Fucose as a Cleavage Product of 2’Fucosyllactose Does Not Cross the Blood-Brain Barrier in Mice

Silvia Rudloff,* Sabine Kuntz, Christian Borsch, Enrique Vazquez, Rachael Buck, Martina Reutzel, Gunter Peter Eckert, and Clemens Kunz

Scope: To further examine the role of the human milk oligosaccharide 2’fucosyllactose (2´FL) and fucose (Fuc) in cognition. Using 13C-labeled 2’FL, the study previously showed in mice that 13C-enrichment of the brain is not caused by 13C-2’FL itself, but rather by microbial metabolites. Here, the study applies 13C1-Fuc in the same mouse model to investigate its uptake into the brain.

Methods and Results: Mice received 13C1-Fuc via oral gavage (2 mmol 13C1-Fuc/kg-1 body weight) or intravenously (0.4 mmol/kg -1 body weight). 13C-enrichment is measured in organs, including various brain regions, biological fluids and excrements. By EA-IRMS, the study observes an early rise of 13C-enrichment in plasma, 30 min after oral dosing. However, 13C-enrichment in the brain does not occur until 3-5 h post-dosing, when the 13C-Fuc bolus has already reached the lower gut. Therefore, the researcher assume that 13C-Fuc is absorbed in the upper small intestine but cannot cross the blood-brain barrier which is also observed after intravenous application of 13C1-Fuc.

Conclusions: Late 13C-enrichment in the rodent brain may be derived from 13C1-Fuc metabolites derived from bacterial fermentation. The precise role that Fuc or 2’ FL metabolites might play in gut-brain communication needs to be investigated in further studies.

1. Introduction

Breastfeeding supports the healthy growth and development of infants.[1,2] Among multiple benefits, improved postnatal cognitive development in children is increasingly discussed to be favored by not yet identified factors in human milk.[2-6] The question of whether sialylated or fucosylated human milk oligosaccharides (HMOs) are involved in these processes has been researched at length.[7-14] Regarding sialic acid and sialyllactose (SL) and its potential effects on the brain we refer to a recent review being in favor of a direct incorporation of these milk components into brain glycoproteins and glycolipids.[15] The authors underline the importance of pig models to address such questions. For example, Obelitz-Ryom and coworkers presented data showing preterm piglets fed SL-supplemented milk had improved learning skills and cognition compared to non-supplemented formula-reared counterparts; however, SL supplementation did not increase the sialic acid (SA) content in the hippocampus or change magnetic resonance imaging (MRI) endpoints, although these pigs upregulated genes related to sialic acid metabolism, myelination and ganglioside biosynthesis in the hippocampus.[10] In contrast, Mudd et al. applied MRI 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.[8] In a previous...
study using a mouse model, however, we did not find a direct incorporation of $^{13}$C-SL or its constituent $^{13}$C-SA into the brain; these molecules were not able to cross the blood-brain barrier.[16] There, we also discussed various factors for the divergent opinions on a direct link between milk oligosaccharides and the brain.

Fucosylated HMOs such as 2‘-fucosyllactose (2’FL) have been the subject of extensive investigation in recent years. The biological importance of fucosylation on host microbe interactions, leukocyte trafficking, cancer metastasis and learning, memory and cognitive processes has been summarized elsewhere.[17] Fucose (Fuc), a major monosaccharide building block of 2’FL, was found to be enhanced in rats receiving oral 2’FL, but not after fucose application.[24,25] Recently, Tosh and coworkers reported that the fucosylation of proteins at the synapse; the rapid degradation of synapsins was used for oral and 0.4 mmol/kg body weight was used for oral and 0.4 mmol/kg body weight for intravenous applications.

2. Experimental Section

2.1. Materials

The study used l-Fucose (Fuc) labeled with the stable isotope $^{13}$C at C1 [1-$^{13}$C1-Fuc] with a $^{13}$C-enrichment of 99% (Elicityl, Crolles, France).

2.2. Dosage Information

In a previous study, physiological doses of the fucosylated oligosaccharide 2’FL were used in the same mouse model.[14] Thus, isomolar doses of $^{13}$C-Fuc, i.e., 2 mmol/kg body weight was used for oral and 0.4 mmol/kg body weight for intravenous applications.

2.3. Animal Models

2.3.1. Intravenous Application of $^{13}$C-Labeled Fuc to Wild-Type NMRI Mice

Male NMRI mice (8-weeks-old, 39 ± 2 g body weight) were purchased from Charles River Laboratories (Sulzdihl, Germany) and housed in groups of five animals with free access to water and food (Altromin Spezialfutter GmbH & Co KG, Lage, Germany). On the day of experiments, animals (n = 5 treated) received 66 mg $^{13}$C-Fuc/kg body weight divided into three equivalent doses every 6 h through the tail vein. Controls (n = 3) received 0.9% saline in the same way. From the time of injection, animals were individually housed in metabolic cages until the end of the experiment 24 h after the first injection.

2.3.2. Oral Application of $^{13}$C-Fuc

Male NMRI mice (8-weeks-old, 38 ± 2 g body weight) were housed as described above. On the day of the experiment, animals received either a single dose of 0.33 g $^{13}$C-Fuc/kg body weight (treated, n = 5 per time point) or saline as the vehicle (controls, n = 3 for the time points 0.5/5 h, 1/3 h, 9, and 15 h) via oral gavage. Time points of controls were consolidated in case the treatments were done on the same day to save animals. As for the intravenous application, the gavage dose was calculated to
Figure 1. Potential pathways of 2’FL and Fuc metabolism and their link to the brain. After oral intake, Fuc or 2’FL is transported through the gut where (i) they may be taken up into the intestinal cells and released intact into the blood to be transported to organs and tissues or (ii) they are subjected to intracellular degradation resulting in various metabolic products which may be further used from the intestinal cells themselves or released to the blood or (iii) Fuc and 2’FL are fermented by gastrointestinal microorganisms leading to microbial metabolites with a high potential for local or systemic effects including effects on the vagus nerve and, hence, influencing brain activity. A direct influence on the brain by Fuc or its metabolites requires that the blood-brain barrier can be overcome. (Images from Motifolio Toolkit (Motifolio Inc, Ellicott City, MD, USA).

be isomolar to the dose of 13C-2’FL given in a previous study. Animals were kept individually in metabolic cages and sacrificed after 0.5, 1, 3, 5, 9, and 15 h. All experiments were carried out by individuals with appropriate training and experience according to the requirements of the Federation of European Laboratory Animal Science Associations and the European Communities Council Directive (Directive 2010/63/EU). Experiments were approved by the regional authority (Regional Authority Darmstadt; V54 – 19 c 20/15 – FU/1056).

2.3.3. Sample and Tissue Collection

At the end of the experiments, the treatment of the animals was done as described previously with a modification of the euthanasia protocol. Briefly, animals were killed individually with CO2 (flux rate 1.4 L/min) until the intertoe reflex and respiration ceased completely. From each animal, a blood sample was taken from the retrobulbar plexus and centrifuged at 1000 x g at 4°C for 10 min to obtain plasma. The abdomen was immediately opened and animals were perfused with saline to avoid plasma contamination of organs. Then, organs were quickly removed (liver, heart, spleen and kidney), the brain was placed on ice while separating the stem, cerebellum and cerebrum. Furthermore, the small intestine (SI) was cut into three pieces of equal length; the large intestine (LI) was taken separately. Intestinal content was collected from each segment. Urine left in the metabolic cages was collected. All samples were snap-frozen in liquid nitrogen and kept at -80°C until analysis.

2.4. Analytical Methods

The biological samples were subjected to Elemental Analysis-Isotope Ratio Mass Spectrometry (EA-IRMS) as described previously. Isotope ratio calculations were done using Elemental Software (IonVantage and Ionos; Elementar UK, Stockport UK) and results were expressed as δ13CVPDB enrichment with VPDB being the international standard Vienna Pee Dee Belemnite from the International Atomic Energy Agency IAEA (Vienna, Austria). It is notable that the natural abundance of 13C reveals negative values for the baseline 13C-enrichment between -23 and -26 in biological fluids and tissues of mice or humans, when the isotope ratios as δ13C are standardized for VPDB.

2.5. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 6.0.7 (GraphPad Software Inc, La Jolla, U.S.A.). Results were expressed as box plots with medians and min to max whiskers. Data were analyzed by ANOVA with multiple comparison test or Student t-test as group comparison between treated animals versus controls for the respective time points. Differences were considered significant at *p < 0.05, **p < 0.01 and ***p < 0.001.
Figure 2. $^{13}$C-enrichment ($\delta^{13}$C in $\%$) in plasma (A) and in brain sections (brain stem, cerebellum, cerebrum; B-D) of wild-type mice receiving an oral dose of $^{13}$C-labeled Fuc (black boxes). For comparison, data from our previous study using an isomolar dose of $^{13}$C-labeled 2´FL (grey boxes) have been added.\cite{14} Data are depicted as box plots with median and min-max whiskers; controls $\delta^{13}$C of the Fuc study are indicated as dotted line. Differences were calculated between the groups receiving an oral dose of $^{13}$C-Fuc and their saline controls for the same time points; they were considered significant at $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

Figure 3. $^{13}$C-enrichment ($\delta^{13}$C in $\%$) in luminal content of intestinal segments (upper panel, A-D) and organs (liver, heart, spleen, kidney) (lower panel, E-H) in wild-type mice receiving an oral dose of $^{13}$C-labeled Fuc (black boxes). For comparison, data from our previous study using an isomolar dose of $^{13}$C-labeled 2´FL (grey boxes) have been added.\cite{14} Data are depicted as box plots with median and min-max whiskers; controls $\delta^{13}$C of the Fuc study are indicated as dotted line. Differences were calculated between the groups receiving an oral dose of $^{13}$C-Fuc and their saline controls for the same time points; they were considered significant at $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. (LI, large intestine; SI, small intestine with section 1, 2, 3).

3. Results and Discussion

3.1. Oral Application of $^{13}$C-Fuc

After oral application of $^{13}$C-Fuc labeled at the same C-atom as $^{13}$C-2´FL, namely C$_1$, EA-IRMS analysis revealed an immediate $^{13}$C-enrichment of plasma at the earliest time point samples were collected, 30 min after dosing (Figure 2A, black boxes). This $^{13}$C-enrichment remained at the same level at all time points. When compared to the data from our previous study using $^{13}$C-2´FL\cite{38} (also refer to Figure 2A, grey boxes), $^{13}$C-enrichment in plasma rose at 2 h after dosing which is in line with findings by Vazquez and coworkers in rat pups receiving a single dose of unlabeled 2´FL.\cite{24} However, $^{13}$C-enrichment of plasma reached its maximum only at 5 h, indicating that at these later time points, an additional uptake of fermentation products of 2´FL carrying the $^{13}$C-label occurred since the $^{13}$C-2´FL dose had already reached the lower gut as discussed previously.\cite{38} In addition, Vazquez and co-workers detected Fuc in serum and found it remained at stable levels in pups and adult rats.\cite{24} With $^{13}$C-labeled Fuc, orally applied Fuc was rapidly absorbed and the $^{13}$C-enrichment levels remained high which may be explained by the absorption of Fuc metabolites at later time points (Figure 2A).

Most interestingly, the immediate rise in the $^{13}$C-enrichment of plasma after $^{13}$C-Fuc application was not associated with a $^{13}$C-enrichment in the tissues, including the brain sections.
enrichment shown in very similar to the course seen for 13C-2′FL (compare black and grey boxes for Fuc and 2′FL, but did occur later (>3 h) and was similar to what has been seen for 13C-2′ FL (compare black and grey boxes for Fuc and 2′FL, respectively, in Figure 3). In contrast to these previous observations, an early 13C-enrichment was observed in all organs except the brain (Figure 3E-H) in parallel to the fast 13C-enrichment in plasma even at the first time point, i.e., 0.5 h after oral application of the dose (Figure 2A).

3.2. Intravenous Application of 13C-Fuc

To prove whether Fuc was able to cross the blood-brain barrier, 13C-Fuc was applied intravenously to bypass the gastrointestinal barrier and to avoid microbial Fuc degradation at the same time. 12 h after the last intravenous dose of 13C-labeled Fuc, small amounts of 13C-Fuc were still found in the plasma, but the majority of 13C-Fuc was excreted via the urine (Figure 4). Most importantly, there was no 13C-enrichment in brain sections as well as in liver, heart and spleen and a low enrichment in kidney which might be due to urinary remnants not taken care of during the tissue preparation (Figure 4).

From the observations described above, we suggest that Fuc was not able to cross the blood-brain barrier since intravenous application of 13C-Fuc did not lead to a 13C-enrichment of brain tissue (Figure 4). In addition, the fast, initial rise in plasma 13C-enrichment after oral 13C-Fuc application may be due to the absorption of 13C-Fuc starting in the small intestine (Figure 3). At later time points (>3 h), however, 13C-enrichment in plasma may derive from both intestinal absorption of intact Fuc and its fermentation products carrying the 13C-label. Since Fuc was not able to cross the blood-brain barrier as described above (Figure 2), the 13C-enrichment observed in brain tissue was most likely due to 13C-labeled fermentation products similar to what we have seen after 13C-2′FL application. The small 13C-enrichment we had observed at early time points in other organs (Figure 3), however, may reflect a minor uptake of intact Fuc to be metabolized or used for glycoconjugate synthesis. We are aware that data regarding the metabolic fate of Fuc and 2′FL are urgently needed to answer the question whether brain composition and/or activity through signaling processes can be influenced by dietary means. In this context, various short chain fatty acids are certainly important factors influencing the gut-brain axis.[12,29] Such studies cannot be performed in infants; hence, we rely on animal studies although application of those data to human physiology requires great care.[40] The current opinion, whether fucosylated or sialylated HMOs can directly be incorporated into the brain is controversial as addressed in the introduction. Our studies with 13C-2′FL, 13C-Fuc, 13C-SL and 13C-SA do not support a direct influence of HMOs on brain composition.[14,16] With regard to Fuc and fucosylated HMOs, there is so far no evidence that a direct transfer and uptake into brain cells occurs in animals or humans.

Previous studies on the metabolic fate of Fuc support our results. In 1964, Coffey and coworkers addressed the metabolic question in rats using 13C as a radioactive label of Fuc.[41] The authors observed a rapid elimination of Fuc in urine after intraperitoneal injection of 14C-Fuc. Similar to this previous study, we also found urine as the major elimination route after oral and intravenous application of 13C-labeled 2′FL or Fuc (Figure 4).

13C-enrichment of brain tissue after oral Fuc application was relatively modest in scope and occurred only at the later time points indicating that Fuc was not readily enriched in the brain as an intact molecule. This conclusion is supported by earlier observations from Harsh et al. (1984) who found an uptake of L-Fuc in brain tumors but not in normal tissue.[42] The authors stated that their data imply a permissive blood-brain barrier in tumors rather than differences in Fuc metabolism. Wiese et al. (1994) observed that the uptake of L-Fuc into eukaryotic cells does not occur through a glucose transporter but potentially through facilitated diffusion.[41] To date, there is little information about an intracellular uptake of Fuc into the brain. GLUT-1 which is essential for the transport of glucose across the blood-brain barrier does not transport Fuc. The SLC database (http://www.bioparadigms.org/slc/intro.htm) lists transporters for metabolically activated carbohydrates such as GDP-Fuc (e.g., SLC35C1), but not for free L-Fuc. Therefore, we assume that if Fuc or 2′FL as such have an effect on brain function, it is more likely to be either through a direct effect of one or more bacterial metabolites transported to the brain or through an effect within the gut, e.g., via stimuli on the vagus nerve. As discussed in our previous publication,[14] the majority of ingested 2′FL reaches the colon where it can be used as a substrate for intestinal bacteria and catalyzed into acetate and lactate, as has been reported for some Bifidobacteria strains.[26,28,44,45] Co-existing bacteria participate in cross-feeding relationships that influence HMO metabolism.[26] Whether
HMO metabolites derived from various bacterial activities exert beneficial effects on the gut–brain axis is a highly relevant question to be addressed by future research.

3.3. Concluding Remarks

Our studies in mice receiving $^{13}$C-labeled Fuc via oral gavage revealed an early rise of $^{13}$C-enrichment in plasma (30 min after dosing) which had not been the case with $^{13}$C-2´FL. However, $^{13}$C-enrichment in the brain does not occur until 3 - 5 h after Fuc intake, when the $^{13}$C-Fuc bolus has already reached the lower gut. These data are consistent with the notion that Fuc was absorbed in the upper small intestine, but could not cross the blood-brain barrier and that the later $^{13}$C-enrichment in the brain may be derived from the uptake of Fuc metabolites resulting from bacterial fermentation as has been seen for 2´FL in the previous study. Metabolites, e.g. deriving from bacterial fermentation in the lower gut, however, can be enriched in tissues, including the brain. These HMO-derived metabolites (SCFA or other organic acids, such as lactic acids) may well affect brain function and composition, but most likely not by directly incorporating intact HMOs or their monosaccharides into brain structures. Thus, any benefit from dietary intake of 2’FL to an organ outside the gastrointestinal tract cannot be explained by absorption of intact fucose. The specific role of Fuc or 2´FL metabolites and to what degree they may be effective in the gut-brain communication needs to be investigated in further studies.

Acknowledgements

The authors are grateful to David Hill, PhD for carefully reading and editing the manuscript and to Cordula Becker and Katrin Koslowski for their excellent technical assistance. The project was financially supported (without external funding) by Abbott Nutrition.

Conflict of Interest

The authors declare no conflict of interest.

Author Contribution

S.R., E.V., R.B. and C.K. designed the study; S.R., C.B., M.R. and G.P.E. conducted research; S.R. performed statistical analysis; S.R. and C.K. drafted the manuscript; the authors read, revised and approved the final manuscript.

Data Availability Statement

Data available on request from the authors.

Keywords

$^{13}$C-labeled fucose, biological fluids, brain, metabolism, microbiota

Received: January 17, 2021
Revised: May 31, 2021
Published online:
[33] S. Rudloff, S. Obermeier, C. Borsch, G. Pohlentz, R. Hartmann, H. Brösicke, M. J. Lentze, C. Kunz, *Glycobiology* 2006, 16, 477.

[34] S. Rudloff, G. Pohlentz, C. Borsch, M. J. Lentze, C. Kunz, *Brit. J. Nutr.* 2012, 107, 957.

[35] V. Dotz, S. Rudloff, C. Meyer, G. Lohchnit, C. Kunz, *Mol. Nutr. Food Res.* 2015, 59, 355.

[36] K. C. Goehring, A. D. Kennedy, P. A. Prieto, R. H. Buck, *PLoS One* 2014, 9, e101692.

[37] L. R. Ruhaak, C. Stroble, M. A. Underwood, C. B. Lebrilla, *Anal. Bioanal. Chem.* 2014, 406, 5775.

[38] S. Kuntz, C. Kunz, C. Borsch, E. Vazquez, R. Buck, M. Reutzel, G. P. Eckert, S. Rudloff, *Mol. Nutr. Food Res.* 2019, 63, 1900035.

[39] C. R. Martin, V. Osadchiy, A. Kalani, E. A. Mayer, *Cell. Mol. Gastroenterol. Hepatol.* 2018, 6, 133.

[40] E. A. Mayer, K. Tillsch, A. Gupta, *J. Clin. Invest.* 2015, 125, 926.

[41] J. W. Coffey, O. N. Miller, O. Z. Sellinger, *J. Biol. Chem.* 1964, 239, 4011.

[42] G. R. Harsh, R. N. Nishimura, B. E. Dwyer, V. A. Levin, *Exp. Neurol.* 1986, 94, 21.

[43] T. J. Wiese, J. A. Dunlap, M. A. Yorek, *J. Biol. Chem.* 1994, 269, 22705.

[44] C. Yamada, A. Gotoh, M. Sakanaka, M. Hattie, K. A. Stubbs, A. Katayama-Ikegami, J. Hirose, S. Kurihara, T. Arakawa, M. Kitaoka, S. Okuda, T. Katayama, S. Fushinobu, *Cell Chem. Biol.* 2017, 24, 515.e5.

[45] T. Katayama, *Biosci. Biotechnol. Biochem.* 2016, 80, 621.