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-acetylenuraminic 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 ST8SialI and ST8SialIV, 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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2 Supported by research grant 302016 from the National Health & Medical Research Council (NH&MRC) of the Commonwealth of Australia and a project grant from Mead Johnson Nutritional USA.

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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-acetylgalactosamine-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) 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.

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FIGURE 1. Schematic representation of the learning area with 8-arm radial maze and the visual cues used for the easy and difficult learning tasks.
sialic acids were measured according to published methods (10, 21). Sialytransferase 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 $E_{target}/E_{reference}$, 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.

**RESULTS**

**Learning performance**

In both the easy and difficult tasks, the sialic acid-supplemented groups reached the learning criterion faster than did the control group ($P = 0.001$, Kaplan-Meier, in task 1 and $P = 0.018$ in task 2; *Figure 2*). In the easy task, only 45% of the control group reached the criterion within 40 trials compared with $>70\%$ in the other groups, but no dose-response effect was observed ($P = 0.122$, Cox regression). In the difficult task, 100% of piglets in group 4 reached the criterion within 30 trials compared with only 70% of the control group, with a clear dose-response effect ($P = 0.003$, Cox regression). The findings were unchanged when adjusted for body weight or rate of weight gain (data not shown).

We also considered the total number of mistakes (ie, the number of times the piglet chose the wrong door) as a measure of learning (*Figure 3A*). Because learning in the first 20 trials of the easy task was likely to be predominantly “trial and error,” we considered trials 21–40 separately. The control group made significantly more mistakes than did the supplemented groups for both tasks ($P = 0.016$ for trials 21–40 of task 1 and $P = 0.048$ for all trials in task 2; *Figure 3B*), but there was no dose-response order. Because the piglets would have used learning in the first task to aid learning in the second, we considered the total number of mistakes made in the first as a covariate of learning in task 2.
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).

Sialytransferase 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 ($P > 0.05$; Figure 7A), and no correlations were observed between mRNA levels of $ST8SIA4$ and total sialyltransferase activity ($P = 0.705$ in the frontal cortex and $P = 0.458$ in the hippocampus). However, sialyltransferase activity in the frontal cortex correlated inversely with the number of mistakes in the easy task ($r = -0.336, P = 0.017; n = 50$). Columns bearing same letters between groups are not significantly different ($P > 0.05$) based on a general linear model (univariate ANOVA) with Bonferroni’s adjustment for multiple comparisons.

### Body weight gain

Mean ($\pm$SE) starting body weight was the same in each group ($2.1 \pm 0.04$ 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.
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 increases after long-term active avoidance conditioning in brain sialic acid incorporation. In particular, ganglioside synthesis increases after long-term active avoidance conditioning 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 than others.

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...
Sources of sialic acid could influence brain biochemistry are proposed mechanisms by which exogenous and endogenous polysialic acid and therefore the need for sialic acid itself. The turn lowering the demand for endogenous synthesis of NCAM—in the preformed substance reduces early learning capacity, in synthesis of sialic acid (18), it is conceivable that a diet deficient learning. Because neonates have limited capacity for de novo increase the synthesis of polysialic acid on NCAM during active respect) (28, 29). Evidence is strong that the concentration of sialic acid in cells regulates the content 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.

We thank Alison Staples, Paul Quaggiotto, and Zia Ahmad for participation in various parts of the study. We thank Maria Makrides, Stewart Truswell, Louise Baur, Emma Whitelaw, Patricia McVeagh, and Justin Harries for comments on the manuscript.

BW took part in the project study design, all data collections, data interpretation, and manuscript preparation; BY took part in the study design of molecular work; MK took part in data collection of sialyltransferase activity and learning behavior test; HH took part in data collection of the molecular work; YS took part in data collection of learning and behavior test; PM and SH took part in the study design of piglet learning behavior test; PP took part in the statistic analysis; JB-M took part in the project study design, data
interpretation, and manuscript preparation. None of the authors had a financial conflict of interest.

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