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Supplementation with a mixture of complex lipids derived from milk to growing rats results in improvements in parameters related to growth and cognition

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Abstract

Alterations in nutritional factors during early development can exert long-term effects on growth, neural function, and associated behaviors. The lipid component of milk provides a critical nutritional source for generating both energy and essential nutrients for the growth of the newborn. The present study, therefore, investigated the hypothesis that nutritional supplementation with a complex milk lipid (CML) preparation, derived from the milk fat globule membrane rich in phospholipids and gangliosides from young rats, has beneficial effects on learning behavior and postnatal growth and development. Male Wistar rat offspring from normal pregnancies were treated from neonatal day 10 until postnatal day 80 with either vehicle or CML at a dose of 0.2% (low) and 1.0% (high) based on total food intake (n = 16 per group). Neonatal dosing was via daily oral gavage, while postweaning dosing was via gel supplementation to a standard chow diet. Animals underwent behavioral tasks related to spatial memory, learning, and cognitive function. Complex milk lipid supplementation significantly increased linear growth rate ($P < .05$), and the improved growth trajectory was not related to changes in body composition as quantified by dual-energy x-ray absorptiometry scanning or altered plasma lipid profiles. Moreover, this effect was not dose dependent and not attributable to the contribution to total energy intake of the CML composition. Supplementation of the CML to growing rats resulted in statistically significant improvements in parameters related to novelty recognition ($P < .02$) and spatial memory ($P < .05$) using standard behavioral techniques, but operant testing showed no significant differences between treatment groups. Supplementation with a CML containing gangliosides had positive growth and learning behavioral effects in young normal growing rats.

Keywords: Rat; Nutrition; Complex milk lipids; Gangliosides; Growth; Behavior; Cognition
Abbreviations: ANOVA, analysis of variance; BMD, bone mineral density; CML, complex milk lipid; DEXA, dual-energy x-ray absorptiometry; IP, intraperitoneally; MWM, Morris water maze; NORT, Novel Object Recognition Task.

1. Introduction

Few other aspects of food supply and metabolism are of greater biologic importance than the feeding of mothers during pregnancy and lactation to ensure the optimal

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outcomes for the infant. The course of pregnancy, childbirth, and lactation, as well as milk composition, and the short- and long-term outcome of the child are influenced by the intake of foods and, particularly, micronutrients. Milk, as the first food the infant receives, plays a wider role of providing basic nutrition, and it delivers bioactive components for protection and development. The lipid component of milk provides a critical nutritional source for generating both energy and essential nutrients for the growth of the newborn. The milk fat globule membrane provides complex lipids, such as phospholipids and gangliosides, which are also likely to contribute important bioactivities during this early critical window of development. In particular, nutritional factors during early development can exert long-term effects on neural function and associated behaviors. A well-documented example of this is the dietary addition during pregnancy and/or infancy with ω-3 long chain polyunsaturated fatty acids, where studies have shown improved intelligence scores of both breast-fed and formula-fed children after ω-3 supplementation [1-3].

The brain is a lipid-rich organ containing mostly complex polar phospholipids, sphingolipids, gangliosides, and cholesterol. These lipids are involved in the structure and function of cell membranes in the brain. There are established species differences in tissue levels of minor lipids such as gangliosides, as well as lactational and seasonal variations in ganglioside profiles of milk across some species [4-7]. For example, human and bovine milk vary in the total amount and the composition of gangliosides; the total amount of gangliosides in mature bovine milk (the source of gangliosides for infant formula) is significantly lower than that in breast milk, and as a consequence, the ganglioside content in bovine milk-based infant formula is also significantly lower compared with human milk [8,9]. It has been calculated that infants fed starter formulas have total ganglioside intakes of 20% of those fed human colostrum or transitional milk [9]. Thus, variation of the bioactive lipids in human and bovine milk, and infant formulas might have some biologic significance regarding neonatal brain development, allergies, and infant growth [8].

The effect of supplementation during the neonatal period with dietary-derived gangliosides and phospholipids on behavioral outcomes is not well documented. Long-term nondietary treatment with gangliosides has been shown to improve learning and memory in various animal models [10-12]. Recent work investigating dietary choline and ganglioside supplementation to young normal rats has shown no significant beneficial effect of supplementation on spatial short-term memory [13]. In contrast, early work by Mei and Zheng [14] has shown that high doses of gangliosides administered intraperitoneally (IP) can enhance learning ability and memory retention in rats at a young age as quantified using the radial arm maze. A more recent trial has shown that intracerebroventricular (ICV) treatment with ganglioside GQ1b improved spatial learning and memory of rats [15].

There have been many studies investigating the effects of dietary bioactive lipids such as ω-3 and ω-6 fatty acids and several of the phospholipid classes. However, most studies on gangliosides to date have examined the effect of *non-dietary* supplementation on behavioral and cognitive outcomes, which are difficult to translate as an effective adjunct to standard nutrition in the human setting because of the nondietary modes of delivery. In the present study, we hypothesize that oral nutritional supplementation with a complex milk lipid (CML), as a natural food-derived source of complex lipids and, in particular, containing gangliosides, in young growing rats will have beneficial effects on learning behavior and postnatal growth and development.

2. Methods and materials

2.1. Animals

Timed mating of outbred Wistar rats were performed at 100 days of age using an estrous cycle monitor (EC-40; Fine Science Tools, San Francisco, Calif). Confirmation of mating was via a vaginal lavage with sterile saline and visualization of spermatozoa under a microscope. Pregnant dams were housed as singletons throughout pregnancy and lactation in standard rats cages with ad libitum access to a standard control diet (Diet 2018; Harlan Teklad, Oxon, UK) (Table 1) and water and a standard 12:12 light/dark cycle. At birth, litter size was adjusted to 8 pups per litter, and offspring were maintained on the same dam until the end of lactation. Male offspring were used, with the balance of the litters made up to 8 with female pups where necessary. Male offspring were used for behavioral analysis to avoid the potential confounders of estrous in female offspring. Pups were left on lactating dams to feed normally and were supplemented with either the CML (Table 1) or water via sham gavage (controls) between 10 and 80 days of age (young adult). The CML was

Table 1
Composition of the standard control diet and the CML composition

Control Diet 2018	
Protein	18.8%
Fat	6.0%
Crude fiber	3.8%
Ash	5.9%
Starch	45%
Sugar	5%
Energy from protein	23%
Energy from fat	17%
Energy from carbohydrate	60%
Metabolizable energy	13.8 kJ

Composition of the standard control diet (Diet 2018, Harlan Teklad). The CML contained 80% fat, 7.7% ash, 7.3% protein, 2% moisture, and 3% lactose. In the fat of the CML, there were 5.9% gangliosides (gangliosides GM3, 0.7%; gangliosides GD3, 5.2%), 50.6% phospholipids (phosphatidyl serine, 13.7%; phosphatidyl choline, 6.5%; phosphatidyl ethanolamine, 13%; phosphatidyl inositol, 9.5%; and sphingomyelin, 6.9%), and 23.5% neutral lipid. Docosahexaenoic acid was supplemented at 450 mg per 100 g, and choline was supplemented at 34 mg per 100 g in the CML.

supplemented at a rate to provide a concentration of 0.2% (low) or 1% (high) wt/wt relative to measured food/milk intake and based on current available literature in the rodent [13,16,17]. Thus, for example, a rat with a standard adult food intake of 25 g per day would receive 48 mg or CML (0.2%, low) or 240 of mg CML (1%, high). Treatment was performed within litter to minimize litter variability. (Two male pups within each litter were randomized on day 10 into control, low-CML, or high-CML treatment groups.) The supplement was administered by oral gavage (once daily at 0:800 hours) using feeding tubes specifically designed for use in rat pups/weanlings (product number FTP 20-30, 20 gauge; Instech Laboratories, Plymouth, PA, USA) from day 10 until weaning at day 22. Dose of CML was adjusted relative to expected milk intake every 2 days during the neonatal treatment period. Dose volume was 0.1 mL in sterile water. This procedure allowed precise dosing of the animals —dietary supplementation by incorporating the CML formulation directly into the maternal diet would lead to unavoidable variation. All animals were weighed (and CML supplementation adjusted according to weight gain), and food intake of dams was monitored daily throughout pregnancy and lactation. Postweaning administration of CML was via gel formulations, and food intakes were measured routinely and dose adjustments made accordingly every 5 days. At the completion of the trial, animals were killed by decapitation while under anesthesia with sodium pentobarbitone (60 mg/kg, IP). All animal studies were performed under the ethical approval of the Animal Ethics Committee at the University of Auckland, Auckland, New Zealand.

2.2. Postweaning gel supplementation

At weaning (postnatal day 22), animals were housed 2 per cage under standard conditions. A total of 32 animals per treatment group (a total of 96 animals) were studied using 2 animals per treatment group from each of 16 litters. The chow diet (ad libitum) was supplemented with CML using gels at the doses defined above based on food intake and adjusted according to changes in dietary consumption. The gels were placed at either end of the feeding platform in each cage, and the gels were preferentially consumed over the chow. There were no significant gel remnants from any of the animals using this technique.

2.3. Complex milk lipid composition

The CML composition was a mixture of complex lipids extracted from bovine buttermilk containing the milk fat globule membrane material (Table 1) (Fonterra Research Centre, Palmerston North, New Zealand) and comprising (by weight) 80% fat (includes 5.2% wt/wt ganglioside GD3, 46% wt/wt phospholipids), 7.7% ash, 7.3% protein, 2% moisture, 3% lactose, and energy of 2730 kJ per 100 g. The total choline and docosahexaenoic acid concentrations were 450 mg per 100 g of CML (0.45%) and 34 mg per 100 g of CML (0.034%), respectively. Thus, the extra energy added to the diet was negligible above that of controls and was calculated to be approximately an additional 1.3 kJ (48-mg CML) and 6.5 kJ (240-mg CML) for the low and high doses, respectively.

2.4. Behavioral tasks

Three types of behavioral tests were performed, which encompassed classic behavioral protocols (Morris water maze [MWM] and Novel Object Recognition Task [NORT]) and operant learning techniques. The MWM paradigm was used to investigate short-term effects on spatial memory, NORT was used to investigate the novelty recognition, and operant techniques were used to quantify longer-term effects on memory and task learning. Because of the need for daily food restriction in the operant animals, the cohort of 96 animals was divided into 2 subcohorts: 48 animals (16 per treatment group) for MWM and NORT analysis and 48 animals for the operant learning paradigm. The MWM and NORT tests were carried out in young postpubertal animals at 60 days of age. The operant learning protocol was initiated at postnatal day 34 as detailed below.

2.5. Morris water maze

This test, developed by Morris [18], is widely used in behavioral research and can be used to quantify spatial working or reference memory. It uses a circular tank filled with water and contains a hidden platform below the water surface. Animals need to find the platform and remember its position using the extra-maze cues. The distance swum to the platform and the time taken in doing so should decrease over testing sessions as the animals learn the location of the platform. Moreover, it is expected that if the animal learned the location of the platform in relation to the extra-maze cues, its initial response on the probe trial will be to swim directly to the quadrant in which it expects to find the platform. The MWM was performed in dimmed lighting conditions, and multiple distant cues around the room were kept in the same location throughout the experiments. All data were collected automatically via computer using an automated tracking system (AnyMaze; Stoelting, Inc., Wood Dale, IL, USA). In brief, a hidden platform was placed into one quadrant of the pool. Rats were placed into the water with its head pointed toward the side of the pool (starting position is randomized each day). The time taken for the rat to reach the platform was recorded with rats guided to the platform if time exceeded 90 seconds. The rat was then allowed to remain on the platform for 10 to 15 seconds. The rat was then removed from the pool and, after a 5-minute waiting period, was replaced in the pool from the same location with the platform in the same location. Each rat was given 4 trials on the first day. On day 2, the platform was inserted in the same location as on the first day, but the start position was randomized. The rats were given 4 trials per day with 5-minute intervals for 4 days until performance was stabilized and latency to find the platform was low (<10

seconds to platform). Performance was expressed as the average time it takes each rat to find the submerged platform. For the spatial probe trial, trained rats were placed into the pool without a platform. The rat was removed after 30 seconds, and the time recorded in the quadrant that previously contained the platform was recorded and expressed as a percentage of total time spent in the pool.

2.6. Novel Object Recognition Task

The NORT is widely used in behavioral research to quantify short-term memory function in rodents [19-22]. Exploration is a typical learning behavior for rats when they have been placed into a novel environment. The amount of exploratory activity decreases over time during the acquisition phase and can be reactivated by introducing a novel object during the testing phase. The increase in exploratory behavior when the environment is altered by changes in the environment, for example, the additional objects being introduced after the acquisition, provides a measure of the memory for familiarity and the recognition of novelty. The tests were carried out during 4 days with 2 trials on each testing day with 2-hour intervals. For animals to be familiarized with the testing arena (90 × 60 × 40 cm), rats spent 10 minutes in the empty arena during each trial on the first day of testing. During the acquisition phase, 4 different objects were placed into each corner of the testing arena 15 cm away from the walls. Rats were allowed to spend 5 minutes exploring all objects in each trial and had 5 trials during the next 3 testing days to become familiarized with the objects. During the testing phase on the third day, one familiar object was replaced by a novel object before the second trial. The time spent on exploring the objects was recorded using a stopwatch. Animal awareness of the environmental change was quantified by observing the changes in exploration activity. The total time of exploration of all objects during the testing phase was analyzed for novelty recognition. The current protocol was applied because of the use of young normal rats where learning ability is expected to be high. Thus, the acquisition of 4 different objects is a more difficult task compared with 2 identical objects, a paradigm that has been used for measuring the novelty recognition of aged rats and rats with potential leaning difficultly after surgical manipulation.

2.7. Operant conditioning

At approximately 34 days of age, rats were placed on scheduled feeding for 2 hours per day, during which, they received the standard laboratory chow in their home cages. In addition to the chow, rats received a block of gel that contained either blank or the low- or high-dose CML as detailed above. The gel was preferentially consumed over the standard chow, and total food intakes were not different between the treatment groups. Behavioral sessions were conducted daily. All rats were placed individually in sound- and light-attenuated operant chambers (ENV-007; Med Associates, St Albans, Vt). One day after the introduction of scheduled feeding, pretraining in the operant chambers started, where rats were trained to respond on levers for 45-mg food reinforcers (Noyes pellets, purified formula; TestDiets, Richmond, IN, USA). After 19 days of pretraining, the main experimental operant procedure commenced [23]. The subjects were exposed to 7 unsignaled reinforcer ratios in each session (eg, 27:1, 9:1, 3:1, 1:1, 1:3, 1:9, and 1:27). The computer controlling all experimental contingencies selected these in a random order, and the reinforcer ratio was changed every 10 reinforcer deliveries (a component). Sensitivity to reinforcement of the generalized matching law [24], or the extent to which response allocation follows changes in the reinforcer ratio, was our measure of learning and behavioral adaptability. Behavioral sessions finished after 60 minutes or after they had received the maximum of 70 reinforcers.

2.8. Dual-energy x-ray absorptiometry scanning

Body composition and bone mineral content and density were assessed in animals after behavioral testing using dual-energy x-ray absorptiometry (DEXA; Lunar Hologic, Waltham, Mass).

2.9. Blood plasma measurements

Blood samples were collected at the completion of the animal trials and centrifuged, and supernatant was removed for subsequent analysis. Lipid profiles were measured in all animals after CML supplementation. All measurements were performed using an automated biochemistry analyzer (Hitachi 902 Autoanalyser; Roche Diagnostics, Indianapolis, Ind).

2.10. Brain ganglioside and phospholipid quantification

Brain tissue from the tested rats was homogenized in water and the gangliosides and phospholipids extracted and partitioned using the method of Svennerholm and Fredman [25]. Gangliosides (GM3, GM2, GD3, GD1a, GD1b, GT1b, and GQ1b) were separated by hydrophilic high-performance liquid chromatography and quantified using a LTQ-Orbitrap mass spectrometer (LTQ-Orbitrap, Thermo Scientific, Waltham, MA, USA). Phospholipids (phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin) were separated by hydrophilic high-performance liquid chromatography and quantified using phospholipid class–specific fragmentations using a tandem mass spectrometer.

2.11. Statistical analyses

All statistical analyses were performed using StatView software package (SAS Institute, Cary, NC, USA). Data were tested for normality and analyzed using 1-way analysis of variance (ANOVA) with treatment as a factor. Behavioral data were analyzed using repeated measures ANOVA (acquisition day × treatment group and factors), and bodyweight was used as a covariate where appropriate. Body weight was

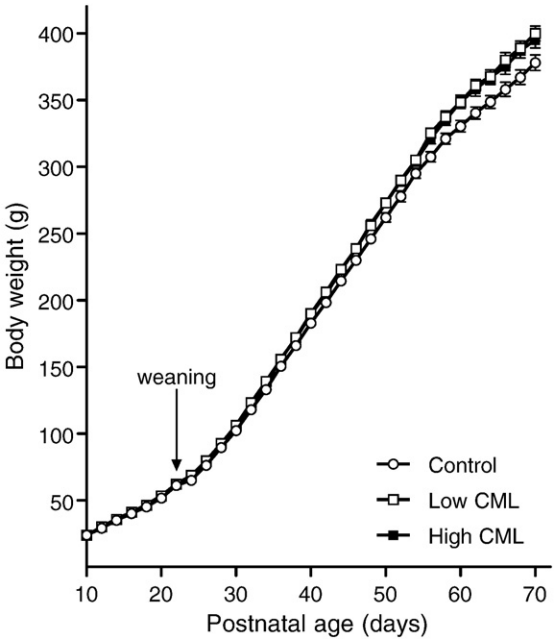

Fig. 1. Postnatal growth curves in control, low-CML–, and high-CML–supplemented animals. n = 16 per group; results are expressed as means ± SEM.

analyzed via repeated measures and final body weight. Post hoc testing used the Fisher protected least significant difference (PLSD) test. Results are expressed as means ± SEM unless otherwise stated.

3. Results

3.1. Body weight gain

At the commencement of neonatal supplementation at postnatal day 10, there were no significant differences in weights between the 3 treatment groups (control 23.8 ± 0.2 g, low CML 23.8 ± 0.3, and high CML 23.8 ± 0.3). At weaning (day 22), there was a small but significant increase ($P < .05$) in body weights in the high-CML–treated animals compared with controls (control, 61.49 ± 0.43 g; low CML, 62.33 ± 0.42 g; high CML, 63.22 ± 0.48 g).

Postnatal growth was significantly increased in CML-supplemented offspring (Fig. 1). This effect was not dose dependent and not ascribable to the added usable energy of the nutritional supplement containing the CML, because this was a nominal ingredient in comparison with overall food consumption. The weight gain was also independent of changes in body composition as assessed by DEXA scanning. There were no significant differences in body fat composition between any of the treatment groups (Table 2), although bone mineral density (BMD) was slightly but significantly increased in the high-CML group compared with controls. Thus, the increased weight gain observed in CML-supplemented animals does not reflect increased adiposity, although there was a slight trend toward an increased fat/lean ratio in low-CML–treated animals compared with controls ($P = .1$). Of note, high-CML–treated animals were also slightly but significantly longer ($P < .05$, nose-anus length) than control animals (Table 2). The enhanced body growth was also independent of any alterations in plasma lipid and total protein profiles (Table 3), although there was a strong trend toward an improved cholesterol profile in low-CML–supplemented animals compared with controls ($P = .07$).

3.2. Morris water maze

Latency to platform was not different between any of the treatment groups at the start of acquisition testing (Fig. 2A,B). The effect of learning over time was $P < .0001$ for all groups. On acquisition day 2, the high-CML–treated animals had a significantly ($P < .05$) reduced latency to platform than controls and low-CML treatment groups. However, by testing day 4, all groups were similar in their ability to locate the platform. Of note, the high-CML animals showed a linear response in latency to platform over the 4 trials held on any given day, a pattern not evident in the low-CML and control groups (Fig. 2A). There were no significant differences in swim speed between any of the treatment groups (Fig. 3A,B). The trend for decreased swim speed over time was $P < .0001$ for all treatment groups. There was an overall trend toward decreased swim speed in high-CML–treated animals ($P = .09$). With swim distance, there was a significant difference in overall swim distance in the high-CML–treated animals vs controls on day 2 of testing, which parallels the shortened time to platform in this group ($P < .05$). Trend for change (decrease) in swim distance over time was $P < .0001$ for all treatment groups (Fig. 3C,D). There were no significant differences in the spatial probe trial (Fig. 4), although there was a trend toward reduced latency to platform in the low-CML–treated group ($P = .09$, Fig. 4C).

Table 2
Body composition data

Measurement	Control	Low CML	High CML	Control vs low CML	Control vs high CML	Low CML vs high CML
Fat (%)	20.9 ± 0.7	22.2 ± 0.8	21.1 ± 0.8	NS	NS	NS
Fat/lean (ratio)	0.266 ± 0.01	0.297 ± 0.02	0.270 ± 0.01	NS	NS	NS
BMD (g/m^3)	0.147 ± 0.001	0.148 ± 0.001	0.150 ± 0.001	NS	$P < .05$	NS
Length (mm)	245.3 ± 1.5	247.5 ± 1.6	249.7 ± 1.4	NS	$P < .05$	NS

Total body fat percentage, fat/lean ratios, BMD, and body lengths (nose-anus). Results are expressed as means ± SEM, n = 16 per group. NS indicates not significant.

Table 3
Plasma biochemistry profiles

Measurement	Control	Low CML	High CML	Control vs low CML	Control vs high CML	Low CML vs high CML
FFA (mmol/L)	0.363 ± 0.06	0.371 ± 0.04	0.366 ± 0.03	NS	NS	NS
Trig (mmol/L)	1.52 ± 0.14	1.74 ± 0.14	1.61 ± 0.13	NS	NS	NS
LDL (mmol/L)	0.29 ± 0.02	0.26 ± 0.02	0.28 ± 0.02	NS	NS	NS
HDL (mmol/L)	1.34 ± 0.06	1.39 ± 0.05	1.31 ± 0.04	NS	NS	NS
LDL/HDL (ratio)	0.22 ± 0.02	0.19 ± 0.01	0.21 ± 0.01	NS	NS	NS
T protein (g/L)	5.55 ± 0.06	5.59 ± 0.07	5.72 ± 0.06	NS	NS	NS

Plasma biochemistry profiles of animals at postnatal day 80 after supplementation with the CML containing gangliosides. Results are expressed as means ± SEM, n = 16 per group. Trig indicates triglycerides; T protein, total protein; NS, not significant; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FFA, free fatty acids.

There was no effect of bodyweight on behavioral outcomes when used in covariate analysis.

3.3. Novel Object Recognition Task

The introduction of a novel object resulted in a significant increase ($P < .05$) in exploring activity during the testing phase in the groups treated with high dose CML (Fig. 5) compared with acquisition phase, and these data suggest an increased awareness of an altered environment after CML supplementation.

3.4. Operant conditioning

The 3 treatment groups were tested for 50 sessions using a procedure that measures short-term learning and adaptation to changing situations [23]. For all rats, sensitivity values increased in a hyperbolic fashion as more reinforcers were obtained. There were no significant group differences in sensitivity to reinforcement ($P > .05$, repeated measures ANOVA). The average numbers of responses per minute are shown in Fig. 6 as an indicator for motivation to respond for food reinforcers. Data from sessions 16 to 50 were included in the analyses. The group differences in response rates were not significant ($P > .5$, ANOVA). Group values of sensitivity to reinforcement and response rates were not significantly different for any of the session blocks (sessions 1-15, sessions 16-35, or sessions 36-50). The amount of learning, indexed by the asymptotic sensitivity to reinforcement attained, increased with age in all 3 groups, but there was no overall difference between the control and the CML-supplemented groups.

3.5. Brain ganglioside and phospholipid analysis

Gangliosides GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b, and GQ1b were quantified in the brains from 80-day-old rats in the control, low-CML, and high-CML groups. None of the individual ganglioside species or total ganglioside concentration showed significant differences between the control and treatment groups (total ganglioside concentration:

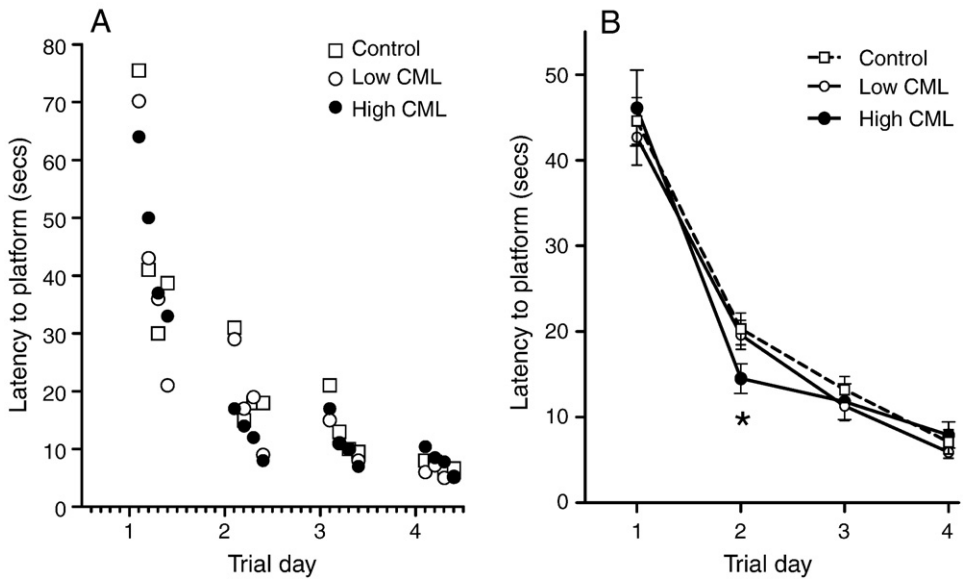

Fig. 2. Morris water maze. (A) Mean latency to platform for each of the 4 individual trials per testing day; (B) mean latency to platform on each day of acquisition testing. Results are expressed as means ± SEM, n = 16 per group. *$P < .05$ for high CML vs control.

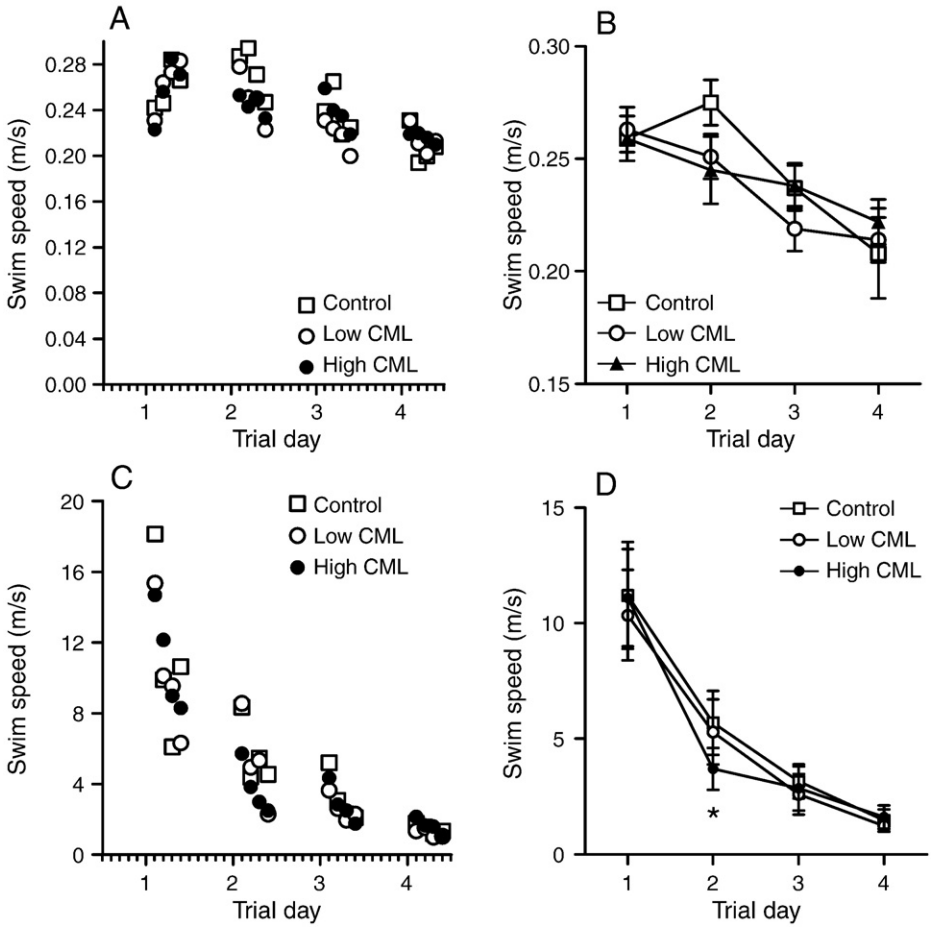

Fig. 3. Morris water maze. (A) Mean swim speed over each of the 4 individual trials per testing day; (B) mean swim speed over the 4 days of acquisition testing in the MWM; (C) average swim distance over each of the 4 individual trials per testing day; (D) average swim distance over the 4 days of acquisition testing in the MWM. Results are expressed as means ± SEM, n = 15 to 16 per group. *$P < .05$ for control vs high CML.

control, 1683 ± 39 μg/g wet weight; low CML, 1668 ± 38; high CML, 1653 ± 36; $P > .05$). Phospholipids PC, PI, PE, PS, and SM were all quantified in the brains from 80-day-old rats in the control, low-CML, and high-CML groups. There were no significant differences in individual species of phospholipids or total phospholipid concentrations between control and treatment groups (total phospholipid concentration: control, 51 ± 1 mg/g wet weight; low CML, 52 ± 1; high CML, 53 ± 1; $P > .05$).

4. Discussion

In the present study, we have investigated the effect of nutritional supplementation with a CML formulation containing bioactive lipids (gangliosides, phospholipids, glycosphingolipids, and other polar lipids) to normal young growing rats on a range of behavioral and physiologic outcomes. To date, the biologic value of CML containing these milk-derived bioactive lipids on behavioral and cognitive outcomes in nondeficit models have yet to be fully elucidated.

Indications of improved behavioral performance were observed in short-term trials of spatial and reference memory using the MWM and NORT tests. In the MWM, there was a significant change in the learning slope during the acquisition phase in the high-CML treatment group, which was paralleled by a decrease in swim distance and a trend toward increased swim speed. However, by completion of the training phase and the spatial probe trial, there were no overall significant differences between any of the treatment groups. In the NORT protocol, CML-treated animals exhibited a significant change in awareness to the changed environment compared with control animals, particularly the group treated with higher dose. The operant-conditioning procedure assessed how the animals distributed their behavior between 2 response alternatives that change frequently with respect to the relative frequencies with which they "pay off." It, thus, provided a measure of behavioral adaptability and learning in a frequently changing environment. The conclusion based on these data from the operant learning protocol was that CML supplementation did not have a significant effect on learning in a frequently changing environment, because there were no significant

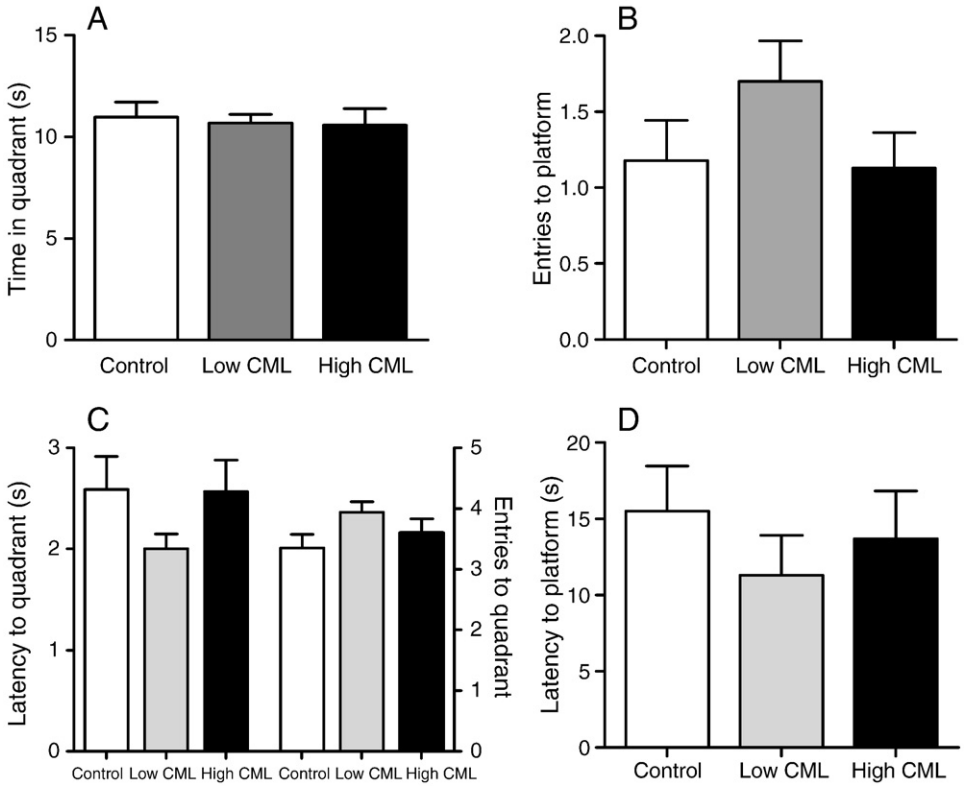

Fig. 4. Morris water maze. Spatial probe trial on day 5 after 4 days of acquisition testing in the MWM. (A) Total time spent in correct quadrant; (B) entries to platform area; (C) latency and number of entries to correct quadrant; (D) latency to platform area. Results are expressed as means ± SEM, n = 16 per group.

group differences in sensitivity to reinforcement. Thus, depending on the observed outcome measure of learning, CML supplementation was shown to have some beneficial effects in tests of acute learning but no overall benefit on long-term memory acquisition. One possible limitation of the operant learning approach is that related to scheduled feeding. Scheduled feeding is known to cause activation of dopamine metabolism in the ventrolateral striatum, known to be important for the regulation of automation of behavior. A scheduled feeding regimen may, therefore, alter the plasticity within neural circuitry regulating motivated learning, which could translate to effects on task learning compared with animals that are fed ad libitum [26,27].

The doses of complex lipids and, in particular, the gangliosides provided by the CML gave levels in the diet that were in a physiologic range and were designed to be

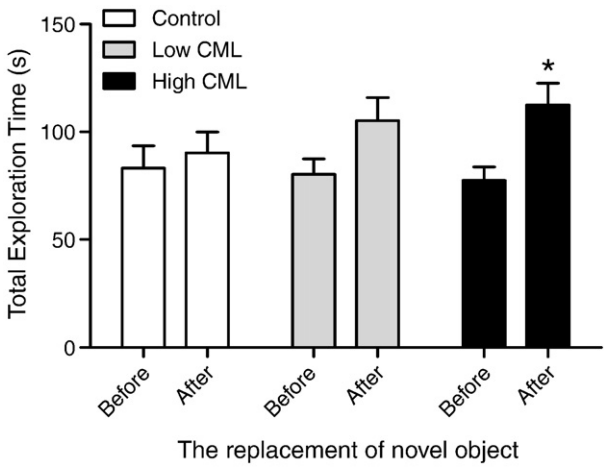

Fig. 5. Novel Object Recognition Task trial: Two-way ANOVA showed a significant overall increase in exploring time after a novel object being introduced across all treatment groups ($P < .05$) but was only significantly different from baseline in the group treated with high dose of CML ($P < .5$, paired t test). Results are expressed as means ± SEM, n = 16 per group.

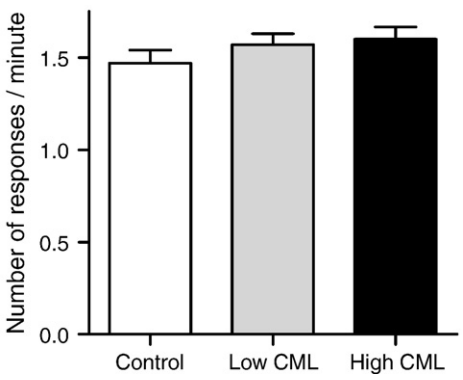

Fig. 6. Operant learning behavior: average lever response rate (per minute) for each experimental group ($P > .05$, ANOVA) over sessions 16 to 50. Results are expressed as means ± SEM, n = 16 per group.

representative of the range expected to be found in human milk [4,28]. Supplementation with the CML led to an unexpected but significant increase in body weight gain (both low- and high-dose treated groups) and length (high dose group). This was not reflected in increased adiposity as assessed by DEXA scanning and was independent of energy intake. The increased body weight gain was also independent of any significant changes in plasma lipid profiles. This unexpected observation on enhanced body and linear growth may relate to specific bioactive ingredients with the CML supplement influencing nutrient partitioning and warranting further investigation.

It is important to note that the present trial was performed in normal young growing rats receiving adequate nutrition via suckling and receiving a balanced postweaning control diet and with no existing learning or cognitive deficits. Under this paradigm, it may be indeed difficult to improve rate of learning above that of controls because of the rapid learning ability in normal young rats. However, the testing efficacy of a dietary CML formulation in a normal model has provided important baseline data and can now be extended to known-deficit paradigms such as that of learning deficits induced by maternal undernutrition [29]. Such a model would be a more "natural" setting in which to test such supplements as opposed to a chemically induced memory deficit such as scopolamine-induced amnesia.

In the present study, the observed effects are representative of the efficacy of the CML as an ingredient, and it is difficult to ascribe the effects observed to any particular components without more data on the mechanisms. Many components found in the CML formulation may contribute to the effects seen. In particular, gangliosides from the same source have been shown to increase brain gangliosides but not other complex lipids such as phospholipids [16]. Nondietary gangliosides have been shown to improve memory and learning in a number of animal models of neurologic deficits [10-12]. In the present study, ganglioside doses in the CML were within the range (0.01%-0.05% of total dietary intake) reported by others [17]. Furthermore, the ganglioside concentrations and ratios and phospholipid levels in brain tissue in the present study are similar to those reported in the literature for the rat [13,16]. The present trial does not show an increase in brain gangliosides as a result of increased CML, and ganglioside intake as has been reported by Park et al [16], but this may directly relate either to the age or strain of the animals studied (weanling Sprague-Dawley animals compared with the present trial in mature adult Wistars) or the higher dose of CML as a source of gangliosides used in the Park et al trial [16]. Similarly, previous work by Wainwright et al [13] has shown changes in ganglioside content independent of changes in learning or behavioral tasks when young animals were given CML as a source of gangliosides. However, in that study, in the Long-Evans rat, artificial rearing techniques were used, and the authors used CML as a source of gangliosides alone as well as in combination with choline chloride administration; an increase in brain gangliosides was only observed for the animals receiving both CML and choline. The work by Mei and Zheng [15] showed benefits of gangliosides on learning and memory, but in that trial, the gangliosides were administered IP and cannot be easily extrapolated to a dietary-based regimen. It could also be speculated that, in the present study, changes in ganglioside and phospholipids were induced early after onset of treatment but were washed out by the time of adult tissue collection. The docosahexaenoic acid and choline levels in the CML supplement are significantly below the published representative effective doses in rats [30-33]. Further work could, thus, investigate the effects of the different components found in CML via an ontogenic approach.

In summary, supplementation with a CML formulation containing the milk fat globule membrane lipids, gangliosides and phospholipids, at physiologically relevant doses to normal young growing rats resulted in an enhanced linear growth profile and some markers of increased learning ability using classic behavioral test platforms. Further investigation of efficacy of this CML formulation, as a natural and acceptable source of dietary gangliosides, and the efficacy of the lipid components as an infant formula supplement is warranted.

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