Effects of Glutamine on Brain Development in Very Preterm Children at School Age

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KEY WORDS
glutamine supplementation, prematurity, brain development, intervention, neonatal infection

ABBREVIATIONS
BW—birth weight
DTI—diffusion tensor imaging
FA—fractional anisotropy
GA—gestational age
SES—social economic status

Mr de Kieviet wrote the article, collected the data, and did the analysis; Dr Oosterlaan critically revised the article and helped in the interpretation of the data and study design; Drs Vermeulen and Lafeber critically revised the article and helped in the interpretation of the data; Dr Pouwels critically revised the article and did the analysis; and Dr van Elburg supervised the project and made a substantial contribution in the study conception and design. All authors approved the current article for submission and publication.

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OBJECTIVES: The amino acid glutamine has been shown to reduce the number of serious neonatal infections in very preterm children, which may benefit long-term brain development. The aims of the current follow-up study were to (1) determine the long-term effects of glutamine-enriched feeding in the first month after birth in very preterm children on measures of brain development at school age, and (2) elucidate a potential mediating role of serious neonatal infections.

METHODS: Fifty-two very preterm children who originally took part in a randomized controlled trial on enteral glutamine supplementation between day 3 and 30 after birth participated at a mean (SD) age of 8.6 (0.3) years. Measures of brain development included volumetric outcomes of major brain structures, as well as fractional anisotropy (FA) values of major white matter tracts.

RESULTS: Glutamine supplementation in the first month was associated with medium-sized increases in white matter ($d = 0.54$, $P = .03$), hippocampus ($d = 0.47$, $P = .02$), and brain stem ($d = 0.54$, $P = .04$) volumes at school age. Exploratory analyses using an uncorrected $P$ value indicated higher FA values of the bilateral cingulum hippocampal tract in the glutamine group. All differences were either strongly associated (hippocampus volume, brain stem volume, and FA values of cingulum hippocampal tract) or completely mediated (white matter volume) by the lower number of serious neonatal infections in the glutamine group.

CONCLUSIONS: Short-term glutamine supplementation after birth increases white matter, hippocampus, and brain stem volumes in very preterm children at school age, mediated by a decrease in serious neonatal infections. Pediatrics 2012;130:e1121–e1127

WHAT’S KNOWN ON THIS SUBJECT: Brain maturation processes of very premature children are adversely affected by serious neonatal infections. Differences in brain development persist into childhood and adolescence, and underpin widespread neurocognitive and behavioral deficits in very preterm children.

WHAT THIS STUDY ADDS: We present evidence for long-term beneficial effects of early nutritional intervention with glutamine in very preterm infants on brain development at 8 years of age, mediated by a decrease in the number of serious neonatal infections.
With advances in neonatal intensive care, the survival of very preterm (gestational age [GA] <32 weeks) children has improved considerably. However, a variety of risk factors associated with preterm birth, including neonatal infections and inflammatory responses, adversely affect normal brain maturation processes in these children.1,2 Consequently, widespread differences in brain development compared with term peers are found, as indicated by overall reduced brain volumes as measured by MRI and reduced white matter integrity as measured by diffusion tensor imaging (DTI).4,5 Unfortunately, these unfavorable differences in brain development appear to persist into childhood and adolescence,6 and increase the risks for poor motor,7 cognitive,8 and behavioral development7,8 in very preterm children.

Over the past decade, the potential protective effects of supplementation of the amino acid glutamine in very preterm children have been studied, including the attenuation of inflammatory response and stimulation of immunity.9 Experimental studies have revealed an important role of glutamine in maintaining the functional integrity of the gut,10,11 which in turn leads to decreased bacterial translocation and systemic spread of bacteria,12–14 and consequently may lead to decreased infectious morbidity. Indeed, 2 studies revealed that glutamine-enriched enteral nutrition between day 3 and 30 after birth decreased the number of neonatal infections in very preterm children, although other studies failed to replicate the beneficial effects of glutamine.15 Nevertheless, a lower number of neonatal infections may potentially be beneficial for long-term brain development in very preterm children.1,2 However, the long-term effects of glutamine supplementation on brain development in very preterm children have not been studied so far.

The aim of the current study was (1) to determine the long-term effects of enteral glutamine supplemented in a randomized controlled trial between day 3 and 30 after birth on measures of brain development of very preterm children at school age by using MRI and DTI, and (2) to elucidate the role of serious neonatal infections on any potential differences in long-term brain development.

METHODS

Sample

In the initial randomized controlled trial, 102 very preterm (<32 weeks) infants received enteral glutamine supplementation (0.3 g/kg per day) or isonitrogenous placebo supplementation (alanine) between day 3 and 30 after birth. All very preterm children admitted to the level III NICU of the VU University Medical Center between September 2001 and July 2003 were eligible for inclusion.16 Of the 102 infants included in the study, 89 infants were alive at 1 year of follow-up and 74 were still participating at 6 years of follow-up. At 7 years of age, parents of all 74 children were contacted and invited to participate in the current study, of which 53 (72%) children successfully completed MRI follow-up. The main reason for dropout was that either the parents or the children felt uncomfortable with the use of imaging techniques. Data of 1 child with porencephaly were excluded because the porencephaly crucially affected the reliability of volumetric and integrity outcomes for this individual. Therefore, data of 52 very preterm children (8.6 years, SD = 0.3; 26 boys) term-born peers from the same classrooms or other schools located in the same area as schools attended by the very preterm children were invited to participate. Social economic status (SES) was determined by classifying the highest level of education in a household with a number ranging from 1 to 4.18 A higher number indicated a higher level of education and a corresponding higher SES.

Procedure

The study was approved by the medical ethical committee of the VU University Medical Center. All parents gave written informed consent for the MRI study. MRI follow-up was performed at the VU University Medical Center. A simulation scanner was used for subjects to get comfortable with the scanner environment and procedures.19

Structural MRI Acquisition and Processing

Structural MRIs were acquired by using a 1.5 Tesla MRI scanner, equipped with an 8-channel phased-array head coil (Siemens Sonata, Erlangen, Germany). Anatomic 3D T1-weighted images were obtained in the sagittal plane with an MPRA (Magnetization-Prepared Rapid Acquisition Gradient Echo) sequence (repetition time = 2730 ms, echo time = 3.7 ms, inversion time = 1000 ms, flip angle = 7°, with a 1 × 1 mm in-plane resolution and slice thickness of 1 mm). We used techniques supplied by the FMRIB software library (FSL) software package version 4.1 (FMRIB Analysis group, Oxford, UK) to extract all brains (brain extraction tool [BET]) and to
automatically segment white matter and gray matter (FMRIB’s Automated Segmentation Tool [FAST]). Subcortical structures, including the thalamus, cerebellum, putamen, nucleus accumbens, amygdala, hippocampus, globus pallidum, brainstem, and caudate nucleus, were automatically segmented by using the FMRIB’s Integrated Registration and Segmentation tool (FIRST). In addition, total subcortical volume and total intracranial volume were calculated. The underlying method for the FAST is based on a hidden Markov random field model and an associated expectation-maximization algorithm, giving robust and reliable outcomes. The FIRST is based on a Bayesian model of shape and appearance of the various subcortical structures, as estimated from the intensities of the T1-weighted images.

**DTI Acquisition and Processing**

DTI images were collected during 1 acquisition with single shot echo planar imaging consisting of 4 volumes without directional weighting and 24 volumes with 24 noncollinear gradient directions (b value = 750 s/mm², repetition time = 7500 ms, echo time = 85 ms, with a 2.5 × 2.5 mm in-plane resolution and slice thickness of 2.5 mm). DTI analysis was performed by using the FMRIB’s Diffusion Toolbox. After eddy current and motion correction, all volumes for each child were screened for the presence of artifacts. If an artifact was present within a volume, this volume was removed for this child, and analyses were conducted on the remaining volumes. Voxelwise statistical analysis of the fractional anisotropy (FA) data were conducted by using Tract-Based Spatial Statistics as supplied by FSL, in which FA maps were nonlinearily registered to the “most representative” subject in the study and transformed into MNI152 standard space to enable group comparison. Next, the mean FA image was created and thinned to create a mean FA skeleton, which represents the centers of all tracts common to the group. A threshold of FA > 0.20 was used to only include major white matter tracts in the brain. Each subject’s aligned FA data were then projected onto this skeleton and the resulting data fed into voxelwise cross-subject statistics by using the Randomize tool in FSL. Randomize carries out permutation-based testing and inference by using Threshold-Free Cluster Enhancement, which is generally more robust than cluster-based thresholding and P values are usually corrected for multiple spatial comparisons. To minimize the possibility that a restricted testing strategy may erroneously lead to the conclusion that no detrimental or beneficial effects of glutamine enriched feeding exists on long-term FA values, additional exploratory analyses were conducted by using an uncorrected P value. Anatomic locations of differences on the skeleton between groups were identified by using the Johns Hopkins University atlas for white matter tracts.

**Statistical Analyses**

Statistical analyses were performed by using PASW Statistics 17.0 (SPSS Inc, Chicago, IL). Pearson correlations were used to explore the effects of gender, SES, GA, BW for GA, and number of serious neonatal infections on brain volumes and FA values. To study the effects of glutamine supplementation, analyses of variances were conducted on brain volumes and FA values with treatment as between group factor, and age, gender, and BW for GA as covariates. In addition, Sobel techniques were used to test whether the number of serious neonatal infections significantly mediated potential differences in brain volumes and FA values. The Sobel test is a statistical method that allows one to determine whether a mediator (the presence of absence of infections) significantly reduces the direct effect of the independent variable (treatment group) on the dependent outcome variable (brain development), and thereby addresses whether the mediation effect is statistically significant. More specifically, linear regressions were performed to determine (1) the direct effect of treatment group on brain development, (2) the indirect effect of treatment group on brain development via differences in the number of serious neonatal infections, and (3) the direct effect of treatment group on brain development after adjustment for the mediating indirect effects. Group differences were quantified in terms of effect sizes (Cohen d) with values of 0.20, 0.50, and 0.80 referring to small, medium, and large effects, respectively. Testing was performed 2-sided and α was set at 0.05.

**RESULTS**

**Sample Characteristics**

The glutamine and placebo group did not differ in gender; SES, or on clinical characteristics, indicating that there were no differences in illness severity (Table 1). However, there was a higher incidence of prenatal steroid treatment in the placebo group (P = .05). In line with the follow-up at 2 years of age, the number of serious neonatal infections was lower in the glutamine group (P = .02). No differences were found between the current group of 52 participants and the 37 children on all clinical characteristics (range d = 0.01–0.20; all Ps > .21).

**Correlation Analyses**

Female gender was significantly associated with smaller gray matter, white matter, cerebellum, and subcortical volume (range r = 0.28–0.37). In
addition, a lower z score of BW for GA was associated with reductions in all brain structure volumes (range $r = 0.54$–0.62). However, SES (range $r = 0.21$–0.27), the use of prenatal corticosteroids (range $r = 0.03$–0.14), and GA (range $r = 0.02$–0.20) were not associated with any of the brain volumes, except for a weak association between GA with total subcortical volume ($r = 0.28$, $P = 0.4$). Hence, in the analysis of treatment effects, gender and BW for GA were implemented as covariates. In addition, age was implemented as covariate in the analyses to adjust for age related developmental differences. Because there was a significant difference in the use of prenatal corticosteroids between both groups, which has been associated with adverse development of the hippocampus, we additionally included the use of prenatal corticosteroids as a covariate in all analyses involving the hippocampus.

**Volumetric Outcomes**

We confirm the well-established differences in brain structure volumes in white matter volume ($d = 0.55$, $P = 0.03$, Table 2), gray matter volume ($d = 0.54$, $P = 0.02$), cerebellum ($d = 0.72$, $P = 0.003$), hippocampus ($d = 0.77$, $P = 0.002$), and total subcortical volume ($d = 1.05$, $P < 0.001$) between the placebo group and term controls, illustrating an equivalent reduction in brain structure volumes in the placebo group as described for other very preterm children. After adjusting for the effects of age, gender, and BW for GA, the glutamine group revealed significantly higher volumes of white matter ($d = 0.54$, $P = 0.03$), brain stem ($d = 0.54$, $P = 0.04$), and hippocampus ($d = 0.47$, $P = 0.02$) compared with the placebo group. In addition, there were trends for higher volumes of the putamen ($d = 0.55$, $P = 0.06$), total subcortical volume ($d = 0.50$, $P = 0.06$), and total intracranial volume ($d = 0.49$, $P = 0.07$) in the glutamine group compared with the placebo group.

**White Matter Integrity Outcomes**

Data of 2 children were excluded from DTI analyses because artifacts were present in $>30\%$ of the volumes. For all included children, on average 97\% of all volumes were found to be suitable for DTI analyses. After adjusting for the effects of age, gender, and BW for GA, FA values of the white matter skeleton were not significantly different between the glutamine group and the placebo group.

Exploratory analyses using a $P$ value uncorrected for multiple comparisons indicated significantly higher FA values in clusters located in the left cingulum hippocampal tract (cluster size = 72 mm$^3$; Montreal Neurological Institute (MNI) coordinates at highest significance: $x = 131$, $y = 80$, $z = 75$) and the right cingulum hippocampal tract (cluster size = 156 mm$^3$; $x = 71$, $y = 85$, $z = 72$) in the glutamine group as compared with the placebo group (Table 2). For both bilaterally located clusters, higher FA values were significantly correlated with a lower number of serious infections ($r = -0.33$, $P = 0.02$; and $r = -0.41$, $P = 0.003$, respectively). In addition, there were no significant clusters with lower FA values in the glutamine group using a $P$ value uncorrected for multiple comparisons.

**Mediation Analyses**

The number of serious neonatal infections was significantly associated with reductions in all brain structure volumes (range $r = 0.32$–0.47), except for the caudate nucleus and the nucleus accumbens. For white matter volume, the number of serious neonatal infections significantly mediated the differences between the glutamine and placebo group ($P < 0.05$), whereas there was a trend toward significant mediation for differences in hippocampus volume ($P = 0.09$) and brain stem volume ($P = 0.05$, Table 3). Nevertheless, differences between the glutamine and placebo group on all 3 brain structures became nonsignificant after correcting for the number of neonatal infections, indicating that the number of serious neonatal infections was significantly associated with reductions in all brain structure volumes.
infections has an important, mediating role in the differences in brain volumes between both groups.

**DISCUSSION**

This study reveals that enteral glutamine supplementation between day 3 and 30 after birth is associated with medium-sized increases in white matter volume (d = 0.54), hippocampus volume (d = 0.47), and brainstem volume (d = 0.54) at school age. The differences in white matter volume are completely mediated by differences in the number of serious neonatal infections between the glutamine and placebo group. Furthermore, differences in hippocampus volume and brainstem volume disappear when taking into account the beneficial effects of a lower number of serious neonatal infections, although the test of mediation just escapes conventional levels of significance. Enteral glutamine supplementation has no statistical effect on FA values of the white matter skeleton. However, exploratory analyses suggested a tendency toward higher FA values in the glutamine group as compared with the placebo group, in particular in the left (d = 0.81) and right (d = 1.06) cingulum hippocampal tract, associated with the lower number of serious neonatal infections in the glutamine group.

Very preterm birth is associated with large reductions in brain structure volumes that persists throughout childhood and adolescence. These reductions in brain development are caused by numerous factors, including inflammation and ischemic events, with accompanying excitotoxicity and free-radical accumulation. It has been hypothesized that these events lead to damage of late-developing granular cells (interneurons) by reducing the population of neurons that will later differentiate into specific brain structures. As a consequence, the morphology of the structures is maintained, but the volume of the structures is reduced. From this perspective, decreasing the number of serious neonatal infections may positively affect brain structure volumes but will have less pronounced effects on the underlying morphology. In addition, there is a striking fourfold increase in cortex volume between week 28 and week 40 of gestation, whereas axon development of the underlying white matter tracts is concentrated before week 28 of gestation and myelination proceeds from 40 weeks onwards. As glutamine is supplemented between 28 and 36 weeks of gestation, this study reveals that enteral glutamine supplementation may lead to a fourfold increase in cortex volume.

**TABLE 2** Volumetric and White Matter Integrity Outcomes in the Glutamine and Placebo Group at School Age

<table>
<thead>
<tr>
<th>Brain Structures</th>
<th>Placebo (N = 30)</th>
<th>Glutamine (N = 22)</th>
<th>Effect Sizea</th>
<th>P</th>
<th>Association With Serious Infections, r (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total white and gray matter, cm³ (SD)</td>
<td>1171.81 (107.57)</td>
<td>1223.87 (142.94)</td>
<td>0.41</td>
<td>.14</td>
<td>−0.42 (0.002)</td>
</tr>
<tr>
<td>Gray matter, cm³ (SD)</td>
<td>705.35 (82.25)</td>
<td>727.38 (85.60)</td>
<td>0.29</td>
<td>.39</td>
<td>−0.38 (0.005)</td>
</tr>
<tr>
<td>White matter, cm³ (SD)</td>
<td>466.46 (49.22)</td>
<td>486.48 (60.44)</td>
<td>0.54</td>
<td>.03</td>
<td>−0.43 (0.001)</td>
</tr>
<tr>
<td>Cerebellum, cm³ (SD)</td>
<td>105.70 (8.47)</td>
<td>110.89 (15.32)</td>
<td>0.41</td>
<td>.13</td>
<td>−0.38 (0.005)</td>
</tr>
<tr>
<td>Subcortical structures, cm³ (SD)</td>
<td>62.87 (5.81)</td>
<td>66.45 (8.19)</td>
<td>0.50</td>
<td>.06</td>
<td>−0.45 (0.001)</td>
</tr>
<tr>
<td>Brainstem, cm³ (SD)</td>
<td>17.60 (2.23)</td>
<td>18.89 (2.51)</td>
<td>0.54</td>
<td>.04</td>
<td>−0.40 (0.003)</td>
</tr>
<tr>
<td>Thalamus, cm³ (SD)</td>
<td>14.56 (1.48)</td>
<td>15.27 (2.07)</td>
<td>0.39</td>
<td>.14</td>
<td>−0.47 (&lt;.001)</td>
</tr>
<tr>
<td>Putamen, cm³ (SD)</td>
<td>10.12 (1.14)</td>
<td>10.87 (1.55)</td>
<td>0.55</td>
<td>.06</td>
<td>−0.36 (0.009)</td>
</tr>
<tr>
<td>Caudate nucleus, cm³ (SD)</td>
<td>7.29 (1.05)</td>
<td>7.41 (1.42)</td>
<td>0.10</td>
<td>.86</td>
<td>−0.25 (0.08)</td>
</tr>
<tr>
<td>Hippocampus, cm³ (SD)</td>
<td>6.89 (0.64)</td>
<td>7.24 (0.84)</td>
<td>0.47</td>
<td>.02</td>
<td>−0.32 (0.02)</td>
</tr>
<tr>
<td>Globus pallidum, cm³ (SD)</td>
<td>3.26 (0.34)</td>
<td>3.45 (0.44)</td>
<td>0.48</td>
<td>.10</td>
<td>−0.39 (0.005)</td>
</tr>
<tr>
<td>Amygdala, cm³ (SD)</td>
<td>2.15 (0.39)</td>
<td>2.32 (0.35)</td>
<td>0.46</td>
<td>.08</td>
<td>−0.32 (0.02)</td>
</tr>
<tr>
<td>Nucleus accumbens, cm³ (SD)</td>
<td>1.00 (0.18)</td>
<td>0.99 (0.29)</td>
<td>0.04</td>
<td>.82</td>
<td>−0.19 (0.19)</td>
</tr>
<tr>
<td>Left cingulum hippocampal tract (FA)</td>
<td>0.241 (0.045)</td>
<td>0.279 (0.048)</td>
<td>0.81</td>
<td>.007</td>
<td>−0.33 (0.02)</td>
</tr>
<tr>
<td>Right cingulum hippocampal tract (FA)</td>
<td>0.285 (0.029)</td>
<td>0.318 (0.033)</td>
<td>1.06</td>
<td>&lt;.001</td>
<td>−0.41 (0.003)</td>
</tr>
</tbody>
</table>

* Cohen’s d between placebo and glutamine group.
† Corrected for age at MRI scan, gender, and BW for GA.
‡ Corrected for age at MRI scan, gender, BW for GA, and use of prenatal corticosteroids.

**TABLE 3** Results of Mediation Analysis for the Number of Serious Neonatal Infections on Differences Between the Glutamine and Control Group in White Matter Volume, Hippocampus Volume, and Brainstem Volume at School Age

<table>
<thead>
<tr>
<th>Brain Structures</th>
<th>Effect Intervention Group on Brain Volumes</th>
<th>Effect Intervention Group on Serious Infections</th>
<th>Effect Serious Infections on Brain Volumes</th>
<th>Test of Mediation †</th>
<th>Effect Intervention Group on Brain Volumes (Adjusted) ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>c (S_x)</td>
<td>P</td>
<td>a (S_y)</td>
<td>b (S_z)</td>
<td>Z</td>
<td>c (S_x)</td>
</tr>
<tr>
<td>White matter</td>
<td>27.35 (12.472)</td>
<td>0.05</td>
<td>0.542 (0.220)</td>
<td>0.02</td>
<td>29.278 (8640)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>420 (172)</td>
<td>0.02</td>
<td>0.542 (0.220)</td>
<td>0.02</td>
<td>290 (1212)</td>
</tr>
<tr>
<td>Brainstem</td>
<td>1241 (5353)</td>
<td>0.04</td>
<td>0.542 (0.220)</td>
<td>0.02</td>
<td>1181 (379)</td>
</tr>
</tbody>
</table>

* Test of mediation as described by Sobel (1982).
† Adjusted for indirect effects of differences in the number of serious neonatal infections; a, b, and c are unstandardized path coefficients adapted from linear regressions; S_x, S_y, and S_z, are the SEs of the path coefficients.
beneficial effects on the number of serious neonatal infections may have particularly acted upon cortex growth, and to a lesser extent on the myelination processes. Indeed, multiple small to medium-sized negative associations are found between the number of serious neonatal infections and volumes of various brain structures. Interestingly, the beneficial effects on brain volumes of lower rates of neonatal infections after glutamine supplementation are evident at school age, >8 years after birth. This finding highlights the potential and long lasting effects of early nutritional interventions after preterm birth on brain development.

The widespread abnormalities in brain development in very preterm children affect their neurocognitive functioning and consequently influence behavioral outcomes.7,8 In a previous study, however, we did not find that the relative increase in volumes of several brain structures in the glutamine group as compared with the placebo group improved neurocognitive performance and behavioral outcomes as measured by motor tasks, cognitive tasks, and behavioral questionnaires.33 Although a weak positive relationship has been reported between brain structure volumes and neurocognitive functioning, it is highly likely that brain structure volumes and hence the number of neurons in a particular brain structure, is not the sole factor that determines neurocognitive and behavioral functioning in very preterm children. Due to the high plasticity of the preterm brain, the loss of brain volume may have induced an increased use of alternative neuronal strategies in the placebo group to compensate for any potential neurocognitive and behavioral effects. Moreover, a range of other factors are involved in neurocognitive and behavioral functioning, including white matter integrity, functional differences in neuronal (network) activity, and environmental factors.36 The exact role of these factors and use of alternative neuronal strategies warrant further investigation.

There are some limitations for this study that need to be taken into account. First, generalizability of the findings is somewhat limited by the modest sample size and the fact that we lost 42% of our cohort for follow-up 8 to 9 years after treatment. However, there are no differences between the original cohort and the current sample. Furthermore, power is sufficient to detect medium-sized differences in brain volumes between the glutamine and the placebo group. Secondly, some caution needs to be taken into account when interpreting the finding of higher FA values in the bilateral cingulum hippocampal tract of the glutamine group. This difference may originate from a liberal testing strategy in our exploratory analyses, though effect sizes of differences in FA values are large for both clusters and present bilaterally. Finally, a reduction in the number of serious neonatal infections after glutamine supplementation in very preterm children has not been described by all studies, which may relate to differences in methodology.37,38 Nevertheless, current findings indicate that future studies into possibilities to reduce the number of serious neonatal infections in very preterm children, for example, using glutamine supplementation, are warranted and may have beneficial effects on long-term brain development.

In this study, we show that glutamine supplementation in the first month after birth increased white matter volume, hippocampus volume, and brainstem volume in very preterm children at school age. Volume differences were related to differences between the glutamine and placebo group in the number of serious neonatal infections in the first months after birth, emphasizing that beneficial effects of early (nutritional) intervention on the number of serious neonatal infections have a long-term effect on brain development at school age.

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