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Metabolic fate and organ distribution of ^{13}C-3′-sialyllactose and ^{13}C-*N*-acetylneuraminic acid in wild-type mice – No evidence for direct incorporation into the brain

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ABSTRACT

Milk sialyllactose (SL) and sialic acids (SA) are considered to be crucial for brain composition and development. To investigate their metabolic fate, we administered ^{13}C-labelled 3′SL (^{13}C-3′SL) and ^{13}C-*N*-acetylneuraminic acid (^{13}C-Neu5Ac) to NMRI mice. From per oral and intravenous (i.v.) applications, an organ specific ^{13}C-enrichment can be excluded. The ^{13}C-enrichment after oral application (o.a.) was lowest in brain tissue and not detectable after i.v. in any organ. The presence of ^{13}C-Neu5Ac in urine after the o.a. of both labelled components demonstrated that ^{13}C-Neu5Ac was taken up by gut epithelial cells. Because plasma ^{13}C-enrichment increased over time, when the oral ^{13}C-bolus had reached the lower gastrointestinal tract, an involvement of intestinal epithelial cells and/or gut microbiota in the metabolism of ^{13}C-3′SL and/or ^{13}C-Neu5Ac could be assumed. Hence, SL or Neu5Ac might influence the gut brain axis by effects within the gastrointestinal tract rather than being directly incorporated into the brain.

1. Introduction

Over the past decade, neutral and acidic human milk oligosaccharides have become regarded as being of great importance for the health of newborn infants (Barton et al., 2019; Bode, 2012; Cabrera-Rubio et al., 2019; James et al., 2019; Jantscher-Krenn et al., 2019; Katayama, 2016; Korpela et al., 2018; Kunz et al., 2017; Larsson et al., 2019; McGuire et al., 2017; Monaco et al., 2018; S. Rudloff et al., 2019; Samuel et al., 2019; Schroten, Hanisch, & Hansman, 2016; Seppo, Autran, Bode, & Jarvinen, 2017). However, a definitive proof of the suggested gastrointestinal or systemic functions in infants is still missing. Although highly interesting, the primary outcomes of the few intervention studies published so far with 2′Fucosyllactose (2′FL) or 2′FL + Lacto-*N*-neotetraose, have been focused upon safety aspects and growth patterns which are amongst the most important prerequisites for new strategies to modify infant formula (Goehring et al., 2016; Marriage, Buck, Goehring, Oliver, & Williams, 2015; Puccio et al., 2017; Sprenger, Lee, De Castro, Steenhout, & Thakkar, 2017). From these observational studies, it could be concluded that both components were safe and led to growth parameters comparable to those of breastfed infants.

Apart from the potential beneficial effects of human milk oligosaccharides (HMO) on the gut microbiota, inflammatory processes, the immune system or, allergies (Kulinich & Liu, 2016; Morozov, Hansman, Hanisch, Schroten, & Kunz, 2018; Plaza-Diaz, Fontana, & Gil, 2018; van den Elsen, Garssen, Burcelin, & Verhasselt, 2019), there is also a great interest in applying individual single neutral or acidic oligosaccharides for other purposes (Kuntz et al., 2019; Oliveros et al., 2018; S. Rudloff et al., 2019). For example, Duncan and colleagues hypothesized that sialic acid could serve as a conditionally essential nutrient for the suckling neonate (Duncan, Raymond, Fuerholz, & Sprenger, 2009). For a substantial period of time, sialic acid has been hypothesized as being an essential nutrient for brain development and cognitive function (Morgan & Winick, 1980; Witt, von Nicolai, & Zilliken, 1979). This

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association has been renewed recently (Fleming, Chichlowski, Berg, Donovan, & Dilger, 2018; Mudd, Fleming, Labhart, Chichlowski, Berg, Donovan, & Dilger, 2017; Obelitz-Ryom et al., 2019; Wang, 2009). The great interest in SL and sialic acids is also reflected by the increasing number of studies involving the feeding of SL to animals, mainly rats or pigs (Fleming et al., 2018; Mudd et al., 2017; Obelitz-Ryom et al., 2019; Oliveros et al., 2018; S. Rudloff et al., 2019; Sakai et al., 2006; Tarr et al., 2015).

Regarding the hypothesized functions of SA and SL the fundamental question as to whether these components are specifically incorporated into the brain, so that they can influence cognition and brain development, remains unclear. More than 70 years ago, it was found that the brain contains a high amount of sialylated glycolipids, mainly gangliosides (Kolter, 2012), in addition to numerous sialylated glycoproteins (Schnaar, Gerardy-Schahn, & Hildebrandt, 2014). Hence, it was intriguing to speculate about the potential to influence the brain composition by orally - given food components such as sialic acids or SL. Witt *et al.* reported in 1979 on the impact of orally or intravenously applied HMO on brain glycoconjugate composition, by comparing ^{14}C-radiolabelled free sialic acids and SL (Witt et al., 1979). The authors reported a preferential incorporation of ^{14}C-SL compared to free ^{14}C-sialic acids in rat brain gangliosides. However, from the data it could not be excluded that the identification of ^{14}C in brain was an unspecific distribution of the radioactive label only. At the same time, Nöhle and Schauer investigated the metabolic fate of an orally or intravenously applied mixture of radiolabelled ^{14}C–^{3}H-Neu5Ac mixture in rats and mice and observed that only very little of the applied ^{14}C- and ^{3}H-radioactivity was retained in the blood, liver, spleen, and kidney, and was excreted with urine. The authors concluded that sialic acids (i.e. Neu5Ac) occurring in food cannot directly be used for the biosynthesis of glycoconjugates on a large scale (Nöhle & Schauer, 1981).

Recently, the discussion concerning the impact of orally applied sialic acids and/or SL on brain composition has resurfaced. Obeliz-Ryom and coworkers presented data that in preterm piglets bovine milk oligosaccharides with SL do not increase the sialic acid content in the hippocampus or change magnetic resonance imaging (MRI) endpoints (Obelitz-Ryom et al., 2019), although supplemented pigs upregulated genes related to sialic acid metabolism, myelination and ganglioside biosynthesis in the hippocampus. In contrast, Mudd and coworkers applied magnetic resonance imaging in young pigs and identified effects in various parts of the brain, which led the authors to conclude that these parts may be differentially sensitive to dietary SL supplementation (Mudd et al., 2017). The authors recommended that in future studies the metabolic fate of SA or SL should be investigated as a priority in order to be able to come to a strong conclusion.

To gain more insight into the metabolic fate of the most common sialic acid, Neu5Ac as well as Neu5Ac linked to Lactose (i.e. SL), we wanted to determine the target organs and tissues of dietary ^{13}C-3′SL and ^{13}C-Neu5Ac. The main objectives were (i) to investigate whether ^{13}C-Neu5Ac and/or ^{13}C-3′SL are absorbed in the intestine and efficiently transported and incorporated into the brain and (ii) to test whether different effects of ^{13}C-3′SL and free ^{13}C-Neu5Ac could be detected. The application of the stable isotopically labelled ^{13}C-Neu5Ac and ^{13}C-3′SL is a unique method of specifically addressing these objectives (Dotz, Rudloff, Meyer, Lochnit, & Kunz, 2015; Kuntz et al., 2019; Silvia Rudloff et al., 2006). The advantage of ^{13}C-IRMS is the unique and specific labelling together with a very high sensitivity to be able to detect traces of a ^{13}C-label in biological fluids or organs. However, it only detects the ^{13}C-label and cannot be differentiated if this ^{13}C-label is part of the applied intact ^{13}C-Neu5Ac molecule or if it is a metabolic product of ^{13}C-Neu5Ac. For an unambiguous detection of the applied and still intact ^{13}C-Neu5Ac in the presence of endogenous Neu5Ac, we additionally used a mass spectrometry (MS)-based strategy. Therefore, blood and urine samples were analyzed by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) for the presence of ^{13}C-Neu5Ac (Bayer et al., 2013; Klein et al., 1997).

2. Materials and methods

2.1. Materials

Isotopically labelled ^{13}C-sialyllactose (α-*N*-Acetyl-[1,2,3-^{13}C3]neuraminic acid-(2 → 3)-β-galactose (1 → 4)-glucose-[3-^{13}C]; 99.5 atom % ^{13}C, chemical purity greater than 95%) and ^{13}C-sialic acid (^{13}C-Neu5Ac (*N*-Acetyl-D-[1,2,3-^{13}C]neuraminic acid; ≥99 atom % ^{13}C, chemical purity ≥ 97%) were obtained from Cambridge Isotope Laboratories Inc., Tewksbury, MA (USA).

2.2. Oral and intravenous administrations

All experiments were performed by persons with appropriate training and experience in accordance with the requirements of the Federation of European Laboratory Animal Science Associations and the Directive of the Council of the European Communities (Directive 2010/63/EU). The experiments were approved by the Regional Authority (Regierungspraesidum Darmstadt, Germany, FU/1056). The methods used for the studies in NMRI mice have been described in detail previously (Kuntz et al., 2019). Briefly, for **oral administration**, 8 weeks old, male NMRI mice were treated with ^{13}C-3′-Sialylactose or ^{13}C-Neu5Ac by oral gavage. The dosage for SL was 1 g/kg body weight and for SA, 200 mg/kg body weight; the control mice received the vehicle (0.9% NaCl) and mice were kept individually in metabolic cages. Although food and water were available ad libitum, the animals' intake was not measurable. Hence, we considered the animals as being in a fasting state. After 1, 3, 5 and 9 h, mice (n = 3 controls, n = 5 treated animals at each time point) were deeply anaesthetized, blood was taken and the body was perfused with 0.9% NaCl to avoid plasma contaminations of organs. Following the perfusion tissues and organs removed, including brain segments (cerebrum, cerebellum, midbrain, and brainstem), liver, heart, spleen and kidneys. Urine and feces were collected, as well. The small intestine was cut in 3 sections of equal length and luminal contents of these sections as well as from the colon were collected (Kuntz et al., 2019). Tissue samples were weighed and snap-frozen in liquid nitrogen and kept at −80 °C until analysis.

For **intravenous administration** of ^{13}C-SL or ^{13}C-Neu5Ac, a stock solution of 100 mg dissolved in 0.9% NaCl was prepared. Young male NMRI mice (5 treated animals, 3 controls, age 8 weeks, weight about 40 g) were individually housed in metabolic cages to facilitate feces and urine collection. Each animal received three times (every 6 h) 2.5 mg of the ^{13}C-3′-Sialylactose or ^{13}C-Neu5Ac by intravenous injection through the femoral vein. Animals were sacrificed 24 h after the first dosage.

2.3. EA-IRMS of biological samples

^{13}C-enrichment in brain and other tissues were determined by Elemental Analysis Stable Isotope Ratio Mass Spectrometry (EA-IRMS) with minor modifications as has previously described for metabolic studies in infants (Dotz et al., 2015; Rudloff, Pohlentz, Borsch, Lentze, & Kunz, 2012) and for animals (Kuntz et al., 2019). Briefly, brain segments were homogenized by using plastic pestles for 1.5 mL-Eppendorf cups. All other tissues were homogenized in a mixer mill (MM400, Retsch GmbH, Haan, Germany) using two steel balls (7 mm diameter) per cup. Aliquots were weighed into tin cups (2–4 mg of tissue; 20 µl of biological fluid) onto Chromasorb (0.5–5 mg, amounts varying between organs and fluids). Then, ^{13}C enrichment was determined as δ^{13}C by Isotope Ratio Mass Spectrometry (IR-MS; Isoprime, Isoprime Limited, Manchester, UK) after total combustion at 920 °C (PyroCube, Elementar, Hanau, Germany) as described earlier (Kuntz et al., 2019).

2.4. LC-MS analysis of ^{13}C-Neu5Ac

5 µl of urine and 15 µl of plasma samples in addition to standards and blanks were lyophilized. To release the sialic acid residues, samples were

hydrolyzed in 200 µl 2 N acetic acid for 90 min at 80 °C. After cooling down to room temperature, samples were dried in a SpeedVac concentrator. Sialic acids were labelled using the DMB-method as previously described (Hara, Takemori, Yamaguchi, Nakamura, & Ohkura, 1987) and subsequently analyzed by LC-MS (Bayer et al., 2013; Klein et al., 1997). Therefore, samples were dissolved in 80 µl DMB-labelling buffer (9 mM sodium hydrosulfite, 0.5 M β-mercaptoethanol, 20 mM trifluoroacetic acid (TFA)) and incubated for 2 h at 55 °C. The reaction was stopped by adding 20 µl 0.2 N NaOH. The fluorescently - labelled sialic acids were directly analyzed using an Accela HPLC-System (Thermo) with an ESI LTQ XL Orbitrap mass spectrometer (Thermo). For chromatography an Accucore C18 column (50 × 2.1 mm, 2.6 µm, Thermo Fisher Scientific) was used. Column temperature was set to 40 °C. Mobile phases consisted of A: H_2O with 0.05% formic acid and B: MeOH with 0.05% formic acid. Flow rate was set for 0.05 mL/min and 15 µl of the derivatized samples, standards as well as blanks were injected and analyzed. Separation was performed by applying the following gradients: G1 (urine samples): 0 min, 15% B; 1 min, 15% B; 7 min, 35% B; 7.5 min, 100% B; 12.5 min, 100% B; 12.6 min 15% B, 17 min, 15% B; G2 (plasma samples): 0 min, 15% B; 1 min, 15% B; 7 min, 65% B; 7.5 min, 100% B; 12.5 min, 100% B; 12.6 min 15% B, 22 min, 15% B. ESI and MS parameters were set to: Capillary Temperature: 275 °C; Sheath Gas: 15; Aux Gas: 1; Sweep Gas: 1; Electron voltage: 4,2 kV. For quantification the extracted ion chromatograms for sodiated DMB-Neu5Ac (*m/z* 448) and sodiated DMB-^{13}CNeu5Ac (*m/z* 451) were used for calculation of the peak areas.

2.5. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 6.0.7 (GraphPad Software Inc., La Jolla, U.S.A.) and results were expressed as box plots with medians and minimum to maximum whiskers or means with SEM (Figs. 1–4) or SD (Fig. 7). Data were analyzed by ANOVA with multiple comparison test or student *t*-test. Differences were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results

3.1. Distribution of orally given SL and Neu5Ac in organs, tissues and biological fluids.

In order to analyze if free Neu5Ac or Neu5Ac attached to lactose (SL) was preferentially incorporated into specific organs and tissues, IR-MS was used after an oral application of ^{13}C-SL or ^{13}C-Neu5Ac as a single dose. The obtained data demonstrated that ^{13}C-enrichment in plasma noticeably increased within the first 3 h after the dosage, staying at this level at 5 and 9 h as well. After ^{13}C-SL application, ^{13}C-enrichment in all four brain sections cerebellum, cerebrum, brainstem and mesencephalon was found to be in parallel to the ^{13}C-increase in plasma, but to a much lower degree (Fig. 1). After ^{13}C-Neu5Ac application, however, ^{13}C-enrichment in the brain followed this trend only in cerebellum and cerebrum; an increase for the brain sections brainstem and mesencephalon was only reached 9 h after the dosage.

Sometimes, a higher ^{13}C-enrichment at t = 1 h compared to the other time points was found which can be explained by the large individual

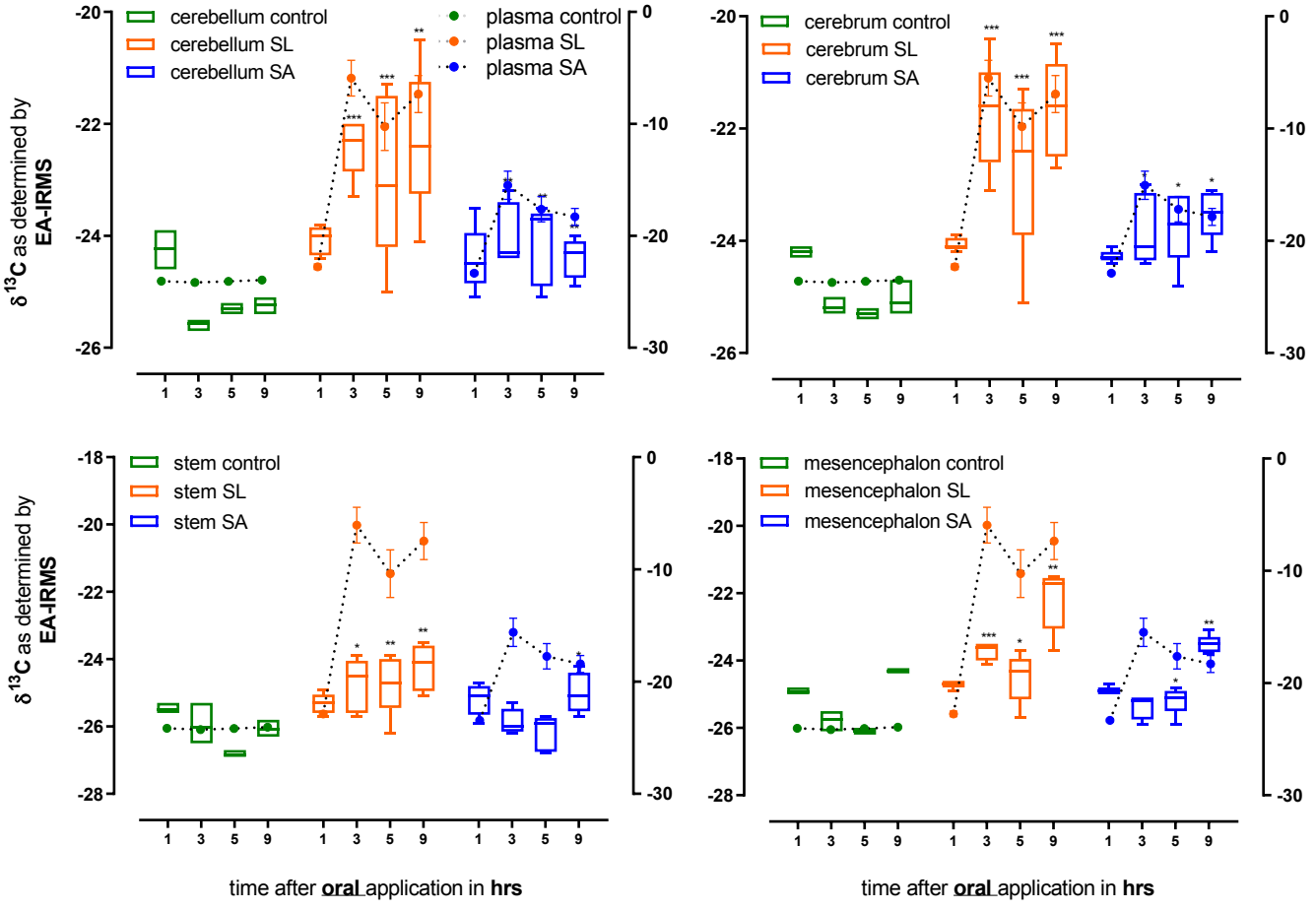

Fig. 1. ^{13}C-enrichment (δ^{13}C in $^{0}/_{00}$) in brain sections (left y-axis) and plasma (right y-axis) from mice receiving an oral dose of ^{13}C-labelled SL (1 g/kg body weight) or ^{13}C-labelled Neu5Ac (200 mg/kg body weight). Data for brain sections are depicted as box plots with median and min–max whiskers; data for plasma are shown as mean ± SEM (differences to the corresponding controls were significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). Controls n = 3; treated, n = 5.

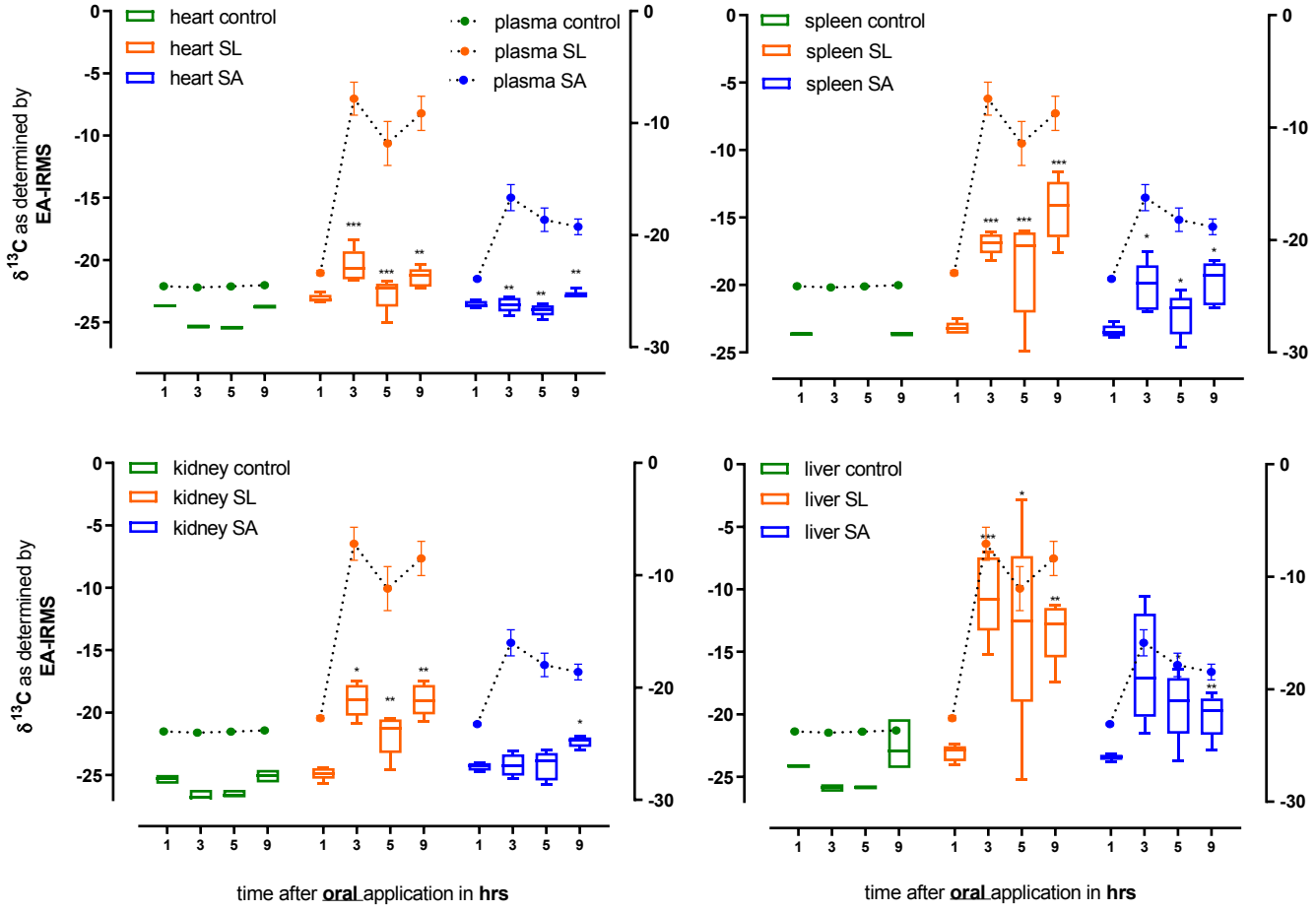

Fig. 2. ^{13}C-enrichment (δ^{13}C in $^{0}/_{00}$) in organs (heart, spleen kidney and liver) (left y-axis) and plasma (right y-axis) from mice receiving an oral dose of ^{13}C-labelled SL (1 g/kg body weight) or ^{13}C-labelled Neu5Ac (200 mg/kg body weight). Data for organs are depicted as box plots with median and min–max whiskers; data for plasma are shown as mean ± SEM (differences to the corresponding controls were significant at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$). Controls n = 3; treated, n = 5.

variation between the animals. This explanation is supported by the data of the control animals which, although not receiving any ^{13}C-labelled material, also demonstrates the high variability.

Analyzing ^{13}C-enrichment in other organs such as liver, heart, spleen and kidney, we observed that ^{13}C-enrichment showed the same time dependency, although reaching a slightly higher ^{13}C-enrichment in liver and spleen (compare Figs. 1 and 2) indicating that ^{13}C-enrichment may not be an organ specific process. However, in comparison to these organs, lower levels of ^{13}C-enrichment were detectable in the brain. Overall, ^{13}C-enrichment after SL application appeared to be higher than that after Neu5Ac dosage (Figs. 1 and 2).

Furthermore, the luminal content of intestinal sections was analyzed. Overall, ^{13}C-enrichment after SL application appeared higher than that after SA dosage (Fig. 3). Whether this was due to a higher ^{13}C-labelling density of SL compared to SA or a better uptake of compounds most likely derived from bacterial metabolism within the gut, remains unanswered.

The ^{13}C-excretion via urine and feces indicated late elimination of ^{13}C, which was found to be time - delayed for urine compared to plasma reaching their maxima at 5 and 3 h, respectively (Fig. 4). In feces, ^{13}C-enrichment followed a comparable time course to that found for urinary ^{13}C-enrichment. In comparison to the tissue and plasma samples considerably more ^{13}C-enrichment was observed. For instance, whereas in brain sections mostly differences between 2 and 4 δ^{13}C in $^{0}/_{00}$ were detectable, in urine values up to 2,000 δ^{13}C in $^{0}/_{00}$ were measured.

In order to test if an uptake of intact ^{13}C-Neu5Ac takes place after oral application of ^{13}C-SL and ^{13}C-Neu5Ac, the DMB-LC-ESI-MS method was applied. Since by IR-MS analysis urine samples revealed the strongest ^{13}C-enrichments in comparison to all organs and tissues, ^{13}C-positive urine samples (3, 5 and 9 h) were used as a control for a possible uptake of intact ^{13}C-Neu5Ac. In urine of control animals only endogenous Neu5Ac was observed. Extracted ion chromatogram (EIC) of sodium adducts ($[M + Na]^+$) of DMB-Neu5Ac (m/z 448) exhibited a peak at 9.8 min, whereas no signal occurred in EIC of m/z values corresponding to DMB-^{13}C-Neu5Ac (m/z 451) (Fig. 5 A). In contrast, after an oral dose of ^{13}C-labelled SL the LC-ESI-MS analysis displayed significant signals for DMB-^{13}C-Neu5Ac in urine samples after 5 h (Fig. 5). Whereas after oral application of ^{13}C-SL, the maximum ^{13}C-Neu5Ac content was reached after 5 h, in the case of orally applied ^{13}C-Neu5Ac, the highest values were detected after 9 h. Thus, the time courses of the LC-MS data were comparable to that of the IR-MS analyses.

In addition, ^{13}C-positive plasma samples (analyzed by IR-MS) were examined for intact ^{13}C-Neu5Ac by DMB-LC-MS analysis. However, the signals for DMB-^{13}C-Neu5Ac were only strong enough for quantification in 3 samples at the time-point 5 h after oral application of ^{13}C-SL (Fig. 6). In the case of orally administered ^{13}C-Neu5Ac, intact ^{13}C-Neu5Ac residues were only detectable in 1 sample at time-point 5 h. Since the signal is close to the limit of detection (poor signal/noise ratio), no quantification is possible (Fig. 6). In contrast to urine, the analyses of plasma samples by LC-MS and IR-MS exhibited different time courses. Whereas IR-MS show similar ^{13}C-values between 3 and 9 h, the obtained LC-MS-results demonstrated that ^{13}C-Neu5Ac is only quantifiable in plasma 5 h after oral application of ^{13}C-SL. Thus, ^{13}C signals obtained after 3 and 9 h by IR-MS originated from other molecules than ^{13}C-Neu5Ac.

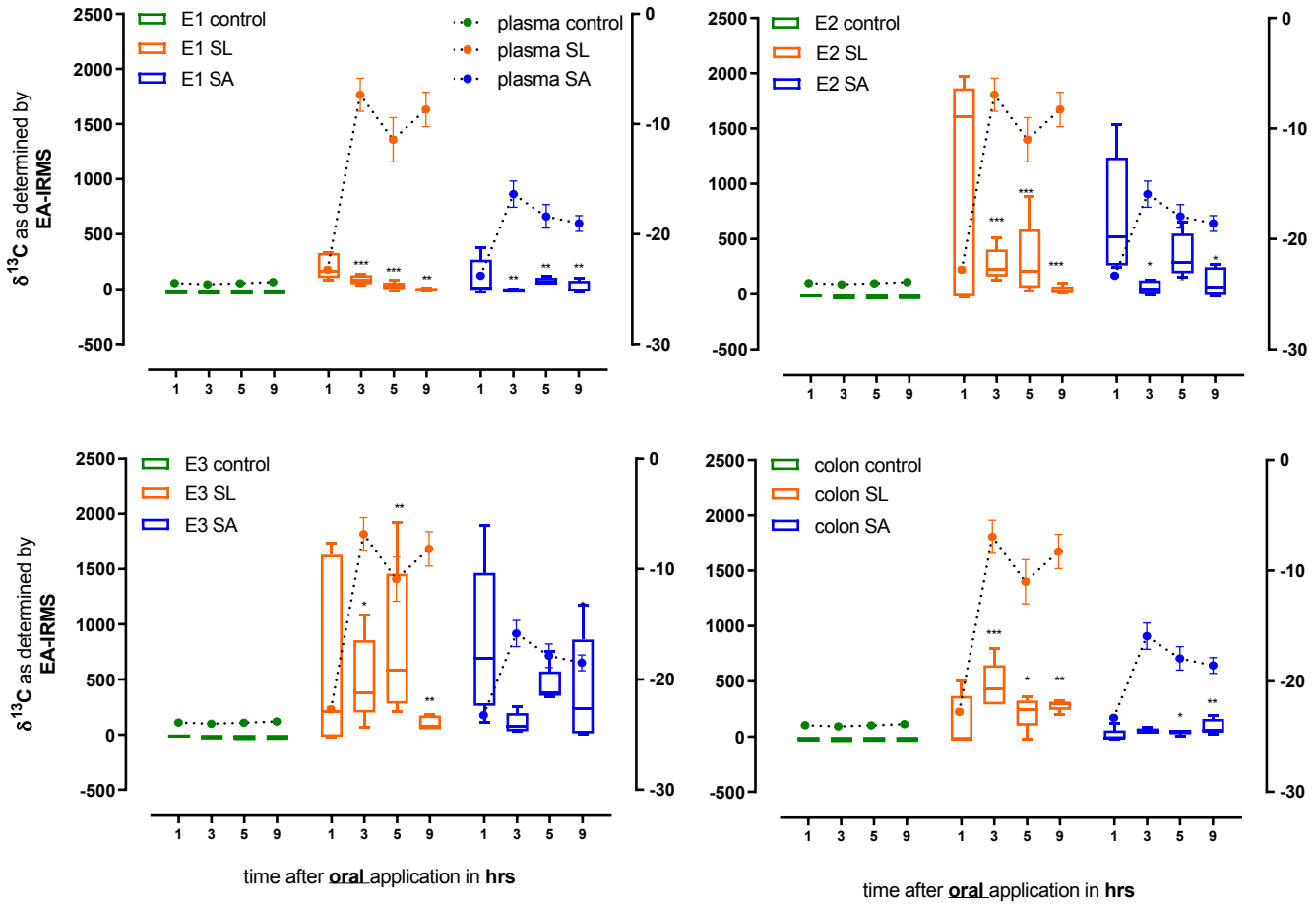

Fig. 3. ^{13}C-enrichment (δ^{13}C in $^{0}/_{00}$) in luminal contents from the small intestinal (sections E1-E3) and the from the colonic contents (left y-axis) and plasma (right y-axis) from mice receiving an oral dose of ^{13}C-labelled SL (1 g/kg body weight) or ^{13}C-labelled Neu5Ac (200 mg/kg body weight); data for luminal contents are depicted as box plots; data for plasma are shown as mean ± SEM (differences to the corresponding controls were significant at *p < 0.05, **p < 0.01 and ***p < 0.001). Controls n = 3; treated, n = 5.

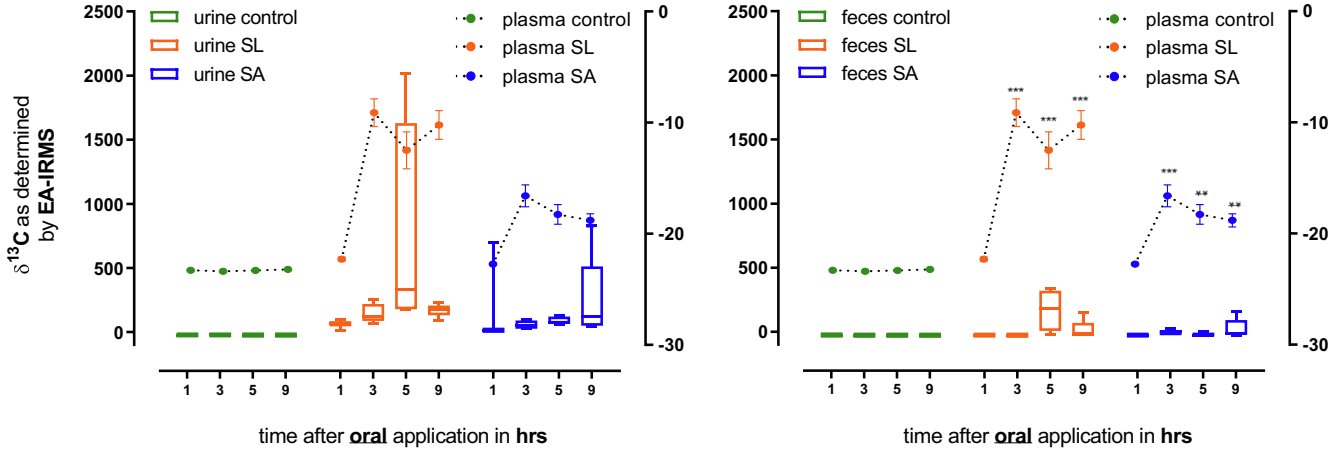

Fig. 4. ^{13}C-enrichment (δ^{13}C in $^{0}/_{00}$) in urine and feces (left y-axis) and plasma (right y-axis) from mice receiving an oral dose of ^{13}C-labelled SL (1 g/kg body weight) or ^{13}C-labelled Neu5Ac (200 mg/kg body weight); data for urine and feces are depicted as box plots, for urine controls min–max is given; data for plasma are shown as mean ± SEM (differences to the corresponding controls were significant at *p < 0.05, **p < 0.01 and ***p < 0.001; controls n = 3; treated, n = 5).

Taken together, the presence of intact ^{13}C-Neu5Ac in urine as well as plasma samples after oral administration of ^{13}C-SL and ^{13}C-Neu5Ac demonstrated that ^{13}C-Neu5Ac was absorbed and transferred into circulation. However, Neu5Ac that reached the circulation seemed to be immediately excreted into the urine.

3.2. Distribution of intravenously given SL and Neu5Ac in plasma, urine and brain segments

To test whether in general free Neu5Ac or Neu5Ac linked to lactose can be transferred from the blood stream into the brain both molecules

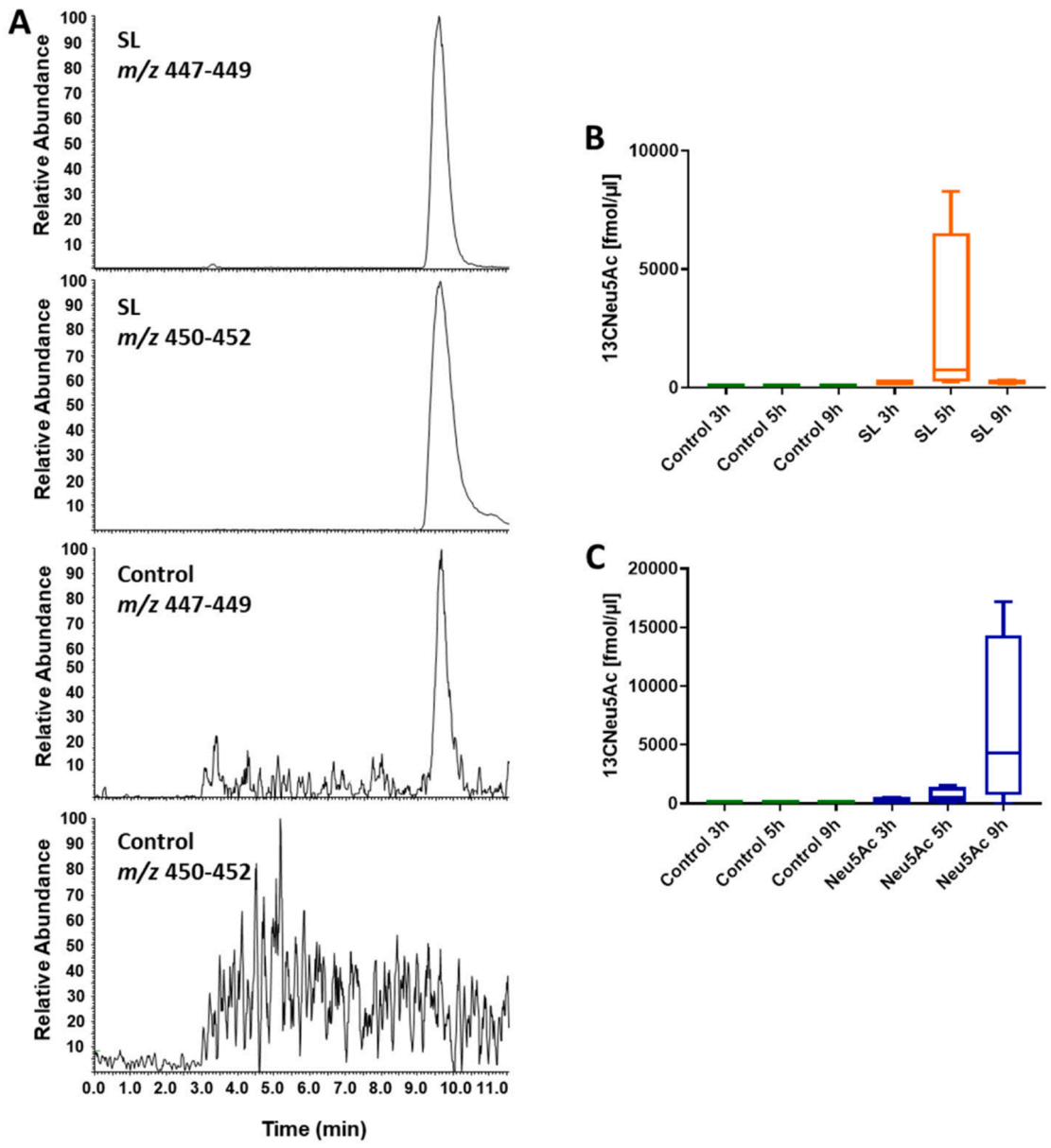

Fig. 5. ^{13}C-Neu5Ac is present in the urine after oral application of ^{13}C- SL and ^{13}C-Neu5Ac. A) EIC of sodium adducts ($[M + Na]^+$) of DMB-Neu5Ac (*m/z* 447–449) and DMB-^{13}C-Neu5Ac (*m/z* 450–452) were generated after LC-ESI-MS analysis using urine samples. Exemplary EIC of control as well as ^{13}C-SL treated animals are displayed for time point 5 h. The obtained signals (peak areas) were used for the quantification of ^{13}C-Neu5Ac. Box and whisker plots (median; min to max) are shown for B) SL (controls $n_{3,\ 5,\ 9h} = 3$; treated, $n_{3\ h} = 5$, $n_{5\ h} = 4$, $n_{9h} = 5$) and C) Neu5Ac treated mice (controls $n_{3,\ 5,\ 9h} = 3$; treated, $n_{3\ h} = 3$, $n_{5\ h} = 4$, $n_{9h} = 4$).

were intravenously applied. Six hours after the last (out of 3) intravenous applications of ^{13}C-SL, ^{13}C-enrichment in plasma had reached baseline levels; the extremely high ^{13}C enrichments of urine verified that the 3 dosages given per animal were excreted. However, there was no ^{13}C enrichment in the brain sections of animals treated with ^{13}C-SL. It is important to note that the putative ^{13}C enrichment in urine of control animals (Fig. 7) was due to an accidental contamination with previously prepared and highly enriched urine samples from treated animals.

In addition, the same procedure was used to test, whether free ^{13}C-Neu5Ac was able to cross the blood–brain barrier. Again, after intravenous application of ^{13}C-Neu5Ac, ^{13}C-enrichment was observed neither in the brain nor in other organs, although residual ^{13}C traces after intravenous application still seemed to be in the circulation.

Since intact ^{13}C-Neu5Ac was only significantly detectable in urine after oral application of ^{13}C-Neu5Ac or ^{13}C-SL, urine samples were additionally analyzed by LC-MS. Again, in urine of control animals only endogenous Neu5Ac and no ^{13}C-Neu5Ac was observed (Fig. 8). However, in urine samples after both intravenous applications, ^{13}C-SL as well as ^{13}C-Neu5Ac, accumulations of ^{13}C-Neu5Ac were detectable. Thus, the intravenous administration of ^{13}C-SL or ^{13}C-Neu5Ac leads to a urinary excretion of intact ^{13}C-Neu5Ac and no uptake into the systemic circulation occurred.

4. Discussion

Supplementation of infant formula with SL, either as one of the two isomers 3′SL and 6′SL alone or in combination, is currently of great interest based on the view that they may influence the gastrointestinal microbiota or affect brain composition (e.g. gangliosides or glycoproteins) and/or brain activity (Fleming et al., 2018; Mudd et al., 2017; Obelitz-Ryom et al., 2019). Several recent publications report the effects of SL on cognition and memory in animal models; however, the question, whether this is a direct effect through the incorporation of SA and/or SL is very controversial. For instance, Jacobi *et al.* reported that

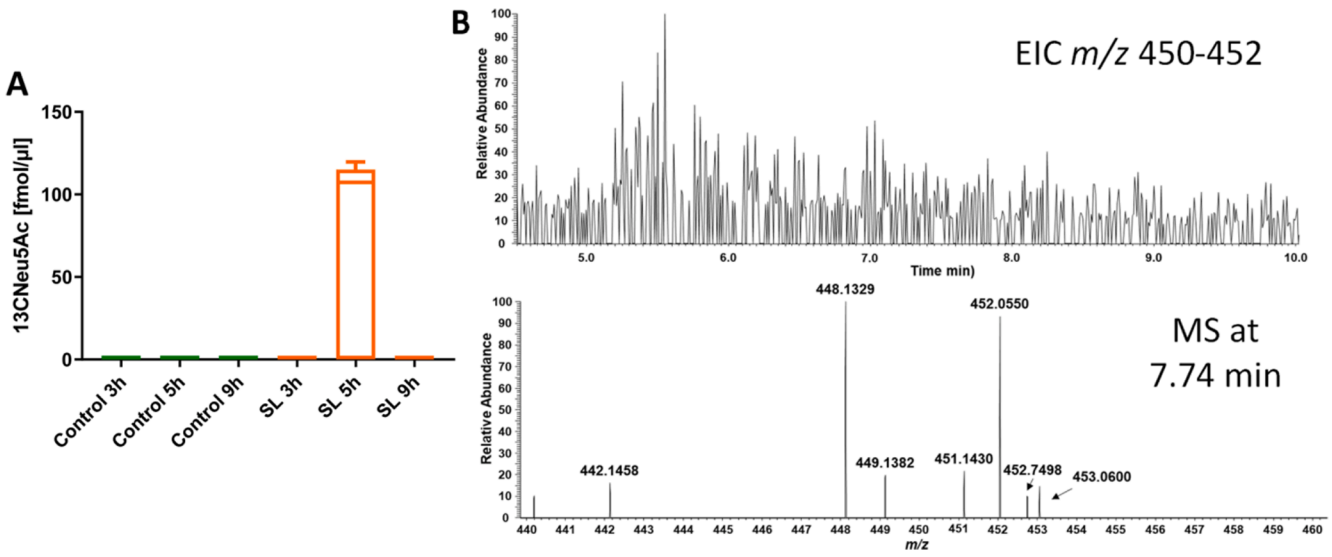

Fig. 6. ^{13}C-Neu5Ac is present in plasma after oral application of ^{13}C-SL and ^{13}C-Neu5Ac. Plasma was investigated for ^{13}C-Neu5Ac by LC-ESI-MS analysis in control animals as well as after oral administration of ^{13}C-SL and ^{13}C-Neu5Ac as described in Fig. 5. (A) The concentration of ^{13}C-Neu5Ac was calculated after application of ^{13}C-SL. Box and whisker plots (median; min to max) are shown (controls $n_{3,\ 5,\ 9\ hrs} = 3$; treated, $n_{3,\ 5,\ 9\ hrs} = 5$). (B) The obtained EIC for sodium adducts of DMB-^{13}C-Neu5Ac (*m/z* 450–452) exhibited no quantifiable signals after oral application of ^{13}C-Neu5Ac. However, in one plasma sample a signal for ^{13}C-Neu5Ac was detectable (*m/z* 451.14) at the retention time of Neu5Ac.

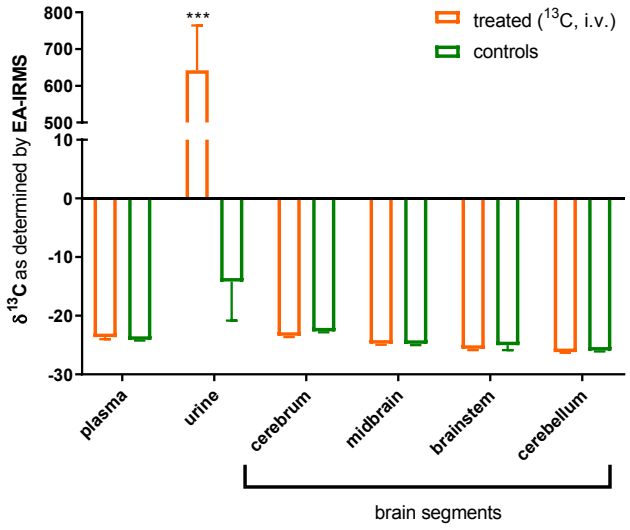

Fig. 7. ^{13}C enrichment (as δ^{13}C in $^{0}/_{00}$) in plasma, urine and brain sections after 6 h from animals either given ^{13}C-SL (treated, n = 8) or 0.9% NaCl (controls, n = 4) intravenously. Results are shown as mean ± SD (differences to the corresponding controls were significant at ***$p < 0.001$).

dietary isomers of SL increased ganglioside SA concentrations in the corpus callosum and cerebellum of formula-fed piglets (Jacobi et al., 2014). In addition, Mudd et al. (2017) reported an influence of dietary SL on bound sialic acids in the prefrontal cortex and a small change in the ratio of free to bound sialic acid in the hippocampus of pigs. The controversy regarding the role of SA and/or SL on brain composition continues as it has been shown that sialylated bovine milk oligosaccharides had no impact on the SA content in the hippocampus of preterm piglets (Obelitz-Ryom et al., 2019).

As a general remark, with regard to the conflicting data, it needs to be noted that there is neither a routine nor a standardized method for HMO quantification available which allows an unequivocal comparison of data from different studies e.g., previous studies, in which a possible uptake of applied Neu5Ac or SL into the brain was investigated, using

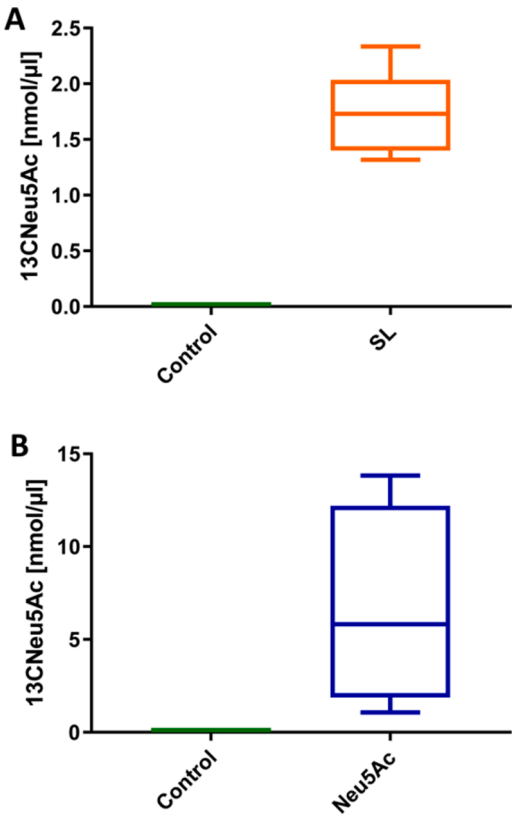

Fig. 8. ^{13}C-Neu5Ac is present in the urine after intravenous application of ^{13}C-SL and ^{13}C-Neu5Ac. EIC of sodium adducts ($[M + Na]^+$) of DMB-^{13}C-Neu5Ac (*m/z* 450–452) were generated after LC-ESI-MS analysis as described in Fig. 5 and the concentration was calculated in urine samples after intravenous administration of A) ^{13}C- SL (controls n = 4; treated, n = 8) and B) ^{13}C-Neu5Ac (controls n = 3; treated, n = 5). Box and whisker plots (median; min to max) are shown.

radiolabelled Neu5Ac. The tissues were tested for their radioactivity without any proof of whether intact Neu5Ac or only the radiolabelled carbons of metabolized Neu5Ac were incorporated. In addition, colorimetric assays using thiobarbituric acid were often applied for SA quantification. However, this reagent is known to be very sensitive to a variety of other substances, e.g. to malondialdehyde and other oxidation products from lipids which also occur in higher amounts in the brain. Thus, such data have to be carefully evaluated due to the likelihood of erroneous values.

To get more information on the metabolic fate of 3′SL or Neu5Ac we combined two different methods: (i) we used IRMS as an exceptionally sensitive method to follow the ^{13}C-enrichment of tissues, feces and biological fluids (blood and urine) after an oral dose of ^{13}C-labelled 3′SL or ^{13}C-Neu5Ac and (ii) LC-ESI-MS analysis of those blood and urine samples to verify the uptake of intact ^{13}C-Neu5Ac.

After the oral application of ^{13}C-SL, as well as ^{13}C-Neu5Ac, plasma - ^{13}C-enrichment started to increase in the first 3 h and stayed at these levels throughout 9 h. However, only a modest ^{13}C-enrichment was found in all organs (liver, heart, kidney and spleen) including the different brain regions. The time course was in parallel to the ^{13}C-enrichment found in plasma (compare Figs. 1 and 2). The data for ^{13}C-Neu5Ac were similar to those found for ^{13}C-SL with minor differences regarding a slightly higher ^{13}C-enrichment in liver and spleen. Also, the highest ^{13}C-enrichment in brainstem and mesencephalon was only reached at 9 h with ^{13}C-Neu5Ac compared to 5 to 9 h after the application of ^{13}C-SL. We conclude from this data that ^{13}C-enrichment is not an organ specific effect but occurs in parallel to the ^{13}C-enrichment in plasma. Simple plasma contaminations of organs could be excluded as the whole animals, after blood was taken, were perfused with 0.9% NaCl before the animals had been sacrificed and the organs separated.

We would like to underline that the statistical analysis has been done with respect to controls as the animal experiments for the different time points could not all be done on one day. Since naturally occurring ^{13}C-enrichment is known to vary, as can be seen for control animals in Figs. 1–4, we preferred to relate the data only to the control animals. Within group variation of data from animals obtained at time differences up to 9 h would blur the significance of the results.

To get more information on the uptake processes of SL and SA, we investigated the link between oral doses of the ^{13}C-labelled components, the enrichment of ^{13}C in the intestine, feces and plasma and the final excretion in urine. Evaluating the ^{13}C-enrichment in plasma, we found that it increased at the time points when the ^{13}C-bolus had reached the lower gastrointestinal tract. The intestinal transit time of the ^{13}C-bolus was fast, reaching the lower part of the intestine after 1 to 3 h. However, based on the LC-MS data, it is clear that significant amounts of intact ^{13}C-Neu5Ac were transferred into circulation later than the observed ^{13}C-enrichment. Only after 5 h, substantial concentrations of ^{13}C-Neu5Ac were detectable in the circulation (compare Figs. 1 and 6), which seems to be directly excreted via the urine. These results suggest that metabolic products of ^{13}C-Neu5Ac were absorbed 3 h after o.a.. This hypothesis is supported by the i.v. application of ^{13}C-3′SL or ^{13}C-Neu5Ac demonstrating that no uptake of these molecules from the blood stream into the brain is possible resulting in the excretion of ^{13}C-Neu5Ac into the urine (Figs. 7 and 8). Thus, no effective incorporation mechanisms exist in the brain for free Neu5Ac or as a part of 3′SL. Since ^{13}C-enrichment of the brain and other organs already occurs 3 h after o.a., our results lead to the reasonable presumption that the ^{13}C -enrichment of organs was not derived from intact ^{13}C-Neu5Ac and that it is more likely that metabolic products from intestinal epithelial cells and/or intestinal microbiota and/or the liver may be absorbed and transported to the organs. It is known that several bacterial species and eukaryotic cells can metabolize Neu5Ac for nutritional purposes (Angata & Varki, 2002; Schauer, 2004; Vimr, 2013). In both cases, lyases cleave pyruvate resulting in the formation of *N*-acetylmannosamine (ManNAc) that can be directly transformed into *N*-acetylglucosamine (GlcNAc). The three ^{13}C of the applied ^{13}C-Neu5Ac are located at C1, C2 and C3 and thus, the cleaved pyruvate will consist of the ^{13}C-isotopes. Pyruvate can be used as a building block for the formation of numerous biomolecules or for the energy metabolism in mitochondria. In addition, pyruvate itself can pass the blood–brain barrier (Cremer, Cunningham, Pardridge, Braun, & Oldendorf, 1979). Already in 1981, Nöhle and Schauer suggested that Neu5Ac in food is prevalently excreted in the urine or metabolized by lyases instead of a direct incorporation into nascent glycoconjugates (Nöhle & Schauer, 1981).

Furthermore, it has to be mentioned that in mammals, in contrast to bacteria, no SA transporters in the cell membrane are known. In mammals, SA seems to be nonspecifically absorbed via the pinocytic/endocytic pathways (Bardor, Nguyen, Diaz, & Varki, 2005) explaining that endothelial cells were unable to efficiently take up the intravenously applied ^{13}C-Neu5Ac. In line with studies which applied the SA *N*-glycolylneuraminic acid (Neu5Gc) instead of Neu5Ac, the results demonstrate that no effective system exists for an uptake of SA in mammals as they exist for glucose or other monosaccharides (Naito-Matsui et al., 2017; Samraj et al., 2015).

Moreover, it should be noted that Neu5Gc, which is, like Neu5Ac, present in substantial amounts in murine blood, appear to be absent in neuronal cells of the brain (Naito-Matsui et al., 2017). The detectable low levels of Neu5Gc in murine brain samples are mostly present in endothelial cells (Naito-Matsui et al., 2017). Therefore, even if very small amounts of ^{13}C-Neu5Ac would be detectable in brain segments in our study, this can most likely be assigned to endothelial cells as it has been recently shown for Neu5Gc (Naito-Matsui et al., 2017). Since Neu5Gc has negative effects on the neuronal system such as alternated locomotor activity, impaired object recognition memory, and disturbed axon myelination, an uptake has to be prevented or Neu5Gc has to be directly metabolized in neuronal cells as reported previously ((Naito-Matsui et al., 2017). A transfer of sialic acids from blood into the brain might bear a high risk as intracellular CMP-SA transporters in addition to sialyltransferases are unable to efficiently distinguish between Neu5Ac and Neu5Gc. Consequently, an incorporation of Neu5Gc into nascent glycoconjugates cannot be prohibited, when Neu5Gc would be present in the cytosol of neuronal cells.

In addition, it might be unnecessary to support the *de novo* synthesis of SA, such as Neu5Ac. Usually the cellular production of Neu5Ac starts with glucose and only one more glucose molecule is necessary to obtain a pyruvate for the generation of the required energy and a second one for the elongation of ManNAc-6-Phosphate (C6-backbone) into Neu5Ac-9-phosphate (C9-backbone) (Schauer, 2004). The amino group at C5 derives from glutamine and the acetyl-residue is transferred from acetyl-CoA. Thus, the de novo synthesis of Neu5Ac does not require great resources or limited molecules deriving from oral intake. So far, it remains unproven as to how ingested sialic acids could directly support the developing brain and neural benefits would be more likely to be from an indirect effect e.g. from breakdown products via a neural pathway.

In sum, our results support the notion that in humans no mechanism seems to exist for an effective utilization of free Neu5Ac as well as Neu5Ac attached to lactose, if one assumes the transferability from mouse to human.

5. Conclusion

In contrast to the i.v. application of ^{13}C-labelled 3′SL or Neu5Ac which did not lead to a ^{13}C-enrichment of any organ, low ^{13}C-enrichments in all organs and tissues could be detected after an oral dosage following the time course of ^{13}C-enrichment found in plasma. Hence, the ^{13}C-enrichment does not seem to be organ specific. The presence of ^{13}C-Neu5Ac in plasma and urine samples demonstrated that ^{13}C-Neu5Ac was taken up by epithelial cells in the gut. However, ^{13}C-Neu5Ac was mainly directly excreted in the urine. As plasma ^{13}C-enrichment increased at the time points when the ^{13}C-bolus had reached the lower gastrointestinal tract, we assume that intestinal epithelial cells, gut microbiota and/or the liver are involved in the metabolism of SL and/or

Neu5Ac. A further indication for this assumption is that an i.v. application of both compounds did not lead to any significant ^{13}C-enrichment in brain or other organs, but were quickly excreted in the urine. Thus, we suggest that mainly metabolic products derived from intestinal microbial activities might be absorbed and transported in small amounts to the organs, whereas transferred Neu5Ac is mainly excreted via urine. Our data rather support the current view that acidic HMOs such as SL or SA or neutral components such as 2′fucosyllactose (Kuntz et al., 2019) or others may have an influence on the gut-brain axis by an effect within the GI tract (e. g. by nervus vagus) rather than being directly incorporated into the brain.

Ethics

All experiments were performed by persons with appropriate training and experience in accordance with the requirements of the Federation of European Laboratory Animal Science Associations and the Directive of the Council of the European Communities (Directive 2010/63/EU). The experiments were approved by the Regional Authority (Regierungspraesidum Darmstadt, Germany, FU/1056).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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