Dietary sialic acid supplementation improves learning and memory in piglets1–3

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ABSTRACT

Background: Sialic acid, a key component of both human milk oligosaccharides and neural tissues, may be a conditional nutrient during periods of rapid brain growth.

Objective: We tested the hypothesis that variations in the sialic acid content of a formula milk would influence early learning behavior and gene expression of enzymes involved in sialic acid metabolism in piglets.

Design: Piglets (n = 54) were allocated to 1 of 4 groups fed sow milk replacer supplemented with increasing amounts of sialic acid as casein glycomacropeptide for 35 d. Learning performance and memory were assessed with the use of easy and difficult visual cues in an 8-arm radial maze. Brain ganglioside and sialoprotein concentrations and mRNA expression of 2 learning-associated genes (ST8SIA4 and GNE) were measured.

Results: In both tests, the supplemented groups learned in significantly fewer trials than did the control group, with a dose-response relation for the difficult task (P = 0.018) but not the easy task. In the hippocampus, significant dose-response relations were observed between amount of sialic acid supplementation and mRNA levels of ST8SIA4 (P = 0.002) and GNE (P = 0.004), corresponding with proportionate increases in protein-bound sialic acid concentrations in the frontal cortex.


KEY WORDS Sialic acid supplementation, learning and memory, gene expression, brain development, piglets

INTRODUCTION

A large body of evidence shows that breastfeeding provides long-term cognitive advantages, particularly for infants born small or premature (1, 2). The question is why? The subject remains controversial because it is difficult to disentangle genetic, environmental, and nutritional factors. The question is one of profound clinical and public health importance. Advances in reproductive technologies have increased the number of infants born early or small for gestational age (3), yet their long-term neurodevelopmental outcomes remain poor (4, 5). Lower academic performance and psychosocial and learning difficulties, particularly problems of visuospatial perception, are common (6). Evidence that nutrient intake per se is critical stems from rare randomized controlled trials that showed increased IQ in premature infants that were fed enriched formulas (7). Identifying key nutrients for cognitive development is therefore an important objective.

Sialic acid, a 9-carbon sugar molecule with a strong negative charge (also known as N-acetyleneuraminic acid), occurs in large amounts as a component of human milk oligosaccharides (8, 9). Sialic acid is also the terminal functional residue of brain gangliosides and glycoproteins, such as the neural cell adhesion molecule (NCAM). The brains of breastfed infants have higher amounts of ganglioside-bound and glycoprotein-bound sialic acid than do formula-fed infants (10). Compared with mature human milk (=0.7 g/L), infant formulas provided little sialic acid (0–0.2 g/L). In animal models, an exogenous source of sialic acid increased learning performance as well as the concentration of sialic acid in the brain frontal cortex (11, 12).

Understanding the biochemical basis of learning, memory, and cognitive development are also important challenges. Changes in the NCAM polysialylation state occur during neurite cell migration, synaptogenesis (13, 14), and learning (15, 16). Two polysialyltransferases (α-2,8-sialyltransferase II and IV, abbreviated as ST8SiaII and ST8SiaIV, respectively) are the key enzymes involved in sialic acid metabolism and have been linked to learning behavior (17). Although the liver can synthesize sialic acid de novo from glucose, the activity of the limiting enzyme, UDP-N-acetylgalactosamine-2-epimerase, is low during the neonatal period (18).

On the basis of this evidence, we hypothesized that an exogenous source of sialic acid could be needed during periods of

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rapid brain growth. Using an appropriate animal model of the human infant, we designed a series of studies to determine whether early sialic acid supplementation increased learning and memory performance, brain protein-bound and ganglioside-bound sialic acid, and mRNA levels of the key enzymes, UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE) and ST8SiaIV.

MATERIALS AND METHODS

Animals

Piglets were chosen because brain structure and function during development resemble that of human infants (19, 20). The newborn piglet is less developmentally mature, and its body weight is relatively small in relation to its mature weight. For this reason, both newborn piglets and low-birth-weight infants are vulnerable to developmental deficits. Importantly, the birth of both species occurs in the midst of the developmental spurt of brain-mass accretion (19). Finally, the pig digestive system shares similar physiology and anatomical structure with human infants and has comparable nutrient requirements. This makes the piglet ideally suited for the coordinated nutritional, metabolic, and molecular investigations.

Three-day-old male domestic piglets (Sus scrofa, Landrace/Large White cross) weighing 1.5-2.4 kg were purchased from a commercial piggery (n = 54), stratified according to weight and litter, and randomly allocated to 1 of 4 treatments. They were housed in pairs in a temperature-controlled room on a 12-h light (0800–2000) and dark (2000–0800) cycle. The home pens contained a “nest” (a rubber tire covered with a towel), a heat lamp, and an identical plastic toy. One piglet died of pneumonia at 2 wk of age, leaving 53 animals in the final analysis. The study protocol was approved by the University of Sydney Animal Ethics Committee.

Casein glycomacropeptide supplementation

Casein glycomacropeptide (CGMP) providing 60 mg sialic acid/g was blended into the pig’s milk replacer (Wombaroo Food Products, Glen Osmond, Australia) at specified concentrations. The amount in the final milk varied according to group: 140 mg/L (group 1, the control group with no added CGMP, n = 14), 300 mg/L (group 2, low dose, n = 13), 635 mg/L (group 3, middle dose, n = 14), and 830 mg/L (group 4, highest dose, n = 12). These concentrations represented an approximate intake of 40, 85, 180, and 240 mg · kg body wt⁻¹ · d⁻¹, respectively, the highest dose being comparable with the amounts present in sow milk (840 mg/L, unpublished data, 2003) and human milk (700 mg/L in mature milk). The milk replacers were formulated so that total protein intake remained the same irrespective of the amount of added CGMP. To maintain normal rates of growth, the piglets received 285 mL milk · kg⁻¹ · d⁻¹ in the first 2 wk of the study and 230 mL · kg⁻¹ · d⁻¹ in the remaining weeks. Feeding times were at 0800, 1300, 1800, and 2230, with an extra 50 mL milk/pig supplied at the last feeding. Body weight, milk intake, and health status of piglets were recorded daily.

Learning ability and memory performance assessment

To reduce stress at the beginning, we allowed both piglets in each pair into the maze for habituation 1 d before the formal test. Formal learning tests began on day 21 with the use of an 8-arm radial maze (Figure 1) adjacent to the home pens. A video camera was installed overhead to record the learning and memory tests. Two learning tests were performed: task 1 and task 2. Both tests had accessible milk (corresponding to their treatment group) in 1 arm, and inaccessible milk in the remaining 7 arms, such that all 8 arms of the maze gave the same olfactory signals. In both tests, a visual cue consisting of 3 black dots was placed randomly on a door with accessible milk in the arm. In task 1 (the easy task), one black dot was placed on the remaining 7 doors. In task 2 (the more difficult task), 2 black dots were placed on the remaining 7 doors. The position of 3 black dots visual cue was changed between trials in a predetermined random order.

All piglets were tested in the maze individually. Forty trials of task 1 were conducted during 5 days (8 trials/d) and 40 trials of task 2 during 6 days. Assessment of learning performance was determined as the number of trials taken to successfully learn the visual cue and the number of mistakes (wrong doors) and successes (correct door) in finding the accessible milk arm during each trial. A mistake was registered each time the piglet entered or put its whole head through a wrong door. A success was registered only when piglets entered the correct door and found the accessible milk. The criterion to learn the visual cue was a maximum of 1 mistake in 3 consecutive trials. Two days after completion of each set of trials, the same task was presented as a “memory test” for one trial only. All the tests were conducted by trained staff members, one of whom was not blinded to the amount of sialic acid intake. Results were corroborated by independent analysis of the video material. On day 34 or 35, the piglets were euthanized; tissues were collected and stored at –80 °C. Frontal cortex ganglioside-bound and protein-bound...
Sialic acids were measured according to published methods (10, 21). Sialyltransferase activity was determined with the use of a modified method by Laroy et al (22). Because stress may influence learning and memory, morning blood cortisol concentration was measured at weekly intervals beginning on day 7 with the use of a commercial kit (Coat-A-Count Cortisol; Diagnostic Products, Doncaster, Australia).

Relative quantification of target gene mRNA

Total RNA was extracted from 3 different areas: hippocampus, frontal cortex, and liver from piglets with the use of the SV total RNA isolation system (Promega, Madison, WI) and was converted into cDNA by reverse transcriptase (Superscript III; Invitrogen, Carlsbad, CA) and random hexamers. The quantitative analysis was performed with the use of real-time polymerase chain reaction (PCR) with SYBR Green Master Mix (Applied Biosystems). Two target pig gene sequences (ST8SIA4 and GNE) were characterized by our group (GenBank accession no. DQ133503 and DQ132898, respectively), whereas the reference gene (pig 18S ribosomal RNA) sequence was retrieved from GenBank (AY265350). All real-time PCRs were set up with the use of the liquid handling robot (epMotion 5070; Eppendorf, Hamburg, Germany) with the total volume of 10 μL and analyzed in triplicate. PCR conditions and primer sequences are available on request. The quantification was performed in ABI Prism 7900 HT Sequence Detector (Applied Biosystems). The mRNA levels were represented by the corresponding cDNA levels, and the relative mRNA levels were expressed as $(E_{target})_{\text{mean}} \cdot C_{i} / (E_{reference})_{\text{mean}} \cdot C_{i}$, where $E_{target}$ and $E_{reference}$ are the PCR efficiencies determined by using an assumption-free LINREGPCR software (version 7.5; Dr JM Ruijter, Academic Medical Centre, Amsterdam, Netherlands) (23) and $C_{i}$ is threshold cycle numbers and was determined with the use of the SDS software (version 2; Applied Biosystems). Between 96% and 100% PCR efficiencies for target and reference genes were observed (data not shown). The individual target mRNA levels were first normalized to its reference gene 18S rRNA at the same location. Finally, the mean value of the control group was considered as a calibrator, and the remaining groups with different amounts of sialic acid supplements were expressed as an $n$-fold ratio in graphs compared with the calibrator. We showed that 18S rRNA is a suitable reference gene in the study because no significant variation was observed in its cDNA levels between the groups and locations (data not shown). Laboratory staff members who undertook the biochemical assays were blinded to treatment.

Statistical analysis

Differences in learning (number of trials needed to learn the visual cue) were compared with the use of Kaplan-Meier survival analysis with Cox regression to examine potential covariates that may influence learning. Comparisons between means (with or without covariates) were performed with the use of the general linear model [univariate analysis of variance (ANOVA)] with Bonferroni’s adjustment for multiple comparisons where appropriate. Pearson’s correlation was used to examine the relation between number of mistakes, body weight, and memory performance. All statistical analyses were completed with the use of SPSS for WINDOWS 11 and 12 (SPSS Inc, Chicago, IL). A significance level of 0.05 was used.
The difference among the groups remained highly significant \((P = 0.002)\), and the dose-response effect was not altered. The findings were similar when the last 20 trials in task 2 were considered separately or when mistakes in trials 1–10 were used as a covariate \((P = 0.016)\).

In studies such as this, the tool by which learning and memory is assessed is critical. If the test is too easy, all animals, including the controls, may learn at a similar rate. If the task is too difficult, only a few animals may learn at all, and it will be harder to establish a dose-response relation. Learning of a difficult task, however, benefits greatly from previous training on an easier version of the same task \((24)\). In the present study, when the learning task was relatively simple, performance was better overall in the 3 treatment groups compared with the control group \((P = 0.001)\), but no suggestion of a dose-response relation was observed \((P = 0.122)\). We could have raised the level of difficulty by making the learning criterion stricter (eg, 1 mistake in 4 or 5 consecutive trials, instead of 3). Interestingly, when we analyzed the data retrospectively by using increasingly strict learning criteria, the dose-response relation was evident, this time in both the easy and difficult tasks (Figure 4).
A limitation of our method is that piglets continued to be tested for the full number of trials (when the memory test was administered), irrespective of whether they reached the learning criterion. This “overlearning” was to a large extent unavoidable. Had we stopped testing those piglets that reached criterion early, the elapsed time between the achievement of criterion and the memory test would have differed among the piglets. Alternatively, had we administered the memory test immediately, the developmental age of piglets would have differed at the time of the memory test and therefore subsequent euthanasia.

**Memory test**

Only piglets that had reached the learning criterion were assessed for their ability to remember the visual cue. Supplemented groups scored more highly than did the control group in the difficult task ($P = 0.036$; Figure 3C) but not the easy one ($P = 0.285$), with no dose-response effect in either case. More mistakes in the learning trials predicted a greater number of mistakes in the memory test in task 2 ($P = 0.029$) but not in task 1 ($P = 0.973$). Body weight, rate of weight gain, and rate of learning did not significantly affect memory performance (data not shown).

**Brain sialic acid concentration**

Protein-bound sialic acid concentration in the frontal cortex was up to 10% higher in supplemented groups than in the control group ($P = 0.001$, ANOVA), showing a significant dose-response relation (Figure 5). Ganglioside-bound sialic acid concentration also increased with dose, but the differences were not statistically significant ($P = 0.307$, ANOVA). On an individual piglet basis, a higher concentration of sialic acid in the frontal cortex tended to predict faster learning and better memory in both tasks, but none of the correlations, whether parametric or non-parametric, were statistically significant.

**ST8SIA4 and GNE expression**

mRNA level of ST8SIA4 was higher in the frontal cortex and hippocampus compared with the liver ($P = 0.001$), whereas the mRNA level of GNE was highest in the liver ($P = 0.001$). Significant dose-response relations were observed between amount of sialic acid supplementation and mRNA level of ST8SIA4 ($P = 0.002$ in the hippocampus; Figure 6A) and GNE ($P = 0.009$ in the liver and $P = 0.004$ in the hippocampus; Figure 6B). In the hippocampus, ST8SIA4 mRNA level was 2.5-fold higher in group 4 than in group 1 ($P = 0.003$), whereas in the liver, GNE mRNA level was 3-fold higher in group 4 than in group 1 ($P = 0.004$). Significant correlations were observed between the concentration of protein-bound sialic acid in the frontal cortex and expression of hippocampus ST8SIA4 ($P = 0.029$) and GNE ($P = 0.012$). On an individual basis, a trend was observed for higher mRNA levels of ST8SIA4 in the hippocampus to correlate with faster learning in task 2 ($P = 0.070$) but not in task 1 ($P = 0.207$).

**Sialyltransferase activity**

In the absence of a specific assay for the ST8SiaIV isoform (NCAM and endo-N were not available at the time of the study),
total sialyltransferase activity was assessed. The assay encompasses the activity of some 20 distinct enzymes responsible for catalyzing the transfer of sialic acid from cytidine 5′-monophosphate sialic acid to the carbohydrate moiety of glycoconjugates (25). No significant differences were observed between the control group and the supplemented groups (\( P > 0.05 \)) based on a general linear model (univariate ANOVA) with Bonferroni’s adjustment for multiple comparisons.

**Body weight gain**

Mean (±SE) starting body weight was the same in each group (2.1 ± 0.04 kg), and animals gained weight at similar rates (Figure 8). Although the control group weighed more than the other groups by the end of the study, differences were not significant on either day 21 (\( P = 0.068 \)) or day 28 (\( P = 0.68 \)) when the easy and difficult trials began, nor was the rate of weight gain (in g/d) significantly different among the groups (\( P = 0.503 \)).

**Plasma cortisol**

As a measure of stress, mean cortisol concentration in each group was highest in the first week and declined significantly over the duration of study with no significant differences among the groups (\( P = 0.285–0.547 \)) (Figure 9). When blood plasma cortisol concentration was used as a covariate during learning, no significant effects were observed (\( P > 0.05 \)).

**Amino acid content**

Although total protein intake was the same in each group, the amino acid pattern varied slightly (Table 1). This difference was unavoidable because the amino acid make-up of CGMP differs from other cow milk proteins, and other sources of sialic acid were less feasible from a practical viewpoint.
DISCUSSION

In the present study, we showed concurrent links among dietary intake, gene expression, brain biochemistry, and learning behavior. In a dose-response manner, supplementary sialic acid was associated with faster learning; higher concentrations of protein-bound sialic acid in the frontal cortex, and 2–3-fold higher mRNA levels of 2 learning-related genes, ST8SIA4 and GNE. Because human milk is a rich source of sialic acid, the findings provide a possible mechanism to explain the link between breastfeeding and higher intelligence.

The strengths and limitations of our study should be noted. Although our findings suggest that sialic acid influences learning and memory, we cannot rule out the possibility that it affects other domains of behavior such as motivation and motor or sensory function, which in turn alters learning ability. Likewise, the 2 learning tests were intended to reflect different levels of difficulty, but interpretation is compromised because the piglets were 2 different ages (and maturation) at the time of testing. The piglets were the same strain from a single farm, but genetically distinct. Additionally, the distribution of different types of gangliosides is needed to achieve adequate power in future studies. In addition, the results of learning performance were confirmed by independent analysis of the video material, the research assistant responsible for feeding and maze testing was not blind to treatment. In addition, even though the total protein concentration of each milk was the same, the amino acid pattern varied slightly (Table 1). Although unlikely, we cannot rule out the possibility that small differences in the intake of specific amino acids caused changes in learning performance.

We examined the possibility that differences in stress responses might influence learning and memory performance. Plasma cortisol concentrations (a crude measure of stress level) did not vary by the groups (Figure 8) and did not correlate with learning performance or memory. Cortisol concentrations declined significantly after the first week and remained constant thereafter, suggesting that the piglets had habituated to their environment by the beginning of week 2. Indeed, the piglets appeared to enjoy the learning trials and were “proud” of their successes.

We also explored the relation between learning performance and differences in brain sialic acid concentration. In the frontal cortex, sialic acid molecules can be lipid bound or protein bound, forming the functional terminal groups of gangliosides and glycoproteins, respectively. In piglets, the concentrations of both forms were lower than that found in human infants (10, 26) and adult pig (21). CGMP supplementation was associated with a 10% higher concentration of both forms of brain sialic acid, but only the protein-bound fraction showed a significant dose-response relation ($P = 0.001$). Because the ganglioside-bound form showed greater interindividual variation, a greater sample size is needed to achieve adequate power in future studies. In addition, the distribution of different types of gangliosides should be determined because some types may be more important to learning and memory than others.

Many studies have shown that learning behavior increases brain sialic acid incorporation. In particular, ganglioside synthesis increases after long-term active avoidance conditioning in

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1 On a dry matter basis, the milk replacer contained 31% protein, 24% carbohydrate, 40% fat, and 5% vitamin and mineral mix. The protein was a casein, whey, soy, and casein glycomacropeptide mixture at a ratio of 36:9:55:0 in milk 1, 36:3.5:53:7.5 in milk 2, 26.5:3.5:52:18 in milk 3, and 19:3.5:52.5:25 in milk 4. The carbohydrate was lactose. The fat was a mixture of milk fat, vegetable oil, and fish oil extract containing 0.65% docosahexaenoic acid and arachidonic acid in the final mix. The vitamin and mineral mix contained ∼1.2–1.6% Ca, ∼0.9–1.3% P, ∼0.7–1.1% salt, 2.30% lysine, 9 mg vitamin A/kg, 90 μg vitamin D3/kg, 9 mg vitamin B-2/kg, 500 mg Mg/kg, 90 mg ferrous iron/kg, 15 mg Mn/kg, 10 mg Zn/kg, 10 mg Cu/kg, 8 mg Se/kg, 0.7 μg I/kg, and 120 μg S/kg.
rats (27), and time-dependent increases in the polysialylation of NCAM occur within 12–24 h of passive-avoidance training (16). NCAM is the main carrier of polysialic acid in the central nervous system, and changes in polysialylation state facilitate memory formation (28, 29). Evidence is strong that the concentration of sialic acid in cells regulates the content of polysialic acid on NCAM (30). Increasing sialic acid availability by overexpressing GNE with the use of GNE-sialuria-transfected cells or by the addition of N-acetyl-D-mannosamine (the product of the GNE reaction and physiologic precursor of sialic acid) dramatically increases the amount of polysialic acid on NCAM (30). The findings of the present study suggest that the reverse is also true; that is, that increases in polysialic acid on NCAM up-regulate sialic acid synthesis. This would explain why the amount of sialic acid supplementation correlated not only with increased learning performance but also with an unexpected increase in GNE expression (P = 0.009 and 0.004 in the hippocampus and liver, respectively). Moreover, at each location (liver, hippocampus, frontal cortex), the level of ST8SIA4 mRNA correlated with the level of GNE mRNA (n = 53, P = 0.0001, 0.038, and 0.016, respectively). This implies that both enzymes work in tandem to increase the synthesis of polysialic acid on NCAM during active learning. Because neonates have limited capacity for de novo synthesis of sialic acid (18), it is conceivable that a diet deficient in the preformed substance reduces early learning capacity, in turn lowering the demand for endogenous synthesis of NCAM-polysialic acid and therefore the need for sialic acid itself. The proposed mechanisms by which exogenous and endogenous sources of sialic acid could influence brain biochemistry are shown in Figure 10. The question our study cannot answer is whether dietary supplements of sialic acid increase neural tissue synthesis in the absence of learning activity. Further studies that incorporate sham and deferred learning treatments are therefore needed.

Because posttranscriptional regulation of the gene could be important, we endeavored to measure actual enzyme activity in the frontal cortex. Unfortunately, the specific activity of ST8SiaIV alone could not be determined because the separation of polysialyltransferase isoforms had not been established at the time of study. Although total sialyltransferase activity did not differ between the control and the supplemented groups, one measure of learning performance (the number of mistakes in the easy task) was loosely related to total enzyme activity (r = −0.336, P = 0.017, Figure 7B) and was still significant when only the last 20 trials were considered (r = −0.282, P = 0.047).

Taken together, our findings provide evidence that sialic acid from a glycoprotein source (CGMP) can facilitate early brain development in young piglets when fed in amounts up to and including the amount present in mature sow milk. We speculate that the large amounts of sialylated oligosaccharides in mature human milk (700 mg/L) may be one mechanism by which breast-feeding promotes higher cognitive performance in children. The relatively small amount of sialic acid in infant formulas (0–200 mg/L) (9) is of concern. Future studies should examine the safety and effectiveness of sialic acid-supplemented formulas to promote brain development in low-birth-weight and premature infants.

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**FIGURE 10.** Proposed mechanism of how dietary sialic acid and learning influence cellular function during brain development. 1) Endogenous sialic acid (Sia) is produced in the cytosol from glucose or the products of glycolysis. The epimerase enzyme of UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE) is responsible for the rate-limiting step, converting UDP-GlcNac to the sialic acid precursor, N-acetyl-D-mannosamine (ManNac). GNE activity is low in neonates (18). 2) In the nucleus, sialic acid is activated to cytidine 5′-monophosphate-sialic acid (CMP-Sia), the substrate for sialyltransferases such as polysialyltransferase α-2,8-sialyltransferase IV (ST8SiaIV). 3) Within the Golgi apparatus, sialic acid residues are transferred onto acceptors, including gangliosides, and the neural cell adhesion molecule (NCAM). The intracellular concentration of sialic acid drives the synthesis of polysialic acid (PSA) on NCAM (30). CMP-Sia exerts negative feedback inhibition on GNE, limiting excess production of free sialic acid (30). 4) Supplementation of the diet with sialic acid bypasses GNE, increasing the amount of sialic acid available for metabolism and hence CMP-Sia for polysialic acid synthesis. 5) Normal brain development and active learning increase the requirement for sialylated structures, including brain gangliosides and NCAM (26, 27). We propose that increased learning or higher ST8SiaIV activity automatically increases the expression of GNE, thereby coupling endogenous sialic acid synthesis to polysialic acid synthesis. Thus, during times of high sialic acid demand (learning and brain growth), the inhibitory feedback of CMP-Sia on GNE is minimized.
interpretation, and manuscript preparation. None of the authors had a financial conflict of interest.

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