Iron Deficiency Impairs Developing Hippocampal Neuron Gene Expression, Energy Metabolism, and Dendrite Complexity

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Abstract
Iron deficiency (ID), with and without anemia, affects an estimated 2 billion people worldwide. ID is particularly deleterious during early-life brain development, leading to long-term neurological impairments including deficits in hippocampus-mediated learning and memory. Neonatal rats with fetal/neonatal ID anemia (IDA) have shorter hippocampal CA1 apical dendrites with disorganized branching. ID-induced dendritic structural abnormalities persist into adulthood despite normalization of the iron status. However, the specific developmental effects of neuronal iron loss on hippocampal neuron dendrite growth and branching are unknown. Embryonic hippocampal neuron cultures were chronically treated with deferoxamine (DFO, an iron chelator) beginning at 3 days in vitro (DIV). Levels of mRNA for \textit{Tfr1} and \textit{Slc11a2}, iron-responsive genes involved in iron uptake, were significantly elevated in DFO-treated cultures at 11DIV and 18DIV, indicating a degree of neuronal ID similar to that seen in rodent ID models. DFO treatment decreased mRNA levels for genes indexing dendritic and synaptic development (i.e. \textit{BdnfVI}, \textit{Camk2a}, \textit{Vamp1}, \textit{Psd95}, \textit{Cfl1}, \textit{Pfn1}, \textit{Pfn2}, and \textit{Gda}) and mitochondrial function (i.e. \textit{Ucp2}, \textit{Pink1}, and \textit{Cox6a1}). At 18DIV, DFO reduced key aspects of energy metabolism including basal respiration, maximal respiration, spare respiratory capacity, ATP production, and glycolytic rate, capacity, and reserve. Sholl analysis revealed a significant decrease in distal dendritic complexity in DFO-treated neurons at both 11DIV and 18DIV. At 11DIV, the length of primary dendrites and the number and length of branches in DFO-treated neurons were reduced. By 18DIV, partial recovery of the dendritic branch number in DFO-treated neurons was counteracted by a significant reduction in the number and length of primary dendrites and the length of branches. Our findings suggest that early neuronal iron loss, at least partially driven through altered mitochondrial function and neuronal energy metabolism, is responsible for the effects of fetal/neonatal ID and IDA on hippocampal neuron dendritic and synaptic maturation. Impairments in these neurodevelopmental processes likely underlie the negative impact of early life ID and IDA on hippocampus-mediated learning and memory.

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Introduction

Iron deficiency (ID) is the most common micronutrient deficiency, affecting an estimated 2 billion people and 40–50% of pregnant women and children worldwide [1, 2]. This is not just a problem in low- and middle-income countries – 18% of pregnant women and 14% of 1- to 2-year-old children in the USA are iron deficient [3, 4]. These population groups are particularly important because the developing fetal and early postnatal brain has greater iron requirements than the mature brain [5]. In both humans and rodents, ID during fetal and early postnatal life impairs learning and memory [6]. Even more concerning is that learning and memory impairments persist into adolescence and adulthood despite prompt iron repletion during infancy, findings supported experimentally in rodent models of fetal/neonatal ID [6].

Declarative learning and memory processing and consolidation depend on proper development of the hippocampus [7]. Hippocampal development, especially neuronal maturation (i.e. axon/dendrite growth and branching and spine/synapse formation), is a metabolically demanding process requiring appropriate levels and intracellular trafficking of iron, ATP, and other energetic substrates [8–11]. Iron provides the catalytic component for several mitochondrial enzymes [12]. Fetal/neonatal ID reduces mitochondrial cytochrome c oxidase activity in a brain-region-specific manner, with the greatest loss of activity occurring in the hippocampus [13] during the early stage of rapid dendrite maturation.

Dendrite and synapse formation is a dynamic process requiring continuous remodeling of the cytoskeleton [14], a process facilitated by actin- and microtubule-associated proteins (e.g. cofilin, profilins, and guanine deaminase) [15–17]. This process also depends on extrinsic signaling from neurotrophins (e.g. brain-derived neurotrophic factor; BDNF) and neurotransmitters [18], which is supported by a variety of synaptic proteins including calcium/calmodulin-dependent protein kinase II-α (CaMKIIα), synaptobrevin 1 (i.e. vesicle-associated membrane kinase 1; VAMP1), postsynaptic density protein 95 (PSD95), and regulators of synaptic protein gene expression (e.g. early growth response protein 1; EGR1) [19–22].

Gene and protein expression for TfR-1 and DMT-1, two iron-responsive proteins that regulate neuronal iron uptake, in the hippocampus increases between postnatal day (P)5 and P15 [23, 24], just prior to the period of rapid hippocampal neuron maturation [25]. Dietary-induced fetal/neonatal ID anemia (IDA) in the rat leads to aberrant apical and basal dendrite branching and thinner spine heads in hippocampal subregion CA1 pyramidal neurons in early postnatal life [26, 27]. CA1 pyramidal neurons from adult formerly IDA rats have shorter branches and a decrease in distal branching despite neonatal iron repletion, indicating that early-life IDA causes long-term impairments in neuronal structure [26]. This manifests electrophysiologically as lower CA1 long-term potentiation expression [28] and behaviorally as impaired performance in spatial and recognition learning/memory tasks in formerly IDA rats [29, 30], suggesting a permanently impaired synaptic function and efficacy.

The rat dietary IDA model is characterized by several secondary pathophysiologies with potential neurotoxic effects, including anemia, tissue hypoxia, hypothyroidism, and divalent metal toxicity [6], making it impossible to determine whether the specific structural defects are due to neuronal iron loss. Genetic mouse models of early-life hippocampal neuron-specific ID, without these potential confounders, are characterized by an abnormal CA1 pyramidal neuron structure accompanied by reduced long-term potentiation and spatial learning/memory deficits in adulthood [8, 31, 32], indicating that the long-term structural and functional deficits seen in IDA rats are specifically due to reduced iron in CA1 pyramidal neurons. However, the specific molecular and cellular effects of hippocampal neuron-specific ID on dendrite growth and branching during the period of rapid hippocampal neuron development are still unknown. Determination of how neuronal iron loss during this early-life period specifically affects dendrite maturation is necessary to reveal the developmental origins of the long-term structural deficits.

We developed a novel culture model of chronic hippocampal neuron ID during peak dendrite development, which generates a similar degree of ID documented in rodent ID models and human iron-deficient newborn infants. Neuronal ID impaired the mRNA expression of genes involved in dendrite and synapse development, as well as mitochondrial function. Both mitochondrial respiration and glycolysis were impaired in iron-deficient neurons. Ultimately, neuronal ID caused an overall reduction in dendritic arborization, with age-dependent differences in the relative contributions of branches and primary dendrites to the total arbor. These data suggest that intrinsic (e.g. energy metabolism) or extrinsic (e.g. synaptic activity) mechanisms may promote dendrite branching in the face of ID and/or iron prioritization to dendrite maturation processes.

ID Impairs Dendrite Maturation
**Materials and Methods**

### Animals

Mice were free access to food and drinking water, and they were housed at a constant temperature and humidity on a 12-hour light:dark cycle. All animal procedures were conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. The local Institutional Animal Care and Use Committee approved these procedures.

### Cell Culture

Primary hippocampal neuron cultures were prepared as previously described [33]. Timed-pregnant CD1 mice were ordered from Charles River Laboratories. The hippocampus was removed from embryonic day 16 (E16) embryos. Pooled hippocampi were incubated at 37°C for 15 min in 0.25% trypsin-EDTA (T4174; Sigma-Aldrich), rinsed 3 times for 5 min in calcium- and magnesium-free Hanks’ balanced salt solution, and resuspended in neuronal plating medium [10 mM HEPES, 10 mM sodium pyruvate, 0.5 mM glutamine, 12.5 μM glutamate, 10% fetal bovine serum, and 0.6% Δ-glucose (Invitrogen); Sigma-Aldrich], rinsed 3 times for 5 min in calcium- and magnesium-free Hanks’ balanced salt solution, and resuspended in neuronal plating medium [10 mM HEPES, 10 mM sodium pyruvate, 0.5 mM glutamine, and 1× penicillin/streptomycin]. Dishes were rinsed 3 times for 5 min in calcium- and magnesium-free Hanks’ balanced salt solution, and resuspended in neuronal plating medium [Neurobasal, 1× B27 (Invitrogen)]. At 3 days in vitro (DIV), the cultures were treated with 67.5 μM 5-fluoro-2‘-deoxyuridine (No. F0503; Sigma-Aldrich)/136 μM uridine (No. U6381; Sigma-Aldrich) (5-FU) to inhibit glia proliferation, allowing a nearly pure neuronal culture. The hippocampus was removed from embryonic day 16 (E16) embryos. Pooled hippocampi were dissociated by trituration with a fire-polished pipette, and then viable cells were counted using trypan blue and a hemocytometer. Cells were plated in neuronal plating medium at 200 cells/mm² in 35-mm dishes containing five 12-mm acid-washed German glass coverslips coated with 100 μg/ml poly-D-lysine and 4 μg/ml laminin. After the cells adhered (1–3 h after plating), the plated medium was replaced with glia-conditioned medium that extended through the major time period of dendrite development, allowing a nearly pure neuronal culture. To create chronic ID control experiments, hippocampal cultures were treated with 10 μM deferoxamine (DFO) (No.14595; Cayman Chemicals), an iron chelator, beginning at 3DIV. Each week, beginning at 7DIV, half of the medium was removed and replaced with fresh glia-conditioned neuronal growth medium containing 1× 5-FU and 1× DFO. To compensate for DFO breakdown, the medium was supplemented with fresh DFO (assuming a 50% breakdown) each week beginning at 10DIV. Cultures were analyzed just after the beginning of dendritic branching (i.e. 11DIV) and during peak branching and synapse formation (i.e. 18DIV) [34].

Postnatal glia cultures were prepared as previously described [35]. The glia medium (10% fetal bovine serum, 2 mM glutamine, and 1× penicillin/streptomycin in EMEM) was changed each week. Glia-conditioned medium was prepared by replacing the glia medium with neural growth medium on confluent glial plates for 24 h. Once the conditioning period was complete, the conditioned medium was removed and replaced with fresh glia medium.

### mRNA Expression Analysis

Total RNA was extracted from hippocampal cultures at 11DIV and 18DIV using a Quick-RNA MicroPrep Kit (Zymo Research) according to the manufacturer’s protocol. Cells were lysed directly in the tissue culture dishes. To eliminate potential genomic DNA contamination, optional on-column digestion of DNA with DNase was performed. RNA integrity and purity were established spectrophotometrically using a NanoDrop spectrophotometer. cDNA was synthesized from 250 or 500 ng of total RNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems) in a 20-μl reaction volume. Quantitative real-time polymerase chain reaction (qPCR) was performed using a 2× FastStart Universal Probe Master (RoX) Kit (Roche Applied Science) and a Stratagene MX3000P qPCR machine. PCR reactions were performed on cDNA equivalent to 10 ng of total RNA according to the manufacturer’s protocol, except that a final volume of 10 μl was used. TaqMan qPCR probes for the genes assessed are described in table 1. Quantification cycle values were determined in the log-linear amplification phase using the qPCR machine’s software. Relative mRNA levels for the genes of interest were calculated relative to an internal calibrator cDNA sample and they were normalized to a reference gene (i.e. TATA box binding protein; Tbp).

### Neuronal Bioenergetics

Eight thousand hippocampal cells were plated in each well of an XF24 cell culture microplate ( Seahorse Biosciences) designed for subsequent bioenergetic analysis. Iron-deficient and iron-sufficient hippocampal neuron cultures were prepared as described above. For these experiments, the DFO concentration was reduced to 6 μM due to increased evaporation in the Seahorse microplate wells compared to 35-mm dishes. At 18DIV, the growth medium was exchanged for Assay Media (unbuffered DMEM plus 25 mM glucose, 1 mM sodium pyruvate, 31 mM NaCl, and 2 mM GlutaMax, pH 7.4 at 37°C) and incubated at 37°C (non-CO₂ incubator) for ~30 min prior to bioenergetic analyses [36]. The real-time oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were simultaneously measured using a Seahorse XF24 Extracellular Flux Analyzer at baseline and following treatment with 1 μM oligomycin (ATP synthase inhibitor), 4 μM FCCP (uncouples oxygen consumption from ATP production), and 1 μM antimycin A/rotenone (ETC complex III and I inhibitors, respectively). The assay protocol is outlined in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000448514). Mitochondrial oxidative phosphorylation is the main determinant of OCR [31]. ECAR is predominantly controlled by lactic acid formation and thus is a specific readout of glycolytic energy metabolism [31]. Key indicators of mitochondrial respiration and glycolysis were calculated, including basal respiration [last baseline OCR – nonmitochondrial respiration (i.e. minimum OCR after antimycin A/rotenone)], maximal respiration (maximum OCR after FCCP – nonmitochondrial respiration), ATP production (last baseline OCR – minimum OCR after oligomycin), spare respiratory capacity (maximal respiration – basal respiration), glycolytic rate (maximum baseline ECAR), glycolytic capacity (maximum ECAR after oligomycin), and glycolytic reserve (glycolytic capacity – glycolytic rate).

### Plasmids and Neuronal Transfection

For neuronal tracing experiments, hrGFP (a ‘humanized’ recombinant green fluorescent protein cDNA from Rotylenchulus reniformis adapted for high-level expression in mammalian cells) was expressed to enable visualization of individual neurons [37]. Briefly, a mammalian expression vector utilizing a CMV-enhanced chicken B-actin promoter to drive hrGFP expression was electro-
Electroporated into hippocampal cells prior to plating using the Lonza Nucleofector II system and an Amaxa Mouse Neuron Nucleofector Kit (No. VAPG-1001; Lonza) as previously described [35]. Electroporated cells were then mixed 1:1 with nonelectroporated cells before plating.

**Immunocytochemistry**

At 11DIV and 18DIV, hippocampal cultures on coverslips were fixed at 37°C with 4% paraformaldehyde/PHEM (60 mM PIPES, pH 7.0; 25 mM K-HEPES, pH 7.0; 10 mM EGTA, and 2 mM MgCl2)/0.12 M sucrose-buffered fixative for 30 min. Coverslips were rinsed 2 times (5 min each) in PBS and then stored in 3% bovine serum albumin (BSA)/PBS at 4°C. The cells were permeabilized using 0.2% Triton X-100 in PBS for 10 min at room temperature, rinsed in PBS for 5 min, and blocked for 30 min in 3% BSA/PBS. The blocking reagent was removed and the cells were incubated in rabbit anti-hrGFP (No. 240142, 1:1,000; Agilent Technologies) and mouse anti-MAP2 (No. ab11268, 1:1,000; Abcam) antibodies overnight at 4°C in a humidified chamber. Coverslips were rinsed in PBS for 5 min and incubated in fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (No. 711-095-152, 1:200; Jackson Immunoresearch) and Alexa Fluor 594-conjugated donkey anti-mouse IgG (No. 715-585-151, 1:200; Jackson Immunoresearch) for 1 h at room temperature in the dark. All antibody mixtures were prepared in 1% BSA/PBS. Coverslips were rinsed for 5 min in PBS, incubated for 30 s in DAPI, and mounted on glass slides with a glycerol-based mounting medium [80% glycerol, 2.5% 1,4-diazabicyclo-(2.2.2)octane, 150 mM Tris; pH 8.0]. Three-channel (hrGFP, MAP2, and DAPI) images of dendritic arbors for individual hrGFP-expressing neurons were collected using a Zeiss Axiovert 200M microscope with a 20× Plan-Apo objective and Openlab software.

**Morphological Analyses**

ImageJ or FIJI was used for all image processing and morphological analyses [38, 39]. For dendrite tracing, MAP2 and hrGFP images for each individual neuron were merged and converted to 8-bit color images. Dendrites were traced using the NeuronJ plugin (http://www.imagescience.org/meijering/software/neuronj/) and quantified using XL-Calculation as previously described [35, 40]. A semiautomated Sholl analysis [41] was performed on the NeuronJ tracings using the Sholl Analysis plugin (http://fiji.sc/Sholl_Analysis). It is estimated that 85–90% of the neurons in the E16 hippocampus are pyramidal neurons [42]. Nonpyramidal neurons were excluded, based on morphology, from dendrite tracing and Sholl analyses.

To determine neuronal density, DAPI and MAP2 images were merged and the number of neuronal nuclei per square millimeter

<table>
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<tr>
<th>Table 1. TaqMan qPCR probes</th>
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<td>Genes</td>
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<td>BdnfVI</td>
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<td>Camk2a</td>
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<td>Cfl1</td>
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<td>Cox6a1</td>
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<td>Egr1</td>
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<td>Psd95</td>
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<td>Slc11a2(Dmt1)</td>
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<td>Ucp2</td>
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<td>Vamp1</td>
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BdnfVI = Brain-derived neurotrophic factor transcript variant VI; Camk2a = calcium/calmodulin-dependent protein kinase IIa; Cfl1 = cofilin; Cox6a1 = cytochrome c oxidase subunit VIII polypeptide 1; Egr1 = early growth response factor 1; Gfa = guanine deaminase; Pfn1 = profilin 1; Pfn2 = profilin 2; Pink1 = PTEN-induced putative kinase 1; Psd95 = post-synaptic density 95; Slc11a2 = solute carrier family 11 member 2; Tbp = TATA box binding protein; Tfr1 = transferrin receptor 1; Ucp2 = uncoupling protein 2; Vamp1 = vesicle-associated membrane protein.
was calculated. Cell health was qualitatively assessed via visual examination of the nuclear morphology [43]. Healthy cells have a relatively equal distribution of chromatin throughout the nuclei (smooth, round nuclei), whereas unhealthy or apoptotic cells undergo nuclear chromatin condensation (crenellated nuclei). In addition, dendrites (MAP2 staining) were visually inspected for signs of poor health such as fasciculation, focal swelling (varicosity), and fragmentation.

**Statistical Analysis**

Statistical analyses and data graphing were carried out using Prism (GraphPad Software) software. Data from 2 or 3 independent cultures were pooled and Student’s t tests were used to determine differences between experimental groups for each parameter. When variances were unequal, ln transformation was used prior to statistical analysis. When variances were unequal and not normalized by ln transformation, the Mann-Whitney U test was utilized. All data are presented as means ± SEM. α = 0.05 was chosen to define significant differences.

**Results**

**Cell Health and Neuronal Density**

MAP2 immunocytochemistry demonstrated healthy neurons with minimal fasciculation, fragmentation, or dendrite swelling throughout both untreated and DFO-treated cultures (fig. 1A shows representative healthy neurons). Based on nuclear morphology, DFO treatment did not alter the percentage of healthy cells (i.e. cells without nuclear chromatin condensation) in 11DIV or 18DIV hippocampal cultures (fig. 1A, insets). The neuron density at 11DIV was not altered by DFO treatment (fig. 1B). However, by 18DIV there was a 29% reduction in neuron density in DFO-treated hippocampal cultures (fig. 1B), suggesting that some neurons died and detached from the coverslip.

**Gene Expression Indexing Iron Status, Neuron Development, and Mitochondrial Function**

The expression of genes/proteins involved in cellular iron uptake is regulated at the level of mRNA stability through iron-regulatory protein binding to iron response element regions, providing sensitive regulation of the cellular iron status [44]. To determine the effect of DFO treatment on the functional, neuronal iron status, qPCR was performed for *Tfr1* and *Slc11a2* (i.e. *Dmt1*), which are iron response element/iron-regulatory protein-regulated genes involved in neuronal iron uptake. Titration of the DFO dosage from 1 to 10 μM demonstrated that 10 μM DFO induces a degree of *Tfr1* and *Slc11a2* upregulation at 18DIV (online suppl. fig. 1) similar to that seen in rodent ID models and human iron-deficient infants [24, 31, 45]. At 11DIV, DFO treatment increased *Tfr1* and *Slc11a2* mRNA levels by 42 and 23%, respectively (fig. 2A). By 18DIV, *Tfr1* and *Slc11a2* mRNA levels were 118 and 43% higher in DFO-treated compared to untreated hippocampal cultures (fig. 2B). DFO has its highest affinity for iron, but it can also chelate other divalent metals (e.g. zinc) [46]. However, DFO treatment did not alter *Znt-1* mRNA levels in 11DIV neurons (online suppl. fig. 2), indicating that the neuronal zinc status was preserved [47].
DFO treatment reduced mRNA levels for genes indexing synaptic plasticity/function and cytoskeletal structural development (i.e. *BdnfVI*, *Camk2a*, *Vamp1*, *Psd95*, and *Egr1*) (C, D), cytoskeletal structural development (i.e. *Cfl1*, *Pfn1*, *Pfn2*, and *Gda*) (E, F), and mitochondrial health (i.e. *Ucp2*, *Pink1*, and *Cox6a1*) (G, H). Relative mRNA levels are calculated relative to an internal control cDNA sample and a reference gene (i.e. *Tbp*). Data from 2–3 independent cultures were pooled and are presented as means ± SEM. The asterisks indicate a statistical difference between groups at a given neuronal age by Student’s t test or the Mann-Whitney U test (p < 0.05). 11DIV: n = 8–13; 18DIV: n = 9.

**Fig. 2.** Effect of DFO treatment on gene expression. Hippocampal neurons cultured from E16 mice were treated with DFO and 5-FU beginning at 3DIV. At 11DIV and 18DIV, cells were collected, total RNA was extracted, and cDNA was synthesized. qPCR was performed for genes indexing neuronal iron status (i.e. *Tfr1* and *Slc11a2*) (A, B), synaptic development, function, and plasticity (i.e. *BdnfVI*, *Camk2a*, *Vamp1*, *Psd95*, and *Egr1*) (C, D), cytoskeletal structural development (i.e. *Cfl1*, *Pfn1*, *Pfn2*, and *Gda*) (E, F), and mitochondrial health (i.e. *Ucp2*, *Pink1*, and *Cox6a1*) (G, H). Relative mRNA levels are calculated relative to an internal control cDNA sample and a reference gene (i.e. *Tbp*). Data from 2–3 independent cultures were pooled and are presented as means ± SEM. The asterisks indicate a statistical difference between groups at a given neuronal age by Student’s t test or the Mann-Whitney U test (p < 0.05). 11DIV: n = 8–13; 18DIV: n = 9.
Pfn1 mRNA levels were 12% lower at 11DIV, but they were not significantly altered at 18DIV. As a preliminary measure of the effect of neuronal ID on mitochondrial function, mRNA levels were quantified for genes encoding mitochondrial proteins important for the regulation of oxidative stress (i.e. Ucp2) [48], mitophagy (i.e. Pink1) [49], and electron transport (i.e. Cox6a1) [50]. Ucp2 and Pink1 mRNA levels were 31 and 41% lower, respectively, in 11DIV DFO-treated cultures, but they were no longer altered in 18DIV DFO-treated cultures (fig. 2 G, H). Cox6a1 mRNA levels were ∼18% lower in both 11DIV and 18DIV DFO-treated cultures (fig. 2 G, H).

**Neuronal Energy Metabolism**

To further assess the effect of neuronal ID on mitochondrial function, real-time OCR and ECAR were measured at baseline and following oligomycin, FCCP, and antimycin A/rotenone treatments (fig. 3A). DFO treatment impaired overall mitochondrial respiration (fig. 3A), reducing basal respiration, maximal respiration, ATP production, and spare respiratory capacity by ∼70% (fig. 3B) without affecting ATP coupling efficiency (data not shown). Likewise, neuronal ID significantly reduced glycolytic rate (40% lower) and capacity (84% lower) with a trend towards less glycolytic reserve (fig. 3C).

**Hippocampal Pyramidal Neuron Dendrite Morphology**

Representative images (and dendrite tracings) of hrGFP-labeled hippocampal neurons from untreated and DFO-treated cultures are shown in figure 4A and B. Sholl analysis revealed a significant decrease in overall distal dendritic arbor complexity in DFO-treated neurons (fig. 4C, D). The number of crossings was significantly lower between 70 and 170 μm at 11DIV and between 90 and 210 μm at 18DIV.

To distinguish between the effects on dendrite number and/or lengths, a detailed analysis of dendrite tracings (fig. 4Ac, Ad, Bc, Bd) was performed. The average number of primary dendrites per neuron (fig. 5A) was not altered at 11DIV but it was 11% lower in DFO-treated neurons at 18DIV (4.98 ± 0.13 in untreated group vs. 4.41 ± 0.12 in DFO-treated group). DFO treatment reduced the length of primary dendrites by 20 and 17% at 11DIV and 18DIV, respectively (fig. 5B). The length of the longest dendrite (an in vitro surrogate for pyramidal neuron apical dendrite length) was 19 and 14% shorter in 11DIV and 18DIV DFO-treated neurons (fig. 5C).

The total number of dendrite branches was 28% lower in DFO-treated neurons at 11DIV, but it was no longer significantly different at 18DIV (fig. 6A). However, DFO treatment reduced the branch length by 20 and 13% at 11DIV and 18DIV, respectively (fig. 6D). Secondary and
tertiary branches were similarly affected by DFO treatment at 11DIV but not at 18DIV. At 11DIV, DFO treatment resulted in 25% fewer and 21% shorter secondary branches and 38% fewer and 36% shorter tertiary branches (fig. 6B, C, E, F). At 18DIV, there was a 14% reduction in secondary branch length (fig. 5E) and a trend (p = 0.06) toward a reduced secondary branch number (fig. 6B) in DFO-treated neurons. There was no significant differ-
ence in tertiary branch number or length at 18DIV (fig. 6C, F). The sum length of all branches was significantly less in both 11DIV (41%) and 18DIV (22%) DFO-treated neurons (fig. 7A). To give an indication of the total dendrite output of DFO-treated and untreated neurons, the sum length of all dendrites (primary, secondary, and tertiary) was calculated. DFO treatment reduced the sum dendrite length by 33 and 25% at 11DIV and 18DIV, respectively (fig. 7B).

**Discussion**

In order to determine the specific effects of neuronal ID on hippocampal neuron dendrite structure during the period of rapid dendrite maturation, we developed a novel primary hippocampal neuron culture model of chronic ID. Chronic iron chelation with DFO allows easy manipulation of the amount of iron available to neurons without modification of neuronal growth medium that is already optimized for long-term survival of hippocampal neuron cultures. It is also preferable over culture of neurons from transgenic mice with hippocampal neuron-specific ID where cells from individual embryos would have to be cultured separately (a prohibitively time-consuming process) to ensure genotype homogeneity. A limitation of this approach is that DFO, despite having its highest affinity for iron, can also chelate other divalent cations (e.g. zinc) at high DFO doses [46]. To account for this, we show that zinc transporter-1 mRNA levels, a measure of cellular zinc status [47], were not altered in 10 μM DFO-treated 11DIV neurons. This culture model of neuronal ID showed a 2-fold increase in Tfr1 mRNA levels at 18DIV (fig. 2B), a time point approximately equivalent to P15, in vivo. This magnitude of Tfr1 upregulation is similar to the effect seen in P15 rats following dietary-induced fetal/neonatal IDA [24] and in nonanemic P15 mice with a late fetal, neuronal specific knockout of DMT-1 [31], indicating a degree of ID similar to that seen in in vivo models that demonstrate dendrite structure ab-
normalities in the hippocampus. The P15 IDA rat has a 60–70% loss of hippocampal iron content and a 40% loss of whole brain iron content [24, 28, 51], which is commensurate with the degree of brain ID documented in human newborns [45]. The altered mRNA expression for genes involved in synaptic development and function (i.e. BdnfVI, Camk2a, Vamp1, and Psd95) and actin and microtubule dynamics (i.e. Cfl1, Pfn1, Pfn2, and Gda) is consistent with alterations in these pathways in the neonatal rodent hippocampus following early-life ID with or without anemia [8, 24, 26, 31, 52]. Thus, our culture model of chronic hippocampal neuron ID during development provides a physiologically and translationally relevant model for the study of the molecular, cellular, and structural developmental processes affected by neuronal iron loss.

Declarative learning and memory processes depend on the proper functioning of hippocampus-dependent circuits [7], which in turn are dependent on the degree of dendritic arborization available for synaptic connectivity [53]. Prior to this study, the specific effect of neuronal iron loss on hippocampal neuron dendrite growth and branching was unknown since previous analyses were qualitative and only assessed in adults. Adult transgenic mice with hippocampal neuron-specific ID [knockout of Slc11a2 (i.e. DMT-1) or conditional overexpression of a dominant negative form of TfR1 beginning on E18.5] have an aberrant CA1 dendritic arbor organization [8, 31]. In the dominant negative TfR1 model, these structural impairments remain present with late iron repletion (i.e. P42) but are rescued with early iron repletion (i.e. P21), indicating a critical postnatal period for intervention [8]. Consistent with the neonatal effects of nutritional IDA on hippocampal neuron dendrite arborization [26, 27, 51], our 11DIV and 18DIV morphology data demonstrate that chronic neuronal ID during the proposed critical period [6, 8] blunts dendritic growth and branching, resulting in an overall reduced dendritic field in developing iron-deficient neurons. Thus, the long-term defects in hippocampal neuron dendritic arborization observed in both IDA and neuronal-specific ID models [8, 26, 31, 51] likely stem from chronic neonatal ID impairing dendrite growth and branching during a period of rapid maturation early in life. These findings also indicate that the early-life effects of ID on dendrite branching are driven by a loss of neuronal iron without the confounding effects of anemia and whole body/brain ID. This demonstrates that the neuron is the site of action and a potential, specific target for intervention, which is important translationally as nonanemic ID is 2–3 times more common than IDA in children [2] and is associated with impaired auditory cortex electrophysiology and recognition memory in human infants [54]. In addition, a low maternal iron intake during pregnancy is associated with impaired gray matter organization in infant offspring, perhaps driven through early-life iron-dependent deficits in dendritic growth and branching [55].

Between 11DIV and 18DIV, chronically iron-deficient neurons appeared to adapt their structural development to the lack of iron. At 18DIV, compared to 11DIV, there
were slightly less severe effects of DFO treatment on neurodevelopmental gene transcript levels and some measures of dendrite branching despite a greater degree of ID. In particular, the number of dendrite branches recovered to near control levels between 11DIV and 18DIV. However at 18DIV, branches remained shorter and there was an accompanying significant decrease in the number of primary dendrites. This suggests either a process of primary dendrite retraction or that iron-deficient neurons with fewer primary dendrites are more likely to survive, as the neuronal density was significantly lower in iron-deficient neurons at 18DIV but not at 11DIV. These data are consistent with prioritization of the limited iron to branch and synapse maturation (at the expense of neuronal and primary dendrite numbers) between 11DIV and 18DIV, when these important neurodevelopmental processes are ramping up.

The mechanisms underlying this prioritization phenomenon may be cell intrinsic or extrinsic. Cell intrinsically, mitochondrial respiration and energy metabolism are potential candidates, as mitochondrial function is directly dependent on iron and it is also critical for dendrite growth [9]. Abnormalities in hippocampal energy metabolism have been documented in the IDA rat with iron deficiency [13, 24, 56] and in adulthood long after iron repletion [57]. Our neuronal bioenergetics findings have demonstrated for the first time that neuronal ID impairs both mitochondrial respiration and glycolysis, indicating complete blunting of the neuronal energetic capacity. These data also suggest that neuronal iron loss, and not anemia or whole body/brain ID, is responsible for these ID-induced hippocampal energy metabolism deficits. Our 11DIV gene expression data demonstrate impairments in mRNA levels for genes implicated in oxidative stress (i.e. Ucp2) and mitophagy (i.e. Pink1), two other important mitochondrially regulated pathways. Ucp2 and Pink1 mRNA levels had recovered to control levels by 18DIV, leading us to speculate that homeostatic mechanisms regulating mitochondrial health (including mitophagy and regulation of mitochondrial reactive oxygen species) may be responsible for the partial recovery of dendrite branching observed in 18DIV iron-deficient neurons. At the same time, impaired mitochondria energy production likely contributes to the continued deficits in dendrite growth in iron-deficient neurons.

Hippocampal neuron dendritic branching is also highly dependent on afferent innervation, especially after the first week in culture [58]. In addition, both axonal arborization and functional synapse formation increase during development, peaking between 14DIV and 21DIV [34, 59]. The fact that Pink1, which regulates dendritic spine formation but not dendritic arborization [16], was the lone cytoskeletal gene altered by DFO at 11DIV to completely recover at 18DIV implies that extrinsic signaling mechanisms may also contribute to the observed compensation. It is possible that intercellular connection-dependent dendrite maturation is not altered in iron-deficient neuronal cultures, accounting for the partial recovery observed between 11DIV and 18DIV. Despite these potential homeostatic mechanisms, iron-deficient neurons remain fundamentally abnormal during this developmental time period, with blunted dendritic fields and impaired neurodevelopmental gene expression.

The most troubling aspect of early-life ID is that the cognitive deficits (including learning and memory impairments) persist into adulthood despite iron treatment in infancy, implying that iron therapy alone is not sufficient for full recovery [6]. Understanding the iron-dependent molecular/cellular mechanisms that directly cause early-life neurological dysfunction will allow future design of more effective therapies to prevent the long-lasting effects of early-life ID. Our findings with a novel culture model of chronic hippocampal neuron ID will allow further interrogation of the specific iron-dependent mechanisms (e.g. mitochondrial respiration) underlying the effects of neuronal iron loss on dendrite maturation.

Acknowledgements

We thank the members of the Georgieff and Lanier labs for their invaluable assistance with culture preparation and tracing analysis. In particular, we would like to thank Justin Campagna, Lanka Dasanayaka, and George Michalopoulos. Grants supporting this research included NIH R01 HD029421.

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