Dietary Sialic Acid and Cholesterol Influence Cortical Composition in Developing Rats1–3

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Abstract

Human milk compared with infant formulas contains considerably more sialic acid (SA) and cholesterol. Because both compounds accumulate rapidly in the frontal cortex in infancy, it has been suggested these compounds may be conditionally essential nutrients for brain development. A limited number of animal studies demonstrate that dietary cholesterol and SA increase cortical cholesterol and SA concentrations, respectively, and enhance learning. No study to our knowledge has examined the effects of simultaneously increasing cholesterol and SA intake on brain cortex composition. Rats were provided with cholesterol (0 or 0.5% of diet weight) from conception until they were killed on postnatal day (P) 32. Litters were culled (P1) to 8 pups, weaned early (P17), and fed a diet (P17–32) with the same amount of cholesterol as the dam for that litter with 1 of 4 amounts of SA from casein glycomacropeptide estimated to provide 0, 20, 40, or 80 mg · kg−1 · d−1 SA. The brain cortex from 10–12 pups (all from different litters) was analyzed for each of the 8 cholesterol-SA groups. SA, cholesterol, and protein concentrations were measured in cortex. Cholesterol exposure from conception to P32 increased cortex weight (P = 0.003) and the concentrations of cortical cholesterol (P = 0.006), protein (P = 0.034), and ganglioside SA (P = 0.02). Independent of cholesterol feeding, SA fed from P17 to P32 increased the cortical ganglioside SA concentration (Ptrend = 0.007). Dietary cholesterol and SA independently contribute to brain cortex composition during early brain development. J. Nutr. doi: 10.3945/jn.112.169508.

Introduction

Human milk contains sialic acid (SA)6 and cholesterol (1,2) and both compounds accumulate rapidly in the human brain during the first year of life (3). Infant formulas contain <25% of the SA found in mature human milk (1,3) and much less cholesterol: 0–300 mg (0–0.78 mmol)/L compared with 1.0–1.3 g (2.6–3.4 mmol)/L (2). Breast-fed compared with formula-fed infants who died of sudden infant death syndrome in the first 7 mo of life had higher concentrations of SA in brain gangliosides and glycoproteins (4), evidence that higher dietary intake of SA may play a role in brain SA accumulation.

Based on analysis of SA in milk oligosaccharides, exclusively breast-fed infants are estimated to consume ~170 mg · kg−1 · d−1 SA during the first 2 wk of life and 20 mg · kg−1 · d−1 from 2 to 7 mo of age (1). N-acetyneuraminic acid (Neu-Ac) is the SA in human milk. Neu-Ac that was administered either i.p. (5–7) or by gavage (7) at ~20 mg · kg−1 · d−1 increased brain ganglioside and glycoprotein SA in young rats. SA was provided for either 30 (5,6) or 8 d (7). Casein glycomacropeptide (CGMP) is a dietary source of SA isolated from cow milk. Piglets fed several amounts of dietary SA from CGMP had higher cortical glycoprotein SA and superior learning and memory compared with controls fed the lowest amount of SA (8).

Cholesterol deprivation and supplementation affect brain cholesterol composition in developing animals. Piglets deprived of dietary cholesterol during the first 4–8 wk of life have a lower cerebral cholesterol concentration as young adults (9), whereas cholesterol supplementation increased brain myelination in mice (10) and cerebral cholesterol and exploratory behavior in piglets selected specifically for low serum cholesterol (11). In humans, total serum cholesterol is linked to measures of cognitive function, such as verbal fluency, concentration, and abstract reasoning (12).

No study to date has evaluated the effects of exposure to dietary cholesterol and SA early in brain development on the composition of rat brain cortex.

Materials and Methods

Animals and diets. Female Long-Evans rats (Blue Spruce, Harlan) were bred with proven male breeders (Blue Spruce, Harlan). They were fed an

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3 Supplemental Table 1 is available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
4 Abbreviations used: CGMP, bovine casein glycomacropeptide; +Ch, 0.5% cholesterol; Neu-Ac, N-acetyneuraminic acid; P, postnatal day; SA, sialic acid; SA-0, SA-20, SA-40, or SA-80, 0, 20, 40, or 80 mg · kg−1 · d−1 sialic acid.
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AIN-93G diet (13) with or without cholesterol (0.5 or 0% by weight) from confirmed d1 (sperm positive) of pregnancy through lactation. The diet without soybean oil was obtained from Harlan Teklad and soybean oil with or without cholesterol was added at the research site. Cholesterol was blended with soybean oil until homogeneous prior to mixing the oil with the powdered formula to ensure that the small amount of cholesterol was uniformly distributed throughout the diet after final mixing. Diets were prepared <2 wk before feeding, stored at −40°C until dispensed, and food cups were changed at 2- to 6-d intervals.

All litters were culled to 8 pups on postnatal day (P)1. On P17, each litter of 8 was divided into 4 pairs of pups, and each pair was separately housed and fed a diet that contained 0, 0.186, 0.372, or 0.742 mg/g SA. The diets were estimated to provide pups with SA (0, 20, 40, or 80 mg · kg−1 · d−1). CGMP was obtained from Tata Co-Operative Dairy Company. The company provided an analysis of the protein (85 g/100 g), carbohydrate (15 g/100 g), and SA (67 mg/g) concentrations of the lot of CGMP. We used the company’s analysis to determine the amount of the product to add to each diet and to adjust diets to the same relative concentration of protein and carbohydrate by adding small amounts of casein and corn starch (Supplemental Table 1). The pups’ diets contained the same amount of cholesterol by weight as the diet of their dams, either 0 or 0.5%, so that there were a total of 8 groups with 4 amounts of SA and with cholesterol (+Ch) or without: SA-0, SA-0+Ch, SA-20, SA-20 +Ch, SA-40, SA-40+Ch, SA-80, and SA-80+Ch. All pups used for brain analyses (n = 10–12) in each diet group came from a different litter to control for differences in intrauterine growth among litters.

The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center (Protocol 2006–1585) and complied with the NRC’s Guide for the Care and Use of Laboratory Animals.

**Quantification of ganglioside- and glycoprotein-bound SA.** All pups were decapitated on P32. Brains were removed from the cranium onto dry ice and samples were stored at −80°C until analysis. We dissected the frontal cortex and used the left half of the frontal cortex for analysis of brain composition from only one of the pups from each pair housed together after weaning. Gangliosides were extracted from the isolated portion of the cortex according to the procedure of Svennerholm and Fredman (14) and glycoproteins were contained in the resulting peller. The concentration of SA in the ganglioside and glycoprotein fractions was determined using the HCl-resorcinal method (15) as modified by Suzuki (16). Samples were analyzed in duplicate and the OD of each was determined using a BioSpec-mini spectrophotometer (Shimadzu) at 620 nm (14). A standard curve derived from standard SA solutions (Matreya) was run with each set of samples. To further control for any daily variation in the colorimetric procedure, each batch of brains analyzed included an equal number of cortices from each of the 8 dietary treatments.

**Analysis of cortical cholesterol and protein.** Cortical cholesterol was analyzed using the Infinity cholesterol assay (Thermo Electron). The concentration of cortical protein was determined by the bicinchoninic acid protein assay (Pierce).

**Analysis of stomach contents for cholesterol.** Coagulated milk was removed from the stomach of pups culled on P1 and stored at −80°C until analysis to estimate if pups of dams fed cholesterol received a higher amount of cholesterol in milk until weaning. Samples of stomach contents were saponified in 10% ethanolic potassium hydroxide (17) and the concentration of cholesterol was determined by the method of Rosenthal et al. (18). The stomach contents from a total of 16 pups born to cholesterol-fed dams and 17 pups born to dams not fed cholesterol were analyzed.

**Statistical analysis of data.** A mixed-model ANOVA with subject as a random effect (SPSS 17.0) was used to evaluate ganglioside SA. The day of analysis was treated as a block to decrease any random error introduced by day-to-day environmental variation (e.g., room temperature and humidity), with cholesterol and SA intake as main effects. Glycoprotein SA data were analyzed using an ANOVA model that treated cholesterol and day as fixed effects and SA intake as a fixed covariate interaction (dietary cholesterol × SA) (SPSS 17.0). All P values were considered significant at the 0.05 level. If an interaction term was nonsignificant, we dropped the interaction term and fitted a mixed-model ANOVA with main effects only. The only post hoc test used was a simple 2-sample comparison of cholesterol, because the effect of day was not of interest and SA was a continuous covariate. The influences of dietary cholesterol on total milk cholesterol, cortical cholesterol, cortical protein, and cortex weight were evaluated using Student’s t distribution (Microsoft Excel 2007).

**Results**

Rats exposed to 0.5% cholesterol during gestation, lactation, and postweaning had a 6.5% larger left cortex (0.31 ± 0.03 vs. 0.29 ± 0.03 g; P = 0.003) and significantly higher concentrations of cortical cholesterol and protein compared with those not exposed to cholesterol (Table 1). Using the mixed-model ANOVA, because cholesterol × SA was found to be nonsignificant (P = 0.56), the effects of cholesterol and SA were considered separately. Overall, the brain cortex ganglioside SA concentration increased with cholesterol feeding (Fig. 1; P = 0.02). Dietary SA did not affect cortex weight; however, it increased the concentration of cortical ganglioside SA (P-trend = 0.007).

The cortical glycoprotein SA concentration at P32 was not influenced by either CGMP or cholesterol intake. The concentration of cortical glycoprotein SA was identical for cholesterol-exposed and not exposed pups (0.84 ± 0.02 μmol/g or 0.26 ± 0.01 mg/g).

The concentration of cholesterol in the stomach contents of newborn pups was greater in pups from dams fed diets with (n = 16) compared to without (n = 17) cholesterol (0.71 ± 0.03 vs. 0.56 ± 0.02 g/L; P = 0.0009).

**Discussion**

In the present study, cholesterol supplementation from conception to P32 significantly increased weight and the concentration of ganglioside SA, protein, and cholesterol in the frontal cortex of rats. It is not possible to determine the period of time in which exposure to cholesterol resulted in the effects, however. Because maternal cholesterol is a source of fetal cholesterol (19), pups in the cholesterol groups may have received greater exposure to cholesterol during gestation. It is reasonable to suppose that the cholesterol groups received greater exposure from P1 to P17 as well, because the coagulated milk contents from pups in the cholesterol-fed groups was higher in cholesterol than that of pups fed by dams not fed cholesterol. By design, pups in the cholesterol groups were fed cholesterol from P17 to P32.

<table>
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<tr>
<th>Table 1</th>
<th>Cortical cholesterol and protein concentration in rat pups pre- and postnatally exposed to diets containing 0 and 0.5% cholesterol 1</th>
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<tr>
<td></td>
<td>Cholesterol</td>
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<tr>
<td>Non-cholesterol diet</td>
<td>11.7 ± 0.2</td>
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<tr>
<td>Cholesterol diet</td>
<td>12.6 ± 0.3</td>
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<td>P value</td>
<td>0.006</td>
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1 Values are mean ± SEM, n = 40–44. All SA groups were pooled within the cholesterol group, because there was no effect of SA and no SA by cholesterol interaction. SA, sialic acid.

2 To convert mg/g to mmol/g, multiply by 0.0026.
In the central nervous system, cholesterol is a key component of neuronal cell membranes, nerve growth cones, and myelin, and it is concentrated in microdomains of neuronal cell membranes that are enriched in gangliosides and thought to function as platforms for signaling pathways. The brain is thought to produce most if not all of the cholesterol it requires (see 20 for review); however, recent evidence suggests there could be a role for an external source of cholesterol (see 21 for review). Such a role could help explain our results and that of others who find effects of dietary cholesterol during development on brain composition and function. For example, piglets deprived of dietary cholesterol during the first 4–8 wk of life had a lower cholesterol concentration in their cerebrum (which includes the frontal cortex) as adults (9). Haque and Mozaffar (10) showed that dietary cholesterol increases the rate of brain myelination in weaning mice. In the only study of dietary cholesterol and brain function, Schoknecht et al. (11) increased the cerebral cholesterol concentration and exploratory behavior among cholesterol-supplemented compared with control pigs selected for low serum cholesterol.

This is the second study to demonstrate that feeding SA from CGMP increases the brain ganglioside SA concentration. It follows an earlier report in pigs (8). An increase in brain SA concentration could influence a number of aspects of brain function. Gangliosides accumulate rapidly in the brain at specific stages of development. These compounds are involved in membrane-related events such as cellular recognition, adhesion, and signal transduction (5), and they function in neural development, synaptic transmission, cognition, and memory formation (1,6,7). Thirty years ago, Morgan and Winick (5,6) reported that well-fed and undernourished rat pups i.p. administered Neu-Ac during the first 30 d of life had higher brain ganglioside and glycoprotein SA concentrations and fewer behavioral abnormalities secondary to malnutrition (6). Only recently is there evidence that the conjugated sources of SA in human milk or food sources such as CGMP can enhance brain ganglioside SA accumulation (4,8) and influence learning (8). Until the commercial availability of CGMP, the lack of a food source of SA limited research in this area.

Future animal studies should consider the stages of brain development that best relate to the stages of human brain development. Vanier et al. (22) showed that the concentration of SA-containing lipids in the frontal cortex of humans increased linearly between 10 and 40 wk of gestation and again after birth. Human infants readily survive after as little as 24 wk of gestation.

A limitation of the study is that SA exposure did not begin until P17, i.e., after the major period of brain ganglioside accumulation in the rat (22). Until P17, pups were fed mothers’ milk, which provides SA (23). Thus, the true ability of dietary SA to increase cortical ganglioside SA is likely underestimated in this study. As previously noted, another limitation of this study is that whereas dietary cholesterol increased cortical weight and the cortical cholesterol, protein, and SA concentrations at P32, we were not able to isolate the timing of the effect of cholesterol feeding.

In summary, this is the first study to our knowledge to show that dietary cholesterol increases not only the cortical cholesterol concentration but also cortical concentrations of SA and protein. The study confirms that dietary SA increases the cortical ganglioside SA concentration in a dose-response manner when fed postnataally during a period of rapid brain development. This increase is in addition to the increase found with cholesterol feeding.

Acknowledgments
S.E.C. designed the feeding study; S.E.C. and B.S.G. conducted the feeding study; S.A.S. and B.S.G. conducted the chemical analyses; B.J.G. and S.A.S. performed the statistical analysis; and S.E.C. and S.A.S. wrote the paper and had primary responsibility for the final content. All authors read and approved the final manuscript.

Literature Cited