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Relation of Neonatal Iron Status to Individual Variability in Neonatal Temperament

ABSTRACT: *The relation between indices of neonatal iron status and individual differences in neonatal temperament were investigated in a sample of 148 low-income Peruvian women and their newborn infants. Using cord blood, at birth we obtained measures of neonatal ferritin, serum iron, and hemoglobin. While neonates were still in the hospital, their behavior during a structured anthropometry examination was videotaped and subsequently coded on four temperament dimensions: activity level, negative emotionality, alertness, and soothability. The same dimensions were coded using a videotape obtained during a subsequent visit to the neonates' homes. Results indicated that lower levels of neonatal hemoglobin and serum iron were related to higher levels of negative emotionality and to lower levels of alertness and soothability. A similar pattern was found for ferritin, but only for females. For the most part, relations between neonatal iron measures and neonatal temperament were linear, operating across the full range of iron values. Our pattern of iron–temperament results could not be attributed to variation in family demographics, low birth weight, gestational age, maternal dietary intake, or markers of neonatal illness and maternal diabetes. Our findings are consistent with prior research with older infants relating iron deficiency to temperament. These results support the importance of increased research on the early functional–behavioral consequences of individual differences in iron status as well as on the mechanisms that underlie such consequences. © 2005 Wiley Periodicals, Inc.* Dev Psychobiol 46: 141–153, 2005.

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Temperament is defined as: ‘‘Biologically rooted individual differences in behavior tendencies that are present early in life and are relatively stable across various kinds of situations and over the course of time’’ (Bates, 1989, p. 4). Implicit in this definition is the hypothesis that we should be able to identify individual differences in at least some domains of temperament even in the neonatal period (Rothbart, Derryberry, & Posner, 1994). Obviously, identifying stable individual behavioral characteristics in the neonatal period is an enterprise fraught with methodological problems. For example, the expression of neonatal temperament may vary depending on the environmental context within which the neonate is assessed (Ricciuti & Breitmeyer, 1988; Wachs, Pollitt, Cueto, & Jacoby, 2004); however, researchers have been able to identify neonatal behavioral patterns fitting temperament dimensions such as irritability, activity level, and alertness (Rothbart, Derryberry, & Hershey, 2000; Wachs et al., 2004). These neonatal temperament dimensions can be reliably scored (Reise, 1983; Ricciuti & Breitmeyer, 1988) and show short-term stability across the first few months of life (Crockenberg & Smith, 1982; Korner, Hutchinson, Koperski, Kraemer, & Schneider, 1981; Worobey, 1986; Worobey & Lewis, 1989). Further, some studies also have demonstrated modest levels of prediction between indices of neonatal temperament and measures of temperament assessed at or after the first year of life (Korner et al., 1985; Newnham et al., 1997; Reise, 1987, 1995).

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Given that there is a biological basis for individual differences in temperament, and that individual differences in neonatal temperament can be reliably measured and have predictive value, an important question is what biological factors are associated with variability in early appearing individual differences in temperament? There has been a substantial body of research showing genetic contributions to temperament (e.g., Braungert, Fulker, & Plomin, 1992; Plomin et al., 1993; Robinson, Kagan, Reznick, & Corley, 1992; Saudino, Plomin, & DeFries, 1996); however, what little evidence that is available also indicates that genetic influences upon temperament appear to be far less during the neonatal period (Reise, 1990). While other studies have related biomedical risk factors to indices of neonatal temperament, reviews indicate that evidence from these studies does not yield a consistent pattern of results (Wachs & Bates, 2001).

Conceptually, intrauterine nutrition as well as the levels of specific nutrients assessed at birth (e.g., iron) also could contribute to individual differences in temperament. Both infrahuman and human data have documented the sensitivity of the fetal central nervous system (CNS) to both general malnutrition and to specific nutritional deficiencies (Morgane et al., 1993; Rao & Georgieff, 2000). In addition, those aspects of CNS structure and neurotransmitter metabolism that have been shown to be influenced by variability in nutritional intake are, in many cases, the same CNS areas and metabolic processes that have been implicated in individual variability in temperament (Wachs, 2000). One specific nutrient that may be of particular relevance for individual differences in temperament is iron. Infrahuman research has shown that the CNS areas affected by pre- and perinatal iron deficiency include those involved in emotional processing (de Ungria et al., 2000). At the human level, Vaughn, Brown and Carter (1986) reported higher levels of irritability in newborns whose mothers were iron deficient. In earlier studies with older infants, Lozoff and colleagues (Lozoff et al., 1998; Lozoff, Wolf, & Jimenez, 1996) reported higher levels of negative affect and lower levels of attention to people and objects in 12- to 24-month-old infants with iron-deficiency anemia, as compared to nonanemic infants. In a more recent article, Lozoff et al. (2003) reported that 6-month-old infants who received iron supplementation were higher on positive affect, were more oriented towards people in their environment, and were more soothable at 12 months of age than were infants who did not receive iron supplementation. While the previous evidence is supportive of a potential link between early iron status and early temperament, little is known about the behavioral consequences of differences in neonatal iron status (Beard & Connor, 2003); in our review of the literature, we were unable to find any studies directly relating measures of neonatal iron status to measures of neonatal temperament. Thus, the focus in the present article is on relations between neonatal temperament and neonatal iron measures.

Using a relatively large sample of pregnant Peruvian women and their newborn infants, we assessed maternal anthropometry, diet, and iron status during the second and third trimesters of pregnancy as well as measures of fetal growth, neonatal anthropometry, neonatal iron status, and neonatal temperament. Because our analyses indicated that variability in neonatal temperament was unrelated to our measures of maternal and neonatal anthropometry, maternal diet, maternal iron status, or fetal growth, we report only our findings for neonatal iron. The lack of significant prediction for our measures of maternal anthropometry, diet, and iron status and fetal and neonatal anthropometry was not unexpected given the nature of our sample. While the overwhelming majority of mothers in our sample had low intakes of iron, calcium, folate, and zinc, this was not a sample characterized by severe malnutrition, severe anemia, or fetal growth retardation (see descriptive data). In addition, there is evidence indicating that fetuses are at least partially buffered against maternal malnutrition, so that unless there is severe maternal malnutrition or severe maternal micronutrient deficiencies, there are not likely to be functional consequences to the fetus (Dobbing, 1990; Mahomed, 2003). For example, while the iron supply of the fetus is basically derived from maternal iron stores during pregnancy (Allen, 1997; Michaelsen, Milman, & Samuelson, 1995; O'Brien, Zavaleta, Abrams, & Caulfield, 2003), measures of neonatal hemoglobin have generally not been found to be related to maternal iron status during pregnancy, even when mothers are anemic (Allen, 2000; Halvorsen, 2000). Given evidence for fetal buffering plus the fact that ours was not a population of severely malnourished mothers or a population characterized by severe intrauterine growth retardation, it is not surprising that our maternal or fetal measures were nonpredictive.

However, there is evidence suggesting that early iron status may be predictive, even in a population that is not severely iron deficient. Individual differences in maternal iron status during pregnancy are related to indices of less severe neonatal iron deficiency such as neonatal serum iron (Agrawal, Tripathi, & Agarwal, 1983), neonatal ferritin (Hokama et al., 1996; Milman, Agger, & Nielsen, 1994), and neonatal serum transferrin receptors (Choi, Kim, & Par, 2000). Further, even in the absence of neonatal iron-deficiency anemia, there is a greater risk of later iron deficiency or iron-deficiency anemia for neonates with reduced levels of iron (Georgieff, Wewerka, Nelson, & Deregnier, 2002) whose mothers were iron deficient during pregnancy (Colmer et al., 1990; Preziosi et al., 1997). In addition, some evidence suggests that neonatal iron status may be predictive of later temperament, even in the absence of severe iron deficiency. Specifically, Tamura et al. (2002) reported that neonates whose ferritin values fell in the lowest quartile of their sample showed significantly lower alertness and tractability (an index of self-regulation) at 5 years of age, as rated by their mothers. Besides these data, there is an almost complete absence of studies investigating relations between neonatal iron status and neonatal behavioral patterns. Given the almost total absence of information, we believe it is important to present our findings on neonatal iron and temperament, if only to suggest the potential importance of additional research in this area.

METHOD

Sample

The Canto Grande Maternal Child Health Center (MCHC) was the site of the study. The MCHC is a state health facility that serves low-income families within Canto Grande. Canto Grande is a low-income, semi-urbanized district located northeast of Lima, Peru, at an altitude of 200 m above sea level. The MCHC is the largest public health facility in the area where mothers living in this district could deliver their babies. The rates of unemployment and underemployment for the local population were considered high according to national census criteria. The mean number of people per household was five. While 97% of the houses in our sample had electricity and 83% had a public water supply, only 65% of houses had access to sewage services.

Pregnant women who lived in the MCHC-Canto Grande district were identified in the first trimester of pregnancy. Criteria for inclusion of pregnant women were: less than 14 weeks/gestation as determined from last menstrual period and confirmed later via ultrasound exam, no significant maternal physical or mental health problems, mother greater than 14 years old, and mother willing to attend the two subsequent prenatal checks programmed for the study. A total of 249 mothers met these inclusion criteria and formed our initial sample; however, 99 mothers of newborns in the initial sample chose to have their baby at a hospital other then MCHC, which was our primary site. While we were able to make arrangements with these other hospitals to assess temperament on these 99 neonates while they were still in the hospital, we were unable to arrange for neonatal blood collection at birth. In addition, for 2 infants born at MCHC, problems in videotaping led to loss of temperament assessments. Thus, our final sample consisted of 148 newborns for whom we had complete data on pregnancy measures during the second and third trimesters and iron status and temperament at birth. Comparison of infants for whom we had complete data versus those for whom we had no data on neonatal iron indicated higher levels of alertness ($t = 4.59$, $p < .01$) and activity level ($t = -3.62$, $p < .01$), as assessed in our structured laboratory procedure, for neonates in the no-iron measures group; however, there were no other group differences in the other six temperament measures or in measures of family SES, prenatal status measures, (e.g., fetal weight, maternal glucose) or measures of newborn status (e.g., gestational age, birth weight). With a sample size of 148, power analysis indicated that we should be able to detect a medium to small effect size for iron with power greater than .80 and a medium to small covariate effect size with power greater than .80.

Prenatal Measures and Procedures[1]

Each trimester, trained dieticians assessed the energy and nutrient intakes of the mothers using standardized 24-hr dietary-recall assessments taken on 2 nonconsecutive days. Intakes were compared with current dietary recommended intakes (e.g., Institute of Medicine, 2000). In terms of specific dietary deficiencies, those nutrients with the greatest probability of inadequacy for women at 10 to 24 weeks and 28 to 30 weeks of pregnancy, respectively, were folate, calcium, iron, and zinc, with most of the population not covering 25% of the recommended intake for these nutrients across each of the trimesters. The levels of maternal dietary intake found in our sample are similar to those reported by Sacco, Caulfield, Zavaleta, and Retamozo (2003) for pregnant women living in another peri-urban area of Lima. Alcohol use during pregnancy was relatively rare, with over 90% of women in our sample reporting no intake of alcohol during pregnancy and less then 1% reporting an intake of more then one drink per week. Over 75% of the women in our sample reported no caffeine intake over the course of pregnancy.

At the end of the first trimester, 8 ml of fasting venous blood were drawn from the mothers during their regularly scheduled prenatal visit to the MCHC; 6 ml of fasting venous blood were drawn at the end of the second trimester of pregnancy. Measures of maternal hemoglobin, serum iron, total iron binding capacity, blood glucose level, and C-reactive protein were obtained from maternal blood samples. Measures of maternal iron status declined significantly across trimesters. While the average level of maternal Hb was greater than 11.0 g/dl ($SD = 1.0$) in the first trimester, by the end of the second trimester the average Hb level had significantly dropped to below 10.5 g/dl ($M = 10.4$, $SD = 1.00$); this latter level is recommended as the cutoff point defining maternal anemia in the later stages of pregnancy (Milman, Byg, & Agger, 2000). Ultrasound measures were taken in the second and third trimesters to assess fetal growth rates. Our ultrasound measurements indicated that fetuses showed adequate growth during the course of pregnancy, with significant gains in estimated fetal weight occurring between Trimesters 1 to 2 and 2 to 3.

Neonatal Measures and Procedures

After the infant was born, using a standardized examination procedure, trained Institute for Nutritional Investigation (IIN)

[1]Because the primary focus in this article is on neonatal iron, we only briefly summarize the measures and procedures used to assess maternal and fetal measures taken during pregnancy. Readers wishing a detailed description of how we assessed and coded our measures of maternal anthropometry, maternal diet, maternal biochemistry, and fetal growth during pregnancy or our measure of neonatal anthropometry can obtain this information by writing to the first author. Readers wishing a detailed description of our nonsignificant findings for these variables also can obtain this information by writing to the first author.

field workers assessed the infant's body weight, length, and skinfold thickness. During the examination, field workers also examined each child for the presence of minor physical anomalies. To assess levels of neonatal hemoglobin, hematocrit, serum ferritin, serum iron, total iron binding capacity, and C-reactive protein, 4 ml of cord blood taken at birth were used. To obtain cord blood, the cord was cut and clamped. The umbilical cord from the placenta to the clamp was held by surgical tweezers, and a needle with a syringe was inserted into the vein. The blood sample was removed, put into a test tube, and centrifuged within 30 min to separate out the serum prior to storage. All blood determinations were performed at the laboratory of the IIN, and a standardized set of procedures were implemented to ensure that cord blood drawn would reach the IIN laboratory without contamination or deterioration. The analytic quality of data from the IIN laboratory has been evaluated and has received high rankings from a number of standardization agencies including the National Institute of Standards and Technology (Gaithersburg, MD, USA) and the Swedish National Food Administration Quality Control Program (Uppsala, Sweden).

Neonatal temperament outcomes were assessed on two separate occasions. The first assessment occurred within 2 days after the infant was born and took place during the structured laboratory procedure assessing neonatal anthropometry and minor physical anomalies. The second assessment occurred after the infant was discharged from the hospital and was an unstructured observation at the infant's home between the Days 3 and 7 of life. In the structured observation, the neonate was undressed and trained examiners weighed the neonate, assessed length using a Franklin plane, and assessed skinfold thickness using calipers. To assess minor physical anomalies, examiners assessed distance between the eyes, whether ears were set low on the head, 5th-finger curvature, palmer crease, and asymmetry in toe size using the procedure developed by Waldrop, Bell, McLaughlin, & Halverson (1978). Because both the anthropometry and the physical anomalies exam required manipulating the neonate in specific ways, this procedure can be regarded as the more stressful of the two examination situations. If the neonate became distressed during the examination, except for holding the child between procedures, no attempts at soothing took place.

The unstructured observation was carried out at home at a time between feedings when the neonate was awake. The neonate was placed on his or her back on a bed or a couch in the home, and mothers were instructed not to touch or talk directly to their child unless instructed to do so by the examiner. If the neonate became distressed, maternal intervention was requested as part of a standardized soothing procedure. In contrast to the structured procedure, no demands were placed on the neonate during the unstructured home observation, and neonatal distress was responded to relatively quickly.

Both structured and unstructured assessments were videotaped. Because of the more intrusive nature of the structured assessments, neonates did not fall asleep during these procedures; therefore, all structured assessments were videotaped in a single session that took between 15 to 20 min to complete. For the unstructured procedure, our goal was to obtain a minimum of 15 min of videotape when the infant was awake. If the infant fell asleep before the 15 min were up, the examiner returned no later than the following day and continued videotaping until we had at least the 15-min minimum.

All videotapes were sent to Purdue University, where they were coded by a trained graduate-student observer or by the Purdue University co-principal investigator. In all cases, coders were unaware of the neonates' iron status. Coding was done using 15-s observational cycles. The coder observed the video until the sound of a recycling timer indicated that 15 s had elapsed. At this time, the coder paused the videotape and recorded the ratings. When the recycling timer indicated that another 15-s cycle was starting, the coder began the videotape and observed for another 15-s cycle. Both coders independently coded a subset of tapes to assess intercoder reliability. Intraclass correlations were used to assess level of intercoder reliability (Bartko, 1976). Across the five dimensions coded (discussed next), the average intraclass $r = .95$, with a range from $r = .91$ to $r = .99$.

Based on the neonatal temperament coding criteria developed by Riese (1987) and by Ricciuti and Breitmeyer (1988), in both assessment contexts we coded neonatal *state* plus four dimensions of neonatal temperament: alertness, activity level, negative emotionality (distress), and soothability. Initial state at the start of the temperament assessment procedure was coded using a 6-point scale ranging from asleep to crying, with midpoint codes being regarded as optimal state. Alertness was coded using a 4-point scale ranging from no visual orienting to high alertness. Activity was the summed score across two codes: head and number of limbs moved plus vigor of movement (coded on a 4-point scale ranging from no movement to very large movements). Negative emotionality was coded using a 4-point scale ranging from no negative affect to intense distress. When neonates were coded as showing moderate or intense distress, soothability was then coded. The soothability code was based on the number of different soothing manipulations it took (range = 0–6) before the neonate returned to a nondistressed state (Detailed coding criteria for each dimension of temperament are available from the principal author upon request.)

Covariate Measures

A culturally relevant SES inventory developed at IIN was administered upon enrollment of the mothers. This instrument included data on education of the parents, construction material of their house, services at home (water, electricity, sewage), number of siblings, occupation of the parents, and household possessions. Measures of maternal dietary intake and maternal caffeine and alcohol use during pregnancy were obtained via dietary recall measures obtained during the second and third trimesters of pregnancy, as described earlier. Measures of maternal C-reactive protein and maternal glucose levels during pregnancy and neonatal C-reactive protein at birth were obtained from blood biochemistry assessments, as described previously. Gestational age was assessed from ultrasounds taken during pregnancy, as also described previously. Measures of birth weight, type of delivery, and neonatal Apgar scores were taken directly from hospital records.

Data Analyses Procedures

In assessing the possible contributions of neonatal biochemistry, we utilized the most direct measures of early iron status: neonatal serum iron, serum ferritin, and hemoglobin levels. Both visual analysis of the distribution and descriptive statistics indicated that hemoglobin (skew = −.300, kurtosis = −.147) and serum iron (skew = .136, kurtosis = .177) approximated a normal distribution. While the distribution for ferritin was shifted to the left (skew = 1.325, kurtosis = 3.173), this distribution was normalized utilizing a square-root transformation (skew = .342, kurtosis = .748); however, given that our results were essentially identical when we used the nontransformed or the square-root transformed ferritin values, for ease of comparison we report the findings for the nontransformed ferritin values.

In contrast to results for older children and adults, far less is known about the functional consequences of deficits in measures of neonatal iron status, particularly with regard to the question of whether a continuous or a discrete (threshold) model is more appropriate. Given the lack of definitive evidence on an appropriate cutoff point, we treated each of our neonatal iron measures as a continuous variable. When significant relations were found between neonatal iron and neonatal temperament measures using a linear model, we then reanalyzed the data to see if a nonlinear model provided a better fit. When a nonlinear model provided a better fit to the data, we inspected the scatter plot to determine what range of neonatal iron values was most predictive.

Given the potential importance of considering context when looking at indices of neonatal temperament, we chose to separately analyze the four neonatal temperament dimensions for each testing situation. Because of deviations from a normal distribution, activity level in the structured situation and alertness in the unstructured observation were transformed using log transformation (thus reversing directionality). For distress, we used a percentage score, reflecting the percent of observational blocks the child was scored as displaying either moderate or high levels of distress. The %score in turn was transformed using a square-root transformation. For soothability, using cluster analysis procedures children were classified into one of three groups reflecting either *low soothability*, *moderate soothability*, or *high soothability* (Details of group-composition criteria may be obtained from the principal author.)

Because of the paucity of previous data on the question of nutritional contributions to variability in neonatal temperament, we felt that greater than usual consideration needed to be given to minimizing Type II errors, in addition to the usual emphasis on avoiding Type I errors. Our analytic goal was to find an approach which minimized the likelihood of capitalizing on chance by using too many comparisons, yet would allow us to look at relatively specific predictor–outcome relations. Because of the many unknowns with regard to relations among our different measures of neonatal iron status and our measures of neonatal temperament, we did not feel that structural equation modeling would be appropriate given that this approach requires having explicit and plausible models of the links among the different predictors and different outcomes (Schumacker & Lomax, 1996). Given our analytic requirements, we chose a two-stage analytic strategy. In the first stage, we computed the canonical correlation between the eight measures of neonatal temperament and the three measures of neonatal iron status. A test of the null hypothesis that all correlations between multiple predictor and multiple criterion variables are zero order is equivalent to a nonsignificant canonical correlation between a set of predictor and a set of criterion variables (Johnson & Wichern, 1982); however, if the overall canonical correlation between our indices of neonatal temperament and our indices of neonatal iron status is significant, this means that at least some dimensions of neonatal iron status are significantly related to neonatal temperament over and above chance levels. To determine which specific nutritional and temperament variables were producing the overall significant canonical correlation in the second stage of data analysis, we computed a series of eight multiple regressions, regressing our neonatal iron measures onto each measure of neonatal temperament. By use of this two-stage strategy, we both minimize the likelihood of capitalizing on chance (initial canonical correlation) while maintaining our ability to identify specific iron–temperament relations (Stage 2 regressions).

In two sets of additional analyses, we used the mediation test model described by Baron and Kenny (1986). According to Baron and Kenny, if a mediating variable is responsible for the relation between predictor and outcome, then statistically controlling for the influence of the mediator will reduce the predictor–outcome relation to nonsignificance. The standard mediation test model described by Baron and Kenny requires that both the predictor and alternative mediating variables be significantly related to both each other and to the outcome variable. Given the stringency of this criteria and the exploratory nature of our research, it was decided to utilize a less stringent set of criteria in our second set of mediation analyses, designed to determine if non-iron variables were responsible for observed relations between our measures of neonatal iron and neonatal temperament. Specifically, variables were chosen for further study in our second set of mediation analysis if they were significantly related to *any one* of our three iron predictors or if they were significantly related to *more then one* of our eight temperament outcome measures.

RESULTS

Descriptive Data

Only 2% of our sample of newborns had birth weights less than 2.5 kg, 66.3% had birth weights between 2.5 and 3.49 kg, and the remaining 31.5% had birth weights greater than 3.5 kg. Only 4 neonates in our sample had 1-min Apgar scores below 7, and only 1 neonate had a 5-min Apgar score below 7. There were only 2 preterm infants in our sample (The range of gestational ages at birth was 32–39 weeks.) Data for the preterm infants were not used in our analyses.

With regard to our measures of neonatal iron, the mean value for serum iron was 148.79 ug/dl (SD = 44.99 ug/dl). The normal range of serum iron in full-term neonates is 125 to 225 ug/dl (Siner & Newman, 2002). A total of 30.8% of our sample had serum iron levels below 125 ug/dl; 3.8% had values above 225 ug/dl. The mean ferritin level in our sample was 153.19 ug/L ($SD = 78.67$ ug/L). The normal range of ferritin in full-term neonates is between 25 to 200 ug/L (Siner & Newman, 1997). Only 1 neonate had a ferritin value below 25 ug/l whereas 22.6% had values exceeding 200 ug/l. The mean hemoglobin level in our sample was 15.6 g/dl ($SD = 1.4$ g/dl). The normal range of hemoglobin values in full-term neonates is 14.0 to 20.0 g/dl (Siner & Newman, 2002). Fifteen percent of our sample had hemoglobin levels below 14 g/dl, and none were above 18.9 g/dl. While neonatal serum iron and ferritin levels were significantly correlated with each other in the expected direction ($r = .21$, $p < .01$), the correlation between neonatal ferritin and hemoglobin, while significant, was negative ($r = -.22$, $p < .01$); serum iron and hemoglobin levels were uncorrelated ($r = -.01$, n.s.).

The means and *SD*s (in parentheses) for neonatal temperament scores are shown in Table 1. As shown, analysis of neonatal temperament in the structured and unstructured contexts revealed that while there were no differences in activity level across the two contexts, there were significantly higher levels of neonatal alertness in the unstructured testing situation, along with significantly higher levels of distress and higher numbers of less soothable neonates in the structured testing situation. Given the differing nature of the testing situations, these significant differences would be expected. As shown in Table 1, analyses also indicated low, but significant, cross-contextual correlations between each of our temperament domains. These results are consistent with results from other studies examining the stability of different measures of neonatal temperament assessed in different contexts in the first week of life which also reported modest, but significant, correlations (St. James-Roberts & Wolke, 1988). There was a trend for neonates to be in a more alert, active state at the start of the unstructured observation session. As would be expected, the correlation of initial neonatal state across testing contexts was nonsignificant ($r = .06$, n.s.).

Neonatal Temperament and Neonatal Iron Status. The overall canonical correlation between our three measures of neonatal iron status (Hb, serum ferritin, and serum iron) and our eight neonatal temperament scores (soothability, alertness, activity, and distress assessed in the structured and unstructured settings) was statistically significant ($Rc = .46$, Bartlett's $\chi^2 = 55.87$, $df = 24$, $n = 148$, $p < .01$). As discussed earlier, significance of the total canonical correlation gives us overall protection against a Type 1 error when we break down analyses to determine what aspects of neonatal iron status and neonatal temperament were driving the significant canonical correlation.

To break down the significant canonical correlation, we ran eight multiple regressions using our three neonatal iron status measures as predictors and individual neonatal temperament dimensions assessed in either the structured or the unstructured setting as outcome variables. Five of the eight regressions were significant (Table 2). For temperament assessed in the structured laboratory situation, neonates with higher hemoglobin levels were rated as more alert. Also shown in Table 2, neonates with higher levels of serum iron were rated as showing less distress and as being less active (activity log transformed, so directionality reversed and higher activity scores mean lower activity). In the unstructured assessment, results again indicated significantly greater alertness for neonates with higher hemoglobin levels (alertness log transformed, so directionality reversed). In addition, neonates with higher levels of serum iron were more likely to be rated as

Table 1. Neonatal Temperament Scores[a]

Variable	Structured (lab)	Unstructured (home)	Mean Differences across Lab and Home Assessments	Lab–home Assessmment Correlations
Alertness (4-point scale)	2.26 (0.73)	3.45 (0.51)	$t = 23.64$**	$r = -.13^{*,a}$
Activity (combined 8-point scale)	5.45 (1.98)	5.51 (2.24)	$t = 0.84$	$r = -.18^{**,a}$
% time blocs showing high or moderate distress	49.15 (27.07)	23.19(17.36)	$t = 18.28$**	$r = .16$**
Soothability group membership	Low = 10.6% Moderate = 69.4% High = 20%	Low = 18% Moderate = 32.4% High = 49.6%	$\chi^2 = 63.38$**	$\chi^2 = 9.29$
Initial state	3.10 (1.55)	3.31 (0.76)	$t = 1.95^t$	.06

**$p < .01$.
*$p < .05$.
[t]$p < .06$.
[a]True directionality of relation reversed due to log transformation of activity in the structured laboratory assessment and alertness in the unstructured home assessment.

Table 2. Significant Regression Analyses Relating Neonatal Iron to Neonatal Temperament Measures[a]

Iron Measure	Temperament Lab Alertness $R^2 = .053$ $F(3, 144) = 2.67$*	β	t
Hemoglobin		.220	2.63**
Serum Iron		.045	0.55
Ferritin		.111	1.30
	Lab Activity $R^2 = .063$ $F(3, 144) = 3.23$*		
Hemoglobin		.052	0.63
Serum Iron		.211	2.57*
Ferritin		.102	1.21
	Lab Distress $R^2 = .102$ $F(3, 144) = 5.44$**		
Hemoglobin		−.126	−1.54
Serum Iron		−.267	−3.32**
Ferritin		−.107	−1.29
	Home Alertness $R^2 = .082$ $F(3, 144) = 4.26$**		
Hemoglobin		−.288	−3.49**
Serum Iron		.008	0.10
Ferritin		−.011	−0.13
	Home Soothability $R^2 = .053$ $F(3, 144) = 2.68$*		
Hemoglobin		−.012	−0.14
Serum Iron		−.227	−2.75**
Ferritin		.092	1.08

$n = 148$ in all analyses.
*$p < .05$.
**$p < .01$.
[a]True directionality of relation reversed due to log transformation of activity in the structured laboratory assessment (low score means high activity) and alertness in the unstructured home assessment (low score means high alertness) or to reverse coding of soothability group (low score means high soothability).

easier to soothe following distress (Negative beta reflects reverse scoring of soothability group.)

We reanalyzed each of the five regressions shown in Table 2 to determine if a better fit to the data could be obtained using a nonlinear model (log, quadratic, cubic) rather than our original linear model. For all analyses involving hemoglobin and for those analyses involving serum iron, distress, and soothability, no improvement was found when a nonlinear analysis was used, suggesting that relations are occurring across the full range of iron values. In only one analysis involving serum iron and lab activity (change in $R^2 = .047$, $p < .01$) was a better fit obtained using a nonlinear cubic model.[2] For serum iron and lab activity, a cubic model suggested little relation between activity level and serum iron in the 85- to 185-ug/dl range. Increased activity level was found for neonates with serum iron levels below 85 ug/dl, and decreases in activity level were found for neonates with serum iron levels greater than 185 ug/dl.

Given evidence cited earlier on the pattern of relations between iron and temperament, our finding that lower serum iron level is related to higher laboratory-assessed activity level seems counterintuitive; however, our findings do suggest an alternative interpretation. As reported earlier, higher serum iron is related to both lower activity level and lower distress in our laboratory assessment. Our results also indicate that higher distress in the laboratory situation is related to higher activity level ($r = -.52$, $p < .01$; activity level log transformed, hence the negative correlation). This pattern suggests the possibility that neonatal distress may be mediating the relation between serum iron and activity level. Mediation occurs when significant relations between a specific predictor (serum iron) and outcome variable (activity level) occur because of the influence of a third (mediating) variable common to both predictor and outcome (distress). To test this mediating hypothesis, using the Baron and Kenny procedure described earlier, we reran the regression between our neonatal iron measures and lab activity, initially entering neonatal lab distress. Under these conditions, the previously significant relation between serum iron and laboratory activity level dropped to nonsignificance in the regression ($t = 1.187$, $\beta = .091$, n.s.). What this result indicates is that neonates with low serum iron levels are more likely to be distressed, and that one of the consequences of greater distress is higher activity level.

Additional Tests for Mediation Effects. A final set of analyses was utilized to assess whether observed relations between neonatal temperament and measures of neonatal iron status could be attributed to an alternative mediating variable other than iron, per se. As described earlier, variables were chosen for further mediation analysis if they were significantly related to *any one* of our three iron predictors or if they were significantly related to *more than one* of our eight temperament outcome measures.

In the initial step of our mediation analysis, based on our review of the literature and reviewers' comments, we identified 10 potential alternative mediating variables that were available in our database and which previous research had related to either neonatal iron or neonatal temperament. Four of the mediating variables chosen had been identified in previous research as potentially relevant to individual differences in neonatal temperament. These variables included *neonatal state* (Sameroff, Krafchuck, & Bakow, 1978), *birth weight* (Garcia-Coll, Halpern, Vohr, Seifer, & Oh, 1992; Riese, 1994; Sajaniemi, Salokorpi, & vonWendt, 1998), *maternal medication during delivery* (Lester, Als, & Brazelton,

[2]Readers wishing to have a copy of our results for the nonlinear regressions can obtain these by writing to the first author.

1982; because we did not have adequate record data of use of anesthetic or analgesics during delivery, we assessed whether the baby was born by C-section as a proxy), and *maternal caffeine intake* during pregnancy (Engle et al., 1999). An additional three variables chosen had been identified in previous research as potentially relevant to individual differences in neonatal iron status. These variables included *family SES* measures (parental education level, home quality, and family possessions: Grantham-McGregor, Fernald, & Sethuraman, 1999), *neonatal biomedical status* (Allen, 2000; Lee & Nieman, 1996: assessed via 1- and 5-min Apgar scores and neonatal C-reactive protein), and *maternal diabetes* (Georgieff, Schmidt, Mills, Radner, & Widness, 1992; Nelson et al., 2000; Petry et al., 1992; measures of maternal blood glucose level obtained during pregnancy were used as a marker variable for risk of maternal diabetes). Three additional measures also were selected based on prior research relating these variables to both neonatal temperament and neonatal iron status. These three additional measures were *gestational age* (Luchtman-Jones, Schwartz, & Wilson, 2002; Ricciuti & Breitmeyer, 1988), *maternal intake during pregnancy of calcium and zinc* (Guiang & Georgieff, 1998; Merialdi, Caulfield, Zavaleta, Figueroa, & DiPietro, 1998), and *maternal alcohol intake* during pregnancy (Miller, Roskams, & Connor, 1995; Weinberg, 1997). In addition to these 10 potential mediators, we also looked at gender as a possible moderator of our findings relating neonatal temperament to neonatal iron measures, given evidence suggesting the possibility of gender differences in both iron metabolism at birth (Choi et al., 2000; Tamura et al., 1999) and infant temperament (Eaton & Enns, 1986; Rothbart, 1989).

In Step 2 of our mediational analysis, we assessed whether the 10 variables selected met our criteria of being related to any one of our neonatal iron measures or to more than one of our neonatal temperament measures. In this analysis, our measures of family SES, maternal dietary intake of calcium and zinc during pregnancy, maternal caffeine intake during pregnancy, C-section birth, neonatal C-reactive protein, and birth weight were dropped from further consideration since they did not meet either of these criteria. Five variables met one of the aforementioned criteria, namely neonatal state (met the Baron & Kenny, 1986, criteria), maternal glucose levels during pregnancy (related to more than one temperament outcome), maternal alcohol intake during pregnancy (related to more than one temperament outcome), neonatal gestational age (related to more than one temperament outcome), and 1-min Apgar score (related to neonatal hemoglobin). These variables were each initially entered into our iron–temperament regressions to determine if our pattern of findings would change after accounting for the influence of state, glucose, alcohol intake, gestational age, and Apgar. While there was modest variation in both betas and significance levels, with one exception all significant iron–temperament relations reported in Table 2 remained significant after accounting for the influence of state, maternal glucose during pregnancy, maternal alcohol intake during pregnancy, gestational age, and 1-min Apgar.[3] The one exception involved the relation between serum iron and laboratory activity level, which dropped below the traditional criterion for statistical significance ($\beta = .137$, $t = 1.68$, $p < .10$) after variance associated with the neonate's initial state at the start of the laboratory session was accounted for.

As discussed previously, we also investigated the question of whether gender moderated our pattern of findings. While there were no gender differences in our iron measures, males were more alert and less distressed than females in the laboratory setting while females were more active in the home assessment. To test for moderation, as recommended by Baron and Kenny (1986), Gender × Iron interaction terms were entered as a second step in those regressions previously identified as significant in our initial analyses. Two significant Gender × Iron interactions were identified. The first was a Gender × Ferritin interaction for alertness assessed in the laboratory situation ($t = -2.29$, $\beta = -.469$, $p < .05$); the second also was a Gender × Ferritin interaction for soothability assessed at home ($t = -2.10$, $\beta = -.431$, $p < .05$). Breakdown of the first interaction indicated that the relation between ferritin and alertness assessed in the laboratory setting was significant for females ($r = .29$, $p < .01$), but not for males ($r = -.08$, n.s.), with the difference between the two correlations statistically significant by *r-z* transform test ($z = 2.34$, $p < .05$). Similarly, the correlation between ferritin and soothability assessed in the home was significant for females ($r = -.26$, $p < .05$), but not for males ($r = -.18$, n.s.), with the difference between the two correlations again being statistically significant ($z = 2.70$, $p < .01$).

DISCUSSION

Until now, the largest body of research on the biological roots of temperament has focused on the genetics of individual differences in temperament. In terms of other potential biological influences, at least for our sample, results indicate little relation between prenatal nutritional factors such as maternal nutritional intake or fetal growth. Our nonsignificant findings for these predictors does not

[3]Readers wishing to have a copy of our results for these mediational analyses or our moderation analysis can obtain these by writing to the first author.

necessarily mean that maternal nutrition during pregnancy is unrelated to individual differences in early temperament. There remains the question of whether such prediction might occur with a sample that is more severely malnourished than the women in our sample.

What our results do suggest is the potential relevance of neonatal iron status to variability in early temperament. Specifically, our findings indicate that lower levels of neonatal hemoglobin are related to lower levels of neonatal alertness, assessed in both a structured laboratory-examination procedure and in an unstructured observation done in the neonate's home. The relation between hemoglobin level and neonatal alertness appears to be linear, operating across the full range of hemoglobin values, with no evidence for a threshold above or below which hemoglobin is unrelated to alertness. Our results also indicate that lower levels of neonatal serum iron are related to increased neonatal negative emotionality, as seen in greater distress and distress-mediated activity level assessed during the laboratory testing and reduced soothability assessed during the unstructured home observation. What appears to be a setting difference (Serum iron predicts distress in the laboratory assessment and soothability in the home assessment.) may well reflect procedural factors, given that examiners did not attempt to soothe neonates during the laboratory assessment while distress was responded to quickly in the home-observation setting. Rather than setting differences, what our findings suggest are differential relations, with hemoglobin related to alertness, which may be an early manifestation of later developing self-regulation processes (Derryberry & Rothbart, 1997) while individual differences in serum iron level are related to indices of negative emotional reactivity. While ferritin was unrelated to any of our temperament measures when assessed as a main effect, our results do suggest the possibility of gender differences, with higher levels of ferritin being related to higher alertness and soothability for females, but not for males.

It is obvious from our regressions that neonatal iron measures offer only a partial explanation of variability in neonatal temperament. Depending upon the specific iron and temperament measures assessed, neonatal iron accounts for between 5 to 10% of unique variance in neonatal temperament. It is possible that the relatively small amount of predictive variance associated with neonatal iron would have been greater had there been more neonates with severe levels of iron deficiency in our population (discussed later); however, at least within this study, our findings with regard to neonatal iron do appear to be robust. As part of our analytic strategy, we considered the possibility that our results were due to the actions of an alternative mediating variable that covaries with neonatal iron status and with individual variability in neonatal temperament. To test for alternative mechanisms besides iron, we first identified a set of 10 potential mediators based on our review of the literature and suggestions from reviewers of this article. We eliminated a number of these potential mediators from consideration (family SES, maternal caffeine, calcium and zinc intake during pregnancy, C-section birth, 5-min Apgar, neonatal C-reactive protein, and birth weight) based on the fact that they were unrelated to any of our three neonatal iron measures and to less than two of our eight neonatal temperament measures. We then recomputed our analyses to determine if our pattern of findings would change after partialling out the variance associated with the remaining mediators that did correlate with any measure of neonatal iron or more than one of our temperament measures: neonatal state, maternal glucose level during pregnancy, maternal alcohol use during pregnancy, 1-min Apgar, and gestational age. With the exception of the relation between serum iron and neonatal activity, all initial significant findings on the relation of hemoglobin and serum iron to neonatal temperament remained significant, even after controlling for the influence of these alternative explanatory variables.

Obviously, elimination of the aforementioned variables as potential mediators of our findings in and of itself does not indicate that we can attribute variability in neonatal temperament primarily to variability in neonatal iron status, in part because of the limited amount of unique variance associated with neonatal iron and because this list of mediating factors is not exhaustive. The impact of other potential mediating variables such as maternal cigarette use during pregnancy, specific drugs taken during pregnancy, or when the umbilical cord is clamped following delivery could not be assessed because this information was not in our database. While we cannot conclusively eliminate all possible mediators as an alternative explanation for our findings, we can conclude that our findings remained robust even after taking into account some of the most likely alternative explanations.

Given that our results relating variability in neonatal temperament to measures of neonatal iron status cannot be easily attributed to alternative non-iron explanations, a critical question is the mechanism that underlies our findings. Any iron-related explanatory mechanisms offered must be considered in relation to the many issues involved in conceptualizing the nature and consequences of measures of individual differences in neonatal iron status (Beard & Connor, 2003). The most obvious explanatory mechanism would be iron deficiency, given what is known about the effects of iron deficiency on CNS development (Beard & Connor, 2003; Rao & Georgieff, 2000) and the relation of CNS function to temperament (Wachs, 2000). Support for iron deficiency as the mechanism underlying our findings can be found in the consistency between our pattern of associations relating neonatal iron measures to increased neonatal distress and reduced neonatal alertness and soothability with results from infrahuman studies and studies with older iron-deficient infants. At the infrahuman level, our findings are consistent with evidence indicating reduced attention to environmental cues in iron-deficient as opposed to iron-sufficient young rats (Beard, Erikson, & Jones, 2002). At the human level, our results indicating reduced attention and increased negative emotionality are consistent with the findings by Lozoff and colleagues (2003; Lozoff et al., 1998; Lozoff et al., 1996) based on studies with iron-deficient anemic toddlers.

However, before attributing our pattern of findings to iron deficiency, it is essential to consider the nature of our population. While there was an increased risk of iron deficiency in our sample, the majority of neonates in our sample were not iron deficient. As illustrated in our descriptive data, while 30% of our sample was below the threshold for normal levels of serum iron and 15% was below threshold for hemoglobin, the mean sample values for serum iron and hemoglobin were above threshold. Further, only 1 neonate was below the normal threshold for ferritin. In addition, our curve fitting analyses indicated that in almost all cases a linear model fit the data, indicating that iron–temperament relations were operating across the full range of iron values rather than being restricted to neonates with the lowest iron values.

Given the nature of our sample, targeting iron deficiency as an explanatory mechanism would require an additional assumption, namely that neonates are highly sensitive to even low levels of iron deficiency. The possibility that early brain development may be highly vulnerable to iron deficiency has been raised by researchers studying infrahuman populations (Youdim, Ben-Shachar, & Yehuda, 1989). Congruent with the conclusion of Youdim and colleagues (1989), Felt and Lozoff (1996) reported reduced orientation and activity levels in 8-day-old rat pups with normal hemoglobin that had been exposed to an iron-deficient intrauterine environment during their early gestational period. Unfortunately, little human evidence is available testing the assumption of high susceptibility to low levels of early iron deficiency, particularly for behavioral–developmental outcomes. As noted earlier, Tamura et al. (2002) showed long-term developmental consequences for neonates with reduced levels of cord serum ferritin; however, Tamura et al.'s (2002) significant findings were restricted to neonates in the lowest quartile of the ferritin distribution (<76 ug/l). Our results with a different age group and outcome measures indicated a ferritin–temperament link only for females, with no evidence supporting the superiority of a nonlinear model. Thus, whether human neonates are particularly susceptible to low levels of iron deficiency remains a critical question that must be dealt with before we can conclude that our findings represent a downward extension of the findings of Lozoff and colleagues (Lozoff et al., 1996, 1998, 2003), or that relations between iron deficiency and individual variability in temperament may be occurring earlier in life then previously suspected.

In terms of other potential mechanisms of iron metabolism besides iron deficiency, exposure to excess iron during the neonatal period has been linked to impaired neurological function during the first year of life, perhaps as the result of oxidation due to free radical toxicity (Buonocore et al., 2003). However, given inadequate maternal dietary iron during the prenatal period, a relatively low frequency of mothers taking iron supplementation during pregnancy and the lack of markers indicating iron overload in neonates in our sample (e.g., excessively high ferritin levels: Guiang & Georgieff, 1998) as well as linear rather than nonlinear threshold patterns relating iron to temperament, iron excess does not seem to be a likely alternative mechanism. A related mechanism, suggested by one reviewers of this article, was that the underlying mechanism could involve iron homeostasis rather then iron deficiency, per se. The importance of developing adequate iron regulation in neonates is well established (Guiang & Georgieff, 1998). It is possible that the negative correlation found between ferritin and hemoglobin levels in our sample may reflect neonatal attempts to regulate iron homeostasis. Based on evidence from both infrahuman and human samples, Georgieff and colleagues suggested that such a negative relation may be biologically plausible, reflecting a reallocation of storage iron into the red-cell mass to meet increased neonatal iron demands (Amarnath, Ophoven, Mills, Murphy, & Georgieff, 1989; Georgieff et al., 1990; Georgieff, Widness, Mills, & Stonestreet, 1989; Guiang, Georgieff, Lambert, Schmidt, & Widness, 1997); however, negative correlations between storage iron and hemoglobin are more likely to occur in preterm neonates undergoing specific stresses that require iron mobilization, such as hypoxemia (Rao & Georgieff, 2002). These conditions do not characterize our sample. Further, in contrast to iron deficiency and iron excess where there are known biological consequences that could act to influence neonatal temperament, the underlying mechanism through which poorly regulated iron homeostasis could translate into variability in neonatal temperament is unclear. Results from infrahuman studies have suggested that when storage iron is depleted in the fetal or neonatal period, physiological regulation processes prioritize remaining iron towards red blood cell production at the expense of organs such as the brain (Guiang et al., 1997). While little human evidence is available on the developmental consequences of such iron-regulation processes, redirection of iron away from the brain could have potential influences on neonatal behavioral patterns.

While much is known from infrahuman studies about the physiological consequences of iron deficiency in the fetal and neonatal period, there is remarkably little evidence on the behavioral consequences, particularly in human populations (Beard & Connor, 2003). While our findings support a link between individual variability in neonatal iron status and individual variability in neonatal temperament, the nature of the process underlying these findings remains a question for future research. The results presented here support the importance of increased research on the early functional–behavioral consequences of individual differences in iron status as well as on the mechanisms that underlie such consequences, particularly in populations where there is a high incidence of more severe early iron deficiency.

NOTES

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