Long-Term Reduction of Hippocampal Brain-Derived Neurotrophic Factor Activity After Fetal-Neonatal Iron Deficiency in Adult Rats

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ABSTRACT: Fetal-neonatal iron deficiency acutely alters hippocampal biochemistry, neural morphology, and electrophysiology accompanied by a downregulation of brain-derived neurotrophic factor (BDNF). These changes provide a cellular and molecular basis for observed short-term learning and memory impairments. However, the etiology of residual, long-term hippocampal neurotransmission abnormalities and learning impairments after treatment remain unclear. Because BDNF modulates learning and memory, we assessed its expression in 65-d-old formerly iron deficient (FID) male rats that had been iron deficient during the fetal-neonatal period and treated with iron since postnatal day 7. BDNF-III and -IV mRNAs and BDNF protein expression remained down-regulated in FID rats when compared with the always iron-sufficient rats. Expressions of BDNF activity-dependent downstream targets (3-hydroxy-3-methylglutaryl CoA reductase and immediate early genes *c-fos*, early growth response gene 1 and 2) were reduced in FID rats. In turn, hippocampal expressions of direct targets of early-growth response genes, including hypoxia-inducible factor 1, dual-specificity phosphatase 4, IGF 2, and myelin basic protein were also diminished in FID rats. Collectively, fetal-neonatal iron deficiency lowers hippocampal BDNF expression and function beyond the period of iron deficiency. These findings may underlie the persistence of learning deficits seen after fetal-neonatal iron deficiency. (*Pediatr Res* 65: 493–498, 2009)

Iron deficiency is a common early-life nutrient deficiency affecting approximately 30–50% pregnancies worldwide, including an estimated 80% of pregnancies in developing countries (1). Late gestational and neonatal iron deficiency in the offspring arises from four maternal conditions during pregnancy: severe iron deficiency anemia, placental vascular insufficiency resulting from maternal hypertension, diabetes mellitus, and cigarette smoking (2–5). In humans, neonatal iron deficiency causes deficits in cognitive function during the period of iron deficiency and poor school performance beyond the period of iron deficiency (6,7). Although certain neurodevelopmental deficits can be corrected with iron treatment, other behavioral and cognitive deficits persist more than 10 y

after iron treatment (8). The neural basis of these long-term deficits remains unclear.

Evidence from animal models suggests that fetal, neonatal, and early postnatal iron deficiency influence myelination, monoamine metabolism, and energy metabolism (9–12). Acutely, fetal-neonatal brain iron deficiency results in impaired neuronal morphology, synaptic transmission, and increased susceptibility to infarction in the neonatal hippocampus (13–15). We have described the specific effects of fetal-neonatal iron deficiency on the expression of neurotrophic factors critical for inducing and maintaining hippocampal differentiation and plasticity (16). Hippocampal brain-derived neurotrophic factor (BDNF) expression is down-regulated whereas nerve growth factor (NGF), epidermal growth factor (EGF), and glial-derived neurotrophic factor (GDNF) are up-regulated during iron deficiency in rats (16).

BDNF regulates multiple aspects of hippocampal development and function (17–19). In particular, induction of long-term potentiation (LTP), a cellular phenomenon associated with memory formation, in the rodent hippocampus rapidly increases BDNF transcript levels (20–22). Suppression of BDNF expression and genetic deletion of BDNF lead to impairment of learning and memory (23,24). BDNF is a complex gene with multiple mRNA species, and transcripts III and IV are the most abundant in rat hippocampus (16). BDNF signaling is mediated by tyrosine-receptor kinase B (TrkB) and p75 neurotrophic receptor (p75^{NTR}) (25). There are two known isoforms of TrkB, long (TrkB_L) and short (TrkB_S). TrkB_S lacks the intracellular signaling domain (26). BDNF binding of TrkB promotes neurite outgrowth and synaptic plasticity, in part, through regulation of activity-dependent immediate early genes *c-fos*, early-growth-response-genes 1 and 2 (*Egr1* and *Egr2*) (27–30). In contrast, BDNF binding of

Abbreviations: BDNF, brain-derived neurotrophic factor; **Dusp4**, dual specificity phosphatase 4; **Egr1 and 2**, early-growth-response-gene 1 and 2; **ERK1 and 2**, extracellular-signal regulated kinase 1 and 2; **FID**, formerly iron deficient; **Hif1 α** , hypoxia inducible factor 1 α ; **HMGCR**, 3-hydroxy-3-methylglutaryl coenzyme A reductase; **ID**, iron deficient; **IS**, iron sufficient; **LTP**, long-term potentiation; **Mbp**, myelin basic protein; **NGF**, nerve growth factor; **p75^{NTR}**, p75 neurotrophic receptor; **P**, postnatal day; **qPCR**, quantitative RT-PCR; **TrkB**, tyrosine-receptor kinase B

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p75^{NTR} facilitates long-term depression and reduces neurite outgrowth (31–33).

Although it is not surprising that multiple neural systems are dysfunctional while the brain is iron deficient, the underlying mechanisms for compromised function long after iron repletion remain unclear. Despite iron treatment beginning in the neonatal period, formerly iron-deficient (FID) adult rats continue to demonstrate deficits on hippocampal-dependent tasks consistent with similar findings in humans (34,35). The behavioral deficits in these rats corroborated the finding of lower LTP expression in a similar model (13). Given its role in modulating synaptic plasticity, we postulated lower BDNF activity in the hippocampus of FID rats. Here, we present evidence that fetal-neonatal iron deficiency continues to downregulate BDNF expression and activity beyond the period of iron deficiency, suggesting a long-term alteration in the programming of BDNF expression. Lower BDNF activity may be an important molecular underpinning for the persistent cognitive deficits in FID rats.

METHODS AND MATERIALS

Animals. Timed-pregnant Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). Fetal-neonatal iron deficiency was induced as previously described to achieve a 40% loss of total brain iron at postnatal day (P) 10 (11), a degree of brain iron deficiency equivalent to that seen in newborn humans (3). In this model, the hippocampus remains iron deficient (ID) through P30 (25% loss) and is iron sufficient (IS) by P56 (13). In brief, pregnant dams were maintained on an ID diet (3 mg/kg iron, Rx247497) from gestational day 2 to P7, after which time the nursing dams were given the nonpurified IS control diet (198 mg/kg iron, Rx 241632), both diets were purchased from Harlan Teklad (Madison, WI). IS control animals were given the IS diet throughout the experiment. Litters were culled to 8 pups/litter, all pups were weaned at P21 and fed IS diet for the duration of the experiment. All animal experiments were performed with the approval of the University of Minnesota Institutional Animal Care and Use Committee.

Iron concentration. Brain iron concentrations were measured as previously described (13). In brief, deeply anesthetized P65 rats (intraperitoneal injection of 100 mg/Kg Beuthanasia) were perfused transcardially with 0.9% NaCl. The hippocampus was dissected and rinsed in saline solution. Hippocampal wet weight was recorded and the tissue was lyophilized for 72 h. Lyophilized tissue was digested with 4:1 nitric/perchloric acid solution for 5 d. Iron concentration was determined by atomic absorption spectroscopy

using a standard curve generated from stock iron standards (Sigma Chemical Co.).

Quantitative RT-PCR (qPCR). P65 male rats were killed by an intraperitoneal injection of Beuthanasia (100 mg/kg). Brains were removed and bisected along the midline. Hippocampus was dissected and flash frozen. Total RNA was isolated from dissected hippocampus using an RNA-isolation kit (Stratagene, La Jolla, CA). Approximately, 4 µg of total RNA was used to generate cDNA using SuperScript III (Invitrogen, Carlsbad, CA) and random hexamer primers, and cDNA was diluted 7-fold. qPCR experiments were performed with 1/2 the manufacturer's recommended volume (Applied Biosystems Inc., Foster City, CA). Data were collected using a MX3000P instrument (Stratagene, La Jolla, CA). Information about the qPCR probes/primers is listed in Table 1.

Immunohistochemistry. Immunohistochemistry was performed as previously described (16). In brief, 20 µm coronal sections were rehydrated in Tris buffer saline pH 7.6 (TBS). Antigen unmasking was performed by immersing sections in hot (95°C) 10 mM Na-citrate pH 8.6. Sections were permeabilized in TBS + 0.2% Triton X-100, incubated in blocking solution (10 g/L BSA diluted in TBS + 0.1% Tween-20), and then incubated in primary antibody overnight at 4°C. Excess antibody was removed with TBS + 0.1% Tween-20 (TBST) rinses (3X). Sections were retreated with blocking solution and, incubated in fluorescence-labeled secondary antibody overnight at 4°C. Sections were washed with TBST and mounted in aqueous mounting media with DAPI (Vector laboratories, Inc., Burlingame, CA). Antibodies included biotin-conjugated anti-BDNF (5 mg/L) chicken polyclonal (A&D system, Minneapolis, MN), anti-NGF (1:100) rabbit monoclonal (Cell Signaling, Danvers, MA), and anti-p75^{NTR} (1:100) rabbit polyclonal (A gift from Dr. William Engeland, University of Minnesota). Fluorescence-labeled secondary antibodies were used according to the manufacturer's recommendation (Invitrogen, Eugene, OR). Sytox Green (Invitrogen) was used to stain nuclear DNA in some experiments. Confocal images were captured with a Nikon Digital-Eclipse C1 microscope system (Nikon, Japan).

Western blot analysis. Protein isolation was performed as described previously (36). Approximately, 31 µg of total protein was loaded and separated in 12% and 4–12% gradient SDS-PAGE gels (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membranes (Pierce, Rockford, IL). Membranes were blocked in blocking buffer for near infrared fluorescent Western Blotting (Rockland, Gilbertsville, PA) for 1 h at room temperature and incubated overnight at 4°C in primary antibody diluted in Blocking Buffer. Membranes were rinsed in PBS with 0.1% Tween-20 (4X) to remove excess antibody and then incubated in fluorescent secondary antibody diluted in Blocking Buffer with 0.1% Tween-20 and 0.01% SDS at room temperature for 45 min, and rinsed 4X in PBS with 0.1% Tween-20 to remove excess antibody. Membranes were imaged with Odyssey Infrared Imaging System (Li-cor Biosciences, Lincoln, NE) and the integrated intensity of the protein of interest was quantified and normalized to actin. The primary antibodies included anti-BDNF (1:1000) rabbit polyclonal (Abcam, Cambridge, MA), anti-TrkB (1:1000) rabbit monoclonal, anti-NGF (1:1000) rabbit monoclonal, anti-p44/42 MAPK (Thr202/Tyr204) (1:1000) rabbit monoclonal (Cell Sig-

Table 1. Identification of mRNA transcripts assessed

Gene name	Gene bank accession #	ABI assay ID	Category
BDNF-III	BC087634	Rn01484928_m1	Signaling molecule
BDNF-IV	NM_012513	Rn02531967_s1	Signaling molecule
TrkB	NM_012731	Rn01441747_m1	Membrane receptor
TrkB _L	NM_012731	Rn00820626_m1	Membrane receptor
p75 ^{NTR}	NM_012610	Rn00561634_m1	Membrane receptor
NGF	XM_227525	Rn01533872_m1	Signaling molecule
EGF	NM_012842	Rn00563336_m1	Signaling molecule
CNTF	NM_013166	Rn00755092_m1	Signaling molecule
GDNF	NM_019139	Rn00569510_m1	Signaling molecule
CTGF	NM_022266	Rn00573960_g1	Signaling molecule
Egr-1	NM_012551.2	Rn00561138-m1	Transcription factor
Egr-2	NM_053633.1	Rn00586224_m1	Transcription factor
c-fos	NM_022197.2	Rn02396760_g1	Transcription factor
HMGCR	NM_013134.2	Rn00565598_m1	CoA reductase
Hif1α	NM_024359	Rn00577560_m1	Transcription factor
Dusp4	NM_022199	Rn00573501_m1	Phosphatase
IGF-II	NM_031511	Rn01454518_m1	Signaling molecule
Mbp	NM_001025293	Rn00566745	Structural protein
Rps18	NM_213557	Rn01428915_g1	Internal control

naling, Danvers, MA), anti-p75^{NTR} (1:2000) rabbit polyclonal, and anti-actin (1:5000) mouse monoclonal (Sigma Chemical Co., St. Louis, MO). Secondary antibodies included Alexa Fluor 680 conjugated anti-mouse (1:12500) (Invitrogen, Carlsbad, CA) and Infrared Dye 800 conjugated anti-rabbit antibody (1:12500) (Rockland, Gilbertsville, PA).

Statistical methods. Data for transcript and protein levels were collected from six male rats for each dietary group. Data were analyzed by a nonparametric Mann-Whitney *U* test, with significance set at $\alpha < 0.05$. Graphs and statistical calculations were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA).

RESULTS

Hippocampal iron status at P65. At P65, the mean hippocampal iron concentration of the FID group was not different from the IS control group (IS: $6.79 \pm 0.78 \mu\text{g Fe/g wet weight}$, $n = 7$; FID: $6.66 \pm 0.84 \mu\text{g Fe/g wet weight}$, $n = 7$; $p = 1.00$).

Decreased BDNF and TrkB expression in P65 FID hippocampus. P65 FID rats had lower BDNF-III and -IV mRNA and protein levels compared with control IS rats (Fig. 1A and B). Expression of the total and long form of the BDNF receptor TrkB was lower in the FID group compared with controls (Fig. 1C). The protein level of TrkB_L in the FID group was similar to IS control, but TrkB_S level was lower (Fig. 1D). TrkB_S is expressed at a higher level than TrkB_L in the control hippocampus, but not in the FID (Fig. 1D, IS). The levels of p75^{NTR} mRNA (Table 2) and protein (data not shown) were not different between groups. The mRNAs of other growth factors important for neuronal function, including ciliary neurotrophic factor, connective tissue growth factor, EGF, GDNF, and NGF, were not different between control and FID P65 rats (Table 2). Protein levels of NGF were also similar between groups (data not shown). Immunohistologic analysis of hippocampal BDNF, NGF, and p75^{NTR} protein revealed no apparent difference in localization between IS control and FID rats (Fig. 2).

Reduced BDNF-regulated target gene expression in FID hippocampus. Transcript levels of several known activity-dependent targets of BDNF signaling were examined to assess BDNF activity in FID hippocampus. These include the 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) and the immediate early genes *c-fos*, *Egr1* and *Egr2* (29,30,37). Consistent with lowered BDNF activity, levels of these target genes were decreased in P65 FID rats (Fig. 3).

Reduced expression of *Egr1*- and *Egr2*-regulated target genes in P65 FID hippocampus. Expression of genes regu-

lated by the transcription factors *Egr1* and *Egr2* was measured to demonstrate the consequences of lower BDNF activity beyond immediate effectors. These included hypoxia-inducible factor 1 α (hif1 α) and dual-specificity phosphatase 4

Table 2. Comparison of hippocampal neurotrophic factor expression in control (IS) and FID P65 rats*

Neurotrophic factor/receptor	P65		Mann-Whitney <i>U</i> test <i>p</i> value
	IS	FID	
CNTF	1.0 ± 0.2	0.8 ± 0.1	0.20
CTGF	1.0 ± 0.4	0.9 ± 0.2	1.00
EGF	1.0 ± 0.2	0.9 ± 0.2	0.73
GDNF	1.0 ± 0.3	0.9 ± 0.3	0.63
NGF	1.0 ± 0.1	1.0 ± 0.1	0.84
p75 ^{NTR}	1.0 ± 0.3	0.8 ± 0.4	0.20

Values are means $\pm$ SD, $n = 4-6$.

* Data represent fold of control (IS).

Figure 2. Localization of BDNF, NGF, and p75^{NTR} in P65 CA1 hippocampus. (A and B) Similar expression pattern of BDNF (Red) and p75^{NTR} (Green) in IS control (A) and FID (B) hippocampus. Arrows show BDNF+ pyramidal CA1 neurons. Arrowheads indicate p75^{NTR}+ neurites. (C and D) NGF protein (Red) expression in IS control (C) and FID (D) hippocampus. Arrowheads show NGF presence in pyramidal neurons. Nuclei counterstained with Sytox Green. P, pyramidal layer; sr, stratum radiatum. Scale bar = 50 μm .

Figure 1. Reduced BDNF and TrkB expression in P65 FID hippocampus. (A) BDNF mRNA levels. (B) BDNF protein levels. (C) Total TrkB and TrkB_L mRNA levels. (D) TrkB protein levels. Data are normalized to control (IS) group. Values represent mean $\pm$ SEM, $n = 4-6$. Asterisks denote significant *p* values. * $p < 0.05$, ** $p < 0.01$.

Figure 3. Down-regulation of BDNF activity-dependent target genes in P65 FID rat hippocampus. Data are normalized to control (IS) group. Values are mean $\pm$ SEM, $n = 4-6$. Asterisks denote significant p values. * $p < 0.05$, ** $p < 0.01$.

(Dusp4), which are targets of Egr1 (38,39), and IGF 2 (IGF-II) and myelin basic protein (Mbp), which are targets of Egr2 (40,41). Consistent with reduced Egr1 and Egr2 levels, transcript levels of these targets were lowered in P65 FID hippocampus (Fig. 4). Furthermore, phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1/2) were increased in FID compared with IS rats (Fig. 4C), in line with decreased Dusp4 phosphatase activity in the FID group (Fig. 4B).

DISCUSSION

We and others have demonstrated that early iron deficiency results in long-term behavioral and cognitive deficits beyond the period of fetal-neonatal iron deficiency anemia. In particular, fetal-neonatal iron deficiency compromises hippocampal-dependent learning and memory formation in adult FID rats (34). Here, we provide evidence that early iron deficiency

results in long-term decreases in BDNF expression and activity without compensatory increases in its receptor TrkB. BDNF and its downstream targets influence a diverse cascade of molecular mediators of synaptic plasticity, which, in turn, form the basis for learning and memory formation (Fig. 5). Reduced hippocampal BDNF expression in FID rats negatively affects this cascade. We speculate that these findings provide molecular bases for the long-term electrophysiologic, morphologic, and ultimately, behavioral abnormalities that persist after fetal-neonatal iron deficiency (8,13,14,34,35,42,43).

BDNF normally induces neuronal HMGR, the rate-limiting enzyme in cholesterol synthesis that facilitates synaptic vesicle formation (37). Lower HMGR expression in the FID hippocampus suggests that vesicle formation may be compromised. Combined with lower levels of synaptobrevin I (36), a protein involved in vesicle fusion (44), this finding may contribute to impaired paired-pulse facilitation and reduced synaptic efficacy (LTP) in this model (13). Paired-pulse facilitation and LTP are important indices of neuroelectrophysiologic events during neurotransmission and LTP is widely accepted as a cellular substrate for learning and memory (45,46).

BDNF also normally induces the expression of c-fos, Egr1, and Egr2, activity-dependent immediate early transcription factors that facilitate LTP in the hippocampus (27). Lower expression of c-fos, Egr1, and Egr2 may contribute to reduced plasticity in the hippocampus of FID rats. Lower c-fos expression not only leads to a reduction in expression of genes necessary for LTP (47,48) but also may influence a feed-forward loop, further reducing BDNF activity (49,50).

Reduced expression of Egr1 was accompanied by lower expression of its known target genes, hif1 α and Dusp4, in the hippocampus of FID rats. Hif1 α is an oxidative-state-dependent transcription factor that regulates chemokine (C-X-C motif) ligand 12 (Cxcl12), an important modulator of synaptic formation (51). Cxcl12 mRNA expression is reduced in FID rats (36). These changes, when combined with similar long-term reductions in postsynaptic density 95 (PSD95) and calmodulin-dependent kinase II α (CamKII α), may account for the abnormal dendritic length and branching in FID hip-

Figure 4. Reduced expression of Egr1 and Egr2 target genes in P65 FID hippocampus. (A-C) mRNA levels of Egr1 targets, Hif1 α (A) and Dusp4 (B). Increased level of phosphorylated ERK1/2 (P-ERK1/2) in FID hippocampus (C). (D and E) Reduced mRNA levels of IGF-II (D) and Mbp (E) in FID hippocampus compared with IS control. Data are normalized to control (IS) group. Values are mean $\pm$ SEM, $n = 4-6$. Asterisks denote significant p values. * $p < 0.05$, ** $p < 0.01$.

Figure 5. Proposed underlying mechanism of lower BDNF activity that leads to decreased neuroplasticity in FID rats. Asterisks denote previously defined factors contributing to lower synaptic plasticity.

pocampal neurons (14,36). DUSP4 (protein) is a dual-specificity phosphatase targeting ERK1 and ERK2, factors phosphorylated during formation of memory (52–54). Reduction of DUSP4 activity could further contribute to impaired synaptic plasticity by dampening neuronal responsiveness to stimulation. Alternatively, sustained ERK1/2 phosphorylation may compensate for already lowered neural plasticity in FID rats.

BDNF-mediated reduction in Egr2 expression resulted in reduced expression of its target genes, Mbp and IGF-II that are important for myelin health and glial contributions to plasticity. Mbp is a complex gene with multiple splice variants, encoding a major component of the myelin sheath of oligodendrocytes. In the adult brain, IGF-II is expressed only in astroglia (55) and regulates myelin-associated protein genes (56). Decreased Mbp and IGF-II expression together may contribute to impairment of myelination and consequently alter neural transmission as seen in P65 FID hippocampus (13). Whole brain Mbp expression is acutely reduced during iron deficiency (57–59); however, the findings in the hippocampus of FID animals provide evidence for a long-term effect of early iron deficiency on the health and function of astrocytes and oligodendrocytes.

The long-term abnormalities in hippocampal function despite iron repletion could be ascribed to changes in structure resulting from lack of iron during critical periods of development or to long-term dysregulation of genes important for experience-dependent plasticity throughout the lifespan. These are not mutually exclusive conditions. We have previously confirmed the former possibility by demonstrating persistently abnormal dendritic structure at P65 in this model (14). Dendritogenesis peaks between P15 and P25 in the hippocampus and iron deficiency during this time period induces substantial alterations in branching and elongation during that time period (14). Complete repletion of hippocampal iron status does not occur in this model until after the end of this critical window and it seems that structural abnormalities induced at the early age remain present in young adulthood.

The current study provides evidence for the second possibility, suggesting a role for iron in long-term programming of hippocampal BDNF and its downstream effectors. The mechanisms mediating the effect are unknown, but may be similar to those involved in the developmental origins of health and disease (60), including epigenetic modifications of genes. Our

results emphasize the concept that provision of nutrients alone is not adequate to maintain optimal brain function. Rather, proper regulation of growth factors is also essential to ensure optimal utilization of nutrients. Long-term dysregulation of these growth factors may thus account for persistent abnormal function despite nutrient repletion.

In summary, our findings begin to provide insights into a possible molecular mechanism underpinning long-term cognitive deficits in this model. In addition, this study suggests that interventions that enhance BDNF activity, such as exercise or selective serotonin reuptake inhibitors (61,62), may be useful as therapeutic approaches to treat long-term effects of fetal-neonatal iron deficiency.

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