Brain ganglioside and glycoprotein sialic acid in breastfed compared with formula-fed infants¹–³

Bing Wang, Patricia McVeagh, Peter Petocz, and Jennie Brand-Miller

ABSTRACT
Background: The concentration of sialic acid in brain gangliosides and glycoproteins has been linked to learning ability in animal studies. Human milk is a rich source of sialic acid–containing oligosaccharides and is a potential source of exogenous sialic acid.

Objective: The aim of the study was to compare the sialic acid concentration in the brain frontal cortex of breastfed and formula-fed infants.

Design: Twenty-five samples of frontal cortex derived from infants who died of sudden infant death syndrome were analyzed. Twelve infants were breastfed, 10 infants were formula-fed, and 1 infant was mixed-fed; the feeding status of the remaining 2 infants was unknown. Ganglioside-bound and protein-bound sialic acid were determined by HPLC. Ganglioside ceramide fatty acids were also analyzed to determine the relation between sialic acid and long-chain polyunsaturated fatty acids.

Results: After adjustment for sex with age at death as a covariate, ganglioside-bound and protein-bound sialic acid concentrations were 32% and 22% higher, respectively, in the frontal cortex gray matter of breastfed infants than in that of formula-fed infants (P < 0.01). Protein-bound sialic acid increased with age in both groups (P = 0.02). In breastfed but not in formula-fed infants, ganglioside-bound sialic acid correlated significantly with ganglioside ceramide docosahexaenoic acid and total n-3 fatty acids.


KEY WORDS Brain cortex, infant feeding, intelligence, long-chain polyunsaturated fatty acids, docosahexaenoic acid

INTRODUCTION
Whether nutrition in early life has long-term influences on neurodevelopment and function is controversial. This subject is of major public health and clinical concern, even in industrialized nations, where premature infants may be particularly vulnerable to nutritional deficits. Numerous studies have suggested that breastfeeding is associated with higher intelligence, but it is difficult to disentangle the effects of social and educational factors from nutritional ones (1, 2). Lucas et al (3, 4) showed in preterm infants that human milk feeding is associated with superior developmental scores at 18 mo and with a higher intelligence quotient at 7.5–8 y. However, clear demonstration of causal effects of early nutrition on neurodevelopment requires strict randomization of groups. In one such trial, preterm boys assigned to be fed standard milk formula showed a 12-point disadvantage in the verbal intelligence quotient compared with those fed nutrient-enriched formula (5).

Experimental studies in animals have shown that brain structure and function are permanently affected by early nutrition. In the rat brain, malnutrition during the first few weeks of life produces a reduction in the extent of dendritic arborization, a decreased content of ganglioside and glycoprotein sialic acid, and corresponding deficits in learning behavior (6). Gangliosides are neuronal membrane glycosphingolipids that are concentrated in the synaptosomes and are important for differentiation (7), synaptogenesis (8), and neurotransmission (9). Gangliosides interact with membrane-bound functional glycoproteins, which modulate their activity and influence the membrane-mediated transfer of information (10). Brain growth and maturation are associated with an increase in gangliosides and sialoglycoproteins (11), whereas advancing age and congenital retardation syndromes are associated with a decrease (12, 13).

Of particular interest are experiments in well-nourished and malnourished animals that have shown that the administration of exogenous sialic acid increases brain ganglioside and glycoprotein sialic acid concentrations (14, 15) and improves learning performance (14). In earlier work, we and others showed that human milk is a very rich source of sialic acid relative to infant formulas (16, 17) and that breastfed infants have 2-fold higher concentrations of salivary sialic acid (18, 19). We postulated that sialic acid might be a conditionally essential nutrient in infancy because of a high demand coupled with a limited capacity for endogenous synthesis (16, 18). If this is true of human infants, then the unusually large amounts of sialic acid in human milk may be an important exogenous source of preformed sialic acid.

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In the present study, we hypothesized that breastfeeding may be associated with higher concentrations of brain ganglioside sialic acid (a biochemical marker of total gangliosides) and glycoprotein sialic acid. Sialic acid and long-chain polyunsaturated fatty acids (LCPUFAs), particularly docosahexaenoic acid (DHA, 22:6n−3), may be linked in brain development. Samples of brain frontal cortex gray matter of 25 infants who died of sudden infant death syndrome (SIDS), whose feeding history had been previously recorded in 21 cases, were analyzed.

SUBJECTS AND METHODS

Brain samples from SIDS infants

Samples of postmortem cerebral tissue derived from 25 infants who died of SIDS between January 1994 and November 1996 were studied. This tissue had been stored at −80 °C as part of a previous study of the effect of dietary LCPUFAs on brain concentrations (20). Approximately 2–5 g frontal lobe, including cortex and underlying white matter, was removed for processing in each case. A previous investigation showed that the proportion of white matter was ~15–20% with the use of a stereologic grid-point counting technique on adjacent sections (20). We were able to further exclude white matter, and the final samples contained < 5% white matter as evaluated by an experienced neuropathologist.

The diagnosis of SIDS was made after death-scene investigation, review of the clinical history, and complete postmortem examinations according to standard definitions (21). The diet, mode of feeding, and gestational age were obtained from the records of the SIDS Association of South Australia or the hospital where death occurred. The study protocol was approved by the Research Ethics Committee at the Adelaide Women’s and Children’s Hospital, the Clinical Investigations (Ethics) Committee at Flinders Medical Centre, Adelaide, South Australia, and the South Australian State Coroner. A breastfeeding index was calculated by expressing the length of breastfeeding as a percentage of age at death (20). Infants with an index ≥ 85% were arbitrarily considered to be breastfed, and those with an index < 30% were considered to be formula-fed. Infants who fell outside these limits were considered to be mixed-fed. Investigators were blinded to the method of infant feeding until all samples were analyzed.

In the present study, 12 infants were breastfed, 10 were formula-fed, and 1 was mixed-fed; the feeding status of 2 infants was unknown. The age at death was not recorded for one formula-fed infant, which left only 9 infants for the age-adjusted data analysis.

Table 1

<table>
<thead>
<tr>
<th>Description of cases</th>
<th>Breastfed</th>
<th>Formula-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 6 M, 6 F)</td>
<td></td>
<td>(n = 9 M, 0 F)</td>
</tr>
<tr>
<td>Gestational age at birth (wk)</td>
<td>39.5 ± 1.7</td>
<td>38.4 ± 4.5</td>
</tr>
<tr>
<td>Age at death (wk)</td>
<td>11.4 ± 7.4</td>
<td>13.1 ± 6.9</td>
</tr>
<tr>
<td>PME interval (h)</td>
<td>34 ± 13.9</td>
<td>32 ± 28.8</td>
</tr>
</tbody>
</table>

1 ± SD. PME, postmortem examination. There were no significant differences between groups, P > 0.05.

2 One formula-fed infant did not have the age at death recorded, which left only 9 infants for the age-adjusted data analysis.

Analysis of ganglioside-bound and protein-bound sialic acid

Ganglioside-bound and protein-bound sialic acids were analyzed separately. Gangliosides were extracted, isolated, and purified from 1 g tissue according to standard methods (22, 23). Briefly, brain frontal cortex (1 g) was homogenized with 3 mL distilled water and added to 10.8 mL methanol with constant shaking. Chloroform (5.4 mL) was added, and the mixture was centrifuged (2000 × g, 30 min, 4 °C) and the top layer removed. The brain residue was reextracted by homogenization in 2 mL water followed by 8 mL chloroform:methanol (1:2, vol:vol) and centrifugation (2000 × g, 30 min, 4 °C). The 2 extracts were combined, 5.2 mL water was added to give a chloroform:methanol:water value (+ tissue) of 1:2:1.4, and then the mixture was centrifuged (2000 × g, 10 min, 4 °C). The upper phase was set aside while 3 mL methanol and 2 mL of 0.01 mol KCl was added slowly to the lower phase and mixed for 2 min and then centrifuged (2000 × g, 15 min, 4 °C). The 2 upper phases were combined, and 1-butanol (0.2 mL) was added to prevent foaming. The mixture was then evaporated and freeze-dried. The dried material was redissolved in 4 mL chloroform:methanol (1:1, vol:vol), sonicated, and centrifuged (3000 × g, 10 min, 4 °C) to remove insoluble material. Sample (2 mL) plus 3 mL chloroform were applied to the prepared silicic acid column. Sulfatides and fatty acids were eluted in 20 mL chloroform:methanol (4:1, vol:vol) at a flow rate 0.5 mL/min. Gangliosides were rapidly eluted in the second fraction with 50 mL chloroform:methanol (1:1, vol:vol) and evaporated until 1 mL remained. The optimal time for release of ganglioside-bound sialic acid was obtained in 0.1 mol trifluoroacetic acid/L, heated for 150 min at 80 °C (23).

The defatted residue (pellet) was used to analyze the protein-bound sialic acid. The optimal time for release of protein-bound sialic acid was based on the published method of 0.05 mol H2SO4/L for 1 h at 80 °C (24). Sialic acid concentrations in both ganglioside and protein fractions were determined by using the HPLC-based, periodate-coupled thiobarbituric acid method (18). In 2 infants (one breastfed and one formula-fed), protein-bound sialic acid measurements were unavailable because the pellet was prematurely discarded. All samples were analyzed in duplicate, and the final concentration of sialic acid in each fraction was expressed in μg/g wet tissue.

Analysis of fatty acids in brain ganglioside ceramide

An aliquot of the ganglioside fraction (500 μL) was dried under nitrogen. After the addition of 2 mL methanol:benzene (4:1, vol:vol), the tubes were mixed by vortex while 200 μL acetyl chloride was added. Polytetrafluoroethylene tape was wound around the top of each tube, and the cap was screwed on tightly to prevent evaporation. Tubes were then incubated for 1 h in a 100 °C oven. The samples were cooled in water, and 6% K2CO3 (5 mL) was added to stop the reaction and neutralize the mixture. After vortex mixing, the tubes were centrifuged at 2000 × g for 10 min at 4 °C and the upper layer containing the fatty acid methyl esters was removed for analysis (25). Fatty acids were then separated and quantified by capillary gas chromatography. The injector and detector temperature were set at 300 °C. A 2-step program was
used to optimize fatty acid methyl ester separation: 170 °C for 2 min with an increase of 10 °C/min to 190 °C for 1 min followed by an increase of 5 °C/min to 220 °C for the remaining 14 min. The running time was 25 min. The resulting chromatograph was analyzed by using CHEMSTATION software (Hewlett-Packard, North Ryde, Australia). Peaks indicating constituent fatty acids were determined by comparing retention times with those of fatty acids in a standard mixture. Individual fatty acids were expressed as a percentage of total fatty acids.

Statistical analysis

Comparisons between breastfed and formula-fed infants were made by using a multivariate general linear model for the components of sialic acid (ganglioside-bound and protein-bound), with adjustment for age at death with sex as a covariate. The significance level was set at 0.05.

The correlations between sialic acid components and fatty acid components before and after adjustment for age were also analyzed. A Bonferroni-type adjustment was made, and a correlation was taken to be significant if \( P < 0.01 \). The \( F \) test was used for the comparison of the slopes of the regression line of ganglioside-bound sialic acid against ganglioside DHA. All analyses were completed by using SPSS for WINDOWS 10 (SPSS Inc, Chicago).

RESULTS

Subject characteristics

Mean gestational age at birth, age at death, and postmortem examination interval were not significantly different between the 2 groups (Table 1). However, formula-fed infants were all male and slightly older when they died. Differences in brain sialic acid concentrations were therefore compared by using a multivariate general linear model with adjustment for age at death with sex as a covariate.

Protein-bound sialic acid

There was a significant positive correlation between protein-bound sialic acid and age at death in breastfed infants (\( P = 0.025 \)) but not in formula-fed infants (\( P = 0.57 \)) (Figure 1). Protein-bound sialic acid in gray matter was 22% higher in breastfed infants than in formula-fed infants after adjustment for age at death with sex as a covariate (\( P = 0.01 \)) (Figure 2).

Ganglioside-bound sialic acid

There was a positive correlation between ganglioside-bound sialic acid and age at death, but the difference was not statistically significant in either group separately or in the 2 groups combined (\( P > 0.05 \)). Interindividual variation in ganglioside-bound sialic acid was larger than that of protein-bound sialic acid (SD = 116 in ganglioside-bound and 29 in protein-bound sialic acid). After adjustment for age at death with sex as a covariate, the average concentration of ganglioside-bound sialic acid in breastfed infants was 32% higher than that in formula-fed infants (\( P = 0.013 \)) (Figure 2).

Brain frontal cortex ganglioside fatty acids

Breastfed infants had a significantly higher percentage of DHA and total \( n-3 \) fatty acids in brain frontal cortex gangliosides relative to formula-fed infants, both before and after adjustment for age at death with sex as a covariate (Table 2). However, there was no significant difference in arachidonic acid (AA, 20:4n-6), total \( n-6 \) fatty acids, LCPUFAs, or monounsaturated fatty acids between the 2 groups (\( P > 0.05 \)).

Correlation of sialic acid with fatty acids in brain gangliosides

In all 25 brain samples, ganglioside-bound sialic acid was significantly correlated with ganglioside ceramide DHA and total \( n-3 \) fatty acids (both \( P < 0.01 \)) but not with AA, total \( n-6 \) fatty acids, or LCPUFAs (all \( P > 0.01 \)) (Table 3). The correlations tended to be stronger in breastfed than in formula-fed infants, particularly with respect to DHA (\( r = 0.81 \) in the...
breastfed infants compared with \( r = 0.32 \) in the formula-fed infants; Figure 3) and total n–3 fatty acids \( (r = 0.82 \) in the breastfed compared with \( r = 0.15 \) in the formula-fed infants). However, the differences were not statistically significant, probably because of the small sample size \( (P = 0.11, \text{two-sided}) \). The 2 regression lines in Figure 3, however, are significantly different from each other \( (F = 3.6, P = 0.048) \). Protein-bound sialic acid was more strongly correlated with DHA \( (r = 0.46, P = 0.028) \) than with AA \( (r = 0.29, P = 0.173) \). The correlation of protein-bound sialic acid and ganglioside DHA was not significantly different between breastfed \( (r = 0.36, P = 0.28) \) and formula-fed \( (r = 0.43, P = 0.25) \) infants; there was no difference between the 2 groups \( (P = 0.88) \) and the 2 regression lines \( (F = 0.55, P = 0.59) \). Total sialic acid (the sum of ganglioside-bound and protein-bound sialic acid) was significantly correlated with DHA, AA, LCPUFAs, and total n–3 and n–6 fatty acids \( (P = 0.0001–0.007) \) (Table 3). The correlations remained significant after adjustment for age. Neither protein-bound nor ganglioside-bound sialic acid correlated with total monounsaturated fatty acids.

### DISCUSSION

In this study of infants who died of SIDS, those who had been largely breastfed had significantly higher concentrations of ganglioside-bound and protein-bound sialic acid in the gray matter of their frontal cortex than did the formula-fed infants. Our findings provide the first evidence of differences in brain sialic acid concentrations between breastfed and formula-fed infants, which suggests differences in brain development and cognition. Ganglioside sialic acid is routinely used as a marker of total ganglioside content \( (12) \), and reduced concentrations are associated with early malnutrition and decreased learning performance in animal studies \( (14) \). There were significant correlations between ganglioside sialic acid content and the

### TABLE 2

Fatty acid composition of gangliosides in the brain frontal cortex of breastfed compared with formula-fed infants who died of sudden infant death syndrome\(^1\)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Breastfed ((n = 12))</th>
<th>Formula-fed ((n = 9))</th>
<th>% by wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHA</td>
<td>2.755 ± 0.339</td>
<td>1.438 ± 0.518(^2)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>3.303 ± 0.554</td>
<td>1.803 ± 0.846</td>
<td></td>
</tr>
<tr>
<td>Total n–3</td>
<td>3.076 ± 0.326</td>
<td>1.511 ± 0.498(^3)</td>
<td></td>
</tr>
<tr>
<td>Total n–6</td>
<td>4.493 ± 0.737</td>
<td>2.823 ± 1.126</td>
<td></td>
</tr>
<tr>
<td>LCPUFA</td>
<td>7.570 ± 1.012</td>
<td>4.334 ± 1.544</td>
<td></td>
</tr>
<tr>
<td>MUFAs</td>
<td>12.78 ± 1.013</td>
<td>9.796 ± 1.546</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) ± SE. Values were adjusted for age at death and sex. DHA, docosahexaenoic acid; AA, arachidonic acid; LCPUFAs, long-chain polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids.

\(^2\) Significantly different from breastfed: \(^3\) \(P = 0.04\), \(^4\) \(P = 0.018\).

### TABLE 3

Correlation of sialic acid and major classes of fatty acids in brain cortex ganglioside ceramide in 25 infants (unadjusted for age)\(^1\)

<table>
<thead>
<tr>
<th>Sialic acid (µg/g wet tissue)</th>
<th>DHA</th>
<th>AA</th>
<th>Total n–3</th>
<th>Total n–6</th>
<th>LCPUFA</th>
<th>MUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gagliaoside-bound</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r )</td>
<td>0.58</td>
<td>0.50</td>
<td>0.54</td>
<td>0.43</td>
<td>0.50</td>
<td>0.163</td>
</tr>
<tr>
<td>( P )</td>
<td>0.002</td>
<td>0.011</td>
<td>0.005</td>
<td>0.033</td>
<td>0.011</td>
<td>0.437</td>
</tr>
<tr>
<td>Protein-bound</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r )</td>
<td>0.46</td>
<td>0.29</td>
<td>0.38</td>
<td>0.20</td>
<td>0.28</td>
<td>0.17</td>
</tr>
<tr>
<td>( P )</td>
<td>0.028</td>
<td>0.173</td>
<td>0.072</td>
<td>0.358</td>
<td>0.020</td>
<td>0.437</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( r )</td>
<td>0.71</td>
<td>0.62</td>
<td>0.59</td>
<td>0.53</td>
<td>0.59</td>
<td>0.14</td>
</tr>
<tr>
<td>( P )</td>
<td>0.0001</td>
<td>0.001</td>
<td>0.002</td>
<td>0.007</td>
<td>0.002</td>
<td>0.495</td>
</tr>
</tbody>
</table>

\(^1\) DHA, docosahexaenoic acid; AA, arachidonic acid; LCPUFAs, long-chain polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids. Significance was set at \( P < 0.01 \) to allow for multiple comparisons.
proportion of DHA, the fatty acid believed to play an important role in higher cognitive function (26).

Our study, therefore, has important implications. The first is that nutrition might influence the availability and incorporation of sialic acid into sialic acid–containing compounds. Early human milk is a rich source of unique sialylated oligosaccharides compared with infant formulas. Although little is known about the digestion and absorption of sialic acid and sialyl lactose, their exogenous administration has been shown to increase brain ganglioside and protein-bound sialic acid concentrations in animals. This increase corresponded to a reduction in the time required to learn a maze in both well-nourished and undernourished rat pups (14). It is therefore conceivable that the previously documented developmental advantages of breastfeeding might be partly explained by a higher ganglioside content in gray matter, which resulted from a higher intake of sialic acid in human milk. Brain development is at a critical stage soon after birth. The head circumference, a crude clinical proxy for an increase in brain volume, increases at a maximal rate of 1.1 mm/d (27). At the cellular level, ganglioside accretion is highest during this period of dendritic arborization and synaptogenesis (28). Growth of new synaptic connections and changes in the strength of thousands of synapses connecting neurons in a network are thought to create the brain substrate for a typical human memory (29). Differences in protein-bound sialic acid between the 2 groups are therefore of particular interest because of the postulated role of sialoglycoproteins, including GP-350, in physiologic processes connected with the mechanism of memory (30). Unlike for ganglioside-bound sialic acid, there was a clear positive correlation between protein-bound sialic acid and age at death in the breastfed group. Sialoglycopeptides are highly concentrated in the synaptosomal fraction of neural tissues, especially gray matter (31). In patients with congenital athyroidism, protein-bound sialic acid in gray matter was 60% lower than in control subjects (13), a relatively greater difference than that seen for ganglioside-bound sialic acid. In the developmentally delayed offspring of mothers with uncontrolled phenylketonuria, there was a more significant reduction of sialoglycoproteins than of specific gangliosides (32). It is therefore possible that protein-bound sialic acid is a more sensitive biochemical marker of brain development than is ganglioside-bound sialic acid.

In general, most of the biological functions of gangliosides are attributed to their oligosaccharide portions. However, their fatty acid ceramide tail may also be important, although the exact reason is unclear. Dietary deficiency of n−3 PUFAs has been associated with a decrease in the amplitude of the electroretinogram (33) and with a significant deficit in the brightness-discrimination learning performance (34). Some studies have shown that supplements of DHA and AA in formula milk improve visual activity and psychomotor performance in premature infants (35, 36). Interestingly, in rat pups, lack of essential fatty acids in the maternal diet during gestation and lactation depressed both brain ganglioside and glycoprotein sialic acid concentrations (37).

In the present study, we found that the proportion of fatty acids as DHA and total n−3 PUFAs in the ceramide tail of the gangliosides was significantly higher in breastfed infants (Table 2). We also found that ganglioside-bound sialic acid in all 25 brain samples was correlated with the proportion of DHA and total n−3 PUFAs in the ceramide tail (P < 0.01). At the time of sample collection, neither LCPUFAs nor DHA was being routinely added to infant formulas. An examination of the dietary groups separately showed that the correlations remained significant only in those infants who were breastfed, particularly with regard to DHA and AA (Figure 3). These associations therefore imply that sialic acid and LCPUFAs are interdependent building blocks for neural tissues involved in higher cognitive function. Conceivably, both sialic acid and DHA work together to increase the fluidity and functionality of neuronal membranes.

The limitations of our study should be considered. The number of infants studied was small, and their sudden death for unexplained reasons suggests that they may not be representative of all infants. Furthermore, differences in age at death and...
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BW, JB-M, and FM designed the study; BW collected the data; BW and JB-M wrote the manuscript; and PP conducted the statistical analysis. None of the authors had any conflict of interest.

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