predict that intracellular signaling effectors of BDNF (i.e. P-Mek, P-Erk) would be lower in the ID group, because the TrkB<sub>1</sub> isoform contains the intracellular signaling domain. These findings may be important in terms of understanding the early antecedents of adult neurological disorders characterized by reduced hippocampal function or early hippocampal degeneration, including Alzheimer and Parkinson’s diseases. Both are characterized by reduced BDNF levels without compensatory increased TrkB expression (25,54). The altered expression of genes involved in the pathogenesis of Alzheimer’s disease as well as elevated susceptibility of brain injury in this same model that we have demonstrated in our previous studies supports this possibility (18,19).

Increased EGF, GDNF, NGF, and p<sup>75</sup>NTR expression in the ID hippocampus suggest alternate signaling pathways in lieu of lower BDNF expression. GDNF has been shown to synergize with BDNF in promoting neuronal survival (55). Increased GDNF transcript levels in P7 and P15 ID hippocampus could minimize the deleterious effects of reduced BDNF by facilitating the survival of hippocampal neurons. Given that GDNF and BDNF are synthesized by astrocytes, but have opposing expression levels in the ID hippocampus, it is likely that BDNF was also up-regulated in astrocytes but was insufficient to compensate for its overall decrease in the ID hippocampus. Additional study is needed to elucidate these differential outcomes and whether the upregulation of GDNF is sufficient to mitigate the effects of lowered BDNF, awaiting genetic models where iron deficiency could be targeted in neurons or astrocytes. Likewise, the upregulation of EGF in P15 ID hippocampus could promote survival at the expense of differentiation as suggested by the reduced Dcx expression in P15 ID hippocampus. Increased EGF levels may also affect astrocytic regulation of glutamine synthesize activity, which would be consistent with our prior finding of increased glutamine levels in the ID hippocampus (12,56).

It is less clear what effects the upregulation of NGF and p<sup>75</sup>NTR may have in the ID hippocampus. ProNGF/p<sup>75</sup>NTR signaling induces neuronal apoptosis, in contrast to the survival effect of the mature-NGF/TrkA signaling system (57,58). Protein levels of proNGF and TrkA were not determined in this study; however, the elevated NGF and p<sup>75</sup>NTR mRNA transcripts and corresponding p<sup>75</sup>NTR protein levels in the ID hippocampus would have predicted an increase in neuronal cell death. Instead, the reduced apoptosis in the ID hippocampus suggests that these increases in NGF and p<sup>75</sup>NTR likely mediate survival, as proposed in other studies (59,60). p<sup>75</sup>NTR promotes high-affinity ligand/receptor binding (e.g. BDNF/TrkB) as well as ligand/receptor retrograde transport (31). The upregulation of p<sup>75</sup>NTR in the ID hippocampus might act to maximize the activity of available BDNF/TrkB, which has been shown to promote spine formation and LTP (33,61,62). Conversely, increased p<sup>75</sup>NTR expression in the ID hippocampus might also increase BDNF/ p<sup>75</sup>NTR signaling, which reduces dendritic branching and synaptic spine formation (32,34). Our findings revealed little if any p<sup>75</sup>NTR and BDNF colocalization in the developing hippocampus, making it unlikely that BDNF/p<sup>75</sup>NTR signaling is a major contributing effector in the ID hippocampus.

In summary, our findings suggest that perinatal iron deficiency lowers BDNF activity associated with delayed neural differentiation in the developing hippocampus. These defects might serve as the basis for the morphologic and functional deficits during the period of iron deficiency. It remains unknown if these alterations are consequences of the lack of neuronal iron per se or of other confounding factors in this anemia model, including hypoxia at systemic and/or cellular levels. Which factors drive neuronal differentiation in the ID hippocampus following iron treatment and, more broadly, how iron deficiency affects the development of glial cells remains to be determined. The current study also suggests the possibility that interventions that enhance BDNF activity may work as a therapeutic approach to mitigate against acute and long-term effects of perinatal iron deficiency.

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