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The effects of breastfeeding versus formula-feeding on cerebral cortex maturation in infant rhesus macaques

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Abstract

Breastfeeding is positively associated with several outcomes reflecting early brain development and cognitive functioning. Brain neuroimaging studies have shown that exclusively breastfed children have increased white matter and subcortical gray matter volume compared to formula-fed children. However, it is difficult to disentangle the effects of nutrition in breast milk from other confounding factors that affect brain development, particularly in studies of human subjects. Among the nutrients provided by human breast milk are the carotenoid lutein and the natural form of tocopherol, both of which are selectively deposited in brain. Lutein is the predominant carotenoid in breast milk but not in most infant formulas, whereas infant formulas are supplemented with the synthetic form of tocopherol. In this study, a non-human primate model was used to investigate the effects of breastfeeding versus formula-feeding, as well as lutein and natural *RRR- α* -tocopherol supplementation of infant formula, on brain maturation under controlled experimental conditions. Infant rhesus macaques (*Macaca mulatta*) were exclusively breastfed, or were fed infant formulas with different levels and sources of lutein and α -tocopherol. Of note, the breastfed group were mother-reared whereas the formula-fed infants were nursery-reared. Brain structural and diffusion MR images were collected, and brain T_2 was measured, at two, four and six months of age. The mother-reared breastfed group was observed to differ from the formula-fed groups by possessing higher diffusion fractional anisotropy (FA) in the corpus callosum, and lower FA in the cerebral cortex at four and six months of age. Cortical regions exhibiting the largest differences include primary motor, premotor, lateral prefrontal, and inferior temporal cortices. No differences were found between the formula groups. Although this study did

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not identify a nutritional component of breast milk that could be provided to infant formula to facilitate brain maturation consistent with that observed in breastfed animals, our findings indicate that breastfeeding promoted maturation of the corpus callosum and cerebral cortical gray matter in the absence of several confounding factors that affect studies in human infants. However, differences in rearing experience remain as a potential contributor to brain structural differences between breastfed and formula fed infants.

Introduction

Breastfeeding is considered a normative reference for infant nutrition. Due to a wide range of short and long-term health benefits, exclusive breastfeeding is recommended for the first 6 months of life [1]. These benefits include lower blood pressure and body mass index [2] and lower risk of diabetes [3], childhood asthma [4], infection [5, 6] and otitis media [7]. In particular, numerous studies have investigated positive neurodevelopmental outcomes associated with breastfeeding including higher intelligence quotient (IQ), cognitive function and educational achievement [8–11]. For example, evidence from one large randomized trial demonstrated that prolonged and exclusive breastfeeding improved cognitive development as revealed by higher IQ scores and teachers' academic ratings for both reading and writing by 6.5 years of age [8]. A prospective cohort study also provided convincing evidence that children with longer breastfeeding durations (>4 months) were more likely to have better adaptability and communication performance than children breastfed less than 4 months [9].

Several nutrients in breast milk play critical roles for early infant central nervous system (CNS) development and influence the cognitive performance during adolescence [10–12]. Thus, differences in nutrient composition between breast milk and infant formula may contribute to the relatively improved CNS developmental outcomes associated with breastfeeding. For example, one component of breast milk, the long-chain omega-3 fatty acid docosahexaenoic acid (DHA), is present at high levels in the brain and retina and accumulates rapidly in the infant brain, a process dependent on both endogenous synthesis and dietary supply [13]. DHA is present in breast milk, and in recognition of its role in visual and cognitive development [14] has been added to infant formulas since 2001. However, persisting cognitive differences identified between breastfed and formula-fed individuals motivate investigations of the numerous additional nutrient differences between infant formula and breast milk. For example, breast milk of well-nourished mothers is higher than formula in lutein [15], a carotenoid which is preferentially accumulated in retina and brain and has been shown to have beneficial associations with visual and cognitive function [16, 17]. Another nutrient preferentially deposited in brain is the naturally occurring stereoisomer of α -tocopherol, *RRR*- α -tocopherol. This form is preferentially present in breast milk whereas most formulas contain synthetic *all-rac*- α -tocopherol, a racemic mixture of 8 stereoisomers, which is less well transported to tissues and has less biological activity [18]. Little information exists on effects of these specific nutrients on brain development.

It is challenging to attribute characteristics of brain development and maturation to nutrition due to potentially confounding effects of other environmental influences. Neuroimaging methods offer the advantage of providing non-invasive measures of brain development, and

thus enable longitudinal characterization of brain growth, which potentially provides high sensitivity to the effects of nutrition. Recently, multiple MRI-based modalities have been used to investigate potential neuroanatomical mediators of the effects of nutrition on cognitive abilities. Measures of brain volume, as well as specific white matter (WM) or gray matter (GM) subregion volumes have implicated breast feeding in anatomical characteristics positively associated with IQ [19, 20]. Deoni et al used a technique termed driven-equilibrium single-pulse observation of T₁ and T₂ (DESPOT) [21] to investigate the influence of breastfeeding on early WM and myelin development in toddlers and young children from 10 months to 4 years of age [22]. Although a preponderance of evidence documents the benefits of breastfeeding, complications associated with studying human populations limit precise determination of the specific biological processes or factors that underlie these benefits. Socioeconomic status and maternal education are known confounding factors that influence outcomes in studies of human subjects, in addition to maternal health, feeding pattern, and duration of exclusive feeding [23, 24]. Biological, socioeconomic, and psychosocial factors, in addition to breastfeeding [25], have also been found to relate to behavioral measures in large-scale, population-based cohort infant studies, while other studies have identified that social and home environmental factors heavily mediate the relationship between cognitive performance and breastfeeding [26, 27]. Due to potential confounding factors, considerable ambiguity and controversy exists regarding the direct effects of breastfeeding on early human brain development (for example, [22, 28]). One strategy adopted in recent studies of prematurely-born infants involves performing MRI measurements early in life (e.g. in infancy or at term-equivalent age), to minimize the magnitude of confounding effects [19, 29–32].

Studies of animal model systems provide an additional approach that can minimize the influence of experimental confounds. Among laboratory animals used to study the effects of nutrition on development, only nonhuman primates approach humans in the complexity of cortical structure and cognitive capacity and have similar developmental trajectories. In addition, nonhuman primates, unlike most other animals, share with humans the selective accumulation of lutein in brain [33]. Therefore this animal model is well-suited for studying the role of dietary lutein in brain development. Further, in animal studies the effects of breastfeeding versus formula-feeding can be longitudinally examined, in infants, while maintaining tightly controlled environmental conditions. In this study, brain development was longitudinally characterized by MRI in rhesus macaques (*Macaca mulatta*) at 2, 4, and 6 months of age. Breastfed infants were compared to infants randomly assigned to one of two formulas with different levels of lutein and different sources/stereoisomer compositions of α -tocopherol. This design avoids many of the experimental confounds affecting studies of nutrition on brain development performed on humans, though one confound that persists in our study is that the breastfed animals were mother-reared whereas the formula-fed animals were nursery-reared. At each age, structural MR and diffusion tensor images were collected to evaluate brain anatomical development, including measures of anisotropy in both WM and cerebral cortical GM. We hypothesized there would be detectable brain differences between breastfed and formula-fed infants. We further investigated whether differences between breastfed and formula-fed animals depended on the amount of lutein supplementation or source of α -tocopherol.

Materials and Methods

Subjects and experimental diets

Twenty-one infant macaque monkeys (*Macaca mulatta*) were studied from birth to six months of age. The measurements presented in this manuscript were part of a larger number of outcomes that were evaluated in these infants, including measures of retinal development and tissue biochemistry. Sample size power calculations were based on outcomes for which infant monkey data were available, including biochemical measures (carotenoid levels in brain and other tissues) and functional measures (behavioral measures of visual acuity), in which sample sizes of 4 to 8 had been sufficient to detect significant differences due to other nutritional manipulations. On the first day of life, each infant was assigned to one of three groups: a breastfed reference group (n=6, 2 males) or one of two nursery-reared formula groups. Randomization among the three groups was stratified by gender and by birth weight below or above the median (400–485 versus 486–550 g for females and 420–506 versus 507–610 g for males). All pregnant and breastfeeding mothers received a standard laboratory diet, Monkey Diet Jumbo 5037 (Lab Diet, St. Louis, MO, USA), plus a variety of fruits and vegetables. For the formula-fed groups, ready-to-feed, nutritionally adequate formulas were manufactured for the study by Abbott Nutrition (Columbus, Ohio). The unsupplemented formula (n=7, 3 males) contained only the carotenoids inherent to the formula lipid components (39 nmol/kg lutein, 2.3 nmol/kg zeaxanthin, and 21.5 nmol/kg β -carotene) and 36.6 nmol/g synthetic *all-rac*- α -tocopherol. The supplemented formula (n=8, 4 males) was identical, derived from the same manufacturing lot, but was supplemented to have a carotenoid content of ~240 nmol/kg lutein, 19 nmol/kg zeaxanthin, 74 nmol/kg β -carotene, and 338 nmol/kg lycopene, plus 22.1 nmol/g natural *RRR*- α -tocopherol. In comparison, the breast milk of the breastfeeding mothers contained mean levels of 250 nmol/kg lutein, 105 nmol/kg zeaxanthin, no detectable β -carotene or lycopene, and a mixture of natural *RRR*- and *all-rac*- α -tocopherol. Additional details are described in Jeon et al [34].

Breastfed infants were housed with their mothers from birth. Formula-fed infants were nursery-reared from one day after birth according to established protocols at Oregon National Primate Research Center (ONPRC), as previously described [34]. After being housed in incubators for the first 2–4 weeks, infants were weaned to cage-housing with an age-matched member of the same diet group, where they were provided with blankets, stuffed toys, a variety of enrichment devices and frequent contact with familiar caretakers. All infant monkeys were fed their designated diet for 6 months.

All procedures complied with the Society for Neuroscience Policies on the Use of Animals and Human in Neuroscience Research and National Institutes of Health (NIH) guidelines, and were approved by the Oregon Health and Science University Institutional Animal Care and Use Committee.

MR image acquisition

MRI scans were acquired at 2, 4 and 6 months. Each subject was scanned *in vivo* with a Siemens Tim Trio whole body 3T MRI system (Erlangen, Germany). At the 2-month time point, MRI data were collected using a circularly-polarized extremity transmit/receive

human wrist RF coil. At the 4 and 6 month time points, MRI data were collected using a customized pediatric rhesus head 8channel volumetric array receive coil (Rapid MR International, Columbus, OH, USA) and “body” RF transmitter. All subjects were sedated for MRI procedures with an initial dose of ketamine (5 mg/kg), intubated, and maintained at ~1% isoflurane anesthesia. Each imaging session included two three-dimensional (3D) T₁-weighted magnetization-prepared rapid gradient-echo (MP-RAGE) images [35], one turbo spin-echo dual-echo image, and four series of diffusion-weighted images (DWI) with corresponding phase-reversed b=0 images (for magnetic susceptibility induced distortion correction), with a total scan time of approximately 1 hour. This relatively short acquisition time for a sedated procedure was desirable because numerous sedated procedures were performed on these young infant animals, and efforts were made to minimize overall sedation time.

For the 3D MP-RAGE imaging sequence, TE/TR/TI = 3.86/2500/1100 ms, flip angle = 12°, voxel sizes were 0.4 mm isotropic and 128 slices were acquired. In-plane image sampling consisted of 216 and 256 data points in the phase-encode and readout directions, respectively. Two such images were acquired in one imaging session and these were rigid-body registered to each other and averaged. For the turbo spin-echo dual-echo imaging sequence, TE1/TE2 = 11/95 ms, TR = 10640 ms, echo train length = 8, slice thickness = 1 mm, in-plane nominal resolution was 0.5 × 0.5 mm, the sampling matrix was 256 × 256, and 60 slices were acquired. For the diffusion MRI sequence, a single-shot echo-planar imaging (EPI) sequence was used to acquire diffusion-weighted data with a spatial resolution of 1.0 × 1.0 × 1.0 mm, in 40 continuous 2D slices. The high spatial resolution utilized in this study facilitates the analysis of diffusion anisotropy within the cerebral cortex [36] and small WM structures of the infant rhesus brain. The diffusion sampling utilized 30 isotropically distributed diffusion weighted directions with b-values of 1000 s/mm² and six b = 0 images. For the wrist coil, TE/TR = 112/7000 ms, FOV = 96 mm and the image acquisition time was 5 min 53 sec. For the 8channel array, TE/TR=97/7000 ms, parallel imaging was performed with an acceleration factor of 2 and acquisition time was 4 min 35 sec. Following each DTI scan, a b = 0 diffusion image with the same geometric parameters except reversed phase-encoding direction was acquired for correction of susceptibility induced distortion [37]. Diffusion tensor images and the reversed phase-encoding b=0 diffusion image acquisition were both repeated four times.

MR image processing

Image processing steps were implemented by individuals (ZL and XW) who were not informed of animal group membership until after brain volumes, T₂ values, and DTI parameters were determined. All image processing and analyses were carried out using the ANTS suite (version 2.1; <http://stnava.github.io/ANTs/>), the FSL suite (version 5.0, <http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/>), Matlab R2016a software (MathWorks Inc., Natick, MA, USA) and SAS (SAS Institute Inc., Cary, NC, USA). In this study, diffusion weighted images were used to calculate DTI parameters such as mean diffusivity (MD), fractional anisotropy (FA), radial diffusivity (RD) and axial diffusivity (AD). Dualecho images were used to calculate water ¹H T₂ maps. T₁-weighted MP-RAGE images were used to construct study-specific brain templates for each age with brain parcellation labels derived from the

INIA19 atlas [38]. The templates provided a common standard reference for group-level analyses and segmentation of these measurements, where all of these measurements were registered to the corresponding templates.

Construction of average brain templates

Due to the growth and maturation of the infant brain over the 2 to 6 months age range, average brain templates were constructed for each time point from MP-RAGE images of the breastfed group using non-rigid image registration methods [39–42]. Prior to the brain template construction, a set of preprocessing operations were performed on the MP-RAGE images to correct various types of artifacts, such as the presence of motion and intensity bias fields, and to extract brain masks [43], as reviewed in Fig. 1a. For each MRI session, the first acquired MP-RAGE image was chosen as a reference and the remaining MP-RAGE images were aligned to it using 12-parameter affine linear registrations with the ANTS registration tool [44]. The reference image and all the aligned images were then averaged to produce the final T_1 -weighted structural image. This averaging operation removed motion artifacts generated among MP-RAGE scans and improved the SNR. For each averaged MP-RAGE image, the intensity bias field was corrected using a B-spline approximation routine and a hierarchical optimization scheme. This processing was implemented by “N4BiasFieldCorrection” tool in ANTS [45]. Next, a registration-based skull-stripping operation was performed for each corrected averaged MP-RAGE image. Skull-stripping occurred successively, beginning with the oldest age (6 months) group, with each brain mask serving as the reference for the corresponding subject in the preceding earlier age group, until finally the earliest age group (2 months) was processed [39]. At the beginning, the INIA19 adult monkey template, which includes a T_1 weighted head image and a brain mask, was used as a reference for 6-month-old brains [38]. All corrected and averaged MP-RAGE images at 6 months of age were b-spline non-linearly registered to the reference head image with “antsRegistrationSyN.sh” tool in ANTS [44]. With the resulting transformation parameters, the reference brain mask was then reversely aligned to each individual MP-RAGE image space to generate the brain mask using a nearest neighborhood interpolation method. For each subject at 4 months of age, the reference was updated with their corresponding corrected and averaged MP-RAGE images at 6 months of age and all individual merged MP-RAGE images were b-spline nonlinearly registered to their references. Then the brain masks at 6 months of age were aligned to MP-RAGE image space at 4 months of age with the resulting transformation parameters. Skull-stripping of MP-RAGE images at 2 months of age was performed using the same method with the updated references with corrected averaged MP-RAGE images at 4 months of age. After skull-stripping of all MP-RAGE images, the process of intensity bias correction for all brain images was repeated using “N4BiasFieldCorrection” tool in ANTS, in which the individual brain mask was used to limit the correction region to improve correction quality [45].

After these preprocessing operations, population-based average brain templates were constructed from the bias intensity corrected brain images of the breastfed subjects for all ages. The construction method, which generally follows the procedures described by Amaral and co-workers [39], is the same for all ages, and therefore the process will be detailed only for the 6-month time point. First, all preprocessed MP-RAGE images of control subjects

were 6-parameter rigid-body aligned to an INIA19 template brain image that was linearly interpolated from 0.5 mm isotropic resolution to 0.4 mm isotropic. The interpolated INIA19 image produced a consistent and “standard” coordinate frame with the same spatial resolution as the raw MP-RAGE images. The normalized brain image intensities were divided by their mean values and then averaged together to update the initial reference. The acquired initial reference image was therefore not biased by variations in intensity or geometry of the individual brain images. Next, all brain images were b-spline non-linearly registered to the updated reference, and the warped images were again demeaned and averaged to provide a next iteration of the reference image. For each nonlinear registration, the sum of voxel displacements for each image was calculated and used to assess for convergence of the template construction [46]. As the registration and averaging steps were iteratively repeated, the process was terminated when displacement sums did not change between two consecutive iterations. The final brain template was obtained by demeaning and averaging all warped images of the final iteration. For construction of the 4-month brain template, the initial reference template was updated by the obtained 6-month template. And similarly, the initial reference for 2-month template was the 4-month template. The 2, 4 and 6 month templates required 5, 4, and 4 iterations, respectively.

The INIA19 brain template is labeled with a detailed cortical GM, WM, and subcortical label map provided through NeuroMaps [38]. This label map was used to parcellate the constructed template images. First, the 6-month brain template was aligned to the INIA19 reference using a bspline non-linear registration. With the resulting transformation parameters, the label map in INIA19 was inversely transferred to the 6-month brain template with nearest neighborhood interpolation. Similarly, the 4-month brain template was aligned to the 6-month template and the 2-month brain template was aligned to the 4-month template using the same registration method. Finally, the label maps for templates of 4- and 2-month brains were obtained through inverse alignments using the resulting transformation parameters. For each brain at each time point, overlays of the registered INIA19 label map on the individual MP-RAGE was visually assessed for accuracy. The selected brain tissue segmentation results listed in Table 1 were determined using the NeuroMaps labels.

Regional DTI measurements and T₂ intensity analysis

The FSL suite was used to calculate FA maps and other DTI parameters from the diffusion-weighted images of each individual animal [47], as illustrated in Fig. 1b. First, the “topup” tool was used to estimate and correct susceptibility induced distortions in raw DTI images with the corresponding reversed phase-encoding b=0 diffusion tensor images [37, 48]. Next, FA maps, an image of the T₂-weighted signal in the absence of diffusion weighting, herein termed “S₀”, and other measurements were calculated by fitting the diffusion tensor to the corrected diffusion data using the “DTIFIT” tool in FSL. The S₀ images were aligned to corresponding T₁-weighted MP-RAGE images with 12-parameter affine linear registration using mutual information as a cost function in the registration procedure. Using the resulting transformation parameters, all measurements were then normalized to the corresponding T₁-weighted images. The individual T₁-weighted brain images were registered to the template images using a b-spline non-linear registration. These transformations facilitated the transfer of the normalized DTI measurements to the template coordinates. All DTI analyses were

performed in the age-appropriate template coordinate frame and the parcellation atlas of templates were applied for segmentation of regions of interest directly. This operation also provided the possibility to achieve the voxel-based statistical analysis, i.e. TBSS in FSL [49], where all DTI results were registered to a template space.

To visualize DTI tensors in the template coordinate space, DTI tensor templates for all three ages were constructed from breastfed animals using DTI-TK (<https://www.nitrc.org/projects/dtitk>), a tensor-based spatial normalization tool [50], following the construction pipelines described in [51]. The final obtained tensor templates were 12-parameter affine aligned to the corresponding T_1 -weighted templates.

White matter maturation, and in particular myelin formation, can further be assessed through reductions in the $^1\text{H}_2\text{O}$ T_2 [52]. Multi-compartmental analyses of MR signal measured at multiple TE values can be used to estimate a parameter termed the myelin water fraction (MWF), which has been shown to reflect myelin content in brain [53]. Deoni and co-workers [21] recently used a related technique termed mcDESPOT to analyze a parameter termed the volume fraction of myelin (VF_M) in age-matched infants and children who had been breastfed, formula-fed, or fed a mixture of breast milk and formula [22], and concluded that breastfeeding promoted WM myelination. The T_2 measurements performed in the present study, based on a dual-echo measurement, could not be used to estimate MWF or VF_M directly; however, we postulated that changes in MWF or VF_M could manifest as changes in T_2 . Thus, T_2 was analyzed here to determine whether group differences could be detected. The T_2 maps of individual animals at all time points were calculated by determining T_2 -relaxation times from the two echo images of the dual-echo imaging sequence using Matlab [54]. The first echo image is herein referred to as the “ T_2 -weighted image” and was aligned to the corresponding T_1 -weighted image with a 12-parameter affine linear registration using mutual information as a cost function. Using the resulting transformation parameters, T_2 maps were normalized to the T_1 -weighted coordinates. As with DTI data, normalized T_2 maps were then transformed to the template coordinates and the age-appropriate template parcellation was applied to analyze the T_2 maps. Parameters determined from DTI and T_2 analyses were averaged throughout regions of interest listed in Table 1.

In order to visualize regions of the cerebral cortical surface that were most sensitive to feeding group, group differences in cerebral cortical FA were subjected to threshold free cluster analysis (TFCE) [55] on voxels within cerebral cortical GM. Corrected p-value maps resulting from randomize/TFCE were projected onto a cerebral cortical surface constructed from the INIA19 template. The surface areas of regions with significant differences were computed using standard functionalities in CARET (v5.65, Washington University School of Medicine, St. Louis, MO, USA).

Evaluation of the potential influence of rf instrumentation on FA measurements

As noted above, a different rf coil was used to acquire the 2-month time point data than for subsequent time points. Therefore, an analysis was conducted to determine whether differences in coil sensitivity, or other data acquisition settings, resulted in systematic differences in measurements of diffusion anisotropy. The signal-to-noise ratio (SNR) in

diffusion MRI data was compared between the 8-channel receiver used at 4 and 6 months and the wrist rf coil used at 2 months. For these comparisons, the ratio of signal intensities within a cortical region of interest (ROI) and a region outside of the head were determined for one of the $b = 0$ images for each monkey. A region of interest was drawn on the corpus callosum, and FA values were averaged within the ROI. Averaged over all 21 monkeys, the SNR was determined to be 20.4 for the wrist coil at 2 months of age, and 30.8 and 30.1 for the 8-channel array at 4 and 6 months of age, for a 50% higher SNR with 8-channel array. In order to investigate whether the two receivers resulted in systematic differences in FA, three animals were scanned twice at the 4 month time point, first using the 8-channel array and then using the wrist coil in the same scanning session. A region of interest was drawn on the corpus callosum, and FA values were averaged within the ROI. The average FA using the wrist coil was 0.65 ± 0.05 , and for the 8-channel array, it was 0.68 ± 0.03 , indicating that FA differences were not apparent using the two coils ($p=0.11$). Therefore, while the 8-channel receiver likely improves precision in DTI measurements due to the higher SNR, evidence of bias in FA values was not found.

Statistics

Two statistical analysis approaches were taken. An ROI-based approach was utilized for all measurements, and this was followed by a voxel-wise approach for analysis of FA. The ROI approach utilized repeated-measures ANOVA as implemented in SAS. The potential outcomes of repeated-measures ANOVA are main effects of age and/or diet group, as well as an age-by-diet group interaction. If statistically-significant main effects were observed (at a level of $p < 0.05$), post-hoc pairwise comparisons were performed using the Tukey-Kramer method, to correct for multiple comparisons, also implemented in SAS. Specifically, if a main effect of age was observed, three pairwise comparisons were performed (2 vs. 4, 4 vs. 6, and 2 vs. 6 months of age), and if a main effect of group was observed, three pairwise comparisons were performed, potentially in addition to the age comparisons (breastfed vs. supplemented, breastfed vs. unsupplemented, and supplemented vs. unsupplemented). If an age-by-diet group interaction was observed, 36 pairwise comparisons were performed, with Tukey-Kramer adjustment, as implemented in SAS. The types of measurements that were analyzed using the ROI approach were brain (or brain region) volume, ROI-averaged T_2 , and ROI-averaged FA. Volume analyses were conducted for total brain volume, fraction of brain volume occupied by cerebral cortical GM, and fraction of brain volume occupied by WM. ROI-averaged T_2 was analyzed within WM, cerebral cortical GM, and within the corpus callosum. ROI-averaged FA was analyzed within cerebral cortical GM, and within 9 sub-regions within the WM, defined in the INIA19 atlas [38]. The WM was subdivided in this way because different fiber systems are known to exhibit different timing of FA changes with development [56]. The 9 sub-regions are WM within the occipital, parietal, temporal, and frontal lobes, the internal and external capsules, and the splenium, body, and genu of the corpus callosum. A schematic of the procedure adopted for the ROI analysis is shown in Figure S1.

In order to examine the regional pattern of DTI group differences in detail, voxel-wise analyses were performed within the WM and cerebral cortical GM. These analyses followed the tract-based spatial statistics (TBSS) [57] procedure, using a WM skeleton generated

using standard TBSS functions, and using a cerebral cortical skeleton generated by identifying voxels intersected by a cerebral cortical surface model. In contrast to the ROI-based analyses, which utilize age-specific template images, the voxel-wise procedure requires each FA map to be nonlinearly registered to a common space. This was not possible for the 2 month FA time point. Due to the large developmental differences in FA between 2 and 4 months of age in the rhesus brain, registration of the FA maps of 2 month old brains to an age-combined FA template was considered unreliable. For example, regions of high diffusion anisotropy in the cerebral cortical GM would align with template WM structures. Therefore, voxel-wise analyses of WM and GM FA were restricted to the 4 and 6 month ages. Voxelbased statistics were computed using “randomize” [58] to test for main effects of age, diet group, or age-by-group interactions, using threshold-free cluster enhancement (TFCE) to detect statistically significant differences. A schematic of the procedure adopted for the voxel-wise analysis is shown in Figure S2.

Results

Brain templates

Three brain templates for 2-, 4- and 6-month-old rhesus macaques were constructed from T₁-weighted images of the 6 breastfed subjects. In Fig. 2a-c, NeuroMaps label maps for cerebral cortical lobes are overlaid on left hemispheres, and major WM regions are overlaid on right hemispheres of an axial plane intersecting the T₁-weighted brain template images for each age. Average T₁-weighted images are shown for the same axial plane in Fig. 2d-f, for T₂-weighted images in Fig. 2g-i, and for FA in Fig. 2j-l. In addition, diffusion tensor templates are shown in Fig. 2 m-o. All templates are shown in the same coordinate system with a common 0.4 mm isotropic spatial resolution. Due to the non-biased template construction, brain sizes are preserved, and slight increases in volume are apparent between 2 and 6 months of age. As a result of tissue maturation over this period, image contrast changes are also observable, particularly within the frontal lobe, between 2 to 6 months. Changes in the average DTI tensor and T₂-weighted image contrast are also apparent over this age range.

Analysis of brain volume

No statistically significant differences were observed between the dietary groups in TBV, or percent of TBV occupied by WM or cerebral cortical GM, i.e. no main effect of diet group, or age-by-diet group interaction, was observed. Table 2 lists the mean total brain volume (TBV) for each group at the three time points and brain growth rates over the 2–4, 4–6 and 2–6 month periods. Volume analysis results are shown in Fig. 3. The WM and cerebral cortex GM volumes were normalized by TBV. Both TBV and the percentage occupation of WM increased significantly from 2 to 4 months ($p < 0.01$) 4 to 6 months ($p < 0.05$). Conversely, the occupation of cerebral cortex GM decreased significantly over the study period with $p < 0.01$, as shown in Fig. 3(c). The rate of brain growth was significantly faster from 2 to 4 months than from 4 to 6 months ($p < 0.05$). From 2 to 4 months, TBV in all groups increased from 7.61% to 9.93%. However from 4–6 months, the brain growth rates slowed significantly to 1.68% to 3.82% ($p < 0.05$). This trajectory is consistent with previous reports for the rhesus macaque [39]. Detailed results for individual TBV and percentage

occupations of WM and cortical GM subregions are listed in the supplementary Tables S1 and S2.

Analysis of the $^1\text{H}_2\text{O}$ T_2

No significant group difference in T_2 values, or age-by-diet group interaction, was observed ($p=0.48$), however, as shown in Fig. 4a, the $^1\text{H}_2\text{O}$ T_2 decreases with age in WM over the 2- to 6-month age range ($p<0.01$), particularly from 2 to 4 months. In order to examine the possibility that $^1\text{H}_2\text{O}$ T_2 differences could specifically be observed in the corpus callosum, as was found in analyses of diffusion anisotropy (see below), repeated measures ANOVA was performed specifically on the corpus callosum, and again no statistically significant group effects were observed, as shown in Fig. 4b. The overall mean T_2 values in all groups decreased from 2 months to 4 months ($p<0.01$) but remained stable from 4 to 6 months. As in WM, the $^1\text{H}_2\text{O}$ T_2 in cerebral cortical GM did not differ between diet groups, but decreased with age between 2 and 4 months ($p<0.01$), as shown in Fig. 4c. From 4 months to 6 months, the mean T_2 values did not change.

Diffusion Tensor Imaging: White Matter

A mid-sagittal FA map is shown in Fig. 5. Repeated measures ANOVA was used to separately evaluate FA in the corpus callosum genu, body, and splenium, as defined in the NeuroMaps label map (overlays in Fig. 5), for all three time points and experimental groups. Main effects of age were observed throughout each subregion (all $p<0.01$), as shown in Fig. 5d-f. Differences in FA between diet groups were also observed in the genu ($p=0.02$) and splenium ($p=0.01$). Post-hoc tests were performed between ages, and for the genu and splenium, between diet groups, with Tukey-Kramer adjustment for multiple comparisons. These analyses revealed that FA was significantly larger at 6 months of age than at 4 months, and larger at 4 months of age than at 2 months (all $p<0.01$). FA in breastfed animals was significantly larger than FA in supplemented formula-fed animals in the genu ($p=0.02$), and FA in both supplemented and unsupplemented formula-fed animals in the splenium ($p=0.01$, and $p=0.02$, respectively). No differences were found between the two formula groups nor among the groups in the corpus callosum body. Due to the use of a different coil at the 2-month timepoint, the same analyses were also performed on the corpus callosum ROIs for 4- and 6-month time points only, and similar main effects of diet group were observed.

Comparisons performed for lobar WM regions of interest lead to the common finding that water FA increased with age between 2 and 4, and between 4 and 6 months; however, differences between groups were not observed in these regions (data not shown). Nor were statistically significant group differences observed throughout the WM in voxel-wise analyses performed on the 4 and 6 months time points using TBSS, following threshold-free cluster enhancement (TFCE) correction for multiple comparisons (data not shown). Thus, the group differences in WM FA in the corpus callosum suggest a particular vulnerability of this structure, but the effect is sufficiently subtle that detection requires averaging over entire ROIs. However, although the effect is small, it should be noted that the relative FA values are highly stable within individuals and reproducible over time, with individual FA values throughout the corpus callosum as a whole exhibiting very strong correlations between the 2, 4, and 6 month time points, as shown in Fig. 5g-i.

Diffusion Tensor Imaging: Cerebral cortical gray matter

In addition to WM, DTI measures were also used to evaluate cerebral cortex GM, as previously described in our studies of macaque prenatal development [59]. Developmental changes in FA within the cortex may provide insight into the reason for the observed difference between feeding groups. During fetal development, water diffusion in the cerebral cortex is highly anisotropic [59], and the direction of least restricted diffusion parallels radially oriented cellular processes such as apical dendrites. In the mature cortex, diffusion anisotropy is reduced, but it is detectable under MR imaging conditions that achieve sufficiently high spatial resolution [36].

As in fetal cerebral cortex, the primary eigenvector is oriented radially for much of the isocortical surface at maturity [36, 60] (but see also [61]). However, although consistent patterns of appreciable diffusion anisotropy are apparent within the cerebral cortex in the template images of Fig. 2j-l, it is important to note that FA values are much lower within the cerebral cortical GM than within WM over the 2 to 6 month age range. Fig. 6a-c shows diffusion tensor primary eigenvectors overlaid on the raw FA maps of a breastfed subject. Diffusion tensor primary eigenvectors are consistently aligned in a radial orientation, indicating that rather than being a consequence of the FA “noise floor”, the observed cortical FA reflects underlying cellular structure.

Repeated measures ANOVA was performed on the cortical FA data obtained at all three ages, and this analysis yielded a significant age-by-diet group interaction ($p=0.016$). Post hoc pairwise tests, performed with Tukey-Kramer adjustment for multiple comparisons, revealed that significant FA reductions with age occurred only in the breastfed group, i.e., cerebral cortical FA at 2 months was significantly larger than at 4 and 6 months, but not in either of the formula-fed groups, as shown in Fig. 6d. As a consequence of, a notable difference is observable between these groups, with cerebral cortical GM FA being lower in breastfed than in formula-fed groups at both 4 and 6 months of age. However, no difference was observed between the two formula groups. As shown in Fig. 6e, measured cortical FA values are highly reproducible between 4 and 6 months of age, with no differences between 4 and 6 months.

In order to assess whether the differences in FA values observed between groups arise from spurious causes associated with low FA values [62], the magnitude of cortical FA was compared to FA measured in isotropic structures. Within the three animals imaged using the wrist coil at 4 months of age, the average FA values within the cortex, the striatum (averaged bilateral ROIs within the caudate and the putamen), and the vitreous of the eyes were 0.195 ± 0.012 , 0.106 ± 0.001 , and 0.039 ± 0.001 when studied using the wrist coil, and 0.182 ± 0.005 , 0.108 ± 0.010 , 0.042 ± 0.001 using the 8-channel array. These findings indicate that cortical FA is larger than the noise floor, which is likely to be approximately 0.04, the value observed in the vitreous cavities of the eyes.

In order to assess whether specific regional patterns exist in the difference in cortical GM FA between breastfed and formula-fed infants, a voxelwise analysis was performed. As described above (Materials and Methods, Statistics), voxelwise analyses require registration of all FA maps to a common reference space, and this operation was not reliable for the data

from 2 months time point, due to developmental differences in FA between 2 months of age and subsequent time points. Therefore the voxelwise analysis was specifically performed on the 4 and 6 months old brains. In contrast to the repeated-measures ANOVA result from the analysis of all three ages, repeatedmeasures ANOVA restricted to the 4 and 6 months data yields a statistically significant main effect of feeding group, but no effect of age, or age-by-group interaction. Similar to the repeated-measures ANOVA, the voxelwise approach reveals clusters of statistically significant differences in cortical GM FA between breastfed and formula-fed infants. The observed clusters are overlaid on cortical surface maps in Fig. 7. Cortical GM FA for breastfed 4 and 6 months old infants is lower than for unsupplemented (Fig. 7a) and supplemented (Fig. 7b) formula-fed groups, but no clusters of significant differences between supplemented and unsupplemented formula-fed groups were observed (data not shown), nor were clusters observed in which cerebral cortical FA for breastfed animals was higher than for formula-fed animals. Further, no differences between the 4 and 6 month ages, or ageby-group interactions were observed. Notably, consistent patterns of FA differences are observed between hemispheres. Further, these patterns are shared between the breastfed vs. unsupplemented and breastfed vs. supplemented formula-fed animals. The clusters of different FA were found in both hemispheres of the lateral and medial frontal cortex, precentral gyrus, postcentral gyrus, the ventral occipital lobe, and throughout the inferior temporal lobe, appearing as patches along the inferior temporal gyrus (Fig. 7 arrows).

Discussion

This multimodal, longitudinal study characterizes rhesus macaque brain maturation from 2 to 6 months of life, and identifies significant differences between breastfed and formula-fed infants. Over this period, brain volume changes were quantified with structural T₁-weighted images, and observed to be consistent with previous similar reports for this species [39]. Cellular-level microstructural changes in both WM and cortical GM were also evaluated by DTI, and myelination was monitored in these regions with measurements of T₂. Notable differences between breastfed and formula-fed animals were found in microstructural development in both WM and cortical GM. Other imaging modalities did not lead to further identification of differences between groups. Among the formula-fed animals, differences were not detected between groups with or without supplementation with carotenoids and the natural stereoisomer of vitamin E.

Absence of brain size differences between formula-fed and breastfed infants

No macroscopic differences were observed between the experimental groups in brain size or in gray and WM volumes. This finding is in contrast to previous studies of human infants and children, in which formula-feeding generally has been associated with smaller brain size than in breastfed individuals [63–65]. A primary difference between this study and previous human studies is the smaller number of subjects available for study here. However, this discrepancy is partially ameliorated by a longitudinal design with three measurements per animal. Although it would require additional subjects to assess whether more subtle differences occur that would replicate previous findings in human subjects, it is noted that

relatively modest age-related changes, on the order of 1% GM or WM percentage of intracranial volume, were detectable in our longitudinal experiment.

Breastfeeding affects white matter maturation

Previously, the $^1\text{H}_2\text{O}$ spin relaxation measurements of Deoni and colleagues [23, 66] indicated different trajectories of myelogenesis between breastfed and formula-fed children over the age range from 10 months to 4 years. Differences in the $^1\text{H}_2\text{O}$ T_2 were not observed between feeding groups in this study, though they were observed between 2 months and subsequent ages. It is likely that the dual echo method implemented here provides less sensitivity to myelin development than the mcDESPOT method. However, the difference found by Deoni et al. in WM maturation is supported by our finding of differences between breastfed and formula-fed infants in maturation-dependent changes in water diffusion anisotropy within the corpus callosum. Anderson and Burggren [28] have challenged the viewpoint presented by Deoni et al., partly on the basis of potentially confounding demographic factors as well as diet composition differences following the breast- or formula-feeding period (but see response, [66]). More generally, studies of breastfeeding versus formula-feeding on human brain and cognitive development have unavoidable confounding factors such as socioeconomic class and maternal education. The study design employed here addresses the concern that differences in WM maturation are influenced by demographic factors, as well as some of the potentially confounding nutritional factors, such as intake of cow's milk, raised by Anderson and Burggren [67]. It is interesting that the differences in WM observed in this study were restricted to the genu and splenium of the corpus callosum. The callosum is a relatively late-developing WM structure, and it is possible that the 2–6 month period is a particularly sensitive time for detecting maturation-related changes within it. As discussed below, Sanchez and co-workers [68] similarly found the corpus callosum, specifically in the caudal body and splenium, to be particularly sensitive to postnatal rearing conditions. The finding here that the splenium and genu, but not the body, exhibit reduced FA in formula-fed infants compared to breastfed controls could reflect regional sensitivity of the callosum to diet or other postnatal influences. However, an additional possibility is that the reduced thickness of the body, compared to the splenium and genu (Fig. 5a-c), lead to greater partial volume averaging within the body, reducing the sensitivity of FA measurements within that region.

Diffusion MRI reveals widespread differences in the cerebral cortex between mother-reared breastfed, and formula fed animals

In addition to maturational differences in WM, water diffusion anisotropy within the cerebral cortical GM was found to decrease with age in breastfed animals but remain constant over the 2- to 6month age range in formula-fed counterparts. In cerebral cortical GM of the mid-gestation rhesus macaque fetus, water diffusion anisotropy is conspicuously high [69], but it decreases throughout the second half of gestation as dendritic arbors gain complexity [59]. Over this period, or the corresponding stage of CNS development in other species [70], abnormally high cortical FA has been observed in multiple animal models of neurodevelopmental disorders [71, 72], and is interpreted to result from aberrant morphological maturation of cortical neurons. However, by the first day of postnatal life in the rhesus macaque, cortical FA has decreased to a value comparable to that observed in the

mature cortex [73]. Thus it is somewhat surprising that FA differences were observed between feeding groups at 4 and 6 months of age. A potential interpretation of the observed FA difference is that the cortical neuropil of breastfed infants continues to elaborate dendritic and axonal arbors postnatally, producing differences in the orientation distribution of cortical cell processes between breastfed and formula-fed animals over the 4–6 month age range [74]. Indeed, classic studies of macaque cortical development show that the first few postnatal months are characterized by rapid synaptogenesis and dendritic arborization, with a peak of synaptic density at 3–4 months of age [75, 76] and sensitivity to sensory input and other environmental factors [77, 78].

It is plausible that formula-feeding and/or nursery rearing attenuates some aspect of morphological development of cells within the cortex, or that breastfeeding and/or maternal rearing provides a stimulus to induce more elaborate cortical differentiation. Other data from this study has shown that, although lutein levels in multiple brain areas of the supplemented formula group were higher than in unsupplemented formula animals, they were still several-fold lower than in breastfed animals [34]. If the absence of lutein in the developing visual system were to constrain cortical development, then one might expect differences to be largest in the cortical regions with highest lutein concentrations, such as the primary visual cortex. Indeed, as shown in Fig. 7, the ventral occipital lobe consistently differed in cortical FA between breastfed and formula-fed groups. On the other hand, if nutritional or other benefits of breastfeeding stimulate brain functional development, then later-developing cortical regions such as frontal and premotor cortical areas may be anticipated to exhibit the most notable differences. In support of this, widespread differences were observed throughout the frontal cortex, extending caudally to the pre- and postcentral gyri (primary motor and somatosensory cortices, respectively) of both hemispheres. Of particular interest are the horizontal bands or patch-like structures of the inferior temporal cortex (Fig. 7, arrows) that resemble cortical patches which, in rhesus macaques, subserve recognition of animate objects such as faces [79, 80]. One possibility is that mother-infant interactions during breastfeeding could stimulate anatomical development of these patches.

Neuroanatomical differences: A consequence of nutrition or experience?

In spite of the precise experimental control achievable with rhesus macaques, it is not possible to distinguish between nutritional and experiential contributions to differences in brain (gray or white matter) maturation between breastfed and formula-fed groups. The maternal rearing of breastfed infants provided a very different social experience from nursery-rearing, which was necessary to control nutrient intake in formula-fed infants, and previous studies have established linkages between social experience and brain structure in nonhuman primates. Most comparable to the present experiment, another study of nursery rearing in male rhesus infants found a reduction in the cross-sectional area of the posterior corpus callosum at 18 months of age compared with those mother-reared in seminatural social groups [68]. Furthermore, in the same study, corpus callosum area was correlated with aspects of cognitive performance at 1–2 years of age. Of specific relevance to the finding of higher cortical diffusion anisotropy in formula-fed animals, reduced dendritic branching of cerebral cortical pyramidal neurons was found in socially isolated 6-month-old macaque monkeys [81]. Although the isolation in that study was a considerably more severe social

deprivation than the nursery-rearing of our formula-fed infants, the results are generally consistent with the group-level cerebral cortical GM differences observed in this study. Inversely, environmental enrichment, including social enrichment, is known to influence cerebral cortical neuron morphology [82]. Additionally, on a macroscopic anatomical scale, a study of chimpanzees found differences between adults that had been mother-reared or peer-reared in WM volume, white:gray matter ratios and cortical folding (i.e., depth of sulci) [83]. The present study confirms and extends these findings, and in particular confirms the special sensitivity of the corpus callosum to early diet and/or experience. Thus, differences in rearing conditions are acknowledged as a potential contributor to the brain differences described herein.

It should be noted that the potential influence of postnatal experience on brain structural differences between breastfed and formula-fed animals does not preclude the potential ability of nutritional supplementation of infant formula to ameliorate the anatomical effect. The finding that serum and tissue lutein concentrations in supplemented formula-fed infants were lower than in breastfed controls [34] suggests that breast milk contains cofactors that contribute to the bioavailability of lutein as well as many other nutrients. Future work directed at restoring the infant brain concentration of lutein and other nutrients to that of breastfed infants would further clarify the potential ability of nutritional supplementation of infant formula to better support optimal brain development.

Conclusion

This longitudinal study found significant brain structural differences between breastfed and formula-fed infant rhesus macaques at 2 – 6 months of age. The specific anatomical characteristics found to be most sensitive to feeding were maturation of the corpus callosum and diffusion anisotropy throughout the cerebral cortical GM. The latter result has not been previously reported in studies of human subjects, potentially because diffusion MRI analyses frequently focus exclusively on WM. Differential morphological properties of cerebral cortical neurons may underlie the observed differences. The primary goal of this study was to identify nutrient supplements that could diminish neuroanatomical differences between breastfed and formula-fed infants. Although infant formula supplementation with carotenoids and natural vitamin E was unable to achieve this goal, the observed effects of formula feeding on the developing brain do provide strong evidence supporting conclusions of human subject studies. Notably, brain differences were observed in the absence of confounding demographic factors such as maternal education and social status. However, it was not possible to disambiguate the effects of differential rearing experience from nutritional differences between breast milk and infant formula. Nevertheless, the brain structural differences observed here confirm and extend previous findings related to anatomical differences between breastfed and formula-fed infants, which contribute understanding of the biological bases of breastfeeding on functional brain development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

An overview of image preprocessing steps for brain volume and DTI analyses. (a) For T_1 -weighted MP-RAGE images, the following operations were performed: (1) averaging with motion correction was used to generate a merged structural image; (2) intensity bias field correction was used to correct the intensity bias in the final structural image; (3) skull-stripping was used to extract brain region. (b) For DTI images, (1) the “topup” function in FSL was used to estimate and correction susceptibility induced distortion; (2) skull-stripping was performed by using an affine transformation of the brain mask to the corresponding MP-RAGE image; (3) the “dtifit” function was used to estimate diffusion tensor parameters, such as FA, MD, eigenvectors and eigenvalues. After these procedures, MRI data were prepared for volumetric analysis and regional analysis of DTI parameters.

Figure 2.

Brain template images for the breastfed group with anatomical labels for 2, 4 and 6 months of age. In (a)-(c), labels are overlaid on axial T₁-weighted brain template slices for 2, 4 and 6 months, respectively. The overlays over the left hemisphere are six cerebral cortex regions: frontal (red), sensory/motor (green), temporal (blue), parietal (yellow), occipital (cyan), and allocortex (magenta); overlays over the right hemisphere are six WM regions: frontal (red), internal capsule (green), temporal (blue), parietal (yellow), occipital (cyan), and external extreme capsule (magenta). Images in (d)-(f) are axial T₁-weighted brain template slices for

2, 4 and 6 months, respectively, (g)-(i) are average T_2 -weighted slices for 2, 4 and 6 months, respectively, (j)-(l) are average FA slices for 2, 4 and 6 months old, respectively, and (m)-(o) are diffusion tensor colormap images, in which the intensity indicates the degree of diffusion anisotropy, and the color indicates the direction of least restricted diffusion, with red corresponding to left/right, green corresponding to rostral/caudal, and blue corresponding to dorsal/ventral. All images are constructed from the breastfed animals and located in the same coordinates with the same locations.

Figure 3.

Brain volume boxplots for breastfed, supplemented and unsupplemented formula-fed animals at 2-, 4- and 6-month time points. All data values are overlaid on boxplots. Outlier data values (greater than $q_3 + 1.5(q_3 - q_1)$, or less than $q_1 - 1.5(q_3 - q_1)$, where q_1 and q_3 are the first and the third quartiles, respectively) are indicated as red “+” symbols. No outliers were excluded from statistical analyses presented in the text. (a) Total brain volumes (TBV), (b) The percentage of TBV occupied by WM and (c) cerebral cortical GM. For all of regions, main effects of age were observed with $p < 0.01$. After 3 post-hoc pairwise comparison,

significant increases were observed for TBV and WM from 2 to 4 months and from 4 to 6 months, and significant reductions were observed for cerebral cortical GM from 2 to 4 months and from 4 to 6 months. No significant differences between diet groups, nor age-by-group interactions, in the analysis of brain volume.

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Figure 4.

Averaged T₂ boxplots from all three groups at three time points. (a) WM, (b) corpus callosum, and (c) cerebral cortical GM. In all regions, main effects of age were observed with $p < 0.01$. Post-hoc analyses with Tukey-Kramer adjustment identified statistically significant reductions in T₂ between 2 and 4 months and between 4 and 6 months. In corpus callosum and cerebral cortical GM, T₂ only decreased significantly between 2 and 4 months. No significant main effects of group or age-by-group interactions were observed. Outlier data

points, as defined in the Figure 3 caption, are indicated as red “+” symbols, but were included in all analyses described in the text.

Figure 5.

Analysis of FA values in the corpus callosum. Panels (a)-(c) show overlays of three corpus callosum regions with FA images for 2, 4 and 6 months of age as underlays (red=splenium, green=body, and blue=genu). Panels (d)-(f) show FA boxplots for all ages and groups in the splenium, body and genu of the corpus callosum, respectively. For all regions, main effects of age were observed with $p < 0.01$ and post-hoc analysis with Tukey-Kramer adjustment indicate that FA increases from 2 to 4 months and from 4 to 6 months. In addition, main effects of diet group were observed in the genu and splenium ($p < 0.01$). Post-hoc analysis

with Tukey-Kramer adjustment indicates that the breastfed group has larger FA in the genu than the lutein supplemented formula-fed group, as well as larger FA in the splenium than the other two formula-fed groups ($p < 0.05$). Outlier data points, as defined in the Figure 3 caption, are indicated as red “+” symbols, but were included in all analyses described in the text. Plots in (g)-(i) are correlations between mean corpus callosum FA values measured at different ages across individual subjects. Values are highly correlated for each pair of time points.

Figure 6.

Panels (a)-(c) show the FA values with overlaid 1st eigenvectors of the same animal from the breastfed group at 2, 4 and 6 months of age, respectively. All FA maps and corresponding eigenvectors were aligned to the template space with a rigid-body linear transformation. The cerebral cortex is associated with radially-oriented diffusion anisotropy. Panel (d) shows a boxplot of cerebral cortical GM FA for each group at three time points. A significant group-by-age interaction was observed with $p=0.016$. In the breastfed group, mean FA was significantly larger at 2 months than at 4 and 6 months ($p<0.01$). However, in formula-fed groups, no significant age-related changes in FA were observed. Outlier data points, as defined in the Figure 3 caption, are indicated as red “+” symbols, but were included in all analyses described in the text. Panel (e) shows the correlations of mean FA values in the cerebral cortex GM for each animal between 4 and 6 months of age.

Figure 7.

Clusters of cerebral cortical surface regions exhibiting significantly different FA between breastfed and formula-fed groups, projected onto the cortical surface. Adjusted p -values are mapped to the cortical surface and are shown in (top row) medial views (middle row) dorsal and ventral views, and (bottom row) lateral views for (a) the test for voxels in which cortical FA in breastfed animals is less than cortical FA in unsupplemented animals and (b) the test for voxels in which cortical FA in breastfed animals is less than cortical FA in supplemented animals. Arrowheads indicate stripes of reduced FA in breastfed animals in inferior temporal lobe observed on both hemispheres (see text for details).

Table 1

Brain ROI Definitions Relative to the Neural Label Maps

ROIs	Labels
<i>Frontal</i>	196, 213, 214, 215, 216, 217, 220, 1196, 1213, 1214, 1215, 1216, 1217, 1220
<i>Sensory motor</i>	111, 147, 1111, 1147
<i>Temporal</i>	37, 45, 54, 58, 1037, 1045, 1054, 1058
<i>Parietal</i>	7, 8, 9, 10, 1007, 1008, 1009, 1010
<i>Occipital</i>	1, 3, 4, 5, 6, 1001, 1003, 1004, 1005, 1006
<i>Allocortex</i>	63, 65, 70, 1063, 1065, 1070
<i>Cerebral cortex</i>	{ <i>Frontal, sensory motor, temporal, parietal, occipital, allocortex</i> }
<i>Occipital WM</i>	2, 1002
<i>Parietal WM</i>	55, 1055
<i>Internal capsule</i>	97, 1097
<i>External extreme capsule</i>	149, 150, 1149, 1150
<i>Temporal WM</i>	151, 1151
<i>Frontal WM</i>	193, 1193
<i>WM</i>	{ <i>Occipital WM, cerebral WM, internal capsule, external extreme capsule, temporal WM, frontal WM</i> }
<i>Splenium corpus callosum</i>	71, 1071
<i>Body corpus callosum</i>	124, 1124
<i>Genu corpus callosum</i>	221, 1221

Table 2

Total brain volume (mean $\pm$ S.D.) at three time points and growth rates during three 854 intervals for breastfed and formula-fed groups.

	Total brain volume (ml)			Growth rate (%)		
	2 months	4 months	6 months	2–4 months	4–6 months	2–6 months
Breastfed	79.40 $\pm$ 3.50	85.43 $\pm$ 3.49	87.66 $\pm$ 3.53	(7.61 $\pm$ 1.34)%	(2.62 $\pm$ 0.67)%	(10.43 $\pm$ 0.90)%
Unsupplemented	78.96 $\pm$ 3.73	86.59 $\pm$ 5.55	89.87 $\pm$ 5.57	(9.60 $\pm$ 2.66)%	(3.82 $\pm$ 2.22)%	(13.79 $\pm$ 3.86)%
Supplemented	78.88 $\pm$ 6.72	86.64 $\pm$ 6.78	88.10 $\pm$ 6.70	(9.93 $\pm$ 2.59)%	(1.68 $\pm$ 1.34)%	(11.77 $\pm$ 2.72)%