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Original Research Article

Effects of iron supplementation on neural indices of habituation in Bangladeshi children

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ABSTRACT

Background: Iron deficiency and anemia have been associated with poor cognition in children, yet the effects of iron supplementation on neurocognition remain unclear.

Objective: We aimed to examine the effects of supplementation with iron on neural indices of habituation using auditory event-related brain potentials (ERPs).

Methods: This substudy was nested within a 3-arm, double-blind, double-dummy, individual randomized trial in Bangladesh, in which 3300 8-mo-old children were randomly selected to receive 3 mo of daily iron syrup (12.5 mg iron), multiple micronutrient powders (MNPs) (including 12.5 mg iron), or placebo. Children were assessed after 3 mo of intervention (mo 3) and 9 mo thereafter (mo 12). The neurocognitive substudy comprised a randomly selected subset of children from the main trial. Brain activity elicited during an auditory roving oddball task was recorded using electroencephalography to provide an index of habituation. The differential response to a novel (deviant) compared with a repeated (standard) sound was examined. The primary outcome was the amplitude of the mismatch response (deviant minusstandard tone waveforms) at mo 3. Secondary outcomes included the deviant and standard tone-evoked amplitudes, N2 amplitude differences, and differences in mean amplitudes evoked by deviant tones presented in the second compared with first half of the oddball sequence at mo 3 and 12.

Results: Data were analyzed from 329 children at month 3 and 363 at mo 12. Analyses indicated no treatment effects of iron interventions compared with placebo on the amplitude of the mismatch response (iron syrup compared with placebo: mean difference (MD) = 0.07μV [95% CI: −1.22, 1.37]; MNPs compared with placebo: MD = 0.58μV [95% CI: −0.74, 1.90]) nor any secondary ERP outcomes at mo 3 or 12, despite improvements in hemoglobin and ferritin concentrations from iron syrup and MNPs in this nested substudy.

Conclusion: In Bangladeshi children with >40% anemia prevalence, iron or MNP interventions alone are insufficient to improve neural indices of habituation.

This trial was registered at the Australian New Zealand Clinical Trials Registry as ACTRN12617000660381.

Keywords: iron, anemia, multiple micronutrients, children, brain function, event-related brain potentials, neurocognition

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Abbreviations used: CRP, C-reactive protein; EEG, electroencephalography; ERP, event-related brain potential; IQR, interquartile range; MD, mean difference; MNP, multiple micronutrient powder; RCT, randomized controlled trial; SD, standard deviation.

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Introduction

An estimated 81 million children 3–4 years of age living in low- and middle-income countries experience low cognitive or socio-emotional development [1]. Adversity during early life, including malnutrition, may influence the long-term cognitive development of children. In fact, a key rationale for the prevention of anemia in young children, including through iron supplementation as recommended by the WHO [2, 3], has been the assumed benefits to child cognition. In South Asia, the prevalence of malnutrition is high; for instance, in Bangladesh, anemia affects 71% of children between 6 and 23 mo of age [4]. Micronutrients, especially iron, may play a critical role in the developing infant brain through effects on myelination, neurotransmission, and synaptogenesis [5, 6]. Yet, the neurophysiologic effects of iron interventions in a population of children facing a high prevalence of iron deficiency and anemia have not been clearly determined.

Measurement of early childhood cognition can be challenging. Parent reports and behavioral tests have proven to be useful in large population surveys but are general measures of child development that may be prone to assessor and respondent bias, and alternative approaches are needed. Event-related brain potentials (ERPs) are neurophysiologic measures that primarily reflect the activity of cortical pyramidal neurons that are time-locked to an event, such as a stimulus presentation [7]. ERPs can be recorded noninvasively at the scalp surface and, unlike behavioral assessments, can directly measure neuronal activity with high temporal resolution without requiring a behavioral response [8]. Furthermore, differences in cognitive functioning detected using ERPs early in life have been shown to be predictive of later intelligence [9–12]. In high-income countries, ERPs have been used to measure neural indices of cognitive functions, such as memory [13, 14], attention [15–17], and executive function [18]. This technique has been seldom used in resource-limited settings [19–22].

Animal studies have demonstrated iron's role in myelination [23–26], neurotransmission [27], dendritogenesis [28], and synaptogenesis [28]. Structural, dopaminergic, neurometabolic, neurochemical, and electrophysiologic functions in the striatum and hippocampus have been shown to be affected by iron deficiency in rats [29–33]. Observational studies in humans show significant cognitive impairments in individuals with iron deficiency, but findings might be vulnerable to confounding [34]. However, previous interventional trials of iron treatment in young children have shown mixed effects, and meta-analyses of randomized trials have not found conclusive evidence of significant effects from iron interventions on early child development [35], perhaps because the measures of cognitive skills utilized in these studies (e.g., Bayley Scales of Infant and Toddler Development, Ages and Stages Questionnaire) are less sensitive to iron-induced improvements. ERP-derived neural indices of memory and attention have emerged as objective and direct measures that are sensitive to iron status [6, 36, 37]. Habituation, which relies on cognitive functions, such as working memory and attention [38–40], describes a broad set of processes that lead to differential responses to recently encountered, predictable, or familiar stimuli compared with novel or surprising ones [41, 42]. Neural indices of habituation reflect the ability to distinguish commonplace events from those that are relatively novel or unusual, which is necessary for directing attention to, and learning from, novel and informative events in a child's environment and are established markers of healthy developmental processes [12, 42–46]. Observational studies have shown associations between children's iron status and neural indices of habituation [36, 37]; however, to our knowledge, no randomized controlled trial (RCT) of iron supplementation has used ERPs to measure the direct effect of improved iron status on neural indices of cognition in young children.

In this randomized placebo-controlled study, we sought to determine the effects of supplementation with iron syrup and iron-containing multiple micronutrient powders (MNPs) on neural indices of habituation using auditory ERPs in Bangladeshi children.

Methods

This neurocognitive substudy was nested within the Benefits and Risks of Iron Supplementation in Children (BRISC) trial (www.anzctr.org.au; ACTRN12617000660381). The BRISC study was a collaboration between the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b), the University of Melbourne, Australia, and the Walter and Eliza Hall Institute, Australia. The parent trial and the neurocognitive substudy were approved by the ethical review committees of icddr,b, and Melbourne Health.

BRISC study randomization and masking

BRISC was a 3-arm, double-blind, double-dummy, individual randomized, superiority trial conducted in the Rupganj Upazila of Narayanganj district, Bangladesh. A detailed explanation of the study characteristics is described elsewhere [47–49]. Briefly, at 8 mo of age ±14 days, 3300 children were randomly selected to receive 3 mo of daily *1)* 12.5 mg iron (ferrous sulfate) syrup + placebo MNPs, or *2)* MNPs (containing iron 12.5 mg as ferrous fumarate, retinol 200 ug, zinc 5 mg, folic acid 0.16 mg, and vitamin C 30 mg) + placebo iron syrup, or 3) placebo iron syrup and placebo MNPs. Exclusion criteria included severe anemia (hemoglobin <80 g/L), drinking water iron content > 1 mg/L, mid-upper-arm circumference <11.5cm, current illness with fever, previously known inherited red cell disorders, or severe developmental disability or delay. Children were randomly assigned to 1 of the 3 arms using a 1:1:1 allocation, stratified by union (local area) of residence and sex. The study team and participants were blinded to the intervention. Assessments were completed in children at baseline, 3 mo postintervention (mo 3), and after a further 9 mo follow-up (mo 12). The Bayley Scales of Infant and Toddler Development, third edition, was used to measure the cognitive, language, and motor development of children at all time points. The study began recruitment in July 2017 and completed follow-up visits in February 2020. Results of the primary study are presented elsewhere [49]. The full BRISC study will henceforth be referred to as the parent study.

Neurocognitive substudy design

Recruitment to the substudy was based on recruitment of up to 5 participants per day, capped at the upper limit of the work capacity for the ERP testing team. Starting in December 2018, using a random number generator, we randomly selected children enrolled in the parent trial to also be included in the neurocognitive substudy starting at the 3-mo postintervention visit. Children who completed the 3-mo postintervention ERP measurement were asked to return for the same measurement at the 9-mo follow-up visit (i.e., 12 mo after the start of the intervention). Additional children were enrolled using random selection to obtain ERP measurements at the 9-mo follow-up visit (Figure 1, Supplementary Figure 1).

Ethical considerations

Before consenting, caregivers were shown all the equipment and given a full explanation of the testing procedures. Written informed consent was obtained from all caregivers of children participating in this substudy. Caregivers were reimbursed for their travel and time.

Experimental paradigm

Children were presented with sequences of auditory tones using a roving oddball design (detailed further in Supplementary Methods) [50]. In this design, a stream of pure sine wave tones was presented through stereo speakers on the left and right side of the child. Thirty different tone frequencies were presented, ranging from 100Hz to 5000Hz (between 100 and 1000 Hz in steps of 100 Hz and between 1000 and 5000 Hz in steps of 200 Hz). At the beginning of the experiment, a tone (randomly selected from the set of tone frequencies) was presented for 200 ms and was repeated after a 300 ms interstimulus interval. After a set number of repetitions, a different tone was presented and then repeated between 4 and 10 times until another new tone was presented. The first tone within a train of tone repetitions was termed a “deviant” tone, whereas the rest were termed “standard” tones (Figure 2).

EEG data acquisition and processing

EEG data were recorded from 32 active scalp electrodes, placed according to the international 10–20 system, using a Brainvision LiveAmp system (Brain Vision, LLC) according to standard procedures. Data preprocessing was conducted using EEGLab v13.4.4 [51] running in Matlab (2018b, The MathWorks) using an established processing pipeline [52] (detailed in Supplementary Methods). The code used for processing the data is available at https://osf.io/gc45j/. EEG data were segmented from −100 ms to 400 ms relative to the onset of each auditory tone and baseline-corrected using the average of the −100 ms to 0 ms time window relative to auditory tone onset. At this step, 9 measurement electrodes spanning bilateral frontal sites were selected based on the observed distribution of (deviant–standard) ERP differences, consistent with previous studies [50, 53, 54].

ERP data analyses

ERPs were then averaged for each tone repetition number separately for each child’s dataset (detailed in Supplementary Methods). Before unblinding, we first identified the time points and channels where (deviant–standard) differences (i.e., mismatch responses) occurred using mass-univariate analyses. From these analyses, we identified our primary time window of interest, which spanned 200–400 ms from auditory tone onset. During this window, deviants evoked more positive-going ERPs at bilateral frontal electrodes (Fz, FC1, FC2, FC5, FC6, F3, F4, F7, and F8) (Supplementary Figure 2, Supplementary Figure 3, Supplementary Figure 4, and Supplementary Figure 5). Mismatch responses were defined as the (deviant–standard) amplitude difference averaged over these time points and electrodes (i.e., as a mean ERP amplitude difference measure). Our study’s primary outcome was the mismatch response at mo 3.

We also derived several secondary outcomes. We calculated mean amplitudes over the 200–400 ms time window evoked by the standards and deviants separately. This was done to probe whether any observed effects on (deviant–standard) difference waves primarily reflected group differences in either deviant- or standard-evoked ERPs, which may index differential processing of novel and repeated sounds, respectively. We also measured the mean amplitude of the auditory N2 component between 90–110 ms from auditory tone onset at channel Fz, consistent with a previous study of infants from the UK and the Gambia that used an auditory oddball design [19]. This component is also

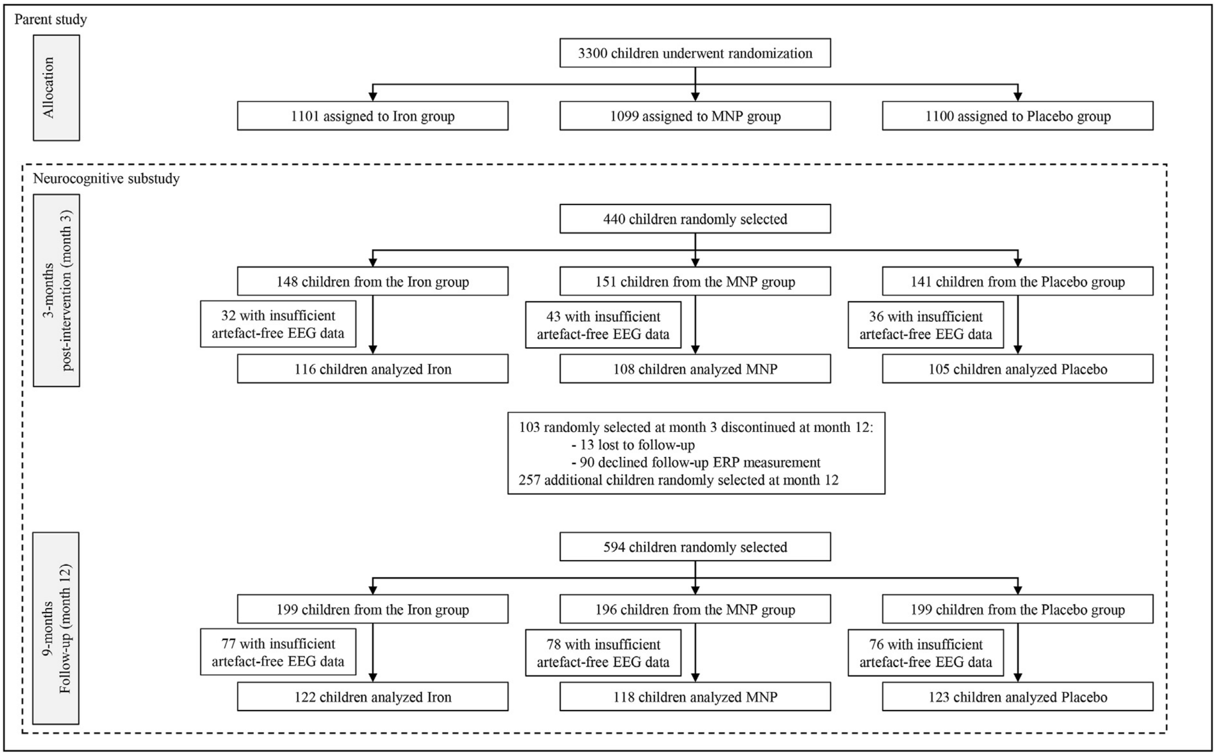

Figure 1. Neurocognitive substudy[1]

[1] EEG, electroencephalography; ERP, event-related brain potential; MNP, multiple micronutrient powder.

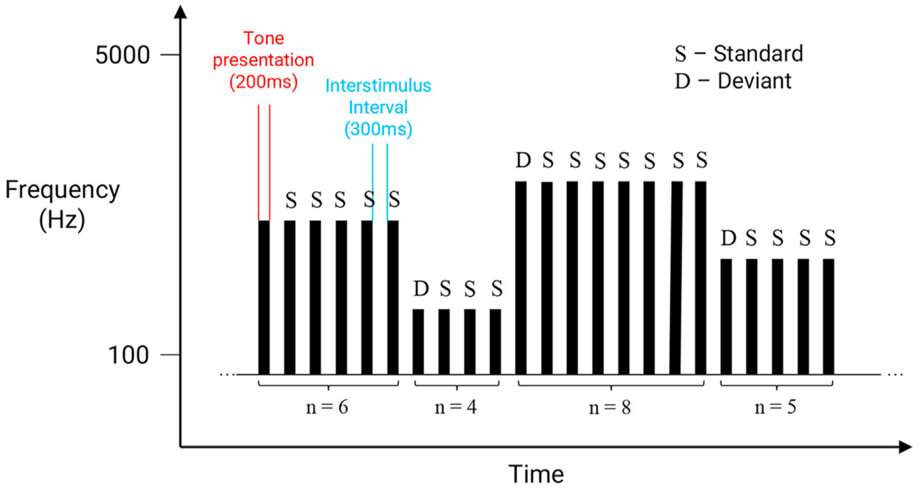

Figure 2. Auditory roving oddball paradigm design[1]

[1] A stream of pure sine wave tones was presented to participants. At the beginning of the experiment, a tone (randomly selected from the set of tone frequencies) was presented for 200 ms and was repeated after a 300 ms interstimulus interval (resulting in a stimulus onset asynchrony of 500 ms). After a set number of repetitions [4–10], a different tone was presented and repeated between 4 to 10 times until another new tone was presented. The first tone of a train of tone repetitions was considered a "deviant" tone, whereas the rest were considered "standard" tones. There were 7 different numbers of auditory tone repetitions [4, 5, 6, 7, 8, 9, and 10]] that were each equally likely to occur. Thirty different tone frequencies were presented, ranging from 100 Hz to 5000 Hz (ranging from 100 Hz to 1000 Hz in steps of 100 Hz and between 1000 Hz and 5000 Hz in steps of 200 Hz). Each child was presented with 588 auditory tones in total, including 84 deviants. S, standard tone; D, deviant tone.

termed early negativity and was reported to be larger in amplitude for deviants compared with standards in both newborns and adults [43, 55]. The same study also reported a reduction in deviant-evoked ERPs from the first to the second half of the experiment, which was interpreted as a general habituation to the (initially novel) stimuli [19]. We also identified the same effect over the 200–400 ms window using mass-univariate analyses and calculated (first half–second half) difference scores for each child to test for differences in longer-term habituation by treatment group.

Sample size

Based on previous literature on associations between iron deficiency anemia and ERPs and the effects of a nutrition intervention on child ERPs [10, 56], a standardized difference of 0.4 was considered an appropriate effect size for the primary outcome at mo 3. A sample size of 120 in each treatment group allows us to detect this effect size in the amplitude of the mismatch response between each intervention arm and placebo, with 80% power at a 2-sided 2.5% alpha level (Bonferroni for 2 comparisons, 2 treatment groups compared with placebo). Data loss for reasons, such as movement and child fussiness of about 20% has been reported [57, 58], and therefore, we aimed to enroll a total of 450 children of those who were planned to attend the month 3 visit of the parent study after initiation of the substudy.

An additional opportunistic sample of 257 children was randomly selected for EEG measurements at mo 12 only. The sample included children who were not assessed as part of the neurocognitive substudy at mo 3 but had recently completed mo 12 measurements for the parent study. Although these children did not have a mo 3 EEG measurement, we assessed them at mo 12 to examine the effects of the intervention after a longer follow-up period, anticipating some participant drop-out between mo 3 and 12.

Statistical analyses

To assess bias in the selection of participants of the neurocognitive substudy, baseline characteristics were compared between children who were enrolled in the substudy and those not, children with sufficient artifact-free epochs and those with insufficient artifact-free epochs, and between treatment groups.

For analysis of the primary outcome, we fitted a linear regression model adjusted for stratification variables used during the randomization of the parent study (union and sex). Treatment effects were estimated as the difference in the mean of the outcome (here, amplitudes of the [deviant–standard] ERP difference waveform, or mismatch response, at mo 3) between the 2 treatment groups. This measure indexes a prominent auditory habituation effect that can be reliably identified in young children [19]. Continuous secondary outcomes were analyzed similarly to the primary outcome and included the mismatch response at mo 12, deviant and standard amplitudes, auditory N2 (deviant–standard) amplitude differences, and deviant mean amplitude in the second compared with first half of the experiment at mo 3 and 12.

Additional analyses were conducted, adjusting for prespecified potential confounders (i.e., baseline family care indicator score [59] and maternal education) and for baseline variables that were not comparable across treatment groups at baseline, in addition to adjusting for union and sex and restricted to those considered compliant with the intervention (i.e., $\geq$70%) (additional models detailed in Supplementary Methods). Preplanned subgroup analyses were run to examine whether treatment effects differed between subgroup categories (detailed in Supplementary Methods)

Analyses described above included complete cases for mo 3 and 12 separately. Additional analyses were conducted, including evaluable data at mo 3 and 12 of children who were recruited at mo 3 using a mixed effects linear regression model, accounting for correlation between repeated measurements using an unstructured variance-covariance and including in the model study visit as a categorical variable, treatment, and treatment by study visit interaction, adjusted for union and sex.

To assess whether iron syrup and MNPs improved hemoglobin and ferritin concentrations in our sample, treatment effects on hemoglobin concentration were estimated as the difference in means between treatment groups, and on ferritin concentration as geometric mean ratios, at mo 3 and 12 using linear regression models with adjustment for stratification variables.

Data were analyzed in Stata/SE 16.1 (StataCorp LLC). All tests were 2-sided using a 5% level of significance unless stated otherwise. Except for the iron interventions versus placebo comparisons of the

Table 1

Participant characteristics at baseline, by treatment group[1]

	Children with sufficient artifact-free EEG data at month 3 (*N* = 329)			Children with sufficient artifact-free EEG data at month 12 (*N* = 363)		
	Iron	MNPs	Placebo	Iron	MNPs	Placebo
	N = 116	*N* = 108	*N* = 105	*N* = 122	*N* = 118	*N* = 123
Household characteristics						
Maternal education (y)	2 (2–3)	2 (2–3)	2 (2–3)	2 (2–3)	2 (2–3)	2 (2–3)
Paternal education (y)	2 (2–3)	2 (2–3)	2 (2–3)	2 (2–3)	2 (2–3)	2 (2–3)
Wealth index						
Quintile 1 (relative poorest) *n* (%)	24/116 (20.7)	26/108 (24.1)	21/105 (20.0)	28/122 (23.0)	33/118 (28.0)	25/123 (20.3)
Quintile 2 *n* (%)	21/116 (18.1)	15/108 (13.9)	19/105 (18.1)	25/122 (20.5)	21/118 (17.8)	22/123 (17.9)
Quintile 3 (relative middle) *n* (%)	30/116 (25.9)	26/108 (24.1)	24/105 (22.9)	26/122 (21.3)	23/118 (19.5)	31/123 (25.2)
Quintile 4 *n* (%)	19/116 (16.4)	12/108 (11.1)	23/105 (21.9)	20/122 (16.4)	19/118 (16.1)	21/123 (17.1)
Quintile 5 (relative wealthiest) *n* (%)	22/116 (19.0)	29/108 (26.9)	18/105 (17.1)	23/122 (18.9)	22/118 (18.6)	24/123 (19.5)
Household Food Secure status[2] *n* (%)	94/116 (81.0)	83/106 (78.3)	86/104 (82.7)	102/122 (83.6)	88/114 (77.2)	105/123 (85.4)
Child characteristics						
General		–				
Female sex *n* (%)	63/116 (54.3)	45/108 (41.7)	53/105 (50.)	65/122 (53.3)	62/118 (52.)	62/123 (50.4)
Age (mo)	8.0 (0.3)	8.0 (0.3)	8.0 (0.3)	8.0 (0.3)	7.9 (0.3)	8.0 (0.3)
Family Care Indicator total score[3]	12.3 (7.3)	13.4 (6.6)	14.4 (7.4)	13.3 (7.7)	13.4 (6.5)	12.7 (6.7)
Laboratory indices						
Hemoglobin concentration (g/L) venous	109.6 (10.7)	109.2 (8.9)	111.7 (8.6)	109.2 (10.0)	109.5 (8.5)	109.7 (9.0)
Anemia venous[4] *n* (%)	54/116 (46.6)	45/102 (44.1)	40/100 (40.0)	60/120 (50.0)	52/111 (46.8)	59/119 (49.6)
Ferritin (ug/L)	20.3 (11.4–35.7)	23.4 (12.6–36.2)	20.5 (14.7–36.3)	22.8 (11.2–43.6)	21.4 (13.8–36.7)	21.4 (12.7–40.4)
Iron deficient[5] *n* (%)	39/115 (33.9)	31/100 (31.0)	19/98 (19.4)	40/117 (34.2)	30/109 (27.5)	30/117 (25.6)
Iron-deficient anemia[6] *n* (%)	29/115 (25.2)	16/100 (16.0)	14/98 (14.3)	32/117 (27.4)	18/109 (16.5)	21/117 (17.9)
C-reactive protein (mg/L)	0.81 (0.39–2.65)	1.05 (0.36–3.52)	0.97 (0.30–2.15)	0.79 (0.36–1.81)	1.09 (0.40–2.89)	1.06 (0.36–2.15)
Inflammation[7] *n* (%)	15/115 (13.0)	19/100 (19.0)	12/98 (12.2)	13/117 (11.1)	21/109 (19.3)	8/117 (6.8)
Child growth						
Length/height-for-age *z*-score	−1.33 (0.98)	−1.35 (1.00)	−1.35 (1.01)	−1.11 (1.04)	−1.24 (1.04)	−1.30 (0.91)
Weight-for-age *z*-score	−0.53 (1.09)	−0.67 (1.08)	−0.54 (0.90)	−0.40 (1.05)	−0.63 (1.01)	−0.48 (0.88)
Weight-for-length/height *z*-score	0.38 (1.09)	0.22 (0.99)	0.40 (0.97)	0.38 (1.05)	0.18 (1.04)	0.42 (0.97)
Child development						
Bayley score[8]						
Cognitive composite	97.2 (8.2)	96.8 (7.5)	97.3 (7.8)	95.9 (8.4)	96.0 (7.1)	95.9 (7.9)
Language composite	90.3 (6.0)	89.4 (6.8)	90.6 (7.7)	88.4 (7.1)	90.1 (6.9)	88.8 (8.2)
Motor composite	93.3 (9.8)	94.1 (9.8)	94.0 (9.9)	93.6 (10.5)	94.4 (10.2)	93.3 (11.0)

[1] Data are presented as mean (SD) or median (IQR) for continuous measures and n/total (%) for categorical measures. Percentages may not total 100 because of rounding. EEG, electroencephalography. Complete participant characteristics at baseline by treatment group are presented in Supplementary Table 1.

[2] Food secure was defined “no” or “rarely” to question 1 and “no” to questions 2–9 on the Household Food Insecurity Assess Scale [66].

[3] Scores on family care indicator total score range from 0 to 42, with higher scores indicating more activities/use of play or reading material [59].

[4] Anemia was defined as hemoglobin <110g/L [67].

[5] Iron deficiency was defined ferritin<12ug/L or <30ug/L if C-reactive protein >5 mg/L [68].

[6] Iron deficiency anemia was defined as anemia and iron deficiency.

[7] Inflammation was defined as C-reactive protein >5 mg/L.

[8] Scores on Bayley were derived as described in Bayley N. Bayley Scales of Infant and Toddler Development: Motor Scale. Psychological Corporation; 2006 [69]. Higher scores indicating better development.

primary outcome, no comparisons were adjusted for multiple testing. For the primary outcome, we used a Bonferroni adjustment for the 2 iron interventions compared with placebo; a P value of <0.025 was deemed statistically significant. Analysis of secondary outcomes and subgroups should be considered exploratory.

Results

Recruitment to the substudy started during the second half of the parent study when approximately two-thirds of the children had completed their mo 3 assessment. Of the 440 children enrolled at mo 3 in the neurocognitive substudy, 329 (74.8%) children provided sufficient artifact-free EEG data. At month 12, 594 children were measured, including 257 newly recruited children, of whom 363 (61.1%) had sufficient artifact-free EEG data (Figure 1, Supplementary Figure 1). A total of 103 participants who underwent assessment at month 3 did not receive a repeated EEG measurement at mo 12 because of loss to follow-up ($N = 13$) or parents declining follow-up EEG measurement ($N = 90$). A total of 138 children had sufficient artifact-free EEG data at both time points.

Baseline comparison between intervention arms

Children enrolled in the neurocognitive substudy were comparable with those in the parent trial in their prevalence of baseline anemia (43.7% in children enrolled at mo 3; 48.9% at mo 12), iron deficiency (28.4% at mo 3; 29.2% at mo 12), and iron deficiency anemia (18.8% at mo 3; 20.7% at mo 12). Baseline stunting (i.e., when the children were 8 mo of age before the intervention), an indicator of a deprived environment, was identified in 25.8% of children enrolled at mo 3, and 19.4% of children enrolled at mo 12. There were differences in baseline iron deficiency anemia between intervention groups for children with sufficient artifact-free EEG data at mo 3 (Table 1, Supplementary Table 1). Among children with sufficient artifact-free EEG data at month 12, there were differences between intervention groups in terms of iron deficiency anemia and inflammation (Table 1). Minor, non-clinically relevant differences in baseline characteristics were observed between children enrolled and not enrolled in the neurocognitive substudy and between children with and without sufficient amounts of artifact-free EEG data (Supplementary Table 2 and Supplementary Table 3). Baseline characteristics were similar between children in the full neurocognitive substudy and those within the analysis restricted to children with compliance ≥70% (Supplementary Table 4).

Improvements in hemoglobin and ferritin concentrations with iron syrup and MNPs

Among children enrolled in the neurocognitive substudy, daily iron syrup improved hemoglobin concentration at mo 3 and 12. Daily MNPs improved hemoglobin concentration at mo 3, but statistically significant improvements were not found at mo 12. Iron syrup and MNPs improved ferritin concentration at both time points (Table 2).

Analyses using EEG measures

There were clear (deviant–standard) ERP amplitude differences spanning 200–400 ms from auditory tone onset (Figure 3A, Supplementary Figure 2, Supplementary Figure 5A) that were largest over bilateral frontal electrodes (Figure 3B, Supplementary Figure 5B). There were no (deviant–standard) differences during the auditory N2 time window (Supplementary Figure 2). There were no treatment effects of either iron intervention on the amplitude of the mismatch response (our primary outcome measure) or any of the secondary EEG outcomes at month 3 (Figure 3A, Table 3). Similarly, at mo 12, no effects of iron interventions were found on any EEG outcomes (Table 3, Supplementary Figure 5). Results were also similar after adjusting for potential confounders or when the analysis was restricted to children in the intervention arm with compliance ≥70% (Supplementary Table 5).

Additional longitudinal analyses in the subset of 376 children with sufficient artifact-free EEG data at months 3 and 12 of those recruited at mo 3 confirmed these findings. Treatment effects were similarly not significant for all EEG outcomes at mo 3 and 12 compared with the primary analyses. (Supplementary Table 6).

In addition, there was no evidence of differences in treatment effects between the subgroups defined by sex of child, anemia, iron deficiency, iron-deficient anemia, family care indicator, wealth index, and food

Table 2

Hematologic outcomes among children included in the neurocognitive substudy[1]

Visit	Outcome	Iron	MNPs	Placebo	Iron vs. placebo		MNPs vs. placebo	
		Mean (SD) or median (IQR)	Mean (SD) or median (IQR)	Mean (SD) or median (IQR)	Estimate (95% CI)	P value	Estimate (95% CI)	P value
Mo 3	N	113	118	109				
	Hemoglobin concentration (g/L)	113.7 (9.7)	112.9 (9.8)	110.2 (10.6)	4.5 (2.3, 6.7)	<0.001	4.2 (2.0, 6.5)	<0.001
	N	111	118	106				
	Ferritin concentration (ng/mL)	33.5 (19.2–55.7)	26.1 (14.5–47.3)	18.7 (9.1–32.5)	1.7 (1.4, 2.1)	<0.001	1.5 (1.2, 1.8)	<0.001
Mo 12	N	114	102	110				
	Hemoglobin concentration (g/L)	113.9 (9.9)	113.6 (10.0)	110.9 (12.0)	2.8 (0.3, 5.4)	0.031	2.3 (−0.4, 5.0)	0.096
	N	110	98	109				
	Ferritin concentration (ng/mL)	16.3 (8.4–28.6)	18.6 (10.8–30.0)	12.1 (6.0–16.5)	1.5 (1.2, 1.8)	<0.001	1.6 (1.3, 2.0)	<0.001

SD, standard deviation; IQR, interquartile range; MNPs, multiple micronutrient powders.

[1] Estimates represent mean difference for analyses of hemoglobin concentration and geometric mean ratio for analyses of ferritin concentration. The analysis sample includes all participants randomized to the neurocognitive substudy with the hematologic outcome measurement at the specified visit. The estimate, CI, and P value were obtained from a linear regression model adjusted for union and sex of the child.

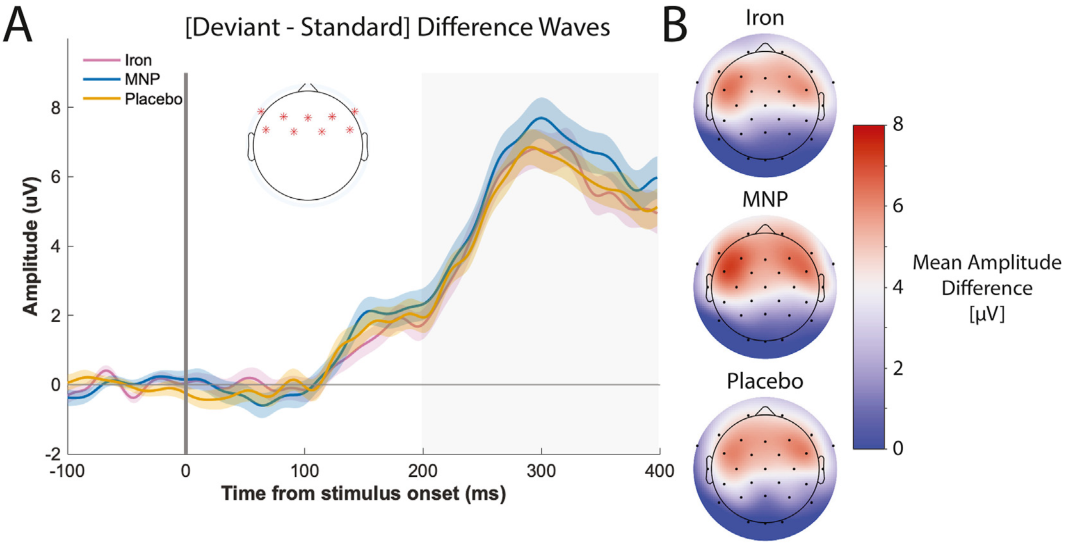

Figure 3. Mismatch responses at month 3 by treatment group[1]. A: Group-averaged [deviant–standard] ERPs by treatment group, averaged across the electrodes marked with asterisks in the scalp map. Shaded areas are SEs around the mean. B: Scalp maps of [deviant–standard] differences for each treatment group, averaged across the time period of 200 ms to 400 ms from auditory tone onset.

[1] *N* iron syrup = 116, *N* MNPs = 108, *N* placebo = 105. ERP, even-related brain potential; MNP, multiple micronutrient powder.

Table 3

Analysis results for the effects of iron syrup and MNP supplementation vs. placebo on EEG outcomes[1]

Visit	Outcome (all units are μV)	Iron	MNPs	Placebo	Iron vs. placebo		MNPs vs. placebo	
		Mean(SD)	Mean(SD)	Mean(SD)	Mean difference (95% CI)	P value[2]	Mean difference (95% CI)	P value[2]
Mo 3	*N*	116	108	105				
	Primary							
	Mismatch response	5.25 (4.94)	5.81 (5.12)	5.19 (4.49)	0.07 (−1.22, 1.37)	0.91	0.58 (−0.74, 1.90)	0.39
	Secondary							
	Deviant waveform amplitude	4.83 (4.75)	5.11 (4.38)	4.93 (4.73)	−0.10 (−1.33, 1.13)	0.87	0.13 (−1.12, 1.38)	0.84
	Standard waveform amplitude	−0.42 (1.90)	−0.70 (2.52)	−0.26 (2.65)	−0.17 (−0.81, 0.46)	0.59	−0.45 (−1.09, 0.19)	0.17
	N2 difference waveform amplitude	−0.11 (3.55)	−0.22 (3.61)	−0.00 (3.12)	−0.13 (−1.05, 0.78)	0.77	−0.20 (−1.13, 0.73)	0.67
	Deviant amplitude—First minus second half of the paradigm	1.57 (6.96)	1.82 (7.19)	0.80 (6.37)	0.74 (−1.09, 2.56)	0.43	1.08 (−0.78, 2.94)	0.25
Month 12	*N*	122	118	123				
	Primary							
	Mismatch response	2.63 (4.91)	2.76 (5.70)	2.29 (4.72)	0.32 (−0.97, 1.61)	0.63	0.46 (−0.85, 1.76)	0.49
	Secondary							
	Deviant waveform amplitude	2.16 (3.91)	2.15 (5.55)	1.64 (3.96)	0.53 (−0.62, 1.67)	0.36	0.50 (−0.65, 1.65)	0.39
	Standard waveform amplitude	−0.46 (2.36)	−0.60 (2.06)	−0.65 (2.24)	0.21 (−0.35, 0.77)	0.46	0.04 (−0.52, 0.61)	0.88
	N2 difference waveform amplitude	−0.04 (4.29)	0.15 (4.19)	−0.04 (3.72)	0.01 (−1.02, 1.04)	0.99	0.19 (−0.85, 1.22)	0.73
	Deviant amplitude-First minus second half of the paradigm	0.80 (7.55)	1.35 (9.26)	0.27 (7.48)	0.52 (−1.53, 2.57)	0.62	1.10 (−0.96, 3.17)	0.30

MNPs, multiple micronutrient powders; SD, standard deviation; CI, confidence interval.

[1] The analysis sample includes all randomized participants with sufficient artifact-free EEG data at the specified visit. The estimate, CI, and *P* value were obtained from a linear regression model adjusted for union and sex of the child.

[2] The *P* value for the primary outcome should be evaluated against a 2.5% significance level to adjust for multiplicity. All CIs and *P* values are presented unadjusted for multiple testing.

security for any of the outcomes at month 3 or month 12 (Supplementary Table7).

Discussion

Defining the role of iron interventions on child neurodevelopment is important to justify the rationale for universal iron interventions as part of anemia control policies in low- and middle-income settings [2, 3, 60] and for screening and treating anemia in high-income settings. In this nested substudy to a placebo-controlled, double-blind, randomized trial in young children living in a low-income setting with a high baseline prevalence of iron deficiency and anemia, we did not detect an effect of iron supplementation (provided through 2 different modes) on neural indices of habituation. We did not find treatment effects for any of the ERP outcomes measured, despite finding improvements in hemoglobin and ferritin concentrations for children given iron syrup or MNPs. These findings are consistent with those of the parent trial, which likewise did not find evidence of an effect of iron interventions on behavioral measures of child development using the Bayley's Scales of Infant and Toddler Development despite substantial reductions in anemia and iron deficiency [49].

Previous studies of iron supplementation in young children in resource-limited settings have generally shown null effects on child development and behavior [34, 61, 62]. However, many were limited by small sample size, lack of placebo control, and, importantly, the use of general child development assessment measures [34]. Observational studies using ERPs to investigate specific neurocognitive processes have reported differences between familiar and novel stimuli in iron-sufficient but not iron-deficient children [36, 37, 63]. However, these studies generally included very small samples (particularly among iron-deficient groups (e.g., $N = 9$–35) [36, 37, 63] and used observational study designs that are susceptible to confounding.

One additional important difference between our study and existing work is that the environmental, nutritional, and psychosocial conditions faced by the children included in our study are likely meaningfully different than those experienced by children in high-income countries, where most prior EEG studies evaluating the impacts of iron status have been conducted. Our study population had potential to benefit from iron supplementation, given the high prevalence of anemia and iron deficiency. The 3-mo intervention duration was in accordance with the WHO guidelines for iron and MNP supplementation in children [3, 64] and was enough to improve hemoglobin and ferritin concentrations at mo 3 and mo 12 [49]. Therefore, any functional effects from the interventions on habituation based on restoration of hemoglobin or iron status should have been detected.

Topographies of the mismatch responses in our study are comparable to effects previously associated with attention orientation. By using a 32-electrode EEG system, we could better characterize the mismatch effect reported by Katus et al. [19]. They found effects over a similar latency and attributed this to another ERP component, the frontal P3, which exhibits a midline fronto-central topography [65]. However, the topography of effects in our study more closely resembled auditory mismatch responses observed over bilateral frontal channels at a similar latency in adults [50, 53, 54]. In these studies, similar mismatch effects were strongest when attention was voluntarily directed to the oddball sequence stimuli and was markedly reduced or not detected when attention was directed away from them. This indicates that the mismatch responses identified in our analyses specifically indexed the orienting of attention to novel or unexpected events, which is a critical process for efficiently identifying and learning from novel occurrences in one's environment.

Our study has several key strengths. This large neurocognitive substudy was conducted within a rigorously designed double-blinded randomized trial and in a field setting where the baseline prevalence of anemia and iron deficiency was high. We used simple yet effective infrastructure for our EEG recording room and trained local psychologists on the equipment and data collection. Our extensive community sensitization activities and thorough informed consent process likely played a role in the study's substantial sample size; we successfully completed EEG recordings while presenting auditory roving oddball sequences in 699 children living in semi-rural Bangladesh. Few EEG studies have been conducted in semi-rural settings in low- and middle-income countries, and our study demonstrates the feasibility of this assessment approach for future clinical studies with neurodevelopmental endpoints. Being novel to this setting, conducting EEG research came with challenges. As in all pediatric EEG research, capping children can be stressful and time-consuming for the child and mother, which likely contributed to 20% of participants declining the follow-up (mo 12) measurement. Fussiness, crying, and movement resulted in over 25% of data loss because of substantial artifacts in the data. However, our data loss was not worse than what has been reported in child EEG studies in well-resourced settings [57, 58]. Although our design did not allow for the examination of mid-latency and slow waves, future research may benefit from using longer intervals between stimuli which allows for exploring these longer latency measures. It would also be useful to examine other important neural indices related to different capacities than the ones measured in our study task and whether they are more sensitive to iron supplementation. Iron supplementation in childhood may be too late or insufficient to improve neurocognition in populations with high iron deficiency anemia, and future research may benefit from examining the effects of iron supplementation during infancy (i.e., before 6 mo of age) or prenatally on child neural indices of habituation.

In conclusion, the results of our rigorously designed study demonstrate that, despite established links between iron availability and neurophysiological development [5, 6], increased iron availability in children under one year of age does not lead to measurable changes in neural indices of habituation in this context. Evidence from this study should be considered when designing public health interventions for South Asian children. Our findings suggest that other forms of adversity faced by children in this region (e.g., lack of early learning opportunities and responsive parenting, illness, and infection) may be critical to consider when designing public health interventions, and there is a need for more evidence on the separate and combined effects of these factors in addition to iron deficiency.

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LML, DF, SB, KAJ, JDH, SBo, and SRP designed the study. LML, DF, SBr, and JJ designed the analysis plan. LML, DF, MIH, JDH, and SRP led the data collection for this study. LML, DF, and JJ provided data management. LML, DF, SBr, and JJ processed and analyzed the

data. LML, DF, BAB, JDH, KAJ, SBo, and SRP provided scientific oversight and study supervision. LML prepared the first draft of the manuscript. All authors critically reviewed and edited the manuscript. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Data Availability

Deidentified individual participant data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajcnut.2022.11.023.

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