Early Supplementation of Phospholipids and Gangliosides Affects Brain and Cognitive Development in Neonatal Piglets^{1–3}

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Abstract

Background: Because human breast milk is a rich source of phospholipids and gangliosides and breastfed infants have improved learning compared with formula-fed infants, the importance of dietary phospholipids and gangliosides for brain development is of interest.

Objective: We sought to determine the effects of phospholipids and gangliosides on brain and cognitive development.

Methods: Male and female piglets from multiple litters were artificially reared and fed formula containing 0% (control), 0.8%, or 2.5% Lacprodan PL-20 (PL-20; Arla Foods Ingredients), a phospholipid/ganglioside supplement, from postnatal day (PD) 2 to PD28. Beginning on PD14, performance in a spatial T-maze task was assessed. At PD28, brain MRI data were acquired and piglets were killed to obtain hippocampal tissue for metabolic profiling.

Results: Diet affected maze performance, with piglets that were fed 0.8% and 2.5% PL-20 making fewer errors than control piglets (80% vs. 75% correct on average; $P < 0.05$) and taking less time to make a choice (3 vs. 5 s/trial; $P < 0.01$). Mean brain weight was 5% higher for piglets fed 0.8% and 2.5% PL-20 ($P < 0.05$) than control piglets, and voxel-based morphometry revealed multiple brain areas with greater volumes and more gray and white matter in piglets fed 0.8% and 2.5% PL-20 than in control piglets. Metabolic profiling of hippocampal tissue revealed that multiple phosphatidylcholine-related metabolites were altered by diet.

Conclusion: In summary, dietary phospholipids and gangliosides improved spatial learning and affected brain growth and composition in neonatal piglets. *J Nutr* 2014;144:1903–9.

Keywords: brain, cognition, 23 ganglioside, phospholipid, piglet

Introduction

Infant formula is considered nutritionally complete and sufficient for supporting growth, but there is concern that it lacks sufficient concentrations of known bioactive components found in breast milk that are important for neural and cognitive development. This is critical because although the WHO recommends exclusive breastfeeding up to 6 mo of age, and continued breastfeeding along with appropriate foods up to 2 y of age, by 6 mo postpartum >50% of infants are formula fed (1). Several reports indicate poorer cognitive development in formula-fed infants than in breastfed infants (2–4). These differences may be related to the high concen-

tration of gangliosides and phospholipids in breast milk compared with formula (5).

Gangliosides are a group of sialic acid glycosphingolipids found in the plasma membrane of cells (6). They are predominantly found in the nervous system where they are involved in neurogenesis, neural repair (7, 8), and learning and memory (6). Phospholipids are found in all plant and animal cell membranes, usually in the form of glycerophospholipids—FAs esterified to a glycerol backbone (9). The backbone can also be made of sphingosine instead of glycerol, forming sphingophospholipids, with sphingomyelin being the most common (9). Sphingomyelin in the membrane of neural cells (e.g., neurons and oligodendrocytes) is important for cell-cell and cell-matrix interactions, cell adhesion, modulation of membrane receptors and signal transduction, and axon myelination (10, 11).

Because human infants experience rapid postnatal brain growth and development, proper nutrition in the early postnatal period is of the utmost importance. Formula, however, is low in

¹ Supported by Arla Foods Ingredients and NIH grant HD069899.

² Author disclosures: H Liu, EC Radlowski, MS Conrad, Y Li, RN Dilger, and RW Johnson, no conflicts of interest.

³ Supplemental Figure 1 and Supplemental Tables 1 and 2 are 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>.

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phospholipids and gangliosides. If these are important for proper brain development, as has been suggested (6, 12, 13), this may partially explain the difference in neurocognitive development that was reported in formula-fed infants than those who were breastfed (2–4). Because exogenous phospholipids such as phosphatidylcholine and lysophosphatidylcholine cross the blood-brain barrier (14), dietary supplementation with gangliosides and phospholipids may be a viable approach to further promote brain development in formula-fed infants.

Therefore, the purpose of the present study was to assess brain development and spatial learning and memory in neonatal piglets fed a nutritionally complete formula or the same formula supplemented with phospholipids and gangliosides. As a gyrencephalic species with brain growth and development similar to humans, the piglet is an excellent translational model for studying neurodevelopment (15–17). The hypothesis was that the lipid-enriched diet would affect brain growth and development and improve spatial learning and memory.

Methods

Animals, housing, and feeding

A total of 24 vaginally delivered Yorkshire piglets were obtained from 6 litters from the University of Illinois swine herd. They were brought to the biomedical animal facility on postnatal day (PD)⁸ 2 (to allow for colostrum consumption from the sow) and housed individually in cages (0.87 m length × 0.87 m width × 0.49 m height). Each cage was positioned in a rack, with stainless steel perforated wall partitions and clear acrylic front and rear doors. In addition, each cage was fitted with flooring designed for neonatal animals (Tenderfoot/NSR; Tandem Products). A toy (plastic Jingle Ball; Bio-Serv) was provided to each piglet. Room temperature was maintained at 27°C, and each cage was equipped with an electric heat pad (K&H Lectro-Kennel heat pad; K&H Manufacturing, LLC). Piglets were maintained on a 12-h light/dark cycle; however, during the dark cycle, minimal lighting was provided. Piglets were handled by the experimenters multiple times per day during cleaning/feeding and daily observations to acclimate them to handling.

Piglets were fed a nutritionally complete, customized milk replacer. The type of fat used in the milk replacer was dried fat 7/60 (Merrick's), and the protein was primarily soy protein isolate. Powdered milk replacer diets were formulated at the University of Illinois and mixed at TestDiet. Upon arrival at the animal care facility, piglets were assigned to 1 of the following 3 dietary treatments, as balanced for birth weight, sex, and litter of origin: 1) control diet with no supplemental phospholipids or gangliosides, 2) control diet with 0.8% Lacprodan PL-20 (PL-20; Arla Food Ingredients), or 3) control diet with 2.5% PL-20. All diets met or exceeded nutrient requirements for young pigs (NRC, 2012) (18) and contained a similar amino acid profile as per the experimental design (Supplemental Table 1).

Milk was reconstituted fresh each morning to a final concentration of 206 g/L with the use of tap water and supplied at a rate of 285 mL/kg body weight (BW; based on daily recorded weights). This amount of feeding allowed for maintenance and growth but prevented complete satiation to ensure that the piglets remained motivated for food rewards in the behavioral task. Water was not provided separately from that used in the milk replacer. Milk replacer was delivered from a reservoir to a stainless steel bowl (secured to the side of each cage) via a peristaltic pump (Control Company). By using this automated delivery system [similar to that described previously (18)], piglets received their daily allotted milk over 18 meals (once per hour), followed by a 6-h food deprivation period before behavioral testing where no milk was provided. All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory

Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

Cognitive testing using a spatial T-maze task

Spatial learning and memory were assessed by using a clear plastic plus-shaped maze (essentially a double T-maze) with extra maze cues, which was previously described and validated (19). The maze consisted of 2 start arms (north and south) and 2 reward arms (east and west). Start arm location was alternated throughout testing, which ensured that the piglet did not solve the task by using an egocentric mechanism (i.e., turning body left or right, striatum-dependent), and instead was forced to adopt an allocentric mechanism (i.e., using extra-maze visual cues to create a spatial map of the room, hippocampus-dependent) for solving the task (20). This task is similar to that used in rodents to assess “place” and/or “direction” learning (21). Starting on PD14, piglets were tested daily between 0800 and 1200 h by one trained experimenter. Piglets completed 10 trials/d (60 s/trial) for a total of 10 d. The first 6 d of testing constituted the acquisition phase, where piglets learned to locate the chocolate milk reward [3 mL of the same milk replacer used for regular feedings with the addition of Nesquik cocoa powder (Nestlé), supplied according to the manufacturer's directions] in a constant place in space, as well as direction (e.g., west reward arm), by using the extra-maze visual cues. Chocolate milk was provided in both reward arms to balance for olfactory cues but was only accessible in the correct reward arm. A performance criterion of 80% correct was applied, which, when reached, would indicate that the piglet had successfully acquired the task. Acquisition was followed by a reversal phase of testing, where the previously incorrect arm (e.g., east) was now rewarded. A video camera was mounted from the ceiling above the arena and used to record piglet movement within the maze. Piglet movement was tracked live by using commercially available software (EthoVision 3.1; Noldus Information Technology).

MRI

On PD28, piglets were transported to the Biomedical Imaging Center at the Beckman Institute and anesthetized by using a telazol + ketamine + xylazine solution [50.0 mg tiletamine plus 50.0 mg zolazepam reconstituted with 2.50 mL ketamine (100 g/L) and 2.50 mL xylazine (100 g/L; Fort Dodge Animal Health)]. An MRI-compatible pulse oximeter was used to monitor piglet vital signs throughout the scanning procedure. Observational records were taken every 15 min after an animal had been anesthetized and through the complete recovery period. After piglets had entered a sustainable plane of deep anesthesia, they were placed in the MRI machine. Piglets were restrained and remained immobilized throughout the MRI scan to prevent motion artifacts in the acquired images.

All MRIs were conducted by using a Siemens Magnetom Trio 3T imager and a 32-channel head coil (Siemens). For structural analyses (i.e., volume of discrete brain regions), anatomic images were acquired by using a 3-dimensional, T1-weighted, magnetization-prepared, rapid gradient-echo sequence with the following parameters: repetition time = 1900 ms, echo time = 2.49 ms, inversion time = 900 ms, flip angle = 9°, matrix = 256 × 256, and slice thickness = 0.7 mm. The final voxel size was 0.7 mm isotropic across the entire head from the tip of the snout to the cervical/thoracic spinal cord junction as described previously (22). All methods for brain region volume estimation and voxel-based morphometry (VBM) analysis can be found in reference 23.

Metabolomic analysis

After MRI procedures were complete, piglets fed the control diet ($n = 7$) and the 2.5% PL-20 diet ($n = 7$) were anesthetized by an intramuscular injection of telazol + ketamine + xylazine (4.4 mg/kg BW). A whole-blood sample was collected by cardiac puncture into evacuated tubes containing EDTA, and piglets were subsequently killed by intracardiac administration of sodium pentobarbital [390 g/L Fatal Plus (Vortech Pharmaceuticals); 1 mL/5 kg BW]; plasma was harvested through centrifugation and subsequently frozen at -80°C . Fresh tissue samples were excised from the left and right hippocampi and flash-frozen in liquid nitrogen. Plasma and hippocampal tissue samples were shipped on dry ice to an external laboratory (Metabolon) for global metabolomic profile analysis. Upon

⁸ Abbreviations used: BW, body weight; PD, postnatal day; PL-20, Lacprodan PL-20; VBM, voxel-based morphometry.

arrival, samples were extracted and prepared for analysis by using standard solvent extraction methods for plasma and hippocampal tissue samples.

The sample preparation process was carried out by using automated equipment (MicroLab STAR system; Hamilton Robotics). Recovery standards were added before the first step in the extraction process for quality control purposes. Sample preparation was conducted by using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into 2 fractions, one for analysis by LC and the other for analysis by GC. Organic solvents were removed from extracted samples (TurboVap; Zymark), and individual samples were subsequently frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS/MS platforms. Also included were several technical replicate samples created from a homogeneous pool containing a small amount of all study samples.

The LC/MS portion of the platform was based on an Acquity UltraPerformance Liquid Chromatography (Waters Corporation) and a Linear Trap Quadrupole mass spectrometer (Thermo-Finnigan), which consisted of an electrospray ionization source and linear ion-trap mass analyzer. The sample extract was split into 2 aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained ≥ 11 injection standards at fixed concentrations. One aliquot was analyzed by using acidic positive ion-optimized conditions and the other by using basic negative ion-optimized conditions in 2 independent injections with the use of separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted by using water and methanol, both containing 0.1% formic acid, whereas the basic extracts, which also used water/methanol, contained 6.5 mmol/L ammonium bicarbonate. The MS analysis alternated between MS and data-dependent tandem MS scans by using dynamic exclusion.

Samples destined for GC/MS analysis were redried under vacuum desiccation for a minimum of 24 h before being derivatized under dried nitrogen with the use of bistrimethyl-silyl-trifluoroacetamide. The GC column was 5% phenyl, and the temperature increase was from 40°C to 300°C in a 16-min period. Samples were analyzed on a fast-scanning single-quadrupole mass spectrometer (Trace DSQ; Thermo-Finnigan) by using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis.

Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. The combination of chromatographic properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity. Additional entities could be identified by virtue of their recurrent nature (both chromatographic and mass spectral).

Statistical analysis

Data analysis was conducted by using the MIXED procedure of SAS (version 9.3; SAS Institute) unless otherwise stated. BW gain and cognitive task data were analyzed by 2-factor (treatment $\times$ day) repeated-measures ANOVA. Brain weight and volume estimation data were analyzed by 1-factor (treatment) ANOVA with the use of the MIXED procedure of SAS. Post hoc paired contrasts were used to further examine treatment effects. Significance was accepted at $P < 0.05$. Values in text are means $\pm$ SEMs.

VBM. The statistical nonparametric methods toolbox was used for statistical analysis (24). This package uses nonparametric permutation and randomization tests that are beneficial when there are a small number of subjects per treatment. An ANCOVA was used for global normalization. No other covariates were used. Voxel-wise data were subjected to 1-factor ANOVA to reveal clusters of voxels that were affected by treatment. These areas were subjected to post hoc analysis (2-sample t tests). Pseudo t -statistic maps were generated by using an uncorrected $P < 0.01$. A threshold of at least 20 edge-connected voxels (clusters) was used.

Metabolomics. After log transformation and imputation with minimum observed values for each compound, a Welch's 2-sample t test was used to identify biochemicals that differed significantly between experimental groups (control vs. 2.5% PL-20) with the use of the open-source statistical program R (11). Differences between treatments were considered significant at the $P < 0.05$ level, and all comparisons were made relative to the control group. Assuming an α level of 0.05, the false discovery rate was estimated with q -values of 0.51 and 0.43 for plasma and hippocampal tissue matrices, respectively.

Results

Brain and BWs. At the beginning of the study the mean BW of piglets was 1.69 ± 0.38 kg. Throughout the study, BWs of control and PL-20-fed piglets (0.8% and 2.5%) were similar ($P = 0.60$; Figure 1A). At PD28, piglets were killed and brains were collected and weighed immediately after excision. Brain weight was affected by treatment ($P < 0.05$), with brain weights of PL-20-fed piglets (0.8% and 2.5%) being higher than those of control piglets ($P < 0.05$; Figure 1B). There was no difference in brain weights in piglets that were fed 0.8% or 2.5% PL-20.

Performance in the spatial T-maze task. Figure 2A, B shows the proportion of correct responses and latency to reward arm, respectively, on each day of the acquisition phase, whereas Figure 2C shows the average proportion of correct responses for the entire acquisition phase. The proportion of correct responses was affected by both day ($P < 0.001$) and diet ($P < 0.05$). As expected, on the first day of acquisition, piglets performed at near chance level. On ensuing days, however, piglets steadily improved, indicating that they learned to use the spatial cues to accurately locate the reward. Overall, compared with control piglets, PL-20-fed piglets, irrespective of supplementation amount, made fewer errors. Latency to reward arm was affected by day ($P < 0.001$), diet ($P < 0.05$), and a day $\times$ diet interaction ($P < 0.05$), with control piglets taking more time than PL-20-fed piglets (0.8% and 2.5%) on day 1 of cognitive testing (no difference between the PL-20 groups). All groups had similar latencies for the remainder of the task. In the reversal phase, there were no differences ($P = 0.67$) in maze performance between control and PL-20-fed piglets (data not shown). All piglets improved over time ($P < 0.001$), but no group reached the performance criterion in the 3-d period.

MRI. The estimated volume of each brain region for each treatment group is shown in Table 1. The estimated volume of

FIGURE 1 Body weight (A) and wet brain weight (B) of control ($n = 8$), 0.8% PL-20-fed ($n = 8$), and 2.5% PL-20-fed ($n = 7$) piglets. Values are least squares means $\pm$ SEMs. Means without a common letter differ, $P < 0.05$. PL-20, Lactrodan PL-20.

FIGURE 2 The proportion of correct responses (A) and latency to reward arm (B) for control ($n = 8$), 0.8% PL-20-fed ($n = 8$), and 2.5% PL-20-fed ($n = 7$) piglets on each day of acquisition in the spatial T-maze task and the average proportion of correct responses for the entire acquisition phase (C). Panel B: *Control piglets were significantly different from PL-20-fed piglets (0.8% and 2.5% PL-20, $P < 0.05$). Panel C: means without a common letter differ, $P < 0.05$. PL-20, Lacprodan PL-20.

every brain region investigated was similar for piglets that were fed 0.8% and 2.5% PL-20. However, compared with controls, piglets that were fed 0.8% PL-20 had a larger internal capsule ($P < 0.05$), putamen ($P < 0.05$), and thalamus ($P = 0.05$) and tended to have more gray matter ($P = 0.07$) and a larger third ventricle ($P = 0.08$).

In the VBM-Diffeomorphic Anatomical Registration using Exponentiated Lie Algebra (DARTEL) analyses (Supplemental Table 2), group comparisons showed that piglets that were fed 0.8% and 2.5% PL-20 had distinct brain areas with more gray and white matter. For example, Figure 3A, B highlights brain areas where diet affected the volume of gray matter and white matter, respectively. The colored scale near the figure helps to discern the intensity of the differences found, with areas labeled in red being more different than those labeled in blue. Supplemental Table 2 lists the coordinates and position of brain areas where gray matter and white matter were affected by diet, as well as the significant findings from all group comparisons.

Compared with control piglets, gray matter volume was increased in piglets that were fed 0.8% and 2.5% PL-20 in 4 discrete areas in the left and right cortex (Supplemental Figure 1A, C, E, G); white matter was increased in 4 brain areas that mapped to the left and right cortex and cerebellum (Supplemental Figure 1B, D, F, H). In addition, compared with piglets that were fed 0.8% PL-20, in piglets that were fed 2.5% PL-20, there were 2 areas with more gray matter that mapped to the right cortex and cerebellum and 2 areas with more white matter that mapped to the left cortex and cerebellum.

Metabolomic profiling of plasma and hippocampal tissue matrices. A total of 267 and 252 metabolites were identified in plasma and hippocampal tissue samples, respectively. Eighteen metabolites were significantly altered in plasma (4 increased, 14 decreased) and 25 metabolites were significantly altered in hippocampal tissue (22 increased, 3 decreased) due to the 2.5% PL-20 diet. In general, there were limited differences in plasma metabolites between experimental groups (Table 2), including circulating metabolites related to phospholipids and gangliosides (i.e., related to the experimental additive). The only plasma phospholipid affected by dietary treatment was 2-arachidonyl-glycerophosphoethanolamine, which was decreased ($P < 0.05$) in piglets that were fed 2.5% PL-20. As a class, all bile acids were lower in 2.5% PL-20-fed piglets relative to control-fed pigs, although none of these differences achieved significance. Piglets that were fed 2.5% PL-20 had increased ($P < 0.05$) concentrations of lactate and decreased ($P < 0.05$) concentrations of deoxycarnitine in both plasma and hippocampal tissue samples; these were the only metabolites affected in both sample matrices.

TABLE 1 Brain region volume of piglets fed a diet containing 0% (control), 0.8%, or 2.5% PL-20 from PD2 through PD28¹

	Volume, mm ³			
	Control	0.8% PL-20	2.5% PL-20	<i>P</i>
Whole brain	71,400 ± 1930	74,980 ± 1800	75,320 ± 2280	0.32
White matter	14,910 ± 400	14,800 ± 410	15,250 ± 400	0.72
Gray matter	34,000 ± 860	36,600 ± 640	34,970 ± 930	0.08
Caudate	550 ± 8	560 ± 14	570 ± 14	0.55
Cerebellum	4850 ± 145	5000 ± 75	4900 ± 126	0.66
Cerebral aqueduct	116 ± 2	116 ± 3	119 ± 2	0.75
Corpus callosum	1015 ± 26	1050 ± 30	1060 ± 48	0.64
Fourth ventricle	150 ± 7	156 ± 8	152 ± 5	0.80
Hypothalamus	546 ± 11	558 ± 11	562 ± 11	0.58
Internal capsule	4570 ± 64 ^b	4800 ± 55 ^a	4680 ± 46 ^{a,b}	0.02
Lateral ventricle	1020 ± 48	1010 ± 32	1020 ± 34	0.98
Left cortex	17,130 ± 373	18,270 ± 332	17,785 ± 430	0.11
Left hippocampus	583 ± 8	600 ± 15	580 ± 13	0.49
Medulla	2590 ± 54	2700 ± 32	2650 ± 60	0.29
Midbrain	3080 ± 25	3115 ± 33	3150 ± 40	0.30
Olfactory bulb	2920 ± 59	3040 ± 63	3040 ± 101	0.41
Pons	920 ± 15	900 ± 21	920 ± 15	0.72
Putamen	1130 ± 28 ^b	1240 ± 25 ^a	1200 ± 28 ^{a,b}	0.02
Right cortex	15,940 ± 365	16,890 ± 289	16,695 ± 396	0.14
Right hippocampus	638 ± 11	682 ± 17	654 ± 19	0.14
Thalamus	2990 ± 43 ^b	3115 ± 30 ^a	3070 ± 27 ^{a,b}	0.05
Third ventricle	192 ± 5	203 ± 5	217 ± 12	0.08

¹ Values are means ± SEMs; control ($n = 8$), 0.8% PL-20 ($n = 8$), and 2.5% PL-20 ($n = 7$). Labeled means in a row without a common letter differ, $P \leq 0.05$. PD, postnatal day; PL-20, Lacprodan PL-20.

FIGURE 3 Representative image of brain areas in which diet (control, 0.8% PL-20, or 2.5% PL-20) affected the volume of gray matter (A) and white matter (B) ($P < 0.01$). (+) indicates directionality (from inferior to superior) within each horizontal slice. Refer to Supplemental Table 2 for more in-depth information on affected areas. PL-20, Lacprodan PL-20.

Of the hippocampal metabolites (Table 3), all 13 lysophosphatidylcholine species measured were increased by an average of 1.6-fold in piglets that were fed 2.5% PL-20-supplemented diets than those fed the control diet. Although only 1-linoleoylglycerophosphocholine was significantly increased ($P < 0.05$) due to PL-20, 7 other species tended to be higher in 2.5% PL-20-fed piglets ($P < 0.10$). Moreover, 2 neuroactive metabolites, acetylcholine ($P = 0.05$) and agmatine ($P < 0.05$), were increased in the hippocampus of 2.5% PL-20-fed piglets relative to the control diet, although neither choline nor phosphocholine was affected by treatment.

Discussion

In the present study, PL-20 was added to piglet formula to increase dietary phospholipids and gangliosides, and outcomes related to brain development and learning and memory were evaluated. Brain weight was greater in piglets fed a diet supplemented with phospholipids and gangliosides. Moreover, VBM revealed multiple brain areas with greater volumes and more gray and white matter in piglets that were fed PL-20. These effects of PL-20 on brain structure were associated with neurochemical changes that were consistent with consuming a phospholipid-rich diet and improved performance in the spatial T-maze task. Thus, dietary phospholipids and gangliosides can affect brain growth, structure, chemistry, and spatial learning in neonatal piglets. How brain and cognitive development of PL-20 piglets compares with that of piglets consuming sow milk cannot be determined from the present study.

Several clinical trials demonstrated better cognitive development in breastfed infants compared with formula-fed infants (2–4), and breastfeeding has been associated with higher intelligence quotient (25). The enhanced cognition in breastfed groups may be due to nutrients and/or bioactive components present in breast milk, environmental factors that relate to breastfeeding, or, most likely, some combination of the diet and environment. The advantage of the present study is that environmental factors were controlled so that diet-specific effects could be studied. The control diet was designed to have minimal phospholipids and gangliosides present, whereas PL-20 was added to the formula to provide phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and phosphatidylserine (9), which naturally occur in both human and sow milk (12). The 0.8% PL-20 concentration was used to mimic breast milk, whereas the 2.5% PL-20 concentration was used to assess the positive or negative effects of providing gangliosides and phos-

pholipids at an amount well beyond what would normally occur. Both supplemented groups showed improvements in cognition when compared with the controls, but there appeared to be little benefit from including PL-20 at the higher amount. This same result was previously seen in mice that were supplemented with either 0.2% (low) or 1% (high) ganglioside/phospholipid-enriched milk (26). In tests measuring novelty recognition and spatial memory, groups that received the supplement scored higher than the controls (26).

In addition to effects on cognition, effects on brain weight were reported after supplementation with gangliosides or phospholipids. For example, maternal supplementation of gangliosides during pregnancy and lactation increased brain weight in rat pups (27). It has been hypothesized that exposure to gangliosides or sphingomyelin during development promotes myelination within the brain, and in addition to enhancing cognition, leads to higher brain weights (10). Park et al. (14) demonstrated that gangliosides and phospholipids added to the diet are readily absorbed and can be found in many tissues throughout the body, including brain.

Previous research using MRI to estimate brain growth in domestic piglets suggests that the piglet brain increases in volume by ~45% from birth to 4 wk of age (22). Thus, the provision of a diet supplemented with phospholipids and gangliosides in the present study occurred during a period of rapid brain growth and development. If gangliosides and phospholipids readily incorporate into developing brain, this could explain our finding that PL-20-fed piglets had a greater brain weight than did control piglets. The increased brain weight in PL-20-fed piglets suggests differences in the volume and/or composition of discrete brain regions.

Herein MRI was used to examine the effects of dietary phospholipids and gangliosides on brain region volumes and composition. The physical size of neonatal pigs allows for quantitative structural MRI with the use of normal clinical scanners (22, 28). Similarly to humans, there are some limitations in

TABLE 2 Changes in plasma metabolites of piglets fed diets containing 2.5% PL-20 relative to controls (0% PL-20) on PD28¹

Plasma metabolite	Fold-change ²	P
Stachydrine	0.57	<0.001
3-Dehydrocarnitine	0.75	0.01
1,5-Anhydroglucitol	0.74	0.01
Caprylate	1.21	0.01
γ-Glutamylisoleucine	1.43	0.02
Homostachydrine	0.81	0.02
N6-acetyllysine	0.81	0.02
Riboflavin	0.80	0.02
Cystine	0.46	0.02
Glycylproline	0.68	0.02
Deoxycarnitine	0.75	0.03
Iosine	1.65	0.03
Prohydroxyproline	0.75	0.03
Leucine	0.72	0.04
3-Hydroxyisobutyrate	0.72	0.04
2-Arachidonoylglycerophosphoethanolamine	0.70	0.04
Urea	0.74	0.05
Lactate	1.98	0.05

¹ PD, postnatal day; PL-20, Lacprodan PL-20.

² Fold-changes expressed as PL-20-supplemented piglets ($n = 7$) relative to control piglets ($n = 7$).

TABLE 3 Changes in hippocampal tissue metabolites of piglets fed diets containing 2.5% PL-20 relative to controls (0% PL-20) on PD28¹

Hippocampal metabolite	Fold-change ²	P
Hydroxyisovaleryl carnitine	0.72	0.001
Isoleucine	1.42	0.003
Alanine	1.22	0.003
Cytidine 5'-diphosphocholine	1.06	0.004
S-Methylglutathione	1.22	0.01
Glycerol 3-phosphate	1.32	0.01
Lactate	1.11	0.01
Deoxycarnitine	0.85	0.02
Agmatine	1.35	0.03
Inositol 1-phosphate	1.13	0.03
6-Oxopiperidine-2-carboxylic acid	1.34	0.03
S-Methylcysteine	1.20	0.03
2-Aminoheptanoic acid	1.68	0.03
Serine	1.13	0.03
Cysteinylglycine	1.19	0.03
Guanosine 5'-diphosphofucose	1.10	0.03
Stearoylcarnitine	1.38	0.03
Desmosterol	1.20	0.03
Malate	1.15	0.04
Erythritol	1.19	0.04
Oleate	1.16	0.04
Glycerol 2-phosphate	1.35	0.04
Pyroglutamine	0.85	0.05
1-Linoleoylglycerophosphocholine	1.73	0.05
Guanosine 5'-monophosphate	1.87	0.05

¹ PD, postnatal day; PL-20, Lacprodan PL-20.

² Fold-changes expressed as PL-20-supplemented piglets (*n* = 7) relative to control piglets (*n* = 7).

using MRI on a developing brain, compared with its use in an adult or older child. Neonatal brain tissue can have within-tissue variability and contrast issues that may make it challenging to discern white and gray matter areas. However, some of these complications can be mitigated by using different segmentation algorithms and by using an atlas specifically designed for our research subjects (22, 29). By using an MRI brain atlas designed for domestic neonatal piglets (22), we determined the volume of 19 discrete brain regions and made voxel-wise comparisons to identify clusters of voxels in PL-20-fed piglets with more or less gray and white matter than control piglets. This approach was recently used in a study on brain development in small-for-gestational-age piglets—a model of intrauterine growth restriction (23). The results revealed in small-for-gestational-age piglets multiple brain areas with reduced white matter than littermate piglets with an average birth weight. In the present study, the volume of several noteworthy brain regions was increased in PL-20-fed piglets, including the putamen and internal capsule. As one of the structures that make up the basal ganglia, the putamen is associated with voluntary motor and procedural learning related to routine behaviors including cognition. The internal capsule is a white matter tract with a large number of motor and sensory fibers that travel to and from the cortex. The VBM-DARTEL analysis corrects for volume and allows for group comparisons to identify differences in the concentration of gray and white matter. This analysis revealed that piglets that were fed 2.5% PL-20 had more gray matter in several brain areas than did piglets fed 0.8% PL-20, including the right insular cortex, which,

like the basal ganglia, is used in motor control in addition to language and homeostatic regulation (30).

Metabolite concentration differences were also found between the 2.5% PL-20-fed piglets and controls. Lactate is an important source of fuel within the brain (31, 32). Glucose is used by astrocytes, which, in turn, produce lactate that is then used by neurons as an important source of fuel (33). In vitro work demonstrates that some cell types preferentially use lactate even in the presence of glucose (34). Increased concentrations of lactate in the brains of 2.5% PL-20-fed piglets could suggest greater energy availability. Lactate is also found as part of the group of metabolites that are connected to glutathione metabolism (cysteine, Cys-Gly, lactate, S-methylcysteine, S-methylglutathione, and S-lactoylglutathione), which were elevated in the 2.5% PL-20-fed piglet hippocampi. It is possible that a general increase in these glutathione pathway metabolites could reflect an improved capacity to respond to oxidative stress. Deoxycarnitine is an intermediate from the breakdown of lysine residues (35). A decrease in deoxycarnitine could reflect a lower rate of protein turnover in the 2.5% PL-20-fed piglets, suggesting a more favorable nutritional status within this group (36). An increase in nucleotide monophosphates (adenosine 2'-monophosphate, adenosine 3' monophosphate, guanosine 5'-monophosphate, and cytosine-2', 3'-cyclic monophosphate) may be consistent with an increase in phosphodiesterase activity. Phosphodiesterases are molecules used in signal transduction and cAMP signaling and are involved in many aspects of cell function (37, 38). Lysophosphatidylcholine and its related species are derived from phosphatidylcholine (39). The increase in the 2.5% PL-20-fed group may be consistent with greater availability of phosphatidylcholine in the diet. Changes in lysophosphatidyl lipids may reflect increased membrane remodeling (9), which is, presumably, active in the developing brain. In addition, a number of other metabolites associated with phospholipid biosynthesis or degradation were also increased in the hippocampus of the 2.5% PL-20-fed group. Cytidine diphosphate-choline, which serves as a substrate in the synthesis of phosphatidylcholine (40), was increased in 2.5% PL-20-fed piglets. This increase in cytidine diphosphate-choline may be a result of increased dietary availability. Increased concentrations of lysophosphatidylcholine degradation products may be consistent with increased phosphatidylcholine availability and membrane remodeling as a result of the PL-20 supplementation. Phosphatidylethanolamine is also a major component of the PL-20 supplement, and some lysophosphatidylethanolamine concentrations in the hippocampi of 2.5% PL-20-fed piglets were elevated compared with control piglets. However, of the 11 lysophosphatidylethanolamine species measured, none achieved significance. Although ganglioside content of the brain was not directly measured, the increase in phosphatidylcholine-related metabolites may indicate that the PL-20 supplement is taken up by the brain. This finding supports the argument that the supplement may be affecting brain composition and structure. Last, acetylcholine was found at higher concentrations in the hippocampi of 2.5% PL-20-treated piglets. However, choline and phosphocholine concentrations were not significantly elevated in the 2.5% PL-20-fed group relative to controls. Increased amounts of acetylcholine, a neurotransmitter, have been linked to improved memory in mice (41) and may explain the differences we see in the learning and memory ability of the PL-20-fed piglets when compared with control piglets.

In conclusion, the present study shows the effects of supplementation of phospholipids and gangliosides on brain and cognitive development in neonatal piglets. The results suggest that the addition of phospholipids and gangliosides to

infant formula may affect brain development and enhance some aspects of cognition in human infants. Because closing the gap in learning differences between breastfed and formula-fed infants is important, further research is warranted.

Acknowledgments

RWJ designed the research; HL conducted the research; MSC, YL, and RND provided essential materials; HL and ECR analyzed data; HL, ECR, and RWJ wrote the manuscript; and RWJ had final responsibility for content. All authors read and approved the final manuscript.

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