A Genetic Developmental Model of Iron Deficiency: Biological Aspects (44357)

ANDREW C. MORSE,*[,1] JOHN L. BEARD,*[,†] AND BYRON C. JONES*

Department of Biobehavioral Health, College of Health and Human Development, The Pennsylvania State University, University Park, Pennsylvania 16802; and Department of Nutrition,† College of Health and Human Development, The Pennsylvania State University, University Park, Pennsylvania 16802*

Abstract. **Numerous studies have demonstrated the negative impact of iron deficiency on growth and development. The present study expands on the published literature by exploring the role of genetics and developmental timing on the impact of iron deficiency on development in two strains of mice. Growth rates, organ weights, and hematological responses to an iron-deficient diet differed by strain and sex. The results from this study provided novel insight into iron metabolism and the impact of iron deficiency in C57 and DBA strains of mice. Future studies should continue to examine the contributions of both genetics and sex to the development of iron deficiency.** [P.S.E.B.M. 1999, Vol 220]

The use of inbred strains of rodents as a tool for understanding iron deficiency is a relatively recent development; the vast majority of animal research in this area has used genetically heterogeneous animal models. The use of genetically defined strains of rats and mice can be useful in linking iron-related phenotypes to candidate gene loci. Because inbred mice exhibit variability in many phenotypes, including iron metabolism, they provide a unique opportunity to better understand the mechanisms of and individual differences in iron metabolism. For example, LeBoeuf, *et al.* used female mice from seven different mouse strains to examine genetic factors involved in the regulation of iron metabolism (1). The results showed that serum iron levels varied as much as two-fold among strains fed the same basal iron diet, whereas the serum transferrin levels remained constant. The implications of these findings suggest that transferrin saturation, but not serum levels of transferrin may be genetically determined in mice. Additionally, hepatic iron stores varied as much as two-fold among the strains, and those stores failed to reflect transferrin saturation, suggesting that different genetic factors control these two measures.

The purpose of this study was to determine if significant variation existed between inbred strains of mice in response to feeding a low-iron diet in early life. Moreover, we wanted to determine if the acquisition of iron by the brain was significantly altered. Since rat models of iron deficiency now clearly show a negative impact of iron deficiency on brain monoamine metabolism (2, 3) we sought to determine if genetics is a significant component.

Materials and Methods

Animal Care. Male and female C57BL/6 (C57) and DBA/2 (DBA) mice from our own colony were used as subjects. Litters were not culled at birth, and mice were weaned into unisex groups of two to three mice per cage at 21 days of age. At 23 days of age, mice were randomly assigned to treatment groups (n = 5–8 mice/group). Siblings were assigned to different treatment conditions. The animals were individually housed in standard 30 × 26 × 23 cm polycarbonate boxes with stainless steel wire covers. Temperature and humidity were maintained at 22°C and 35%–45%, respectively, with a light cycle of 0700 L:1900 D. Mice were allowed free access to demineralized water and iron-adequate or iron-deficient diets for a period of 1–5 weeks. Glass food jars (5 × 5 cm) and stainless steel covers with 2.5-cm diameter openings were placed in the back corner of the cage, opposite the water. Cages, water, and diet were changed on a weekly basis with feed refills oc-

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[1] To whom requests for reprints should be addressed at The Scripps Research Institute, Department of Neuropharmacology, 10550 North Torrey Pines Rd.-CVN7, La Jolla, CA 92037. E-mail: amorse@scripps.edu

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curring every 2–3 days. Mice and food jars were weighed every other day from 23 days of age to determine rates of growth and food consumption. All mice were fasted overnight for a minimum of 10 hr and a maximum of 13 hr prior to the day of testing.

Experiment 2: Sensitive period study. Mice were randomly assigned to one of two groups. Group 1 received the iron-deficient diet for the first 2 weeks post-weaning, followed by 3 weeks of the iron-adequate diet. Group 2 received the iron-adequate diet for the first 2 weeks, the iron-deficient diet for the subsequent 2 weeks, concluding with the iron-adequate diet in the final week.

Use of animals was approved by the Pennsylvania State University's Animal Care and Use Committee (IACUC).

Diet Preparation. The iron-adequate group was fed a powdered diet formulated to meet all nutritional requirements of the growing mouse (Table I) and contained ~35 mg Fe/kg diet. The iron deficient group received the same diet but without iron. The residual concentration was <8 mg Fe/kg diet.

Analytical Procedures. *Necropsy procedure.* Mice were anesthetized with Halothane (Halocarbon Laboratories, River Edge, NJ). Blood was collected by cardiac puncture and stored in heparinized tubes on ice. The animals were trans-cardiac perfused with cold phosphate buffered saline (PBS) until the effluent was clear (30 ml over 2 min). Following decapitation, the brain, heart, liver, and spleen were harvested and placed on dry ice. All tissues were frozen at −80°C until analyzed.

Hemoglobin and hematocrit procedures. Blood collected during the necropsy from the heart of the animals was analyzed fresh for hemoglobin and hematocrit. Hemoglobin concentrations were determined by the method of Drabkin (4) using commercial reagents (Kit No. 252-A, Sigma Chemical Co., St. Louis, MO) on 10 μl whole fresh blood. Heparinized microcapillary tubes were filled with whole blood then spun for 3 min in a microcapillary centrifuge. The remaining blood was centrifuged at 3,000 rpm for 15 min at 4°C, and cells were separated. Plasma was frozen at −80°C.

Serum iron, total iron binding capacity (TIBC), and transferrin saturation procedures. Blood collected during the necropsy of the animals was analyzed as frozen plasma for iron, TIBC, and transferrin saturation by standard methods (5).

Tissue iron determination. Nonheme iron concentrations in brain, heart, liver, and spleen were measured spectrophotometrically by the method of Torrance and Bothwell (6). The harvested organs were dissected into four equally sized sections and digested at 65°C for 20 hr prior to colorimetric analysis (6).

Dietary iron determination. Iron concentrations in the diets (mg/kg diet) were measured using flame atomic absorption spectrophotometry (5100 PC, Perking Elmer, Exton, PA) by the method of Christian and Feldman (7).

Data analysis. Data were analyzed by analysis of variance for a three between subjects (strain, sex, treatment) experiment. Unequal cell sizes were evaluated by unweighted means solutions for sums of squares. All main effects and interaction terms were considered significant at $P < 0.05$.

Table I. Composition of Powdered Diet

Ingredient	Amount (g/kg diet)
Cornstarch	700
Vitamin-free casein[a]	200
Hydrogenated corn oil	50
Mineral mix[b]	40
Vitamin mix[c]	10

[a] ICN Biochemicals (Cleveland, OH).
[b] Modified AIN-76 mineral mix provides the following (g/kg mineral mixture): calcium phosphate dibasic, 440; magnesium oxide 21; sodium chloride, 65; potassium sulfate, 46; potassium citrate, 193; potassium iodide, .01; manganese carbonate, 3.1; copper carbonate, .3; zinc carbonate, 1.4; chromium potassium sulfate, .48; sodium selenate anhydrous, .01; sucrose, 103; DLMethionine, 75; Choline Bitartrate, 50. Ferrous sulfate heptahydrate (6.2 g/kg diet) was added to the iron adequate diet.
[c] AIN-76 vitamin mixture (ICN Biochemicals).

Results

Body Weight. Overall, C57 mice had more rapid growth rates than DBA mice, and iron deficient animals had lower growth rates than those fed a control diet. There was no interaction between dietary treatment and strain or sex indicating that growth within or between genotype was insensitive to dietary iron (Fig. 1).

Organ Weight. After adjusting for body weight, male heart weights were greater than females, [$F(1,160) = 4.6$, $P < 0.05$] and iron-deficient animal heart weights exceeded those of the controls, [$F(1,160) = 78.9$, $P < 0.0001$]. No significant differences in brain weight were seen due to iron-deficient diet.

Hemoglobin and Hematocrit. *Developmental (Weeks 0–5 of dietary treatment).* Hemoglobin and hematocrit varied by strain and sex with additional different responses to duration of dietary treatment (interactions of strain × diet [$F(4,160) = 4.4$, $P < 0.05$] and strain × sex × diet × weeks [$F(4,160) = 5.8$, $P < 0.001$]). Females had higher overall hemoglobin concentrations than males [$F(1,160) = 4.4$, $P < 0.05$], whereas DBA mice had higher hematocrit values of $F(1,160) = 6.9$, $P < 0.01$. Patterns of hemoglobin depletion over time were similar to those of hematocrit (Fig. 2).

Repletion studies. Iron-deficient diet decreased hemoglobin an hematocrit in this study, and all hematological parameters rapidly returned to levels comparable to controls within 1 week of dietary refeeding (data not shown). Hematological measures were unaffected by the prevention of copraphagia as neither Hb nor Hct differed from the corresponding normally housed animals (data not shown).

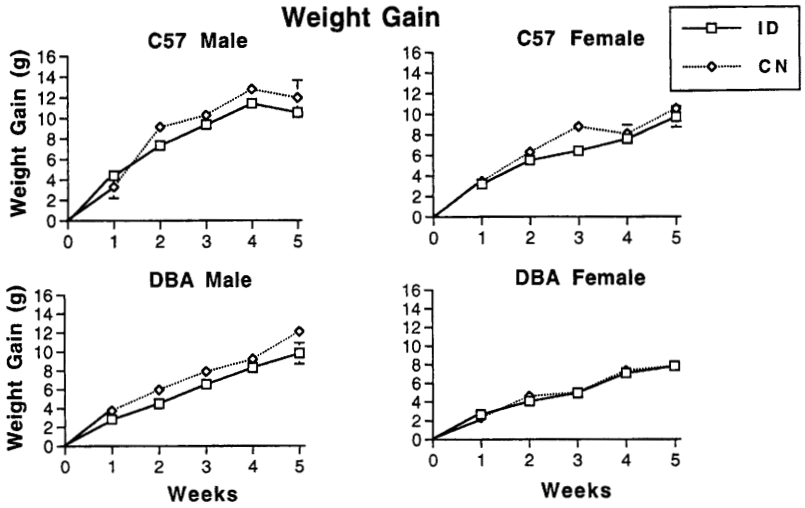

Figure 1. Weight gain for C57 and DBA mice fed iron-deficient (ID) or iron-adequate (CN) diets over 5 weeks. Mice were 23 days old at the initiation of dietary treatment. Weight gain was calculated as a difference score, with baseline weights subtracted from the weight at Weeks 1–5. Weight gain differed by strain [$F(1,156) = 69.9$, $P < 0.0001$], sex [$F(1,156) = 79.9$, $P < 0.0001$], dietary treatment [$F(1,156) = 15.4$, $P < 0.0001$], and week of treatment [$F(4,156) = 127.9$, $P < 0.0001$]. There were significant interactions of strain × sex × weeks [$F(4,156) = 3.6$, $P < 0.01$].

Serum Iron, TIBC, and Transferrin Saturation. Serum iron concentration fell rapidly as a result of dietary treatment with a significant interaction of strain × diet [$F(4,152) = 4.3$, $P < 0.05$]. DBA mice had higher serum iron concentrations compared to C57 mice (Fig. 3).

Total iron binding capacity (TIBC) was greater in C57 mice than DBA mice, and iron-deficient animals had roughly twice the binding capacity of control mice. As expected, this difference increased with the duration of dietary treatment. Serum transferrin saturation response to a low nondiet also differed significantly between strains [$F(1,152) = 4.1$, $P < 0.05$] and sex [$F(4,152) = 2.5$, $P < 0.05$]. Overall, DBA mice had higher transferrin saturation than C57 mice (Table II).

Liver and spleen iron. DBA mice had significantly higher levels of liver and spleen iron overall in the control group and seemed to deposit more iron earlier than C57 mice. However, the response to a low-iron diet was similar in both strains (Fig. 4).

Heart iron. Heart iron concentrations increased within 1 week in control animals regardless of strain or sex. DBA mice showed a rapid increase up to their highest level by Week 1, followed by a significant drop in the following weeks with females at a plateau of ~150 μg Fe/g. However, male DBA mice had a second significant elevation at 5 weeks of dietary treatment. DBA iron-deficient mice also increased during the first week, then dropped back down to baseline levels. Hearts of C57 iron-deficient mice had iron concentrations below baseline levels within 2 weeks, whereas the control C57 mice hearts never increased iron concentrations beyond the baseline levels. There were no significant differences between the baseline scores for strain or sex (data not shown).

Brain iron. Brain iron concentrations were insensitive to depletion in either strain nor sex until the fifth week of dietary treatment when the iron concentrations were lower in ID mice than in CN mice in all treatments but the DBA males. All strains showed an increase in brain iron concentration over the first 2 weeks, followed by a precipitous drop in the third week with another increase in the fourth and fifth weeks. DBA males ended up with higher brain iron

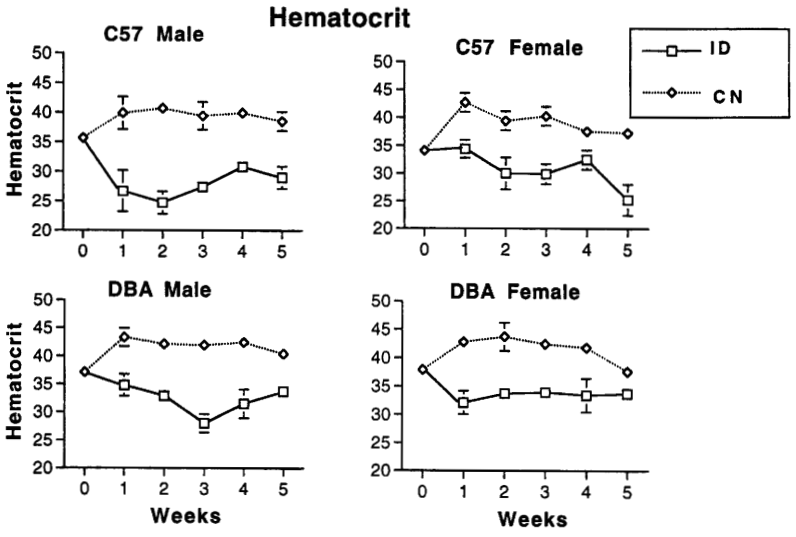

Figure 2. Hematocrit values for C57 and DBA mice. Conditions were as described in Figure 1. DBA mice had higher Hematocrit values than did C57 mice [$F(1,160) = 6.9$, $P < 0.01$].

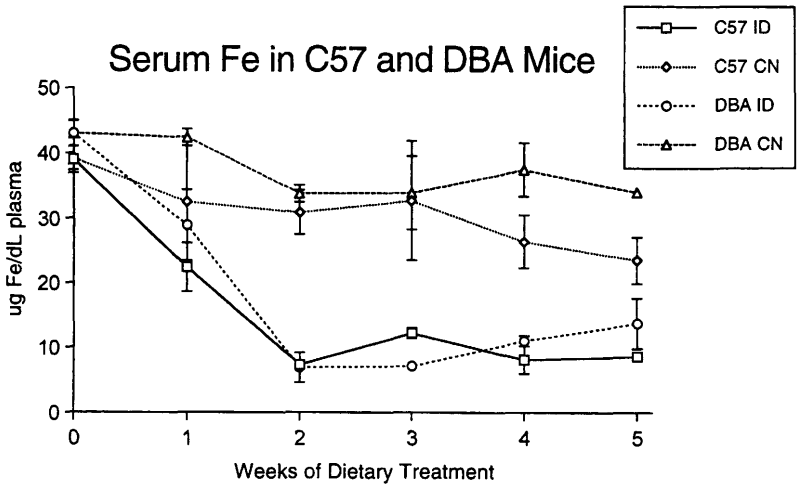

Figure 3. Serum iron levels for C57 and DBA mice. Conditions were as described in Figure 1. Serum Fe levels were higher overall in DBA mice compared to C57 mice [$F(1,103) = 14.1$, $P < 0.001$].

concentrations in both ID and CN conditions than their C57 counterparts (data not shown).

Discussion

The biological impact of iron deficient studied in genetically defined mice, C57 and DBA strains, extends findings from the rat literature (2, 5, 8). The genetic components of the response to a dietary deficiency in iron can be identified partially by using these highly inbred strains. LeBoeuf and colleagues used eight inbred strains of mice to demonstrate that these eight strains clustered into three distinct groups with respect to regulation of plasma iron when fed a standard chow diet (1). While that study had a focus on adaptation to high-iron diets, the current study examined low-iron diet adaptations.

Poor growth rates in iron-deficient animals are related to poor fed efficiency rather than anorexia (9, 10). The basis of the poor growth in iron deficiency is not established though it is well known that iron is required for RNA synthesis and fundamental functioning of many enzymes and proteins (11). Rao and Jagadeesan demonstrated reduced feed efficiency in three different groups of rats fed iron-deficient diets for up to 6 weeks (12). Fischer 344 and Wister strains of rats were adapted to the low-iron diets with a clearly lower feed efficiency over the last 3–6 weeks of dietary treatment, whereas the Sprague-Dawley rats showed little difference in feed efficiency from control rats until the final week of the experiments. These studies demonstrated that nutritional studies in rodents may provide variable results depending on the genetic makeup of the rodents studied. BALB/c mice fed low-iron or control diets identical to the ones in this study also showed significant growth failure with 6–8 weeks of dietary treatment (Malicki EA *et al.*, unpublished data).

Growth rate differences in the current experiment were apparent between the mouse strains with C57 mice gaining weight more quickly than DBA mice. Growth in DBA females was unaffected by dietary iron deficiency despite the clear indication that they too were iron deficient based on liver iron and hematological indicators. The interaction of strain and sex in determining the response of growing mice to low-iron diets is generally unexplored, though BALB/c female mice showed similar growth failure as male BALB/c mice (Malicki EA *et al.*, unpublished data). It is possible that growth of female rats exposed to low-iron diets is less affected than the growth in male rats due to lower iron requirements for growth than to any specific effect of sex (13, 14).

A previous study of iron intake in inbred mice showed a dramatic difference in liver, heart, and spleen iron accumulation in response to a high-iron diet (1). In that study, mice fed normal amounts of dietary iron had a two-fold variation in liver iron content that was not reflected in any difference in transferrin saturation. Three weeks of feeding

Table II. Comparison of Week 1 Versus 5 TIBC and Percentage of Transferrin Saturation

Strain	Sex	Diet	TIBC		% Saturation	
			Week 1	Week 5	Week 1	Week 5
C57	Male	ID	34.2 (10.9)	62.2 (19.2)	27.8 (15.2)	12.8 (2.9)
		CN	40.9 (3.0)	30.9 (10.6)	57.3 (6.5)	79.7 (35.9)
	Female	ID	55.7 (4.7)	94.3 (23.5)	46.4 (8.0)	46.4 (28.9)
		CN	32.5 (8.4)	54.3 (3.8)	111.0 (14.4)	51.0 (5.3)
DBA	Male	ID	56.9 (4.9)	72.4 (12.0)	61.0 (9.7)	15.9 (4.3)
		CN	31.11 (7.9)	36.6 (5.6)	118.0 (9.6)	100.9 (18.8)
	Female	ID	39.8 (16.3)	59.3 (13.6)	41.1 (6.6)	44.6 (20.2)
		CN	29.4 (7.6)	40.4 (6.7)	108.4 (20.6)	94.6 (16.7)

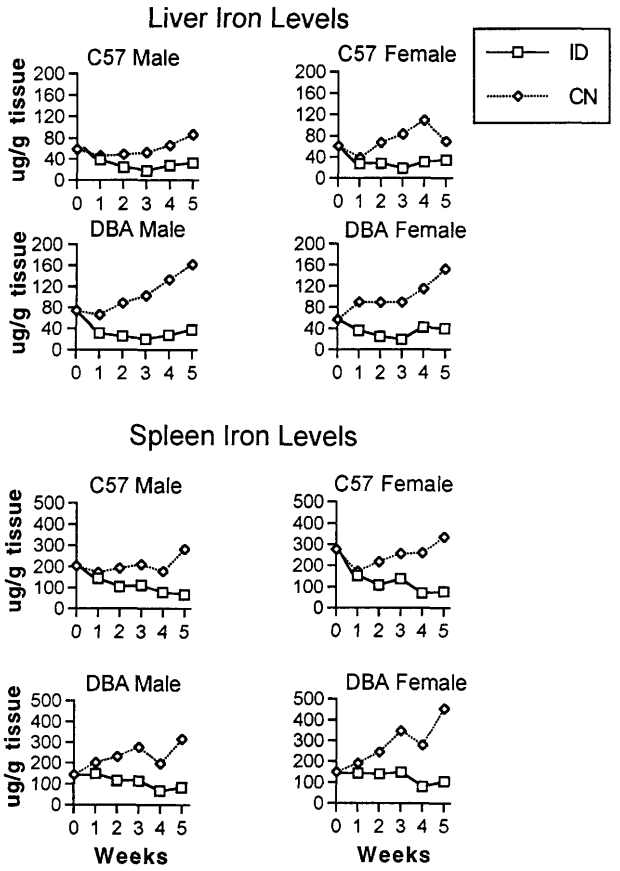

Figure 4. Liver and spleen iron levels for C57 and DBA mice. Conditions were as described in Figure 1.

female C57, BALB/c, DBA/2 and AKR mice revealed that C57 and DBA tended to group together with respect to liver iron content whereas BALB/c and AKR mice had about 75% higher liver iron contents despite identical iron-containing diets. Genetic background did not have the same influence on heart iron content however, as the C57 and AKR mice grouped together, and the BALB/c and DBA/2 mice were similar. Hematocrits were higher in the AKR mice and similar in all other groups. However, LeBoeuf and colleagues studied only female mice so effects of sex could not be determined. In contrast, using males the current study showed higher liver and spleen iron concentrations in DBA than in C57 mice when the mice were fed normal levels of dietary iron. When LeBoeuf and colleagues fed high levels of dietary iron as a 2% carbonyl iron diet (2,000 ppm Fe) for up to 8 months, the BALB/c mice had significantly greater accumulation of iron in liver and spleen than did C57 mice. The current experiment showed that feeding a low-iron diet results in similar diminutions in liver and spleen iron content in both C57 and DBA mice. Bioadaptive responses that protect organ reserves from depletion of storage iron thus appear to be unaffected by genetic differences in the genetic control of iron accumulation in times of iron excess. An interesting example of this is seen when comparing liver and spleen iron levels in the final weeks of iron depletion (Fig. 4). As spleen iron levels decreased in Weeks 4 and 5, liver iron levels increased suggesting that the spleen may be selectively donating iron to the liver to preserve critical liver function.

Indeed, LeBoeuf and colleagues examined plasma iron levels and transferrin levels in eight strains of mice fed a 200-ppm Fe diet and observed a 120% difference between lowest and highest plasma Fe across genetic strains. The interaction of cellular storage of iron and acquisition of iron are linked through the well-described iron response element (IRE) and iron response proteins (IRP1,2) (15, 16). Whereas these regulatory proteins or the products of their regulation, ferritin and transferrin receptor, were not measured in either study, it appears likely from the tissue iron content data in both the LeBoeuf article and the current study that genes regulating tissue iron responses to variations in dietary iron are affected by genotype. Further evidence for this is provided by the rat studies of Rao and Jagadeesan (12). Identical iron-deficient diets fed to Fischer 344, Wistar, and Sprague-Dawley rats resulted in differences in hemoglobin and serum iron by strain with the Fisher rats having the lowest hemoglobin and serum iron and the Sprague-Dawley rats the highest. Differences were as great at 20% between rat groups.

There are several points of agreement between the current study and that of LeBoeuf and colleagues (1). In both studies the DBA mice had overall higher plasma iron concentrations than the C57 mice and also higher transferrin saturations. However, in contrast, we demonstrated significantly higher TIBC concentrations in C57 than in DBA mice whereas the previous investigators did not observe any difference. The additional iron provided by their control diet may have contributed to a differential regulation of TIBC that was somehow not reflected in plasma iron concentration. In both studies, transferrin saturation was elevated in the DBA mice compared to the C57 mice.

There are little data regarding brain growth and iron content in inbred mice (17). Brain weight was also unaffected by dietary iron deficiency in either strain or gender. This observation is similar to that in rats (2, 18). In contrast, a study by Oloyede *et al.* found that animals fed a diet deficient in iron or essential fatty acids (EFA) alone did not affect the brain weight, but when combined, the iron deficiency appeared to exacerbate the effects of EFA deficiency and result in lower brain weight (19). However, the brain was quite resistant to iron depletion, which is an observation similar to some studies in rats (20) but not others (2, 21). The imposition of iron deficiency within the first 10–20 days of life seemingly results in irreversible changes in brain growth and iron acquisition, whereas iron deficiency after this critical period does not have long-lasting effects on brain size or iron content (22). The decline in brain iron content in postnatal life is in agreement with previous studies in rats in which brain iron content declined up through postnatal Day 45 (23). The increase in iron concentration in both controls and ID animals in all groups during the first 3 weeks of dietary intervention is similar to the effect of low-

iron diets on brain iron content in BALB/c mice (Malicki EA *et al.,* unpublished data). That is, there is sufficient iron in the diet to allow some increase in brain iron content, but insufficient amounts to normalize content. Consequences of a diminished iron content have been suggested to be decreased dopamine D_2 receptor densities, increased extracellular dopamine, and altered behavior (3, 24, Morse *et al.,* unpublished data).

In summary, this study reaffirms some previous observations and extends these studies to include the influence of gender on the genetic influence on responses of mice to low-iron diets. Specifically, organ iron contents, plasma iron concentrations, and transferrin saturations were all impacted by the genetic backgrounds of the test animals. Future studies will need to incorporate the genotype and sex of test animals in the interpretation of their iron metabolism studies.

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