Fetal Iron Deficiency and Genotype Influence Emotionality in Infant Rhesus Monkeys

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Abstract

Background: Anemia during the third trimester of fetal development affects one-third of the pregnancies in the United States and has been associated with postnatal behavioral outcomes. This study examines how fetal iron deficiency (ID) interacts with the fetal monoamine oxidase A (MAOA) genotype. MAOA metabolizes monoamine neurotransmitters. MAOA polymorphisms in humans affect temperament and modify the influence of early adverse environments on later behavior.

Objective: The aim of the study was to advance translation of developmental ID research in animal models by taking into account genetic factors that influence outcomes in human populations.

Methods: Male infant rhesus monkeys 3–4 mo old born to mothers fed an ID (10 ppm iron) diet were compared with controls (100 ppm iron). Infant monkeys with high- or low-transcription rate MAOA polymorphisms were equally distributed between diet groups. Behavioral responses to a series of structured experiences were recorded during a 25-h separation of the infants from their mothers.

Results: Infant monkeys with low-transcription MAOA polymorphisms more clearly demonstrated the following ID effects suggested in earlier studies: a 4% smaller head circumference, a 39% lower cortisol response to social separation, a 129% longer engagement with novel visual stimuli, and 33% lesser withdrawal in response to a human intruder. The high MAOA genotype ID monkeys demonstrated other ID effects: less withdrawal and emotionality after social separation and lower “fearful” ratings.


Keywords: rhesus, anemia, MAOA polymorphism, social behavior, infant

Introduction

Research in human populations has demonstrated that developmental iron deficiency (ID), even when corrected by supplements, is associated with long-term effects on multiple behavioral domains, including cognitive, socioemotional, and gross and fine motor functions (1, 2). The majority of this research has been directed at ID in infants and toddlers, but more recently, fetal ID, assessed through maternal hematology, is being identified as a source of deviation in neurobehavioral function of infants and children (3–8). Animal models have been important in identifying prenatal ID effects on postnatal behavior and exploring possible mechanisms (9–15). The nonhuman primate is a particularly appropriate animal model for human fetal development based on the extended period of in utero brain maturation common to primates and the similar approaches to behavioral assessment during infant and juvenile stages of development. Our research in rhesus monkeys indicates that socioemotional components of behavior are most affected by fetal ID (10, 11, 15).

In the course of pursuing developmental ID research in the rhesus monkey, we discovered that the monoamine oxidase A (MAOA) genotype interacts with fetal ID in determining later behavioral consequences in juvenile monkeys (16, 17). MAOA polymorphisms were classified by their level of MAOA transcription (high-MAOA, low-MAOA). Particularly outstanding
was the enhanced emotionality demonstrated by the low-MAOA ID group as juveniles (16, 17). The current report looks at emotionality in infant rhesus monkeys as influenced by prenatal ID and MAOA genotype.

Potential MAOA by ID (MAOA × ID) interactions in infant monkeys were examined in a test battery for 3-to-4-mo-old rhesus monkeys, the BioBehavioral Assessment (BBA). This test battery assesses the response of infants to maternal separation and a series of environmental challenges. It is conducted in most infant monkeys born at the California National Primate Research Center (CNPRC), >4000 infant monkeys to date. It was previously shown to reflect genetic and environmental influences (18–23), including 2 studies of developmental ID (11, 24).

**Methods**

**Assurance of compliance with animal codes.** All procedures followed the Guide for the Care and Use of Laboratory Animals of the US National Research Council (25). The CNPRC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Protocols for this project were approved before implementation by the UC Davis Institutional Animal Care and Use Committee.

**Animals, diet, and genotyping.** Details of diet composition, animal care and housing, and reproduction have been reported (16, 26). Rhesus monkey dams were fed a semipurified diet fortified with iron sulfate at 10 ppm (ID) or 100 ppm [iron-sufficient (IS)] during gestation. The Estimated Nutrient Requirement for iron in macaques is 100 ppm (27). Groups were balanced for age, weight, and parity (Supplemental Table 1). Experimental diets were initiated at pregnancy detection (gestation day 38) and discontinued after birth when the dams were transferred to a standard commercial monkey diet (Monkey Diet Jumbo 5037, LabDiet). By the third trimester (gestation day 150), the ID dams had significantly lower hemoglobin, hematocrit, RBC volume (mean corpuscular volume), hemoglobin content (mean corpuscular hemoglobin), and serum ferritin and higher transferrin (total iron binding capacity) (15). Infants from ID pregnancies did not differ at birth from controls in hemoglobin, hematocrit, mean corpuscular volume, or markers of iron status (ferritin, serum iron, transferrin saturation) (16). However, erythrocyte population dynamics in the postnatal period were found to reflect the prenatal ID (15). After birth, ID dams and any anemic control dams were administered iron dextran as usually used by veterinarians (10 mg/kg intramuscularly, once per week for 28 d), and infant monkeys from ID dams were supplemented with oral iron once a week beginning at 2 wk of age for 4 wk (2 mg/kg Enfamil Fer-In-Sol, Mead Johnson Nutrition). Infants were reared by their mothers in double cages with another mother-infant pair in indoor housing. They were removed from this environment for BBA assessment at 3–4 mo of age.

Genotyping was obtained from colony records. Genotyping for variable number of tandem repeat (VNTR) polymorphisms in the upstream regulatory region of the rhesus MAOA gene (n=MAOA-LPR) is conducted routinely in infant rhesus monkeys at CNPRC by the Veterinary Genetics Laboratory (VGL) with the use of PCR with MAOA-forward and MAOA-reverse primers (16, 28, 29). All PCR reactions included a negative (no DNA template) and 2 positive (MAOA genotypes 5/5 and 6/7) controls. Genotyping via fragment size analysis used VGL’s STRand software. The overall genotyping error rate at the VGL is <0.5%.

MAOA is an X-linked gene with at least 5 different VNTR polymorphisms, resulting in 13 different female genotypes and 5 different male genotypes in rhesus monkeys. The male IS and ID infants in our study were identified as hemizygous for low-MAOA (7 VNTRs) or high-MAOA (4, 5, or 6 VNTRs) polymorphisms, resulting in 4 groups: high-MAOA IS (n=5), high-MAOA ID (n=5), low-MAOA IS (n=5), and low-MAOA ID (n=4). One infant (in the ID group) was excluded from the study due to an ambiguous MAOA VNTR (6.5 repeats) of unknown relevance to MAOA expression. The study was conducted in 2 yearly replicates each containing 2 to 3 monkeys in each of the MAOA × ID subgroups.

**Growth assessments.** Growth trajectories can indicate a general developmental delay, which is important to interpretation of behavioral measures. Morphologic examinations conducted during the first year of life included body measurements (30), tooth eruption (31), and facial morphometry (32, 33).

**BBA.** The BBA involves separation from the home environment and participation in a series of tests as previously described (11, 24). Two evaluations reported here (Holding Cage Observation, Human Intruder Test) represent a primary assessment of response to stress. A third test, the Preferential Look Test, assesses response to novel stimuli as well as visual recognition memory. Another BBA test, response to videotapes of an aggressive adult male monkey, was reported previously for this cohort along with other social tests at later ages (16).

For the Holding Cage Observation, the infant monkeys were monitored directly for a 5-min period immediately and 22 h after social separation. Behavior was coded by using an ethogram (The Observer software; Noldus Information Technology). Multivariate analysis of observations from >1400 infant monkeys tested with the BBA yielded 2 main factors, activity and emotionality, reflecting 2 principal responses to stress: inhibition of activity (withdrawal) and elicitation of emotional facial expressions and vocalizations. These behavioral responses to maternal separation previously demonstrated modulation by serotonin transporter linked polymorphic region (SHTTLPR; serotonin transporter) polymorphism genotypes in rhesus monkeys (34, 35).

For the Human Intruder Test, four 1-min trials were conducted, which differed in proximity of the observer (far: 1 m; near: 0.3 m) and the amount of eye contact (profile, stare). Behavior was recorded by using an ethogram. Behaviors were compiled into an index of withdrawal and an index of distress. For the Preferential Look Test, 7 pairs of pictures of monkeys were displayed on a monitor in front of the cage. First, duplicate versions of one of the pictures were displayed on the right and left sides of the screen (familiarization trial) for 20 s. Then, the novel picture was displayed with the now-familiar picture for two 8-s test trials (preference trial), once to the left and once to the right of the familiar picture. Looking frequencies and durations were recorded during each trial. Novelty preference was calculated as the duration of looking at the novel picture as a percentage of total looking time during the 2 test trials.

During the 25-h BBA period, 4 blood samples were obtained for serum cortisol analysis by RIA (Siemens Medical Solutions Diagnostics) through the CNPRC Endocrine Core (36). The first sample was taken at 1100 h, 2 h after separation of the infant from its home environment, reflecting the initial response to separation and relocation to the BBA testing area. The second sample was taken at 1600 h at the trough of the diurnal cortisol cycle. Subsequent samples reflected the cortisol suppression by dexamethasone injection and stimulation by adrenocorticotropic hormone injection.

**Statistical analysis.** Effects evaluated were prenatal diet group (ID and IS), MAOA genotype category (high-MAOA and low-MAOA expression), and MAOA × ID interaction. Most variables were analyzed by ANOVA or repeated-measures ANOVA (RMANOVA) by using general linear models (JMP9.0; SAS Institute). Planned comparisons compared ID and IS groups within each MAOA genotype group. Correlations were evaluated with Pearson correlation coefficients. The threshold for significance was P < 0.05. Effects identified as a trend (0.05 < P < 0.065) were also examined in some cases by using the planned comparisons.

**Results**

**Growth assessments.** Head size (width, length, and circumference) appeared to be sensitive to the experimental design. ID infant monkeys, and particularly the low-MAOA ID monkeys, had smaller head size measures (Table 1). An MAOA × ID interaction appeared for head circumference at birth (P = 0.029; low-MAOA ID < IS, P = 0.0006), along with an ID effect trend at birth (P < 0.06, ID < IS). An ID effect on head circumference was also seen at 12 mo (P = 0.015, ID < IS). There was an
interaction for head length at 6 mo of age (MAOA × ID interaction by ANOVA, $P = 0.039; ID < IS, P = 0.049$), and an ID main effect for head width at 12 mo of age ($P = 0.040, ID < IS$). Gestation length was a covariate in the birth and 6-mo analyses. There were no effects on most other morphometric measures during the first year of life (Supplemental Table 2), with the exception of an effect of ID on foot length that was seen across all ages (ID < IS). Facial morphometry and tooth eruption were not affected (data not shown).

**Cortisol response.** Figure 1 shows an ID effect in that cortisol response to separation and during pharmacologic challenge of the adrenocortical system was lower in the ID group than in the IS group (RMANOVA: $P = 0.006$, ID < IS). An MAOA × ID interaction was also seen; the ID effect of lower cortisol was seen primarily in the low-MAOA group (RMANOVA, $P = 0.003$; low-MAOA ID < IS, $P = 0.01$). Responses of the adrenocortical system to dexamethasone and adrenocorticotropic hormone (ACTH) were not significant. However, the emotional distress pattern indicated a genotype-specific effect, with the high-MAOA group showing a reduction in emotionality similar to that seen in the Holding Cage Observation, but the low-MAOA group showing more emotionality. An additional measure in the Human Intruder Test was time spent on the side of the cage nearest the intruder. There was a MAOA × ID × condition interaction ($P = 0.012$). The low-MAOA ID group spent the most time near the intruder under the more challenging “stare” conditions.

**Preferential Look Test.** Picture sets were excluded if the monkey failed to look at the pictures during the familiarization trial or the preference trials. Groups did not differ in this respect. An MAOA × ID interaction was seen in the amount of looking during the familiarization trials that preceded each test ($P = 0.05$, ID, iron-deficient; IS, iron-sufficient; MAOA, monoamine oxidase A.

### TABLE 1

| Head size measurements across the first year of life in prenatally IS or ID rhesus monkeys with high and low MAOA transcription rate polymorphisms$^1$ |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | **High-MAOA**   | **Low-MAOA**   | **P (ANOVA effects)** |
| **IS**                         | **n = 5**       | **n = 5**       |                  |
| Head circumference, cm         |                 |                 |                  |
| Birth                          | 20.4 ± 0.2      | 20.6 ± 0.3      |                  |
| 6 mo                           | 23.7 ± 0.1      | 23.6 ± 0.4      |                  |
| 12 mo                          | 25.3 ± 0.2      | 25.3 ± 0.3      |                  |
| Head length, mm                |                 |                 |                  |
| Birth                          | 70.24 ± 1.10    | 70.66 ± 2.81    |                  |
| 6 mo                           | 80.18 ± 1.12    | 82.03 ± 1.47    |                  |
| 12 mo                          | 84.38 ± 0.77    | 85.37 ± 1.40    |                  |
| Head width, mm                 |                 |                 |                  |
| Birth                          | 52.56 ± 0.54    | 54.73 ± 0.95    |                  |
| 6 mo                           | 64.84 ± 0.36    | 63.87 ± 0.64    |                  |
| 12 mo                          | 68.47 ± 0.48    | 68.20 ± 0.75    |                  |

$^1$ Values are means ± SEMs. See text for detailed statistical analysis. *Different from IS low-MAOA monkeys, $P < 0.05$. ID, iron-deficient; IS, iron-sufficient; MAOA, monoamine oxidase A.
FIGURE 2  Infants were IS or ID in utero and differed in MAOA genotype (high-MAOA or low-MAOA). Mean values for each variable are shown. (A) Activity in the holding cage, represented by inverted Activity factor z scores. Less activity indicates increased withdrawal. (B) Emotionality z scores in the holding cage after separation and relocation. (C) Duration of withdrawal behaviors as a percentage of total observation time in the Human Intruder Test. “Stare” includes “stare far” and “stare near” conditions, which are the more stressful of 4 orientation-distance conditions. (D) Number of distress behaviors (facial expressions and vocalizations) during the “stare near” condition of the Human Intruder Test. (E) Duration of looking during the 20-s familiarization trial of the Preferential Look Test. (F) Duration of looking at a novel picture as a percentage of total looking time during the preference trials of the Preferential Look Test. A diet group (ID) effect of \( P < 0.05 \) is indicated by an * on the x-axis; a genotype (MAOA) effect of \( P < 0.05 \) is indicated by an * on the graph symbol legend; an interaction effect of genotype and diet (MAOA × ID, \( P < 0.05 \)) is indicated by an * at the intersection of lines on graph. \( P \) indicates an effect trend of \( P < 0.12 \). Statistical details and pairwise comparisons are provided in text, and \( P \) values are indicated on the graph. High-MAOA, high MAOA transcription rate genotype; ID, iron deficient; IS, iron sufficient; low-MAOA, low MAOA transcription rate genotype; MAOA, monoamine oxidase A; MAOA X ID, MAOA by ID.

0.035; low-MAOA ID > IS, \( P = 0.025 \)) (Figure 2E). In low-MAOA monkeys, ID increased the duration of looking. There were no effects of ID or MAOA on preference for the novel picture overall or for individual sets of pictures (Figure 2F). Behaviors reflecting emotionality were not determined for this test.

Unexpectedly, an MAOA effect was seen for right-left preference during the familiarization trial when 2 identical pictures were shown. The high-MAOA group looked at the picture on the left 49.4% of the time compared with a 61.7% preference for the low-MAOA group (\( P = 0.003 \); data not shown).

Discussion

Nonhuman primates provide a valuable and well-proven model for understanding the development of the brain and behavior. In particular, primates have in common an extended period of fetal brain development during the third trimester, the time when ID most often appears in pregnant women. In the United States, the CDC (37) reports that 33% of pregnant women receiving federal aid develop anemia in the third trimester and the incidence can be >50% in developing countries (38, 39). Although offspring of anemic mothers are not themselves anemic at birth, changes in population dynamics of RBCs are seen in both children and rhesus monkeys after fetal ID (40). In addition, the impact of the fetal iron deprivation on brain development can be detected in infancy and later in childhood development, as demonstrated in human studies (3–6, 41–43), in previous work using the rhesus monkey model (11, 16, 17, 44), and in the present report.

Generalization of the effects reported in this nonhuman primate model of third-trimester ID must take into account some of the limitations of the model. This is a single-nutrient deficiency model, whereas women who are ID in the third trimester likely experience other nutrient deficiencies as well. Furthermore, women with third-trimester anemia are likely to have been ID before conception. Finally, ID in the monkey dams was corrected promptly at birth, a treatment that may not be universally available in human populations.

With regard to postnatal growth, ID effects on foot length and head size may indicate a more general, mild linear growth restriction. In the present study, head size effects emerged most clearly in the low-MAOA ID group and a more prolonged developmental persistence (12 mo postnatally) was suggested. Head circumference in neonates is correlated with most other indices of growth, such as long bone length and foot length (45). We did not obtain measures of long bone length. It may be notable that foot length was also influenced by ID and that foot length at birth correlated highly with head circumference (\( r = 0.74 \)) in our sample. The high correlation with head circumference was also demonstrated in human neonates (46). The effect of fetal ID on head size in the current study is consistent with previous studies in rhesus monkeys (11) in which head width and head length at birth, 1 mo, and 4 mo postnatally were lower in ID infants.

Lower plasma cortisol concentration in response to the social separation and relocation was seen as a main effect of fetal ID, as well as a fetal ID × MAOA interaction in the present study. The degree of cortisol activation in the current study may have allowed the emergence of the ID effect, which was not seen in our other ID studies (11, 24). Cortisol response to the BBA test has been shown to reflect variations in the pretest environment of the monkeys (18, 36). In our studies of developmental ID, the average BBA cortisol for nursery-reared infant monkeys was 60 ± 2 μg/mL (11), for indoor mother-reared infants (current study) was 69 ± 3 μg/mL, and for the outdoor socially reared infants was 80 ± 2 μg/mL (24). It is also possible that MAOA influence was necessary to bring out this ID effect because it was most prominent in the low-MAOA ID group.

In response to fetal ID, the behavioral response to separation and relocation was diminished and this was seen more clearly in the high-MAOA infant monkeys. Both effects [less withdrawal (more activity), less emotionality], along with lower cortisol, suggest a reduced stress response in ID infants, with the reduced inhibition of activity seen primarily in the high-MAOA group. The initial behavioral response to separation and relocation, observed within 2 h of separation in the Holding Cage Observation, was characterized by withdrawal (low activity) and emotional facial and vocal expressions. The reduced withdrawal and emotionality of the ID infant monkeys has face value agreement with lower cortisol values jointly pointing to a lower immediate stress response to separation and relocation.
Some common sense translation for these quantitative behavioral differences in responsiveness was provided in the temperament ratings conducted by the BBA tester at the conclusion of the BBA session. ID infants were rated less “fearful” but only within the high-MAOA genotype.

The Human Intruder Test was similar to the Holding Cage Observation in showing reduced withdrawal in ID infant monkeys of both genotypes, and less emotionality in the high-MAOA ID monkeys. The new finding related to genotype was that the emotionality index showed an effect in the opposite direction (more emotionality) in the low-MAOA infants. The low-MAOA ID infant monkeys also spent more time near the human intruder. The hypothesis developed to explain the greater emotionality in these situations is that low-MAOA ID infants, like high-MAOA ID infants, are less inhibited in initially engaging an emotionally arousing stimulus but also stay engaged longer, resulting eventually in a greater emotional response.

The Preferential Look Test provides an index of novelty preference, the only cognitive assessment in the ID. No ID effects on novelty preference were seen. In general, suggestions of impaired cognitive function have not as yet emerged from the study of fetal ID in rhesus monkeys, although performance of cognitive tasks can be influenced (17).

Some data from the human MAOA and ID literature are consistent with the findings in this rhesus monkey study. Recently, blunted adrenocortical response to venipuncture was reported in children who had been ID in infancy (47). Studies of cortisol response to stress in low- and high-MAOA genotypes in humans found differential interaction with catechol O-methyltransferase (COMT) genetic variations in determining cortisol response (48, 49) and blunting of cortisol response in caregivers with low-MAOA genotypes (50). Early gene × environment influences on externalizing behaviors in response to stressful challenges were reported in humans (51). The single study of MAOA polymorphisms in human infants found less inhibition of visual engagement with a threatening stimulus in infants with low vs. high expression MAOA polymorphisms (52). Residual effects of early postnatal ID were also identified for externalizing behavior (53).

In human studies, environmental events interacting with MAOA genotype to determine later behavior usually are those occurring in childhood (54–56). However, recently, an interaction of prenatal environmental stressors with MAOA genotype in determining infant behavior (negative emotionality) has appeared in the literature (57). In the human studies, individuals with the low-transcription MAOA polymorphism are those who demonstrate heightened emotionality and conduct disorder when they have previously experienced an early environmental stressor.

Because of the diverse and numerous biological functions of iron, there are many pathways along which ID and MAOA transcription could interact to influence brain function. Monoamine neurotransmitter synthesis requires the iron-dependent enzymes tyrosine and tryptophan hydroxylase, but little is known about the role of these neurotransmitters during fetal brain development and even less is known about the effects of ID or MAOA genotype on fetal monoamine neurotransmitter synthesis and action. Although the MAOA gene is known to be important to monoamine metabolism (58), the MAOA VNTR polymorphisms have been difficult to relate to brain MAOA activity (59) or to changes in monoamine metabolites in humans (60–63). Because rodents do not have these polymorphisms, nonhuman primates may be a valuable model for tracing the relation between altered transcription, monoamine metabolism, and behavioral characteristics associated with the MAOA VNTR polymorphisms.

The research reported here suggests that MAOA polymorphism may be an important modifier of the deleterious impact of fetal ID on later behavior functions and diet may be a previously unrecognized source of variability in human studies of MAOA genotype and behavior. Although there is interest in the origin of psychopathology in gene × environment interactions, another concept emphasizes the role of gene × environment interactions in creating the population diversity valuable for human adaptation (64). With this in mind, further study of early nutrition in connection with common gene polymorphisms may open up new approaches to the understanding of both individual differences and of childhood behavior disorders.

References


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