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To cite this article: Ashley B. Breton, Julia A. Fox, Mary P. Brownson & Matthew D. McEchron (2015) Postnatal nutritional iron deficiency impairs dopaminergic-mediated synaptic plasticity in the CA1 area of the hippocampus, *Nutritional Neuroscience*, 18:6, 241-247, DOI: 10.1179/1476830514Y.0000000121

To link to this article: <http://dx.doi.org/10.1179/1476830514Y.0000000121>



Published online: 28 Mar 2014.



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Postnatal nutritional iron deficiency impairs dopaminergic-mediated synaptic plasticity in the CA1 area of the hippocampus

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Objectives: Developmental iron deficiency (ID) has been shown to put children at risk for compromised learning and memory capacity, and it has also been shown to impair hippocampus-dependent forms of memory as well as hippocampal synaptic transmission. Catecholamines are known to play a pivotal role in memory consolidation, and studies have demonstrated that perinatal ID alters dopaminergic systems in various brain areas. It is not known, however, whether perinatal ID impairs dopaminergic synaptic plasticity in learning and memory structures such as the hippocampus. The objective of the present study was to examine dopaminergic-mediated synaptic efficacy in the hippocampus of mice subjected to an ID or control (CN) diet.

Methods: The present study used electrophysiological brain slice methods to examine dopaminergic-mediated synaptic efficacy in the hippocampus of mice subjected to an ID or CN diet from postnatal day (P) P0 through P20. Hippocampal brain slices were prepared in young (P26–30) and adult animals (P60–64). Synaptic efficacy was measured in CA1 neurons by examining population spike amplitude. Slices were treated with the dopaminergic agonist SKF-38393.

Results: Slices obtained from young and adult CN mice exhibited a long-lasting increase in synaptic efficacy as the result of SKF-38393 perfusion while the young and adult ID slices showed little or no increase.

Discussion: The present study demonstrates that postnatal ID produces long-lasting impairments in dopaminergic-dependent synaptic plasticity in the hippocampus. These impairments may play a role in the learning and memory deficits known to result from ID.

Keywords: Iron, Deficiency, Postnatal, Hippocampus, Learning, Dopamine, Catecholamine, Synaptic

Introduction

Developmental iron deficiency (ID) is the leading nutritional disorder in the world, affecting one in every four to five babies in developing countries.¹ A study conducted in the United States demonstrated that 90% of pregnant women reported consuming less than two-third of the recommended daily allowance of iron, and 22% were classified as having ID anemia.² These findings raise serious concerns about developmental iron nutrition because many studies have demonstrated that early periods of ID can put children at risk for compromised learning and memory capacity.³

The hippocampus is a brain structure that is critical for the learning and storage of memories, and may be particularly vulnerable to perinatal ID. For example, previous work has demonstrated that weanling rats

placed on an ID diet for 2 weeks exhibited reduced iron concentrations in the hippocampus, while other brain regions showed more resistance to iron depletion.⁴ The same study also revealed that developmental ID produced a greater increase in transferrin levels in the hippocampus, when compared with other brain regions, suggesting that the hippocampus may be especially vulnerable to early ID.⁴ In addition, perinatal ID has also been shown to produce altered synaptic function, dendritic growth, and synapse formation in the hippocampus.⁵ Animal studies have demonstrated that perinatal nutritional ID impairs hippocampus-dependent forms of memory.^{6,7} A number of these studies have also demonstrated that perinatal ID may produce irreversible long-term deficits in hippocampus-dependent learning.^{6,8}

Catecholamines, such as norepinephrine and dopamine, are known to play a pivotal role in memory consolidation.^{9,10} Systemic infusion of dopamine antagonists has been shown to impair memory

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consolidation in a number of learning tasks.^{11,12} Dopamine agonists have been shown to facilitate memory consolidation in several learning tasks, including hippocampus-dependent learning tasks.^{11,12} These findings demonstrate that dopamine plays an important role in memory consolidation.

Dopamine is also an important modulator of synaptic plasticity in the CA1 region of the hippocampus. A number of studies have demonstrated that application of the D1/D5 receptor agonist, SKF-38393, induces long-term potentiation of field excitatory postsynaptic potentials in the CA1 region of hippocampal slices.^{13,14} Similarly, D1/D5 receptor activation has been shown to facilitate tetanic-dependent synaptic potentiation.¹⁴ In a parallel fashion, hippocampal synaptic potentiation is blocked by administration of dopamine D1/D5 receptor antagonists.¹⁴

Previous studies have demonstrated that perinatal ID alters dopaminergic systems in various brain areas^{15,16}; however, none of these studies have focused specifically on the hippocampus. For example, monoamine synthesis and catabolism appear to be altered in overall brain tissue as a result of ID.¹⁵ Other work has demonstrated that ID during early lactation causes an up-regulation of the neurotransmitter dopamine, with the most notable differences seen in the caudate putamen.¹⁷ These findings suggest that dopaminergic systems may be particularly sensitive to early periods of ID.

Recent work has demonstrated that perinatal nutritional ID impairs noradrenergic-mediated increases in synaptic efficacy in the CA1 region of the hippocampus of rats.⁸ It is not known, however, whether perinatal ID impairs dopaminergic-mediated synaptic plasticity in the hippocampus. Therefore, the goal of this study was to determine if postnatal ID impairs dopaminergic-mediated synaptic transmission within the hippocampus, and to determine if these impairments in dopamine function are long lasting and permanent. Experiments in this study also sought to determine if postnatal ID impairs synaptic efficacy induced by activation of intracellular cAMP, a downstream mechanism of postsynaptic dopamine receptor activation.¹⁸

Materials and methods

All procedures used in this study were approved by The University of Wyoming Animal Care and Use Committee and were in accordance with NIH Guide for the Care and Use of Laboratory Animals.

Subjects and diets

Matched pairs of iron deficient (ID) and control (CN) diet C57/BL6 male mouse pups were used in this study. Mouse pups were bred within the animal vivarium at the University of Wyoming. The ID and CN

diets were purchased from Research Diets (New Brunswick, NJ). The iron content of the ID diet was 3 ppm whereas the CN diet was 90 ppm. Prior to breeding and during gestation dams were maintained on a CN diet. Dams were placed on an ID or CN diet on postnatal day 0 (P0) and this diet was maintained through P20. All animals were placed on the CN diet on P21 and this diet was maintained until animals were sacrificed for electrophysiological recordings. All pups were weaned on P21. Each CN or ID group in this study was formed from a minimum of seven litters, and only male mice were used for the data in this study.

Hematocrit collection

During decapitation blood was collected to determine the percentage of red blood cells in serum. Blood was collected at P26–30 and P60–64, the two age groups used for hippocampal slice recording. Blood was also collected at P20, 1 day before all animals were placed on the CN diet. The P20 mice were randomly selected from the litters used to form the slice recording groups; however, the P20 mice were not used for any slice preparation. Blood was collected from each animal in two 75 mm heparinized capillary tubes and centrifuged (IEC MB Centrifuge, Needham Heights, MA) for 4 minutes. The mean percentage of red blood cells was calculated from the two capillary tubes.

Hippocampal slice preparation

Efforts were made to use matched pairs of ID and CN mice on the day of electrophysiological recording. Matched pairs of mice were used on 50% of the recording days, while only individual ID or CN pups were used on the remaining 50% of recording days. Slice preparation occurred at either a young age (P26–30) or an adult age (P60–64), and the researcher performing the recordings (ABB) was blind to treatment group (i.e. CN or ID) at the time of all recordings. Prior to brain extraction mouse pups were deeply anesthetized using isoflurane (MWI Veterinary Supply, Meridian, ID) in 100% O₂. Mice were then decapitated. The brain was removed quickly (~25–30 seconds) and placed in ice cold pre-aerated (95% O₂, 5% CO₂) dissecting artificial cerebrospinal fluid (ACSF) solution. Each hippocampus was dissected from the brain and transverse slices (400–500 μ m thickness) were prepared through the hippocampus with a vibrating slicer. Slices were placed in aerated ACSF at room temperature for at least 1 hour prior to recording.

Extracellular population spike recordings

During electrophysiological recordings, hippocampal slices were submerged in a recording chamber and continuously perfused at 2.2 ml/min with aerated ACSF at room temperature (~23°C). A computer

controlled analog stimulus isolation unit (A-M Systems 2200; Carlsborg, WA) was used to deliver constant current bipolar pulses (0.1 ms/phase) through two Teflon insulated tungsten wires (76 μ m diameter each) bonded together. Stimulation was delivered to the Schaffer collaterals in order to evoke field potentials in the CA1 region. Somatic field potentials were recorded extracellularly with a glass pipette filled with 9% NaCl. Field potentials were amplified (1000 \times ; A-M Systems Model 1800; Carlsborg, WA), filtered between 300 Hz and 5 kHz, and sampled by a DT3010 data acquisition board (Data Translation; Marlboro, MA) at 20 kHz.

Measures of synaptic efficacy were based on the population spike amplitude of the CA1 somatic field potential. The amplitude of the population spike was defined as the difference between the peak negative voltage and the subsequent peak positive voltage. The input–output (IO) curves were measured by delivering a range of current intensities between 100 and 800 μ A in 50 μ A steps (30 seconds between). Following the IO curve measurement, slices were examined for responses to various drugs. During all subsequent recordings stimulation pulses were delivered at an intensity of 50% of the maximum population spike amplitude observed during the IO curve measurement. Pulses were delivered every 30 seconds for a 10 minute baseline period, followed by a 10 minute drug perfusion period, then a 60 minute washout period with ACSF alone in the bath. Several slices from each group depotentiated by more than 25% below baseline as the result of drug infusion into the bath (range 2–8 slices per group). There was no significant group difference in the number of slices that depotentiated. Thus, slices that depotentiated by 25% were not included in the final analyses.

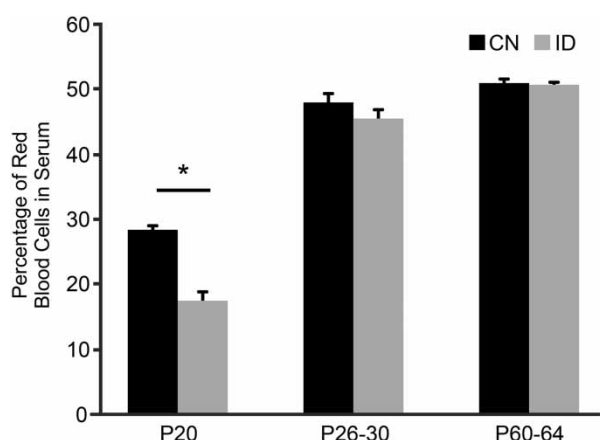


Figure 1 Hematocrit measures showing percentage of red blood cells in serum from P20, P26–30, and P60–64 mice. Values are means \pm SEM. Asterisk indicates differences between CN and ID animals, $P = 0.0001$.

Solutions and drugs

All drug concentrations used in this study were based on previous reports of response efficacy^{14,18} and pilot testing in our laboratory to achieve the maximum amount of potentiation of population-spike amplitude. Dopaminergic-dependent synaptic plasticity was examined using the dopamine receptor agonist (\pm) – SKF-38393 hydro-chloride (SKF; Sigma, St Louis, MO). SKF was prepared daily and used at a final concentration of 20 μ M for P26–30 mice and 40 μ M for P60–64 day mice. cAMP-dependent synaptic plasticity was examined using a mixture of forskolin and rolipram (FSK + R; 25 μ M forskolin and 50 μ M rolipram in ACSF). Forskolin (Sigma, St Louis, MO) was prepared in a stock solution (50 mM, in DMSO) and stored at -80°C until used at a final concentration of 25 μ M in the bath ACSF. Rolipram (Sigma, St Louis, MO) stock solution (0.1 mM, in distilled water) was stored at -80°C until used at final concentration of 50 μ M in the bath ACSF with forskolin.

The dissecting ACSF solution for the SKF experiment consisted of (in mM) 1.2 NaH_2PO_4 , 3.6 KCl, 25 NaHCO_3 , 11.6 glucose, 1.2 MgCl_2 , 234 sucrose, and 2.5 CaCl_2 , at pH 7.4. The standard ACSF bath used for the SKF experiment was composed of (in mM) 125 NaCl, 1.1 NaH_2PO_4 , 2.5 KCl, 26.0 NaHCO_3 , 24.2 glucose, 1.0 MgCl_2 , and 2.7 CaCl_2 , at pH 7.4. For the FSK + R experiment the dissecting solution contained 1.2 MgCl_2 and 1.3 CaCl_2 , while all other concentrations remained the same. The standard ACSF bath for the FSK + R experiment contained 0.5 MgCl_2 and 5.3 CaCl_2 (in mM), while all other concentrations remained the same.

Statistical analyses

Measures of synaptic efficacy were averaged into a baseline period preceding drug perfusion, a drug perfusion period, and six periods following drug perfusion. Repeated measures ANOVAs were used to examine measures of synaptic efficacy using an eight level repeated measure factor (baseline, drug, and six periods post-drug), and a between group factor with two levels (CN and ID). Significant interactions were followed up with Newman–Keuls *post-hoc* tests. Hematocrit values were analyzed using a standard *t*-test. An alpha level of 0.05 was used to determine significance for all analyses. Statview software v5.0 (Cary, NC) was used for all analyses.

Results

ID and anemia

Measurements of ID anemia were assessed using hematocrit (i.e. percent red blood cells in serum) values obtained during different stages of postnatal development. Hematocrits were obtained from ID ($n = 10$

pups from six litters) and CN ($n = 10$ pups from five litters) pups at P20. The P20 time point reflects the level of anemia the day before the ID animals were switched back to the CN diet. The hematocrit values obtained at P20 revealed that 20 days of postnatal ID produces a significant level of anemia when ID pups were compared with CN pups (Fig. 1), $t(18) = 6.78$, $P = 0.0001$. Hematocrits were also obtained from ID ($n = 18$ pups from six litters) and CN ($n = 17$ pups from eight litters) mice at P26–30. The P26–30 time point reflects the level of anemia 6–10 days after ID diets were switched to a normal iron CN diet. The P26–30 mark also reflects the level of anemia on the approximate day that young slices were prepared. These hematocrit values revealed that pups subjected to early postnatal ID were no longer anemic at P26–30 when compared with the CN pups (Fig. 1), $t(33) = 1.39$, $P = 0.18$. Finally, hematocrit

values were obtained from ID ($n = 15$ pups from eight litters) and CN ($n = 15$ pups from 10 litters) P60–64 mice. This time point reflects the level of anemia approximately 40 days after ID diets were switched to a normal CN diet. The P60–64 mark also reflects the level of anemia on the approximate date that the adult slices were prepared. Hematocrit values obtained at P60–64 revealed that adult mice subjected to early postnatal ID were not anemic when compared with the CN adults (Fig. 1), $t(28) = 0.64$, $P = 0.53$.

ID impairs dopaminergic-dependent synaptic plasticity

Young (P26–30) hippocampal slices were subjected to SKF perfusion to examine dopaminergic-mediated increases in synaptic efficacy. Postnatal ID, implemented from P0 through P20, impaired SKF-induced increases in synaptic efficacy in the CA1 region of young hippocampal slices. Specifically, perfusion of 20 μM of SKF resulted in an increase in population spike amplitude in CN slices but the same concentration produced only a slight increase in population spike amplitude in ID slices which quickly returned to baseline (Fig. 2A). An ANOVA with the factors diet (CN and ID) and periods (eight periods) revealed a significant interaction, $F(7,161) = 3.53$, $P = 0.0015$. Follow-up tests revealed a significant group difference during the drug perfusion period and the third and fourth post-drug washout periods.

Adult (P60–64) hippocampal slices were subjected to SKF perfusion to determine if the impairments in synaptic efficacy in the young ID group in Fig. 1A could be reversed with a normal iron diet extending into adulthood. These adult animals received the same dietary protocol as the young animals, ID from P0 through P20, followed by a return to a normal iron diet. Similar to young slices, postnatal ID impaired SKF-induced increases in synaptic efficacy in the CA1 region of adult slices. Moreover, perfusion of 40 μM of SKF resulted in increases in population spike amplitude in adult CN slices but the same concentration resulted in no change in spike amplitude in adult ID slices (Fig. 2B). An ANOVA with the factors diet and periods revealed a significant interaction, $F(7,126) = 2.83$, $P = 0.0086$. Follow-up tests revealed a significant group difference during the final four washout periods with the CN slices showing larger population spike amplitudes when compared with the ID group.

ID does not impair cAMP-dependent synaptic plasticity

We sought to determine if postnatal ID impaired cAMP-dependent potentiation. Application of FSK + R produced a sharp increase in population

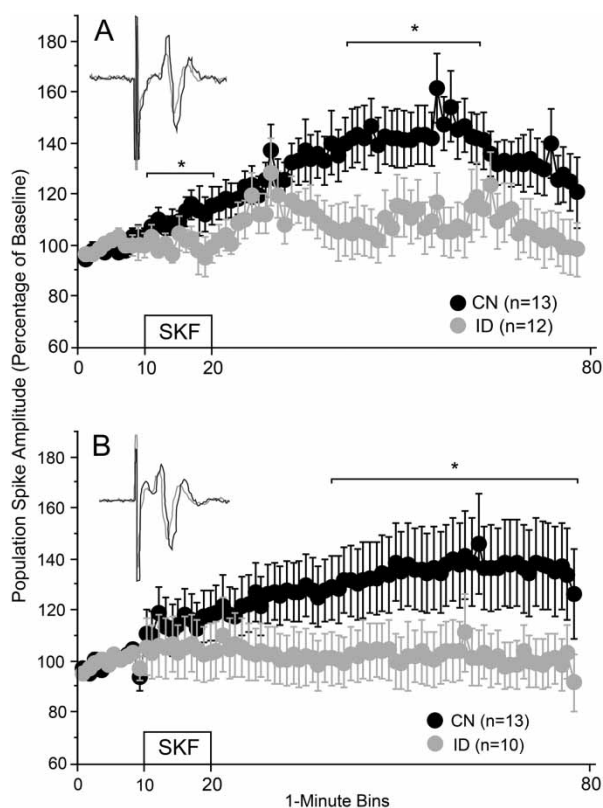


Figure 2 Population spike amplitude recorded from the CA1 area of hippocampal slices from young P26–30 male mice (A) and adult P60–64 male mice (B) before, during, and after perfusion SKF (20 μM in A and 40 μM in B). The 10 minutes SKF perfusion period is indicated by the box starting at the 10 minutes mark. Insets show representative population spikes before SKF perfusion (light) and at the end of the washout period (dark). Values are means \pm SEM. The CN group in panel A was formed from 13 slices obtained from seven litters. The ID group in panel A was formed from 12 slices obtained from seven litters. The CN group in panel B was formed from 13 slices obtained from 10 litters. The ID group in panel B was formed from 10 slices obtained from seven litters. Asterisk indicates different from CN at these times, $P < 0.05$.

spike amplitude in the young (P26–30) CN slices. Although more gradual, FSK + R also produced an increase in the population spike amplitude in the ID slices (Fig. 3A). An ANOVA with the factors diet and period did not reveal an interaction, $F(7,119) = 1.46$, $P = 0.19$, or a main effect $F(1,119) = 4.10$, $P = 0.06$. Although significance was not achieved, there appeared to be group differences during the drug delivery period. A follow-up t -test revealed a group difference during the drug delivery period with a larger population spike amplitude in the CN slices when compared with ID slices, $P = 0.0001$.

We also sought to determine if cAMP-dependent potentiation was impaired in adult (P60–64) animals subjected to early postnatal ID (i.e., ID from P0 through P20). Application of FSK + R produced a similar sharp increase in population spike amplitude in the adult CN and ID slices (Fig. 3B). An ANOVA

with the factors diet and period revealed no interaction, $F(7,126) = 0.714$, $P = 0.66$, or main effect of treatment, $F(1,126) = 1.04$, $P = 0.32$. In summary, the data from Fig. 2 suggest that the impairments in dopaminergic-dependent synaptic plasticity in the ID animals shown in Fig. 1 were not due to impairments in intracellular cAMP signaling, but rather were due to impairments at the level of the dopamine receptor.

Discussion

This study sought to determine if early postnatal ID produced impairments in dopaminergic-mediated synaptic efficacy in the hippocampus. Postnatal nutritional ID reduced dopaminergic-mediated increases in synaptic efficacy in the CA1 region of the hippocampus of young mice tested between P26 and P30. These results suggest that early exposure to ID results in impaired dopaminergic-mediated synaptic efficacy. Dopamine has been shown to be a critical neurotransmitter for the consolidation of memories.^{11,12} Thus, the results of the present study provide evidence that early periods of ID may reduce learning and memory capacity through dopaminergic or catecholaminergic mechanisms within the hippocampus.

This study also sought to determine if early postnatal ID produced long-lasting adulthood impairments in dopaminergic-mediated synaptic efficacy. Adult mice age P60–64 subjected to ID from P0 through P20 continued to show impaired dopaminergic-mediated synaptic efficacy, despite being placed back on a CN diet for over 40 days. Our previous work has demonstrated that similar periods of nutritional ID produce long-term impairments in hippocampus-dependent learning in adult rats.^{6,8} The findings in the present study suggest that postnatal ID can produce irreversible and persistent impairments in dopaminergic-mediated synaptic efficacy in the hippocampus.

The present study may provide one of the few examples of a physiological mechanism which is impaired by early periods of ID and which remains impaired into adulthood. Only a handful of other studies have identified similar physiological mechanisms which are impaired by developmental nutritional ID and continue to show impairments into adulthood. For example, Georgieff's group conducted a study in which rats were maintained on an ID diet from gestational day 2 until P7, then animals were returned to a CN diet. Using this model of nutritional ID they obtained field excitatory postsynaptic potentials from CA1 and found deficits in synaptic function and efficacy when measured at P65.⁵ Their study also revealed that this model of ID produced reductions in hippocampal iron concentrations when measured at P30, but not at P65. Their work raises the possibility that

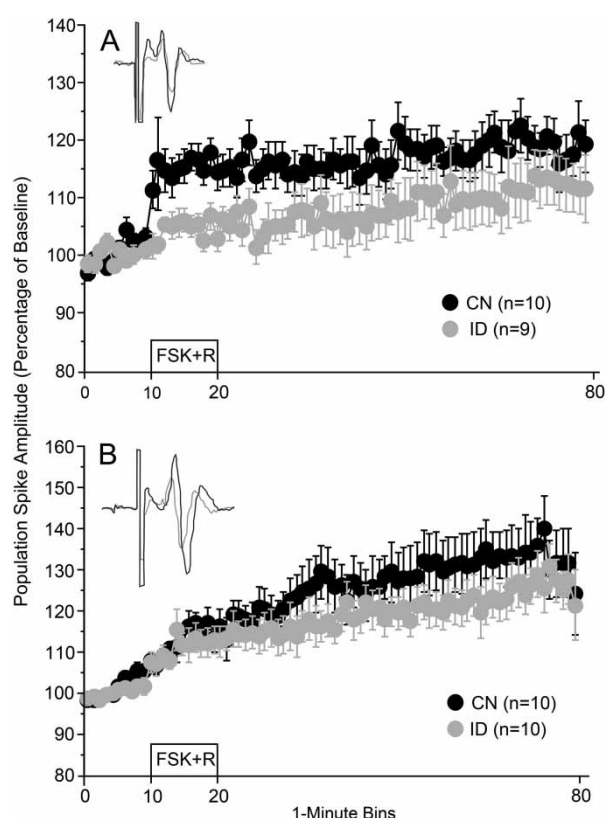


Figure 3 Population spike amplitude recorded from the CA1 area of hippocampal slices from young P26–30 male mice (panel A) and adult P60–64 male mice (panel B) before, during, and after perfusion of forskolin and rolipram (FSK + R). The 10 minutes FSK + R perfusion period is indicated by the box starting at the 10 minutes mark. Insets show representative population spikes before FSK + R perfusion (light) and at the end of the wash out period (dark). Values are means \pm SEM. The CN group in panel A was formed from 10 slices obtained from seven litters. The ID group in panel A was formed from nine slices obtained from seven litters. The CN group in panel B was formed from 10 slices obtained from seven litters. The ID group in panel B was formed from 10 slices obtained from seven litters.

the reductions in dopaminergic synaptic plasticity in the ID hippocampus in the present study may have been modulated by alterations in hippocampal synaptic efficacy or perhaps reduced hippocampal iron concentrations. Unfortunately, hippocampal iron concentrations were not measured in the present study. Another study by the Georgieff group used a similar model of gestational nutritional ID in rats and measured dendritic branching in CA1 and messenger RNA transcript levels of proteins that modify actin and tubulin dynamics.¹⁹ Their study demonstrated that early gestational ID results in a proximal shift in dendritic branching in CA1 at P65 along with a reduction in transcript levels of proteins that modify actin and tubulin. Their findings raise the possibility that the reductions in dopaminergic synaptic plasticity in the ID hippocampus in the present study may have been modulated by altered dendritic structure.

The present study adds to the growing body of evidence suggesting that iron nutrition is critical for the development of normal catecholamine function in the brain.^{8,15,16,20,21} One of our previous investigations used an approach similar to the present study to demonstrate that perinatal ID impairs noradrenergic-dependent synaptic efficacy in the hippocampus.⁸ Unlike the dopamine results in the present study, however, our previous norepinephrine study suggested that perinatal ID may not produce permanent and irreversible deficits in noradrenergic-dependent synaptic efficacy. Thus, the present study along with our previous work may suggest that dopaminergic synaptic mechanisms are more sensitive and vulnerable to early periods of ID when compared with noradrenergic synaptic mechanisms.

Hematocrit measures were collected at various time points to determine how postnatal ID affects systemic levels of anemia throughout development. The hematocrit measures revealed that the ID mice were anemic at P20, just prior to being placed back on a CN diet. However, at the time of hippocampal slice recordings (i.e., P26–30 and P60–64) anemia was no longer present. Therefore, the impairments in dopaminergic-mediated synaptic plasticity revealed in this study were most likely not the result of anemia at the time of recordings. It is important to mention, however, that it is possible that the hippocampus could remain ID for a lag period following the resolution of systemic anemia, as shown in other studies.⁴ Nevertheless, the results in this study provide evidence that early exposure to ID anemia causes prolonged and perhaps irreversible deficits in dopaminergic-mediated synaptic efficacy.

The present study also examined cAMP-dependent synaptic potentiation in ID and CN mice. Forskolin is an adenylylate cyclase activator, which is used to elevate endogenous cAMP, and its degradation was

prevented by the phosphodiesterase inhibitor, rolipram.¹⁸ The present study showed that FSK + R produced similar potentiation in the ID and CN groups when tested in young (P26–30) and adult (P60–64) slices. These findings suggest that the impairments in dopaminergic signaling in the ID animals were not due to impairments in intracellular cAMP signaling, but rather may be due to impairments at the level of the dopamine receptor or the G-protein.

Acknowledgments

This work was funded by National Institutes of Health grant R01 HD 050423 (M.D.M.).

Disclaimer statements

Contributor Statement

M.D.M. designed research, wrote paper, and has primary responsibility for final content. A.B.B. wrote paper, analyzed statistical data, and conducted all electrophysiological slice research. J.A.F. and M.P.B. maintained diets, mouse development, and assisted with solution preparation.

Funding

National Institutes of Health.

Conflict-of-Interest

None.

Ethics Approval

All procedures used in this study were approved by The University of Wyoming Animal Care and Use Committee and were in accordance with NIH Guide for the Care and Use of Laboratory Animals.

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