Iron Deprivation during Fetal Development Changes the Behavior of Juvenile Rhesus Monkeys$^{1,2}$

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Abstract

Sensitive periods for induction of behavioral impairments by developmental iron deficiency were studied in a nonhuman primate model. Rhesus monkey infants were deprived of iron prenatally ($n=14$) via the dam's diet ($10 \mu g$ Fe/g) or postnatally (birth–4 mo, $n=12$) via infant formula ($1.5 mg$ Fe/L). They were compared with controls ($n=12$) with adequate dietary iron throughout development in a series of cognitive tests and related assessments from 6 to 12 mo of age, a developmental stage corresponding approximately to 2–4 y of age in humans. Health, growth, and hematological status were not affected. Auditory brainstem response and white matter volumes in the cerebrum were similarly unaffected. Male infants in the prenatally deprived group had reduced spontaneous daytime activity relative to controls, as monitored by actimeter. On cognitive tests, prenatally deprived juveniles had similar level of correct responding, but showed more completed trials, and shorter latencies during early phases of the tests. Juveniles deprived of iron as infants showed a similar pattern of behavioral change, but most differences from controls were not as great. Inadequate iron nutrition during pregnancy was reflected in the juvenile period primarily as attenuated inhibitory response. This finding may be relevant to individual differences in temperament or to behavior disorders in children involving reduced inhibitory control. J. Nutr. 137: 979–984, 2007.

Introduction

One goal of treatment of anemia in infants, as well as of routine iron supplementation, is to safeguard brain development (1). To prevent or reverse behavioral deficits associated with developmental iron deprivation, the sensitive periods of brain development must be better understood. Because iron deficiency develops slowly, it is possible that iron deprivation affects the brain without induction of anemia or prior to the induction of anemia.

Among the behavioral effects of interest are affective outcomes, including those linked to childhood behavior disorders. As the genetic contribution to these disorders is better defined, it becomes more apparent that early environmental factors also play a role (2).

Our research used a rhesus monkey model in which single nutrient iron deprivation could be produced at well-defined periods of brain development (prenatal and postnatal), with all other nutritional and environmental conditions held constant. Third trimester iron deficiency was produced in the mothers by the prenatal deprivation (3) and ferritin stores were reduced in infants by the postnatal deprivation (4), but we did not detect frank anemia in either group of infants at any time. Behavioral consequences of both prenatal and early postnatal iron deprivation were noted from birth to 4 mo of age, a developmental period approximately equivalent to birth–18 mo of age in human infants, as reported previously (4). In this report, we evaluated long-term effects by studying growth, hematology, and iron status and cognitive behaviors in 6- to 12-mo-old infants beginning 2 mo after discontinuation of the postnatal iron deprivation. This stage of development is approximately equivalent to a 1.5- to 4-y-old human infant/child. In addition, we conducted several noninvasive assessments of brain maturation.

Materials and Methods

Assurance of compliance with animal codes. All protocols were approved prior to use by the University of California, Davis Institutional Animal Care and Use Committee and followed the requirements of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. Monkeys were housed at the California National Primate Research Center (CNPRC)$^5$, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited vivarium.

General. The subjects were 38 male and female rhesus macaques (Macaca mulatta) divided into 2 cohorts born 2 y apart. The use of 2 cohorts allowed internal replication and some additions to the assessments in the

1 Supported by NIH grants P01 HD39386, Betsy Lozoff, principal investigator, and RR00169.
2 Supplemental Tables 1 and 2 are available with the online posting of this paper at jn.nutrition.org.
3 Abbreviations used: ABR, auditory brainstem response; CNPRC, California National Primate Research Center; COD, concurrent object discrimination; CSF, cerebral spinal fluid; D2R, dopamine D2 receptor; DNMS, delayed nonmatch to sample; DR, discrimination reversal; WGTA, Wisconsin General Testing Apparatus.
2nd cohort based on findings in the 1st cohort. Monkeys were born at the CNPRC and raised in the primate nursery until weaning at 4 mo of age, as previously described (4). Briefly, dams were fed a diet (3) either low or adequate in iron during pregnancy depending on group assignment (Table 1). At birth, infants were transferred to the primate nursery and fed exclusively until 4 mo of age formula that was either iron deficient (Similac Advance, Ross Products, 1.5 mg Fe/L) or iron adequate (Similac Advance with iron, 12 mg Fe/L). Infants of dams fed the low-iron diet were given iron-sufficient formula, whereas the infants of dams fed an iron-adequate diet were subdivided into 2 groups, 1 given iron-sufficient and 1 given iron-deficient formula (Table 1). These subdivisions were based on stratification for parity and preconception weight.

At weaning (4 mo), monkeys were transferred in groups of 4 to double stainless steel adult cages (120 × 65 × 79 cm) with a divider that allowed for separation of monkeys when needed. Housing conditions in the cageroom have been described previously (5). From 4 to 6 mo of age, they were transitioned from formula to a solid diet and were adapted for later behavior tests.

During the evaluations reported here (Table 2), twice daily feedings with commercial monkey diet occurred at 0700 and 1500 and feedings were supplemented with daily forage enrichment (small food items could be retrieved from recessed wells in a plastic board) and twice weekly distribution of fresh produce. Iron content was not restricted and was identical for all monkeys.

**Background assessment.** Hematology and iron status were assessed at 6, 8, 10, and 12 mo of age. Complete blood counts, including hemoglobin, were supplemented with assays for serum ferritin, transferrin receptor, iron, and zinc protoporphyrin, as previously described (3). We recorded body weights monthly. We took morphometric measurements at 8, 10, and 12 mo under ketamine anesthesia (10 mg/kg, i.m.) (3). The health record of each infant from 4 to 12 mo of age was summarized by type of health observation (skin/ rash, stool, trauma, weight/feeding, other) and incidence of veterinary treatment. After the completion of all Wisconsin General Testing Apparatus (WGTA) testing, each home cage of 4 monkeys was observed for 30 min for the occurrence and frequency of stereotypic behavior, as described previously (5).

We obtained several noninvasive brain measures in the older juvenile monkeys. Auditory brainstem response (ABR) testing at 8 mo of age utilized the Nicolet Bravo system (Nicolet Biomedical) with needle electrodes and procedures previously developed for infant monkeys (6). Monkeys were anesthetized with Telazol (3–8 mg/kg, i.m.) during the 15-min test period. Cerebral spinal fluid (CSF) samples obtained at the end of the experiment (12 mo of age) were analyzed by HPLC for the dopamine metabolites 3,4-dihydroxy-phenylacetic acid and homovanillic acid and the serotonin metabolite 5-hydroxy-indole acetic acid (5,7). MRI images were also obtained at 12 mo of age in anesthetized monkeys (metatomidine, i.m., 20–40 µg/kg; atropine, i.m. 40 µg/kg) using a GE Signa CV/I 1.5 Tesla magnet and a 15-cm human knee coil. T1/T2 weighted images were imported into NIH Image software. Noncerebral regions were stripped according to previously established methods for infant monkeys, volume intensities were normalized, and intensity ranges were established for white matter, gray matter, and cerebrospinal fluid. We analyzed 35–40 coronal sections to obtain volume measures.

**Activity monitoring.** Monkeys wore a small actimeter (PAM2, Individual Monitoring Systems) over a 48-h monitoring period at 8 mo of age, as described previously (4,8). Cagemates were separated during monitoring. Activity counts were summarized over 2-min intervals.

**Behavioral assessments conducted in cohort 2 only.** Based on data from the 1st cohort, a reward delay task (5) was added after WGTA testing for the 2nd cohort. Similar tasks are used to assess impulsivity in children (11). Briefly, an opaque sliding screen was slowly moved 2.5 cm

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<td>Fe, µg/g diet</td>
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<th>Schedule of assessments for rhesus monkey iron deficiency study</th>
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<tr>
<td>Stereotypy observation</td>
<td>12–18</td>
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**Spatial maze.** A maze test originally developed for aged monkeys (9) was used to assess spatial working memory and behavior from 6 to 8 mo in the monkeys. Monkeys were adapted to the maze during daily 10-min sessions to a criterion of 6/8 treat retrievals from the lidded boxes located around the periphery of the maze platform. If the monkeys did not progress in 13 sessions through any of 5 successive adaptation goals, training was discontinued. If monkeys met the criterion, testing began at ~6 mo of age. Visual cues (cardboard shapes of various sizes and colors) were hung on the wall behind each well. Each time a monkey opened the well, we recorded the latency and the well number (1–8), the animal was pulled back to the center of the maze platform with a tether and then was released again. A total of 8 well visits or 10 min was allowed. An error was defined as opening a well that had already been visited.

**Cognitive testing in the WGTA.** The majority of the testing took place in the infant WGTA test environment (5), where monkeys were trained to reach out of a test cage and displace objects on a tray in front of them to retrieve a food reward (small marshmallows or raisins) from a well. Monkeys were not food deprived; however, the morning food ration and enrichment were withheld until the completion of testing. The delayed nonmatch to sample (DNMS) test has been described previously (5). The visual discrimination reversal (DR) task used a green paper box and a white plastic container as the stimulus objects. We first trained monkeys to displace each object to retrieve a food reward. When they met the criterion (8/10 correct responses for each object), monkeys were maintained with 1×/wk training sessions (fully covered condition) until testing began at 8 mo. For the test, monkeys were presented with both objects, 1 of which was designated correct and baited out of sight with a food reward. Twenty 30-s trials with 5- to 10-s intertrial intervals were conducted each day for a maximum of 20 sessions. If monkeys met a criterion of 18/20 correct responses, we conducted a reversal in the next session in which the previously incorrect object was designated as correct. Each time criterion was met, a reversal was performed until either 4 reversals were completed or the 20-session maximum was reached. The concurrent object discrimination (COD) presented the monkeys with the same 20 pairs of objects in the same order each day. One object of each pair was always designated correct and baited with a food reward. Twenty 30-s trials with 30-s intervals between trials were conducted each day. We recorded the number of correct and incorrect responses, balk (no response) trials, and latency to respond for each session.

**Behavior ratings.** Following each session of DR, COD, and DNMS, the monkey’s behavior was rated in 8 categories [adapted from (10)]: object orientation, distractibility, goal directedness, irritability, activity, impulsivity, inhibition, and stereotypic behavior. Raters were unaware of the experimental group and tester reliabilities were obtained prior to testing.

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at a time over 7 2-s intervals to reveal an empty plastic box covering a food reward. Monkeys were required to wait until the screen was completely removed to displace the box and take the treat. The test consisted of 40 30-s trials with 10-s intertrial intervals.

For cohort 2, we made additional WHTA behavior ratings for uncompleted trials. Monkeys were rated on a scale of 0 to 3, indicating the frequency and/or intensity of the behavioral state during uncompleted trials (scared, distracted, and apathetic). Also, ratings of emotionality in the spatial maze were made during an additional 10-min session conducted following the last spatial maze training session. Each animal’s behavior was rated in 5 categories ranked from most to least emotional (running in circles around the maze; running with no pattern; lay; sit; explore) and the amount of time spent in each behavior was estimated (0, 25, 50, 75, and 100%). Each degree of emotionality (5 to 1) was multiplied by the amount of time and added to obtain an emotionality score.

**Statistical analysis.** Most endpoints were analyzed by ANOVA using general linear models (SAS Institute) with iron deprivation (control, prenatal, and postnatal) as the independent variable. Sex, cohort, birthweight, and gestation length were screened as potential covariates and added to the analysis if they were related to the dependent variable. Post hoc tests compared each deprived group to the control group using least-squares means contrasts. Incidence data were analyzed by contingency analysis (chi-square or Fisher’s exact). Values in text are means ± SEM and differences were significant at \( P < 0.05 \).

**Results**

**Assessments not affected by iron deprivation.** Iron deprivation did not adversely affect weight, weight gain, morphometrics, hematology, iron status variables (Supplemental Table 1), or incidence of health problems. The iron-deprived infants did not differ from controls in the incidence of home cage stereotypy behaviors. Similarly, there were no detectable effects of iron deprivation on assays of CSF catecholamine metabolites, electrophysiological measures of the ABR, and MRI quantitation of white matter in the cerebral cortex (Supplemental Table 2). The MRI analysis yielded a surprising finding: indications of postnatal stroke in 1 of the prenatally deprived monkeys as reflected in an elongated cerebral ventricle and reduced white matter volume. Increased risk for stroke has been documented in anemic human infants (12,13). The juvenile monkeys exhibited distress in the large open room containing the spatial maze and less than one-half the monkeys completed the test (control, 42%; prenatally deprived, 25%; postnatally deprived, 25%; \( P > 0.05 \)). Thus, it was not possible to evaluate effects on spatial learning and memory.

**Activity monitoring.** Because initial analysis indicated group by sex interactions for many activity measures, we evaluated activity separately for males and females. Activity during the day was 29% lower in the prenatally deprived males than in controls (\( P = 0.02 \); Fig. 1). The postnatally deprived males tended to have lower activity (\( P = 0.06 \)) and also spent less time active during the night than did controls (\( P < 0.002 \), sex as covariate). Duration of diurnal sleep and wake states, and the number of transitions between wake and sleep, did not differ by groups in either sex.

**Cognitive testing in cohorts 1 and 2.** Previously iron-deprived monkeys were not impaired in learning the cognitive tasks (Table 3). However, the prenatally deprived group completed significantly more trials early in the DR test sessions (Fig. 2 A) and this was associated with earlier attainment of the learning criteria (\( P = 0.02 \)) and completion of more reversals (\( P = 0.055 \)) in the fixed number of sessions allowed for DR. Nonetheless, the prenatally deprived infants did not learn faster than the control infants, taking into account the lower number of trials on which controls responded. For example, although the prenatally deprived group had a higher percent correct in the 1st 40 trials of DR (\( P = 0.008 \)), they did not differ from controls in the percent correct on the 1st 40 trials on which responses were made (\( P = 0.30 \)). In the 2nd test of the series, COD, prenatally deprived infants also performed more trials over all 20 sessions than controls (\( P = 0.02 \)).

Prenatally deprived infants also had shorter response latencies (\( P = 0.001 \)) during the 1st 10 sessions of the DR test and over all 20 sessions of the COD test (\( P = 0.04 \)) (Fig. 2 B). During these tests, the control infants did not always participate by responding on a trial and responses that were made were often delayed (long latency). The differences in completed trials and response latencies dissipated as the control infants adapted to the test situation; by the last test in the series, DNMS, groups did not differ in the number of trials completed or the response latencies.

Prenatally deprived monkeys also differed from controls in behavior ratings obtained after cognitive test sessions. Over all sessions of all tests, the prenatally deprived offspring were rated less distractible than controls (\( P = 0.049 \), sex included as a factor). Over the 1st 10 DR sessions, the most sessions completed by all the monkeys, prenatally deprived infants demonstrated more object orientation (\( P = 0.0015 \)) and goal directedness (\( P = 0.003 \)) and less distractibility (\( P = 0.02 \)) than controls.

**TABLE 3** Learning of 3 cognitive tasks by juvenile rhesus monkeys deprived of iron prenatally or postnatally by dietary restriction

<table>
<thead>
<tr>
<th>Group</th>
<th>Prenatal deprivation</th>
<th>Control</th>
<th>Postnatal deprivation</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>DR, sessions to criterion, ( n )</td>
<td>3 ± 1*</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>DR, reversals, ( n )</td>
<td>3.1 ± 0.4</td>
<td>2.0 ± 0.4</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>COD, correct last session, %</td>
<td>73 ± 3</td>
<td>64 ± 4</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>DNMS, correct last one-half, %</td>
<td>68 ± 4</td>
<td>64 ± 4</td>
<td>62 ± 4</td>
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*Values are means ± SEM. *Different from control, \( P < 0.05 \).
controls. Postnatally deprived infants also demonstrated more object orientation than controls \( (P = 0.02) \).

**Behavioral tests conducted in cohort 2 only.** Prenatally deprived monkeys of the 2nd cohort had shorter latencies of response in the reward delay task (Fig. 3) than the other 2 groups. There was a significant effect of group on latency of response on the last 10 trials of the 40-trial session, but neither the pre-nor postnatal groups differed from controls. The prenatal group often responded immediately, before screen removal began (Fig. 3B) \( (P = 0.03) \). Moreover, matched pair testing (Fig. 3A) showed that the prenatally deprived monkeys failed to improve in delaying their response after the 2nd block of trials, whereas control and postnatally deprived monkeys continued to improve from the 2nd to the last block of trials \( (control, P = 0.04; postnatal, P = 0.0055) \). Sex was a covariate in these analyses.

We found a marginal group effect for the emotionality rating score in the spatial maze room \( (P = 0.05) \); the prenatal deprivation group had a lower distress score than the other 2 groups (Fig. 3C). Sex was included as a factor in this analysis. Behavior ratings on uncompleted trials in the WGTA demonstrated that the predominant behavioral state associated with failure to respond in the whole group was distraction \( (48\% \text{ of sessions}, \text{followed by } \text{apathy} \ (36\%) \text{ and fear ("scared") } (16\%).\) Fewer sessions were scored scared in the prenatally deprived group \( (7\%) \) than the control group \( (27\%; P = 0.0006) \) (Fig. 3D).

**Discussion**

This experiment characterizes the behavior associated with iron deprivation in juvenile monkeys with no other associated nutritional or environmental deprivation or history of illness. The mild, brief, and isolated iron deprivation would likely not be detected clinically in human infants. Nonetheless, under the controlled conditions of the experiment, a characteristic pattern of behavioral change was seen for the previously iron-deprived juveniles. Although performance of cognitive tasks was not impaired, prenatally deprived juveniles showed more choice responses and shorter response times than controls, particularly in the early sessions of the structured test series, suggesting less behavioral inhibition in new and challenging environments. Behavioral ratings conducted after the test sessions indicated that the prenatally deprived group was more goal-directed, more object-oriented, and less distractible than controls.

Prenatally deprived males had somewhat lower spontaneous activity in the home cage. Reduced activity is a well-known correlate of anemia in both adults and infants/children \( (14) \). At a younger age, just prior to discontinuation of the postnatal deprivation, there was a similar reduction in activity in the prenatally deprived group (both males and females). This reduction in activity occurs long after the lack of iron could be considered to directly influence oxygen delivery. However, long-term changes in iron content \( (15–19) \) could potentially influence oxidative metabolism in the brain.

The reduced inhibition of response seen in prenatally deprived monkeys requires further study in the context of developmental cognitive theory and disruption of specific brain systems. Perseveration and selective attention deficits, often associated with frontal lobe damage in the nonhuman and human primate literature \( (20,21) \), were not seen in the prenatally iron-deprived monkeys, who performed well in DR and were rated low on distractibility. Two different types of inhibition, effortful and reactive, have been described by Eisenberg \( (22–24) \) in connection with behavioral regulation in children. The deficit of the prenatally iron-deprived monkeys seems more related to reactive control or impulsivity, especially as reflected in reward delay tasks. Deficits in impulsivity have been related to childhood behavior disorders \( (25) \). Finally, prenatally iron-deprived juveniles might be seen as lacking negative emotions, such as fear and anxiety, which are normally engaged in encounters with novel environments such as the stressful maze environment. Monkeys with aspiration lesions in the amygdala demonstrate less fear of a snake, a natural fear-evoking stimulus in monkeys \( (26) \), as well as reduced inhibition of response to novel objects or persons \( (27) \). As in our iron-deprived juveniles, this difference diminished as controls lost their initial wariness.
Further investigation of this iron deprivation syndrome may provide information on underlying disruption in development of brain systems such as the amygdala.

The behavioral characteristics of the prenatally iron-deprived monkeys could also be interpreted in the framework of research assessing temperament in humans [including children (28)], particularly the dimensions of novelty seeking and harm avoidance, which demonstrate genetic linkages (29) and are associated with risk for addiction and psychopathology (30,31). These temperament qualities have also been studied in rats and linked to underlying differences in brain neurochemistry (32). The iron-deprived infants could be characterized as demonstrating high novelty seeking and low harm avoidance in the various structured test situations.

Reduced activity and reactive control could be established by statistical analysis in prenatally deprived juveniles but were less strong in the postnatally deprived juveniles. It is unclear whether this indicates a different sensitivity of in utero brain development or a more complex syndrome when deprivation occurs later in development.

Dopamine systems are also potentially involved in the behavioral effects of prenatal iron deficiency. Evidence suggests that iron deficiency during brain development can cause a long-term or permanent decrease in dopamine D2 receptor (D2R) in the basal ganglia (33). Recently, positron emission tomography studies that used the D2R ligand [11C] raclopride found changes in raclopride binding after administration of methylphenidate that correlate with attention deficit hyperactivity disorder symptom severity (34–36). Decrease in D2R in basal ganglia is one of the oldest and most consistent findings in the brains of iron-deficient rodents. This deficit in D2R reversed with iron repletion. However, more recent studies found irreversible changes in D2R in striatum of rats deprived of iron during brain development then repleted with iron for 4 wk (37). At the functional level, this group also demonstrated a reduced behavioral effect of the D2R receptor antagonist raclopride in iron deficiency (15). Studies of D2R in iron-deficient nonhuman primates might prove valuable.

In summary, juvenile monkeys deprived of iron before birth had altered behavioral regulation in learning and memory tasks, characterized by lack of initial inhibition of responding and shorter latencies. In a delayed reward task, where reduced behavioral inhibition was a liability rather than an asset, they were less successful than controls in obtaining rewards. In relation to conceptualization of childhood behavior, they demonstrated lower reactive control, greater impulsivity, reduced harm avoidance, and greater novelty seeking.

Acknowledgments
MRI analysis was conducted by Carolyn Nolte under the direction of Julia Hamstra in the laboratory of Dr. David Amaral at the MIND Institute. Ferritin analyses for monkeys were established and conducted by the Endocrine Core laboratory at CNPRC under the direction of Dr. Bill Lasley. Mary Roberts of CNPRC advised in adapting the spatial maze and testing in the laboratory of Dr. John Beard.

Literature Cited


