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A mouse model and ^{19}F NMR approach to investigate the effects of sialic acid supplementation on cognitive development

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Researchers have observed that a sialic acid (Sia)-supplemented neonatal diet leads to improved cognition in weanling piglets. However, whether cognitive improvement appears with different physiological backgrounds and persists into adulthood is not known. Here, we have established a convenient mouse model and used an ^{19}F NMR approach to address these questions, test the conditionally essential nutrient hypothesis about Sia supplementation, and assess the prospect of measuring Sia metabolism directly *in vivo*. Indeed, the neonatal mouse brain uptakes more Sia than the adult brain, and Sia supplementation of neonatal mice improves the cognitive performance of adult mice. The non-invasive ^{19}F NMR approach and viable mouse model opens unique opportunities for clarifying the interplay of nutritional supplementation, metabolism, and cognitive development.

Keywords: bioenergetics; brain; cognition; metabolism; NMR; nutrition

Poor human nutrition from conception to 24 months after birth can impair development and leave a lasting scar into adulthood. In fact, it poses a marked risk in cognitive development, since brain growth during the neonatal period exceeds all other organs or body tissues [1]. However, when nutritional intervention focuses on just correcting caloric insufficiency, it overlooks specific precursors promoting cognitive development. Indeed, newborn piglets receiving casein glycomacropeptide (CGMP) supplement learn markedly better than the control group, even though both groups receive an equivalent caloric diet [2,3]. Similarly, supplementing the 2–6 months old formula-fed infants with bovine milk fat globule membrane (MFGM) narrows the cognitive development gap between breast milk-fed and formula-fed infants [4]. Breast milk-fed infants display a better cognitive performance than formula-fed infants. Because both CGMP and MFGM contain a considerable fraction of sialic acid (Sia), researchers have hypothesized Sia or interchangeably termed neuraminic acid (Neu), a 9-carbon sugar acid as the causative agent.

Positing a role for Sia in cognitive development appears consistent with its major presence in brain [5]. Over 80% of gangliosides, a class of complex sphingolipids constituting ~ 10% of all brain lipids, contains conjugated Sia [6]. NCAMs (neural cell adhesion molecules) contain polysialic acid (PSia) comprising of 50–400 Sia units. Both gangliosides and PSia-NCAM influence cell-cell adhesion and migration, axon path finding, synaptic connectivity, neural plasticity, and cell signalling. As such, Sia associates closely with cognitive and memory development [2].

Abbreviations
CGMP, casein glycomacropeptide; MFGM, milk fat globule membrane; Neu, neuraminic acid; PSia, polysialic acid; Sia, sialic acid; TFA, trifluoroacetic acid.

Nevertheless, how Sia promotes cognitive development still poses unsettled questions. Incongruent with a key molecule targeting the brain, over 90% of added Sia gets rapidly excreted into the urine of adult rats [7–9]. In contrast, neonatal rat brain uptakes more Sia than adult brain [10]. Such age-dependent difference in Sia metabolism has spawned the supposition that Sia derived from the mother's milk or from supplementation serves as a conditionally essential nutrient to compensate for an incompletely developed *de novo* Sia biosynthesis pathway [11].

Even though experiments with a piglet model have established that Sia improves learning at weaning, the long-term effect remains uncertain. Pigs grow rapidly from 2 kg at birth to about 4 kg at weaning (21 days) and reaches 130 kg as an adult. Assessing any lasting cognitive improvement in mature pigs, which have received Sia supplementation during the neonatal period, would challenge any research design and demand extensive, unique resources. Still, whether cognitive enhancement observed at weaning persists into adulthood poses a critical question. Moreover, the interplay of diverse genetic background on the capacity of Sia role to promote cognitive development remains undetermined. Using a mouse model and labelling Sia with stable isotopes would open opportunities to investigate the biochemical mechanism underlying Sia's role in cognitive development throughout the lifetime of the animal and to identify any genetic contribution.

The present study has examined the feasibility of using a mouse model and ^{19}F Sia. Instead of using radioisotopic ^{3}H or ^{14}C labelled Sia to track Sia biodistribution and metabolism, the experiments have synthesized and have utilized ^{19}F Sia. Indeed, neonate mice uptake more Sia than adult mice. Moreover, feeding Sia to mouse pups from postnatal day 1 (P1) to P14 improves the cognitive performance in corresponding adult mice at P60. Sia supplementation during the neonatal period has then a lasting effect on the cognitive performance in adulthood. The results confirm the utility of using an ^{19}F labelled Sia in animal studies and suggest the feasibility for future human studies. A mouse model can then track the impact of Sia supplementation during the neonatal period on cognitive performance at different stages of growth and development [12].

Materials and methods

Sia supplementation

All animal procedures followed the UC Davis IACUC approved guidelines. Newborn C57/BL6 mice (Charles River) received Sia (Carboynth, powder, >98%) twice a day (total of 240 mg·kg^{-1}·day^{-1}) for 14 days (from P1 to P14) orally administered as solution droplets (10 μL). Control mice received an equal volume of water. Each group had 9 pups. To avoid any sex-related bias on cognitive development test, an equal number of male and female pups in each cage were fed either the Sia solution or water. After weaning, the mice were separated into male and female colonies, lived under identical environment, and were fed *ad libitum* on the same chow until they were tested at 60 days of age (P60). The dams and the weaned mice ate the same standard chow (Newco, Rancho Cucamonga), which comprised of LabDiet constituents: https://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028 021.pdf. The standard rodent chow did not contain any Sia.

Cognitive measurements

A wire grasp test compared neurodevelopment of Sia-fed and control mice at P13 [13]. At P60, a combined water and radial arm maze test, conducted at UC Davis Mouse Biology Program, measured the ability of the control and Sia-fed mice to learn and to recall the location of an escape platform in daily sessions of five trials over a 6-day test period [14].

The maze comprised of six arms; 77.47 cm (30.5 in) diameter, 20.32 cm (8 in) high, 25.4 cm (10 in) long, 14.29 cm (5-5/8 in) wide) in a swimming pool maintained at 21 °C. All the arms opened into a middle free area of 27.94 cm (11in) diameter. A 6.35 cm (2.5 in) escape platform stood 1.0 cm (0.39 in) below the water surface in a designated arm. Tempura paint added to the water obscured the platform, so that mice must learn from room cues (room furniture, poster, ceiling mounted cameras and the investigator) the location of the escape platform, once they got released from one of the maze arms. In daily sessions of five trials per day over a 6-day period, the tester released the mice in a prescribed sequence that randomized the release and escape platform arms' spatial relationship. The time required for a mouse to find the escape platform constituted the latency. In each test day, the tester started with first four consecutive trials with only an inter-trial interval of 30s. The procedure familiarized the mouse to different cues. The fifth trial, 30–40 min after the fourth trial, measured the animal's ability to learn how to use different cues to find the escape platform. Over 6 days, the tester repeated the same protocol and recorded the latency and any errors. The analysis used the scored errors (e.g. animal did not leave the start arm or clung to the maze) as a factor. The latency over advancing test days reflected an index of learning.

Sia biodistribution

For the Sia biodistribution experiments with P6-P60 mice, pH adjusted ^{19}F-Sia dissolved in isotonic saline was administered via intraperitoneal (IP) injection. P6, P17 and P60 mice received IP injections of ^{19}F-Sia. The volume of 5-N-TFA-Neu solution was adjusted to achieve an expected Sia

concentration of ~ 20 mM in the blood, given the mouse blood volume of ~ 7 mL/100 g body wt. Ninety min after the injection of ^{19}F-sialic acid, the mice were anesthetized with 75 mg·kg^{-1} ketamine, and blood samples were collected via transthoracic cardiac puncture into the heparinized tubes, which were immediately centrifuged to obtain plasma samples. Brain, kidney, and urine samples were subsequently obtained.

For P1 mice, whose skulls had not hardened sufficiently, Sia was injected directly into the brain lateral ventricles using a 5 μL Hamilton syringe with a 33-gauge needle to achieve ~ 5 mM ^{19}F-Sia concentration, derived from an estimate of the total brain volume. The injection site was first cleaned by ethanol wipes. Ninety min after injection, the brains were excised and frozen immediately [15,16].

All tissue samples were snap-frozen immediately in liquid nitrogen after dissection and stored at −80 °C until use [17]. For analysis, the tissue was homogenized in phosphate buffered saline (PBS) with a Bullet Blender homogenizer (Next Advance, Troy, NY, USA) and centrifuged at 3000 *g*. All steps were carried out at 4 °C. The supernatant was then collected for ^{19}F NMR analysis. No ^{19}F NMR signals were detected from re-suspended pellets. Some 5-N-TFA-Neu stored at −80 °C cleaved to form free TFA in solution. Since endogenous tissue contains no detectable trace of ^{19}F molecules, the analysis considered the integrated ^{19}F signal of Sia and TFA to represent the total Sia concentration.

NMR spectroscopy

The NMR analysis was performed on a Bruker Avance 500 NMR spectrometer equipped with a broadband ^{1}H-X probe. The X broadband coil measured the ^{19}F signal. ^{19}F acquisitions employed a 90° pulse and WALTZ proton decoupling sequence. A typical signal acquisition used a 120 ppm spectral width and 64K data points. The free induction decays (FID) were zero-filled and multiplied by an exponential window function.

D$_2$O was added to all samples to create a 10% D$_2$O solution, which enabled the deuterium lock. All ^{19}F NMR determination started with the measurement a standard sample containing 1 mM trifluoroacetic acid (TFA) and 5-N-TFA-Neu. The TFA signal was referenced to −75.89 ppm and the 5-N-TFA-Neu signal was detected at 0.13 ppm down-field from TFA at −75.76 ppm. The acquisition and processing parameters were saved in references files and used for subsequent experiments for that day.

Measurements of tissue samples then began using the acquisition, chemical shift calibration, and processing parameters established for the external standard. The number of scans achieving adequate signal to noise was as follows: external standard, 16; urine, 128; kidney, 2048; and brain, 24 192. Upon completing NMR measurement on the tissue sample, another experiment re-measured external standard containing ^{19}F Sia and TFA to verify that the spectral calibration had not changed.

The concentration of 5-N-TFA-Neu in the tissue sample was calculated by integrating the Sia peak in the tissue sample and by referencing to the external standard signal from 1 mM 5-N-TFA-Neu. Over the course of the experiments, the integration values of external standard signals showed no variation. Correcting for the overall tissue weight and sample dilution factors led to an estimate of the tissue concentration of 5-N-TFA-Neu.

Statistical analysis

Data are presented as means ± standard error (SEM). Statistical significance was determined by the standard Student's *t* test ($P < 0.05$).

Results

Sia supplementation does not alter the growth rate of the mouse pups. Both the control and Sia-fed mouse pups show the same postnatal growth rate as reflected in the identical body weight gain of 0.32 g·day^{-1} over 15 days, Fig. 1. At P60, the control and Sia-fed mice have also indistinguishable body weights (Control male, 24.5 ± 0.4 g; Sia fed male, 23.9 ± 0.3 g; control female, 20.0 ± 0.4 g; Sia-fed female, 19.7 ± 0.4 g). Similarly, the tail lengths show no significant difference (Control male, 80.8 ± 0.5 mm; Sia fed male, 80.5 ± 0.4 mm; control female, 79.6 ± 0.2 mm; Sia-fed female, 79.5 ± 0.5 mm).
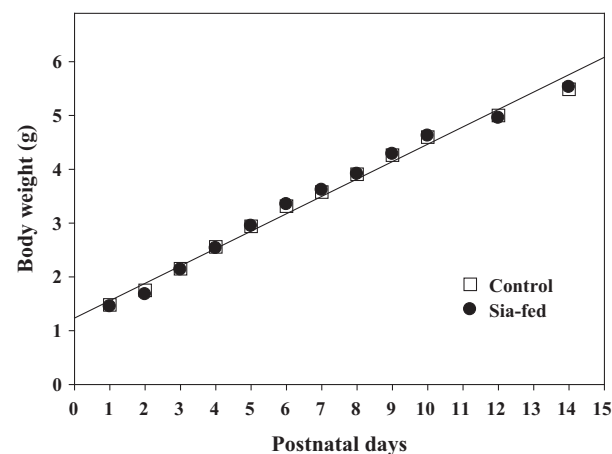

Fig. 1. Postnatal weight gain of mice with and without Sia supplementation. Mean body weights of the Sia-fed and control mouse pups were obtained from the total weight of each group at a given postnatal day divided by the number of pups in that group. Sia-fed and control pups have identical growth rates of 0.32 g·day^{-1}.

Figure 2A shows the ^{19}F spectra of a standard solution containing 5-N-TFA-Neu (5-N-TFA-Sia) and TFA. The 5-N-TFA-Neu signal appears at −75.76 ppm, while the TFA peak resonates at −75.88 ppm. The very small peak at −75.81 ppm has no assignment and arises most likely from a synthesis contaminant. The spectra from urine samples obtained from P60 animals, receiving IP injection of 5-N-TFA-Neu, reveal the major Sia peak at −75.76 ppm and the contaminant peak at −75.81 ppm, Fig. 2B. Unlike the standard solution, which contains both F-Sia and TFA, the tissue samples for the NMR measurement does not contain any TFA to avoid spectral overlap with any endogenously produced TFA. Spectral calibration relies on signals obtained from an external reference containing 5N-TFA-Neu and TFA.

Figure 3 shows the ^{19}F spectra from tissue extracts of kidney and brain obtained from P60 animals 90 min after IP injection of 5-N-TFA-Neu. Relative to a 40× expanded vertical scaling of the kidney spectrum, the brain spectrum shows only a very small signal from 5-N-TFA-Neu. The brain uptakes much less Sia than the kidney. Two small unassigned peaks appear at −75.0 and −75.2 ppm in the kidney spectrum. Indeed, Sia distributes predominantly to kidney and urine in P60 mice, Table 1. The kidney/brain ratio of Sia increases from 10.5 to 24 between P6 and P60.

Urine contains 27 700 times more Sia than brain in P60 mice.

The neonatal brain, however, takes up much more Sia than adult brain. With direct injection of 5-N-TFA-Neu injection into P1 brain, the analysis shows that brain takes up 0.97 mM of Sia. Figure 4 shows the brain only uptakes 0.16 mM Sia in P6 mice after IP injection of 5-N-TFA Neu. At P17 and P60, the brain uptakes an almost undetectable amount of Sia. Sia uptake in kidney also declines with age. The amount of Sia found in kidney falls from 2.54 mM at P6 to 0.24 mM at P60, Table 1.

Figure 5 shows the cognitive performance of the control and Sia-fed mice at P60. The latency measures the time the mouse requires to reach the escape platform. A shorter latency time indicates a better performance in the radial arm water maze test. As adult mice (P60), mice fed Sia from P1 to P14 exhibit a significantly better cognitive performance than the control group. At day 1, the latency time of control mice (38.3s) and Sia-fed mice (28.8s) differs by 9.5s. By day 6, the latency difference widens between control (31.2s) and Sia-fed mice (20.1s) to 11.1s. Relative to day 1, the latency in the control group has decreased by 19%. In contrast the latency in the Sia fed group has decreased by 30%. The linear regression analysis of latency vs days reveals $Y = -1.4X + 39.8$ ($r = 0.69$)
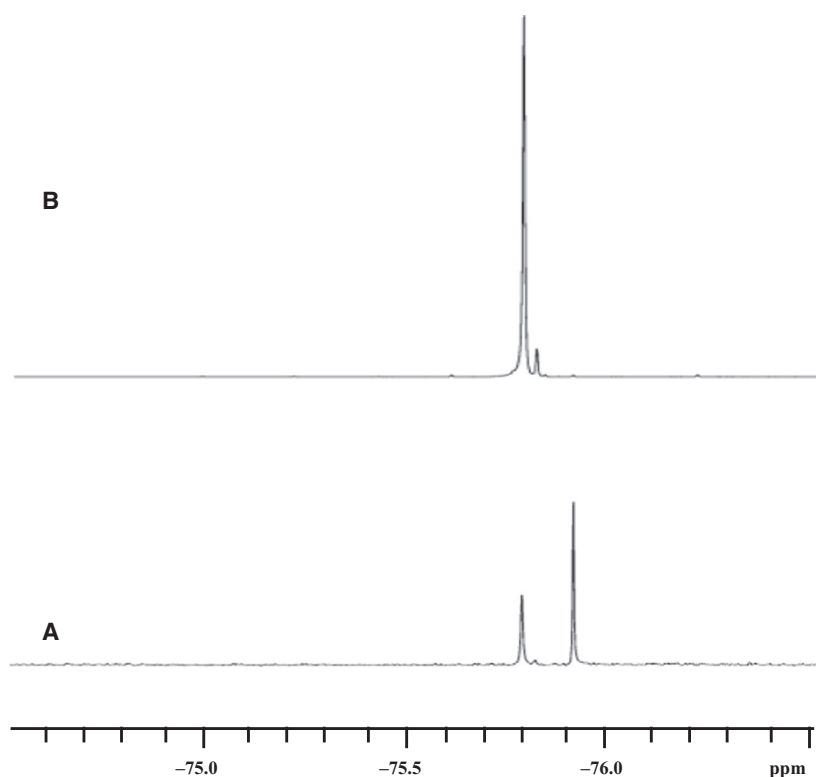

Fig. 2. ^{19}F spectra of (A) standard solution containing 1 mM 5-N-TFA-Neu and 2 mM trifluoroacetate (TFA) NS = 16 and (B) urine from animals 90 min after the addition of 5-N-TFA-Neu. The spectra display the dominant 5-N-TFA-Neu peak at −75.76 ppm, while the TFA signal appears at −75.88 ppm. NS = 128. A small peak at −75.81 originates from an unidentified contaminant observed in the reference spectrum of 5-N-TFA-Neu. The spectra are not normalized. NS, number of scans.
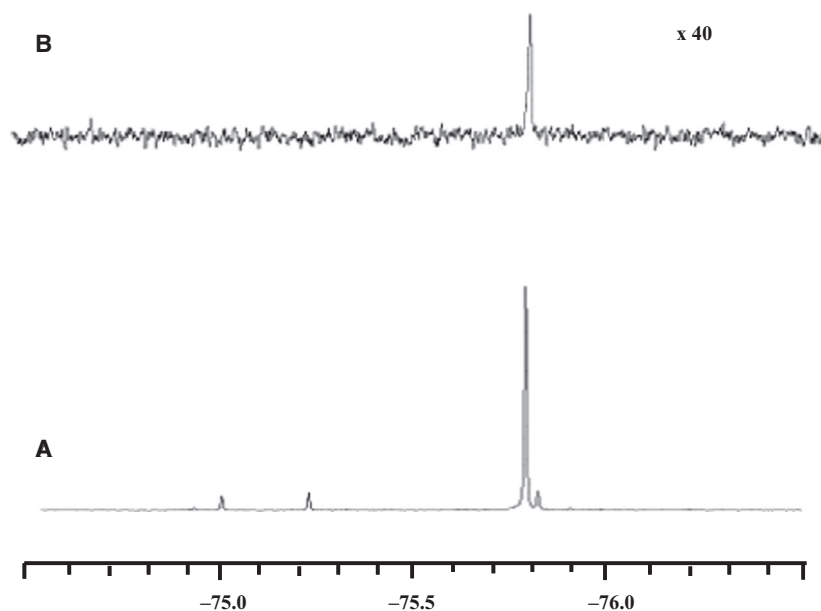
Fig. 3. ^{19}F spectra of tissue extracts and blood plasma obtained from animals 90 min after the addition of 5-N-TFA-Neu: (A) Kidney NS = 2048 (B) Brain NS = 24 192. Kidney spectra show an intense signal of 5-N-TFA-Neu peak at −75.76 ppm. The brain spectra (40× the vertical scale of the kidney spectra) show a less intense signal of Sia. The spectra are not normalized. NS, number of scans.

Table 1. Tissue concentration of F-Sia (mM).

	P1[b]	P6	P17	P60
Brain	0.97 ± 0.10	0.16 ± 0.03	0.02 ± 0.004	0.01 ± 0.01[b]
Kidney		2.54 ± 0.46	0.21 ± 0.06	0.24 ± 0.08
Kidney/ Brain[a]		15.9	10.5	24.0
Urine				277 ± 35

[a]Kidney/brain ratios obtained from a previous report on P3 rats given oral dosage: ^{14}C Sia, 1.18 and ^{14}C Sia-lactose, 0.88 [9]. P3 piglets given ^{14}C Sia yield a ratio of 7.3 [8]. Estimated kidney/brain ratios from P20 mice given oral dosage: ^{14}C Sia, 0.75-1 and IV/IP ^{14}C, 5.13-12.33 [7]. [b]P1 ($n = 7$), P6 ($n = 5$), P17 ($n = 5$), P60 ($n = 6$).

Fig. 4. Brain incorporation of Sia was significantly higher at P6 and P17, compared with adult brain.

for the control group and $Y = -1.7X + 30.5$ ($r = 0.63$) for the Sia fed group. $Y =$ latency; $X =$ days. The regression analysis confirms that Sia fed mice has steeper decline in latency over successive days relative to the control mice.

Discussion

Sialic acid

During the neonatal period, an incompletely developed *de novo* Sia biosynthesis can lead to Sia deficiency, which in turn reduces the conjugated Sia precursor pool. A decreased level of conjugated Sia alters the formation of properly conjugated NCAM and gangliosides required for cognitive and memory development [18,19]. Fr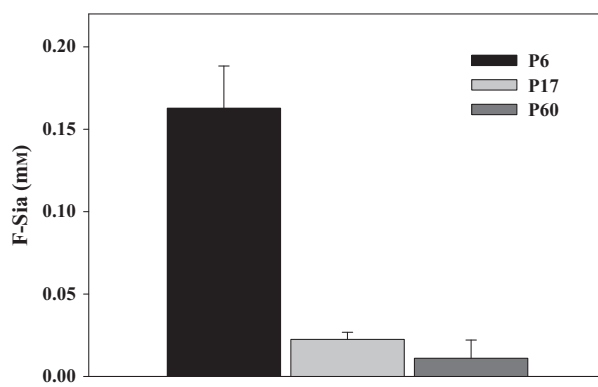om this vantage, Sia supplementation during the neonatal period offsets a precursor deficiency by providing a conditionally essential nutrient.

Given that bacterial sialidase or neuraminidase in the gut must cleave the Sia conjugate and release free Sia into the blood stream, supplementing the diet with just free Sia could potentially bypass the sialidase step and provide directly Sia to the brain for gangliosides and NCAM formation, which facilitates proper neural, cognitive, and memory development [2,5,6,20].

In addition to serving as a precursor, exogenous Sia can also trigger an upregulation of gene expression coding for key regulatory enzymes in the Sia biosynthetic pathway: GNE (N-acetylglucosamine (GlcNac)-2-epimerase)/N-acetylmannosamine (ManNac) kinase, a bifunctional enzyme regulating cellular Sia formation rate and polysialyltransferases (PSiaT), an enzyme
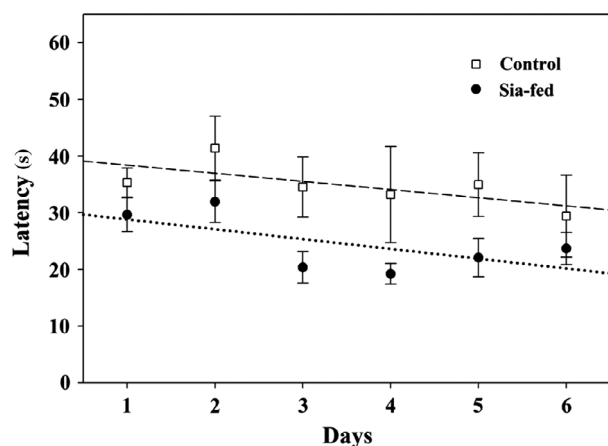
Fig. 5. The latency in the radial arm water maze test shows the Sia-fed mice display a better cognitive ability than control mice at P60. Sia-fed mice exhibit a lower latency in learning the maze from day 1. At day 1, the latency time of control mice (38.3s) and Sia-fed mice (28.8s) differs by 9.5s. At day 6, the latency difference widens between control (31.2s) and Sia-fed mice (20.1s) to 11.1s. Relative to day 1, the latency in the control group has decreased by 19%, while the regression line for the plots of latency vs. days yield the following: control: $Y = -1.7X + 30.5$ ($r = 0.63$). Sia fed: $Y = -1.4X + 39.8$ ($r = 0.69$). Y = latency and X = days.

regulating the synthesis of the polySia glycotope on the NCAM [5,21]. Sia supplementation will compensate any Sia deficit and stimulate key enzymatic activities to increase the flow of Sia toward molecular conjugation.

Nevertheless, adult mice (P60) receiving IP injection of Sia excrete over 90% into urine [7–9]. However, P1 mice uptake 100 times more Sia to brain than adult P60 mice [10]. With IP injection of Sia, P6 brain uptakes 16 times more Sia than P60 brain. At P17 corresponding to the time of weaning, brain uptake of Sia has already dropped by a factor of 8. Sia retention by kidney also shows an age-dependent response. From P6 to P17, Sia level in kidney drops almost 10 times from 2.5 to 0.2 mM. At P60, kidney Sia level remains at 0.2 mM. The age-dependent difference in Sia biodistribution followed by ^{19}F precursor method thus supports the notion that Sia serves as a conditionally essential nutrient in neonates [11].

Because intraventricular and ip injection will introduce Sia to the brain in distinct ways, an ambiguity exists in interpreting the Sia uptake in P1 vs P6-P17 animals. Unfortunately, the thin tissue surrounding the abdomen of P1 mice and the hardened skull of P6-P17 mice preclude the use of a consistent intraventricular or ip injection approach. Future experiments must clarify the Sia uptake in P1-P5 mice relative to P6-P17 brain to establish a better age-dependent Sia metabolism profile.

^{19}F NMR approach

The ^{19}F-labelled Sia precursor provides an alternative approach to investigate Sia biodistribution and metabolism. ^{19}F has almost 100% natural abundance, a nuclear spin of ½ nuclear, and 94% of the ^{1}H sensitivity. The body contains < 0.01% endogenous ^{19}F molecules. Because tissue does not contain any significant ^{19}F molecules, no background signal will interfere with the detection of the biodistribution/metabolism of the ^{19}F precursor.

The stable ^{19}F isotope contrasts sharply with the standard tracer methodology using radioactive ^{14}C or ^{3}H. ^{19}F nuclei also differ from the synthesized ^{18}F nuclei used in positron emission tomography (PET) experiments, which have only a 110 min half-life [22]. Using ^{19}F NMR overcomes the low signal sensitivity, broad lines, and spectral background interference in the conventional ^{1}H and ^{13}C methodological approaches. Indeed, researchers have used ^{19}F NMR to measure cellular oxygenation, pH, and ion status *in vivo* [23].

Employing ^{19}F labelled precursors, however, must assess the impact of substituting the electronegative ^{19}F for ^{1}H, since the substitution can affect the enzyme activity, which would confound any interpretation. However, substituting an ^{19}F in the 5 position of the molecule 5-N-TFA-Neu, where the ^{19}F substitutes for the 3 protons in the CH_3 of the N-acetyl group bound to carbon 5 (C5) of Neu, literature studies have detected no change in key enzyme activity in the Sia metabolism pathway. A trifluoroacetyl (TFA) group attached to the 5 position of Sia (5-N-TFA-Neu) does not alter any biochemical function. In fact, modifying the C5 position (5-N-TFA) of Neu has become a standard methodology to synthesize unusual Sia molecules to monitor enzyme or cellular activity [12,24,25]. The unique features of the Sia molecule and the Sia metabolism pathway permit the use of the ^{19}F Sia analogue to probe the underlying mechanism. Experiments can then utilize the ^{19}F NMR approach to monitor both the acute and chronic impact of Sia supplementation in tissue extract and potentially *in vivo* [26,27].

Conditionally essential nutrient

Liver has the capacity to synthesize Sia *de novo* from simple sugar precursors. Such capacity would raise the question about the need for any exogenous Sia. However, newborn infants have immature livers, and the *de novo* biosynthesis of Sia during early development may not meet the demand of the rapidly growing brain [11].

The abundance of Sia conjugated proteins and oligosaccharides in mother's milk supports the

supposition that Sia serves as a key precursor for cognitive development [2,3]. Of the 200 identified human milk oligosaccharides (HMOs), over 50% contains conjugated Sia. Sia-oligosaccharides account for 0.1% (wt/vol) of breast-fed infant diet in the first 2 weeks of life. Even though Sia content declines rapidly, it continues to represent a significant component in human milk even at the 7th month of lactation [28].

Because of the presence of Sia in the neonatal diet, studies have drawn attention to the low amount of Sia in infant formulas relative to the high amount of Sia in breast milk. Breast milk contains much more Sia than infant formulas [29]. Because breast milk-fed infants perform much better in cognitive test than formula-fed infants, researchers have posited a role for Sia. The narrowing of the cognitive gap between bovine milk fat globule membrane (MFGM) supplemented infants, which also contains a rich source of Sia, and formula-fed infants supports the Sia hypothesis [4]. Consequently, many nutritionists have recommended Sia supplementation during the neonatal period to offset potential deficiency from an immature *de novo* Sia biosynthesis [30].

Consistent with Sia as a conditionally essential nutrient, the present study shows neonate brain uptakes Sia much more than adult brain [3,11]. P1 brain uptakes the most Sia. Even though less than the P1 brain, P6 brain still uptakes Sia. However, by P17, brain uptake of Sia reduces dramatically. That low level of Sia uptake persists in the P60 mice. From P17 to P60, experiments can hardly detect any significant brain uptake of Sia. The observation agrees with an age-dependent Sia biodistribution and metabolism.

Sia supplementation

Since conjugated Sia in the mother's milk still requires bacterial sialidase (neuraminidase) action in the gut to release the free Sia into the blood stream, supplementing with just free Sia could potentially facilitate distribution Sia to various tissues [20]. Cells can certainly uptake free Sia and incorporate into gangliosides and glycoproteins [31–34]. Sia can cross the blood-brain barrier and readily reach the brain [8]. Studies have shown that administering directly free Sia into the animal will enhance the biodistribution to brain [7–9]. In fact, P15 to P21 rodents receiving Sia supplementation increase significantly the amount of sialylated gangliosides [30].

The amount of Sia biodistributed to brain, however, depends upon age. In adult mice, the present study's results agree with literature reports and shows that the IP injected 5-N-TFA-Neu distributes predominantly to urine [7,9]. Brain uptakes only a very small amount. In contrast, neonatal brain (P6) takes up about 16 times more Sia than adult brain. P1 brain uptakes 27 000 times more Sia than adult brain. The age-dependent uptake of Sia supports the essentially conditional nutrient hypothesis.

Sia supplementation and cognitive performance

Because neonates have the highest demand for exogenous Sia, introducing Sia to rat or mouse weanlings would miss a critical development time point and underestimate the importance of dietary Sia. Sia feeding does not alter any energy balance or nutritional supply and demand. Sia supplementation does not add or subtract from a mouse's caloric requirement. Control and Sia-fed mice display the same growth rate. Both the control and Sia supplemented mice show the same growth rate and have the same body weight gain and tail length growth (tail length data not shown). A wire grasp test at P13 cannot detect any neurodevelopment difference (data not shown) [13].

However, mice supplemented with Sia from P1 to P14 perform much better as adults (P60) in the radial arm water maze test than the control animals. On test day 1, Sia-fed mice (P60) already show a latency 9.5s faster than the control mice (P60). On test day 6, the latency of the Sia-fed mice has widened to 11.1s. Sia fed mice display a 21% faster rate of change in latency over the cognitive test period than the control mice. In summary, Sia fed mice already perform better than control mice on the first day and learn faster over 6 days.

The observation agrees with the test results of neonatal piglets receiving from P3 to P21 casein glycomacropeptide (CGMP) containing 60 mg Sia/g. CGMP supplemented piglets at P21 exhibit much better cognitive performance in an 8-arm radial maze than the control piglets [8]. However, no reports have conducted follow-up tests to determine if the piglet's cognitive enhancement at P21 persists into adulthood. The present study shows that Sia supplementation during their neonatal period can impact cognition in adulthood.

Even though the specific mechanism remains unclear, exogenously administered Sia can enter the neonate brain and potentially serve as a conditionally essential nutrient to form conjugated brain molecules, such as gangliosides, NCAM, and other Sia-conjugated molecules. Indeed, studies have shown that Sia supplementation increases the production of Sia conjugated gangliosides in the brain, which supports Sia's proposed role in establishing synaptic pathways [10,35,36]. In fact, neural cell membranes contain 20 times more Sia than other membranes [5,21,37]. The

negatively charged Sia may then facilitate transmitter molecules binding to the synaptic membrane, because most neurotransmitters are positively charged. Indeed, the synaptosomal fraction contains more than 40% of total Sia found in the brain, and Sia contributes to the overall negative charge of the membrane [5,21,37]. In contrast, older animals do not show any significant transport or incorporation of exogenous Sia in the brain.

Alternatively, recent studies have suggested that Sia and PSia can also influence signal transduction and interact with neurotrophic factors, such as brain derived neurotrophic factor (BDNF) [19,38,39]. Indeed, Sia supplementation may impact humans more than other mammals, because adult human brain contains two- to fourfold more Sia than other mammals, including chimpanzees [36]. Even though the present study provides no meaningful insight into the mechanism by which Sia affects the direct (Sia as a precursor) vs. indirect (Sia as a modulator) mechanism in promoting cognitive function, it has demonstrated that studies can now proceed with a mouse model and with the use of ^{19}F Sia as a precursor.

Conclusion

The present study has established the feasibility of using a mouse model and ^{19}F NMR to measure the biodistribution/metabolism of ^{19}F-labelled Sia precursors and to investigate the biochemical mechanism underlying the observed cognitive improvement associated with Sia supplementation. Sia-fed and control mouse pups show the same growth rate, indicating that Sia supplementation has no effect on the caloric intake and utilization. However, mice receiving Sia supplementation as neonates perform significantly better than the control group in cognitive tests as adults. Sia uptake in the brain depends upon age and supports the hypothesis that Sia serves as a conditionally essential nutrient in early brain development.

Acknowledgements

Author's contribution

YC and TJ contributed to the conception and design of research; YC, RM, RB, QG, BL, TJ performed experiments; YC, RM, RB, QG, BL, and TJ discussed, analyzed, and interpreted experiments; YC and TJ prepared figures, analyzed the data, incorporated comments, and wrote drafts of manuscript; YC, RM, RB, QG, BL, and TJ reviewed and approved the final version of the manuscript.

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